Identification and characterization of Risp:
a new nucleocytoplasmic shuttle protein interacting with the HIV-1 Rev protein

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To my Family

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1. Introduction

1.1. HIV-1

Human immunodeficiency virus (HIV), identified in 1983, is the etiological agent of the acquired immunodeficiency syndrome (AIDS) (Barre-Sinoussi et al., 1983; Coffin et al., 1986; Gallo et al., 1983; Levy et al., 1984). Two types of HIV are known: the most common HIV-1, which is responsible to the world-wide AIDS epidemic and the immunologically distinct HIV-2 (Clavel et al., 1986), which is much less common and less virulent (Ariyoshi et al., 2000; Ariyoshi et al., 1999), but produces clinical findings similar to HIV-1 (Wilkins et al., 1993). The HIV-1 type itself includes three groups M with subtypes A to K, and group O, which have different geographic distributions but all produce similar clinical symptoms. The basic pathology in AIDS is the loss of $CD4^+$ lymphocytes and a variety of disorders in immune function, leading to the onset of opportunistic infections like Pneumocystis Carinii pneumonia and malignancies as Kaposi's sarcoma or B-cell lymphomas (Levy, 1998).

HIV is a member of the lentivirus genus of the Retroviridae family. Like all retroviruses, HIV virions contain two identical copies of a single stranded RNA genome which are used as templates by the RNA-dependent DNA polymerase [Reverse Transcriptase (RT)] for production of DNA. Retroviruses were traditionally divided into three subfamilies, based primarily on pathogenicity rather than on genome relationship (oncoviruses which cause neoplastic disorders, spumaviruses which give cytopathic effect in tissue culture but apparently not associated with any known disease and lentiviruses which induce slowly progressing inflammatory, neurological and immunological diseases). Recently the international committee on the taxonomy of viruses has recognized seven distinct genera in the Retroviridae family (Table 1.1) (Fields and Knipe, 1996). Members belonging to the family of Retroviridae are widespread in nature and have been detected in many vertebrate species. Retroviruses show a great diversity in the interaction with their hosts. In fact, infection with some retroviruses can result in fatal diseases like AIDS, malignancies, neurological disorders and other pathological manifestations. Other retroviruses in contrast induce only benign viremia without apparent effects and some can even become established in the germ-line, where they persist as endogenous proviruses (Fields and Knipe, 1996).

Genus	Example isolates	Comments
Avian-leukosis-sarcoma (s)	Rous sarcoma virus (RSV)	exogenous, oncogene-containing
	Rous associated virus 0	endogenous, benign
	Rous associated virus 1 to 50	exogenous, cause B-lymphoma, and other diseases
Mammalian C-type (s)	Feline leukemia virus	exogenous, causes T-cell lymphoma, immunodeficiency and other diseases
	Simian sarcoma virus	exogenous, oncogene-containing
B-Type viruses (s)	Mouse mammary tumor virus	endogenous and exogenous, causes mostly mammary carcinoma
D-Type viruses (s)	Mason-Pfizer monkey virus	exogenous, unknown pathogenicity
HTLV-BLV viruses (c)	Human T-cell leukemia virus lymphoma (HTLV)-1 and -2	exogenous, causes T-cell and neurological disorders
Lentiviruses (c)	Equine infectious virus (EIAV)	exogenous, causes autoimmune hemolytic anemia
	Visna/maedi virus	causes encephalopathy and lung disease in sheep
	Caprine arthritis-encephalitis virus (CAEV)	causes encephalopathy and immune deficiency
	Bovine immunodeficiency virus (BIV)	causes lymphadenopathy and neurological disorders
	Feline immunodeficiency virus (FIV)	causes immune deficiency
	Simian immunodeficiency virus (SIV)	causes immune deficiency and encephalopathy
	Human immunodeficiency virus (HIV)-1 and -2	causes immune deficiency and encephalopathy
Spumaviruses (c)	Simian foamy virus (SFV)	exogenous, apparently benign
	Human foamy virus	exogenous, apparently benign

Table 1.1 Retroviruses Genera. (s) Indicate simple and (c) complex retroviruses.

The lentiviruses are exogenous, non-oncogenic retroviruses causing persistent infections leading to chronic diseases with long incubation periods (lenti for slow). They usually infect cells of the immune system (dendritic cells, macrophages, T lymphocytes) and cause cytopathic effects in permissive cells, such as syncytia and cell death by apoptosis and necrosis.

The prototype members of the lentivirus family were the slow visna virus, the equine infectious anemia virus (EIAV) and the caprine arthritis-encephalitis virus (CAEV). More recent isolates include the related human and simian immunodeficiency viruses

(HIV and SIV) and the more distantly related feline and bovine immunodeficiency viruses (FIV and BIV) (Table 1.1). Like the human T-cell leukemia virus (HTLV) family of primate onco-retroviruses, lentiviruses are complex retroviruses (Cullen, 1991). The significant characteristic of the complex retroviruses is the ability to regulate their own expression via virally encoded protein factors not found in other retroviruses. This property has been proposed to be essential for the long-term association of the complex retroviruses with the host and the generation of chronic active infections. The lentiviral complexity is reflected in their replication cycle, which reveals intricate regulatory pathways, unique mechanisms for viral persistence (Tang et al., 1999) and the ability to infect non-dividing cells.

1.1.1. Genome and morphology of the mature virion

Like all other retroviruses, the proviral DNA of HIV-1 (~ 9.2 kb) has three coding regions *gag*, *pol*, and *env*, two long terminal repeats (LTRs) with transcriptional regulatory elements, and one primary transcript (Fig. 1.1 and 1.2) [for review see (Frankel and Young, 1998; Turner and Summers, 1999)].

The RNA genome is flanked by two short redundant (R) sequences at both termini with adjacent unique sequences, U5 and U3, found at the 5’ and 3’ ends, respectively.

The *gag* gene encodes the large precursor polyprotein p55 that is cleaved in four proteins: the matrix p17 (MA), the "core" capsid p24 (CA), the nucleocapsid p7 (NC) and the p6 (Freed, 1998).

The *pol* gene encodes the synthesis of three important enzymes that function at different times during the replicative cycle. The reverse transcriptase/ribonuclease H complex p51and p66 (RT) acts in the early steps of the virus replication to form a double-stranded cDNA of the virus RNA. The integrase p32 (IN) mediates integration of the viral cDNA into the host chromosomal DNA. The protease p10 (PR) is responsible for the cleavage of the viral Gag and Gag-Pol polyproteins during the maturation of the viral particle.

The *env* gene directs the production of an envelope precursor protein gp160, which undergoes cellular proteolytic cleavage into the outer envelope glycoprotein gp120 (SU), responsible for binding to $CD4^+$ receptors, and the transmembrane glycoprotein gp41 (TM), which catalyzes the fusion of HIV to the target cell's membrane.

In addition, HIV-1 has at least six more genes encoding viral proteins with regulatory functions (*tat* and *rev*) or accessory functions (*nef*, *vif*, *vpr* and *vpu*), for reviews see (Cullen, 1998; Emerman and Malim, 1998; Frankel and Young, 1998; Kjems and Askjaer, 2000; Piguet and Trono, 1999; Pollard and Malim, 1998; Trono, 1995).

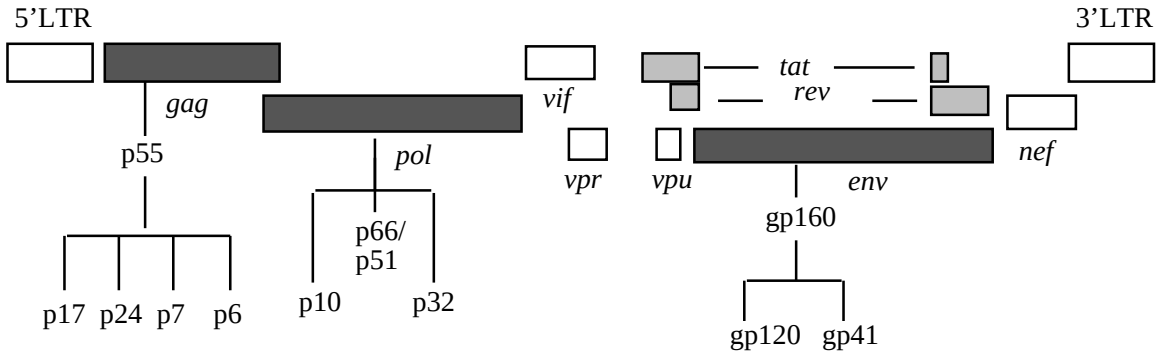


Fig. 1.1 HIV-1 genomic organization. Like all other retroviruses, HIV-1 has three structural genes *gag*, *pol* and *env* (heavily shaded), which are flanked by the long terminal repeats (LTR's). In addition it has six more genes, including two regulatory genes *tat* and *rev* (stippled) and four accessory genes *nef*, *vif*, *vpr* and *vpu* (white).

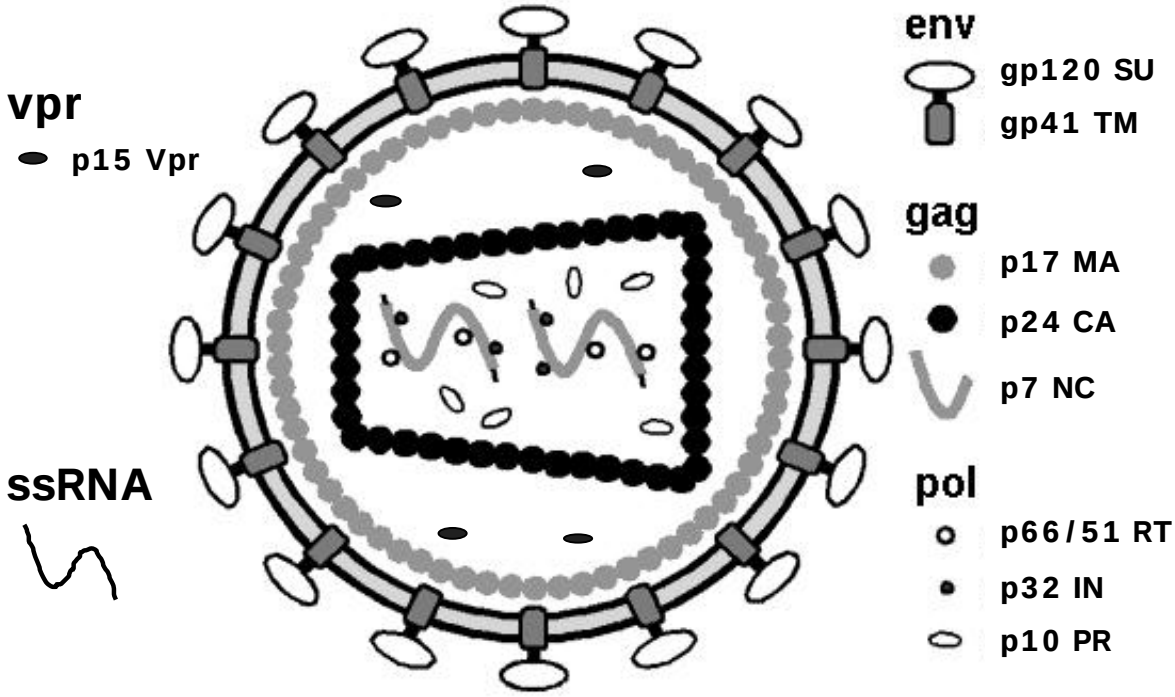


Fig. 1.2 HIV-1 mature virion structure. Modified from WebPath resource collection (Klatt, 2000). Typical lentivirus particles are spherical, about 80-110 nm in diameter, and consist of a lipid bilayer membrane surrounding a conical core. The two identical single-stranded RNA (ssRNA) molecules, of about 9.2kB each, are associated with the nucleocapsid proteins p7gag (NC). They are packed into the core along with virally encoded enzymes: reverse transcriptase (RT), integrase (IN), and protease (PR). P24gag comprises the inner part of the core, the capsid (CA). The p17gag protein constitutes the matrix (MA) which is located between the nucleocapsid and the virion envelope. The viral envelope is produced by the cellular plasma membrane and contains the protruding viral Env glycoproteins: gp120 surface glycoprotein (SU) and gp41 transmembrane protein (TM). Among the accessory proteins

encoded by HIV-1, certainly Vpr and perhaps Nef and Vif are packaged into virions, although the precise location have not yet been elucidated. Neither the other accessory protein Vpu nor the regulatory proteins Tat and Rev have been detected in virion particles.

1.1.2. Replication cycle

General features of the HIV-1 replication cycle are shown in Fig. 1.3. The HIV-1 replication cycle begins with the recognition of the target cell by the mature virion. The major targets for HIV-1 infection are cells bearing the HLA class II receptor, CD4, on their cell surfaces. These include T-helper lymphocytes and cells of the monocyte/macrophage lineage including microglia cells in the brain. The virus-CD4 binding occurs via specific interactions between the viral outer envelope glycoprotein gp120 and the amino-terminal domain of CD4 (Dalgleish et al., 1984; Klatzmann et al., 1984). These interactions are sufficient for binding but not for infection. Subsequently the virus glycoprotein gp120 interacts with additional cell-surface proteins to promote fusion of the viral and cellular membranes. These coreceptors have recently been identified to be members of the chemokine receptor family and include CXCR4 and CCR5 (Alkhatib et al., 1996; Deng et al., 1996; Doranz et al., 1996; Moore, 1997). The initial binding of HIV to the CD4 receptor is mediated by conformational changes in the gp120 subunit, followed by a conformational change in the gp41 subunit, stimulated by the chemokine receptors, that allows fusion and subsequent entry of HIV. Various strains of HIV differ in their use of chemokine coreceptors. There are strains of HIV known as T-tropic strains, which selectively interact with the CXCR4 chemokine coreceptor of lymphocytes, while M-tropic strains of HIV interact with the CCR5 chemokine coreceptor of macrophages and dual tropic HIV strains that infect both cell types (Littman, 1998; Moore, 1997). HIV-1 infection of CD4 negative cells, such as neural cells, has also been reported (Clapham et al., 1989; Harouse et al., 1989; Kozlowski et al., 1991; Kunsch et al., 1989) but the mechanisms of HIV entry are still unclear.
Membrane fusion is followed by a poorly understood uncoating event that allows the intracellular reverse transcription.

The viral RNA is transcribed in the cytosol into double stranded DNA by the reverse transcriptase RT (Hansen et al., 1987; Muesing et al., 1985). This enzyme possesses three enzymatic activities: RNA-dependent DNA polymerase, DNA-dependent DNA polymerase, and ribonuclease H (RNase H). The reverse transcription process takes place within a large nucleic acid-protein complex known as the preintegration complex (PIC) by the assistance of the accessory protein Vif (von Schwedler et al., 1993) and the nucleocapsid protein NC (Darlix et al., 1993). Once synthesized, the

viral DNA is transported to the nucleus of the infected cell as part of the PIC that appears to include tightly condensed viral nucleic acids and the IN, MA, RT, and Vpr proteins. In contrast to other retroviruses, that require cell division and concomitant breakdown of the nuclear envelope to gain access to the nuclear compartment, the lentiviral PIC is actively imported into the nucleus during the interphase (Bukrinsky et al., 1992; Lewis and Emerman, 1994). Nuclear import of the PIC seems to be directed by the accessory protein Vpr (Fouchier et al., 1997; Heinzinger et al., 1994), the Gag matrix protein MA (Bukrinsky et al., 1993; von Schwedler et al., 1994) and the integrase IN (Gallay et al., 1997). Vpr does not contain a conventional nuclear localization signal (see later) but appears to function by connecting the PIC to the cellular nuclear import machinery (Fouchier et al., 1998; Popov et al., 1998a; Popov et al., 1998b). The role of the MA protein in nuclear translocation of PIC remains controversial (Fouchier et al., 1997; Freed et al., 1995). The ability of lentiviruses such as HIV-1 to utilize active transport mechanisms for translocation of the PIC into the nucleus, allows these viruses to infect non-dividing cells such as differentiated macrophages, quiescent T lymphocytes and possibly neurons.

In the nucleus, IN catalyzes covalent integration of the viral DNA into the host genome, where it resides permanently as a provirus. An important modification as a result of reverse transcription and integration is the duplication of the U5 and U3 sequences in the LTR, such that the provirus now is flanked by tandemly repeated sequences U3-R-U5 with important regulatory functions.

The regulation of the HIV-1 transcription involves a complex interplay between cis-acting DNA and RNA elements present within the chromatin-associated proviral LTRs, cellular transcription factors and the viral regulatory protein Tat (transcriptional transactivator). In an arrangement similar to that of several inducible cellular promoters, the HIV-1 promoter, which is located in the U3 region of the 5’LTR, contains a TATA box and binding sites for several cellular DNA-binding transcription factors, such as NF-kB, Sp1 and TBP (Jones and Peterlin, 1994). It is highly inducible and responds to the activation status of the infected cell. NF-kB is the major inducible cellular activator. It is well established that many cells in the lymphoid tissue of infected individuals are latently infected (Pantaleo et al., 1993), even though the viral replication in the body is always active. In resting T-cells, the activity of the HIV-1 promoter is minimal, leading to viral quiescence in infected primary cells. Therefore, viral activation is associated with cell activation.
The transcription of the provirus by the cellular RNA polymerase II results in a primary transcript that may serve three distinct functions: 1) it constitutes genomic

RNA that is incorporated into the virion; 2) it serves as template for translation (Gag and Gag-Pol); 3) it functions as the precursor RNA for the production of diverse subgenomic mRNAs (Fig 1.3).

As mentioned before, HIV-1 encodes two essential regulatory proteins Tat and Rev, which increase viral gene expression at the transcriptional and post-transcriptional levels, respectively. HIV-1 mRNA expression is biphasic and can be divided into early (Rev-independent) and late (Rev-dependent) stages (Kim et al., 1989; Pomerantz et al., 1990). First, shortly after the infection of cells, multiply spliced (~ 2kb) RNA species are formed from the primary transcript and three proteins are produced: Tat, Rev and Nef, therefore referred as early gene products (Schwartz et al., 1990). Tat [for reviews see (Cullen, 1998; Emerman and Malim, 1998; Rubartelli et al., 1998)], greatly increases transcription from the HIV promoter, by binding to a cis-acting target sequence, the trans-activator response element (TAR), which is located at the 5' end of the nascent viral RNA transcript (Berkhout et al., 1989; Dingwall et al., 1989; Rosen et al., 1985). Tat recruits two cellular factors to this complex: cyclin T and cyclin-dependent protein kinase-9 (Cdk9). Cyclin T is proposed to bind directly Tat and to increase its affinity for the TAR RNA (Wei et al., 1998). Cdk9 phosphorylates the RNA polymerase II transcription complex and thus stimulates transcriptional elongation (Herrmann and Rice, 1995; Reines et al., 1996; Wei et al., 1998). Rev (regulator of expression of the virion), which accumulates during the early phase of expression, initiates late gene expression by binding a unique RNA element located in the *env* coding region of HIV-1, the so called Rev-responsive element (RRE). This interaction promotes the stability and transport of unspliced (~ 9 kb) and partially spliced (~ 4 kb) HIV-1 mRNAs out of the nucleus. These mRNAs are responsible for the production of the viral enzymes and structural proteins (Daly et al., 1989; Felber et al., 1989; Hadzopoulou-Cladaras et al., 1989; Hammarskjold et al., 1989; Malim et al., 1989c). Therefore Gag, Pol, Env, Vif, Vpr, and Vpu proteins are referred to as late HIV-1 proteins.

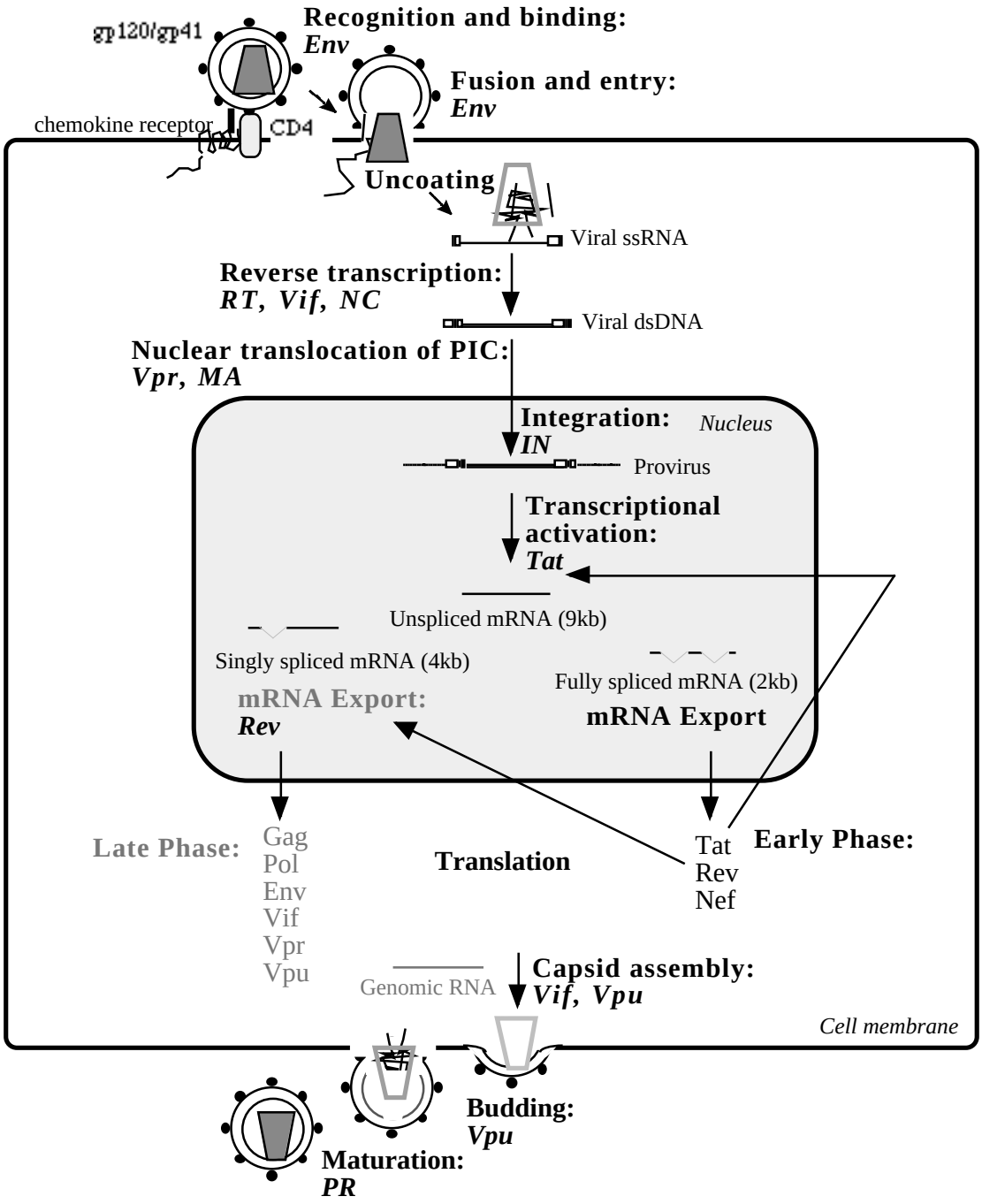


Fig. 1.3 Replication cycle of HIV-1. Modified from Ludwig (Ludwig, 1999). Each fundamental step is presented in bold. Names in *italic* refer to viral gene products involved in the specific steps. HIV-1 gene expression is stimulated by HIV-1 Tat and Rev, which act at transcriptional and post-transcriptional levels, respectively, and can be divided into two phases. The early phase is Rev-independent and the later phase is Rev-dependent (text in gray). Rev stabilizes and mediates export of singly spliced and unspliced RNA transcripts out of the nucleus into the cytoplasm.

Controversial functions have been assigned to the Nef (negative factor) protein. First, it was assumed to have an inhibitory function in the transcription of HIV-1 genes (Ahmad and Venkatesan, 1988). However, recent studies have contradicted this concept by showing that Nef is important for virus replication. Among its various functions (Piguet and Trono, 1999), it enhances viral expression in quiescent cells and mediates lymphocyte chemotaxis and activation at sites of virus replication (Kestler et al., 1991; Kirchhoff et al., 1995; Koedel et al., 1999; Miller et al., 1994; Swingler et al., 1999).

The Env precursor polyprotein (gp160) is synthesized in the endoplasmatic reticulum (ER) where it is glycosylated and appears to oligomerize to a trimeric structure post-translationally (Wyatt et al., 1998; Wyatt and Sodroski, 1998). Thereafter, it is cleaved to produce the non-covalently associated (gp41 TM - gp120 $SU)_3$ trimeric glycoprotein complex, which is transported to the cell membrane for virus assembly. Vpu is thought to enhance this process and inhibit a premature trapping of CD4 to Env in the ER by binding CD4 molecules, which are also synthesized in the ER, and directing them to the ubiquitin-proteasome degradation pathway (Margottin et al., 1998; Schubert et al., 1998; Strebel et al., 1988; Willey et al., 1992a; Willey et al., 1992b). Similarly, the accessory protein Nef facilitates the routing of CD4 from cell-surface and Golgi apparatus to lysosomes, resulting in endosomal degradation and preventing inappropriate interaction with Env (Aiken et al., 1994). In addition, both Vpu and Nef can down-regulate expression of MHC class I molecules. The down-regulation of CD4 and MHC class I molecules on the surface of infected cells also helps infected cells to evade immune responses of the host, such as killing by cytotoxic T lymphocytes (Collins et al., 1998; Kerkau et al., 1997).

During synthesis of the Gag polyprotein by ribosomes, a translational frameshift may occur, resulting in generation of smaller amount of Gag-Pol precursor polyproteins, which associate with the Gag polyprotein at the cellular membrane. The N-terminally myristoylated MA domain of the Gag/GagPol polyproteins directs insertion of the Gag precursors into the cellular membrane and interacts with the cytoplasmic tail of gp41 resulting in the anchoring of Env to the viral particle (Dorfman et al., 1994). Approximately 1200 to 2000 copies of Gag precursor bud to form an immature particle, which encapsidates two copies of the unspliced viral RNA genome, by the ability of NC to interact with nucleic acids. Vif and Vpu proteins have been reported to play a role in packaging of the nucleoprotein core and in virion release, respectively (Hoglund et al., 1994; Lamb and Pinto, 1997). Concomitantly or immediately following the external budding, the cleavage of the Gag/Gag-Pol polyproteins by the

virally encoded PR produces the structural proteins MA, CA, NC as well as the independent enzymes PR, RT and IN. This final step primes new virus particles for the next round of infection and is termed maturation.

1.2. Pathways of nuclear transport: a general overview

As mentioned above active nuclear translocation of HIV nucleic acids, as well as of PIC, and Tat and Rev proteins is essential for HIV replication. Therefore, a general overview of cellular nuclear import and export pathways will be given before discussing the Rev protein in more detail.

Eukaryotic cells are characterized by distinct nuclear and cytoplasmic compartments, which are separated by the nuclear envelope, a double membrane that is continuing in the endoplasmatic reticulum. The genetic information is contained in the nucleus, whereas protein synthesis occurs in the cytoplasm. This separation offers additional opportunities to control gene expression, but requires a large amount of energy to assure controlled bi-directional exchange of macromolecules between these two compartments.

For example RNAs, synthesized in the nucleus, are almost all actively exported to the cytoplasm. A large number of proteins with nuclear functions are actively transported into the nucleus. In addition some RNA molecules, including many uridine-rich small nuclear RNAs (UsnRNAs) are transported in both directions as a part of their assembly into small nuclear ribonucleoproteins (snRNP).

Transport into and out of the eukaryotic cell nucleus occurs through nuclear pore complexes (NPCs), large structures spanning the nuclear envelope and consisting of ~50-100 different proteins that are often commonly called nucleoporins. Many recent reviews have considered NPC composition, their assembly, structure and function (Davis, 1995; Doye and Hurt, 1997; Fabre and Hurt, 1994; Talcott and Moore, 1999). Generally, proteins can enter and exit the nucleus through NPC, either by diffusion or by active mechanisms, which are saturable, energy-dependent and which involve specific signal-mediated transport. For recent reviews see (Gorlich, 1997; Gorlich and Kutay, 1999; Kaffman and O'Shea, 1999; Mattaj and Englmeier, 1998; Nigg, 1997). The structural constraints of the NPC dictate that proteins smaller than 40 kDa may enter the nucleus by passive diffusion. However, RNAs and many small proteins, such as histones or the 18 kDa Rev protein, are imported by a regulated signal-mediated pathway. Generally, proteins require a targeting signal called nuclear localization signal (NLS) for active transport into the nucleus. Export from the nucleus into the cytoplasm requires a nuclear export signal (NES). RNA-substrates for nuclear export, like messenger RNAs, ribosomal RNAs, small nuclear RNAs and transfer RNAs are

probably exported from the nucleus as RNA-protein (RNP) complexes, rather than as naked RNA (Izaurralde and Mattaj, 1995; Nakielny et al., 1997; Stutz and Rosbash, 1998). This indicates that RNA transport is mediated by specific signals provided by the specific cellular factor with which each RNA is associated. Many proteins are capable of both entering and exiting the nucleus. These are nucleocytoplasmic shuttle proteins and they contain NLS and NES signals, which may be contained in separate domains of the protein. However, nucleocytoplasmic shuttling can also be controlled by a class of unique transport signals known as NSSs (nucleocytoplasmic shuttling signals) or be indirectly mediated by interaction with proteins that are transported into and out of the nucleus.

The first two NLSs characterized were the “bipartite” signal in nucleoplasmin [KRPAATKKAGQAKKKK; (Dingwall et al., 1982)] and the “classical” signal in Simian Virus 40 (SV40) large T antigen [PKKKRKV; (Kalderon et al., 1984a; Kalderon et al., 1984b)]. A large number of other NLSs have been identified during the last years, and the NLSs of most nuclear proteins were found to contain a basic amino acid cluster in their primary amino acid sequences, even though there might be no obvious consensus sequences. The classical monopartite NLS contains a lysine-rich stretch of amino acids, whereas the bipartite NLS is composed of two lysine-rich stretches separated by a short amino acid spacer. In contrast, the Rev and Tat NLS signals [RRNRRRRWRER (Kubota et al., 1989; Perkins et al., 1989) and RKKRRQRRR (Subramanian et al., 1990)] are strikingly arginine-rich.

Several years ago, the analysis of the nucleo-cytoplasmic shuttling pathways of Rev and the cyclic AMP dependent protein kinase inhibitor (PKI), led to the identification of two nuclear export signals, short leucine-rich amino acid sequences [LQLPPLERLTLD (Fischer et al., 1995; Wen et al., 1995); LALKLAGLDI (Wen et al., 1995)]. Since then, an increasing number of proteins have been shown to also contain leucine-rich NES. By comparing all of these sequences, a consensus for leucine-rich NESs has been defined ($LX_{2-3}LX_{2-3}LXL$), characterized by four characteristically spaced hydrophobic residues (Kjems and Askjaer, 2000). Many of these proteins are implicated in RNA metabolism: the viral HIV-1Rev, HTLV Rex (Hakata et al., 1998), Herpesvirus ICP27 (Sandri-Goldin, 1998), Influenzavirus NS2 (O'Neill et al., 1998), Adenovirus E1-34 kDa (Dobbelstein et al., 1997); the cellular TFIII (Fridell et al., 1996b), Gle1p (Murphy and Wente, 1996), FMR-1 (Fridell et al., 1996a). Many other leucine-rich NES containing proteins are involved in the regulation of other critical cellular functions: including PKI, Actin (Wada et al., 1998), MAPKK (Fukuda et al., 1997), p53 (Stommel et al., 1999). The Rev NES is

transferable to heterologous proteins, and mutations in this domain abolish the ability of Rev to shuttle between the cytoplasm and the nucleus. In addition, Rev NES-mutants exhibit a *trans*-dominant negative phenotype capable of repressing wild-type Rev function as well as HIV replication (Malim et al., 1989b).

NSS consist of both, NLS and NES, directing both nuclear import and nuclear export (Michael, 2000). The majority of proteins known to contain NSS interact with mRNA, a fact that has stimulated speculations that NSS-proteins are involved in mRNA nuclear export. The first NSS was found in the heterogeneous nuclear hnRNP A1 protein (Michael et al., 1995; Siomi and Dreyfuss, 1995), one of the major pre-mRNA/mRNA binding proteins in eukaryotic cells. A protein, although one of the most abundant in the nucleus, that shuttles continuously between the nucleus and the cytoplasm, via its sequence termed as M9 (NQSSNFGPMKGGNFGGRSSGPYGGG GQYFAKPRNQGGY), which has no similarity to any other NLS or NES, and mediates the nuclear export of cellular mRNA.

Nucleocytoplasmic exchange is mediated by soluble transport receptors that are thought to shuttle continuously between nucleus and cytoplasm. Many of these receptors have now been identified. Interestingly, it emerged that both import and export events are mediated by a related family of shuttling transport factors, which are referred as importin β superfamily (Table 1.2). In humans, the importin β superfamily consists of at least 21 members, which recognize various nuclear transport signals and cargo molecules, comprising cellular (Gorlich and Kutay, 1999) and viral proteins (Whittaker and Helenius, 1998).

Generally, the process of nuclear protein import can be divided into three steps: first, an energy-independent docking to the cytoplasmic face of the NPC, second, an energy-dependent translocation through the NPC, followed by the third step of nuclear release of the cargo. Although proteins carrying different NLSs probably follow the same overall pathway, interesting differences at the docking step exist according to the different types of NLS [see reviews (Gorlich and Kutay, 1999; Kjems and Askjaer, 2000; Mattaj and Englmeier, 1998)].
The mechanism for import of lysine-rich NLS proteins and the arginine-rich NLS Rev has been studied intensively.
A protein carrying the classical lysine-rich NLS, which is found in the majority of nuclear proteins, is recognized by a cytoplasmic receptor that consists of the importin α/importin β heterodimer. Importin α provides the NLS-binding site (Adam and

Gerace, 1991; Weis et al., 1995) and serves as a bridge between substrate proteins carrying the classical NLS and the actual import factor importin β.

Transport receptors [alternative names]	Function	References
Importin (Imp) β [Karyopherin β1]	Import of ribosomal proteins, HIV Rev, HIV Tat, HTLV Rex	1-4
Imp β/Imp α complexes	Import of proteins containing the classical NLS	5-6
Imp β/Snurportin complexes	Import of UsnRNPs	7
Transportin 1 [Importin β2 Karyopherin β2]	Import of hnRNPs, Tap, ribosomal proteins, nucleolin C23	8-10
Exportin 1 [Crm1]	Export of proteins containing leucine-rich NESs, UsnRNAs, Snurportin 1	11-14
CAS	Export of Importin α	15
Exportin t	Export of tRNA	16-17

Table 1.2 Human transport receptors of the importin β superfamily. Modified version of Table 1 in (Gorlich and Kutay, 1999). References: 1 (Henderson and Percipalle, 1997); 2 (Jakel and Gorlich, 1998); 3 (Truant and Cullen, 1999); 4 (Palmeri and Malim, 1999); 5 (Gorlich et al., 1995); 6 (Imamoto et al., 1995); 7 (Huber et al., 1998); 8 (Pollard et al., 1996); 9 (Truant et al., 1999); 10 (Yaseen and Blobel, 1997); 11 (Fornerod et al., 1997); 12 (Fukuda et al., 1997); 13 (Ossareh-Nazari et al., 1997); 14 (Paraskeva et al., 1999); 15 (Kutay et al., 1997); 16 (Arts et al., 1998); 17 (Kutay et al., 1998).

Importin β, via a direct interaction with the nucleoporins of the NPC, drives the translocation of the complex into the nucleus (Gorlich et al., 1995; Moroianu et al., 1995). Once the import complex reaches the nuclear face of the NPC, the direct binding of nuclear RanGTP (see later) to importin β finally terminates the translocation and the dissociation of the importin heterodimer complex, releasing the import substrate in the nucleoplasm.
In contrast, Rev, as well Tat and Rex, which all contain an arginine-rich NLS, have been shown to interact directly with importin β, suggesting that importin α is not essential for the import of these proteins (Henderson and Percipalle, 1997; Palmeri and Malim, 1999; Truant and Cullen, 1999).

Another essential component and common regulator of the nucleocytoplasmic transport is the previously mentioned small Ran-TC4 GTPase (Dahlberg and Lund,

1998; Gorlich, 1997; Kutay et al., 1997; Mattaj and Englmeier, 1998; Melchior et al., 1993). Like all G proteins, Ran switches between a GDP- and a GTP-bound state by nucleotide exchange and GTP hydrolysis. It is proposed that Ran provides the energy necessary for translocation through the pore via GTP hydrolysis and provides directionality of the transport process by virtue of the asymmetry of RanGTP/RanGDP, that exists across the nuclear membrane (Izaurralde et al., 1997). In fact, Ran is mainly complexed with GDP in the cytoplasm and with GTP in the nucleus. Transport receptors are RanGTP-binding proteins that respond to this gradient by loading and unloading their substrates in the appropriate compartment. Importins bind their cargoes in the cytoplasm, release them upon binding to RanGTP in the nucleus and return to the cytoplasm without their cargo, as RanGTP complexes (importin β) or exported by a specialized exportin, namely CAS (importin α,) (Gorlich and Kutay, 1999; Izaurralde et al., 1997; Jakel and Gorlich, 1998; Kutay et al., 1997). Unlike importins, which bind their substrates only in the absence of RanGTP (i.e. in the cytoplasm), export receptors like Crm1(chromosome region maintenance, also known as exportin 1) bind their cargoes preferentially in the nucleus forming ternary complexes with their substrate and RanGTP but not RanGDP (Fornerod et al., 1997; Gorlich and Kutay, 1999; Kutay et al., 1997; Kutay et al., 1998). The trimeric complex is then rapidly transferred to the cytoplasm where it is disassembled, and the free export receptors can re-enter the nucleus and bind the next cargo molecule.

Unlike many substrates for protein import, which are individual proteins carrying a NLS and thus are relatively small and simple, nuclear export substrates are often large and complexes as RNA-protein particles. The molecular mechanism for Rev-mediated nuclear export has been studied intensively and a great part of the knowledge on general nucleocytoplasmic transport relies on analysis of Rev as a prototypic shuttling protein. The presence of leucine-rich NES is a necessary prerequisite for Rev and the other numerous proteins containing this signal, to be recognized by Crm1, a shuttling export receptor related to importin β superfamily (Table 1.2)(Fornerod et al., 1997; Fukuda et al., 1997; Neville et al., 1997; Ossareh-Nazari et al., 1997; Stade et al., 1997).

1.3. The Rev protein

1.3.1. Rev function and features of Rev-responsive RNAs

The translation of messages containing introns is very unusual in eukaryotic cells. Indeed, a mechanism which retains incompletely spliced RNAs in the nucleus until

splicing is complete (Cullen, 1998), ensures that only the correct protein is expressed. Retroviruses such as HIV employ a variety of different splicing events and overlapping reading frames to express a large array of mRNAs (over 30) and proteins (at least 15) from a single primary transcript. The HIV-1 RNA sequence contains at least 4 different 5' splice sites and 8 different 3' splice sites, which are used alternatively (Neumann et al., 1994; Purcell and Martin, 1993; Schwartz et al., 1990). In the HIV-1 replication cycle, as mentioned before, the structural proteins are produced from incompletely spliced and unspliced mRNAs. To bypass the cellular nuclear retention of incompletely spliced viral transcripts, HIV-1 expresses the protein Rev. This protein mediates the nuclear export of unspliced and singly spliced viral mRNAs by binding to and multimerizing on a specific recognition element located within the second intron on the viral RNA, the RRE (Emerman et al., 1989; Felber et al., 1989; Hammarskjold et al., 1989; Malim et al., 1989c).

Rev regulated-expression of incompletely spliced mRNAs has a significant effect on the temporal expression pattern of the individual HIV-1 genes. At the initial stage of HIV-1 expression, the concentration of Rev is low, and incompletely spliced viral mRNAs are subjected to nuclear retention. They are either spliced to completion or subjected to degradation. The fully spliced mRNAs encoding Tat, Rev, and Nef are not retained in the nucleus but are exported to the cytoplasm by the normal mRNA export pathway and therefore are Rev-independent. Subsequently, Rev enters the nucleus, binds to its target RNA, and at a certain threshold concentration, and through interactions with cellular host factors, mediates cytoplasmic expression of unspliced (*gag, pol*) and singly spliced (*env, vif, vpr, vpu*) viral messages. Therefore this phase of HIV-expression is Rev-dependent. The temporal delay of expression of mRNA encoding the structural proteins Gag and Env has been considered to play a role for maintenance of viral latency and to prevent elimination of infected cells by the immune system (Kjems and Askjaer, 2000; Levy, 1998).

Rev-like transactivator proteins have been identified in all known lentiviruses (Kiyomasu et al., 1991; Malim et al., 1989a; Schoborg et al., 1994; Tiley et al., 1990) and within human oncoretroviruses, the human T-cell leukemia viruses type-I and type II (HTLV-I and -II) (Hanly et al., 1989; Hidaka et al., 1988). In HTLV-I and -II, the protein is known as Rex and the *cis*-acting RNA target is termed Rex response element (RXRE). In addition, a protein with Rev-like function has been identified in the human endogenous provirus HERV-K (Lower et al., 1995; Yang et al., 1999).
In contrast, simple retroviruses lack a Rev equivalent protein and can bypass the nuclear retention of intron-containing mRNAs by the action of a *cis*-acting structured

RNA target, termed constitutive transport element (CTE), that together with cellular host factors activates the transport of unspliced viral mRNA to the cytoplasm. The CTE was initially discovered in the Mason-Pfizer monkey virus (Bray et al., 1994) and similar elements have subsequently been found in other retroviruses (Ogert et al., 1996; Tabernero et al., 1997; Zolotukhin et al., 1994). In the case of the Mason-Pfizer monkey virus two host proteins, TAP (Gruter et al., 1998; Kang and Cullen, 1999) and RNA Helicase A (Li et al., 1999; Tang et al., 1997), have been identified and proposed for different roles in the CTE-RNA export. Interestingly, although the CTE and Rev/RRE can functionally substitute for each other in mediating nuclear RNA export, they appear to act via distinct pathways and different mechanisms (Kang and Cullen, 1999; Pasquinelli et al., 1997; Saavedra et al., 1997).

Rev-responsive RNAs contain two distinct features required for regulation: first the *cis*-acting sequence-specific target for Rev, RRE, and second, elements that ensure nuclear retention of an accessible pool of RNA to serve as a substrate for Rev.
The RRE is a highly structured RNA element (351 nucleotides) that resides within the *env* intron and is therefore present in all ~9kb and ~4kb mRNAs. The Rev-RRE interaction has been studied extensively, both *in vitro* and *in vivo*. The RRE contains extensive secondary structure, including several stem loops. The initial and primary specific sequences within the RRE that determine Rev responsiveness are located in the second stem loop from the 5' end, SLIIB, (Bartel et al., 1991; Battiste et al., 1996). The RRE serves as the docking site for Rev on RNAs that are confined to the nucleus. In fact the RRE is the only HIV-1 sequence that needs to be transferred to a heterologous RNA for Rev-responsiveness to be acquired. Even though the RRE harbours only a single high-affinity Rev-binding site, *in vitro* binding and footprinting studies have clearly demonstrated that multiple Rev molecules bind to a single RNA, forming complexes $(Rev)_n$-RRE (Daly et al., 1989; Heaphy et al., 1990; Kjems et al., 1991a). The observation that Rev function requires the sequential binding of multiple Rev molecules to the RRE provides a biochemical explanation for the observed threshold effect for Rev function *in vivo*.

The splice sites of HIV-1 are inefficiently used by the cellular splicing machinery, and thus have been proposed to permit accumulation of a pool of unspliced RNAs in the nucleus. The nuclear accumulation of precursor RNAs is not typical for cellular genes. They are normally retained in the nucleus by the interaction of splicing factors until they are either rapidly spliced to completion, and efficiently exported to the cytoplasm as fully processed mRNAs, or they are degraded (Izaurralde and Mattaj, 1995; Nakielny and Dreyfuss, 1997). It has also been proposed that the Rev-responsive

RNAs are retained in the nucleus by the interaction of splicing factors and that Rev itself could regulate a dissociation of splicing components and pre-mRNA (Chang and Sharp, 1989; Lu et al., 1990; Stutz and Rosbash, 1994). This hypothesis is supported by inhibition of splicing by Rev in *in vitro* experiments (Kjems et al., 1991b). However, more recently, it has been shown that Rev is able to induce the export of both spliceable and non-spliceable RRE-containing pre-mRNAs, regardless of the location of the RRE within the RNA, suggesting that Rev can function independently of splicing (Fischer et al., 1999).

Besides the inefficient splicing of primary transcripts, particular sequence elements are proposed to be important for the control of Rev-responsive mRNAs processing. They have been termed *cis*-acting repressive sequences (CRSs) and/or instability sequences (INSs) (Cochrane et al., 1991; Maldarelli et al., 1991; Rosen et al., 1988; Schneider et al., 1997; Schwartz et al., 1992a; Schwartz et al., 1992b). These sequences are distributed throughout the regions of HIV-1 that form introns in *gag*, *pol* and *env*, and function by promoting degradation of these transcripts. When appended in *cis* to heterologous reporter genes they inhibited expression of those genes. This inhibition was counteracted only by the positive effect of the Rev-RRE interaction. These elements did not contain any known HIV-1 splice sites and acted independently of splicing. It was found that these sequences have an unusually high adenine-uracil content, a common feature among cellular mRNAs with short half-lives, such as those encoding a number of proto-oncogenes or cytokines (Chen and Shyu, 1995; Jacobson and Peltz, 1996). However, unlike the HIV ~9kb and ~4kb transcripts, these cellular mRNAs are degraded in the cytoplasm as fully processed transcripts. Nevertheless, the identification of nuclear cellular factors which are able to interact with the HIV-1 cis-acting repressive sequences raise the possibility that the CRS elements could assist nuclear sequestration and nuclear degradation of RNA by formation of RNA-protein complexes not accessible to nuclear export pathways (Olsen et al., 1992).
Therefore, the mechanisms underlying the nuclear accumulation of HIV-1 precursor mRNAs and their marked nuclear degradation in the absence of Rev still remain to be clarified.

1.3.2. Domain structure of Rev

The HIV-1 Rev is an 18 kDa phosphoprotein containing 116 amino acids that, as previously described, has a number of attributes essential for viral replication. It is capable of being imported into the nucleus, where it binds specifically to incompletely spliced mRNAs containing the RRE, forming multimers $(Rev)_n$-RNA, and it drives

nuclear export of these mRNAs. These numerous activities are directed by at least two functional domains that have been delineated by extensive mutagenesis studies (Hope et al., 1990; Malim et al., 1989b; Malim et al., 1991) (Fig. 1.4). The amino-terminal domain contains a basic arginine-rich motif (aa 35-51: RQARRNRRRRWRERQRQ), which mediates both RNA-binding (Bohnlein et al., 1991; Hope et al., 1990; Kjems et al., 1992; Malim et al., 1990) and nuclear/nucleolar localization (Bohnlein et al., 1991; Cochrane et al., 1990; Kubota et al., 1989; Perkins et al., 1989; Venkatesh et al., 1990). This is, closely flanked on both sides by two sequences (aa 12-29 and aa 52-60) that are responsible for the multimerization of Rev (Malim and Cullen, 1991; Olsen et al., 1990; Szilvay et al., 1997; Thomas et al., 1998; Zapp et al., 1991). The carboxy-terminal region contains a leucine-rich domain (aa 73-84: LQLPPLERLTLD) originally coined the activation domain, because it was thought to bind necessary Rev-interacting proteins (Hope et al., 1992; Malim et al., 1989b; Mermer et al., 1990) and recently, discovered to function as a nuclear export signal (Fischer et al., 1995; Meyer and Malim, 1994; Wen et al., 1995).

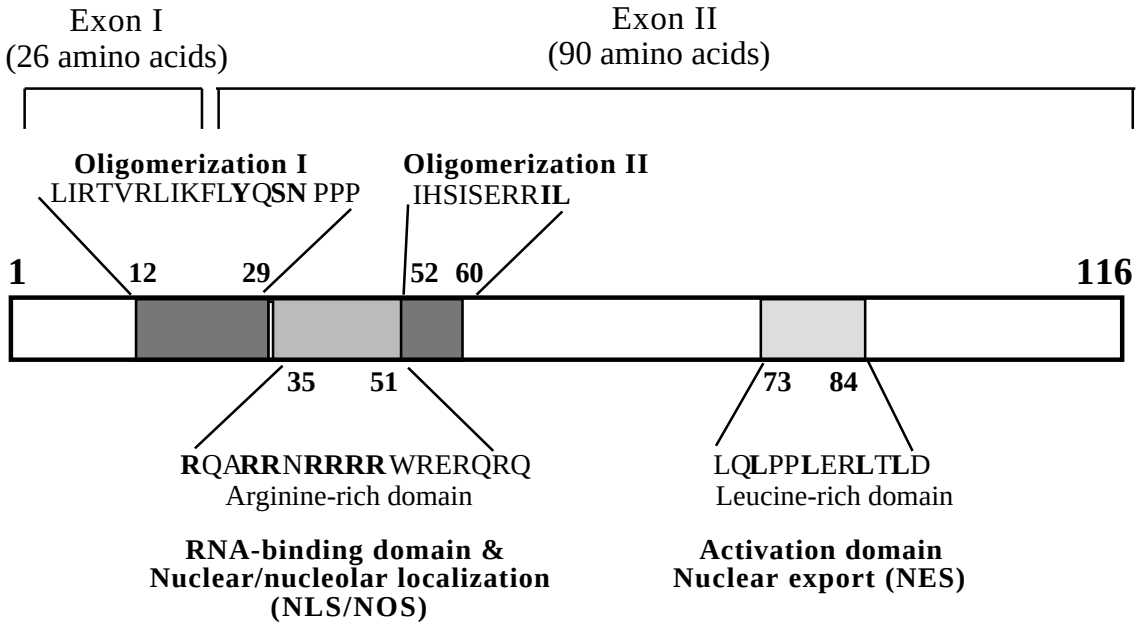


Fig.1.4 Structure of HIV-1 Rev protein. Rev is composed of several domains harboring distinct functions. The N-terminal basic domain including residues 35-51 is involved in RRE binding and in nuclear/nucleolar localization. Sequences flanking the basic domain (aa12-29 and aa 52-60) are essential for multimerization of Rev. The leucine-rich part in the C-terminus (aa 73-84) acts as a nuclear export signal. Mutational analysis showed the presence of amino acids (in bold) essential for Rev function (Hope et al., 1990; Kjems and Askjaer, 2000; Malim et al., 1989b; Thomas et al., 1998).

1.3.3. Cellular cofactors interacting with Rev and RRE

The observation that murine fibroblasts do not support Rev (Trono and Baltimore, 1990) and Tat functions (Winslow and Trono, 1993) was ascribed to lack of essential human factors in murine cells. Both defects could be complemented by fusion with human cells or addition of selected human chromosomes (Shukla et al., 1994; Shukla et al., 1996; Trono and Baltimore, 1990; Winslow and Trono, 1993). Whereas human cyclin T was identified as a candidate host-cell factor for Tat activity (Wei et al., 1998), human host-cell factors capable of restoring Rev activity in rodent cells have not yet been identified.

Many groups tried to isolate and characterize possible Rev-interacting cofactors essential for the post-transcriptional regulation. Besides the recent identification of importin β and Crm1, during the last ten years several other cellular proteins have been reported to interact with Rev and different mechanisms have been proposed to activate Rev function (Table 1.3). These include in chronological order B23, eIF-5A, the splicing factor ASF/SF2-associated protein p32 and several nucleoporins including Rip/Rab. However, although these factors are thought to be Rev-interacting stimulatory cofactors, their actual role in Rev function and support of virus replication is still unknown.

B23 was the first protein identified to interact with Rev, using affinity chromatography (Fankhauser et al., 1991). B23 is one of the major nucleolar phosphoproteins and it may be involved in the process of maturation of ribosomal structures. Rev often accumulates in the nucleolus (Cochrane et al., 1990) and this may reflect the ability of Rev to bind the nucleolar B23 protein. Although B23 was found to stimulate the import of Rev it appears to be neither necessary nor sufficient for this, suggesting that B23 may act as a molecular chaperone by preventing aggregation of proteins and assisting the nuclear import of Rev (Szebeni and Olson, 1999).

The cellular protein eIF-5A, which is the only known protein containing the amino acid hypusine, was shown to cross-link to the Rev/RRE complex *in vitro* (Ruhl et al., 1993). A transdominant negative mutant of eIF-5A, which retained its ability to bind Rev, caused a block of Rev nuclear export as well as HIV replication in lymphocytes (Bevec et al., 1996). Interestingly, although eIF-5A was originally proposed to be a translation initiator, the normal function of the protein is unknown. EIF-5A was shown to interact also with the ribosomal protein L5, which is implicated in the 5S rRNA export pathway (Schatz et al., 1998). Therefore, eIF-5A is one of the Rev-interacting factors that have been suggested to support RNA export.

Cellular factor (localization)	Identifying method	Source	Interaction site	Suggested function in viral replication	References
B23 (nucleolar)	affinity chromatography	HeLa	basic domain of Rev and Tat	chaperon nucleolar import	(Fankhauser et al., 1991; Szebeni and Olson, 1999)
eIF-5A (cytoplasmic and nuclear)	cross-linking experiments	HeLa nuclei extracts	activation domain of Rev and Rex	nuclear export and translation	(Bevec et al., 1996; Ruhl et al., 1993)
YL2 /p32 (primarily mitochondria)	yeast -2 hybrid	murine embryonic tissue	basic domain of Rev and Rex.	splicing synergistic stimulation on HIV transcription	(Luo et al., 1994; Tange et al., 1996)
	co-precipitation assay and protein footprinting	in vitro human protein			
90% = **Tap (Tat associated protein)**			activation domain of Tat	HIV transcription activator	(Yu et al., 1995)
Nucleoporins: (nuclear, NPC)					(Bogerd et al., 1995; Farjot et al., 1999; Fritz and Green, 1996; Fritz et al., 1995; Stutz et al., 1996; Stutz et al., 1995; Zolotukhin and Felber, 1999)
hRip = Rab	yeast -2 hybrid	HeLa, CEM-T cells	activation domain of Rev and Rex	nuclear export	
yRip1p1 = Nup42		yeast			
Nup98, Nup153, Nup214		yeast, human			
NLP-1		HeLa			
Crm1/Exportin 1 (nuclear)	yeast -2 hybrid	yeast	activation domain of Rev and Rex	nuclear export	(Fornerod et al., 1997; Neville et al., 1997)
	in vitro binding	human			
Importin β (cytoplasmic)	in vitro binding	human	basic domain of Rev, Rex and Tat	nuclear import	(Henderson and Percipalle, 1997; Palmeri and Malim, 1999; Truant and Cullen, 1999)

Table 1.3 Cellular cofactors proposed to interact with the HIV-1 Rev protein.

A murine homologue (YL2) of a human ~32 kDa protein (p32), normally associated with the cellular splicing factor SF2/ASF (Krainer et al., 1991), was identified as a Rev-interacting factor by the yeast-two hybrid selection method (Luo et al., 1994). A functional significance of this interaction was proposed by *in vivo* studies where a transient over-expression of YL2 lead to a potentiation of Rev activity while antisense YL2 transcripts reduced it. In addition, the p32-Rev binding was confirmed by *in vitro* coprecipitation experiments and p32 modulated the inhibitory effect of Rev on *in vitro* splicing (Tange et al., 1996). SF2/ASF itself has also been shown to interact with the RRE *in vitro* in a Rev-dependent manner (Powell et al., 1997), raising the possibility that the p32/SF2/ASF complex may bind co-operatively to the Rev/RRE complex. Therefore, it has been proposed that the interaction of Rev with proteins involved in the splicing process, such as p32, may participate in the removal of splicing factors from intron-containing RNAs and that the arrested complex may subsequently function as a substrate for Rev-mediated nuclear export.

An additional proposed cofactor engaged in the Rev transactivation pathway is the RNA helicase A. It was originally identified as a protein binding to the CTE of type D retroviruses (Tang et al., 1997), and subsequently observed also to bind RRE independently of Rev *in vivo* (Li et al., 1999). In experiments with RRE-containing reporter genes an over-expression of the RNA helicase A lead to increased Rev-mediated expression, as well as HIV replication, suggesting a regulatory role of this protein at the post-transcriptional level. RNA helicase has been implicated in the release of mature mRNA from the spliceosomes (Ohno and Shimura, 1996) and contains a bi-directional NSS, using a Crm1-independent export pathway (Tang et al., 1999). Therefore, it was proposed that RNA helicase A acts at a step prior to nuclear export in the Rev transactivation pathway, supporting the release of premature unspliced or incompletely spliced viral mRNAs from the splicing machinery (Li et al., 1999).

Although the involvement of Crm1 in nuclear export as a receptor for NES-bearing proteins is now well documented, the mechanism of translocation of the RRE-Rev-Crm1-Ran complex through the NPC is still unknown. As mentioned before, the NPC consist of different nucleoporins, which are often characterized by domains that contain numerous repeats of the di-peptide phenylalanine-glycine (FG-repeat domain) (Davis, 1995; Doye and Hurt, 1997). Many FG-repeat containing nucleoporins have been shown to interact with Rev and have been proposed to support its nucleocytoplasmic trafficking. The first nucleoporin identified interacting with the Rev NES was isolated by two independent groups using the yeast two-hybrid screening; it was termed

hRip/Rab (Rev interacting protein/Rev activation domain-binding protein (Bogerd et al., 1995; Fritz et al., 1995). Rip/Rab has been shown to be an inessential protein in S. cerevisiae, to exhibit nucleoplasmic as well as NPC localization and to modestly enhance Rev function when over-expressed in transfected cells (Bogerd et al., 1995; Fritz et al., 1995). In the beginning, Rip/Rab was proposed to be the export cofactor for Rev NES. However, recent studies convincingly demonstrated that the export receptor for the leucine-rich NES is Crm1, and that this protein probably mediates the interaction observed between Rev with Rip/Rab and with other Rev-interacting nucleoporins (Neville et al., 1997). Rev and/or Rev NES also interacted by two-hybrid analysis with the FG-repeat regions of a number of additional distinct yeast and vertebrate nucleoporins (Farjot et al., 1999; Fritz and Green, 1996; Stutz et al., 1996; Stutz et al., 1995). Factors that interact with Rev are summarized in Table 1.3.

1.4. Aim of the work

As mentioned above, the binding of the Rev/RRE complex to cellular cofactors is assumed to be essential for Rev function. Although several cellular proteins have been reported to interact with Rev, their actual role in supporting Rev function and virus replication is still unknown. Only recently, it started to become clear that importin β and Crm1 are directly involved in the Rev nuclear transport. However, several aspects involved in the complexity of the multiple functions of Rev still have to be clarified. For instance, it is interesting to note that the Rev protein may behave differently in certain cell types. In fact, some studies, carried out in the group directed by Dr. Brack-Werner, showed that astrocytes constitute the first example of a human cell type with an impaired Rev-response (Brack-Werner, 1999; Ludwig, 1999; Ludwig et al., 1999; Neumann et al., 1995). Astrocytes, well-known long-lived cells that perform crucial functions in the central nervous system, are known to be target cells for non-productive HIV persistence. The failure of the intracellular milieu of astrocytes to support function of Rev, which may be a fundamental mechanism underlying the astrocyte-specific restriction of virus production, could be due to either lack of necessary positive cellular cofactors, or to the presence of negative cellular cofactors capable of repressing Rev/RRE regulation.

This astrocyte behaviour strongly supports the idea that the function of Rev and, consequently, the latent infection may be controlled by still unknown cellular mechanisms.

Based on these considerations, the aim of my work was to study the role of Rev-interacting cellular proteins and signals in Rev in controlling the function of Rev in the host cell. To this end, I pursued the following goals:

- Identification of new factors interacting with HIV-1 Rev protein by yeast two-hybrid screening;
- analysis of their expression and intracellular localization in several human cells including astrocytes;
- analysis of their role and influence on Rev function and virus production;
- analysis of the presence of possible new functional signals in the Rev sequence.

2. Materials and Methods

2.1. List of abbreviations and Glossary

A	Adenine, or adenosine or alanine
AA = aa	Amino acid(s)
Ab	Antibody
AB/AM	antibiotic-antimycotic solution
Aqua bidest.	Water double deionized filtered trough Millipore filters
Aqua dest.	Water deionized
Ag	Antigen
AIDS	Acquired immunodeficiency syndrome
Ala = A	Alanine
AMP	Adenosine monophosphate
APS	Ammonium peroxidisulfate
Arg = R	Arginine
Asn = N	Asparagine
Asp = D	Aspartic acid
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BBB	Blood brain barrier
bp	Base pair(s)
BSA	Bovine serum albumine
B42	Bacterial transcription factor
C	Cytosine, or Cytidine or cysteine
°C	Degree Celsius
CAT	Chloramphenicol acetyltransferase
CD	Cluster of differentiation
cDNA	Complementary DNA, synthetic DNA transcribed from a specific RNA through the action of the reverse transcriptase
cfu	Colony forming units
Ci	Curie, radioactive unit
CM	Complete minimal dropout medium for yeast
CMV	Cytomegalovirus
CNS	Central nervous system
cpm	counts per minute
CTP	Cytidine triphosphate
CX	Cycloheximide
Cy 3/5	Fluorochrom-conjugate
Cys = C	Cysteine
dATP	Deoxyadenosine triphosphate
D	Aspartic acid
DMEM	Dulbecco's Modified Eagle Medium
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dCTP	Deoxycytosine triphosphate
DEPC	Diethyl pyrocarbonate
dGTP	Deoxyguanosine triphosphate
DMSO	Dimethyl sulfoxid
dNTP	Deoxynucleotide triphosphate
DTAF	Dichlorotriazinyl amino fluorescein
dTTP	Deoxythymidine triphosphate
DTT	Dithiothreitol

E	Glutamic acid
E.coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
EGY48	Yeast strain
ELISA	Enzyme linked immunosorbent assay
ER	Endoplasmatic reticulum
EtBr	Ethidium bromide
EtOH	Ethanol
FCS	Fetal calf serum
g	Gram
g	Relative centrifugal acceleration
G	Guanine, or guanosine or glycine
Gal	Galactose
GAL1	Yeast promoter
Gal4	Yeast transcription factor
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
Glc	Glucose = dextrose
Gln = Q	Glutamine
Glu = E	Glutamic acid
Gly = G	Glycine
GTP	Guanosine triphosphate
h	Hour(s)
HEPES	N-(2-Hydroxyethyl)piperazine-N'-2-ethan sulphuric acid
His = H	Histidine
HIV	Human immunodeficiency virus
HTLV	Human T-cell lymphotropic virus
Ile = I	Isoleucine
IN	Integrase
Insert	DNA fragment that has been inserted into a vector plasmid
IPTG	Isopropyl-ß-D-thio-galactopyranoside
KAc	Potassium acetate
kb	Kilobase(s)
KCl	Potassium chloride
kDa	Kilodalton
KH_2P0_4	Potassium dihydrogenphosphate
l	Liter
LB	Luria-Bertani bacterial medium
Leu = L	Leucine
LexA	Bacterial transcription factor
LMB	Leptomycin B
LTR	Long terminal repeat
Lys = K	Lysine
M	Molar
ml	Milliliter
µl	Microliter
Met = M	Methionine
$MgCl_2$	Magnesium chloride
$MgS0_4$	Magnesium sulfate
min	Minute(s)
mix	Mixture
MOPS	4-Morpholine-propanesulfonic acid
mRNA	Messenger RNA
MW	Molecular weight
N	Asparagine

NaAc	Sodium acetate
NaH_2P0_4	Sodium dihydrogenphosphate
Na_2HP0_4	Disodium hydrogenphosphate
NES	Nuclear export signal
$(NH_4)_2S0_4$	Ammonium sulfate
NLS	Nuclear localization signal
NPC	Nuclear pore complex
NSS	Nucleocytoplasmic shuttling signal
Nt	Nucleotide
OD	Optical density
O/N	Over night
ORF	Open reading frame, section of DNA sequence that begins with a start codon and ends with a stop codon
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
Pen/Strep	Penicillin/Streptomycin
Phe = F	Phenylalanine
PIC	Preintegration complex
PR	Protease
Pro = P	Proline
R	Arginine
Raff	Raffinose
RFB	Reilly-Finkel-Biskis murine leukemia virus
RISP	Rev interacting shuttle protein
RNase	Ribonuclease
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RRE	Rev response element
RSV	Rous sarcoma virus
RT	Room temperature
RT	Reverse transcription, or reverse transcriptase
RT-PCR	Method for amplifying cDNA from an RNA template
SDS	Sodium dodecyl sulfate
sec	Second(s)
Ser = S	Serine
SSC	Saline-sodium citrate buffer
SSPE	Saline sodium phosphate buffer
T	Thymine, or thymidine or threonine
TAE	Tris-acetate-EDTA buffer
Taq	Thermus aquaticus (-Polymerase)
TE	Tris-EDTA buffer
TEMED	N,N,N',N'-Tetramethylethylendiamine
Thr = T	Threonine
TLC	Thin layer chromatography
TNE	Tris-NaCl-EDTA buffer
Trp = W	Tryptophan
Tris	Trishydroxyaminomethan
tRNA	Transfer RNA
TTP	Thymidine triphosphate
Tyr = Y	Tyrosine
u	Unit
U	Uracil, or uridine
UV	Ultraviolet light

Val = V	Valine
Vector	Plasmid or phage chromosome used to carry cloned DNA segment
Vol	Volume
v/v	Volume:volume ratio
W	Tryptophan
WB	Western blot
wt	Wild type
w/v	Weight:volume ratio
X-gal	5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside
Y	Tyrosine
YNB-AA/AS	Yeast nitrogen base (without amino acids and ammonium sulfate)
YPD	Yeast extract, peptone, dextrose

2.2 Materials

2.2.1. Chemicals, kits and materials

1-kb-DNA molecular weight marker	Life Technologies, Eggenstein
Acetyl-CoA	Roche Molecular Biochemicals, Mannheim
Acrylamide/Bis solution	BioRad, Munich
Actinomycin D	Sigma, Deisenhofen
Agar (Bacto-agar)	Difco, Detroit MI, USA
Agarose (SeaKem)	FMC Bio Products, Rockland USA
Amino acids (yeast two hybrid)	Sigma, Deisenhofen
Ampicillin	Sigma, Deisenhofen
Ampuwa® H_20	Fresenius, Bad Homburg
Antibiotic-antimicotic solution	Life Technologies, Eggenstein
α[32P]dCTP [#PB10205, 3000Ci/mmol]	Amersham Buchler, Braunschweig
Aprotinine	Sigma, Deisenhofen
Aqua bidest.	Millipore, Eschborn
Alexa™ 488 protein labeling kit	Molecular Probes, Oregon, USA
Alexa™ 568 protein labeling kit	Molecular Probes, Oregon, USA
BCA Protein Assay Reagent Kit	Pierce, Rockford, USA
Bio-Spin® chromatography columns	BioRad, Munich
β−Mercaptoethanol	Sigma, Deisenhofen
Bromophenol blue	Merck, Darmstadt
Bovine serum albumine	Sigma, Deisenhofen
Calf instestinal alkaline phosphatase	Roche Molecular Biochemicals, Mannheim
CAT Elisa	Roche Molecular Biochemicals, Mannheim
Cell count chamber MD Kova Raster 10	Madaus Diagnostik, Munich
Cell culture medium DMEM with Glutamax	Life Technologies, Eggenstein
Cell culture medium MEM-D-Val	Life Technologies, Eggenstein
Cycloheximide	Sigma, Deisenhofen
Cellphect Transfection Kit	Pharmacia Biotech, Freiburg
Coomassie-Brilliant-Blue R-250	Serva, Heidelberg
Chloroform	Merck, Darmstadt
DEPC	Sigma, Deisenhofen
Dextrose extra pure	Sigma, Deisenhofen
DMSO	Sigma, Deisenhofen
DNA (type III sodium salt form salmon testes)	Sigma, Deisenhofen
DNA Ligase buffer 5X	Life Technologies, Eggenstein
DNAse	Sigma, Deisenhofen
dNTPs	Pharmacia Biotech, Freiburg
DTT	Sigma, Deisenhofen
Enhanced chemiluminescence (ECL) assay	Amersham Buchler, Braunschweig

EDTA	Sigma, Deisenhofen
Electroporation cuvettes	BioRad, Munich
Ethanol	Merck, Darmstadt
Ethidium bromide	Serva, Heidelberg
Ethyl acetate	Merck, Darmstadt
Expand™ High Fidelity PCR System	Roche Molecular Biochemicals, Mannheim
Fast CAT® Green (deoxy) CAT kit	Molecular Probes, Oregon, USA
Filter 0.22µm	Nalgene, USA
Filterpaper, Whatman 3mm	Whatman, Maidstone, GB
Filterpaper, extra tick 7.5x10cm	BioRad, Munich
Fetal calf serum (FCS)	Life Technologies, Eggenstein
"	Seromed, Berlin
Formaldehyde 37%	Sigma, Deisenhofen
Formamide	Merck, Darmstadt
Fugene™ 6 Transfection reagent	Roche Molecular Biochemicals, Mannheim
Galactose	Sigma, Deisenhofen
Gentamycin	Life Technologies, Eggenstein
Glass beads (425-600 µ #G8772)	Sigma, Deisenhofen
Glutathione Sepharose™ 4B	Pharmacia Biotech, Freiburg
Glycerol	Merck, Darmstadt
Glyoxal	Sigma, Deisenhofen
Kaleidoscope prestained standard	BioRad, Munich
HIV-1 p24 Ag Assay	Coulter Immunotech, Krefeld
Hoechst 33342 (Bisbenzimide)	Molecular Probes, Oregon, USA
IPTG	Roche Molecular Biochemicals, Mannheim
Isopropanol	Merck, Darmstadt
Leptomycin B	Provided by Dr. Mattaj, Heidelberg
Leupeptine	Sigma, Deisenhofen
Lithium acetate	Sigma, Deisenhofen
L-Glutamine (cell culture)	Life Technologies, Eggenstein
Lysozyme	Sigma, Deisenhofen
Luciferase Assay System	Promega, Madison, USA
Methanol	Merck, Darmstadt
Methylene blue	Merck, Darmstadt
MOPS	Merck, Darmstadt
Nitrocellulose membrane	BioRad, Munich
Nylon filter (Hybond)	Amersham
NucleoSpin PCR purification kit	Macherey-Nagel, Oensingen, Schweiz
Paraformaldehyde	Sigma, Deisenhofen
Pepstatine	Sigma, Deisenhofen
Polyethylenglycol (PEG) 4000	Baker, USA, and Merck, Darmstadt
Pen/Strep	Life Technologies, Eggenstein
Phenol	Life Technologies, Eggenstein
Phenol/Chloroform	Applied Biosystems, Weiterstadt
Plastic material for cell culture	Greiner, Labortechnik, Frickenhausen
PMSF	Sigma, Deisenhofen
Polaroid s/w Film 667	Polaroid, Offenbach
Proteinase K	Roche Molecular Biochemicals, Mannheim
Qiagen® Plasmid Maxi Kit	Qiagen, Hilden
Qiagen® RNEasy Mini Kit	Qiagen, Hilden
Raffinose	Difco, Detroit MI, USA
Rainbow-SDS-Protein standard	Promega, Madison, USA
Rapid DNA Ligation Kit	Life Technologies, Eggenstein
Rediprime™ II random prime labeling system	Pharmacia Biotech, Freiburg
Restriction enzymes	Roche Molecular Biochemicals, Mannheim

“	NewEngland Biolabs, Schwalbach
SDS	Löwe Biochemika, Munich
“	BioRad, Munich
SOC medium	Life Technologies, Eggenstein
T4 DNA Ligase	Life Technologies, Eggenstein
TEMED	BioRad, Munich
Thiamine (vitamine B1)	Sigma, Deisenhofen
TLC plastic sheets Silica gel 60	Merck, Darmstadt
Topo TA cloning® kit	Invitrogen, De Shelp, Netherlands
Tris	Löwe Biochemika, Munich
Triton® X-100	Sigma, Deisenhofen
Trypan blue	Merck, Darmstadt
Trypsin-EDTA solution	Biochrom, Berlin
Tween® 20	Sigma, Deisenhofen
UlTRAhyb™ Hybridization Solution	Ambion, Austin, USA
Uracil	Sigma, Deisenhofen
Yeast extract (Bacto-yeast extract)	Difco, Detroit MI, USA
Yeast nitrogen base (YNB-AA/AS)	Life Technologies, Eggenstein
X-gal	Löwe Biochemika, Munich
Zetaprobe® GT Nylon membrane	BioRad, Munich

2.2.2. Bacteria *E.coli*

· BL21	Pharmacia Biotech, Freiburg
· XL1-blue supercompetent cells	Stratagene, Heidelberg
· KC8	Provided by Dr. W. Kolanus, Munich
· TOP10F’One Shot competent cells	Invitrogen, De Shelp, Netherlands

2.2.3. Yeast cells

· EGY48 (Gyuris et al., 1993)	Provided by Dr. W. Kolanus, Munich

2.2.4. Human cells

· HeLa:
Human cervical adenocarcinoma cell line with epithelial morphology [American Type Culture Collection (ATCC) CRL-7923, Rockville, Maryland]

· HEK293:
Human embryonal kidney cell line (ATCC CRL-1573, Rockville, Maryland)

· Jurkat T cells:
T-cell line (Provided by Dr. W. Kolanus, Genzentrum, Munich)

· 85HG66:
Human CD4-negative astrocytoma cell line described in (Brack-Werner et al., 1992; Stavrou et al., 1987)

· U87MG (U87):
Human astrocytoma-/glioblastoma cell line (ATCC HTB-14, Rockville, Maryland)

· U138MG (U138):
Human glioblastoma cell line (ATCC HTB-16, Rockville, Maryland)

· Primary fetal astrocytes (H4/96):
Human astrocytes prepared from myelencephalon-mesencephalon of a 9-week-old fetus as described in (Aloisi et al., 1992) provided by Dr. F. Aloisi, ISS, Rome, Italy.

2.2.5. Antibodies

The following antibodies were used for indirect immunofluorescence and/or western blot analysis:

· Mouse monoclonal anti-GST, IgG1 (Biozol)
· Mouse monoclonal anti-HIV-1Tat, IgG1a (Dr. J. Karn, MRC AIDS)
· Rabbit polyclonal anti-GFP serum (Dr. George Pavlakis, NCI, Maryland, USA)
· Rabbit polyclonal anti-HIV-1Tat, IgG (Dr. Barbara Ensoli, ISS, Rome, Italy)
· Rabbit polyclonal anti-HIV-1 Rev (#29994 T.B.I.) (Dr. George Pavlakis, NCI, Maryland, USA)
· Rat monoclonal anti-Risp, IgG2a (5D3, 5D4, 8B6, 8B8) and IgG1 (6F11, 6F12, 7B4, 8A11) (Dr. Elisabeth Kremmer, GSF, Munich)
· Rat monoclonal anti-GST, IgG1 (Dr. Elisabeth Kremmer, GSF, Munich)
· Cy3-conjugated goat anti-human IgG (Dianova)
· Cy3-conjugated goat anti-rabbit IgG + IgM (Dianova)
· Fluorescein (DTAF)-conjugated goat anti-rat IgG + IgM (Dianova)
· Peroxidase-conjugated goat anti-mouse IgG + IgM (Dianova)
· Peroxidase-conjugated goat anti-rabbit IgG + IgM (Dianova)
· Peroxidase-conjugated goat anti-rat IgG + IgM (Dianova)

2.2.6. Plasmids

The following mammalian expression plasmids were used for human cell transfections, the yeast and *E.coli* shuttle plasmids were used in the yeast two hybrid search and the prokaryotic pGEX-3X expression plasmid was used to produce GST-fusion proteins (for an overview see Fig. 2.1). To check whether the insertion of the interested gene was correct, all plasmids constructed for this work were sequenced (Sequiserve, Munich).

2.2.6.1. Mammalian expression plasmids:

2.2.6.1.1. HIV-1 regulatory gene-expression plasmids

· pBsRev

Bluescript vector pBSPL that expresses the HXB2- *rev* gene with synthetic exon 1 under the control of the HIV-1 LTR promoter (Neumann et al., 1995), (provided by Dr. G. Pavlakis, NCI, Maryland, USA).

· pBsRevM10BL

Bluescript vector pBSPL that expresses the HXB2-synthetic *rev* gene containing the M10 mutation in the activation domain at the amino acids position 78-79 LE—> DL (Malim et al., 1989) and an additional mutation BL resulting in the addition of 4 amino acids EDLP between position 82-83 (Stauber et al., 1995), (provided by Dr. G. Pavlakis, NCI, Maryland, USA).

· pcRev

pBC12/CMV vector that expresses the HXB3-*rev* gene (Bogerd et al., 1995; Malim et al., 1988), (provided by Dr. B. Cullen, Durham, USA).

· pcRevM5

pBC12/CMV vector that expresses the M5 mutation in the NLS/RNA-binding domain of the HXB3-Rev at the amino acids position 38-39 RR—> DL (Malim et al., 1989) (Thomas et al., 1998), (provided by Dr. B. Cullen, Durham, USA).

· pcRevM5-sg143

pFred143 vector (see 2.2.6.1.4.) that expresses the fusion protein RevM5-GFP under the control of the CMV promoter. The RevM5-ORF starting from the second codon of pcRevM5 was PCR amplified (5' primer 23957 and 3' primer 23958) and fused in frame to an enhanced fluorescence mutant of GFP into the pFred143 vector (see later). The *revM5* gene was inserted into a unique NheI restriction site immediately downstream of the ATG start codon of GFP. A Gly-Ala-Gly hinge region was inserted between the last Rev-codon and the following GFP-ORF to allow correct folding of both protein domains (F. Ceccherini-Silberstein, GSF, Neuherberg).

· pCsRev-sg143

pFred143 vector that expresses the fusion protein Rev-GFP under the control of the CMV promoter. The Rev-ORF starting from the second codon of pBsRev was PCR amplified and was inserted into the unique NheI restriction site immediately downstream of the ATG start codon of GFP. A Gly-Ala-Gly hinge region was inserted between the last Rev-codon and the following GFP-ORF to allow correct folding of both protein domains (Ludwig et al., 1999), (Dr. M. Neumann, GSF, Neuherberg).

· pcTat

pBC12/CMV vector that expresses the HXB3-*tat* gene (Malim et al., 1988), (provided by Dr. B. Cullen, Durham, USA).

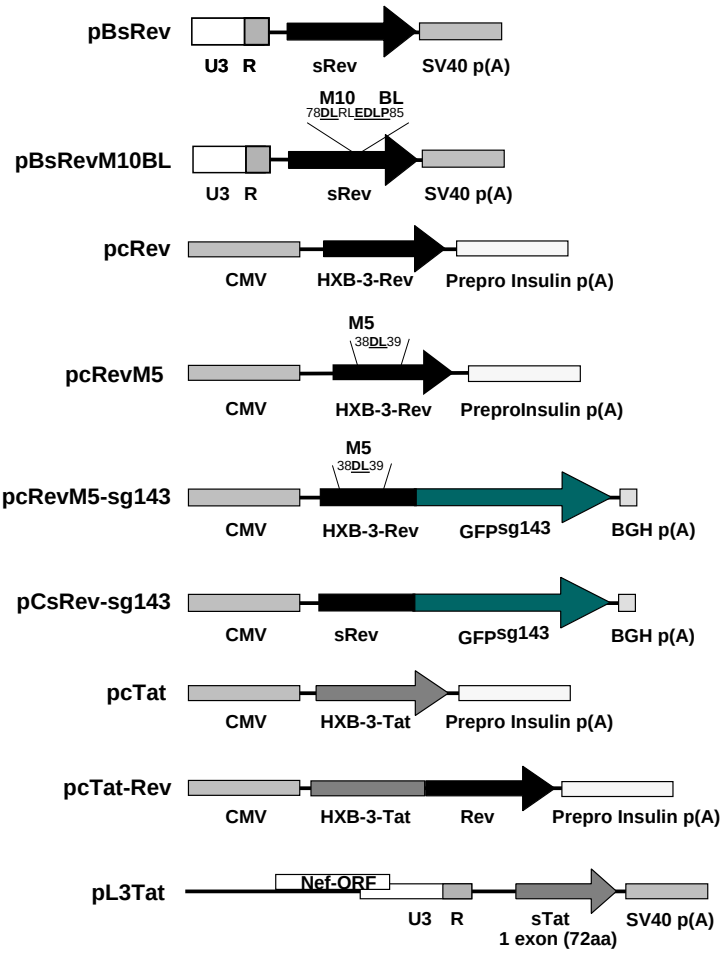
· pcTat-Rev

pBC12/CMV vector that expresses a fusion protein consisting of the full length Tat protein linked to the first amino acid of the Rev ORF (Tiley et al., 1992), (provided by Dr. B. Cullen, Durham, USA).

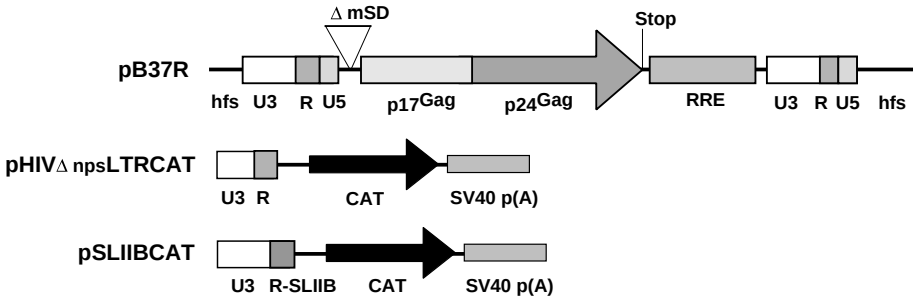
Fig. 2.1 Schematic overview of expression plasmids used for transient transfection assays. The transcription of HIV-1 derived genes was directed either by the HIV-LTR (U3/R region) or the immediate early CMV promoter. pB37RGag contains *gag* encoding sequences (p17 and p24) and the complete RRE (330bp *Sty*I fragment) and lacks the major splice donor site (mSD) of HIV-1, (hfs= human flanking sequences). pHIVΔnpsLTRCAT contains partial Nef sequences upstream of the LTR (nps = nef partial sequence). The transcription of GFP and human-derived genes was directed by the CMV promoter. Transcription of the luciferase gene was directed either by the LTR of the Rous Sarcoma virus (pRSVluc) or from the murine leukemia virus-related RFB-virus (pGL3-RFB). Transcription of LexA- or B42AD-fusion genes was directed by the yeast ADH1 or the inducible GAL1 promoter respectively.
Poly (A) signals (p(A)) for transcription termination were derived from SV40, bovine growth hormone (BGH), ADH1, or insulin genes. For further details on plasmids including references, see the text.

A. HIV-1 expression plasmids

(a) Rev- and Tat- expression plasmids

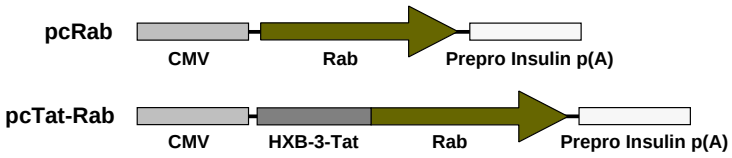


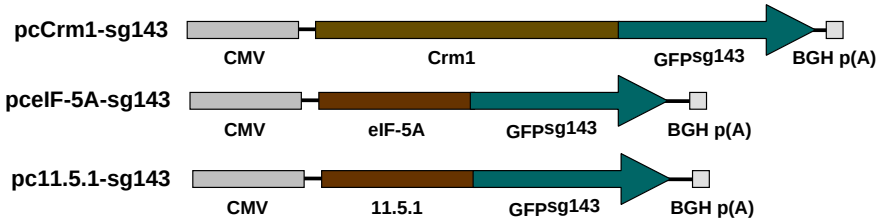
(b) Rev- and Tat-response plasmids



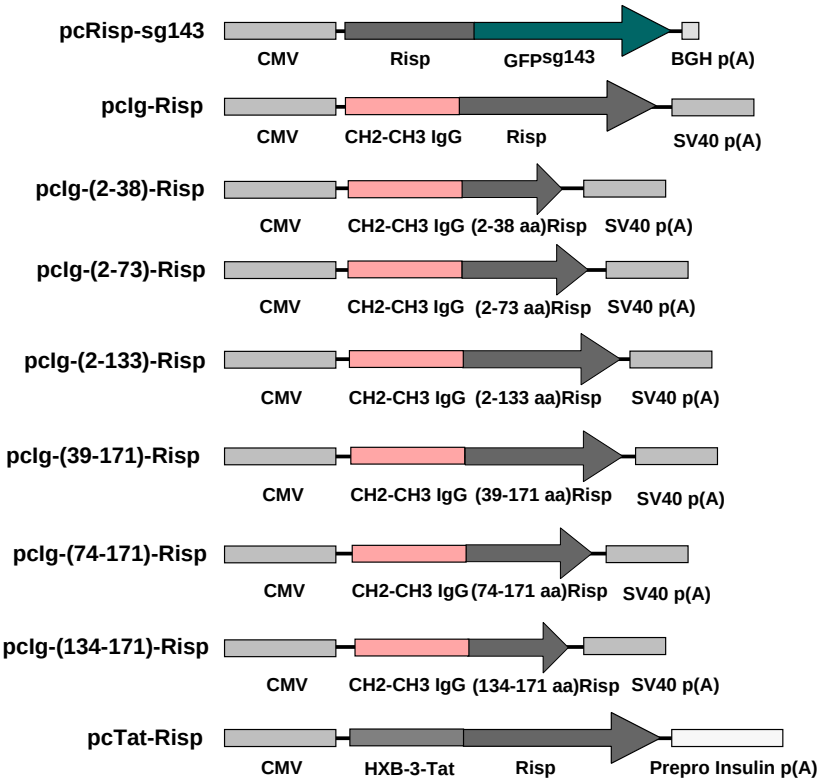
B. Rev-interacting cellular factors expression plasmids

(a) Known cellular factors expression plasmids

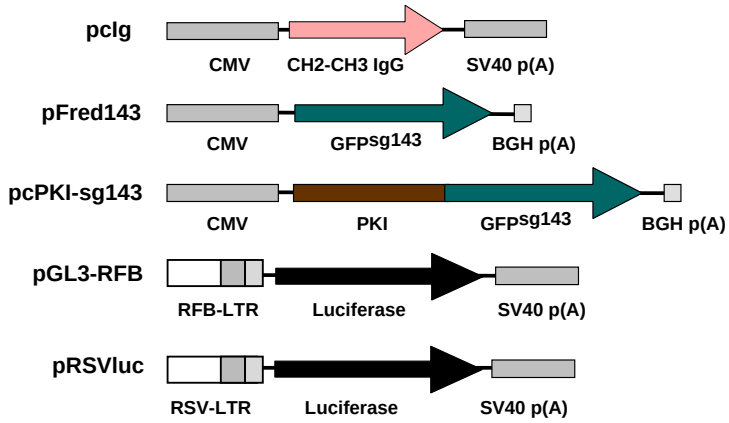




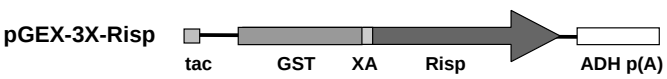
(b) Risp related expression plasmids



C. HIV-unrelated expression plasmids

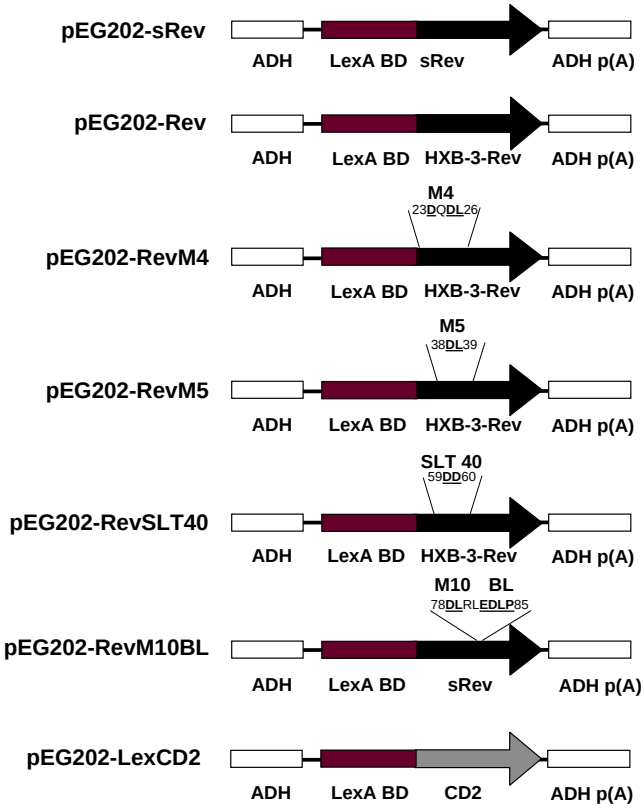


D. Bacterial expression plasmids

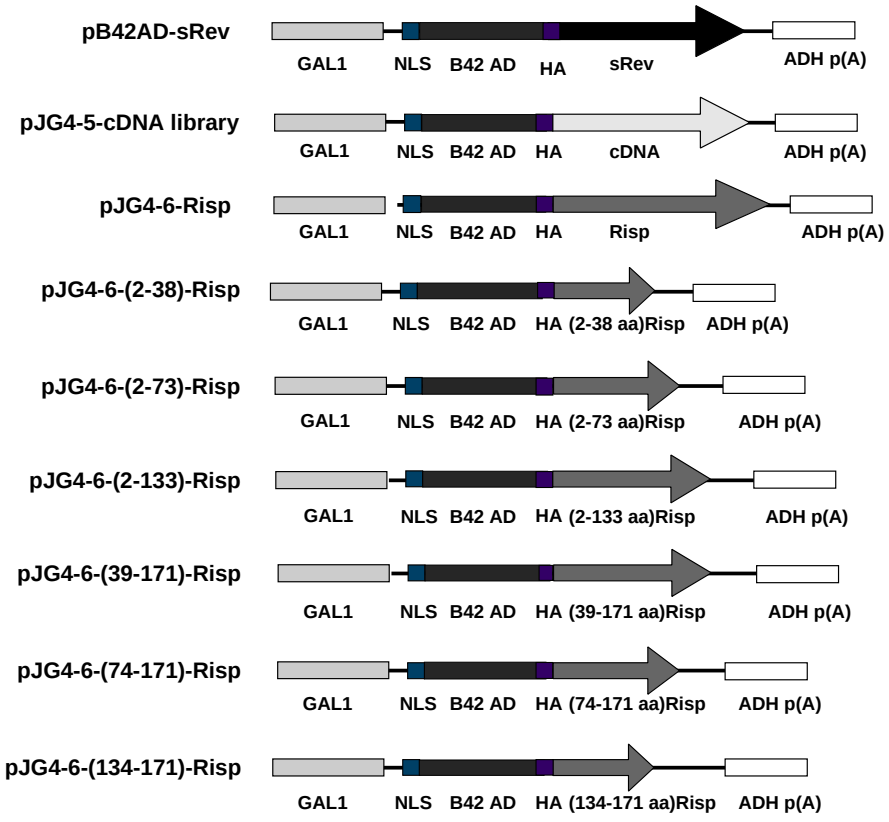


E. Yeast two hybrid expression plasmids

(a) Bait-expression plasmids



(b) Prey-expression plasmids



· pL3Tat

pBR322 vector that expresses the HXB2-*tat* gene (1 exon) under the control of the HIV-1 LTR promoter (Felber et al., 1990; Neumann et al., 1995), (provided by Dr. G. Pavlakis, NCI, Maryland, USA)

2.2.6.1.2. HIV-1 Rev- and Tat-response plasmids

· pB37R

Rev response plasmid that contains HXB2-Gag encoding sequences (p17 and p24) and the complete RRE (330bp *Sty*I fragment) and lacks the major splice donor site (mSD) of HIV-1. The expression of the plasmid is under the control of the HIV-1 LTR promoter (therefore it is Rev and Tat dependent) (Ludwig et al., 1999; Neumann et al., 1995), (provided by Dr. G. Pavlakis, NCI, Maryland, USA).

· pHIVΔnpsLTRCAT

Tat response plasmid that directs expression of the chloramphenicol acetyltransferase (CAT) reporter gene from the HIV-1_{BRU} LTR in a pBR322-derived vector (pSB1). Nef sequences upstream of the LTR were deleted (nps = nef partial sequence) (Ludvigsen et al., 1996), (Dr. A. Ludvigsen, GSF, Neuherberg).

· pSLIIBCAT

Tat-Rev response plasmid that directs expression of the CAT reporter gene from a modified HIV-1 LTR. The TAR RNA stem-loop in HIV-1 LTR linked to CAT is replaced with the RRE-derived stem loop IIB (SLIIB), the minimal RNA target sequence for HIV-1 Rev (Bogerd et al., 1995; Tiley et al., 1992), (provided by Dr. B. Cullen, Durham, USA).

2.2.6.1.3. Expression plasmids for Rev-interacting cellular factors

· pcRab

pBC12/CMV vector that expresses the human *rab* gene (Bogerd et al., 1995), (provided by Dr. B. Cullen, Durham, USA).

· pcTat-Rab

pBC12/CMV vector that expresses a fusion protein consisting of the full length Tat protein linked to the first amino acid of the Rab ORF (Bogerd et al., 1995).

The NcoI/HindIII fragment containing the full-lenght *rev* gene was replaced with a NcoI/HindIII PCR product (5' primer 17355 and 3' primer 17356) containing the full-lenght *rab* gene into the pcTat-Rev plasmid (F. Ceccherini-Silberstein, GSF, Neuherberg).

· pcCrm1-sg143

pFred143 vector that expresses the human *crm1* gene fused in frame to the enhanced fluorescence mutant of GFP under the control of the CMV promoter (Dr. M. Neumann, GSF, Neuherberg).

· pceIF-5A-sg143

pFred143 vector that expresses the human *eIF-5A* gene fused in frame to the enhanced fluorescence mutant of GFP under the control of the CMV promoter (Dr. M. Neumann, GSF, Neuherberg).

· pc11.5.1-sg143

pFred143 vector that expresses the 11.5.1 cDNA (one of the two new yeast two hybrid positive Rev-interacting clones isolated in this study) fused in frame to the enhanced fluorescence mutant of GFP under the control of the CMV promoter. The 11.5.1 -ORF starting from the second codon was PCR amplified from the pJG4-5-11.5.1 plasmid (5' primer 15759 and 3' primer 15760) and was inserted into the unique NheI restriction site. A Gly-Ala-Gly hinge region was inserted between the last 11.5.1-codon and the following GFP-ORF to allow correct folding of both protein domains (F. Ceccherini-Silberstein, GSF, Neuherberg).

2.2.6.1.3.1. Risp (= 16.4.1 artificial protein) expression plasmids

· pcRisp-sg143

pFred143 vector that expresses the *risp* gene fused in frame to the enhanced fluorescence mutant of GFP under the control of the CMV promoter. The *risp* gene starting from the second codon was PCR amplified from the pJG4-5-16.4.1 plasmid using (5' primer 16552 and 3' primer 15762). The Risp PCR product contains few additional nucleotides to include the "missing N-terminal part" (8 amino acids) of the EST 343275 5' clone (Fig. 3.9). The Risp PCR product was inserted into the unique NheI restriction site and a Gly-Ala-Gly hinge region was inserted between the last Risp-codon and the following GFP-ORF to allow correct folding of both protein domains (F. Ceccherini-Silberstein, GSF, Neuherberg).

· pcIg-Risp

pRK5/CMV derived vector containing the 78g78 multi cloning sites (Zeitlmann et al., 1998) that expresses the *risp* gene fused in frame to the C-terminus of the CH2 and CH3 heavy chain domains from human IgG_1(see 2.2.6.1.4.). The Risp-ORF starting from the second codon was PCR amplified from the pcRisp-sg143 plasmid (5' primer 18733 and 3' primer 18510) and was inserted into the pcIg-Sam68 plasmid (provided by Dr. W. Kolanus, Genzentrum, Munich) using the MluI/NotI cloning site after the removal of the MluI/NotI Sam68 insert (F. Ceccherini-Silberstein, GSF, Neuherberg).

· pcIg-(2-38)-Risp

pRK5/CMV derived vector that expresses the N-37 amino acids of Risp fused in frame to the CH2 and CH3 IgG_1 domains. A 121 nucleotide MluI/NotI fragment containing a TAG stop codon was PCR amplified from the pcRisp-sg143 plasmid (5' primer 18733 and 3' primer 19538) and was inserted into the pcIg-Sam68 plasmid using the MluI/NotI cloning site (F. Ceccherini-Silberstein, GSF, Neuherberg).

· pcIg-(2-73)-Risp

pRK5/CMV derived vector that expresses the N-72 amino acids of Risp fused in frame to the CH2 and CH3 IgG_1 domains. A 226 nucleotide MluI/NotI fragment containing a TAG stop codon was PCR amplified from the pcRisp-sg143 plasmid (5' primer 18733 and 3' primer 19539) and was inserted into the pcIg-Sam68 plasmid using the MluI/NotI cloning site (F. Ceccherini-Silberstein, GSF, Neuherberg).

· pcIg-(2-133)-Risp

pRK5/CMV derived vector that expresses the N-132 amino acids of Risp fused in frame to the CH2 and CH3 IgG_1 domains. A 406 nucleotide MluI/NotI fragment

containing a TAG stop codon was PCR amplified from the pcRisp-sg143 plasmid (5' primer 18733 and 3' primer 19540) and was inserted into the pcIg-Sam68 plasmid using the MluI/NotI cloning site (F. Ceccherini-Silberstein, GSF, Neuherberg).

· pcIg-(39-171)-Risp

pRK5/CMV derived vector that expresses the C-133 amino acids of Risp fused in frame to the CH2 and CH3 IgG_1 domains. A 409 nucleotide MluI/NotI fragment containing a TAG stop codon was PCR amplified from the pcRisp-sg143 plasmid (5' primer 20817 and 3' primer 18510) and was inserted into the pcIg-Sam68 plasmid using the MluI/NotI cloning site (F. Ceccherini-Silberstein, GSF, Neuherberg).

· pcIg-(74-171)-Risp

pRK5/CMV derived vector that expresses the C-98 amino acids of Risp fused in frame to the CH2 and CH3 IgG_1 domains. A 304 nucleotide MluI/NotI fragment containing a TAG stop codon was PCR amplified from the pcRisp-sg143 plasmid (5' primer 20818 and 3' primer 18510) and was inserted into the pcIg-Sam68 plasmid using the MluI/NotI cloning site (F. Ceccherini-Silberstein, GSF, Neuherberg).

· pcIg-(134-171)-Risp

pRK5/CMV derived vector that expresses the C-38 amino acids of Risp fused in frame to the CH2 and CH3 IgG_1 domains. A 124 nucleotide MluI/NotI fragment containing a TAG stop codon was PCR amplified from the pcRisp-sg143 plasmid (5' primer 20819 and 3' primer 18510) and was inserted into the pcIg-Sam68 plasmid using the MluI/NotI cloning site (F. Ceccherini-Silberstein, GSF, Neuherberg).

· pcTat-Risp

pBC12/CMV vector that expresses a fusion protein consisting of the full length Tat protein linked to the first amino acid of the Risp ORF.

The NcoI/HindIII fragment containing the full-lenght *rev* gene was replaced with a NcoI/HindIII PCR product (5' primer 17357 and 3' primer 17358) containing the full-lenght *risp* gene into the pcTat-Rev plasmid (F. Ceccherini-Silberstein, GSF, Neuherberg).

2.2.6.1.4. HIV-unrelated expression plasmids

· pBSPL

Bluescript derived vector used as inert carrier plasmid to increase the quantity of DNA for transfections with calcium phosphate procedure (Schwartz et al., 1990), (provided by Dr. G. Pavlakis, NCI, Maryland, USA).

· pcIg

pRK5/CMV derived vector that expresses the constant CH2 and CH3 domain segments (768 bp) from a human IgG1 (provided by Dr. W. Kolanus, Genzentrum, Munich).

· pFred143 (KH1035)

Vector that directs the expression of an enhanced fluorescence mutant GFP under the control of the CMV immediate early promoter (Stauber et al., 1998), (Dr. M. Neumann, GSF, Neuherberg).

· pcPKI-sg143

Vector that directs the expression the human cyclic AMP dependent protein kinase inhibitor (*PKI*) gene (Wen et al., 1995) fused in frame to the enhanced fluorescence mutant of GFP under the control of the CMV promoter (Dr. M. Neumann, GSF, Neuherberg).

· pGL3-RFB

pGL3-basic vector that directs the expression of *luciferase* gene under the control of the C-type murine leukemia virus-related RFB-virus LTR promoter (Frech et al., 1997) (provided by Dr. T. Werner, AG BIODV, GSF, Neuherberg).

· pRSVluc

pBR322 vector that directs the expression of *luciferase* gene under the control of the C-type Rous sarcoma virus (RSV) LTR promoter (de Wet et al., 1987), (provided by Dr. G. Pavlakis, NCI, Maryland, USA).

2.2.6.2. Prokaryotic expression plasmids

· pGEX-3X

pGEX vector for inducible, high-level intracellular expression of genes as fusion with Schistosoma japonicum glutathione S-transferase (GST) under the control a *tac* promoter (inducible with IPTG 1-5mM). The vector includes an internal *lac* I^q gene for use in any *E. coli* host and a coding region for factor Xa protease recognition site for cleaving the desired protein from the fusion protein (Pharmacia Biotech).

· pGEX-3X-Risp

pGEX-3X vector that directs the inducible expression of *risp* gene fused in frame to GST-factor Xa. The Risp-ORF starting from the second codon was PCR amplified from the pcRisp-sg143 plasmid (5' primer 17746 and 3' primer 17747) and was inserted into the pGEX-3X vector using the BamHI/EcoRI cloning site (F. Ceccherini-Silberstein, GSF, Neuherberg).

· pCR® 2.1-Topo

Vector supplied linearized with single 3' thymidine (T) overhangs for TA cloning. It provides a highly efficient insertion of Taq polymerase-amplified PCR products with 3' A overhangs. The cloning site is situated into a laczα fragment (571bp) and is flanked by two EcoRI sites, which allow easy analysis of the plasmid by enzymatic digestion. The presence of any insert interrupts the *lacz* gene and allow a blue/white screening in the presence of X-gal (Invitrogen).

2.2.6.3. Yeast two hybrid expression plasmids

2.2.6.3.1. Bait-expression plasmids

· pEG202 LexA-fusion plasmid

Yeast and *E.coli* shuttle multicopy vector containing the yeast 2µm origin of replication and the HIS3 selectable marker gene, the full-length coding region (amino acids 1 to 202) of the bacterial DNA-binding protein LexA, flanked by the yeast strong constitutive alcohol dehydrogenase ADH1 promoter and terminator as well as a polylinker available for insertion of bait coding sequences. The plasmid contains also the ampicillin resistance gene (AmpR) and the pBR origin (ori) of replication to

allow propagation in *E.coli* (Golemis et al., 1999) (provided by Dr. W. Kolanus, Genzentrum, Munich).

· pEG202-sRev (sense and antisense)

TheHXB2-Rev-ORF starting from the second codon of pBsRev was PCR amplified and inserted in frame with LexA into the unique restriction site EcoRI into the pEG202 vector (in sense and antisense orientation), (Dr. A. Ludvigsen, GSF, Neuherberg).

· pEG202-Rev (sense)

The HXB3-Rev-ORF starting from the second codon of pcRev was PCR amplified (5' primer 19777 and 3' primer 19776) and inserted in frame with LexA into the unique restriction site EcoRI into the pEG202 vector (F. Ceccherini-Silberstein, GSF, Neuherberg).

· pEG202-RevM4 (sense and antisense)

Rev expression plasmid containing the M4 mutation in the I multimerization domain at the amino acids position 23-25-26 YSN—> DDL (Malim et al., 1989). The RevM4-ORF starting from the second codon of pcTat-RevM4 (Thomas et al., 1998) ((kindly provided by Dr. J. Hauber, University of Erlangen) was PCR amplified (5' primer 19777 and 3' primer 19776) and inserted in frame with LexA into the unique restriction site EcoRI into the pEG202 vector (in sense and antisense orientation), (F. Ceccherini-Silberstein, GSF, Neuherberg).

· pEG202-RevM5 (sense)

Rev expression plasmid containing the M5 mutation in the NLS/RNA-binding domain (Malim et al., 1989). The RevM5-ORF starting from the second codon of pcRevM5 was PCR amplified (5' primer 19777 and 3' primer 19776) and inserted in frame with LexA into the unique restriction site EcoRI into the pEG202 vector (in sense orientation), (F. Ceccherini-Silberstein, GSF, Neuherberg).

· pEG202-RevSLT40 (sense)

Rev expression plasmid containing the SLT40 mutation in the II multimerization domain at the amino acids position 59-60 IL—> DD (Thomas et al., 1998). The RevSLT40-ORF starting from the second codon of pcRevSLT40 (kindly provided by Dr. J. Hauber, University of Erlangen) was PCR amplified (5' primer 19777 and 3' primer 19776) and inserted in frame with LexA into the unique restriction site EcoRI into the pEG202 vector (in sense orientation), (F. Ceccherini-Silberstein, GSF, Neuherberg).

· pEG202-RevM10BL (sense and antisense)

Rev expression plasmid containing the M10BL mutant NES region of Rev. The RevM10BL-ORF starting from the second codon of pCsRevM10BL-sg143 was PCR amplified (5' primer 19777 and 3' primer 21116) and inserted in frame with LexA into the unique restriction site EcoRI into the pEG202 vector (in sense and antisense orientation) (F. Ceccherini-Silberstein and C. Bickel, GSF, Neuherberg).

· pEG202-LexCD2

Unspecific bait control plasmid expressing the cytoplasmic domain of CD2 fused in frame to LexA (provided by Dr. W. Kolanus, Genzentrum, Munich).

2.2.6.3.2. Prey-expression plasmids

· pJG4-5 library plasmid

Yeast and *E.coli* shuttle vector that contains a yeast expression cassette that includes sequences that encode 106 amino acids fusion moiety, consisting of the SV40 virus large T antigen NLS (PPKKKRKVA), the B42 transcription activation domain (88-residues) and the hemagglutinin (HA) epitope tag (YPYDVPDYA). Expression of the sequences is under the control of the GAL1 inducible yeast promoter and the terminator sequences from the yeast *ADH1* gene. The plasmid contains also the TRP1 selectable marker and the 2µm origin of replication to allow propagation in yeast, and the ampicillin resistance gene (AmpR) and the pUC origin (ori) of replication to allow propagation in *E.coli* (Golemis et al., 1999; Gyuris et al., 1993), (provided by Dr. W. Kolanus, Genzentrum, Munich).

· pJG4-5-cDNA library

pJG4-5 vector that expresses a Jurkat T cell cDNA library inserted into the unique EcoRI and XhoI sites so that encoded proteins are expressed fused in frame to the B42 transcription activation domain at their amino terminus (Kolanus et al., 1996) (provided by Dr. W. Kolanus, Genzentrum, Munich).

· pJG4-5-11.5.1

pJG4-5 vector that expresses the 11.5.1 Jurkat T cell cDNA encoded protein able to interact in the yeast two hybrid system with the HIV-1 Rev protein.

· pJG4-5-16.4.1

pJG4-5 vector that expresses the 16.4.1 Jurkat T cell cDNA encoded protein able to interact in the yeast two hybrid system with the HIV-1 Rev protein.

· pJG4-6

pJG4-5-derived vector with the original NotI restriction site removed and containing an insert in the EcoRI/XhoI cloning site with a MluI and NotI restriction site at the 5' and 3' extremity respectively. Vector used to express encoded proteins fused in frame to the B42 transcription activation domain at their amino terminus using the MluI/NotI cloning site (provided by Dr. W. Kolanus, Genzentrum, Munich).

· pJG4-6-Risp

pJG4-6 vector that expresses the full lenght of Risp fused in frame to the B42 transcription activation domain. The Risp-ORF starting from the second codon of pcRisp-sg143 plasmid was PCR amplified (5' primer 18733 and 3' primer 18510) and inserted into the pJG4-6 vector using the MluI/NotI cloning site (F. Ceccherini-Silberstein, GSF, Neuherberg).

· pJG4-6-(2-38)-Risp

pJG4-6 vector that expresses the N-37 amino acids of Risp fused in frame to the B42 transcription activation domain. A 121 nucleotide MluI/NotI fragment containing a TAG stop codon was PCR amplified from the pcRisp-sg143 plasmid (5' primer 18733 and 3' primer 19538) and was inserted into the pJG4-6 vector using the MluI/NotI cloning site (F. Ceccherini-Silberstein, GSF, Neuherberg).

· pJG4-6-(2-73)-Risp

pJG4-6 vector that expresses the N-72 amino acids of Risp fused in frame to the B42 transcription activation domain. A 226 nucleotide MluI/NotI fragment containing a

TAG stop codon was PCR amplified from the pcRisp-sg143 plasmid (5' primer 18733 and 3' primer 19539) and was inserted into the pJG4-6 vector using the MluI/NotI cloning site (F. Ceccherini-Silberstein, GSF, Neuherberg).

· pJG4-6-(2-133)-Risp

pJG4-6 vector that expresses the N-132 amino acids of Risp fused in frame to the B42 transcription activation domain. A 406 nucleotide MluI/NotI fragment containing a TAG stop codon was PCR amplified from the pcRisp-sg143 plasmid (5' primer 18733 and 3' primer 19540) and was inserted into the pJG4-6 vector using the MluI/NotI cloning site (F. Ceccherini-Silberstein, GSF, Neuherberg).

· pJG4-6-(39-171)-Risp

pJG4-6 vector that expresses the C-133 amino acids of Risp fused in frame to the B42 transcription activation domain. A 409 nucleotide MluI/NotI fragment containing a TAG stop codon was PCR amplified from the pcRisp-sg143 plasmid (5' primer 20817 and 3' primer 18510) and was inserted into the pJG4-6 vector using the MluI/NotI cloning site (F. Ceccherini-Silberstein, GSF, Neuherberg).

· pJG4-6-(74-171)-Risp

pJG4-6 vector that expresses the C-98 amino acids of Risp fused in frame to the B42 transcription activation domain. A 304 nucleotide MluI/NotI fragment containing a TAG stop codon was PCR amplified from the pcRisp-sg143 plasmid (5' primer 20818 and 3' primer 18510) and was inserted into the pJG4-6 vector using the MluI/NotI cloning site (F. Ceccherini-Silberstein, GSF, Neuherberg).

· pJG4-6-(134-171)-Risp

pJG4-6 vector that expresses the C-38 amino acids of Risp fused in frame to the B42 transcription activation domain. A 124 nucleotide MluI/NotI fragment containing a TAG stop codon was PCR amplified from the pcRisp-sg143 plasmid (5' primer 20819 and 3' primer 18510) and was inserted into the pJG4-6 vector using the MluI/NotI cloning site (F. Ceccherini-Silberstein, GSF, Neuherberg).

· pB42AD-sRev

The HXB2-Rev-ORF starting from the second codon of pBsRev was PCR amplified (5' primer 8726 and 3' primer 9585) and fused in frame to the B42 transcription activation domain using the unique restriction site EcoRI into the pB42AD vector (pJG4-5 derivate-vector, Clontech), (F. Ceccherini-Silberstein, GSF, Neuherberg).

2.2.7. Primers

Oligonucleotides used for DNA amplification and sequence analysis (Table 2.1) were designed using the software programs Gene Construction Kit™2 and OligoEdit and were kindly synthesized in the institute AG BIODV (GSF, Neuherberg).

2.2.8. Buffers and Solutions

All the solutions used for cell culture were sterilized (autoclaved or filtered with 0.22µm filters).

Actinomycin D stock
Actinomycin D 0.1%
Ampuwa H_20
Sterilized by filtration. Aliquots 100 µl; stored -20°C

Ampicillin stock
Ampicillin 100mg/ml
Ampuwa H_20
Aliquots 500 µl; stored -20°C

Breaking buffer (rapid isolation of plasmid DNA from yeast)
Triton-X-100 2%
SDS 1%
NaCl 100mM
Tris/HCl 10mM (stock 1M pH 8.0)
EDTA 1mM, (stock 0.5M pH 8.0)

Buffer A (CellPhect; Pharmacia Biotech)
$CaCl_2$ 0.5M
in HEPES 0.1M pH 7.0

Buffer B (CellPhect; Pharmacia Biotech)
NaCl 0.28M
NaH_2PO_4 0.75mM
Na_2HPO_4 0.75mM
in HEPES 0.05 M pH 7.0

Buffer P1 resuspension buffer (Qiagen)
Tris/HCl 50mM pH 8.0
EDTA 10mM
RNAse A 100 µg/ml

Buffer P2 lysis buffer (Qiagen)
NaOH 200mM
SDS 1%

Buffer P3 neutralisation buffer (Qiagen)
Potassium acetate 3M pH 5.5

Buffer QBT equilibration buffer (Qiagen)
NaCl 750mM
MOPS 50mM
EtOH 15%
Triton X-100 0.15%

Buffer QC wash buffer (Qiagen)
NaCl 1M
MOPS 50mM pH 7.0
EtOH 15%

Buffer QF elution buffer (Qiagen)
NaCl 1.25M

Tris/HCl 50mM pH 8.5
EtOH 15%

Cellular lysis buffer for Western Blot (WB) 5X
Tris/HCl 250mM (stock 1M pH 6.8)
SDS 10%
Glycerin 7.5%
ß-Mercaptoethanol 2.5%
Bromophenol blue 0.1%

Cell culture lysis reagent 5X 30 ml (Luciferase, Promega)
Tris/H_3PO_4 125 mM pH 7.8
EDTA 10mM
DTT 10mM
Glycerol 50%
Triton X-100 5%

Cycloeximide stock
Cycloeximide 0.2%
Ampuwa H_20
Sterilized by filtration. Aliquots 100 µl; stored -20°C

Denhardt's solution 50X
BSA (fractionV) 1%
Ficoll (type 400) 1%
Polyvinylpyrrolidone 1%
Aqua bidest.
Sterilized by filtration (0.45µm). Stored -20°C

DNA loading buffer
Bromophenol blue 0.25%
Xylencyanol FF 0.25%
Ficoll in Aqua bidest. 15%
Ampuwa H_20

Dithiothreitol (DTT)
DTT 1M
Sodium acetate 0.01M pH 5.2
Sterilized by filtration (0.22µm). Aliquots 1 ml; stored -20°C

Ethidium bromide stock
Ethidium bromide 1% (w/v)
Aqua bidest.

Formaldehyde 3.7%
Formaldehyde 3.7% (stock 37%)
in PBS

Glycerol solution
Glycerol 65% (v/v) sterile
MgSO4 0.1 M
Tris/HCl 25 mM pH 8.0
Ampuwa H_20

Glyoxal-mixture for Northern Blot
Glyoxal 14.7% (stock 40%; aliquots 50 µl; stored -20°C)
Na_2HPO_4 10mM (stock 1M pH 7.2)

DMSO 50%
Ampuwa H_20

Hybridization solution for Southern Blot
SSPE 5X
Denhardt's solution 10X
SDS 2%
Freshly denatured, sheared salmon sperm DNA 0.01%
Ampuwa H_20
Sterilized by filtration (0.45µm)

IPTG
Isopropyl-ß-D-thio-galactopyranoside 0.2%
Ampuwa H_20
Sterilized by filtration (0.2µm). Aliquots 1 ml; stored -20°C

Leptomycin B stock
Leptomycin B 40µM
Ampuwa H_20
Sterilized by filtration. Aliquots 100 µl; stored -20°C

Lithium acetate stock solution 10X pH 7.5
Lithium acetate 1M
Acid acetic
Ampuwa H_20
Sterilized by filtration

Luciferase assay reagent 7mg lyophilized (Promega)
When re-hydrated with 10 ml of luciferase assay buffer:
Coenzyme A (lithium salt) 270µM
Luciferin 470µM
ATP 530µM
stored –80°C

Luciferase assay buffer 10 ml (Promega)
Tricine 20mM
$(MgCO_3)_4Mg(OH)_2 \cdot 5H_2O$ 1.07mM
$MgSO_4$ 2.67mM
EDTA 0.1mM
DTT 33.3mM

Na_2HPO_4 1M pH 7.2
Na_2HPO_4 1M
H_3P0_4 85%
Ampuwa H_20
Autoclaved

Paraformaldehyde 3.7%
Paraformaldehyde 3.7%
in PBS (dissolved at 60°C, filtered with paper filter)

50% PEG 4000
PEG 4000 (or 3350) 50%
Ampuwa H_20

Phosphate buffer saline (PBS) pH 7.4
NaCl 140mM

KCl 5.4mM
Na_2HPO_4 x $2H_2O$ 9.7mM
KH_2PO_4 2mM
Aqua bidest.

Reagent A (BCA kit, Pierce) 1000 ml

Na_2CO_3 1%
$NaHCO_3$ 1%
BCA-Na_2 1%
Sodium tartrat 0.16%
in NaOH 0.2N

Reagent B (BCA kit, Pierce) 25 ml

$CuSO_4$ 4%

RNA hybridization buffer for glyoxal method

SDS 7%
Na_2HPO_4 0.5M (stock1M pH 7.2)
Ampuwa H_20

RNA loading buffer for glyoxal method

Bromophenol blue 0.4%
Glycerol 50% (stock 50% in Ampuwa H_20, autoclaved; aliquots 1 ml; stored -20°C)
Na_2HPO_4 10mM (stock 1M pH 7.2)

Sodium dodecyl sulfate (SDS) 10%

Sodium dodecyl sulfate 10% (w/v)
Aqua bidest.
Autoclaved

SDS-electrophoresis running buffer 10X pH 8.3

Tris Base 250mM
Glycin 1.92M
SDS 1% (w/v)
Aqua bidest.

SDS-protein running gel	**12%**	**10%**
Tris/HCl (stock 1.5M pH 8.8)	2.5 ml	2.5 ml
Acrylamide + Bisacrylamide (stock 30%)	4 ml	3.33 ml
SDS (stock 10%)	0.1 ml	0.1 ml
Aqua bidest.	3.3 ml	4 ml
TEMED	5 µl	5 µl
APS (stock 10%)	0.1 ml	0.1 ml

SDS-protein stacking gel	**4.4%**
Tris/HCl (stock 1M pH 6.8)	3.5 ml
Acrylamide + Bisacrylamide (stock 30%)	0.75 ml
SDS (stock 10%)	0.05 ml
Aqua bidest.	3.5 ml
TEMED	5 µl
APS (stock 10%)	0.09 ml

Saline sodium citrate buffer (SSC) 20X

NaCl 3M
Sodium citrate 0.3M pH 7.0
DEPC-treated Aqua bidest.

Autoclaved

Saline sodium phosphate buffer (SSPE) 20X pH 7.4
NaCl 3M
$NaH_2PO_4 \cdot H_2O$ 0.2M
EDTA 20mM
DEPC-treated Aqua bidest.
Autoclaved

Stripping buffer for WB
Tris/HCl 62.5mM (stock 1M pH 6.7)
SDS 2%
ß-Mercaptoethanol 100mM
Aqua bidest.

Stripping buffer for Northern Blot
SSC 0.1X
SDS 2%

TBS-T wash buffer 5X pH 7.5
Tris Base 250mM
NaCl 750mM
Tween 20 0.1% (1X)
Aqua bidest.

TE buffer
Tris/HCl 10mM (stock 1M pH 8.0)
EDTA 1mM
Aqua bidest.

TNE buffer pH7.4
Tris/HCl 40mM (stock 1M pH 7.4)
EDTA 1mM (stock 0.5M)
NaCl 150mM
Aqua bidest.

Transfer buffer pH 8.3
Tris Base 25mM
Methanol 20%
Glycine 1.44% (w/v)
Aqua bidest.

Tris acetate EDTA (TAE) buffer 50X pH 8.0
Tris-acetate 2M
NaCl 0.5M
EDTA 50mM (stock 0.5M pH 8.0)
Aqua bidest.

Triton X-100 cell lysis buffer
Triton X-100 0.5%
in PBS

Trypan blue solution
NaCl 0.9%
Trypan blue dyestuff 0.5%
Aqua bidest.

X-gal

5-Bromo-4-chloro-3-indolyl-ß-D-galactopyranoside 2%
in 100% N,N-dimethylformamide
Wrapped in aluminium foil; stored -20°C

2.2.9. Media

2.2.9.1. Bacterial media

E.coli bacteria were grown in Luria Bertani (LB) medium. Transfected bacteria containing the ampicillin resistance gene cells were grown in LB containing ampicillin (Amp) at the concentration of 50-100µg/ml.

LB medium pH 7.5

Bacto-tryptone 1%
Bacto-yeast extract 0.5%
NaCl 0.5%
Aqua bidest.
Autoclaved

LB agar plates

LB medium 1000 ml
Bacto agar 15 g
Autoclaved
Amp 100 mg

SOC medium

Complete *E.coli* medium without selection (Stratagene or Life Technologies)

Glycerol freezing solution

80% Glycerol 2/3
10mM $MgCl_2$

2.2.9.2. Yeast media

Ingredients for liquid or solid media were dissolved in deionized water, mixed until completely dissolved and autoclaved in bottles (500-1000 ml). Amino acids (dropout solutions) were prepared in water as stock solutions, filtered (filters 0.22µm) and each added (8.3 ml/liter) as requested to the complete minimal (CM) media after have been autoclaved.

Dropout solutions

Amount in dropout powder (g)		Final concentration (mg/ml)	Stock (mg/100 ml)	
Adenine (hemisulfate salt)	40	500	2.5	RT
L-arginine (HCl)	1.2	20	240	4°C
L-aspartic acid	6.0	100	1200	RT
L-glutamic acid	6.0	100	1200	RT
L-histidine	1.2	20	240	4°C
L-leucine	3.6	60	720	RT
L-lysine (mono-HCl)	1.8	30	360	4°C
L-methionine	1.2	20	240	4°C
L-phenylalanine	3.0	50	600	RT
L-serine	22.5	375	4500	4°C
L-threonine	12.0	200	2400	4°C

L-tryptophan	2.4	40	480	4°C
L-tyrosine *	1.8	30	180	RT
L-valine	9.0	150	1800	4°C
Uracil	1.2	20	240	RT

*16.6 ml/liter of L-tyrosine was added.

Gal/Raff/CM liquid **solid**

YNB-AA/AS 0.17%
$(NH_4)_2S0_4$ 0.5%
Galactose 2% + Agar 2%
Raffinose 1% + NaOH 0.01%
Autoclaved
Dropout solutions

Glc/CM liquid **solid**

YNB-AA/AS 0.17%
$(NH_4)_2S0_4$ 0.5%
Dextrose 2% + Agar 2%
Autoclaved + NaOH 0.01%
Dropout solutions

Glc/CM X-gal plates

YNB-AA/AS 0.17%
$(NH_4)_2S0_4$ 0.5%
Dextrose 2%
Agar 2%
NaOH 0.01%
Autoclaved
Dropout solutions
KH_2P0_4 0.07M pH 7.0 (Stock 0.7M)
X-gal 0.004% (stock 2% in N,N-dimethylformamide)

Gal/Raff/CM X-gal plates

YNB-AA/AS 0.17%
$(NH_4)_2S0_4$ 0.5%
Galactose 2%
Raffinose 1%
Agar 2%
NaOH 0.01%
Autoclaved
Dropout solutions
KH_2P0_4 0.07M pH 7.0 (Stock 0.7M)
X-gal 0.004% (stock 2% in N,N-dimethylformamide)

YPD medium liquid **solid**

Yeast extract 1%
Peptone 2% + Agar 2%
Dextrose 2% + NaOH 0.01%

Medium-A 5X

$(NH_4)_2S0_4$ 0.5%

KH_2P0_4 2.25%
K_2HP0_4 5.25%
Sodium citrate x $2H_20$ 0.25%
Autoclaved

Minimal vitamin B1, Trp- plates (1 liter)

Agar 2%
Aqua bidest up to 800 ml
Autoclaved
+ 200 ml Medium-A (stock 5X, autoclaved)
+ 1 ml MgS04 (stock 1M, autoclaved)
+ 10 ml Galactose (stock 20%, autoclaved)
+ 0.1 ml Vitamin B1 (stock 0.5%, filtered)
+ 16.6 ml L-uracil (240 mg/100 ml, filtered)
+ 16.6 ml L-histidine (240 mg/100 ml, filtered)
+ 5.5 ml L-leucine (720 mg/100 ml, filtered)
+ Amp 100 mg

2.2.9.3. Cell culture media

Dulbecco's modified Eagle medium (DMEM)

with Glutamax I, w/o Na-Pyruvat, 4.5g/l Glucose (Life Technologies)

Complete DMEM

DMEM
Fetal calf serum 10% (heat-inactivated)
AB/AM solution 1% (= 100 units Penicillin, 100µg Streptomycin, und 0.25µg Fungizone®/ml, Life Technologies)

DMSO freezing medium

Complete DMEM 45%
Fetal calf serum 45%
DMSO 10%

2.3. Molecular biology methods

Many of the molecular biology procedures were performed as described in (Ausubel et al., 1999; Sambrook, 1989).

2.3.1. Plasmid DNA isolation from *E.coli*

2.3.1.1. QIAGEN maxi preparation

The plasmids used in the transfection experiments were prepared with the Qiagen maxiprep kit, according to the manufacturer's instructions. Qiagen plasmid purification protocols are based on a modified alkaline lysis procedure, followed by binding of plasmid DNA to QIAGEN anion-exchange resin under appropriate salt and pH conditions. RNA, proteins, dyes, and low molecular weight impurities are removed by a medium salt wash. Plasmid DNA was eluted in a high salt buffer, concentrated and desalted by isopropanol precipitation.

Briefly, plasmids were prepared from *E.coli* XL-1 bacterial cultures grown in the presence of a selective antibiotic, generally ampicillin.
A single colony (or 50 µl of a glycerol stock) was inoculated into 5 ml of standard LB medium in the presence of ampicillin (50 µg/ml), and grown for 4-5 h at 37°C with vigorous shaking (260 rpm). The miniculture was diluted 1:100 into a larger volume of selective medium and grown to saturation (O/N) at 37°C with gently shaking (100 rpm).
The bacterial cells were harvested and centrifuged at 4000 rpm for 20 min at 4°C in Sorvall RC-5 with GS-3 rotor. The pellet was resuspended in 10 ml of Buffer P1 containing RNase (1 mg/10 ml) and transferred into non-glass tubes for rotor SS3H. 10 ml of Buffer P2 were added, mixed gently, and the mixture was incubated at room temperature (RT) for 5 min maximum. 10 ml of ice-cold Buffer P3 were added, mixed gently and the mixture incubated on ice for 20 min. The suspension was centrifuged at 16000 rpm 30 min at 4°C and the supernatant was transferred into new tube and recentrifuged at 16000 rpm 15 min at 4°C. Qiagen column was equilibrated with 10 ml Buffer QBT. Subsequently, the supernatant from the cell lysate was applied to the column. Plasmid DNA bound to QIAGEN anion-exchange resin was washed twice with 30 ml Buffer QC and eluted with 15 ml Buffer QF, collecting it in a 50 ml Falcon tube. The DNA was precipitated with 0.7 vol of RT isopropanol, incubated 5 min at RT and centrifuged at 5000 rpm for 30 min. The DNA pellet was washed with 15 ml of 70% ethanol, vortexed, centrifuged 15 min at 5000 rpm, dried for 5-30 min and redissolved in 1 ml of sterile Ampuwa water. The DNA was stored at -20°C.

2.3.1.2. QIAGEN mini preparation

This procedure, which isolates rapidly plasmid DNA from bacteria using the alkaline lysis method followed by isopropanol precipitation, was used during the cloning process to isolate small amount of DNA from a large number of clones.
Briefly, each single colony was inoculated into 3 ml of LB medium (plus 100µg/ml Amp) in glass culture tubes and grown O/N at 37°C with vigorous shaking (260 rpm). The next day 1.5 ml of miniculture was transferred in Eppendorf tube and bacterial cells were harvested centrifuging at 14000 rpm for 2 min.
The pellet was resuspended in 250 µl of ice-cold Buffer P1 containing RNase (1 mg/10 ml) and 250 µl of Buffer P2 were added, mixed gently, and the mixture was incubated at RT for 2-3 min. 250 µl of ice-cold Buffer P3 were added and mixed gently. The mixture was incubated on ice for 5-10 min and then centrifuged at 14000 rpm 10 min. The supernatant (~800 µl) was transferred in a new Eppendorf tube and 0.7 vol of isopropanol was added. The suspension was vortexed and recentrifuged at 14000 rpm 15 min. The pellet was washed with 500 µl of 70% ethanol, vortexed, centrifuged 5 min at 14000 rpm, dried for 5-30 min and redissolved in 50 µl of sterile Ampuwa water. The DNA was stored at -20°C.

2.3.2. Qualitative and quantitative DNA analysis

To determine the quality and yield, the DNA was measured in an UV spectrophotometer (Pharmacia Biotech) at 260 and 280 nm, normally diluting 2 µl in

100 µl of Ampuwa water. The DNA concentration was calculated by the spectrophotometric conversion $1A_{260}$ unit of double-stranded DNA = 50µg/ ml (Sambrook, 1989). In addition, the ratio A_{260}/A_{280} was measured. Preparation with a DNA concentration below of 0.2µg/ml and a ratio below of 1.5 was discarded.
To ensure the correct preparation of the DNA plasmids, the DNAs were analyzed by one or more restriction enzyme digestions.

0.5-1 µg DNA (from maxiprep) or 5-10 µl DNA (from 50 µl miniprep) were digested into 1.5 ml Eppendorf tube in 20 µl of total volume containing:
1 µl of enzyme (10U)
2 µl of buffer (10X)
x µl of Ampuwa water up to 20 µl

The mixture was spun down and incubated at 37°C (or recommended temperature) for 2 h. 0.8-1.2% agarose gel in TAE 1X buffer with ethidium bromide (final concentration 20 µg/100 ml) was prepared. 2 µl of DNA loading buffer (10X) was added and mixed to each DNA sample and all content was loaded on the gel and run for 1-2 h at 50-100 V. The 1-kb-DNA molecular weight marker (Life Technologies) was used as a standard marker.

2.3.3. DNA transformation into bacteria cells

Two different protocols were used for bacterial transformation. The electroporation procedure was performed to transform electrocompetent BL21 cells (see 2.3.3.1), used to produce expression of GST fusion protein, and electrocompetent KC8 cells (see 2.4.8.), used in the yeast two hybrid system. The heat shock protocol was performed to transform chemically competent cells such as Epicurian coli XL1-blue supercompetent cells (see 2.3.3.3) and TOP10F' cells (see 2.3.4.2).

2.3.3.1. Preparation of electrocompetent cells

A single bacterial colony was inoculated in 10 ml of LB medium in a small glass tube and incubated O/N at 37°C shaking. The next morning the O/N culture (10 ml) was inoculated in 200 ml in 1l flask and cells were grown for 2 h to reach OD_{600} ~0.5. Subsequently, (working always on ice) the cells were transferred into Beckman centrifuge-tubes and centrifuged 15 min at 4000 xg in precooled GS-3 rotor at 4°C. Each cell pellet was washed first in 100% vol with cold and sterile Ampuwa water, then in 50% vol Ampuwa water and finally in 10 ml of cold 10% glycerol (filtered). Cells were re-centrifuged 10 min at 4000 xg, resuspended in 600 µl of cold 10% glycerol, aliquoted (45 µl / sterile Eppendorf tube), frozen in N_2 and stored in -80°C freezer.

2.3.3.2. Plasmid DNA transformation with electroporation

For electroporation, BL21 bacteria cells and the pGEX-3X-Risp plasmid DNA were thawed on ice.

1 µl of plasmid DNA (50-100ng) was added to thawed cells (45 µl) and mixed. The cells were incubated on ice 30 sec and added to ice-cold electroporation cuvette. The cells were shocked by 1 pulse of 1.8 kV in 3-4msec. After each pulse immediately 900 µl of RT SOC medium were added and the suspension was transferred back to the original microtube, then incubated 50 min at 37°C shaking. After 1 min of centrifugation at 2000 rpm, 800 µl of medium was removed, the rest was resuspended and 100 µl of suspension was plated on prewarmed LB/Amp plates and incubated at 37°C O/N.

2.3.3.3. Plasmid DNA transformation in Epicurian coli XL1-blue supercompetent cells

The transformation of Epicurian coli XL1-blue supercompetent cells (Stratagene) was performed according to the manufacturer's instructions. Briefly, cells were thawed on ice (~ 15 min) and aliquots of 100 µl of cells, for each transformation (included one for the vector religation control), were transferred into prechilled 15 ml Falcon 2059 polypropylene tubes. 1.7 µl of the provided β-mercaptoethanol was added to each tube, mixed gently keeping the tube on ice and incubated for 10 minutes, mixing occasionally. 1 ng of transforming plasmid DNA or 5 µl of a typical 10 µl ligation mixture were added to the cells, mixed gently and incubated on ice for 30 min. The cell/DNA mixtures for exactly 45 seconds were heat shocked at 42°C in a water bath. Cells were transferred immediately on ice and incubated for 2 min. 900 µl of RT SOC medium were added to each tube. The tubes were incubated at 37°C for 1 h with shaking at 250 rpm. Cells were then concentrated by centrifuging at 1000 rpm for 5 min. The pellet was resuspended in 200 µl of SOC medium and 100 µl were spreaded on prewarmed LB/Amp plates and incubated O/N at 37°C.

2.3.4. Cloning of PCR products

All the new plasmids generated in this work were prepared by subcloning into the appropriate recipient vector the gene of interest amplified by PCR and containing the compatible cloning restriction sites.

Briefly, the strategy followed was:

- Design and synthesize 5'and 3'-end PCR primers with the desired restriction sites
- Perform the PCR with the appropriate primers and DNA template
- Clone the PCR product into the pCR®-TOPO® vector
- Transformation of DNA into TOP10F' cells
- Analysis of TOP10F' transformants (blue/white selection)
- Plasmid DNA minipreps
- Identification of clones containing the correct DNA insert (restriction analysis)
- Insert DNA sequence analysis
- Preparation of the vector and the insert for cloning (preparative gel/DNA purification)
- Ligation of plasmid vector and insert DNAs
- Transformation of DNA into *E.coli* XL1-blue supercompetent cells

- Plasmid DNA minipreps
- Identification of clones containing the correct DNA insert (restriction analysis)
- Insert DNA sequence analysis
- Store the correct plasmid as DNA and bacteria glycerol stock at -80°C.

2.3.4.1. Amplification of DNA by polymerase chain reaction (PCR)

To amplify DNA the Expand™ High Fidelity PCR system (Roche Molecular Biochemicals) was used. The system is composed of a unique enzyme mix containing thermostable Taq DNA and Pwo DNA polymerases (Barnes et al., 1994). This powerful polymerase mixture is designed to give PCR products with high yeld, high fidelity and high specificity. The final product has blunt ends and 3' single A overhangs. The DNA amplification was performed according to the manufacturer's instructions. First, the cycle conditions in the Thermocycler (Perkin Elmer Cetus Gene Amp 9600) have been adjusted. Generally the PCR reaction (used for PCR fragment with length up to 1.5 kb) has been performed at the following conditions:

1.	94°C	2 min	denaturation of template	
2.	94°C	30 sec	denaturation	20 cycles
3.	65°C	30 sec	annealing	
4.	72°C	1 min	elongation	
5.	72°C	10 min	prolong elongation	
6.	4°C	∞	reaction end	

Subsequently, two master mixes were prepared (in a PCR room, on a laminar flow cabinet) into sterile microfuge tubes on ice as follows:

- Mix 1:

dNTPs (10mM)	8 µl
5' Primer (20µM)	0.75 µl
3' Primer (20µM)	0.75 µl
Sterile Ampuwa water	up to 49 µl

- Mix 2:

10X Expand HF buffer (with 15mM $MgCl_2$)	10 µl
Expand HF enzyme mix	0.75 µl
Sterile Ampuwa water	up to 50 µl

Outside the PCR room, working on ice, mix 1 and mix 2 were pipetted into a thin-walled PCR tube, 1 µl of template DNA (100 ng) was added and immediately the sample was placed in the thermocycler and the PCR performed. At the end of the PCR, 5 µl for each sample was analyzed on a 0.8-1% agarose gel.

2.3.4.2. TOPO TA Cloning®

The TOPO TA cloning® (Invitrogen) provides a highly efficient, one-step cloning strategy for the direct insertion of Taq polymerase-amplified PCR products into a

plasmid vector. The kit provides a plasmid vector pCR2.1-TOPO supplied linearized with single 3‘ thymidine (T) overhangs for TA cloning and Topoisomerase. Since PCR products generated with Taq polymerase or Expand™ High Fidelity enzyme mix are with blunt ends and 3’ single A overhangs, ligation of the pCR2.1-TOPO vector with a PCR product is very efficient. The TOPO TA cloning® was performed according to the manufacturer’s instructions.
Generally 1 µl of fresh PCR product, 1 µl of pCR2.1-TOPO vector and 3 µl of sterile Ampuwa water were mixed and incubated at RT for 5 min. Subsequently the DNA mixture was placed on ice and transformed into One Shot™ TOP10F’ cells thawed on ice.
2 µl of 0.5M β-mercaptoethanol were added to each vial of competent cells and mixed by stirring gently with the pipet tip.
2 µl of the TOPO cloning™ reaction were added to each vial of competent cells, mixed gently and incubated on ice for 30 min. Cells were incubated at 42°C for 30 sec, transferred immediately to ice and incubated for 2 min. 250 µl of RT SOC medium were added and the vials were put in bacterial culture glass tubes and incubated at 37°C for 30 min with shaking at 250 rpm. 100 µl from each transformation were spread on LB/Amp plates prewarmed and prespreaded with IPTG (40 µl of 100mM) and X-gal (40 µl of 40mg/ml) and incubated O/N at 37°C.

The next morning ~12 white or light blue colonies were picked for DNA plasmid miniprep analysis (digestion and subsequent sequence analysis). The plasmid containing the correct DNA insert was stored as DNA miniprep or maxiprep at -20°C.

2.3.4.3. Vector and insert preparation

In this study DNA was purified from agarose gels using a modified “Freeze Squeeze”method (Tautz and Renz, 1982).
Both vector (2-5 µg DNA) and pCR2.1-TOPO-PCRinsert (2-5 µg DNA or 10 µl DNA miniprep) were digested with the compatible restriction enzymes in 20-40 µl for 3-4h. If the ends of the prepared vector were identical (e.g., following a single digestion), the vector DNA was treated for 30 min at RT with 1 µl of calf intestinal alkaline phosphatase (Roche Molecular Biochemicals) to remove the phosphate groups from the 5‘ends to prevent self-ligation of the vector.
Both linearized vector and insert were separated by agarose gel electrophoresis using 0.8% high-quality SeaKem agarose. The DNA fragments were then isolated by cutting the smallest possible volume of gel slice.

2.3.4.3.1. DNA recovery form agarose gel using the freeze squeeze method

To isolate DNA from agarose gel a modified “Freeze Squeeze” method was performed. Briefly, the gel slice of interest was incubated into 1.5 ml sterile Eppendorf tube with 1 ml of sodium acetate buffer (NaAcetate 0.5M, EDTA 1mM, pH 5.2) at RT for 30 min. Subsequently, the gel slice was dried with a paper towel and transferred to a perforated 0.5 ml autoclaved freeze squeeze Eppendorf tube containing siliconized glasswool (Whatman GF/C, φ=2.5cm) and frozen in liquid N_2 for 1-2 min. The freeze squeeze tubes were placed into 1.5 ml sterile tubes without lid and were

centrifuged at maximum speed, 14000 rpm for 10 min RT. The DNA flow-through was transferred into a new sterile Eppendorf tube and DNA was precipitated with 2.5 vol ethanol 100%. After 3 min of incubation at RT, samples were centrifuged at 14000 rpm 15 min, and DNA pellet was washed with ethanol 70%, dried and dissolved in 5-30 µl of sterile Ampuwa water.

2.3.4.4. Ligation of plasmid vector and insert DNAs

After the vector and insert DNAs have been purified, the concentration of DNA was estimated by agarose gel electrophoresis along with molecular weight standard (1 µl sample). The ligation reaction was performed using the T4 DNA Ligase (Life Technologies) according to the manufacturer's instructions. The typical ligation reaction was performed at RT for 2 h, with 50-200 ng of vector DNA (molar ratio of vector to insert 1:3) in 10 µl of final volume:

1-2 µl of vector
3-5 µl of insert
2 µl of DNA Ligase buffer (5X)
1 µl of T4 DNA Ligase
x µl of Ampuwa water up to 10 µl

To monitor the efficiency of ligation of vector with insert, the linear vector was subjected to ligation in the absence of the DNA insert.
At the end of the reaction, the mixture samples were placed on ice and 5 µl were transformed into chemically competent cells (*E.coli* XL1-blue supercompetent cells).

2.3.5. Analysis of RNA by Northern hybridization

To analyze the size and the expression of the Risp RNA, Northern analysis was performed. Total RNA was isolated by cellular lysates, run on an agarose gel, transferred on nylon membrane and detected by hybridization with a radiolabeled DNA-probe. To successfully isolate and analyze intact RNA some important precautions were performed: effective disruption of cells, denaturation of nucleoprotein complexes, inactivation of endogenous ribonuclease (RNase) activity and purification of RNA away from contaminating DNA and protein (Sambrook, 1989). All disposable plasticware were sterile. Many solutions as well as nondisposable plasticware were pretreated with diethyl pyrocarbonate (DEPC) 0.05% O/N before sterilization.

2.3.5.1. QIAGEN isolation of total RNA

The isolation and purification of RNA from cell lysates was performed using the Qiagen RNEasy mini kit according to the manufacturer's instructions. Briefly, cells were washed with sterile PBS, trypsinized, diluted in the complete medium, counted and centrifuged at 1000 rpm for 5 min. 10 µl of the provided β-mercaptoethanol were added per 1 ml of RLT lysis buffer. Cell pellets were resuspended well with RLT buffer (350 µl / for up to $5x10^6$ cells or 600 µl / for up to $1x10^7$ cells) and frozen at -

80°C. Frozen lysates were thawed at 37°C for 10 min and were transferred directly into QIAsheder columns sitting in 2 ml collection tubes and centrifuged at maximum speed for 2 min. Samples were collected into new tubes and 1 vol of ethanol 70% was added, creating conditions which promote selective binding of RNA to the RNEasy membrane. 700 µl of sample were applied to RNEasy mini spin column sitting in 2 ml collection tubes and centrifuged at 10000 rpm for 15 sec to allow absorption of RNA to the membrane. If the volume of lysate cells was exceeding 700 µl, all the rest of suspension was applied to the same RNEasy spin column, using the same collection tube but discarding each time the flow-through.
The columns were washed with 700 µl of RW buffer and centrifuged at 10000 rpm for 15 sec. After having transferred the RNEasy spin columns into 2 ml new collection tubes, 500 µl of RPE buffer (containing ethanol) were added and centrifuged at 10000 rpm for 15 sec. Reusing the collection tubes and discarding the flow-through, the columns were recentrifuged at maximum speed for 2 min to dry the RNEasy membrane. The RNEasy spin columns were transferred into 1.5 ml collection tubes and RNA was eluted with two centrifugation steps at 10000 rpm for 1 min with 40 µl of sterile Ampuwa water and stored at -80°C.
After this step all procedures were performed keeping RNA samples on ice.
Yield and quality of RNA were analyzed by UV spectrophotometer. The concentration of RNA was determined by the spectrophotometric conversion $1A_{260}$ unit of single-stranded RNA = 40 µg / ml. The absorbance ratio A_{260}/A_{280} was determined and good RNA preparations were considered exhibiting a ratio A_{260}/A_{280} grater than 1.8 (Sambrook, 1989). RNA (1.5 µl) was also analyzed by agarose gel electrophoresis and the 28S and 18S ribosomal RNA bands were observed.

2.3.5.2. Agarose gel electrophoresis (glyoxal method)

To perform Northern analysis, a modified glyoxal method (Ausubel et al., 1999; Sambrook, 1989) was performed. Two hours before starting, the run chamber, combs and tray were incubated with 0.5% SDS in TE + Proteinase K (10-20 mg/l) for 30-60 min and after washed with DEPC-treated millipore water.
20 µg of total RNA were dried in a speed vac and resuspended in 20 µl of Glyoxal-mixture, mixed well by pipetting and incubated for 1 h in a water bath at 50°C (mixing every 30 min for 30 sec).

- **Glyoxal mixture for 10 samples**:
  64 µl Ampuwa H_20
  2 µl Na_2HPO_4 1M pH 7.2
  100 µl DMSO
  34 µl Glyoxal (40%)

  200 µl

After 1 h of incubation on ice, 6µl of RNA loading buffer were added and samples were loaded into the previously prepared 1% agarose gel without EtBr (because it

interferes with glyoxal). The gel was run for 4-5 h at 75-80V. The electrophoresis was performed until the bromophenol blue has migrated 2/3 of the gel.

- **Loading buffer**
  1 ml 50% Glycerol (autoclaved, 1 ml aliquots stored at -20°C)
  10 µl Na_2HPO_4 1M pH 7.2
  0.4 g Bromophenol blue
- **Agarose gel**
  Agarose 1%
  Na_2HPO_4 10mM pH 7.2
  Ampuwa H_20
- **Running buffer**
  Na_2HPO_4 10mM pH 7.2

2.3.5.3. Transfer of RNA to membrane

At the end of the electrophoresis, the gel was soaked in NaOH 0.05M for 5-15 min to partially hydrolyze RNA. The gel was then rinsed in sterile DEPC-treated Millipore water. In the meanwhile twelve pieces of Whatman 3MM paper and one piece of nylon membrane (Zeta–Probe® GT blotting membrane, BioRad) were cut to the exact size of the gel, soaked first in sterile DEPC-treated Millipore water and after in SSC 10X (prepared with DEPC-treated Millipore water and autoclaved). The transfer of RNA to membrane was performed by capillar blotting O/N at RT (Sambrook, 1989). To control the RNA on the filter, the membrane was first incubated for 10 min in a solution of sodium acetate 0.3M pH 5.2 containing 0.02% methylen blue and after washed in SSC 2X for 2-5 min. The filter was photographed, baked at 80°C for 1 h and stored at RT.

2.3.5.4. Probe hybridization

Before the hybridization step, the filter was washed in SSC 0.2X and SDS 1% at RT for 20-30 min, changing several times the buffer.
The filter was then placed in a heat sealable glass tube and pre-hybridized for 1 h in rotation at 65°C in 10 ml of hybridization solution. The DNA probe was prepared, denatured at 100°C for 5 min, quick chilled on ice and added in 7-10 ml of fresh prepared and prewarmed hybridization solution at the final concentration of 10^6 cpm/ml. The filter was hybridized in continuous rotation O/N at 65°C. It was washed twice with 50-100 ml of wash buffer I at 65°C for 20-30 min and twice with 50-100 ml of wash buffer II at 65°C for 20-30 min. The radioactive filter was wropped in plastic (without allowing to dry) and normal autoradiography was performed. To reprobe the filter, it was washed in a large volume of stripping buffer at 95°C for 20 min.
As a positive control for RNA levels, the filter was re-hybridized with a GAPDH [α^{32}P]-labeled probe, using a GAPDH insert of 1.3 kb (PstI-PstI) from a GAPDH expression pBSPL-plasmid (kindly provided by Dr. M. Stuerzl, GSF).

- **Hybridization solution**
  Na_2HPO_4 0.5M pH 7.2
  SDS 7%
- **Stringency wash buffer I**
  Na_2HPO_4 40mM pH 7.2
  SDS 5%
- **Stringency wash buffer II**
  Na_2HPO_4 40mM pH 7.2
  SDS 1%
- **Stripping wash buffer**
  SSC 0.1X
  SDS 0.5%

To increase the sensitivity of blot hybridization UltRAhyb™ Ultrasensitive Hybridization Solution (Ambion) has been also tried once. This solution maximizes the sensitivity by increasing hybridization signal without increasing background. According to the manufacturer's instructions, the UltRAhyb™ was preheated at 68°C before use and 10 ml were used to prehybridize the filter for 30 min at 42°C. The hybridization was performed at 42°C O/N and filters were washed at 42°C twice with SSC 2X and SDS 0.1% for 5 min and twice with SSC 0.1X and SDS 0.1% for 15 min.

2.3.5.5. Radioactive labeling of DNA

2.3.5.5.1. Radioactive PCR

One of the methods performed to incorporate 32P in the DNA probe was a PCR using α[32P] dCTP with standard cycle conditions (see 2.3.4.1). The PCR reaction mixture was prepared by adding mix 1 to mix 2:

- Mix 1:

dATP (10mM)	1 µl
dGTP (10mM)	1 µl
dTTP (10mM)	1 µl
α[32P] dCTP (0.1mM)	7 µl
5' Primer (20µM)	1 µl
3' Primer (20µM)	1 µl
Sterile Ampuwa water	11 µl
DNA (10ng/µl)	1 µl

- Mix 2:

10X Expand HF buffer (with 15mM MgCl2)	4 µl
Expand HF enzyme mix	0.75 µl
Sterile Ampuwa water	10.25 µl

The PCR product was subsequently centrifuged and purified using NucleoSpin PCR purification kit (Macherey-Nagel). 60 µl of TE buffer pH 8.0 and 400 µl of NT2 buffer were added to the 40 µl of PCR and mixed. The sample loaded into a NucleoSpin tube previously placed into a 2 ml centrifuge Eppendorf was centrifuged at 6000 xg for 1 min. After discarding the flow-through, NucleoSpin tube was washed twice with 700 µl of NT3 buffer (containing ethanol) at maximum speed for 1 min. It was dried

by centrifugation and DNA was eluted in 40-70 µl of NE buffer by centrifugation at maximum speed for 1 min. 2 µl of probe were diluted in 10 ml of scintillation fluid and the specific activity of the probe was measured in a liquid scintillation counter (Kontron).

2.3.5.5.2. Rediprime™ II random prime labeling system

Alternatively, the system Rediprime™ II (Pharmacia), which uses random sequence hexanucleotides to prime DNA synthesis on denatured template DNA at numerous sites along its length, was used to radiolabel DNA. Using Klenow fragment of DNA polymerase I, non-radioactive nucleotides were substituted with the radiolabeled equivalent in the reaction mixture, and newly synthesized DNA was made radioactive. According to the manufacturer's instructions, 50 ng of DNA was diluted in 45 µl of TE buffer pH 8.0 in a 1.5 ml sterile centrifuge tube, heat denatured at 95-100°C for 5 min and snap cooled on ice for 5 min. After a brief spin down, all the DNA denatured was added to the reaction Rediprime™ II tube. 5 µl of α[32P] dCTP were added, mixed by pipetting up and down about 12 times, and the reaction mixture was incubated at 37°C for 10 min. The reaction was stopped by adding 50 µl of TE buffer and DNA was denatured by heating to 95-100°C for 5 min and snap cooled on ice for 5 min. After a brief spin down, the radiolabeled DNA was purified using Bio-Spin Chromatography columns (BioRad) according to the manufacturer's instructions.

2.3.6. Analysis of DNA by Southern hybridization

Risp DNA analysis was performed using positively charged nylon membrane (Clontech's ZOO-BLOT) containing EcoRI-digested genomic DNAs derived from 9 eukaryotic species: human, monkey (Rhesus), rat (Sprague-Dawley), mouse (Balb/c), dog, cow, rabbit, chicken and yeast (*Saccharomyces cerevisiae*). The hybridization was performed according to the manufacturer's instructions. The hybridization solution was first warmed at 50°C and used to prehybridize the filter for 6 h at 65˚C. 100µg/ml of freshly denatured, sheared salmon sperm DNA were added to the prehybridization and hybridization solution. The hybridization was performed at 65˚C O/N with 1-2 x10^6 cpm/ml radioactively labeled DNA probe. The hybridized filter was washed under non stringent conditions for detection of genomic sequences with low homology. First it was washed with SSC 2X and SDS 0.05% for 40 min at RT with several changes of wash solution and after for 40 min at 63˚C. The radioactive filter was wropped in plastic and normal autoradiography was performed.

2.4. Yeast two hybrid system

2.4.1. Background

To identify new Rev-interacting factors the interaction trap/two-hybrid system has been used (Fields and Song, 1989; Golemis et al., 1999; Gyuris et al., 1993). This method is a useful sensitive approach to detect protein-protein interactions that uses yeast as a test vehicle and transcriptional activation of a reporter system. The two-hybrid approach takes advantage of the modular domain structure of eukaryotic

transcription factors. This modular structure consist at least of two distinct functional domains, one that directs binding to specific DNA sequences and one that activates transcription. The DNA binding and activation domains need not to be covalently attached to each other for activation; and they can be exchanged between transcription factors and retaining the function. In this work the interaction trap developed by Gyuris et al. has been used (Brent and Finley, 1997; Gyuris et al., 1993) that consists of three critical components (Fig. 2.2):

1) The vector pEG202 (see 2.2.6.3.1.) that directs the expression of the HIV-1 Rev bait protein fused to the DNA-binding domain of the bacterial LexA transcription factor.

2) The pJG4-5 library plasmid (see 2.2.6.3.2.) that directs the conditional expression of a Jurkat T cell cDNA library fused at their amino termini to a moiety containing three domains: the SV40 nuclear localization signal, the activation domain of the bacterial B42 transcription factor, and the hemagglutinin (HA) epitope tag. The activation-tagged cDNA-encoded protein is expressed from the conditional yeast GAL1 promoter, which is induced by galactose and repressed by glucose.

3) A trap that uses the EGY48 yeast strain expressing two reporter genes with binding sites for the LexA DNA-binding domain in the promoter. One reporter is the yeast LEU2 gene that has its normal upstream regulatory sequences replaced by three high affinity LexA operators and integrated into the yeast chromosome. The yeast growth is limited in the absence of leucine unless the LexAop-LEU2 gene is transcribed. The other reporter gene is the bacterial LacZ, which can be expressed by the plasmid pJK103 or by pSH18-34. These LacZ reporters contain part of GAL1 promoter plus various number of LexA operators, 1 in the PJK103 and 8 in the pSH18-34 reporter gene plasmids. In addition to the mutation in the endogenous LEU2 gene, EGY48 strain carries mutations in three other marker genes (*his3*, *trp1*, and *ura3*) that are needed to allow selection of the plasmids used in the interaction trap. The *his3* mutation is complemented by the *HIS3* gene on the pEG202-bait expression vector. The *trp1* and *ura3* mutations are respectively complemented by the *TRP1* gene on the pJG4-5 library plasmid, and the *URA3* gene on the lacZ pJK103 or PSH18-34 reporter plasmid (Fig. 2.2).

The preparation and the characterization of the Rev-LexA expression plasmid as well as part of the test screening was made by Dr. Alexandra Ludvigsen and Petra Willnauer, members of IMV/GSF.

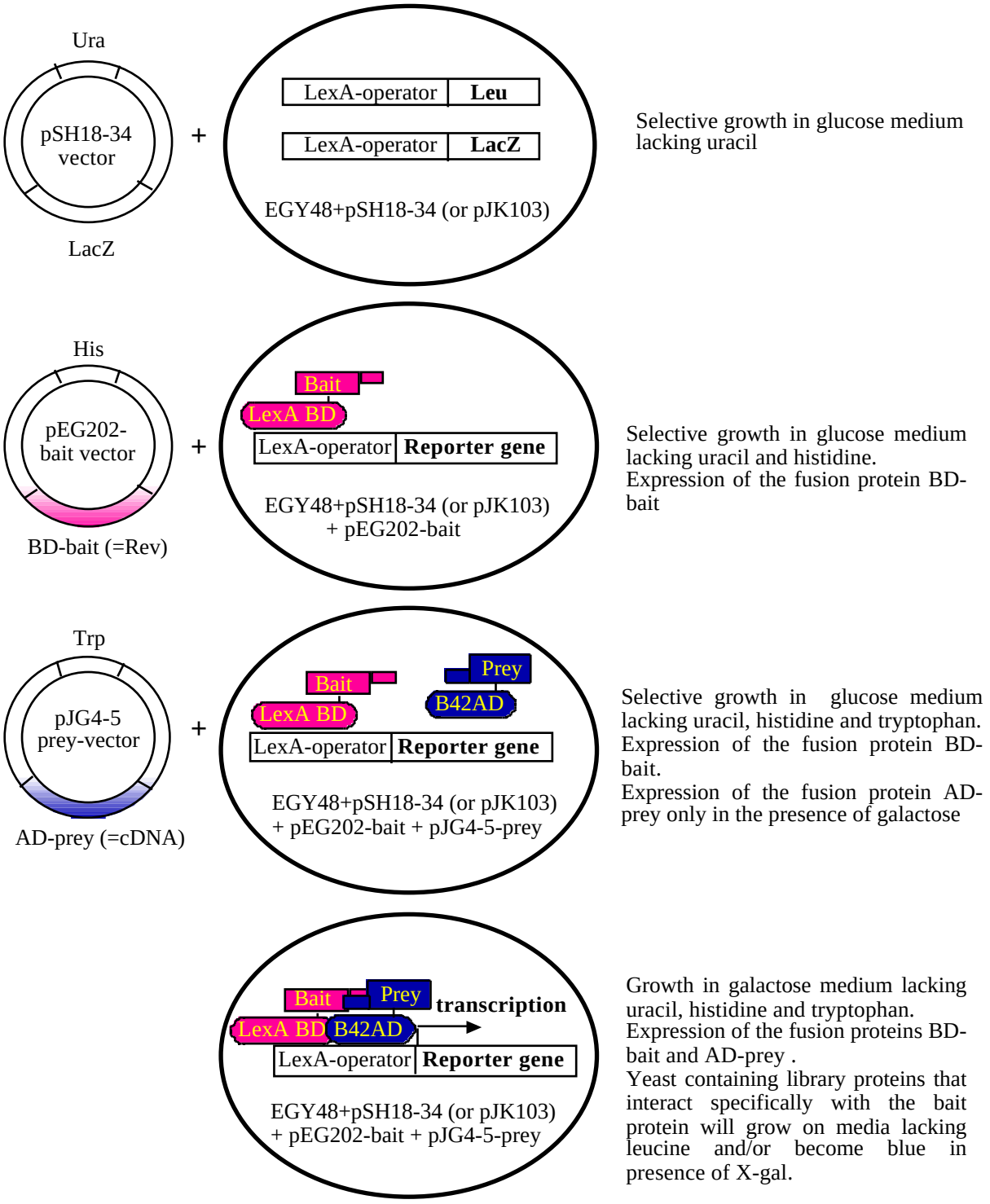


Fig. 2.2 The principle of the interaction trap. In glucose medium the GAL1 promoter is repressed and no cDNA-encoded protein is made. The expression of Rev (bait) protein fused with the binding domain of LexA and the corresponding binding to LexA operators upstream of the reporter genes LEU2 and LacZ are not enough to activate their transcription and the yeast does not grow in the absence of leucine. When the yeast is grown on galactose medium, activation-tagged cDNA-encoded proteins are expressed, and those that interact with Rev activate transcription of the reporter genes LEU2 and LacZ, allowing the yeast to grow in galactose medium lacking leucine and form blues colonies on galactose X-gal plates.

Brielfly, the main steps of the interaction trap performed to identify new Rev-interacting factors were:

- Construction of bait protein plasmids (pEG202-sRev sense, pEG202-sRev anti-sense, pEG202-LexCD2)
- Transformation of yeast EGY48 cells with pSH18-34 or pJK103 reporter plasmid
- Transformation of EGY48/pSH18-34 and EGY48/pJK103 cells with pEG202-sRev sense plasmid
- Characterization of bait protein expression and activity
- Large scale transformation of EGY48/pSH18-34/pEG202-sRev and EGY48/pJK103/ pEG202-sRev cells with pJG4-5 Jurkat-cDNA library plasmid
- Collection of primary transformants (8 x 24x24-plates), stored 25 aliquots 1-ml at -80°C
- Screening for Rev-interacting clones:
  Thaw one aliquot and plated on 20 100-mm Gal/Raff U-, H-, W-, L- plates
  Collection of clones after 2d, 3d, 4d
- Test for Gal-dependence:
  Stop GAL1 promoter induction by plating clones in Glc/U-, H-, W- plates
  Plated on Gal/Raff U-, H-, W-, L-; Gal/Raff U-, H-, W-, X-gal; Glc/ U-, H-, W-, L-; and Glc/ U-, H-, W-, X-gal plates
  Selection of clones by growth into Gal/Raff L- and blue in Gal/Raff X-gal plates
- Isolation of plasmid-DNA from yeast clones and transformation in KC8-bacteria
- Selection of pJG4-5 library plasmid:
  Replica-plating on VitB/W- andVitB/W-, H- plates with KC8-bacteria
  Selection of clones by growth into VitB/W- plates and no growth into VitB/W-, H- plates
- Isolation of plasmid-DNA by miniprep, restriction analysis
- Screening for specific Rev interacting proteins:
  Transformation of cDNA library plasmids into yeast:
  EGY48 / pSH18-34 / Rev sense
  " " / Rev as
  " " / LexCD2
  Selection of transformants for each cDNA clone (growth in Glc/ U-, H-, W- plates)
  Plated on Gal/Raff U-, H-, W-, L-; Gal/Raff U-, H-, W-, X-gal; Glc/ U-, H-, W-, L-; and Glc/ U-, H-, W-, X-gal plates
  Selection of clones by growth into Gal/Raff L- and blue in Gal/Raff X-gal plates
- DNA maxiprep of positive clones and sequence analysis

2.4.2. Determination of yeast cell density

The density of cells in culture was determined spectrophotometrically by measuring its optical density (OD) at 600 nm. For reliable measurements, cultures were diluted such that the OD_{600} was <1. In this range, each 0.1 OD_{600} unit correspond to ~ $3x10^6$ cells/ml.

2.4.3. DNA transformation into yeast cells using lithium acetate procedure

The lithium acetate method is based on the fact that alkali cations make yeast competent to take up DNA and provides high transformation efficiency of 10^4 to 10^6 transformants/µg.

At least two days before the experiment a single EGY48 yeast colony was inoculated in 5 ml of medium (YPD or CM dropout medium according to the yeast culture) and grown O/N to saturation at 30°C. (Some times the saturated overnight culture was stored for few days at 4°C and used <2 weeks).

The night before transformation an appropriate amount (5 to 25 µl) of the saturated culture was inoculated into a 1 l sterile flask containing 50 ml medium and grown O/N at 30°C to reach ~$1x10^7$ cells/ml (OD_{600} 0.3 to 0.5). At this point cells were diluted to $2x10^6$cells/ml (OD_{600} ~0.1) in fresh medium and grown for another 1 to 2 generations (2 to 5 h).

After that procedure, the yeast cells were harvested by centrifuging 5 min at 5000 rpm in Sorvall GS rotor at RT, resuspended in 10 ml sterile Ampuwa water, transferred to smaller centrifuge tubes and centrifuged again 5 min at 7000 rpm in SS-34 rotor at RT. Pellet cells were resuspended in 1.5 ml buffered lithium solution, freshly prepared as follows:

1 vol 10X TE buffer, pH 7.5
1 vol 10X lithium acetate stock solution
8 vol Sterile Ampuwa water.

Cells were incubated for 1 h at 30°C and after were transformed. (Some times the yeast cells were stored at 4°C in this solution and used <2 weeks).

For each transformation, 200 µg freshly denaturated high-molecular-weight carrier DNA with 5 µg transforming DNA were mixed into a sterile 1.5 ml microcentrifuge tube. Total volume of DNA was kept <20 µl. Immediately 200 µl of yeast suspension and 1.2 ml PEG solution freshly prepared as follows, were added:

1 vol 10X TE buffer, pH 7.5
1 vol 10X lithium acetate stock solution
8 vol 50% PEG.

Cells were shaken 30 min at 30°C; heat shocked 15 min at 42°C and centrifuged 5 sec at RT. Finally, yeast cells were resuspended in 200 µl to 1 ml of 1X TE buffer freshly prepared. 200 µl of yeast suspension were plated onto CM dropout plates, incubated at 30°C until transformants were appearing (normally 2-5 days).

2.4.3.1. Preparation of single-stranded high-molecular-weight carrier DNA

Addition of denatured (single-stranded) high-molecular-weight carrier DNA is critical to achieve high transformation efficiency using the lithium acetate method.
1 g of DNA (type III sodium salt form salmon testes; Sigma D1626) was dissolved in 100 ml TE buffer (pH 8.0) and was stirred O/N at 4°C. The viscous solution of DNA was subsequently sonicated several times at 75% power for 30 sec. An aliquot of DNA was run on a 0.8% agarose gel to check the size distribution of sonicated fragments. The sonification was considered complete when the fragments were distributed in a range from 2 kb to 15 kb, with a mean size of ~7 kb.
The DNA was extracted firstly with 1:1 (vol/vol) phenol/chloroform and then with chloroform alone. Afterwards the DNA was precipitated with 1/10 vol of 3M sodium acetate (pH 5.2) and 2.5 vol of ice-cold 100% ethanol centrifuging 15 min at 12000 xg.
The DNA pellet was washed with 70% ethanol, centrifuged, dried and resuspended in sterile TE buffer to a 10mg/ml final concentration.
The suspension was stirred O/N at 4°C and after a boiling-denaturation for 2-3 min was transferred in ice-water bath. Finally, the DNA was aliquoted into sterile tubes and stored at -20°C.

2.4.4. Preparation and test of the Rev bait protein

Firstly, pEG202 bait plasmids expressing wt Rev, in sense and antisense orientation, and the cytoplasmic domain of CD2 were produced. In a second step, using the lithium acetate procedure, the yeast strain EGY48/pJK103 containing the two LexA operator-responsive reporters (leucine and LacZ) was transformed with the plasmids pEG202-sRev sense, pEG202-sRev antisense and pEG202-LexCD2. In parallel, EGY48 yeast cells were transformed with the pSH18-34 plasmid in presence and absence of the bait plasmids pEG202-sRev sense, pEG202-sRev antisense and pEG202-LexCD2. Since the *lacZ* reporter gene expression plasmids pSH18-34 and pJK103 and the bait expression plasmid pEG202 harbor the *URA3* and *HIS3* genes respectively (see 2.2.6.3.1.), transformants were selected by the growth in Glc/CM Ura-, His-, dropout plates.
Series of control experiments were performed to establish that the bait protein is made as stable protein in yeast and that it does not appreciably self-activate transcription of the LexA operator-based reporter genes.
The test for self-activation was performed picking a colony of EGY48 yeast containing pBait and pSH18-34/or pJK103 reporter plasmids into 500 µl sterile water. 100 µl of suspension were diluted into 1 ml sterile water and a series of 1/10 dilutions were prepared in sterile water to cover a 1000-fold concentration range. 100 µl from each tube (undiluted, 1/10, 1/100, 1/1000) were spreaded out on 100-mm Gal/Raff Ura-, His- and Gal/Raff Ura-, His-, Leu- dropout plates and then incubated at 30°C. In addition, background of LacZ expression in the selection strains was also tested. For this selection 100 µl from each yeast suspension undiluted was plated on Glc/CM Ura-, His-, X-gal plates. The growth of cells was monitored for several days. Yeast

strains that grew well on plates with leucine and did not grow on plates lacking leucine, and were white on plates containing X-gal were chosen for the further use.
To confirm expression of Rev protein each yeast strain harboring the bait plasmid was analyzed by western blot. A single colony of EGY48 yeast containing pBait and pSH18-34/or pJK103 reporter plasmids was grown in 5 ml of Glc/CM Ura-, His- liquid dropout medium O/N at 30°C. 1/10 of the O/N culture was inoculated into a fresh 5ml culture and incubated at 30°C for 5-6 h (OD_{600} = 0.5-1). After that 1.5 ml of yeast culture was transferred into microcentrifuge tubes, the yeast were spun down and the pellet dissolved in 50 µl of western blot lysis buffer.

2.4.5. Large scale cDNA library transformation

In order to obtain a high number of primary transformants expressing an individual library plasmid, each yeast strain was transformed in large scale in 30 different sterile tubes.
Each yeast selection strain EGY48/pSH18-34 and EGY48/pJK103 containing the pEG202-sRev sense plasmid was grown in 20 ml of Glc/CM Ura-, His- liquid dropout medium O/N at 30°C.
The next morning, the cultures were diluted into 1 l sterile flasks into 300 ml of Glc/CM Ura-, His- liquid dropout medium to $2x10^6$cells/ml (OD_{600} = 0.1) and incubated at 30°C to reach $1x10^7$ cells/ml (OD_{600} ~ 0.5).
After that step, the yeast cells were harvested by centrifuging 5 min at 1000-1500 xg in a low-speed centrifuge at RT, resuspended in 30 ml sterile Ampuwa water, transferred to 50 ml conical tubes and centrifuged again 5 min at 1000 xg at RT.
The cells were resuspended in 1.5 ml 0.1 M lithium acetate/TE buffer.
For each yeast selection strain transformation, 50 µg high-quality sheared salmon sperm carrier DNA with 1 µg pJG4-5 library DNA plasmid were added in 30 sterile 1.5 ml microcentrifuge tubes (total volume of library and carrier DNA was kept <20 µl). Immediately 50 µl of the resuspended yeast solution and 300 µl of sterile 40% PEG 4000 were added and after inverting the tubes, cells were shaken 30 min at 30°C.
40 µl of DMSO were added, the tubes were inverted and heat shocked 10 min at 42°C.
All the content of 28 tubes plus 360 µl from the other 2 tubes was mixed and plated undiluted on 8 24x24-cm Glc/CM Ura-, His-, Trp- dropout plates (~1.2 ml/plate). The remaining 40 µl of the 2 tubes were used to make a series of 1/10 dilutions in sterile water (1/10, 1/100, 1/1000) and spreaded on 100-mm Glc/CM Ura-, His-, Trp- dropout plates. All the plates were then incubated at 30°C for 2-3 days expecting colonies. The dilution series were performed to calculate the transformation efficiency.

2.4.5.1. Preparation of sheared salmon sperm DNA (sssDNA)

200 mg of high-quality salmon sperm DNA (type III sodium salt form salmon testes; Sigma D1626) were dissolved in 20 ml TE buffer (pH 7.5) and were stirred O/N at 4°C. The viscous solution of DNA was subsequently sheared by sonicating 2-3 times at 75% power for 30 sec. A small aliquot of DNA was run on a 0.8% agarose gel to check the size distribution of sonicated fragments. The sonification was considered

complete when the fragments were distributed in a range from 2 kb to 15 kb, with a mean size of ~7 kb.
The sssDNA was extracted by mixing with an equal volume of TE-saturated buffered phenol in a 50 ml conical tube, shaking vigorously and centrifuged 5-10 min at 3000 xg. The extraction was repeated using 1:1 (vol/vol) buffered phenol/chloroform and once with chloroform alone. Afterwards the DNA was transferred in tubes suitable for high-speed centrifugation and precipitated with 1/10 vol of 3M sodium acetate (pH 5.2) and 2.5 vol of ice-cold 100% ethanol centrifuging 15 min at 12000 xg.
The DNA pellet was washed with 70% ethanol, centrifuged, dried and resuspended in sterile TE buffer to 10mg/ml final concentration.
The resuspension was denatured for 20 min in a 100°C water bath then immediately transferred in ice-water bath. Finally, the DNA was aliquoted in sterile tubes and stored at -20°C.

2.4.5.2. Collection of primary transformants
The 8 24x24-cm Glc/CM Ura-, His-, Trp- dropout plates containing the primary transformants from each strain were cooled for several hours at 4°C to harden the agar. Wearing gloves and using sterile glass microscope slides, yeast cells were gently scraped and collected in 50 ml conical tubes. The cells were washed twice with sterile TE buffer and centrifuged 5 min at 1000-1500 xg RT. The pellet of cells was resuspended in 1 vol glycerol solution and frozen in aliquots (1ml) at -70°C.
To determine the replating efficiency 200 µl of 1 ml aliquot of frozen transformed yeast was diluted 1/10 in Gal/Raff/CM Ura-, His-, Trp- dropout medium and incubated with shaking for few hours at 30°C to induce the GAL promoter on the library. Serial dilutions of yeast cells were plated on 100-mm Gal/Raff/CM Ura-, His-, Trp- dropout plates and incubated at 30°C until colonies were visible. Yeast colonies were counted and the number of cfu per aliquot of transformed yeast was determined.

2.4.6. Screening for interacting proteins
To select for transcription of the *LEU2* reporter gene, 200 µl of each transformed yeast strain were incubated O/N in 300 ml of Gal/Raff/CM Ura-, His-, Trp- medium. Cultures were plated on 20 100mm Gal/Raff/CM Ura-, His-, Trp-, Leu- dropout plates and incubated 2-4 days at 30°C.
3 series of new Gal/Raff/CM Ura-, His-, Trp-, Leu- master dropout plates picking colonies at day 2, 3 and 4 were prepared.
To test for Gal dependence of the Leu and LacZ phenotypes (to confirm that they are attributable to expression of the library-encoded proteins) the GAL1 promoter was turned off in the presence of glucose. For this purpose the Leu^+ clones were replated from the Gal/Raff/CM Ura-, His-, Trp-, Leu- dropout plates to 100-mm Glc/CM Ura-, His-, Trp- dropout plates and incubated at 30°C until colonies were growing.
With all yeast clones replica plating was performed using different dropout selection plates:

Gal/Raff/CM Ura-, His-, Trp-, Leu-
Glc/CM Ura-, His-, Trp-, Leu-
Gal/Raff/X-gal/CM Ura-, His-, Trp-
Glc/X-gal/CM Ura-, His-, Trp-.

The colonies were considered positive if they were blue on Gal/Raff/X-gal/CM Ura-, His-, Trp- but not on Glc/X-gal/CM Ura-, His-, Trp- dropout plates and if they grew on Gal/Raff/CM Ura-, His-, Trp-, Leu- but not on Glc/CM Ura-, His-, Trp-, Leu- dropout plates.

2.4.7. Rapid isolation of plasmid-DNA from yeast

Plasmid DNA is released from yeast transformants along with chromosomal DNA in a rapid protocol by vortexing cells with glass beads in the presence of detergents, phenol, chloroform, and isoamyl alcohol.
Each single yeast colony was inoculated into 2 ml of medium in sterile glass tubes. The yeast cells were grown O/N at 30°C at 200 rpm.
From each of the O/N cultures 1.5 ml was transferred in a new microcentrifuge tube and spun 5 sec at high speed RT. The supernatant was removed and the pellet was briefly vortexed. Cells were resuspended in 200 µl of breaking buffer (see 2.2.8.). 0.3 g of glass beads (0.45-0.52 mm acid-washed, ~200 µl) and 200 µl of phenol/chloroform/isoamyl alcohol (25:24:1) were added and vortexed for 2 min at highest speed. The suspension was centrifuged 5 min at high speed at RT, and 50 µl of the aqueous layer was transferred in a new microcentrifuge tube and stored at -20°C.

2.4.8. Transformation of plasmid DNA in *E.coli* KC8 by electroporation

Firstly *E.coli* KC8 were made electrocompetent and aliquoted at -80°C (see 2.3.3.1).
To perform the electroporation with plasmid DNA, KC8 bacteria cells and DNA were thawed on ice.
2 µl of plasmid DNA was added to thawed cells, mixed, and added to ice-cold cuvettes. The cells were shocked by 1 pulse of 1.8 kV in 3-4msec. After each pulse immediately 900 µl of SOC medium were added and the suspension was transferred back to the original microtube, then incubated 1 hour at 37°C shaking. After 2 min of centrifugation at 5000 rpm, 800 µl of medium was removed, the rest was resuspended and 50 µl of suspension was spreaded in LB/Amp plates and incubated at 37°C O/N.

2.4.9. Selection of library plasmid in *E.coli* KC8 using vitamin B1 plates

The inability of *E.coli* KC8 to grow in absence of tryptophan was used to select bacteria containing the library plasmid with the yeast *TRP1* gene on the pJG4-5 library plasmid. Bacteria containing library plasmids were selected by growing on minimal plates supplemented with vitamin B1, ampicillin, uracil, histidine, and leucine and lacking tryptophan. Firstly 30-40 transformed bacteria by each LB/Amp plate were transferred in a special scheme in a new LB/Amp master plate and incubated at 37°C O/N. Using sterile nylon filters replica plating was performed. The nylon filters

were cautiously brought first in contact with clones on the masterplate and subsequently put on minimal vitamin B1, Trp- plates and on minimal vitamin B1, Trp-, His- plates.
Clones containing the pJG4-5 library plasmid were selected by the growth on plates lacking tryptophan. Bacteria growing on plates in the absence also of histidine were expressing the *HIS3* gene by the bait pEG202 plasmid and were excluded.
Single bacteria clones containing the pJG4-5 library plasmid were picked from the minimal vitamin B1, Trp- plates and transferred in new LB/Amp plates to isolate DNA and analyzed by enzyme digestion.

2.4.10. Screening for specific interacting proteins

To select only specific Rev-interacting proteins the rescued library plasmid DNAs were retransformed in the original yeast strains containing the bait plasmids (expressing Rev in sense and antisense orientation and the unspecific bait CD2).
DNAs were separately transformed using the lithium acetate procedure in:

EGY48/pSH18-34/pEG202-sRev sense
pEG202-sRev antisense
pEG202-LexCD2

or in:

EGY48/pJK103/ pEG202-sRev sense
pEG202-sRev antisense
pEG202-LexCD2.

Each transformation was plated on Glc/CM Ura-, His-, Trp- dropout plates and incubated at 30°C for 2-3 days until colony growth was detected. Four to six independent colonies from each transformation plate were replated on Glc/CM Ura-, His-, Trp- master dropout plates and incubated O/N at 30°C. With the same series of yeast clones replica plating was performed using the following different selection plates:

Gal/Raff/CM Ura-, His-, Trp-, Leu-
Glc/CM Ura-, His-, Trp-, Leu-
Gal/Raff/X-gal/CM Ura-, His-, Trp-
Glc/X-gal/CM Ura-, His-, Trp-.

Clones were considered positive when growing without Leu and showing X-gal conversion only in the presence of galactose and not of glucose.

2.4.11. Sensitive and semi-quantitative Lac Z dot-blot assay

For some clones LacZ activity was also/only analyzed using a sensitive and semi-quantitative dot-blot assay (Essers and Kunze, 1996).
After the transformation, yeast cells were resuspended in 600 µl of 1x TE buffer freshly prepared. 200 µl were inoculated in 5 ml of Glc/CM Ura-, His-, Trp- dropout medium and incubated at 30°C 1-3 days until the OD_{600} >1. Yeast cells that grew more rapidly were kept at 4°C. Once each yeast clone was with the OD_{600} >1, 500 µl

of each were added in 5 ml of fresh dropout medium Glc/CM Ura-, His-, Trp-, Gal/Raff Ura-, His-, Trp- and Gal/Raff/CM Ura-, His-, Trp-, Leu-. The cultures were then incubated for few (3-5) hours until they reached the same OD_{600} ~ 0.8 to obtain equal cell densities. Aliquots of 25 µl, 100 µl and 500 µl were transferred onto nylon filter by vacuum filtration in a standard dot-blot chamber. The 25 and 100 µl suspension volumes were diluted with sterile water to achieve a more even distribution of cells within the dot area. The nylon filters were submerged in liquid nitrogen for 10 sec to crack the cells and then placed onto X-gal plates (100mM Na2PO4 pH 7.0, 10mM KCl, 1mM MgSO4, 1mM DTT, 14 g/l agar, 40-200 mg/l X-gal). The filters on the plates were incubated at 37°C until the blue colour was clearly visible (O/N - 24 h).

2.5. Biochemical methods

2.5.1. Cell culture

Cells were maintained under standard cell culture conditions in Dulbecco's modified Eagle medium, with Glutamax-I, without Na-Pyruvat, and with 4.5 g/l of Glucose. Media were supplemented with 10% fetal calf serum, 1% antibiotic-antimycotic solution (100 units of penicillin, 100µg streptomycin and 0.25µg fungizone/ml). Cells were kept in culture for serial passages (maximum 50), and were expanded 1:5 – 1:10 twice weekly by trypsinization. Primary fetal astrocytes (H4/96 passages 3-4) (kindly provided by Dr F. Aloisi, ISS, Rome) were maintained under standard cell culture conditions in Minimal essential medium D-valine (Life Technologies) containing 10% fetal calf serum, Glutamine 2mM and Gentamicin 1% (Aloisi et al., 1992).

2.5.1.1. Plasmid-DNA transfection of adherent cells

Cells were transfected either by calcium phosphate ($CaPO_4$) coprecipitation method with a commercially available kit (CellPhect; Pharmacia,) or using FuGENE™ 6 transfection reagent (Roche Molecular Biochemicals) according to the manufacturer's instructions. One day before transfection, cells were seeded at densities between 4-8x10^5 cells per 60-mm diameter dish or at densities between 1-4x10^5 cells per 35-mm diameter dish depending on the cell line. Plasmids were purified with Qiagen DNA purification columns according to the manufacturer's instructions.

2.5.1.1.1. Calcium phosphate method

Using the $CaPO_4$ coprecipitation the correct amount of DNA was mixed with $CaCl_2$ and dropped onto a phosphate buffer containing Hepes solution. At the correct pH of 7.1 a $CaPO_4$ precipitate is formed. Part of the phosphate backbone of the DNA takes part in this process leading to a coprecipitation of the DNA.
DNA was always filled up with pBSPL bluescript vector to total amount of 17 µg/60-mm diameter dish or 8.5µg/35-mm diameter dish. In each experiment, the same plasmid mixture was added to at least two dishes of cells.
After warming up all reagents to RT, in a laminar flow cabinet the DNA mixtures were prepared. In sterile tubes the DNA plasmids (17µg total/plate) were added and diluted in Ampuwa water (120 µl/plate). After a short spinning down, to the same Eppendorf

tubes the appropriate amount of buffer A (120 µl/plate) was added. The mixture was incubated at RT for 10 min, in the same time, in 50 ml Falcon tubes the appropriate amount of buffer B (240 µl/plate) was added. The DNA mixture was added slowly and dropwise to buffer B, which was subjected to constant air stream with a pipet aid. The mixture was incubated at RT for 15 min and subsequently dropwise to cells. Cells were incubated with DNA precipitates for 4 h, after a short check at the microscope, cells were washed twice with prewarmed DMEM w/o serum and refed with complete medium. Cells were harvested 48 h after the washing step.

- Rev response assays were typically performed with

Gag reporter plasmid pB37R	5µg
Tat and/or Rev expression plasmids	1µg
Luciferase expression plasmid	1µg
Bluescript vector	up to 17µg

- Tat response assays were typically performed with

CAT reporter plasmid pHIVΔnps-LTR-CAT	2µg
Tat expression plasmids	1µg
Luciferase expression plasmid	1µg
Bluescript vector	up to 17µg

- Tat-Rev response assays were typically performed with

CAT reporter plasmid pSLIIB-CAT	2µg
Tat and Rev/or Tat-Rev expression plasmids	1µg
Luciferase expression plasmid	1µg
Bluescript vector	up to 17µg

To assay Rev response (Ludwig, 1999; Ludwig et al., 1999), 48 h after transfection, cells were washed twice with PBS, incubated 10 min at 37°C in TNE 1X 1 ml / plate and harvested. 200 µl of the cell suspension were transferred in 1.5 ml sterile Eppendorf tubes and used to measure luciferase activity: the cells were centrifuged 15 min at 35000 x*g*, resuspended in 100 µl of 1X "Cell culture lysis buffer" (Luciferase Assay System, Promega) and stored at -20°C. The remaining cells were transferred in other 1.5 ml sterile Eppendorf tubes and used to measure the total protein content (BCA kit, Pierce) and the p24Gag production (p24ELISA, Coulter). Cells were centrifuged 15 min at 35000 x*g*, lysed by three freeze-thaw cycles (-80°C - 37°C) in 400 µl 0.5% Triton X-100/PBS, cleared by centrifugation 10min at 35000 x*g* and stored at -20°C.

For analysis of Tat or Tat-Rev response (Ludwig, 1999; van Empel, 2000), CAT reporter gene expression was quantified either on the protein level by ELISA (Roche Molecular Biochemicals) or by a functional CAT assay (Molecular Probes). The CAT ELISA was performed with cell lysates prepared as described above.

2.5.1.1.2. FuGENE method

For localization analysis, cells were seeded into glass-bottomed dishes (35-mm diameter) and transfected using FuGENE™ 6 transfection reagent.
Generally, the transfection mixture consisted in 0.5-2.0 µg of effector plasmid with 3 times more (in µl) of FuGENE in 100 µl/60-mm diameter or 50 µl/35-mm diameter dishes.
In sterile Eppendorf tubes, serum-free DMEM was added to dilute FuGENE to 100 µl. The necessary amount of FuGENE was added directly into the medium without touching the plastic of the tube and incubated for 5 min at RT. After that, the FuGENE/medium mix was dropped in other sterile Eppendorf tubes containing already the plasmids and after a gentle mix by tipping the tubes, incubated for 15 min at RT. The mix was added to the cells drop by drop and 24-48 h later the cells were examined.

In some experiments, cells were incubated with several drugs:

- with the dye Hoechst 3342 (2µg/ml) for 10 min to allow easy identification of nuclei
- with cycloheximide (25µg/ml) for 2h to block new protein synthesis
- with actinomycin D (5µg/ml) for 2h to block RNA synthesis
- with leptomycin B (10nM) for 2h to block Crm1-dependent nuclear export.

2.5.2. Indirect immunofluorescence

Indirect immunofluorescence was essentially performed as described elsewhere (Ludwig et al., 1999). Briefly, cells were washed with PBS and fixed for 10 min at RT with paraformaldehyde (or formaldehyde) 3.7%. After two washes with PBS, cells were permeabilized for 10 min with PBS/Triton-X100 0.5%.
Primary antibodies were diluted in PBS in concentrations ranging between 1:20-1:100 and secondary antibodies were diluted in PBS 1:50-1:200. Cells were incubated with the respective antibodies at RT for 30 min. After each antibody-incubation, cells were carefully washed several times with PBS. Finally cells were protected with few drops of slow fade light mounting mix (Molecular Probes) and observed in an inverted research fluorescence microscope (Axiovert 135TV Zeiss, Germany). Negative control plates were treated identically except that they were not incubated with the first antibody. The excitation wavelength 493 nm was used for GFP, DTAF and Alexa-green analysis, whereas wavelength 580 nm was used for cyanine 3 and Texas red detection and wavelength 357 nm for Hoechst 33342.

2.5.3. Fluorescence microscopy and imaging of living cells

Microscopy of fixed and living cells expressing GFP-fusion proteins was performed as described extensively in (Lee et al., 1999; Neumann et al., 2001). For GFP-analysis in living cells, cells were seeded into glass bottom petri dishes (Mattek Corp., Ashland, MA), transfected as described above and observed 24-48 hours later. Medium was exchanged against complete medium without phenolred and dishes put

on an inverted fluorescence microscope with a mounted environmental chamber. Images were taken at 10x, 20x, 32x or 40x magnification using a cooled 12 bit CCD-camera (Quantix, Photometrics, AZ). Images were taken at subsaturating conditions (i.e., pixel intensity < 4095). Filters, shutters and camera were controlled by a Macintosh PowerPC computer using IPLab Spektrum 3.2 software. Care was taken to ensure that images used for comparative purposes were taken under identical lighting conditions, exposure and gain settings. Images were manipulated with IPLab Spectrum 3.2, Adobe Photoshop 5.0 and Adobe Illustrator 8.0.

2.5.4. Cellular quantification of GFP fluorescence

The procedure for the quantification of GFP fluorescence in cells expressing GFP fusion proteins was performed using IPLab spectrum as described in (Lee et al., 1999; Neumann et al., 2001). Briefly, images were taken under non-saturating conditions of the CCD detector. For each cell to be quantified the total fluorescence signal of the entire cell and of its nuclear compartment were measured. Transferring cellular and nuclear outlines from phase and Hoechst 33342 images respectively to GFP fluorescent images assisted the delineation of cellular boundaries. Background fluorescence was determined by measuring a signal of an extra area GFP-negative and by calculation of mean background signal per pixel. Total fluorescent signal measured for each cell segment was corrected for background fluorescence and for its area. The corrected signal value of the entire cell segment was set to 100% and proportions of nuclear and cytoplasmic signals calculated.

2.5.5. Western Blot

To analyze protein expression a SDS polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblotting procedure has been performed.

2.5.5.1. SDS polyacrylamide-gel electrophoresis (SDS-PAGE)

First cellular protein extracts were prepared. Cell cultures were washed with cold sterile PBS, trypsinized, diluted in PBS, centrifuged at 1000 rpm for 5 min, rewashed in PBS, counted, centrifuged and resuspended in cellular lysis buffer WB 1X (see 2.2.8.) 100 µl/1.5x10^6 cells.

After 5 min of denaturation at 100°C and a brief spin down, 20 µl of samples and 10 µl of standard molecular weight marker were loaded in home-made or ready (BioRad) SDS gels and run into a gel-electrophoresis chamber (BioRad) in the presence of SDS electrophoresis running buffer 1X pH 8.3 (see 2.2.8.). The running conditions were fixed at 120V/20mA for the first 20-30 min and after at 150V/50mA for 40-60 min. Different gels were used to separate proteins with molecular weight:

- < of 50 kDa, resolving gels with 12% of acrylamide/bisacrylamide,
- > of 50 kDa, resolving gels with 10% of acrylamide/bisacrylamide,
- > of 20 and < of 250 kDa, resolving Tris-HCl ready gels 4-15%.

2.5.5.2. Immunoblotting

Proteins separated on SDS-PAGE were transferred to nitrocellulose membranes (BioRad) using a Semi-dry-Electroblotter (Sartorius). Gels were soaked first in sterile Millipore water and after in transfer buffer pH 8.3 1X for 30 min. Six pieces of Whatman 3MM paper and one piece of nitrocellulose membrane were cutted to the exact size of the gel, soaked first in water and after wards in transfer buffer. The transfer occurred in 1h 30 min at ~ 50V/200mA (mA= cm^2 membrane x 0.8). Filters were blocked in the dark in the presence of BSA 1% in TBS-T wash buffer pH 7.5 1X (see 2.2.8.) at 4°C O/N. Filters were then incubated with the respective antibodies diluted in BSA 1% in TBS-T wash buffer (1:50 – 1:5000) at RT for 1h in continuous agitation, washed with TBS-T wash buffer once for 15 min and twice for 5 min in continuous agitation. Filters were stained with the secondary anti-mouse, anti-rabbit or anti-rat antibodies horseradish peroxidase-conjugate (1:100 – 1:2000) and finally stained proteins were detected by the enhanced chemiluminescence (ECL) assay (Amersham).

2.5.6. Total protein determination (BCA test)

The determination of protein amount in the cellular lysates was performed using the bicinchoninic acid (BCA) kit from Pierce according to the manufacturer's instructions. This system combines the well-known biuret reaction (proteins reducing Cu^{2+} in an alkaline medium to produce Cu^{1+}) with the higly selective detection reagent BCA. BCA in fact, is in the form of its water-soluble solution salt, as sensitive, stable and highly specific reagent for the cuprous ion (Cu^{1+}) and forms a complex BCA- Cu^{1+}-BCA exhibiting a strong absorbance at 562 nm.
Briefly, dilutions in water 1:2 for a series of 8 calibration standards (2000–15.8 µg/ml) from a stock of BSA (2mg/ml) were prepared. In a microtiter plate, per well, in duplicate were added 10 µl of protein standards, water (blank) or cell lysate samples. 200 µl of a mixture of working reagents (50 parts of reagent A with 1 part of reagent B) were added per well and the plate was incubated at 37°C for 30 min. The absorbance was measured using a microelisa reader plate (BioRad) at 570 nm. A standard curve was prepared by plotting the absorbance values obtained on the x-axis against the specific standard protein concentrations on the y-axis, performing linear regression analysis with the computer programs CA-Cricket Graph III or Microsoft Excel 5.0. Using the standard curve, the protein concentration for each unknown protein sample was determined using Microsoft Excel 5.0.

2.5.7. HIV-1 p24 antigen capture ELISA

The quantitative production of HIV-1 Gag in the cell lysates was determined by a commercial HIV-1 p24-antigen ELISA kit from Coulter according to the manufacturer's instructions. Briefly, dilutions 1:2 for a series of 6 calibration standards (125–3.9 pg/ml) from a purified HIV-1 p24 antigen reagent (625 pg/ml) were prepared. 200 µl of p24 standards, 0.5% Triton X-100 in PBS (blank), cell lysate samples (undiluted and/or diluted 1:10, 1:100 and 1:1000) were added to the designated wells, prebound to the surface with a murine monoclonal antibody anti-

HIV-1 p24 antigen. The plate was incubated at 37°C for 1 h. Following a wash step, 200 µl of reconstituted biotinylated human anti-HIV-1 IgG reagent were added to each well and the plate was incubated at 37°C for 1 h. Following another wash step, 200 µl of reconstituted streptavidin-horseradish peroxidase were added and the plate was incubated at 37°C for 30 min. Following a wash step, 200 µl of a substrate reagent containing tetramethylbenzidine (TMB) were added and the plate was incubated at RT in the dark for 30 min. In the presence of peroxidase and hydrogen peroxide, TMB is complexed to form a blue color. Finally, the reaction was stopped adding 50 µl of H_2S0_4 4N. Using a microelisa plate reader the absorbance was measured at 450 nm with reference wavelenght at 570. A standard curve was prepared by plotting the absorbance values obtained on the x-axis against the p24 standard concentrations on the y-axis, performing linear regression analysis with the computer softwares CA-Cricket Graph III or Microsoft Excel 5.0. Using the standard curve, the concentration of HIV-1 p24 antigen for each sample was determined using Microsoft Excel 5.0 software. Samples with absorbance values higher than the highest point on the standard curve (125 pg/ml) were retested more diluted. The levels of Gag were standardized to 100 µg of total protein.

2.5.8. Luciferase Assay

As control for transfection efficiency, the production of the luciferase reporter gene was measured using a luciferase assay provided from Promega. The principle of the assay is based on the activity of the luciferase enzyme, derived from the coding sequence of the *luc* gene cloned from the firely *Photinus pyralis* (De Wet 1985 PNAS). The enzyme catalyzes a reaction using D-luciferin and ATP in the presence of oxygen and Mg^{2+} resulting in light emission.
First, the luciferase assay reagent was reconstituted adding 10 ml of luciferase assay buffer to the vial containing the lyophilized luciferase assay substrate and brought at RT. Once re-hydrated the luciferase assay reagent was stored at -80°C. 10 µl of RT cell extract was transferred in 5 ml clear polystyrol tubes (Sarstedt) and transferred in an automated injection luminometer (AutoLumat LB 953, Berthold) setted to inject 100 µl of luciferase assay reagent substrate to each tube. The light intensity was measured for 10 sec after 6 sec of interval after each substrate-injection and is proportional to luciferase concentration. The light units from each sample were standardized to 100µg of total protein.

2.5.9. CAT ELISA

The quantitative determination of the chloramphenicol acetyltransferase (CAT) in transfected cells was performed using a commercial CAT ELISA kit from Roche Molecular Biochemicals, according to the manufacturer's instructions. Briefly, the CAT enzyme stock solution was reconstituted adding 0.5 ml of Ampuwa water. A series of 7 dilutions 1:2 to produce CAT enzyme standards (1000-15 pg/ml) were freshly prepared adding 40 µl of CAT stock solution in 3.96 ml sample buffer (PBS containing blocking reagents). 200 µl of CAT standards, sample buffer, or cell lysate samples (undiluted and/or diluted 1:2, 1:10 and 1:100) were added to the designated

wells precoated with a goat polyclonal antibody anti-CAT. The plate was incubated at 37°C for 1h. Following a wash step, 200 µl of a reconstituted and freshly diluted antibody anti-CAT conjugated to digoxigenin were added per well and the plate was incubated at 37°C for 1h. Following a wash step, 200 µl of reconstituted and freshly diluted antibody anti-digoxigenin conjugated to peroxidase were added per well. The plate was incubated at 37°C for another 1 h. Following the final wash step, 200 µl of a peroxidase substrate containing ABTS were added, and incubated at RT for 30-40 min until color development (green color). Using a microelisa plate reader the absorbance of the samples was measured at 405 nm with reference wavelenght at 490. A standard curve was prepared by plotting the absorbance values obtained on the x-axis against the CAT standard concentrations on the y-axis, performing linear regression analysis with the computer softwares CA-Cricket Graph III or Microsoft Excel 5.0. Using the standard curve, the CAT concentration for each sample was determined using Microsoft Excel 5.0 software. The levels of CAT were standardized to 100µg of total protein.

2.5.10. CAT Assay

To determine the CAT-activity a fluorescent CAT assay (Fast CAT® Green kit, Molecular Probes) has been used according to the manufacturer's instructions. Briefly, 48 h after transfection, cells were washed twice with PBS, incubated 10 min at 37°C in TNE 1X 1 ml/plate, transferred in 1.5 ml sterile Eppendorf tubes and centrifuged 5 min at 10000 x*g*. The cells were resuspended in 100-300 µl Tris/HCl 0.25M pH 7.4, lysed by 3 freeze-thaw cycles (-80°C/37°C), cleared by centrifugation at 4°C at 35000 x*g* and stored at -80°C. The total protein content and luciferase activity were determined. Depending on the luciferase values, 10-30 µg of total protein was transferred in new 1.5 ml sterile Eppendorf tubes and diluted to a final volume of 65 µl with Tris/HCl 0.25M pH 7.4. Cell lysates were then incubated at 37°C in a thermo block with 5 µl fluorescence-labeled chloramphenicol substrate (freshly rehydrated with 1 ml of methanol). Subsequently, 10 µl of acetyl-CoA solution (9mM final concentration) were added and the probes were incubated 2h-O/N at 37°C in the dark. The reaction was stopped adding 1 ml of cold (4°C) ethyl acetate. After a brief vortex (20 sec) to mix both phases, the samples were centrifuged 2 min at 35000 x*g* to achieve a good phase separation. The organic phase (~ 950 µl) containing the chloramphenicol was transferred in new Eppendorf tubes. The green substrate was evaporated to dryness using a speed-vacuum and resuspended in 10 µl of ethyl acetate. Each sample was dropped onto a silica gel TLC plate and dry. The TLC plates were placed in a chromatography tank pre-equilibrated with 200 ml of chloroform:methanol (85:15) and subjected to thin-layer-chromatography for 35-45 min. Acetylated and non-acetylated chloramphenicol forms were separated and quantified using a STORM™ fluorescence scanner (Molecular Dynamics, Germany) using a Molecular Dynamics-Software (Image Quant v1.2).
Inductions reflect the ratio of CAT-levels or conversion-rates, respectively, standardized to total protein amount in the presence or absence of Tat-expression plasmid.

2.5.11. Preparation and purification of Risp-GST protein

To prepare and purify Risp-GST fusion protein it has been cloned the *risp* gene in the pGEX-3X vector (see 2.2.6.2), and electrocompetent *E.coli* BL21 cells were transformed (see 2.3.3.2) with the constructed plasmid pGEX-3X-Risp. Transformant cells were selected in LB/Amp plate and two clones were picked and diluted into 50 ml of LB/Amp medium and incubated O/N at 37°C. The next day 50 ml of culture were diluted 1:10 in 500 ml of LB/Amp medium and incubated at 37°C for 1-2 h (OD_{600} ~0.8). The expression of the fusion protein was induced by incubation of cells with IPTG (1mM) for other 3-4 h. Cells were centrifuged at 4000 rpm at 4°C for 15 min and the pellet stored at -20°C. Cells were then resuspended in a freshly prepared lysis buffer, vortexed, incubated on ice for 15 min and lysed by mild sonification.

· Lysis Buffer:

10 ml PBS containing
- 10 µl Leupeptin (1mg/m H_20)
- 10 µl Pepstatin (5mg/ml DMSO)
- 10 µl Aprotinin (1mg/ml H_20)
- 10 µl DNase (1mg/ml 50% glycerol)
- 20 µl PMSF (0.2M in isopropanol)
- 26 µl $MnCl_2$ (1M)
- 260 µl $MgCl_2$ (1M)
- 0.01% Lysozyme

After the sonification, 1 ml of NaCl 5M and 500 µl of Triton X-100 20% were added and the mixture was vortexed and incubated on ice for 10 min to facilitate the solubilization of the proteins. The suspension mixture was centrifuged at 14000 rpm at 4°C for 20 min and the supernatant transferred in 50 ml Falcon tube. The purification of the GST fusion protein was performed with glutathione Sepharose 4B (Pharmacia) according to the manufacturer's instructions. Bacterial lysate was incubated with glutathione Sepharose 4B (500 µl / 50 ml lysate) and the Risp-GST protein was eluted using glutathione 15 mM. The integrity of the purified protein was examined in SDS 12% PAGE and Coomassie blue staining. The best fraction of the eluted protein was extensively dialyzed against cold PBS (O/N), quantified using the BCA protein determination assay (see 2.5.6.) and stored at -80°C.

2.5.12. BSA labeling and peptides conjugation

Synthetic peptides were obtained from commercial sources (Sigma-Genosys, Ltd, Pampisford, UK) and were conjugated with BSA previously labeled with the fluorescent Alexa fluor 488 reactive dye (Molecular Probes) according to the manufacturer's instructions.

The following peptides were synthesized:

· CGG-LQLPPLERLTLD amino acids 73-84 of HIV-1 Rev wild-type,
· CGG-LQLPP**DL**RLTLD amino acid positions 73-84 of HIV-1 Rev**M10** mutant,
· CG-LQLPPLE**A**LTLD amino acid positions 73-84 of HIV-1 Rev-**A** mutant,

- CG-LQLPP**DLA**LTLD amino acid positions 73-84 of HIV-1 Rev**M10-A** mutant,
- CG-LESNLFDDNIDIFADL amino acids 86-105 of Risp,
- CG-KPKEKSKKKVEAK amino acids 106-118 of Risp.

2.5.13. Cellular microinjection

One day before microinjections, 1-3x10^5 cells were seeded into glass-bottomed dishes (35-mm diameter). BSA-Alexa Fluor 488 labeled-conjugate peptides (~1mg/ml) or purified Risp-GST protein (~1mg/ml) were microinjected into cytoplasm or nuclei of cells using a manual injector system (Eppendorf). As internal injection control, peptides or purified protein were comicroinjected in the presence of unconjugate BSA-Alexa Fluor 568 labeled (~1mg/ml). After two hours of incubation at 37°C, cells were fixed and analyzed in an inverted fluorescence microscope.

2.6. Computer programs

Program	Origin	Application
Adobe Illustator®8 (Mac)	Adobe Systems, USA	Graphic images
Adobe PhotoShop®5.02 (Mac)	Adobe Systems, USA	Conversion of scanned files
Ca Cricket Graph II and III (Mac)	Cricket Software, USA	Linear regression analysis, diagrams
ClarisWorks 4.0 and 5.0 (Mac)	Claris Corporation, USA	Table calculations, graphic images
Claris Draw 1.0v4 (Mac)	Claris Corporation, USA	Graphic images
DIALIGN (PC)	AG BIODV, GSF	Multiple sequence alignment
DNA-Strider (Mac)	C. Marck, France	DNA and protein sequence analysis
EndNote 3.1 (Mac)	Niles Software, USA	Bibliography organizer
GCG (PC)	AG BIODV, GSF	Find patterns
Gene Construction Kit ™2 (Mac)	Textco Corp. USA	DNA and plasmid sequence presentation
GraphPad PRISM 2.0 (Mac)	GraphPad Software, USA	Statistical analysis (Mann-Whitney-U test)
IPLab Spectrum 3.2.4 (Mac)	Scanalytics, USA	Microscope fluorescence images analysis
Microsoft Word 98 (Mac)	Microsoft Corporation, USA	Text processing
Microsoft Excel 98 (Mac)	Microsoft Corporation, USA	Linear regression analysis, table calculations, diagrams
Microsoft Internet Explorer 4.5 (Mac)	Microsoft Corporation, USA	Internet search
OligoEd (Mac)	AG BIODV, GSF	Primers analysis

Analysis on Internet	**WWW Address**	**Application**
BEAUTY (BLAST Enhanced Alignment Utility)	http://dot.imgen.bcm.tmc.edu:9331/seq-search/protein-search.html	DNA or protein sequence homology search
BLAST search	http://www.ncbi.nlm.nih.gov/BLAST/	DNA or protein sequence homology search
BLOCKS search	http://dot.imgen.bcm.tmc.edu:9331/seq-search/protein-search.html	Motif blocks search
GeneStream align	http://www2.igh.cnrs.fr/bin/align-guess.cgi	Alignment and local alignment of two sequences
PATTIMPROT search	http://pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_pattern.html	Pattern search
PROSITE pattern search	http://www2.ebi.ac.uk/ppsearch/	Protein motifs search
PSORT II search	http://psort.nibb.ac.jp/form2.html	Prediction of protein sorting signals and subcellular localization sites
PubMed	http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=PubMed	National library of medicine’s search

3. Results

3.1. Identification of two HIV-1 Rev-interacting proteins by yeast two-hybrid selection

To identify new HIV-1 Rev-interacting proteins we performed a yeast two-hybrid protein interaction assay. The Rev protein served as a bait and a Jurkat T-cell cDNA library encoding possible Rev-interacting factors supplied the prey (Fig. 3.1).

3.1.1. Preparation and test of Rev as a bait protein

With standard subcloning techniques, *rev* cDNA was inserted into the polylinker of the pEG202 plasmid (see 2.2.6.3.1.) to produce an in frame LexA-Rev fusion protein. Briefly, the HXB2- *rev*-ORF in pBsRev was amplified beginning at the second codon by PCR and inserted in frame with LexA into the unique EcoRI restriction site of the pEG202 vector in sense and antisense orientation. The cytoplasmic domain of CD2 fused to LexA was used as an unspecific bait control (pEG202-LexCD2, see 2.2.6.3.1.).

Using the lithium acetate procedure the yeast strain EGY48/pJK103, containing the two LexA operator-responsive reporters (leucine and LacZ), was transformed with the plasmids pEG202-sRev sense, pEG202-sRev antisense and pEG202-LexCD2. In parallel, EGY48 yeast cells were transformed with the pSH18-34 plasmid in presence and absence of the bait plasmids pEG202-sRev sense, pEG202-sRev antisense and pEG202-LexCD2. Since the lacZ reporter gene expression plasmids pSH18-34 and pJK103 and the bait expression plasmid pEG202 harbor the URA3 and HIS3 genes respectively (see 2.2.6.3.1. and 2.4.1) transformants were selected which grow in glucose medium lacking uracil and histidine. To investigate whether Rev as a bait protein activates transcription of the reporter genes in the absence of the "prey" protein, each yeast transformant was diluted 100 and 1000-fold in sterile water and plated on Gal/Raff Ura-, His- and Gal/Raff Ura-, His-, Leu- plates. In this test galactose medium was used because the following screening of the cDNA library was carried out on galactose plates to induce expression of the activation-tagged cDNA protein.

The growth of cells was monitored for several days. All yeast clones grew well to a similar density on the Gal/Raff Ura-, His- plates. However, in the absence of leucine some yeast clones did not grow at all and some showed weak growth. In addition background LacZ expression in the selection strains was tested. Therefore each yeast transformant was plated on Glc/CM Ura-, His-, X-gal plates. The absence of blue colonies confirmed that the *lacZ* gene was inactive in the presence of glucose.

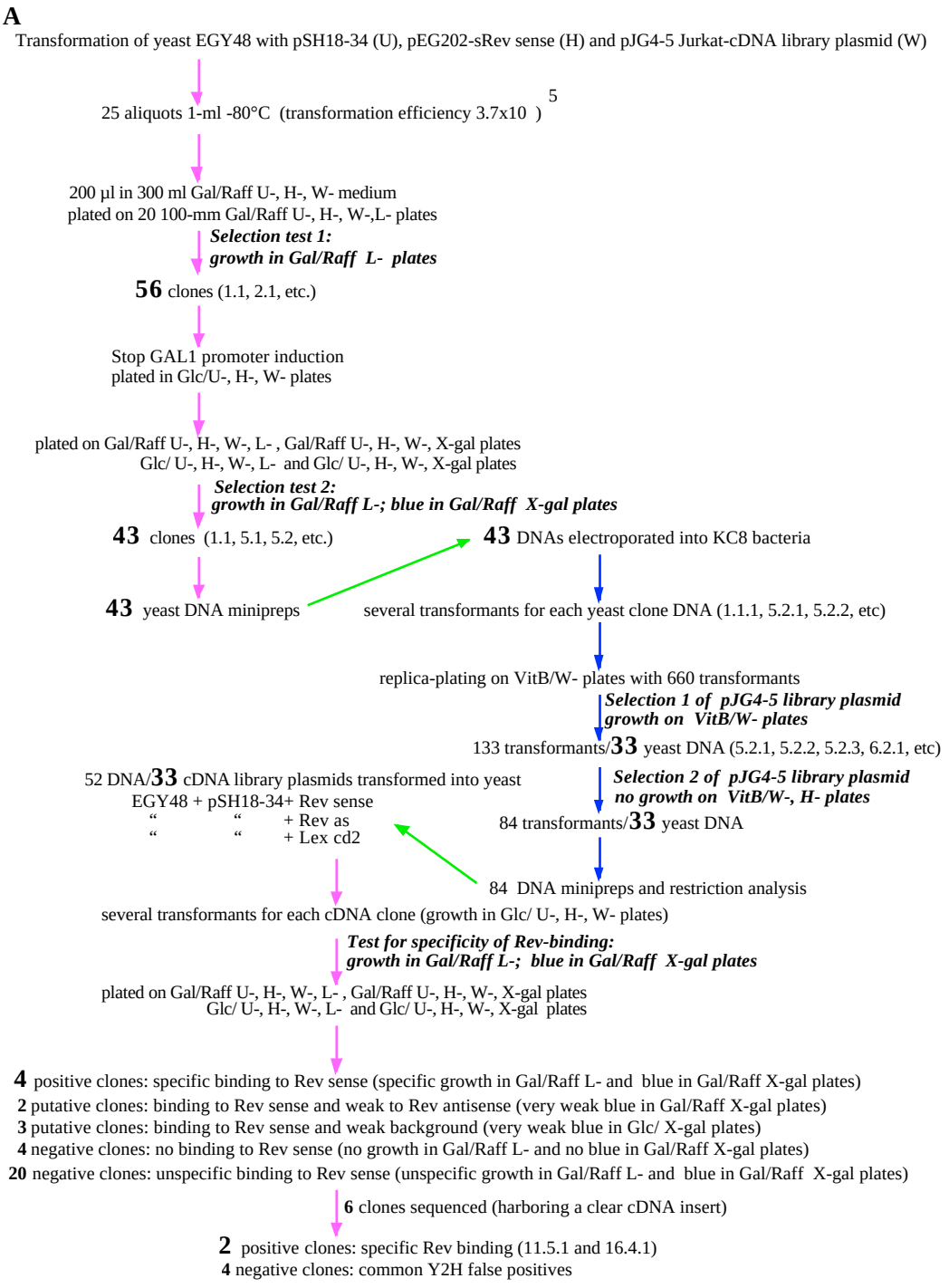
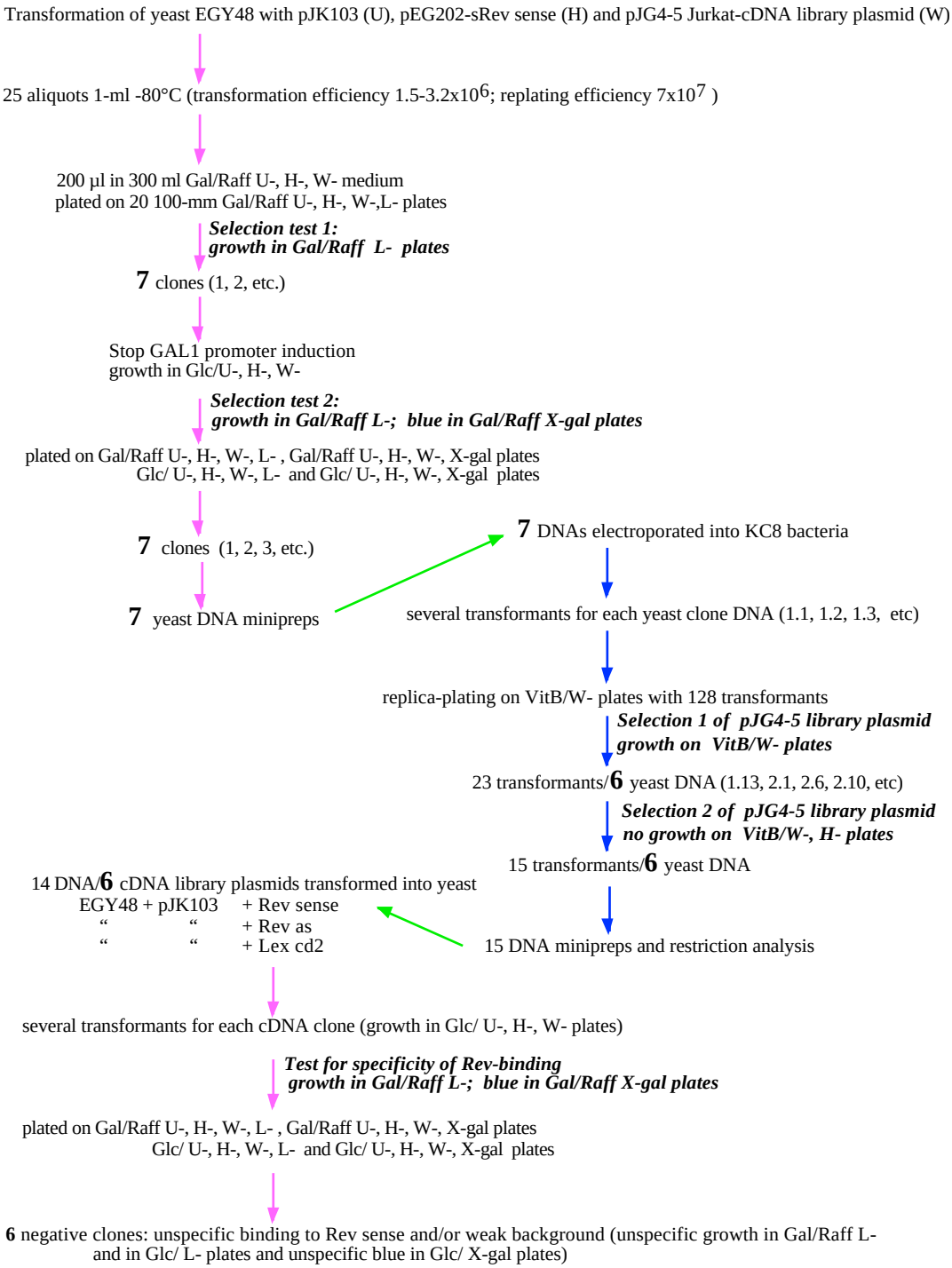


Fig. 3.1 Flow diagrams for the identification of two Rev-interacting cDNA-encoded proteins.

B


Transformation of yeast EGY48 with pJK103 (U), pEG202-sRev sense (H) and pJG4-5 Jurkat-cDNA library plasmid (W)
25 aliquots 1-ml -80°C (transformation efficiency 1.5-3.2x10^6; replating efficiency 7x10^7)
200 µl in 300 ml Gal/Raff U-, H-, W- medium
plated on 20 100-mm Gal/Raff U-, H-, W-,L- plates
Selection test 1:
growth in Gal/Raff L- plates
7 clones (1, 2, etc.)
Stop GAL1 promoter induction
growth in Glc/U-, H-, W-
Selection test 2:
growth in Gal/Raff L-; blue in Gal/Raff X-gal plates
plated on Gal/Raff U-, H-, W-, L- , Gal/Raff U-, H-, W-, X-gal plates
Glc/ U-, H-, W-, L- and Glc/ U-, H-, W-, X-gal plates
7 clones (1, 2, 3, etc.)
7 yeast DNA minipreps
7 DNAs electroporated into KC8 bacteria
several transformants for each yeast clone DNA (1.1, 1.2, 1.3, etc)
replica-plating on VitB/W- plates with 128 transformants
Selection 1 of pJG4-5 library plasmid
growth on VitB/W- plates
23 transformants/6 yeast DNA (1.13, 2.1, 2.6, 2.10, etc)
Selection 2 of pJG4-5 library plasmid
no growth on VitB/W-, H- plates
15 transformants/6 yeast DNA
15 DNA minipreps and restriction analysis
14 DNA/6 cDNA library plasmids transformed into yeast
EGY48 + pJK103 + Rev sense
“ “ + Rev as
“ “ + Lex cd2
several transformants for each cDNA clone (growth in Glc/ U-, H-, W- plates)
Test for specificity of Rev-binding
growth in Gal/Raff L-; blue in Gal/Raff X-gal plates
plated on Gal/Raff U-, H-, W-, L- , Gal/Raff U-, H-, W-, X-gal plates
Glc/ U-, H-, W-, L- and Glc/ U-, H-, W-, X-gal plates
6 negative clones: unspecific binding to Rev sense and/or weak background (unspecific growth in Gal/Raff L-
and in Glc/ L- plates and unspecific blue in Glc/ X-gal plates)

These results showed that the corresponding binding of Rev-LexA fusion protein to the LexA operators upstream of the reporter genes LEU2 and lacZ was not enough to activate their transcription. Therefore, it was concluded that the Rev protein is suitable as a bait protein for the yeast two-hybrid assay. One of the Rev sense, Rev antisense and LexCD2 expressing yeast clones, which didn't show any background activity, was chosen for further use.

Yeast strain	LacZ reporter	Bait plasmids
EGY48	pSH18-34	pEG202-sRev sense
"	"	pEG202-sRev antisense
"	"	pEG202-LexCD2
"	pJK103	pEG202-sRev sense
"	"	pEG202-sRev antisense
"	"	pEG202-LexCD2

Table 3.1 Various transformants of EGY48 yeast strain were created to identify new HIV-1 Rev-interacting factors.

To confirm expression of Rev protein each yeast strain harboring the bait plasmid was analyzed in a western blot. Therefore protein extracts of the yeast clones were separated on a SDS polyacrylamide gel. The gel was blotted and filter-bound protein detected with a polyclonal Rev-specific antibody (see 2.2.5. and 2.5.5.). As a control extracts from HeLa cells transfected with a Rev expression plasmid (pBsRev) were used.

Only yeast clones harboring Rev in sense orientation showed a clear band around 42 kDa corresponding to the Rev-LexA fusion protein.

3.1.2. Selection of Rev-interactors from library transformants

The interactor hunt was performed in two consecutive large plating steps. First, the pJG4-5 plasmid containing the human Jurkat T-cell cDNA library fused to the B42 activation domain (kindly provided by Dr. W. Kolanus) was introduced into both yeast selection strains EGY48/pSH18-34 and EGY48/pJK103 containing the pEG202-sRev sense plasmid. In order to obtain a high number of primary transformants expressing an individual library plasmid, each yeast strain was transformed in large scale in 30 different sterile tubes (see 2.4.5.). To check the transformation efficiency 2 times 40 µl of both yeast strains were used as described in 2.4.5.2. The transformation efficiency expressed as colony forming units (cfu) was $3.7x10^5$ for EGY48/pSH18-34 and 1.5-$3.2x10^6$ for EGY48/pJK103. The remaining

content of the 30 tubes was pooled, plated undiluted on eight 24x24-cm Glc/CM Ura-, His-, Trp- dropout plates and incubated at 30˚C for 2-3 days until colony growth could be detected. In this first plating step, glucose was used as a sugar source to allow growth of all yeast clones harboring the pJG4-5 plasmid. In the presence of glucose the inducible yeast GAL1 promoter that directs expression of cDNA-encoded proteins fused to the B42 activation domain is not activated. This allows amplification of all transformants with heterologous cDNAs. Therefore, cDNAs encoding proteins possibly toxic to the pJG4-5-containing yeast cells were not lost for the following interactor hunt. Primary transformants from each strain were collected in 25 ml sterile TE buffer, washed and resuspended in 1 vol glycerol solution and frozen in 1 ml aliquots at -70˚C (see 2.4.5.2.). Replating efficiency was determined. The cfu was $7x10^7$. In this second step the presence of galactose induces expression of activation-tagged cDNA proteins. To select for transcription of the LEU2 reporter gene, 200 µl of each transformed yeast strain were incubated O/N in 300 ml of Gal/Raff/CM Ura-, His-, Trp- medium. Cultures were plated on twenty 100mm Gal/Raff/CM Ura-, His-, Trp-, Leu- dropout plates and incubated at 30˚C.

After four days 7 clones from the EGY48/pJK103 strain and 56 clones from the EGY48/pSH18-34 strain were detected. These clones were collected on Gal/Raff/CM Ura-, His-, Trp-, Leu- master plates. Each clone was identified by a number (e.g. clone 1.1 = clone number 1 on plate number 1).

1.1	1.2	2.1	4.1	**5.1**	**5.2**	**6.1**	**6.2**	**6.3**	6.4
6.5	8.1	9.1	9.2	**10.1**	**10.2**	10.3	**11.1**	**11.2**	**11.3**
11.4	**11.5**	**11.6**	**12.1**	**12.2**	**13.1**	**13.2**	**14.1**	**14.2**	**14.3**
14.4	**14.5**	14.6	**14.7**	**15.1**	**15.2**	**15.3**	16.1	**16.2**	**16.3**
16.4	16.5	**16.6**	**17.1**	**17.2**	17.3	**17.4**	**18.1**	**18.2**	**18.3**
19.1	**19.2**	**19.3**	**19.4**	**20.1**	**20.2**				

Table 3.2 56 positive clones from the first and second selection test (EGY48 /pSH18-34 strain). Clones that grew in Gal/Raff/CM Ura-, His-, Trp-, Leu- and not in Glc/CM Ura-, His-, Trp-, Leu- plates and were blue on Gal/Raff/X-gal/CM Ura-, His-, Trp- and not on Glc/X-gal/CM Ura-, His-, Trp- plates are expressed in bold.

To confirm that the growth of these clones in the absence of leucine was actually due to expression of the activation-tagged cDNA proteins, a second test for galactose dependence was done. The GAL1 promoter was turned off by adding glucose to the medium and leucine selection eliminated before reinduction. For this purpose the Leu^+ clones were replated from the Gal/Raff/CM Ura-, His-, Trp-, Leu- dropout plates to 100-mm Glc/CM Ura-, His-, Trp- dropout plates and incubated at 30˚C until

colonies were growing. With all yeast clones replica plating was performed using different selection plates to investigate for LacZ expression and galactose dependence.

At this juncture 7 out of 7 and 43 out of 56 clones grew in the absence of leucine and showed X-gal conversion (=blue colonies) only in presence of galactose and not glucose as sugar source. The 43 positive clones are shown in bold in the Table 3.2.

3.1.3. Specificity test

Detection of galactose-dependent Leu^+ and $LacZ^+$ yeast cells demonstrated that the reporter genes were activated by expression of the activation-tagged cDNA-encoded protein. The next step was to examine that activation of the reporters was due to specific interaction of this cDNA-encoded protein with Rev, and not due to a non-specific interaction with LexA operators in the promoters, or with some part of the transcription machinery. To verify that the cDNA-encoded proteins were interacting specifically with Rev, library plasmids were isolated from the Leu^+ $LacZ^+$ yeast clones and selected in KC8 bacteria. The plasmids were reintroduced into the original selection strains EGY48/pSH18-34 and EGY48/pJK103 expressing Rev sense, Rev antisense as well as the unrelated bait LexCD2 to perform the final specificity test.

3.1.3.1. Isolation and classification of library plasmids

From each of the 7 (EGY48/pSH18-34) and 43 (EGY48/pJK103) positive yeast clones library plasmids were isolated by performing a rapid yeast plasmid preparation and the isolated DNA was transformed into competent KC8 bacteria by electroporation. KC8 is an E.coli strain that contains a mutation in the *trpC* gene (trpC-9830 mutation). The inability of these bacteria to grow in absence of tryptophan was used to select bacteria containing the library plasmid with the yeast TRP1 gene on the PJG4-5 plasmid. Transformed bacteria were isolated by plating on LB/Amp plates and incubated at 37˚C O/N.

Thirty-five of 43 yeast DNAs were successfully transformed, while 8 were not expressed in KC8 bacteria. Bacteria containing library plasmids were selected by growing on minimal plates supplemented with vitamin B, ampicillin, uracil, histidine, and leucine and lacking tryptophan. Out of 660 individual KC8 transformants plated on the selection plates lacking Trp, 133 transformants with 33 yeast (EGY48/pSH18-34 strain) plasmids grew on the plates. In order to obtain pJG4-5 library plasmid DNA in the absence of the pEG202-sRev sense plasmid a second selection test was performed. The 133 $KC8^+$ bacteria were replica plated on vitamin B plates lacking

tryptophan and histidine. Bacteria growing on plates in the absence also of histidine were expressing the HIS3 gene by the bait pEG202 plasmid and were excluded.
Eighty-four KC8 bacterial clones were selected by their disability to grow in absence of histidine. DNA was isolated and then analyzed by restriction with different enzymes. Each DNA clone was identified by a number (e.g. clone 5.2.2 = clone number 2 of original yeast clone number 5.2). Examples of the 84 $KC8^+$ clones are shown in the Table 3.3.
From the 7 yeast (EGY48/pJK103 strain) DNAs that were successfully transformed in KC8 bacteria, 128 individual transformants were analyzed in order to obtain pJG4-5 library plasmid DNAs. 23 KC8 transformants with 6 yeast DNAs grew on vitamin B, Amp, Ura, His, Leu, Trp-, and 15 KC8 bacteria were selected by not growing in plates lacking histidine. From these bacteria DNA was isolated, and analyzed by restriction enzyme digestion.

$Yeast^+$ clones	Growth in KC8				$KC8^+$ clones
	Transformants LB/amp	Master plate LB/amp	VitB/Trp-	VitB/Trp-, His-	
5.2	22	17	3 /17	1 /3	5.2.2
6.1	5	5	0	0	0
6.2	50	16	3 /16	3 /3	6.2.1, 6.2.2, 6.2.3
6.5	110	30	10 /30	7 /10	6.5.1, 6.5.2, 6.5.3, 6.5.5, 6.5.6, 6.5.7, 6.5.8
10.2	7	7	0	0	0
11.1	20	20	0	0	0
11.2	117	37	3 /37	3 /3	11.2.1, 11.2.2, 11.2.3

Table 3.3 Isolation of cDNA library plasmids. A set of DNA isolated from EGY48/pSH18-34/pEG202-sRev sense/pJG4-5-Jurkat-T-cDNA library yeast strain was transformed in KC8 bacteria. From each transformation group several KC8 transformants were obtained. A variable number of bacteria was tested by selection growth on plates lacking tryptophan and/or histidine to isolate the pJG4-5 cDNA-library plasmids. Examples of $KC8^+$ clones containing library plasmid are shown.

Each bacterial miniprep DNA was digested with EcoRI and XhoI enzymes to control the insert size of the library plasmids and to exclude possible identical cDNA inserts. Not every DNA preparations showed clear restriction fragments. However each yeast cDNA (33 from EGY48/pSH18-34 and 6 from EGY48/pJK103 strains) was represented by at least one KC8 DNA. DNAs that were used for the consecutive screening experiments are shown in the Table 3.4.

A

5.2.2	6.2.1	6.2.2	6.5.1	6.5.2	6.5.3	6.5.6	11.2.1	11.2.2
11.3.1	11.3.2	11.4.1	11.4.2	11.4.12	11.5.1	11.5.2	11.6.1	11.6.4
12.1.1	12.1.2	13.1.1	13.1.2	13.2.8	13.2.12	14.1.1	14.3.2	14.5.1
14.5.2	14.7.1	15.1.1	15.1.2	15.2.2	15.3.1	16.2.1	16.3.10	16.3.15
16.4.1	16.6.3	17.1.3	17.2.2	17.4.3	17.4.4	18.1.2	18.2.1	19.1.2
19.1.3	19.2.3	19.3.2	19.4.20	19.4.21	20.1.2	20.2.3		

B

1.13	2.1	2.6	2.10	3.6	3.7	4.1	4.2	4.3
5.3	5.10	5.17	7.12	7.14				

Table 3.4 DNA used to test specificity of Rev-binding. A) 52 KC8 clones harboring 33 cDNA library plasmids (EGY48/pSH18-34 strain); B) 14 KC8 clones harboring 6 cDNA library plasmids (EGY48/pJK103 strain).

3.1.3.2. Selection of positive yeast clones in the Rev-binding test

Spurious background was already removed by the previously described series of controls. In order to eliminate other classes of false positives the rescued library plasmid DNAs were retransformed in the original yeast strains containing the bait plasmids.

The 52 purified DNAs (Table 3.4 A) were separately transformed in:

EGY48/pSH18-34/pEG202-sRev sense
pEG202-sRev antisense
pEG202-LexCD2.

The 14 purified DNAs (Table 3.4 B) were separately transformed in:

EGY48/pJK103/ pEG202-sRev sense
pEG202-sRev antisense
pEG202-LexCD2.

Each transformation was plated on Glc/CM Ura-, His-, Trp- dropout plates and incubated at 30˚C for 2-3 days until colony growth was detected. Four to six independent colonies from each transformation plate were replated on Glc/CM Ura-, His-, Trp- master dropout plates and incubated O/N at 30˚C. With the same series of yeast clones replica plating was performed using the following different selection plates:

Gal/Raff/CM Ura-, His-, Trp-, Leu-
Glc/CM Ura-, His-, Trp-, Leu-
Gal/Raff/X-gal/CM Ura-, His-, Trp-

Glc/X-gal/CM Ura-, His-, Trp- plates.

For some clones LacZ activity was also/only analyzed using a sensitive and semi-quantitative dot-blot assay (see Mat and Meth.).
Clones were considered positive when growing without Leu and showing X-gal conversion only in the presence of galactose and not of glucose.

None of the 6 tested cDNA-encoded proteins expressed in the EGY48-pJK103 strains showed specific binding to Rev.
Some yeast clones showed growth or blue colonies also in the presence of the unspecific bait or with the Rev antisense bait. Other yeast showed a weak background on plates containing glucose.

Among the 33 cDNA encoded proteins expressed in EGY48-pSH18-34, 5 clones (two of which represented a same cDNA library plasmid) have been identified to bind specifically to Rev and 5 clones showed "putative" specific binding to Rev (Table 3.5).

The 5 positive clones grew in the absence of leucine and were blue only in plates containing galactose and in the presence of Rev in sense orientation. The 5 "putative" positive clones showed either very weak blue background on Glc/X-gal plates or very weak blue background in yeast expressing Rev in antisense on Gal/Raff/X-gal plates. As mentioned before, one or two DNAs for each cDNA library plasmid was used for the screening experiments. However, with the exception of the pair clones 11.5.1 and 11.5.2, the other pairs of KC8 DNAs representing the same cDNA encoded protein gave different results (Table 3.5).

Other 20 clones were considered as false positives by growing in the absence of leucine and showing blue color also in plates containing glucose and not only in the presence of Rev in sense orientation. The remaining 4 clones were considered as negative because in both specificity tests they were clearly negative.

Positive clones	Interaction with Rev	cDNA EcoRI/XhoI insert
6.2.1	putative/unspecific	~ 400 bp + 300 bp inserts
6.2.2	negative	ND
11.2.1	putative/unspecific	~ 1200 bp insert
11.5.1	clear/specific	~ 500 bp insert
11.5.2	clear/specific	ND
11.6.1	putative/unspecific	no insert
11.6.4	unspecific	ND
12.1.1	negative	ND
12.1.2	clear/specific	no insert
13.1.1	clear/specific	no insert
13.1.2	unspecific	ND
15.2.2	putative/unspecific	~ 300 bp + 100 bp inserts
16.3.15	putative/unspecific	~ 1400 bp insert
16.3.10	unspecific	ND
16.4.1	clear/specific	~ 800 bp insert

Table 3.5 Positive clones in the test for specificity of Rev-binding. In bold are shown clones that grew without leucine and showing X-gal conversion only in the presence of galactose. Only from the clear and putative positive clones DNA was isolated and then analyzed by restriction enzyme analysis.

Restriction analysis was performed with the DNA of 4 positive clones (the clone 11.5.2 was not analyzed, because considered identical to 11.5.1) and the 5 "putative" positive clones. Maxiprep DNA of each KC8 bacteria clone was digested with EcoRI/XhoI and AluI enzymes to analyze the insert size. After separation of EcoRI/XhoI digested DNA on a 2% agarose gel 2 out of the 4 positive clones and 1 out of the 5 "putative" positive clones did not show any insert (Table 3.5).

In summary, 6 yeast clones were isolated in the test for specificity of Rev-binding. Each contained an EcoRI/XhoI cDNA library insert encoding protein able to bind specifically (2 clones) and not clear specifically (4 "putative" clones) the HIV-1 Rev protein.

3.1.3.3. Sequence analysis of positive yeast clones

The 6 clones harboring a EcoRI/XhoI insert in the library plasmid were sent to a company (Medigene) to perform sequence analysis using the primers 20634 (Table 2.1). NCBI's BLAST search (see 2.6.) of available sequence databases suggested that

the “putative” positive clones could be considered as unspecific Rev-binding clones (Table. 3.6). In fact, their sequences were very similar to sequences of proteins generally indicated as common false positive in the yeast two-hybrid assay (Hengen, 1997). The clone 11.5.1 was identified to contain the cDNA for a previously reported nuclear single-strand DNA binding protein dbpB (Sakura et al., 1988). Regarding the clone 16.4.1, computer analysis using the non-redundant database of GenBank EST division revealed initially a 92% sequence identity with Soares fetal heart NbHH19W Homo sapiens cDNA 343275 5’ clone. Subsequently, BLAST search revealed a high identity (99%) with the C-terminal part of a human large brain cDNA clone for KIAA0592 protein (Nagase et al., 1998) and two other human cDNA clones for hypothetical proteins (accession number CAB45765 and CAB43380, submitted 1999). However, BLAST search failed to identify any proteins with known function with significant or partial homology to the predicted 16.4.1 protein sequence. Figure 3.2 shows the final comparison of 16.4.1 clone with previously reported genes. Figures 3.3 A and B show the alignment results of the two clones 11.5.1 and 16.4.1 with the corresponding database clones.

Clone	Interaction with Rev	cDNA bp	Sequence identity with	
6.2.1	putative/unspecific	680	ribosomal protein s7	100%
11.2.1	putative/unspecific	1260	heat shock protein	99.8%
11.5.1	clear/specific	935	h DNA binding protein dbpB	99.9%
15.2.2	putative/unspecific	420	zinc finger proteins	85%
16.3.15	putative/unspecific	1570	HLA-DR associated protein	100%
			phosphatase 2A inhibitor I2PP2a	100%
			h Set gene	99.3%
16.4.1	clear/specific	696	h Soares fetal heart clone 343275 5’	92%
			h male brain 3’end cDNA clone for KIAA0592 protein	99%

Table 3.6 Summary of BLAST search with the sequences of the positive clones interacting with Rev. The ribosomal proteins, zinc finger containing proteins, heat shock proteins are common false positives in the yeast two-hybrid system (Hengen, 1997).

In summary, using the yeast two-hybrid system, the screening of a Jurkat T-cell cDNA library led to the identification of two Rev-interacting cDNAs encoding proteins. Computer analysis of available sequence databases revealed a high percentage of identity (99.9%) with the C-terminal part of a previously reported human nuclear single-strand DNA binding protein dbpB to the clone 11.5.1 (Sakura et al., 1988).
*Clone 16.4.1 showed high sequence identity (99%) with the C-terminal part of a human large brain cDNA clone for KIAA0592 protein (Nagase et al., 1998) and with additional sequences representing subsections of KIAA0592 isolated from fetal heart (*clone 343275 5*'), adult testes (CAB45765) and from fetal kidney (CAB43380) tissues (Fig. 3.2). In addition, no other known proteins with significant or partial homology were identified.*

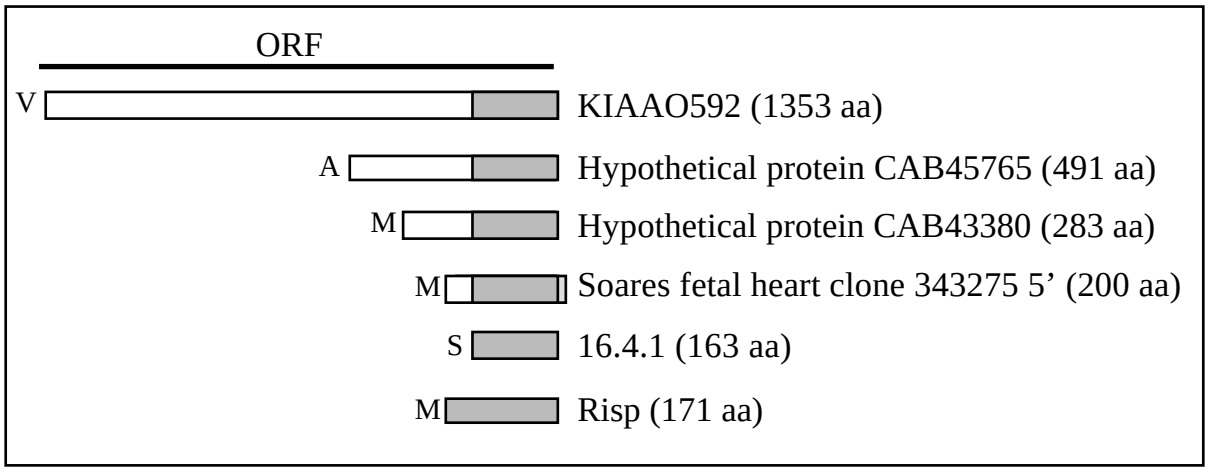


Fig. 3.2 Comparison of 16.4.1 clone with previously isolated genes with unknown function. Risp represents the 16.4.1 cDNA-encoded protein plus 8 amino acids missing from the N-terminal part of the Soares fetal heart clone. The single letters on front of each cDNA-encoded protein bar indicate the first coding amino acid. Dark gray shading represent the location of Risp fragment.

```
>_ 11.5.1 cDNA                                              935 nt vs.
>_ h DNA binding protein cDNA                              1452 nt
scoring matrix: , gap penalties: -12/-2
58.4% identity;         Global alignment score: 2196
                                                                  30
11.5.1 ----------------------------------------------------------CAA
                                                                  :::
_      .... TAACCATTATAGACGCTATCCACGTCGTAGGGGTCCTCCACGCAATTACCAGCAA
             550       560       570       580       590       600
              40        50        60        70        80        90
11.5.1 AATTACCAGAATAGTGAGAGTGGGGAAAAGAACGAGGGATCGGAGAGTGCTCCCGAAGGC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
_      AATTACCAGAATAGTGAGAGTGGGGAAAAGAACGAGGGATCGGAGAGTGCTCCCGAAGGC
             610       620       630       640       650       660
           100       110       120       130       140       150
11.5.1 CAGGCCCAACAACGCCGGCCCTACCGCAGGCGAAGGTTCCCACCTTACTACATGCGGAGA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
_      CAGGCCCAACAACGCCGGCCCTACCGCAGGCGAAGGTTCCCACCTTACTACATGCGGAGA
             670       680       690       700       710       720
           160       170       180       190       200       210
11.5.1 CCCTATGGGCGTCGACCACAGTATTCCAACCCTCCTGTGCAGGGAGAAGTGATGGAGGGT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
_      CCCTATGGGCGTCGACCACAGTATTCCAACCCTCCTGTGCAGGGAGAAGTGATGGAGGGT
             730       740       750       760       770       780
           220       230       240       250       260       270
11.5.1 GCTGACAACCAGGGTGCAGGAGAACAAGGTAGACCAGTGAGGCAGAATATGTATCGGGGA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
_      GCTGACAACCAGGGTGCAGGAGAACAAGGTAGACCAGTGAGGCAGAATATGTATCGGGGA
             790       800       810       820       830       840
           280       290       300       310       320       330
11.5.1 TATAGACCACGATTCCGCAGGGGCCCTCCTCGCCAAAGACAGCCTAGAGAGGACGGCAAT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
_      TATAGACCACGATTCCGCAGGGGCCCTCCTCGCCAAAGACAGCCTAGAGAGGACGGCAAT
             850       860       870       880       890       900
           340       350       360       370       380       390
11.5.1 GAAGAAGATAAAGAAAATCAAGGAGATGAGACCCAAGGTCAGCAGCCACCTCAACGTCGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
_      GAAGAAGATAAAGAAAATCAAGGAGATGAGACCCAAGGTCAGCAGCCACCTCAACGTCGG
             910       920       930       940       950       960
           400       410       420       430       440       450
11.5.1 TACCGCCGCAACTTCAATTACCGACGCAGACGCCCAGAAAACCCTAAACCACAAGATGGC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
_      TACCGCCGCAACTTCAATTACCGACGCAGACGCCCAGAAAACCCTAAACCACAAGATGGC
             970       980       990      1000      1010      1020
           460       470       480       490       500       510
11.5.1 AAAGAGACAAAAGCAGCCGATCCACCAGCTGAGAATTCGTCCGCTCCCGAGGCTGAGCAG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
_      AAAGAGACAAAAGCAGCCGATCCACCAGCTGAGAATTCGTCCGCTCCCGAGGCTGAGCAG
            1030      1040      1050      1060      1070      1080
           520       530       540       550       560       570
11.5.1 GGCGGGGCTGAGTAAATGCCGGCTTACCATCTCTACCATCATCCGGTTTAGTCATCCAAC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
_      GGCGGGGCTGAGTAAATGCCGGCTTACCATCTCTACCATCATCCGGTTTAGTCATCCAAC
            1090      1100      1110      1120      1130      1140
           580       590       600       610       620       630
11.5.1 AAGAAGAAATATGAAATTCCAGCAATAAGAAATGAACAAAAGATTGGAGCTGAAGACCTA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
_      AAGAAGAAATATGAAATTCCAGCAATAAGAAATGAACAAAAGATTGGAGCTGAAGACCTA
            1150      1160      1170      1180      1190      1200
           640       650       660       670       680       690
11.5.1 AAGTGCTTGCTTTTTGCCCGTTGACCAGATAAATAGAACTATCTGCATTATCTATGCAGC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
_      AAGTGCTTGCTTTTTGCCCGTTGACCAGATAAATAGAACTATCTGCATTATCTATGCAGC
            1210      1220      1230      1240      1250      1260
           700       710       720       730       740       750
11.5.1 ATGGGGTTTTTATTATTTTTACCTAAAGACGTCTCTTTTTGGTAATAACAAACGTGTTTT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
_      ATGGGGTTTTTATTATTTTTACCTAAAGACGTCTCTTTTTGGTAATAACAAACGTGTTTT
            1270      1280      1290      1300      1310      1320
           760       770       780       790       800        935
11.5.1 TTAAAAAAGCCTGGTTTTTCTCAATACGCCTTTAAAGGTTTTTAAATTGTTTCATAT…..
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
_      TTAAAAAAGCCTGGTTTTTCTCAATACGCCTTTAAAGGTTTTTAAATTGTTTCATAT…..
            1330      1340      1350      1360      1370      1452
```

Fig. 3.3 A Alignment between 11.5.1 and a section of the gene encoding human binding protein B. The stop codon is shown in green.

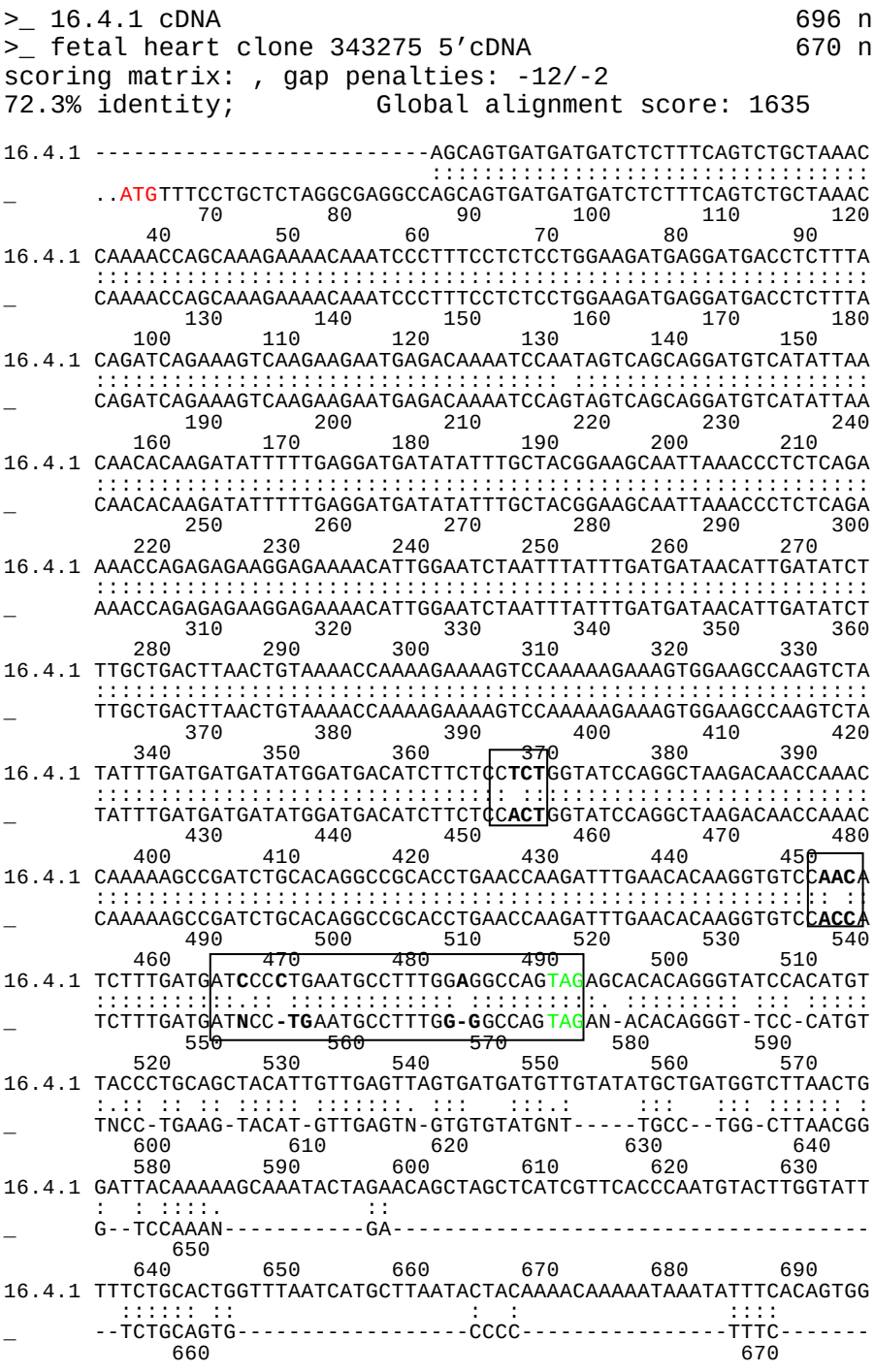


Fig. 3.3 B Alignment between 16.4.1 and 343275 5' clones. The different codons of the two clones are pointed out in bold letters and by boxes. The start and the stop codons are shown in red and green respectively.

3.2. Computer sequence data analysis of the 11.5.1 and 16.4.1 clones

After identification of the 4 "putative" unspecific Rev binding cDNAs as false positive clones, the 11.5.1 and the 16.4.1 cDNA sequences were analyzed in more

detail employing several computer programs. At the beginning of this analysis the cDNA clone for KIAA0592 protein had not yet been reported. Using FASTA and BLAST search it was difficult to identify any significant homology of the 16.4.1 clone with known proteins. However a very weak and partial homology appeared with some RNA-/DNA- binding proteins as shown in the table 3.7.

Protein			Similarity with 16.4.1 clone		
Name(source)	Size (aa)	Function	Location (aa)	%Identity	Score
RNA Helicase *a*	671	RNA binding	2-78	32.5%/77aa[a]	91
Cylicin *h*	598	spermatogenesis	4-119	24.6%/118aa	86
MECpGB-2 *h*	486	DNA binding	122-147	42.3%/26aa	76
Chitin synthase 3 *n*	960	glycosyltransferase	145-161	66.7%/18aa	74
MAP1.2 *m*	2464	microtubule binding	86-146	26.2%/61aa	74
U1 snRNP A *y*	298	RNA binding	34-158	20.5%/127aa	67
Nucleolin C23 *r*	713	DNA/RNA binding	8-53	28.3%/46aa	65
Nopp140 *r*	734	nucleolus shuttle	63-143	24.7%/81aa	66
Histone H5 *x*	196	DNA binding	68-134	22.9%/70aa	49
Fibrillarin *h*	321	RNA binding	16-27	54.5%/11aa	45

Table 3.7 Similarity of 16.4.1 clone to protein with known function. Initial letters were used to indicate the species source as (a) Arabidopsis Thaliana, (h) human, (m) mouse, (n) neurospora crassa, (r) rabbit, (y) yeast Saccharomyces cerevisiae, (x) Xenopus laevis. The similarity analysis was performed using local alignment program. Location indicates the corresponding region in 16.4.1 sequence. Score indicate the highest score obtained for each query protein and is correlated with the location and % identity. aa[a] indicates the number of amino acids overlapping.

3.2.1. BCM search launcher program

3.2.1.1. BEAUTY search results

One of the preferred programs was BEAUTY (BLAST Enhanced Alignment Utility) in the BCM search launcher. This program is an enhanced version of the NCBI's BLAST database search tool. It integrates multiple biological information resources into sequence similarity search results, offering detection of weak, but functionally significant, matches in BLAST database searches.

One of the databases used in the search was the CRSeqAnnot database. This database (78,469 sequences) contains full-length protein sequences with family and conserved region information. Other full-length sequences that contain at least one annotated domain/site are also listed.

Performing the query with the 11.5.1 cDNA-encoded protein, a considerable number of DNA-binding proteins with high scores were represented as shown in the Table 3.8.

However performing the query with the 16.4.1 cDNA-encoded protein, different proteins appeared on the resulting list. Unfortunately none of them was represented with high scores (max score was 61). Nevertheless the list contained shuttle proteins (nop140=nucleolus-cytoplasm shuttle phosphoprotein, nucleolin c23) and RNA-/DNA-binding proteins (u1 small nuclear ribonucleoprotein A, methyl-CPG-binding protein 2, etc) as shown in the Table 3.9.

Sequences producing High-scoring Segment Pairs:	Score	Smallest Sum Probability
Similar to dbpB/YB-1 of mouse	191	1.1e-38
CCAAT-binding transcription factor I subunit A - mouse	312	3.5e-36
DNA-binding protein B	**312**	**5.0e-36**
transcription factor YB-1 - Mouse	304	4.4e-35
Y box binding protein-1 (y-box transcription binding factor I)	296	5.7e-34
DNA-binding protein	251	8.4e-28
Nuclease sensitive element binding protein-1	251	8.5e-28
…etc…..		

Table 3.8 Beauty search results of 11.5.1 clone. Among the proteins proposed to share some similar function with the 11.5.1 clone it was shown with high score the DNA binding protein dbpB (written in bold).

Sequences producing High-scoring Segment Pairs:	Score	Smallest Sum Probability
M-phase inducer phosphatase (mitosis initiation)	49	0.045
Microtubule-associated protein 1B	40	0.87
Nopp140 protein A – rat nucleolus-cytoplasm shuttle phosphoprotein	50	0.95
Cylicin.	61	0.97
Methyl-CPG-binding protein 2 (MECP-2 protein)	54	0.999997
U1 small nuclear ribonucleoprotein A	37	0.999999
Minor capsid protein	56	1.000000
Initiation factor eIF-2 beta chain - rabbit	49	1.000000
Elongation factor 1 gamma, EF-1 gamma	55	1.000000
RNA-directed RNA polymerase (EC 2.7.7.48)/coat fusion protein	44	1.000000
Chitin synthase 2 (chitin-UDP acetyl-glucosaminyl transferase	54	1.000000
Nucleolin (protein C23).	51	1.000000
Histone H2B	41	1.000000
…etc…..		

Table 3.9 Beauty search results of 16.4.1 clone.

3.2.1.2. BLOCKS search results

A BLOCKS search (see 2.6.) with the sequence of the 11.5.1 and 16.4.1 clones was performed to receive more information on which family of proteins they could belong to.

However performing the query with both 11.5.1 and 16.4.1 cDNA-encoded proteins, few member of family proteins appeared on the resulting list and only with low scores, suggesting low significance of these results.

Using the program at different times, first in September 1997 and the second time in March 2000, different results were obtained. Probably this is due to a different number and subgroups of sequences representing the blocks, to a new version of the program and as well to the low scores obtained in both searches with both clones. Within the first search performed in 1997 the query was compared to 4754 block databases. Within the second search performed in the 2000 the query was compared to 10532 block databases.

10 possible hits reported

```
Alkylbase DNA glycosidases
Floricaula / Leafy protein
HIV Tat domain signature **
HIV Rev protein     ***
Mas20 protein import receptor signature
Ribosomal protein L2 proteins
Phosphatidylinositol transfer proteins
Polysaccharide deacetylase
Protamine P1 proteins
Synapsins proteins

**
TAT_HV1RH          50      KKRRQRRGPPQGSQTHQ
                              | |||||   |
     11.5.1        88      yRpRfRRGPPrQRQPRE
    ---------------------------------------------------
***
REV_HV1A2|P04623   26      NPPPSPEGTRQARRNRRRRWRERQRQIR
                                 |||  | ||  |||        |
11.5.1             14      gsesaPEGqAQQRRpyRRRRfppyymrR
```

Table 3.10 BLOCKS search results of 11.5.1 clone. List of hits founded in both searches (1997 and 2000).

7 possible hits reported

```
p53 tumor antigen proteins.
Plasmid replication protein
Flavivirus glycoprotein
Methyl-CpG binding domain
Heat shock hsp90 proteins family
General diffusion Gram-negative
AAA-protein family proteins.
```

Table 3.11 BLOCKS search results of 16.4.1 clone. List of hits founded in both searches (1997 and 2000).

Interestingly, among the hits proposed for the clone 11.5.1 appeared the NLS block of both HIV-1 Rev and Tat. Suggesting that the cDNA-encoded protein 11.5.1 contain an arginine-rich region with weak similarity with the NLS of both HIV-1 Rev and Tat proteins (Table 3.10).

Among the hits proposed for the clone 16.4.1 (Table 3.11), several proteins with DNA-binding function were listed. Confirming in some way the results already obtained by the BEAUTY search, which were suggesting weak similarity with some RNA-/DNA-binding proteins.

3.2.1.3. PROSITE pattern search results

To define the possible presence of already known motifs in 11.5.1 and 16.4.1 sequences a PROSITE pattern search using the 1994 EMBL Data Library based on the MacPattern program was performed.

Regarding the 171 aa in the 11.5.1 clone 5 hits were obtained (Table 3.12 A), suggesting one glycosylation, one amidation and three phosphorylation sites.
Regarding the 163 aa in the 16.4.1 clone 11 hits in the sequence were obtained (Table 3.12 B), suggesting one glycosylation, and ten phosphorylation sites.

A

1 match ASN-glycosylation	159: NSSA	
1 match PKC-phospho-site	4: TRR	
2 matches CK2-phospho-site	23: SAPE	161: SAPE
1 match Amidation	49: YGRR.	

B

1 match ASN-glycosylation	39: NETK				
5 matches PKC-phospho-site	9: SAK	70: SQK	96: TVK	103: SKK	129: TTK
5 matches CK2-phospho-site	1: SSDD	2: SDDD	45: SQQD	52: TTQD	111: SIFD

Table 3.12 PROSITE search results of 11.5.1 (A) and 16.4.1 (B) clones.

3.2.2. Alignment of 16.4.1 with already known NLSs and NESs

The results of the sequence analyses indicated that the 16.4.1 protein could be a member of a new family of nucleus-cytoplasm shuttle proteins involved in RNA-/DNA-binding. Active nuclear import and export of proteins are generally known to be mediated by signal-mediated transport. An increasing number of NLSs and NESs have been identified in the last years (see 1.2.). To identify the presence or possible similarities to one of these signals in the 16.4.1 cDNA-encoded protein, PSORT II

program search (see 2.6.) as well a direct alignment or local alignment with the more known NLS and NES was performed. PSORT II in fact is a web program that predicts sorting signals and localization sites (as cleavage sites, trans-membrane segments and nuclear localization signals) of proteins from their amino acid sequences.

Parts of the results are shown in Fig. 3.4 A and Fig.3.5 and suggest that the 16.4.1 sequence does not contain the "classical" signals known for mediating nucleus-cytoplasm transport. However the presence of a lysine-rich cluster ***KPKEKSKKKVEAK*** *at 98 aa position that could possibly function as NLS or nucleolus localization signal (NOS) was detected.*

A

```
NUCDISC: discrimination of nuclear localization signals NLS Score: -0.22
 PKEKSKK (3) at  99
PROSITE DNA binding and ribosomal protein motifs:  none
NNCN: Reinhardt's method for Cytoplasmic/Nuclear discrimination
Results of the k-NN Prediction        k = 9/23
      60.9 %: nuclear
      21.7 %: cytoplasmic
       8.7 %: cytoskeletal
       4.3 %: mitochondrial
       4.3 %: plasma membrane
>> prediction for QUERY is nuc (k=23)
```

B

```
NUCDISC: discrimination of nuclear localization signals NLS Score:  1.87
 RRRR (5) at   30 and 129
 RRRP (4) at  130
 PYRRRRF (5) at   28
 PRFRRGP (5) at   84
 PPQRRYR (3) at  117
 PQRRYRR (4) at  118
PROSITE DNA binding and ribosomal protein motifs:  none
NNCN: Reinhardt's method for Cytoplasmic/Nuclear discrimination
Results of the k-NN Prediction        k = 9/23
      65.2 %: nuclear
      13.0 %: cytoskeletal
       8.7 %: cytoplasmic
       4.3 %: Golgi
       4.3 %: plasma membrane
       4.3 %: vesicles of secretory system
>> prediction for QUERY is nuc (k=23)
```

Fig. 3.4 PSORTII search results of 11.5.1 (A) and 16.4.1 (B) clones.

Performing the PSORTII search with the amino acid sequence of 11.5.1 clone several arginine-rich signals were suggested on the resulting list (Fig. 3.4B). Confirming the results already obtained by the BLOCKS search, which were suggesting that the cDNA-encoded protein 11.5.1 contain an arginine-rich region with weak similarity with the NLS of both HIV-1 Rev and Tat proteins.

```
Results from Lalign  GeneStream
using matrix file : blosum50 , gap penalties: -14/-4

16.4.1 compared to classical basic NLSs
·  SV40 T antigen  pkkkrkv (monopartite NLS)          max score: 28
66.7 % identity in 6 aa overlap      score: 28
                  99
          16.4.1  PKEKSK
                  :::: :
          SV40    PKKKRK
66.7 % identity in 6 aa overlap      score: 26
                 102
          16.4.1  KSKKKV
                  :.:.::
          SV40    KKKRKV
·  Nucleoplasmin  krpaatkkagqakkkk  (bipartite NLS)      max score: 14
·  c-myc          paakrvkld                            max score: 14
·  Rev            nrrrrwre                             max score: 17
·  Tat            rkkrrqrrr                            max score: 22

16.4.1 compared to NESs (leucine-rich)
·  PKI            lalklagldi                           max score: 14
·  Rev            lpplerltld                           max score: 19
·  TFIIIA         slvldklti                            max score: 18
·  IkBa           qqlgqltlenl                          max score: 18
·  p53            mfrelnealel                          max score: 22

16.4.1 compared to other signals important for NLS and/or NES
·  Importin α rmrkfknkgkdtaelrrrrvevsvelrkakkdeqilkrrnv
             import signal (= Importin β-binding domain)   max score: 27
38.5 % identity in 13 aa overlap     score: 25
                   98
          16.4.1    KPKEKSKKKVEAK
                    : :.:.:  .: .
          Importin α KFKNKGKDTAELR
                    4
·  hnRNP protein A1 nqssnfgpmkggnfggrssgpyggggqyfakprnqggy
             import signal = export signal (M9)           max score: 29
23.5% identity in 17 aa overlap       score: 29
                   128
          16.4.1    KTTKPKSRSAQAAPEPR
                    ... : . ..:   .::
               M9   RSSGPYGGGGQYFAKPR
                     17
```

Fig. 3.5 Lalign GeneStream results of 16.4.1 clone. Bold letters label residues that have been shown to be particularly important for signal function. 16.4.1 contains a lysine-rich sequence.

3.2.3. Identification of a common motif with other Rev-interacting factors

3.2.3.1. Alignment of 16.4.1 with already known Rev-interacting factors

Additionally, possible similarities between the sequences of 16.4.1 and 11.5.1 cDNA-encoded proteins and sequences of already known Rev-interacting factors (Table 1.3) have been investigated using alignment programs.

A thorough analysis of the results showed that the 16.4.1 sequence exhibits a high percentage of identity with many other Rev-interacting factors in the same N-terminal region. Data are shown below.

```
Results from Lalign  GeneStream
using matrix file: blosum50 , gap penalties: -14/-4

·  16.4.1 compared to hCrm1 (1071 aa)                          max score: 40
          14
   16.4.1 PAKKTNPFPLLE
          : ..:: : ::.         50% identity in 12 aa overlap    score: 36
   Crm1   PEHRTNFFLLLQ
        842

·  16.4.1 compared to heIF-5A    (154 aa)                      max score: 37
          14
   16.4.1 PAKKTNPFPLLEDEDDLFT
          :  : : : :.  .:  .. 31.6% identity in 19 aa overlap  score: 35
   eIF-5A PNIKRNDFQLIGIQDGYLS
          82

·  16.4.1 compared to hp32 (278 aa)                           max score: 45
           8
   16.4.1 QSAKPKPAKKTNPFPLL
          :  .: :   : :: ::   47.1% identity in 17 aa overlap  score: 45
   p32    QLLQPAPRLCTRPFGLL
         26

·  16.4.1 compared to hRab/Rip (562 aa)                       max score: 72
           2
   16.4.1 SDDDLFQSAKPKPAKKTNPF
          :....  .: :. :.:::::   40% identity in 20 aa overlap score: 52
   Rab    STNPFVAAAGPSVASSTNPF
        432

·  16.4.1 compared to yNup42=yRip1p (430 aa)                   max score: 49
           1
   16.4.1 SSDDDLFQSAKPKPAKKTNPFPLLE
          .:... : .:.  :   :.::  :.   32% identity in 25 aa overlap
   Rip1p  NSNNSAFGAASNTPLTTTSPFGSLQ                          score: 49
        164
```

Fig. 3.6 Lalign GeneStream results of 16.4.1 clone. Bold letters label residues in the 16.4.1 sequence that are highly conserved in the alignment with other Rev-interacting proteins.

3.2.3.2. Definition of a new specific motif shared between several Rev-interacting factors

Based on these results two consensus motifs for the Rev-interacting factors (Crm1, eIF-5A, hRab/Rip, p32, yRip1p and 16.4.1) have been designed (Fig. 3.7). The common motif consists of 12 amino acids and includes 4 highly conserved residues. The consensus motif 2 was designed for maximal conservation. Even allowing one amino acid mismatch to each protein it appears stricter than motif 1 (Fig. 3.7). To analyze the specificity of the motif for the Rev-interacting factors, the two motifs were scanned against all available sequence databanks using two different programs: Find patterns available in GCG program and PATTINPROT search available in Network Protein Sequence (NPS) web site.

Identical results were obtained using both programs. Of all sequences analyzed (SwissProtPlus + PIR + SPTrembl = 514652 sequences), the only proteins identified as containing the motifs 1 and 2 were the known Rev-interacting factors plus the hypothetical proteins containing the 16.4.1 sequence (Table 3.13 and Table 3.14).

Pattern			mismatches
(PV)(AELNR)(KHILST)(KCRST)(TR)(NRS)(PDF)F(PGLQ)(LST)(LIN)(EAGQS)			0
Sequence databank	**n° proteins**	**matches**	**sequence**
SwissProtPlus (209668 seq.)	7:		
	1 h	Crm1	PEHRTNFFLLLQ
	3 h,c,r	eIF-5A	PNIRRNDFQLIG
	1 h	Rab	VASSTNPFQTNA
	1 h	Rip	VASSTNPFQTNA
	1 y	Nup42/yRip1p	PLTTTSPFGSLQ
PIR (107076 seq.)	7:		
	3 h,c,r	eIF-5A	PNIRRNDFQLIG
	1 h	p32	PRLCTRPFGLLS
	1 h	Rab	VASSTNPFQTNA
	1 h	Rip	VASSTNPFQTNA
	1 y	Nup42/yRip1p	PLTTTSPFGSLQ

Table 3.13 RESULTS using Find patterns in GCG program with pattern consensus motif 1. For some proteins more than one match was proposed for the same protein indicating different species source as human (h), chicken (c), rabbit (r), yeast (y).

Detection of a common 12 amino acids motif in the Rev-interacting proteins

h16.4.1	163 aa	^{14}**P**	A	K	K	**T**	**N**	P	**F**	P	L	L	E^{25}
hCrm1	1071 aa	^{842}**P**	E	H	R	**T**	**N**	F	**F**	L	L	L	Q^{853}
heIF-5A	154 aa	^{82}**P**	N	I	K	**R**	**N**	D	**F**	Q	L	I	G^{93}
hp32	278 aa	^{32}**P**	R	L	C	**T**	**R**	P	**F**	G	L	L	S^{43}
hRab/Rip	562 aa	^{444}**V**	A	S	S	**T**	**N**	P	**F**	Q	T	N	A^{455}
yRip1p	430 aa	^{177}**P**	L	T	T	**T**	**S**	P	**F**	G	S	L	Q^{188}

Consensus motif 1	**[PV]**[AELNR][KHILST][KCRST]	**[TR]**	**[NRS]**	[PDF]	**F**	[PGLQ]	[LST]	[LIN]	[EAGQS]
Consensus motif 2	**P** [AELNR][KHILST][KCRST]	**T**	**N**	[PD]	**F**	[GLQ]	[LST]	[LIN]	[EAGQS]

Fig. 3.7 Primary sequence of the minimal motif domain of selected Rev-interacting factors. The common motif consists of 12 amino acids and includes 4 highly conserved residues (shown in bold). Consensus motif 1 is based on the presence of all amino acids included in each protein domain. Consensus motif 2 allows one mismatch in each protein sequence. The amino acids corresponding to the mismatches are underlined.

Pattern	mismatches
P (AELNR)(KHILST)(KCRST) T N (PD) F (GLQ)(LST)(LIN)(EAGQS)	1

Sequence databank	n°proteins	matches	sequence
SwissProtPlus/ SPTrembl (407576 seq.)	7 Rev-interacting proteins + 3 proteins containing 16.4.1 sequence:		
	1 h	Crm1	PEHRTN**f**FLLLQ
	3 h,c,r	eIF-5A	PNIR**r**NDFQLIG
	1 h	Rab	**v**ASSTNPFQTNA
	1 y	Nup42/yRip1p	PLTTT**s**PFGSLQ
	1 h	p32	PRLCT**r**PFGLLS
	1 h *	KIAA0592 prot.	PAKKTNPF**p**LLE
	1 h *	Hypothet.30.5kDa	PAKKTNPF**p**LLE
	1 h *	Hypothet.53.3kDa	PAKKTNPF**p**LLE

Table 3.14 RESULTS using PATTINPROT with pattern consensus motif 2. For some proteins more than one match was proposed, indicating different species source as human (h), chicken (c), rabbit (r), yeast (y). The amino acids corresponding to the mismatches are expressed in small letters and in bold. The three matches with * are proteins containing the 16.4.1 sequence (Fig.3.2).

Query patterns containing partial modifications of one or more amino acids in the consensus sequence led to isolate the Rev-interacting proteins plus a variable increasing number of different proteins (Table 3.15).

Nevertheless, among all the Rev-interacting proteins, importin β, B23 and the two recently identified Rev-interacting nucleoporins Nup98 and NLP-1 were not present in this consensus motif. With the exception of Nup98, these proteins did not show a high percentage of identity with the motif sequence of the N-terminal region of 16.4.1. Therefore, the similarities of the 16.4.1 sequence (PAKKTNPFPLLE) with the importin β, B23, and NLP-1 protein sequences have been investigated with local alignment program. Each sequence showing the highest similarity with 16.4.1 motif has been used to create new motifs allowing the alignment of the additional proteins (Fig. 3.8). Moreover, since Rev itself is a Rev-binding protein, Rev similarity with the 16.4.1 sequence (PAKKTNPFPLLE) has been investigated and a Rev-modified motif has been created. Moreover, a modification of the consensus motif allowing the alignment of other proteins Rev-related such as Sam68 (Reddy et al., 1999) (see paragraph 3.6.), or 16.4.1-related such as Nopp140 or fibrillarin protein has also been investigated. The subsequent screening analysis among all the sequences available in the database, using the Nup98- or Rev-modified motif led to identify the already previously selected Rev-interacting proteins plus the Nup98 protein or Rev itself.

Rev-interacting motif		P [AELNR][KHILST][KCRST]T	N [PD]	F [GLQ] [LST] [LIN] [EAGQS]	Score
hNup98	920 aa	894**P** **A** **S** q **T**	t **P** l	**Q** m a l905	39
HIV-1Rev	116 aa	21f **L** y q s	**N** **P** p	p n p **E**32	27
hImportinβ	876 aa	306**P** p e h **T**	s k	**F** y a k **G**317	23
hNLP1	423 aa	95f g **L** **S** e	**N** **P**	**F** a **S** **L** **S**106	26
hSam68	443 aa	68**P** p **S** a **T**	g **P** d	a **T** v **G**79	37
rNopp140	703 aa	278**P** **L** **K** **K** **T**	a **P** k	k q v v289	52
hB23	294 aa	85**P** t v **S** l	g g	**F** e i t p96	22
hFibrillarin	321 aa	204a k **K** **R** **T**	**N** i	i p v **I** **E**215	45
Motif Nup98-modified		**P** [AELNR][KHILST][KCqRST] **T**	**N**t [PD]	**F** [GLQ] [LmST][LaIN][EAGlQS]	a+0
Motif Rev-modified		**P**f[AELNR][KHILSTy][KCqRST]**T**s	**N** [PD]	**F** [GLpQ][LnST][LINp][EAGQS]	+0
Motif Imp.β-modified		**P**[AELNpR][KeHILST][KChRST]**T**	**N** [PDk]	**F** [GLQy][LaST][LkIN][EAGQS]	+1#
Motif NLP1-modified		**P**f [AEgLNR][KHILST][KCRST]**T**	**N** [PD]	**F** [GaLQ] [LST][LIN] [EAGQS]	+1*
Motif Sam68-modified		**P** [AELpNR][KHILST][KaCRST]**T**	**N**g [PD]	**F** [GaLQ] [LST][LINv][EAGQS]	+2*″
Motif Nopp140-modified		**P** [AELNR][KHILST][KCRST] **T**	**N**a [PD]	**F** [GkLQ][LSTq][LINv][EAGQSv]	+2
Motif B23-modified		**P** [AELNRt][KHILSTv][KCRST]**T**	**N**g [PDg]	**F** [GeLQ][LiST][LINt][EAGpQS]	+3″
Motif Fibr.-modified		**P** [AEkLNR][KHILST][KCRST] **T**	**N** [PDi]	**F**i [GLpQ][LSTv][LIN] [EAGQS]	+4

Fig. 3.8 Modification of the Rev-interacting motif allowing the presence of additional Rev-interacting and non-related proteins. For each protein the region with highest similarity with the 16.4.1 motif sequence PAKKTNPFPLLE was selected. Score regards the alignment of the sequence with 16.4.1 full-length or PAKKTNPFPLLE sequence. The amino acids conserved in the motif are shown in bold, while in small letters are shown the additional amino acids. In each protein sequence one mismatch is allowed. The amino acids corresponding to the mismatches are underlined. [a] indicates the number of unrelated proteins selected in addition to the Rev-interacting proteins and the query protein using the Pattinprot search among the Swissprot and Sptrembl 456338 sequences.
#= Hypothetical 31.3 kDa protein (NPSA sptr||Q9X6Z7). * = Putative lipoprotein periplasmic GNA2132. ″ = Adenyl cyclase.

Pattern	mismatches	n°proteins	matches	sequence
P(AELNR)(KHILST)(KCRST)T N(PD)F(GLQ)(LST)(LIN)(EAGQS)	**2**	35:	10 Rev-interacting proteins (RIPs) + 25 different proteins:	
			Rab-R protein	PL**p**STNPFQ**p**NG
			Capsid protein VP1	**g**LTS**i**NDFGTLA
			Adenyl cyclase	PESST**g**D**t**LTNS
			…etc.	
P **X** **X** **X** T N(PD) F(GLQ)(LST)(LIN)(EAGQS)	1	25:	10 RIPs + 15 different proteins:	
			CTD-binding SR-like prot.RA8	PPNVTNP**a**GLLG
			TDC operon trans. activator	PCDMT**s**PFGSN
			…etc.	
P **X** **X** **X** T N(PD) F(GLQ)(LST)(LIN) **-**	1	65:	10 RIPs + 45 different proteins:	
			RNA binding protein	PARST**t**PFLLN
			RNA polymerase	PTSL**l**NDFLSI
			RNA replicase polyprotein	PKGK**r**NPFLTL
			ATP-binding cassette transp.	PYSHTNDFLS**s**
			Gag polyprotein	PANK**n**NPFQSL
			DNA mismatch repair prot.MSH2	PQGP**q**NPFGSN
			…etc.	
- **X** **X** **X** T N(PD)F(GLQ)(LST)(LIN)(EAGQS)	1	334:	10 RIPs + 324 different proteins:	
-(AELNR)(KHILST)(KCRST)T N(PD)F(GLQ)(LST)(LIN)(EAGQS)	1	19:	10 RIPs + 9 different proteins	
P(AELNR)(KHILST)(KCRST)**X** N(PD)F(GLQ)(LST)(LIN)(EAGQS)	1	15:	10 RIPs + 5 different proteins	
P(AELNR)(KHILST)(KCRST)T **X**(PD)F(GLQ)(LST)(LIN)(EAGQS)	1	16:	10 RIPs + 6 different proteins	
P(AELNR)(KHILST)(KCRST)T N(PD)**X**(GLQ)(LST)(LIN)(EAGQS)	1	19:	10 RIPs + 9 different proteins	
-(AELNR)(KHILST)(KCRST)**X** **X**(PD)**X**(GLQ)(LST)(LIN)(EAGQS)	**0**	983		
-(AELNR)(KHILST)(KCRST)**X** **X**(PD)**X**(GLQ)(LST)(LIN)(EAGQS)	**1**	>5000		

Table 3.15 Results using PATTIMPROT. Different query pattern with one or more amino acids modified in the consensus motif 2 was used for each search. 0, 1 or 2 mismatches were allowed. The amino acids modified in the query are expressed by a wild card bold and underlined. The amino acids corresponding to the mismatches are expressed in small letters and in bold.

The screening among the database of the importin β- or NLP-1-modified motif led to identify the Rev-interacting proteins, comprising importin β or NLP-1 and the addition of only 1 protein (Fig. 3.8). On the contrary, the screening of B23-, Sam68-, Nopp140-, or fibrillarin-modified motif led to select in addition to the Rev-interacting proteins a variable number (2-4) of other proteins (Fig. 3.8). As a control, Rev-unrelated proteins such as glial fibrillary acid protein (GFAP) or fibronectin were also tested in the motif alignment. The screening of GFAP- or fibronectin-modified motif led to select in addition to the Rev-interacting proteins and GFAP or fibronectin, 5 and 13 additional proteins respectively (data not shown).

In summary, computer sequence analysis of 11.5.1 clone suggested that the 11.5.1 cDNA-encoded protein could belong to a transcription factors family, probably as C-terminal part of the DNA binding protein dbpB. In addition, the presence of four separated arginine-rich regions, some of which have a weak similarity with the HIV-1 Rev and Tat NLS, suggested the presence in the 11.5.1 clone of arginine-rich NLS.
Computer sequence analysis of the 16.4.1 cDNA encoded protein showed weak and partial homology with some RNA-/DNA-binding proteins, some of which show a nucleocytoplasmic activity. The presence of several serines and threonine residues suggested ten hypothetical phosphorylation sites. Although no known signals for mediating the nucleus-cytoplasm transport were recognized, the presence of a lysine-rich cluster, with hypothetical NLS or NOS function was observed. Moreover, several Rev-interacting proteins, such as Crm1, eIF5-A, hRab/Rip, yRip1p and p32, showed a high homology with the N-terminal region of 16.4.1. Based on these results a consensus motif consisting of 12 amino acids and 4 highly conserved residues was proposed. This motif has been shown to be highly specific. Scanning it against all available sequence databanks (407576 sequences analyzed), the only proteins identified to contain that motif were the above mentioned Rev-interacting proteins (and Rev itself).

3.3. 16.4.1 expression analysis

The initial results indicated that only the clone 16.4.1 (and not the 11.5.1) shared a common motif with other already known Rev-interacting factors. Moreover, the 16.4.1 cDNA encodes a protein with an unknown function, so that further studies were concentrated on this clone. As described above, at the beginning the highest similarity of the sequence of 16.4.1 cDNA was identified in the non-redundant database of GenBank EST division with the Soares fetal heart NbHH19W Homo sapiens cDNA clone 343275 5'. Differences between the two sequences were found in both C- and N-terminal regions. The cDNA-translated sequence of the clone 343275 5' contains 8 amino acids more in the N-terminal region and several amino

acids more in the C-terminal region than the cDNA-translated from the 16.4.1 sequence (Fig. 3.9). Alignment of the two cDNA sequences (Fig. 3.3) showed that only few bases were different and that two nucleotides were missing in the EST-clone. The absence of these two nucleotides led to a frame shift in the 343275 5'-ORF, eliminating the stop codon present in the sequence of 16.4.1 at position 490 bp. The 16.4.1 clone was thought to be part of the full-length cDNA expressed by the 343275 5' clone in the EST database. It is known that often the sequences present in the GenBank EST division are not very precise especially at the 3' end.

Therefore a new version of 16.4.1 DNA was created to encode a new protein (Fig. 3.9) containing the "missing N-terminal part" (8 amino acids) of the Soares fetal heart cDNA-encoded protein and terminating with the C-terminal part of 16.4.1. To investigate the expression of this new Rev-interacting protein in the cell, analysis of the gene, and expression on RNA and protein level were performed.

- **Soares fetal heart NbHH19W Homo sapiens clone 343275 5'** **200 aa**
- **16.4.1** **163 aa**

- MFPALGEA**SSDDDLFQSAKPKPAKKTNPFPLLEDEDDLFTDQKVKKNETKS**S**SQQDVILTTQDI**
- -------**SSDDDLFQSAKPKPAKKTNPFPLLEDEDDLFTDQKVKKNETKS**N**SQQDVILTTQDI**

- **FEDDIFATEAIKPSQKTREKEKTLESNLFDDNIDIFADLTVKPKEKSKKKVEAKSIFDDDMDD**
- **FEDDIFATEAIKPSQKTREKEKTLESNLFDDNIDIFADLTVKPKEKSKKKVEAKSIFDDDMDD**

- **IFS**T**GIQAKTTKPKSRSAQAAPEPRFEHKVS**T**IFDDP**ECLWGQZNTGFPCSZSTCZVCVCCLAZRVQ
- **IFS**S**GIQAKTTKPKSRSAQAAPEPRFEHKVS**N**IFDDP**LNAFGGQ*

- RSAVPL

- **16.4.1 artificial protein = Risp** **171 aa**

MFPALGEA**SSDDDLFQSAKPKPAKKTNPFPLLEDEDDLFTDQKVKKNETKS**N**SQQDVILTTQDIFE**
DDIFATEAIKPSQKTREKEKTLESNLFDDNIDIFADLTVKPKEKSKKKVEAKSIFDDDMDDIFSS
GIQAKTTKPKSRSAQAAPEPRFEHKVSNIFDDPLNAFGGQ*

Fig. 3.9 Sequences of 16.4.1 and its correlates. Identical amino acids presents in 16.4.1 and clone 343275 5' of the EST database are indicated in bold. * indicates the stop codon. As described in 3.5.4., 16.4.1 artificial protein fused to GFP is a nucleo-cytoplasmic shuttle protein. Therefore it was termed Risp (=Rev-interacting shuttle protein).

3.3.1. Analysis of the 16.4.1 gene across species

A *16.4.1* DNA was used to probe a positively charged nylon membrane (Clontech´s ZOO-BLOT) containing EcoRI-digested genomic DNAs derived from 9 eukaryotic species: human, monkey (Rhesus), rat (Sprague-Dawley), mouse (Balb/c), dog, cow, rabbit, chicken and yeast (*Saccharomyces cerevisiae*) (see 2.3.7.). The *16.4.1* DNA probe was amplified by PCR from pcRisp-sg143 using the primers 17746 and 17747

(Table 2.1), purified and [α^{32}P]-labeled with [α^{32}P]-dCTP using the random prime labeling system of Pharmacia (see 2.3.6.5.2.). After O/N hybridization at 65˚C the hybridized filter was washed under non-stringent conditions for detection of genomic sequences with low homology. Three days exposure in the Fuji cassette at RT led to detection of several bands. Although some bands were stronger (mouse, dog), others weak (rat, cow) and some missing (rabbit, yeast), we concluded however *16.4.1* gene is conserved across different species (Fig. 3.10 A). Moreover, since several large EcoRI-fragments are shown in the filter, this suggests that the 16.4.1 sequence could be part of a larger gene.

3.3.2. Gene expression in human cells

To analyze 16.4.1 expression RNA from different human cells was prepared (see 2.3.6.1.). The following tumor cell lines were used: Jurkat T-cells (the source of the yeast two-hybrid cDNA library), HeLa, HEK 293, U87, U138 and 85HG66. In addition, primary human astrocytes and microvascular endothelial cells were used. Ten µg of total RNA were transferred by Northern blot to Zeta-probe GT nylon membranes (see 2.3.6.3). A *16.4.1* DNA amplified by PCR with primers 17357 and 17358 (see Table 2.1) and purified was [α^{32}P]-labeled. The filter was hybridized O/N at 65˚C and then was washed (as described in 2.3.6.4.). In every sample a weak unique band a little bit lower than the 28S rRNA was observed, after 5 hours of exposure at RT suggesting a size for the cDNA around 4.6-5 kb (Fig. 3.10 B). The same weak band was observed after 7 days exposure with a x-ray film at – 80˚C.
In addition as a positive control for RNA levels, the filter was stripped (see 2.3.6.4.) and re-hybridized with a probe for a housekeeping gene (GAPDH [α^{32}P]-labeled probe freshly prepared). After O/N exposure at RT a strong unique band lower than the 18S rRNA around 1.6 kb was observed in every RNA sample.
Based on these results, it appeared that the mRNA expressed in the cells and recognized by *16.4.1* probe was larger than the 343275 cDNA sequence in the EST-database. In fact a more recent BLAST search showed that 16.4.1 cDNA could be the C-terminal part of the KIAA0592 clone (4623 bp). This clone lacks a methionine codon at the 5'end (Fig. 3.11) indicating that the sequence is incomplete. Therefore the results indicated that 16.4.1 is expressed in the context of an mRNA 4.6-5 kb in length. Moreover, the weak signals of mRNA recognized by the probe leads to the suggestion that the constitutive expression of this 16.4.1/unknown gene is not very high in cells growing under normal conditions.

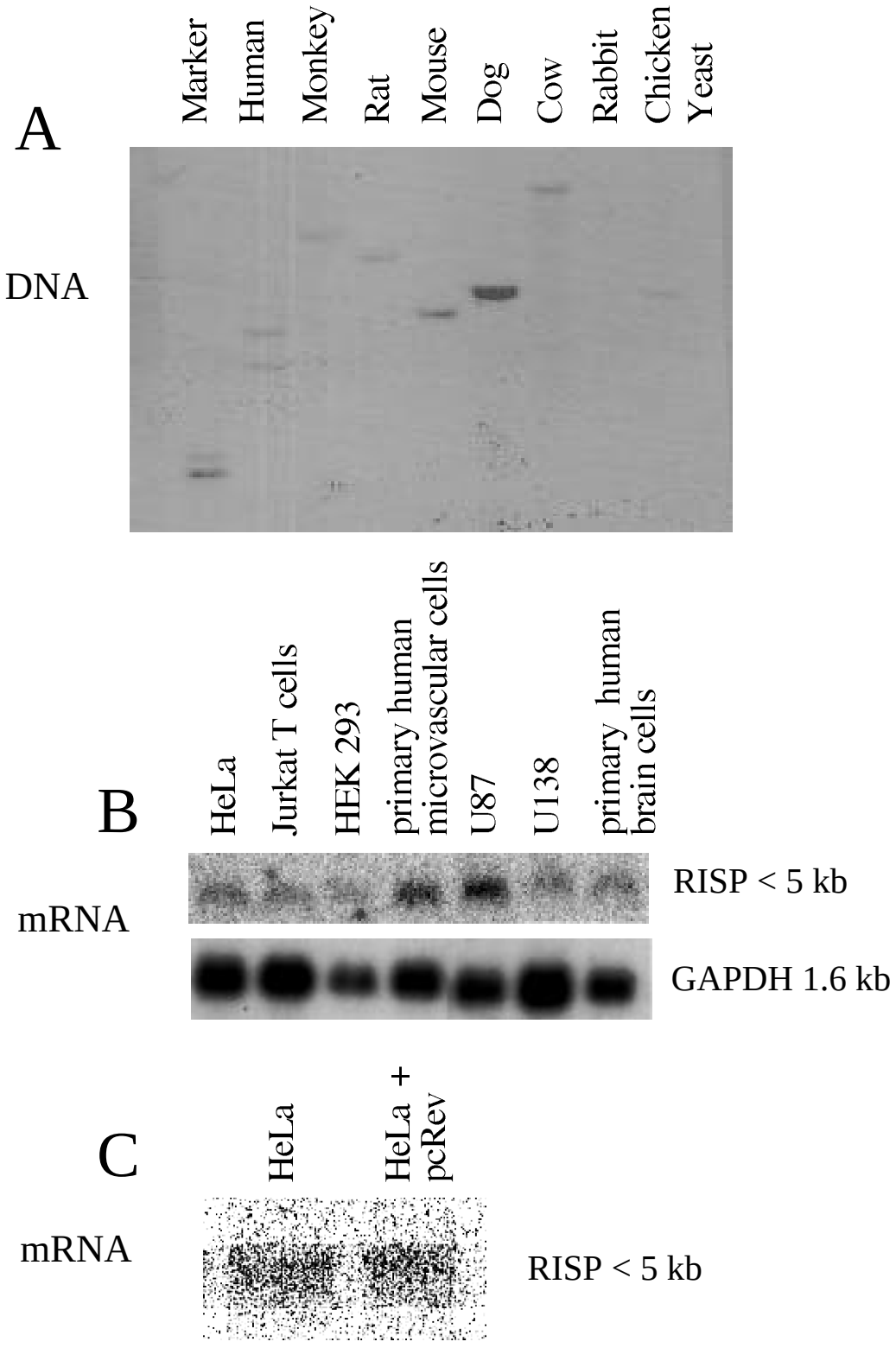


Fig. 3.10 Analysis of *16.4.1* gene expression. (A) Southern Blot analysis of genomic DNAs extracted from the indicated species (Zoo-Blot from Clontech). (B and C) Northern Blot analysis of total RNA extracted from the indicated human cells. For both analysis a *16.4.1* [α32P]-labeled probe was used.

KIAA0592 **1353 aa**

VTPRAAWLGLGFRGSAVLGLCWQPRSPPSRAAGMMNRTTPDQELVPASEPVWERPWSVEEIRRS
SQSWSLAADAGLLQFLQEFSQQTISRTHEIKKQVDGLIRETKATDCRLHNVFNDFLMLSNTQFI
ENRVYDEEVEEPVLKAEAEKTEQEKTREQKEVDLIPKVQEAVNYGLQVLDSAFEQLDIKAGNSD
SEEDDANGRVELILEPKDLYIDRPLPYLIGSKLFMEQEDVGLGELSSEEGSVGSDRGSIVDTEE
EKEEEESDEDFAHHSDNEQNQHTTQMSDEEEDDDGCDLFADSEKEEEDIEDIEENTRPKRSRPT
SFADELAARIKGDAMGRVDEEPTTLPSGEAKPRKTLKEKKERRTPSDDEEDNLFAPPKLTDEDF
SPFGSGGGLFSGGKGLFDDEDEESDLFTEASQDRQAGASVKEESSSSKPGKKIPAGAVSVFLGD
TQVFGAASVPSLKEPQKPEQPTPRKSPYGPPPTGLFDDDDGDDDDDFFSAPHSKPSKTRKVQST
ADIFGDEEGDLFKEKAVASPEATVSQTDENKARAEKKVTLSSSKNLKPSSETKTQKGLFSDEED
SEDLFSSQSASNLKGASLLPGKLPTSVSLFDDEDEEDNLFGGTAAKKQTLSLQAQREEKAKASE
LSKKKASALLFSSDEEDQWNIPASQTHLASDSRSKGEPRDSGTLQSQEAKAVKKTSLFEEDKED
DLFAIAKDSQKKTQRVSLLFEDDVDSGGSLFGSPPTSVPPATKKKETVSEAPPLLFSDEEEKEA
QLGVKSVDKKVESAKESLKFGRTDVAESEKEGLLTRSAQETVKHSDLFSSSSPWDKGTKPRTKT
VLSLFDEEEDKMEDQNIIQAPQKEVGKGCDPDAHPKSTGVFQDEELLFSHKLQKDNDPDVDLFA
GTKKTKLLEPSVGSLFGDDEDDDLFSSAKSQPLVQEKKRVVKKDHSVNSFKNQKHPESIQGSKE
KGIWKPETPQANLAINPAALLPTAASQISEVKPVLPELAFPSSEHRRSHGLESVPVLPGSGEAG
VSFDLPAQADTLHSANKSRVKMRGKRRPQTRAARRLAAQESSEAEDMSIPRGPIAQWADGAISP
NGHRPQLRAASGEDSTEEALAAAAAPWEGGPVPGVDTSPFAKSLGHSRGEADLFDSGDIFSTGT
GSQSVERTKPKAKIAENPANPPVGGKAKSP**MFPALGEASSDDDLFQSAKPKPAKKTNPFP**
LLEDEDDLFTDQKVKKNETKSSSQQDVILTTQDIFEDDIFATEAIKPSQKTREKEK
TLESNLFDDNIDIFADLTVKPKEKSKKKVEAKSIFDDDMDDIFSSGIQAKTTKPKS
RSAQAAPEPRFEHKVSNIFDDPLNAFGGQ

Fig. 3.11 Sequences of KIAA0592. The amino acids present also in Risp are shown in bold. Phenylalanine residues expressed as dipeptide motif FG (8) and FS (13), as well as runs of serine are indicated (14).

To analyze a possible effect of Rev on the mRNA expression of this unknown gene, RNA was extracted from HeLa and the astrocytoma cells 85HG66 non transfected and transfected with pcRev. The RNA was blotted and analyzed as described above. After 5 days of exposure in the Fuji cassette in every sample, a weak band a little bit lower than the 28S rRNA was detected and no quantitative differences were observed between transfected and non-transfected cells (Fig. 3.10 C). Since similar levels of 28S rRNA expression were detected for each RNA preparation, an additional positive control for RNA levels was not used. However a second hybridization with a different 16.4.1 DNA probe and different washing conditions showed the presence of two additional strong bands in the Rev-transfected cells around the 18S rRNA and one larger than the 28S rRNA (data not shown).

For the analysis of protein production, several rat monoclonal antibodies against Risp were prepared. To achieve this, the *risp* gene was cloned into the pGEX-3X vector to allow expression and purification of Risp-GST protein (see 2.2.6.2.). The *risp* gene was amplified by PCR from pcRisp-sg143 using the primers 17746 and 17747 (see Table 2.1) and inserted into a BamHI/EcoRI restriction site. Four pGEX-3X-Risp

plasmid clone preparations were sequenced and all contained the Risp insert in the right correct frame with the *GST* gene. Two plasmids (4.1 and 4.2) were transformed into E.coli (BLB21) by electroporation. Expression and purification of Risp-GST was performed as described in 2.5.11. Both Risp-GST preparations were successful: 1.53mg/ml of fusion protein was obtained with the clone 4.1 and 1.77 mg/ml with the clone 4.2. One mg of Risp-GST protein from the 4.1 clone was sent to Dr. Elisabeth Kremmer (GSF) to immunize rats. After several weeks, nine different rat monoclonal antibodies were obtained (Table 3.16). Four out of nine antibodies recognized exogenous expression of Risp protein by immunofluorescence procedure (see 2.5.2.) in cells transfected with a Risp expression plasmid. However, with the same anti-Risp antibodies and the same immunofluorescence protocol in HeLa and in U87 cells non-transfected a weak unspecific fluorescence signal was observed.

Different dilutions (1:200, 1:500, 1:1000, 1:2000, 1:5000) of three monoclonal antibodies against Risp (8A11, 8B6 and 5D3), which tested positive in immunofluorescence staining, were also assessed by western blot analysis using cell lysates from HeLa and U87 cells (Table 3. 16). All three antibodies were able to recognize exogenous expression of Risp fusion proteins (Risp-GFP and Risp-IgG ~ 60 kDa, Tat-Risp ~ 42 kDa). An additional weak band around 220 kDa was observed in some, but not all cell lysates (Fig. 3.12). In addition, Michael Krappmann tested all antibodies by western blot analysis using the recombinant Risp-GST protein. Seven out of nine antibodies recognized the fusion recombinant protein and some of them showed also a unspecific band around 220 kDa (Table 3. 16).

	Immunofluorescence		Western Blot				
Antibodies	Dilutions	Risp-IgG	Dilutions	Risp-GFP	Tat-Risp	*Risp-GST	~ 220 KD
3E10 (?)	1:2 1:20 1:100	- - -	1:200	N.D.	N.D.	++	
5D3 (R2a) = #7	1:50	+	1:200 1:500 1:1000	++++ +++ ++	+++ - -	+++	+ - -
5D4 (R2a)	1:50	-	1:200	N.D.	N.D.	+++	-
6F11 (G1)	1:2 1:20 1:100	- - -	1:200	N.D.	N.D.	-	-
6F12 (G1)	1:50	-	1:200	N.D.	N.D.	-	-
7B4 (G1)	1:50	-	1:200	N.D.	N.D.	++	((+))
8A11 (G1) = #4	1:50	+	1:200 1:500 1:1000	++ + +	+ + -	+++	- (+)? (+)?
8B6 (R2a) = #1	1:50	+	1:200 1:500 ** 1:2000	+++ +++ +++	- - +	+++	+ + -?
8B8 (R2a)	1:50	+	1:200	N.D.	N.D.	+++	+++

Table 3.16 Summary of results obtained using several anti-Risp rat monoclonal antibodies. Nine different anti-Risp antibodies were tested using U87 cells transfected and non-transfected with pcIG-Risp plasmid (1µg) after fixation and permeabilization by immunofluorescence technique. As positive control a Cy3-conjugated goat anti-hIgG was used. Anti-Risp antibodies were also tested by western blot analysis to recognize Risp fusion protein in cellular extracts or * using 10µg/ml of recombinant Risp-GST protein. Proteins extracted from U87 and HeLa** cells ($0.8x10^5$ cells/60 mm plate) non- transfected and transfected with 1µg of pcRisp-sg143 (Risp-GFP), pcIG-Risp (Risp-IgG) or pcTat-Risp (Tat-Risp) plasmid were separated by gradient SDS-PAGE. The gel was blotted and protein detected with the indicated rat monoclonal anti-Risp antibodies at different dilutions followed by a second peroxidase-conjugated antibody (1:2000). (+) indicates weak signal. R2a indicates rat IgG2a, and G1 IgG1.

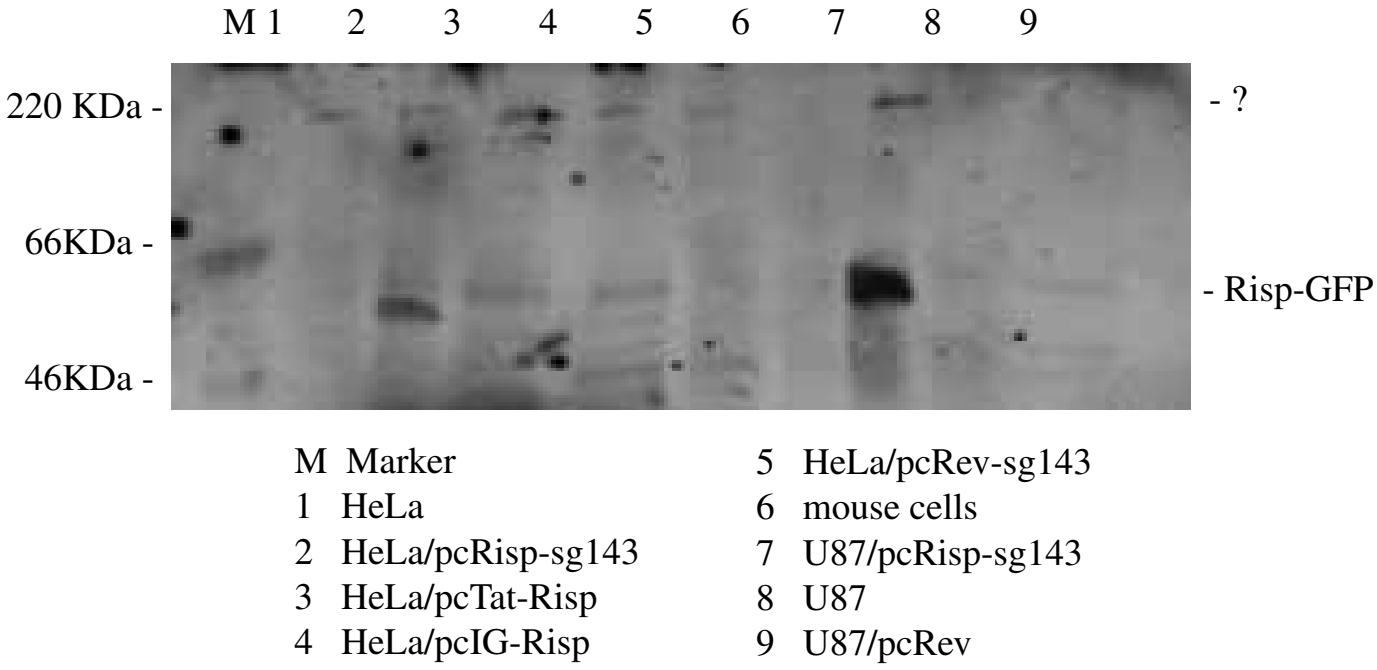


Fig. 3.12 Western Blot analysis of Risp. Proteins extracted from HeLa, U87 or mouse cells (0.8 x105 cells/60 mm plate) non transfected or transfected with 1µg of the indicated plasmids were separated by gradient SDS-PAGE. The gel was blotted and protein detected with the 8B-6 rat monoclonal anti-Risp antibody at dilution 1:500.

In summary, to investigate the cellular expression of Risp, analysis on the DNA-, RNA- and protein level was performed.
The Southern blot analysis using a commercial blot, which contains genomic DNAs derived from 9 eukaryotic species indicated that the risp gene is common among different species. The Northern blot analysis showed a main, however weak, mRNA band around the size of 4.6-5 kb, indicating that the risp probe recognized a larger mRNA than the expected risp of 513 bp. This supports the hypothesis that Risp could be part of a larger protein such as the predicted KIAA0592 (4623 bp; 1353 aa) (Nagase et al., 1998).
Analysis of Risp protein expression was performed with immunofluorescence and western blot analysis. Several tested monoclonal anti-Risp antibodies were able to recognize only exogenous expression of Risp fusion proteins (IgG-Risp, Risp-GFP or Tat-Risp). No specific staining was observed in untransfected cells, suggesting that the available monoclonal Risp antibodies are not sensitive enough to recognize the constitutive expression of the protein.

3.4. Characterization of domains responsible for Rev-Risp binding

3.4.1. *In vivo* Rev-binding assay in yeast cells

To define the regions involved in the interaction of Risp with Rev and vice versa, the yeast two-hybrid approach was used again. Several segments of Risp were cloned into the yeast pJG4-6 vector to encode acid blob B42 activation domain fusion proteins (see Fig. 2.1E). In addition, a set of previously described Rev mutants within different domains of Rev protein was cloned into the yeast pEG202 expression plasmid to encode LexA fusion proteins (see Fig. 2.1E). The plasmids were transformed into the yeast selection strains EGY48/pSH18-34 and EGY48/pJK103 and the Rev-binding specificity test was performed.

3.4.1.1. Preparation of several prey plasmids containing different regions of Risp

To locate the domain of Risp involved in the Rev-binding using the yeast two-hybrid system, full-length or parts of the *risp* cDNA were fused to the B42 activation domain of pJG4-6 expression plasmid. As described in 2.2.6.3.2. section, this vector is very similar to the previously used pJG4-5; the only difference is, that in the pJG4-6 expression plasmid the cloning site is represented by the restriction enzymes MluI/NotI instead of EcoRI/XhoI in the pJG4-5. Several cDNAs encoding N-terminal or C-terminal peptides or the full-length Risp protein were generated by PCR amplification using Risp-GFP as DNA template. Each 5' primer contained a MluI restriction site and each 3' primer a NotI site (see Table 2.1). Seven constructs generated with standard subcloning techniques were sequenced and each confirmed correct plasmid was kept for further use (Fig. 3.13).

3.4.1.2. Determination of Risp region interacting with Rev

In order to identify which region of Risp is involved in the Rev-binding, three independent experiments were performed. In each experiment EGY48/pSH18-34 and EGY48/pJK103 yeast cells expressing the bait proteins were transformed with all the different Risp-prey expression plasmids. Rev protein in sense orientation was used as a bait whereas Rev antisense and the cytoplasmic domain of CD2 were used as unspecific bait controls.

Each transformation mix was plated on Glc/CM Ura-, His-, Trp- dropout plates and incubated at 30˚C for 2-3 days. The growth in these plates allowed selection of only successfully transformed yeasts harboring both bait and prey expression plasmids. To perform the Rev-binding specific test, six independent colonies from each transformation plate were mixed in 1ml of sterile water, diluted 100 and/or 1000-fold and plated on Gal/Raff/CM Ura-, His-, Trp-, Leu- and Glc/CM Ura-, His-, Trp-, Leu-

plates. The growth of cells was monitored for several days. Clones were considered positive when the number of yeast clones growing in Gal/Raff/CM Ura-, His-, Trp-, Leu- plates was higher than 100 and when no significant growth was observed in Glc/CM Ura-, His-, Trp-, Leu- plates. After 4-5 days of incubation at 30˚C none or maximal up to 5 transformants grew in the absence of leucine in glucose plates. A limited number (< 10) of yeast clones encoding the first or the last 38 aa of Risp [transfected with pJG4-6-(2-38) or pJG4-6-(134-171)] was able to grow on galactose plates lacking leucine regardless of which bait protein was co-expressed. However a high number (200-2000) of yeast clones expressing Risp or 16.4.1 cDNA encoded protein or the other segments of Risp were able to grow in galactose plates lacking leucine in presence of Rev sense as bait protein. In presence of the unspecific bait only a limited number (< 31) of yeast clones grew in absence of leucine. Results of one individual experiment are shown Table 3.17.

Figure 3.13 shows Risp regions that interact with Rev and represents the summary of all three experiments. It is not clear, which is the minimal sequence part of Risp required for the Rev-interaction. Nevertheless, we can assume that the binding domain is located in the middle of the Risp protein between amino acids 39-133. In addition it was shown that the N-terminal region of Risp harboring the motif (PAKKTNPFPLLE, 22-33aa) common to Risp and to other Rev-interacting factors is not essential for the Rev-binding.

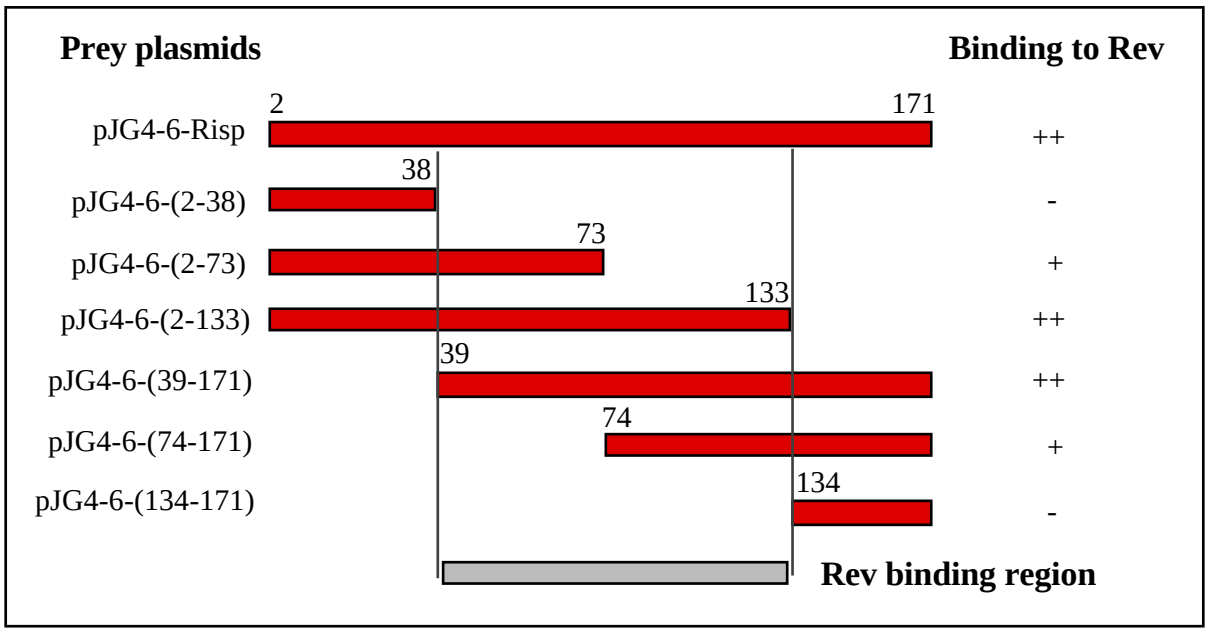


Fig. 3.13 Risp regions interacting with Rev. Summary of three experiments.

Yeast strains	Plasmids	Transformants on Glc/CM Ura-, His-, Trp-	Glc/CM Leu-	Gal/Raff Leu-	
EGY48/pSH18-34/ pEG202-sRev sense	pJG4-5-16.4.1	15	0	300	+
	pJG4-6-Risp	648	1	1040	++
	pJG4-6-(2-38)	320	0	2	-
	pJG4-6-(2-73)	31	0	200	+
	pJG4-6-(2-133)	74	0	704	++
EGY48/pSH18-34/ pEG202-LexCD2	pJG4-5-16.4.1	12	0	24	-
	pJG4-6-Risp	94	0	31	-
	pJG4-6-(2-38)	160	0	6	-
	pJG4-6-(2-73)	5	0	3	-
	pJG4-6-(2-133)	3	0	3	-
EGY48/pJK103/ pEG202-sRev sense	pJG4-5-16.4.1	1	0	0	-
	pJG4-6-Risp	34	0	800	++
	pJG4-6-(2-38)	248	1	0	-
	pJG4-6-(2-73)	9	0	368	+
	pJG4-6-(2-133)	23	0	1360	++
EGY48/pJK103/ pEG202-sRev antisense	pJG4-5-16.4.1	19	0	2	-
	pJG4-6-Risp	252	0	30*	-
	pJG4-6-(2-38)	164	0	1	-
	pJG4-6-(2-73)	46	0	6	-
	pJG4-6-(2-133)	34	0	0	-
EGY48/pJK103/ pEG202-LexCD2	pJG4-5-16.4.1	136	0	0	-
	pJG4-6-Risp	200	0	1	-
	pJG4-6-(2-38)	224	0	0	-
	pJG4-6-(2-73)	82	0	0	-
	pJG4-6-(2-133)	104	0	0	-

Table 3.17 Selective yeast growth in absence of leucine. Results of one experiment. +/++/- indicate yeast growth and therefore strength of binding: + = binding, ++ = stronger binding, - = no binding. * indicate growth of yeast cells very small.

3.4.1.3. Preparation of several bait plasmids containing different Rev mutants

Several mutants of Rev (RevM4, RevM5, RevSLT40, Rev M10BL; see Fig. 2.1E) were cloned in the pEG202 bait expression plasmid. As described in 2.2.6.3.1. each Rev mutant was generated by PCR amplification of a cDNA template with the respective mutation. Both 5'and 3' primers used contained an EcoRI restriction site to allow insertion in frame with LexA into the unique EcoRI restriction site of the pEG202 vector (Table 2.1) in sense and antisense orientation. Seven Rev-bait plasmids generated with standard subcloning techniques were sequenced and each correct plasmid was kept for further use (see Fig. 3.15).

The plasmids containing the RevM10BL mutation were prepared at a later time point and analyzed by Christian Bickel. Using the lithium acetate procedure both yeast strains EGY48/pSH18-34 and EGY48/pJK103 were transformed with the different bait expression plasmids. All transformants were selected for growth in Glc/CM Ura-, His- plates. Although the pEG202 bait plasmid is very big (~ 10 kb) and the efficiency of transformation was not very high as is shown in the Table 3.18, it was possible to isolate at least one yeast strain harboring a Rev mutant for each bait construct.

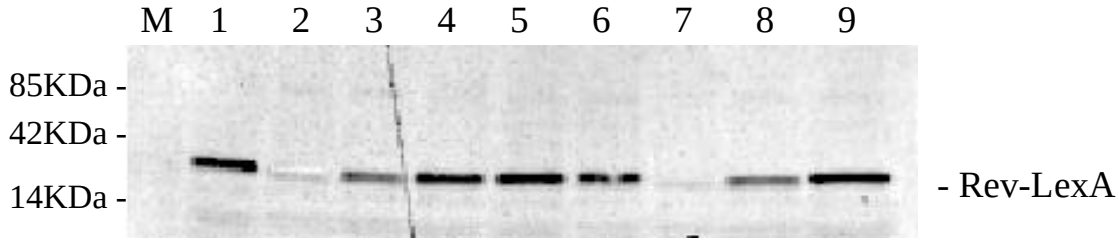
Yeast strains	Bait plasmids	Transformants
EGY48/pSH18-34	pEG202-Rev sense	4
EGY48/pSH18-34	pEG202-RevM4 sense	0
EGY48/pSH18-34	pEG202-RevM4 antisense	1
EGY48/pSH18-34	pEG202-RevM5 sense	10
EGY48/pSH18-34	pEG202-RevSLT40 sense	6
EGY48/pJK103	pEG202-Rev sense	2
EGY48/pJK103	pEG202-RevM4 sense	6
EGY48/pJK103	pEG202-RevM4 antisense	1
EGY48/pJK103	pEG202-RevM5 sense	31
EGY48/pJK103	pEG202-RevSLT40 sense	6

Table 3.18 Yeast transformants with different Rev-bait expression plasmids.

To investigate whether these Rev proteins do not activate transcription of the reporter genes without the prey constructs by themselves, the leucine- and X-gal-bait test were performed. One clone of each yeast transformant was incubated O/N in Glc/CM Ura-, His- medium. Half ml of the yeast suspension culture was diluted 100

and 1000-fold in sterile water and plated in parallel on Glc/CM Ura-, His-, Leu-, Gal/Raff Ura-, His-, Leu- and Gal/Raff Ura-, His-, X-gal plates. The growth of cells was monitored for several days. All yeast clones on the Gal/Raff Ura-, His-, X-gal plates grew well and were white, without showing any transcription activity of the lacZ gene. In addition in the absence of leucine in both glucose and galactose plates no yeast transformants grew. Only in the case of yeast EGY48/pJK103 expressing the plasmid pEG202-RevM4 sense a very low background (4 clones) of growth in Gal/Raff Ura-, His-, Leu- plates was detected.

To demonstrate, that the Rev mutants are expressed as full-length proteins, each of these LexA-Rev fusion proteins were analyzed by western blot using a polyclonal Rev-specific antibody. Only yeast clones containing Rev in sense orientation showed a band around 42 kDa with similar intensity as the Rev-LexA fusion protein (Fig. 3.14).



M Marker
1 pSH18-34/pEG202-Rev
2 pSH18-34/pEG202-RevM4
3 pSH18-34/pEG202-RevM5
4 pSH18-34/pEG202-RevSLT40
5 pJK103/pEG202-Rev
6 pJK103/pEG202-RevM4
7 pJK103/pEG202-RevM4 antisense
8 pJK103/pEG202-RevM5
9 pJK103/pEG202-RevSLT40

Fig. 3.14 Yeast expressing the Rev-LexA fusion proteins. Same amount of proteins extracted from EGY48 yeast cells transformed with the indicated Rev-bait expression plasmids were separated by 12% SDS-PAGE, transferred to nitrocellulose, and then subjected to western blot analysis using a rabbit anti-Rev specific antibody (1:1000).

3.4.1.4. Determination of a region in the N-terminal part of Rev interacting with Risp

In order to identify the region in Rev responsible for binding to Risp the plasmids pJG4-6-Risp and pJG4-6-(39-171) were transformed into the yeast strains containing the bait plasmids shown below:

EGY48/pSH18-34/pEG202-Rev sense
pEG202-RevM4 antisense
pEG202-RevM5 sense
pEG202-RevSLT40 sense
pEG202-RevM10BL sense
EGY48/pJK103/pEG202-Rev sense
pEG202-RevM4 sense
pEG202-RevM4 antisense
pEG202-RevM5 sense
pEG202-RevSLT40 sense
pEG202-RevM10BL sense

Each transformation mix was plated on Glc/CM Ura-, His-, Trp- dropout plates and incubated at 30˚C for 2-3 days until colony growth was detected. To perform the leucine test, six independent colonies from each transformation plate were mixed in 1ml of sterile water, diluted 100 and 1000-fold and plated on Gal/Raff/CM Ura-, His-, Trp-, Leu- and Glc/CM Ura-, His-, Trp-, Leu- plates.
The growth of cells was monitored for several days. After 4 days of incubation at 30˚C none of the yeast transformants grew in the absence of leucine in plates containing glucose. However in plates containing galactose the expression of activation-tagged-Risp protein was induced and yeast clones containing Rev wild type, RevM4 and RevM10BL in sense orientation grew very well in absence of leucine. Yeast clones containing the other Rev mutants as bait proteins were all negative.

Figure 3.15 shows Rev mutants that interact with Risp analyzed in three independent experiments.
It is not clear, which is the minimal portion of Rev protein required for the Risp-binding. Nevertheless, it can be assumed that Risp is a cellular cofactor that interacts with Rev in a region that overlaps the two domains of RNA binding/NLS (34-50aa) and the second multimerization region (52-60aa). In fact two Rev mutants, that affect the RNA binding region and the NLS (RevM5) and the second multimerization region (RevSLT40), were unable to interact with the Risp fusion protein. On the contrary, two Rev mutants affecting the first multimerization region (RevM4) and the effector domain (RevM10BL) were able to bind Risp.

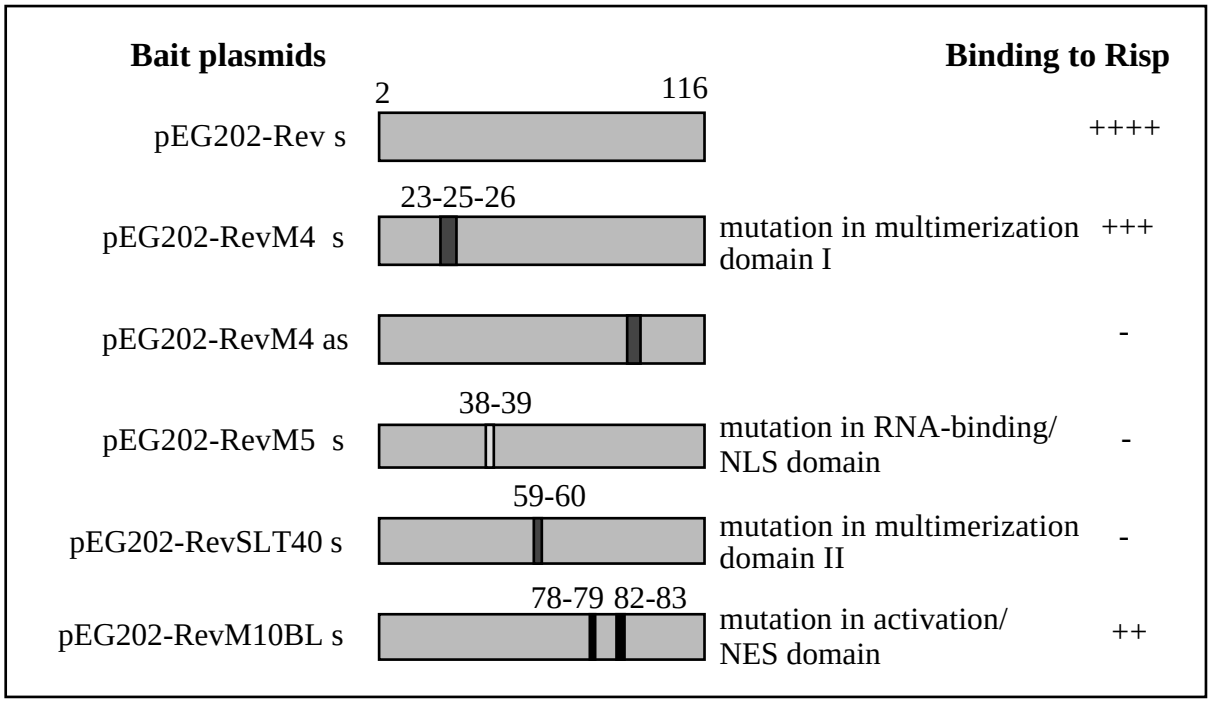


Fig.3.15 Rev mutants interacting with Risp. Summary of three individual experiments.

3.4.2. *In vivo* Rev-binding assay in mammalian cells

To assess interaction of Rev and Risp in human cells, we performed a mono-hybrid assay developed by B.R. Cullen and his collaborators (Bogerd et al., 1995; Tiley et al., 1992). This mammalian *in vivo* assay uses the HIV-1 *tat*-RNA sequence-specific transcriptional activator to provide an indirect measure of the assembly of a protein complex on a RNA target sequence. pSLIIBCAT was used as indicator construct and pcTat-Rab or pcTat-Risp in presence of pcRev were used as activator plasmids (see Fig. 2.1). Tat activates gene expression from the HIV-1 LTR after binding the cis-acting RNA target sequence TAR. In the pSLIIBCAT plasmid the TAR RNA stem-loop in HIV-1 LTR linked to CAT is replaced with the RRE-derived stem loop IIB (SLIIB), the minimal RNA target sequence for HIV-1 Rev. Trans-activation of the HIV-1 LTR in pSLIIBCAT is mediated by a Tat-Rev fusion protein (Fig. 3.16). Rev is required to direct binding to the specific SLIIB RNA target and Tat to activate the transcription. Therefore, in this assay Rev is provided in its wild-type form while Tat is fused to a Rev-binding cellular protein (Rab or Risp). Interaction of Rev with the cellular proteins fused with Tat recruits Tat to the HIV-1 LTR promoter element and activates expression of CAT.

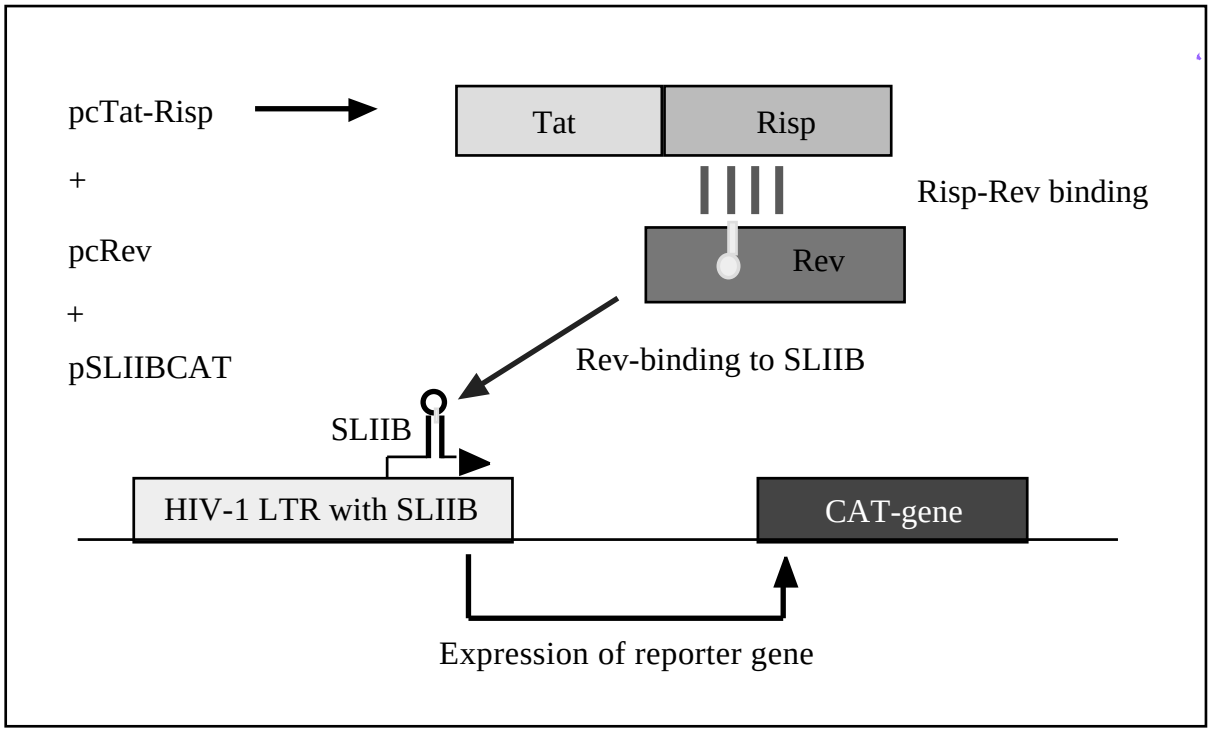


Fig. 3.16 ***In vivo*** **mono-hybrid assay for Rev-binding: SLIIB-CAT.** Cells are transfected with the Tat/Rev-response plasmid pSLIIBCAT in presence of the activator plasmids pcRev and pcTat-Risp. Stimulation of CAT expression is directed by recruitment of Tat-Risp to the LTR by the interaction of Risp with Rev. Binding of the ternary complex to the LTR is mediated by binding of Rev to SLIIB.

3.4.2.1. Preparation of pcTat-Risp plasmid

pSLIIBCAT, pcTat-Rev and pcRev expression plasmids were kindly provided by Dr. B.R. Cullen. PcTat-Risp plasmid as well as pcTat-Rab, used as positive control, were generated by substituting the *rev* gene in the pcTat-Rev expression plasmid by *risp* and *rab*. The *rev* gene was removed by cleavage with NcoI/Hind III and replaced with a PCR-generated cDNA sequence of *risp* and *rab*. For both DNAs primers containing a unique NcoI site at the 5' end and a unique Hind III site at the 3' end (see Table 2.1) were used. The constructs generated with standard subcloning techniques were sequenced and each correct plasmid was kept for further use. To demonstrate expression of the fusion proteins Tat-Risp and Tat-Rab, HEK293 and HeLa cells were independently transfected with the respective expression plasmids (1µg DNA/60mm plate/0.8x10^5cells) and cellular protein extracts were analyzed by western blot analysis. As a control cells non transfected or transfected with 1µg DNA of pL3Tat, pcTat-Rev and pcTat-sg25 were used. Therefore cellular protein extracts were separated on a gradient SDS polyacrylamide gel and protein expression was detected with anti-Tat specific antibodies. Three different anti-Tat antibodies (a rabbit polyclonal (ABT10) kindly provided by Dr. G. Sutter, a rabbit polyclonal kindly

provided by Dr. B. Ensoli and a mouse monoclonal (MRC) kindly provided by Dr. G. Levi) were used in three different western blot experiments. Cell lysates transfected with expression plasmids for Tat fusion proteins and Tat alone displayed several bands around 13-16 kDa and no differences were observed. However using rat monoclonal anti-Risp antibodies a strong band around 42 kDa corresponding to the Tat-Risp fusion protein was observed (Fig. 3.17) only in cells transfected with the pcTat-Risp plasmid. In addition, the fusion protein Tat-Risp was recognized also by immunofluorescence using anti-Risp antibodies.

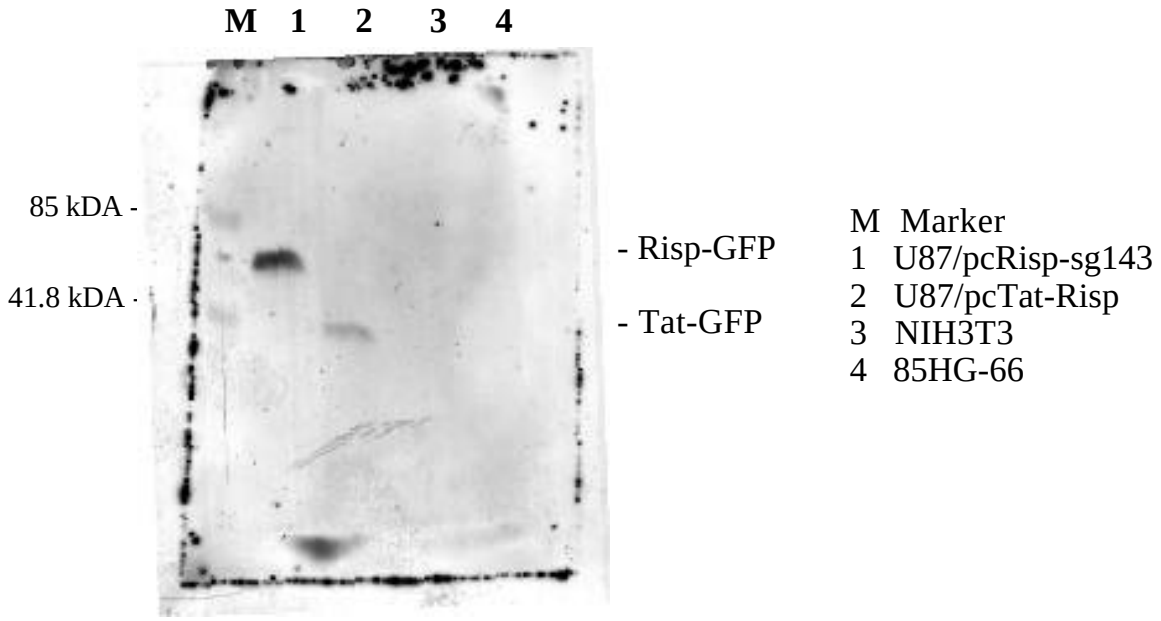


Fig. 3.17 Western Blot analysis of Tat-Risp. U87 cells were transfected with 1µg of the indicated expression plasmids and collected 48h posttransfection. Proteins extracted from the indicated cells were separated by gradient SDS-PAGE, transferred to nitrocellulose, and then subjected to western blot analysis using the rat monoclonal IgG1 anti-Risp antibody 8B6 (dilution 1:1500).

3.4.2.2. Tat transactivation assay with Tat-Risp expression plasmid

Before testing the Rev-binding capacity of Risp and Rab expressed as Tat fusion proteins, the functionality of the Tat moiety of the fusion protein was analyzed. HeLa cells were transfected with a Tat-response plasmid pHIVΔnpsLTRCAT (see Fig. 2.1) alone and in combination with pcTat, pcTat-Risp or pcTat-Rab expression plasmid. All transfection reaction mixtures included the HIV-independent expression plasmid

pGL3-RFB containing the luciferase gene under the control of a C-type retroviral RFB-LTR (see Fig. 2.1). Activation of HIV-LTR-dependent CAT expression was determined measuring CAT-activity with a fluorescent dye labeled chloramphenicol substrate provided by a FAST-CAT Kit (see 2.5.10.). Briefly, cells were harvested 48h after transfection and lysed by 3 freeze-thaw cycles. Total protein content and luciferase activity were determined. Five to 10 µg of total protein was incubated overnight with fluorescent dye labeled chloramphenicol and acetyl-CoA. Reaction was stopped by ethylacetate and a thin-layer-chromatography was performed. Acetylated and non-acetylated chloramphenicol was quantified using a STORM™ fluorescence scanner and the conversion-rates were determined. Results from two separate experiments are shown in the Fig. 3.18. Similar levels of luciferase values were obtained in each experiment indicating the same range of transfection efficiency.

With the different Tat expression plasmids the same range of induction of CAT expression were observed.

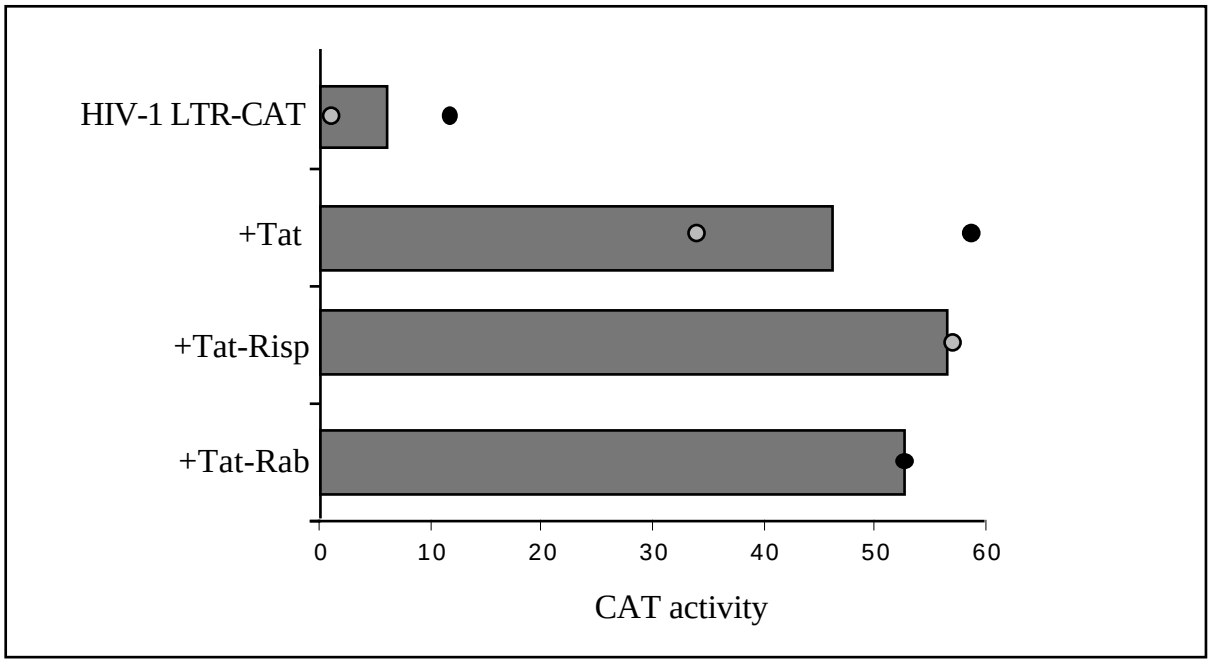


Fig. 3.18 Transactivation of HIV-1 Tat. Tat-response was assayed by determining the effect of Tat on expression of the reporter gene CAT under the control of the HIV-1 LTR. Results from two separate experiments are shown. HeLa cells were transfected with Tat-response plasmid pHIVΔnpsLTRCAT (2µg) alone and in combination with a Tat expression plasmid (1µg): pcTat and pcTat-Risp in one experiment and pcTat and pcTat-Rab in another experiment. All transfection mixtures included pGL3-RFB (1µg) as control for transfection efficiency. Both luciferase and CAT expression were normalized to protein levels. Dots show values for CAT activity in single transfection experiments performed in duplicate; bars show the average values. CAT activity was determined as percentage of CAT converted to an acetylated form.

3.4.2.3. SLIIB-binding assay with Tat-Risp

After demonstrating that Tat-Risp and Tat-Rab efficiently activate HIV-1 LTR transcription, the SLIIB-binding assay was performed. HeLa cells were transfected with pSLIIBCAT indicator plasmid (2µg) in combination with a Tat expression plasmid (1µg). PcTat alone was used as a background control, pcTat-Rev as a positive control and pcTat-Risp or pcTat-Rab were tested in presence and absence of pcRev (1µg). All transfection mixtures were co-transfected with the plasmid pGL3-RFB (1µg) as a control for heterologous gene expression. Forty-eight hours after transfection cells were harvested and lysed. Total protein content and luciferase activity were determined (see 2.5.6. and 2.5.8.). CAT expression was measured by a specific ELISA (see 2.5.9.) or by the functional FAST-CAT assay (see 2.5.10.). Results from a representative experiment are presented in Fig. 3.19. Since luciferase expression varied 2-5 fold between cells transfected with different plasmid mixtures, CAT induction levels were normalized also to luciferase activity as it is shown in the figure.

Neither Tat, Tat and Rev, Tat-Rab nor Tat-Risp alone were able to increase CAT expression over to basal levels seen in cells transfected with the pSLIIBCAT indicator plasmid in the presence of the Tat protein alone. Only the simultaneous expression of both Rev and Tat-Rab in cells induced a detectable ~ 16-fold induction of CAT expression. The simultaneous expression of Rev and Tat-Risp induced only a slight increase of induction of CAT expression compared to that with Tat alone.

In summary, although it is not yet clear which is the minimal sequence part of Risp or Rev required for the Risp-Rev interaction, we can assume that the binding domain in Risp is located in the middle of the protein between amino acids 39-133. It can be assumed that Risp interacts with Rev in a region that overlaps the two domains of RNA binding/NLS (34-50aa) and the second multimerization region (52-60aa). In addition, independently from the two different sources of plasmid pBsRev or pcRev used to express the Rev protein in the yeast cells, no difference in the ability of Risp to bind Rev was observed. In fact the Rev sequence of the two plasmids even if they rather differ at the DNA level, show higher identity at amino acid level and no differences in the known functional domains are present.

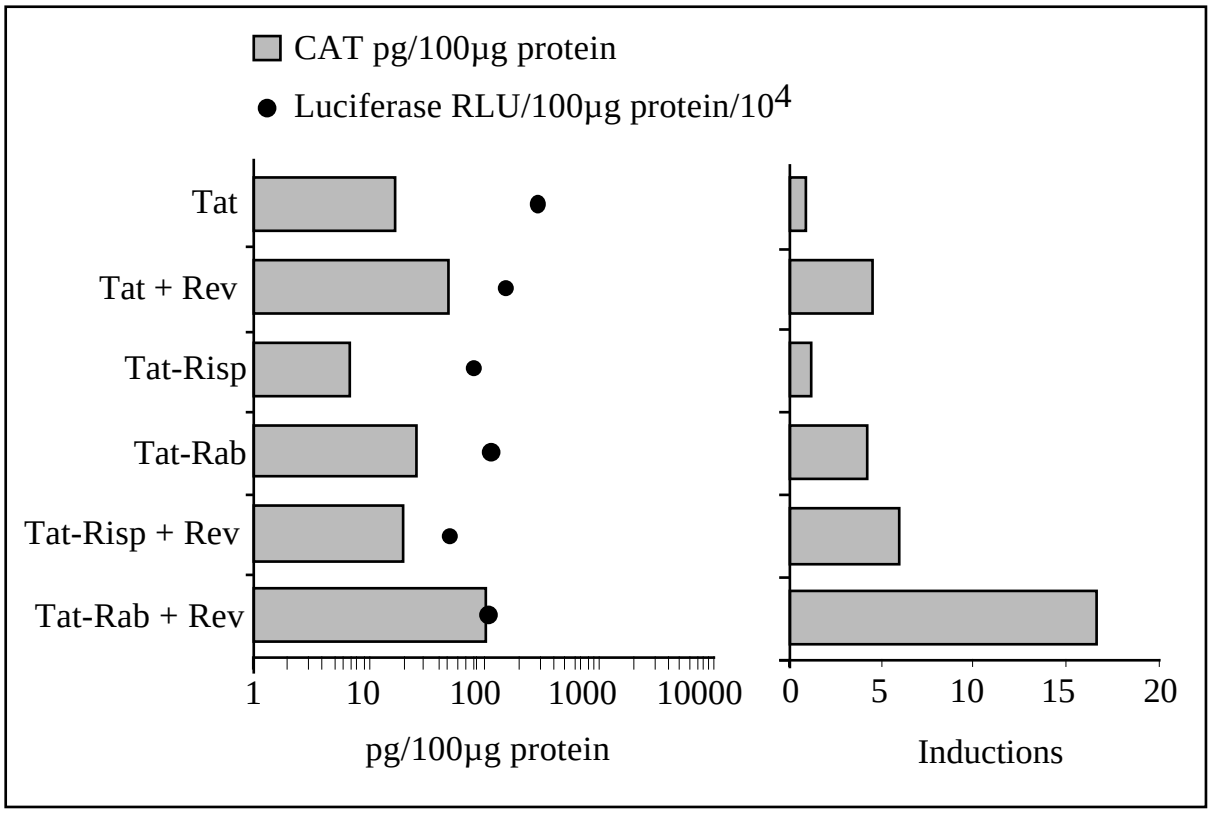


Fig. 3.19 Tat-Risp is not able to induce efficient SLIIB-CAT activation. HeLa cells were transfected with the Tat/Rev-response plasmid pSLIIBCAT (2µg) in combination with a Tat expression plasmid (1µg) in presence or absence of pcRev (1µg). All transfection mixtures included pGL3-RFB (1µg) as a control for transfection efficiency. Results from a representative experiment performed in duplicate are shown. Dots represent luciferase activity (RLU = relative light units) normalized to protein levels and divided by $1x10^4$. The expression of CAT was quantified by the CAT-ELISA. The induction is the activation of CAT expression referred to that obtained in the presence of Tat alone (set to 1) and normalized to protein and luciferase levels.

3.5. Functional study of Risp

3.5.1. Competition of Tat-Rev binding to SLIIB RNA by Risp-GFP

There are several possible explanations for the inability of Tat-Risp to activate the SLIIB-CAT expression in the presence of Rev. One is that the Risp-protein could bind to the RNA binding domain of Rev and therefore prevent the binding of the Rev protein to its SLIIB RNA target. To test this hypothesis a Rev-SLIIB-binding assay was performed in the presence of a Risp expression plasmid at different concentrations.

First, *risp* gene was amplified by PCR from the pJG4-5-16.4.1 plasmid using the primers 16552 and 15762 (Table 2.1) and inserted into the unique NheI restriction site in pFred143 vector (see 2.2.6.1.). After several restriction analyses a pcRisp-sg143 plasmid preparation was sequenced. Sequence analysis confirmed the presence of the

intact *risp* insert in sense and in frame with the *GFP* gene; therefore it was used for the further experiments.

Cells were transfected with the pSLIIBCAT reporter plasmid (2µg) in combination of pcTat (1µg) or with pcTat-Rev (1µg) in presence and absence of variable amounts (0.01-5µg) of pcRisp-sg143. All transfection mixtures included pGL3-RFB or pRSV-luc plasmids (1µg) as a control for luciferase gene expression In five independent experiments HeLa cells were transfected using the FuGENE or calcium phosphate transfection method (see 2.5.1.1.). Since the Rab protein is known to bind to the Rev-protein in its activation domain, the pcRab plasmid (1-5µg) was used in three experiments as a negative competitor control. Moreover, since Risp is expressed as a GFP-fusion-protein, the GFP alone was also analyzed in four experiments using pFred143 plasmid (0.5-5µg). A molar excess of RevM10 protein was used as a positive competitor control using pBsRevM10BL plasmid (3µg). 48h after transfection cells were harvested and lysed; total protein content and luciferase activity were determined. CAT expression was measured by ELISA or by the FAST-CAT assay. Different results for the CAT induction were obtained in the five experiments. A summary of the results is shown in Table 3.19. One experiment was not included in the table because the CAT expression induced by Tat-Rev in the presence of any other plasmids was highly and unspecifically reduced.

Plasmids	µg	Stimulation of CAT expression (fold-induction)					% Induction
	Experiments:	1[a]	2	3	4	Average[b]	Mean[c]
pSLIIBCAT	2						
" + pcTat-Rev	1	187	73.8	20.1	15.1	74	100
" + "+ pcRisp-sg143	0.5	ND	ND	18.8	11.3	15.1	84.2
	1	25.6	12.7	19.6	7.5	16.3	44.3
	2	ND	9.6	8.4	13.2	10.4	47.4
	5	0.3	15.1	ND	ND	7.7	10.3
" + "+ pFred143	0.5	ND	ND	13.7	9.3	11.5	64.7
	1	ND	52.3	15.5	4.0	23.9	58.2
	2	ND	59.3	6.6	4.0	23.3	49
	5	ND	11.7	ND	ND	11.7	15.8
" + "+ pcRab	1	104.7	72.3	ND	ND	88.5	76.9
	5	ND	55.7	ND	ND	55.7	75.4
" + "+ pBsRevM10BL	3	0.9	2.3	ND	ND	1.6	1.85

Table 3.19 Reduction of SLIIB-CAT activation by expression of the Risp-GFP protein. HeLa cells were co-transfected with the Tat/Rev-response plasmid pSLIIBCAT (2µg) and pcTat or pcTat-Rev expression plasmid (1µg). PGL3-RFB or pRSV-luc plasmid (1µg) were used as a control for transfection efficiency. PcRisp-sg143, pFred143, pcRab and pBsRevM10BL plasmids were added at different concentrations. Results from four experiments

performed in duplicate are shown. The induction is the activation of CAT expression in the presence of Tat-Rev. [a)] indicates the use of the FuGENE transfection method while in the other experiments the calcium phosphate transfection method was employed. [b)] represents the average of the CAT induction values of all experiments. [c)] represents the average of the inductions calculated in each experiment where the induction with Tat-Rev is set to the 100%.

In cells transfected with the pSLIIBCAT indicator plasmid, high levels of CAT activation were obtained in the presence of the fusion protein Tat-Rev compared to the basal levels observed in the presence of the Tat protein alone. Nevertheless, a wide variation of CAT activation levels between the different experiments was observed. The induction levels in fact varied between a minimum of 15-fold to a maximum of 187-fold (see Table 3.19). Also luciferase expression varied between cells transfected with different plasmid mixtures (2-10 fold) and between the different experiments (2-20 fold).

However, luciferase activity was very high in each experiment, suggesting a variation in transfection efficiency. A clear correlation between low values of CAT and low luciferase expression was not found. Therefore CAT inductions were normalized only to protein levels and not to luciferase activity.

The results obtained with these four experiments suggest still probably unspecific inhibition of Rev-SLIIB binding in the presence of Risp.

In fact the presence of the Rab protein as well as the GFP alone, that both in principle should not compete in the Rev-SLIIB binding, inhibited the Tat-Rev induced CAT activation in some experiments. The presence of increasing amounts of Risp-GFP expression plasmid in the first two experiments showed a higher and specific reduction of the Tat-Rev induced CAT activation in comparison with the Rab/GFP controls. Nevertheless, in the other two experiments, where Tat-Rev inductions were not very high, a limited and similar reduction of CAT activation in the presence of Risp-GFP and of GFP alone was observed.

The effect of the Risp-GFP-, GFP- and Rab-protein after transfection of 1 µg of DNA of expression plasmid on the CAT activation is shown in Fig.3.19. The CAT activation by Tat-Rev is set to 100%. Although the addition of all these proteins partially inhibited the Tat-Rev-induced CAT activation, the highest level of reduction was observed with the Risp-GFP protein. A major reduction effect of Risp-GFP compared to the other two proteins was observed when the fold-activation was expressed by the median value of all values obtained in every experiment (Fig. 3.20). The median and mean values of CAT activation in the presence of Risp-GFP was 33 and 44%, in the presence of GFP 71 and 58% and in the presence of Rab 77 and 77%.

Unfortunately, the limited number of experiments excludes the possibility of a statistical analysis of the present data. More experiments have to be performed to statistically confirm the observed slight but specific reduction of the binding of Rev to its RNA target SLIIB by the Risp protein.

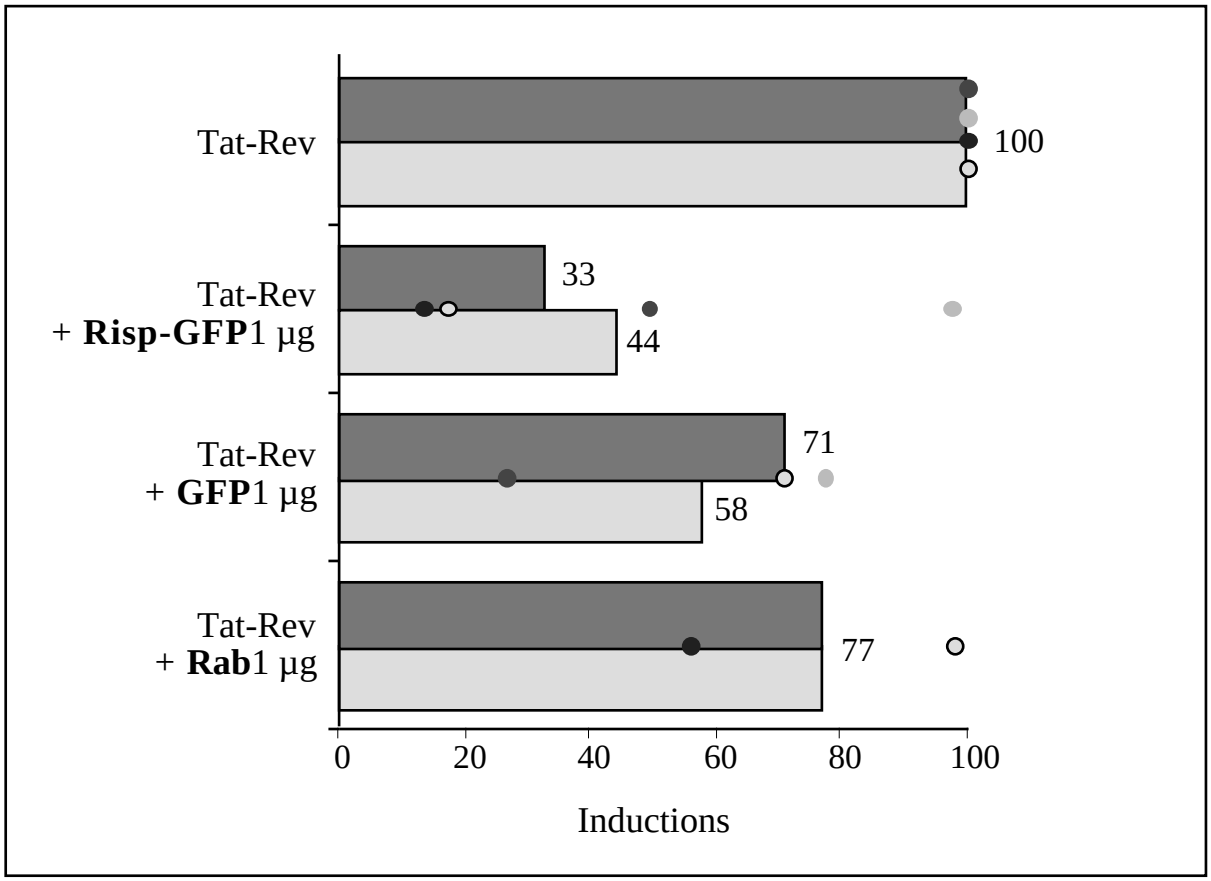


Fig. 3.20 Reduction of SLIIB-CAT activation by Risp-GFP. HeLa cells were co-transfected with the Tat/Rev-response plasmid pSLIIBCAT (2µg) and pcTat or pcTat-Rev expression plasmid (1µg) in presence and absence of pcRisp-sg143, pFred143 or pcRab plasmids (1µg). PGL3-RFB or pRSV-Luc plasmid (1µg) were used as a control for transfection efficiency. CAT activation by Tat-Rev is set to 100 %. Dots show single transfection experiments performed in duplicate; red bars show the average values and blue bars show the median values of all the experiments.

3.5.2. Effect of Risp-GFP on LTR transactivation

Since the Risp-GFP- and the GFP-protein, although to a low extent, were able to inhibit CAT activation in the Rev-SLIIB binding assay as previously shown, the effect of these proteins on HIV-1 LTR driven CAT expression was also analyzed. HeLa cells were transfected with the Tat-response plasmid pHIVΔnpsLTRCAT (2µg) alone and in combination with pcTat (1µg) in presence and absence of pcRisp-sg143 or pFred143 plasmid (0.5, 1 and 2µg). PGL3-RFB plasmid (1µg) was used as a control for transfection efficiency. After 48h of transfection cells were harvested and lysed;

total protein content and luciferase activity were determined. CAT expression was measured by the FAST-CAT assay. Results obtained from one experiment are shown in the Fig. 3.21.

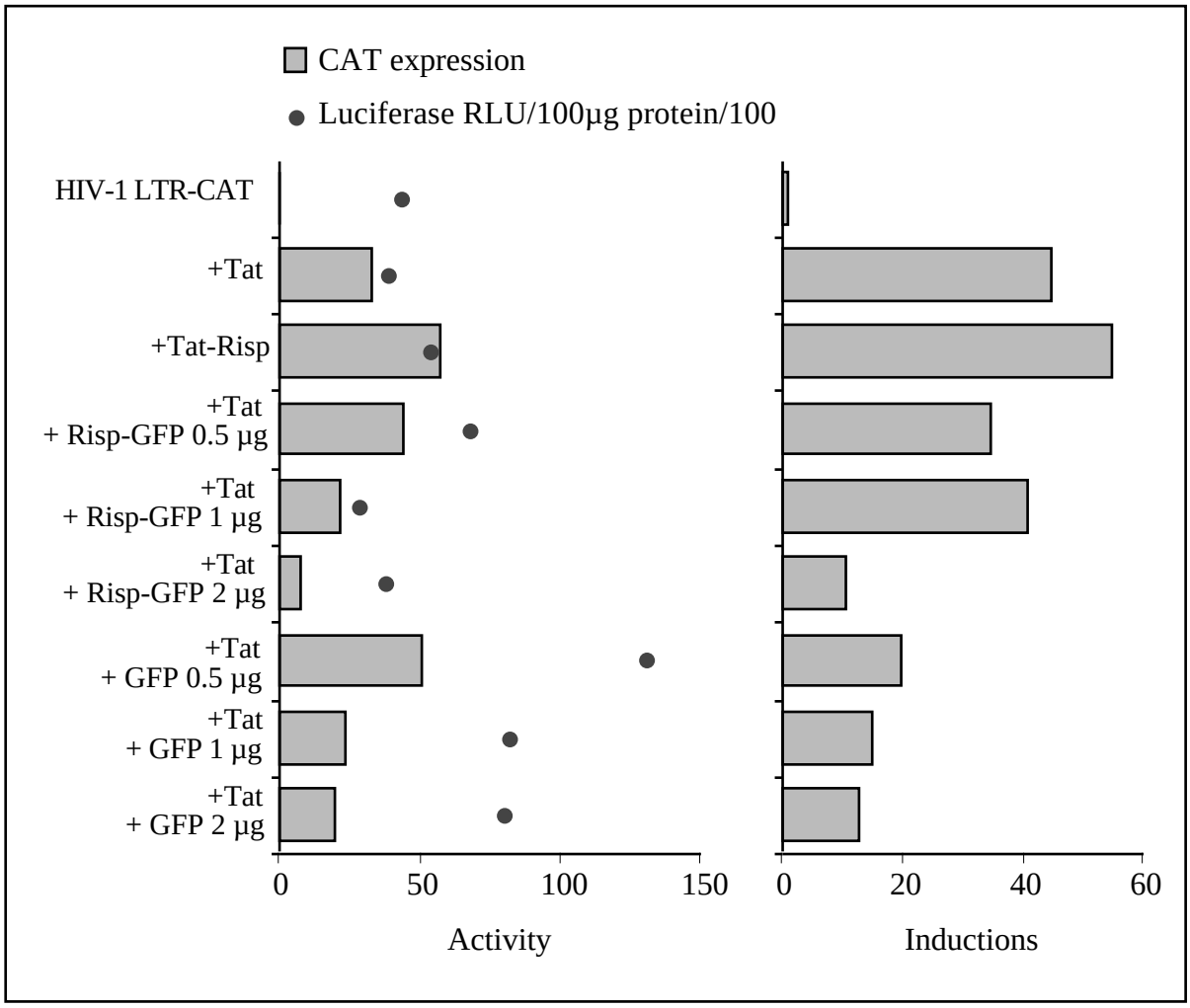


Fig. 3.21 HIV-1 LTR-CAT activation. HeLa cells were transfected with the Tat-response plasmid pHIVΔnpsLTRCAT (2µg) alone and in combination with a Tat expression plasmid (1µg): pcTat or pcTat-Risp. PcRisp-sg143 and pFred143 plasmids were added at different concentrations as shown in the figure. PGL3-RFB plasmid (1µg) was used as a control for transfection efficiency. Both luciferase and CAT expression were normalized to protein levels. Results from a representative experiment performed in duplicate are shown. Dots represent luciferase activity divided by 100. The inductions represent the x-fold activation of CAT expression compared to the basal expression obtained in the absence of Tat. All values are normalized to protein and luciferase levels.

Since luciferase expression varied 2-4 fold between cells transfected with different plasmid mixtures, CAT induction levels were normalized also to the luciferase activity as shown in the figure.

The presence of up to 1µg plasmid DNA for Risp-GFP did not markedly influence Tat-dependent transactivation of the HIV-1 LTR. 0.5µg Risp-GFP DNA led to 23% and 1µg Risp-GFP DNA to 11% reduction of CAT activity. However the presence of GFP expression plasmid also reduced Tat-mediated induction of CAT activity (≥50% of reduction).

3.5.3. Influence on HIV-1 Gag production by overexpression of Risp-GFP

The previously described preliminary experiments showed that the binding of Risp to Rev in its RNA binding domain partially prevented the binding of Rev protein to its SLIIB RNA target. Additional experiments to test Rev function and therefore the production of Rev-dependent viral proteins in the presence of Risp-GFP expression plasmid were performed.

Using transient transfection, Rev function has been determined by assaying the expression of Rev-dependent gag genes with and without Risp-GFP. HeLa cells were transfected using the FuGENE or calcium phosphate coprecipitation with the HIV-1 subgenomic Gag-expression plasmid pB37R (see Fig. plasmids; 5µg) with and without of pcRev expression plasmid (1µg) and Risp-GFP expression plasmid (1-2µg). In all transfection mixtures, the pRSV-luc plasmid (1µg) was included as a control for luciferase gene expression. Moreover, pFred143, pcRab and pcCrm-1-sg143 plasmids (1-2µg) were also used as controls. Forty-eight hours after transfection cells were harvested and lysed. Levels of total protein, luciferase activity and Gag antigen were measured (see 2.5.6-2.5.8.). Low levels of Gag synthesis were obtained without Rev, which of course increased in the presence of Rev. The media of the Rev-inductions obtained in the three experiments was 252 fold.

Results from three independent experiments are presented in Fig. 3.22. The Rev-inductions are set to 100% and normalized also to luciferase activity. The overexpression of Risp-GFP reduced the induction of Gag production by Rev from 30% (1µg plasmid) to 70% (2µg plasmid), whereas the overexpression of GFP or Rab proteins had only a very marginal effect (up to 10%). On the contrary, the overexpression of Crm1-GFP increased Rev-inductions up to 70% (2µg plasmid). The limited number of experiments excludes the possibility of a statistical analysis of the present data. Therefore, more experiments have to be performed to statistically confirm the observed slight and specific reduction of production of the Rev-dependent viral proteins in the presence of Risp.

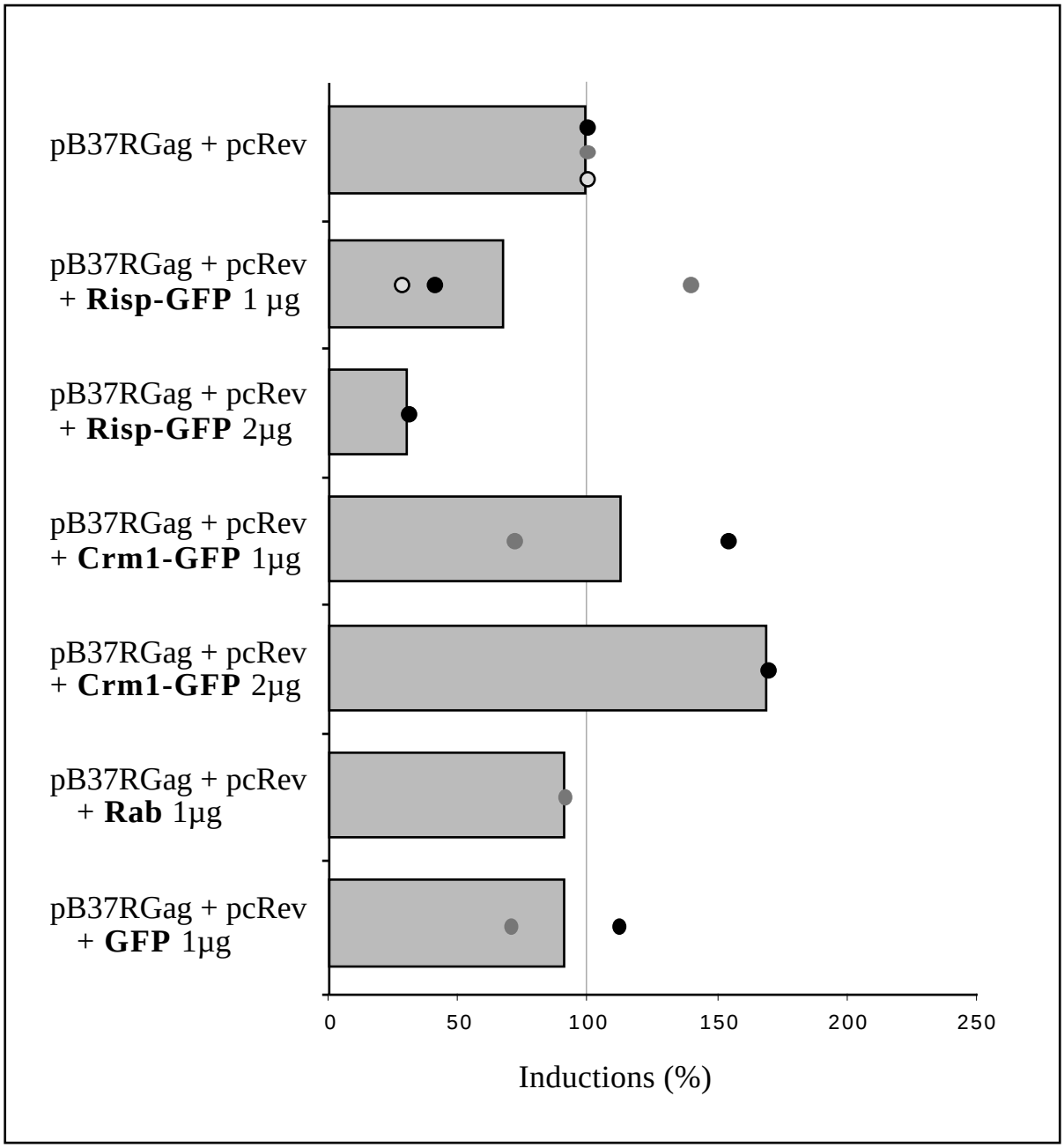


Fig. 3.22 Diminished Rev-dependent stimulation of Gag production in the presence of Risp-GFP. HeLa cells were co-transfected with the Rev-response plasmid pB37R (5µg) without or in combination with Rev-expression plasmid pcRev (1µg) in presence or absence of pcRisp-sg143, pcCrm1-sg143, pcRab or pFred143 plasmid (1µg). Transfection mixtures also included a control plasmid for heterologous gene expression (pRSVluc; 1µg). Gag protein levels were measured in transfected cells by ELISA and the ratio of Gag produced with and without Rev determined (inductions). The Rev-inductions are set to 100%. Dots show single transfection experiments performed in duplicate; blue bars show the average values. All values were normalized to protein and luciferase levels.

3.5.4. Shuttling properties of Risp

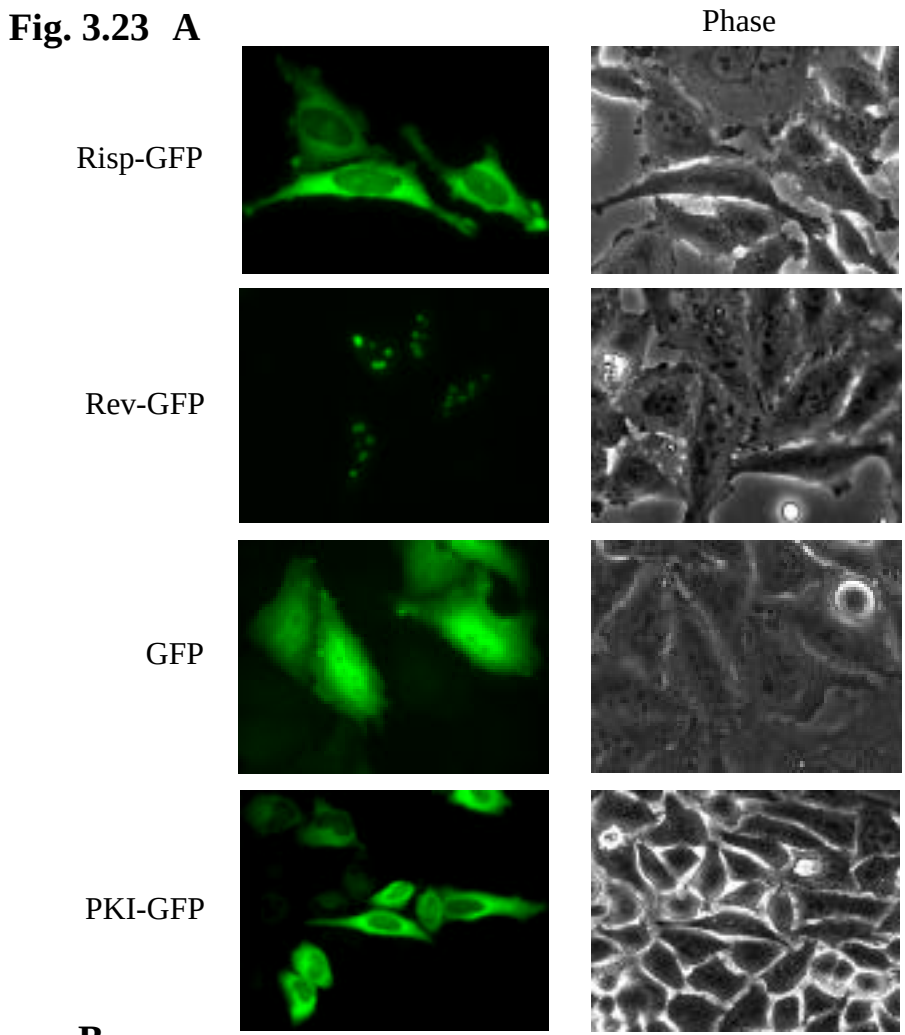
3.5.4.1. Localization of Risp-GFP in HeLa cells

To examine the intracellular localization of Risp, cells were transfected with the plasmid containing the *risp* gene fused to GFP-encoding sequences (pcRisp-sg143, see 2.1B). The Risp-GFP localization was compared to the intracellular localization of Rev-GFP and GFP alone using the pCsRev-sg143 or the pFred143 expression plasmid respectively (see 2.2.6.1.). Both Rev- and Risp-GFP open reading frames in the fusion constructs do not contain an internal translation initiation site downstream of the *rev*- *risp*-sequences to rule out a possible synthesis of unfused GFP.
Expression of a single Risp-GFP fusion protein was confirmed by western blot analysis of lysates of HeLa, U87 or HEK293 cells transfected with pcRisp-sg143 plasmid using antibodies against GFP or Risp (Fig. 3.17, Fig. 3.24).
A comparison of intracellular localization of Risp-GFP, Rev-GFP and GFP 24h after transfection in HeLa cells is shown in Fig. 3.23 A.

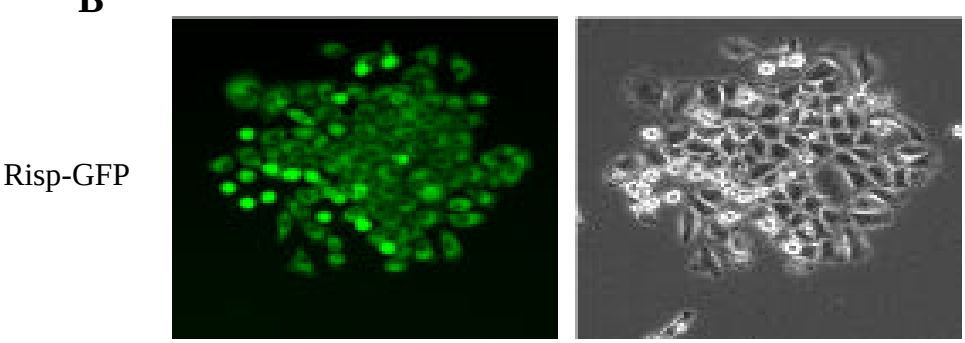
As expected, HeLa cells transfected with pCsRev-sg143 plasmid showed a predominantly nuclear/nucleolar distribution of Rev-GFP, whereas after transfection with the pFred143 plasmid the GFP-protein was distributed in both cytoplasmic and nuclear compartments as already known.
On the contrary, cells expressing the Risp-GFP-protein revealed a strong cytoplasmic fluorescence, indicating that Risp sequesters GFP in the cytoplasm.

The cytoplasmic accumulation of Risp-GFP similar to that observed previously with proteins with a strong NES, like PKI (Wen et al., 1995), suggests that Risp itself could contain an NES or may interact with a protein containing a NES. Localization of the PKI-GFP fusion protein in HeLa cells transfected with pcPKI-sg143 expression plasmid (see 2.2.6.1.4.) is shown in Fig. 3.23A. The accumulation of Risp-GFP in the cytoplasm of HeLa cells was also observed in stable pcRisp-sg143-transfected cells (Fig. 3.23B).
To obtain information of the intracellular distribution of Risp-GFP, a quantification of Risp-GFP fluorescence in nuclear and cytoplasmic compartments of living cells was performed. As described in 2.5.4. total and nuclear fluorescence intensities were quantified for single cells, corrected for background fluorescence and the proportion of cytoplasmic fluorescence calculated. In addition, intracellular distribution of Rev-GFP, PKI-GFP and GFP for nuclear and cytoplasmic control proteins was quantified. Fig. 3.23 C shows the percentage of cytoplasmic fluorescence obtained with the different GFP-tagged proteins for 9-20 HeLa cells.

Fig. 3.23 A



B



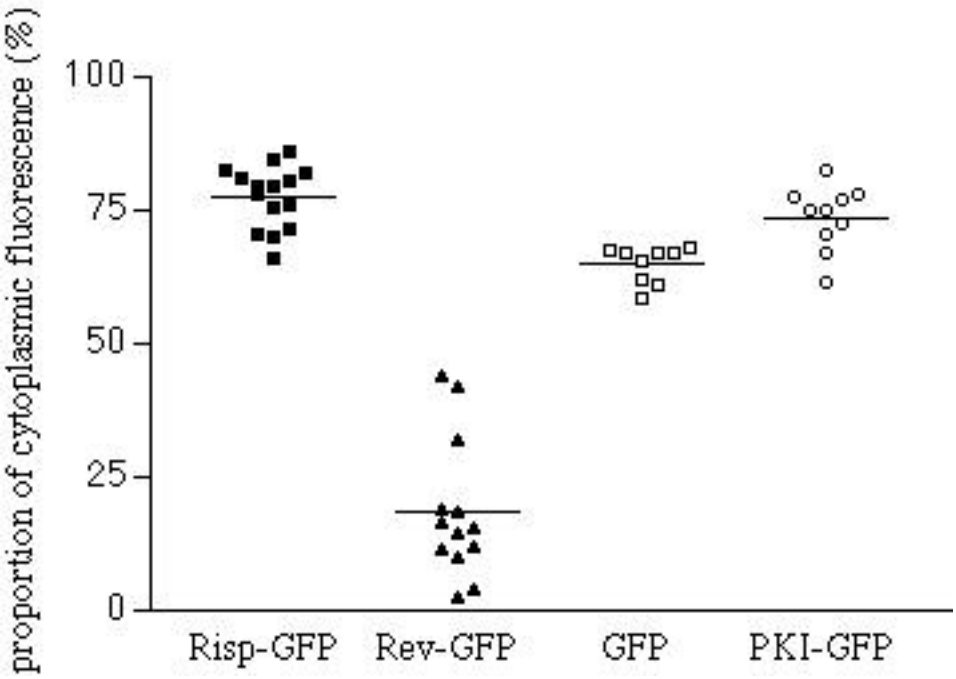


Fig. 3.23 Localization of Risp-GFP. (A) HeLa cells were transfected with 1µg of pcRisp-sg143, pCsRev-sg143 or pFred143 and pcPKI-sg143 expression plasmids. Images of living cells were taken 24h after transfection as described in Mat and Meth. Phase indicates phase-contrast image. (B) Risp-GFP localization in HeLa cells stable transfected. (C) Quantification of the proportion of cytoplasmic fluorescence in HeLa cells expressing Risp-GFP, Rev-GFP, GFP and PKI-GFP 24h posttransfection. Symbols indicate values for single cells and bars mean values for each protein.

Approximately 77% of Risp-GFP was contained into the cytoplasm, whereas 18% of Rev-GFP, 65% of GFP and 74% of PKI-GFP were cytoplasmic. The difference between Risp-GFP and the other proteins is highly significant with the exception of the comparison with PKI-GFP ($p < 0.0001$ (Risp-GFP/Rev-GFP); $p = 0.0002$ (Risp-GFP/GFP); $p = 0.1272$ (Risp-GFP/PKI-GFP); Mann-Whitney t-test for non-parametrically distributed values).

To analyze the intracellular distribution of Risp-GFP with an additional method, HEK 293 cells were transfected with 1µg of pcRisp-sg143 plasmid. Nuclear and cytoplasmic cellular protein fractions were extracted and then subjected to western blot analysis. The protein extracts were separated on a SDS polyacrylamide gel and protein expression was detected with an anti-GFP antibody.

The nuclear and the predominantly cytoplasmic localization of the Risp-GFP-protein were confirmed (data not shown). However, since protein quantification

for the nuclear and cytoplasmic extracts was not performed, these results could confirm only a qualitative nuclear presence of Risp-GFP.

3.5.4.2. Nuclear accumulation of Risp-GFP in the presence of leptomycin B

Several proteins containing leucine-rich NES sequences have been shown to bind to Crm1, a cellular protein involved in nuclear export (see 1.2.) (Fornerod et al., 1997). To assess, whether the cytoplasmic accumulation of Risp-GFP was associated with the presence of NES-Crm1 dependent, the intracellular distribution of Risp-GFP in the presence of leptomycin B was analyzed. Leptomycin B, a *Streptomyces* product, is a potent inhibitor of Crm1, and it is commonly used to block Crm1-mediated nuclear export of proteins (Bogerd et al., 1998; Kudo et al., 1998; Wolff et al., 1997).

Two hours treatment of pcRisp-sg143-transfected cells with 10 nM leptomycin B led to an increase in nuclear fluorescence approximately of the 15%, indicating a clear visible shift of Risp-GFP localization (Fig. 3.24). This difference is highly significant ($p < 0.0001$).

This experiment demonstrated that Risp-GFP is a shuttling protein that can enter and exit the nucleus. Blockage of the nuclear export leads to a nuclear accumulation of Risp-GFP. In addition, these results indicate that this new cellular protein depends on Crm1 as export receptor.

As a control, pcPKI-sg143- and pFred143-transfected cells were also treated with 10nM leptomycin B for 2h and the corresponding fluorescence distribution was quantified (Figs. 3.24 and 3.25).

Fig. 3.24 Localization of Risp-GFP after treatment with leptomycin B. HeLa cells were transfected with 1µg of pcRisp-sg143 or pcPKI-sg143 expression plasmids. Leptomycin B (LMB) was added at the concentration of 10nM 24h after transfection, and images of living cells were taken 2h later.

Fig. 3.25 Quantitative analysis of the localization of GFP fusion proteins in the presence of leptomycin B. Quantification of the proportion of cytoplasmic fluorescence in HeLa cells expressing Risp-GFP, GFP and PKI-GFP before and after LMB treatment. Symbols indicate values for single cells and bars mean values for each protein. Significance between data sets determined by calculating two-tail P values, using the Mann-Whitney U test is also shown.

Fig. 3.24

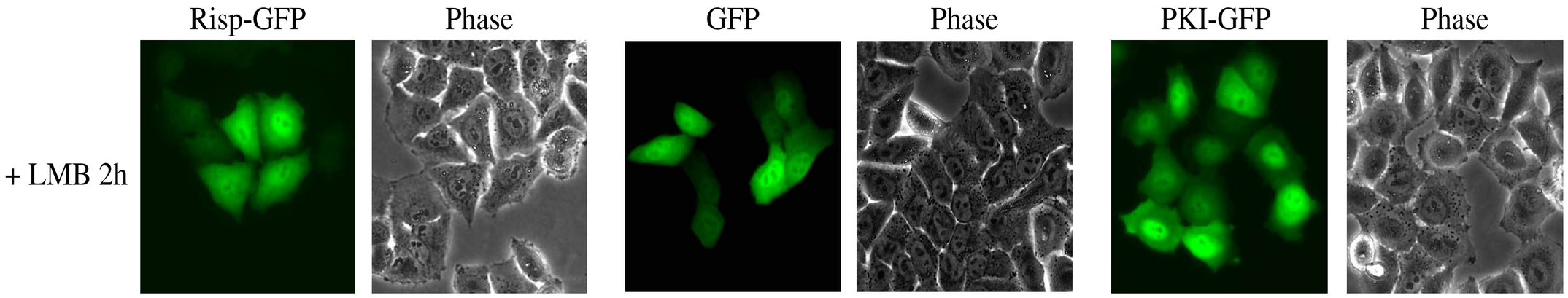
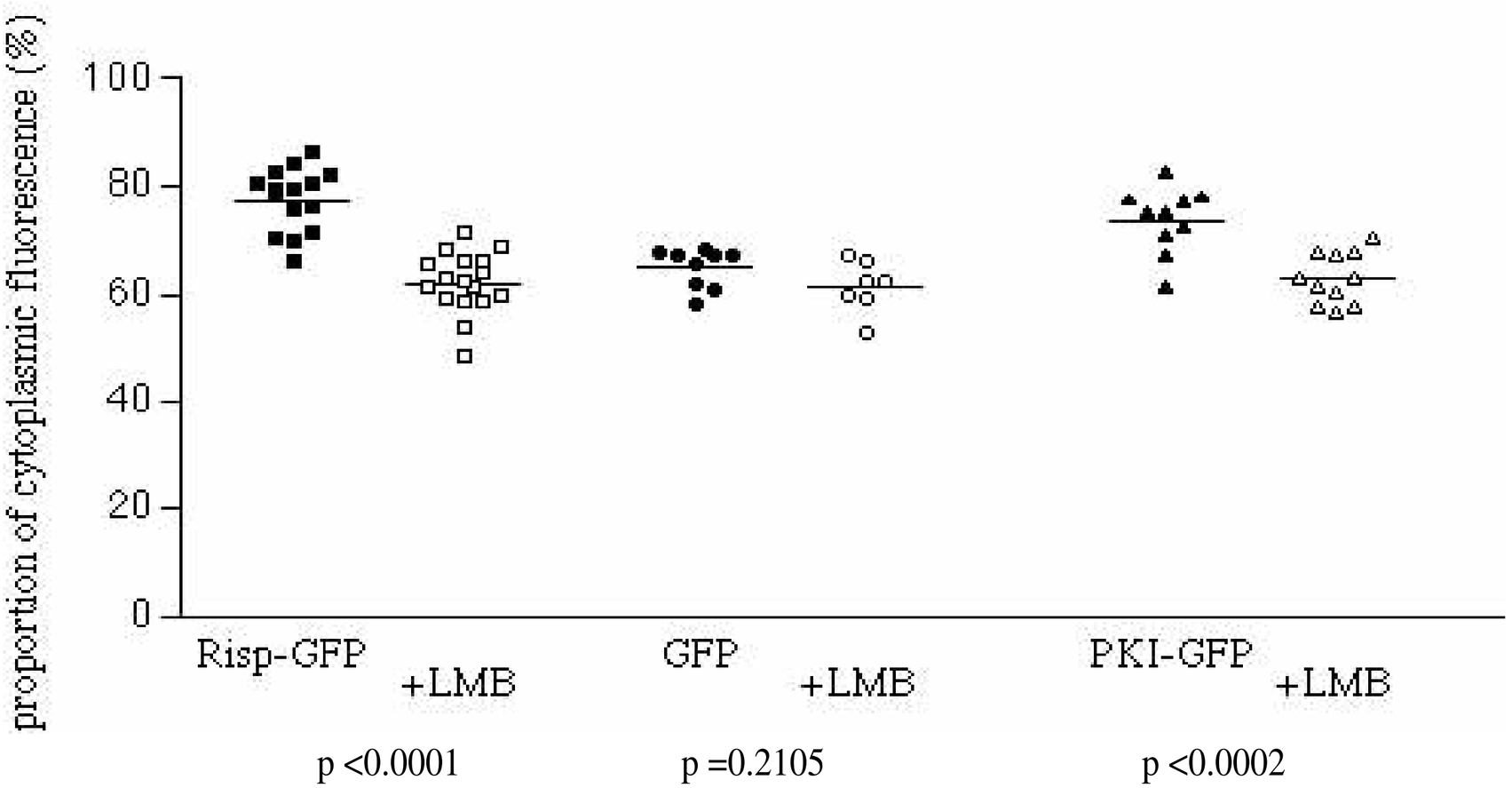


Fig. 3.25



To examine the intracellular distribution of other Rev-interacting proteins, cells were transfected with pcCrm1-sg143, pceIF-5A-sg143 and pc11.5.1-sg143 expression plasmids (see Fig. 2.1B). A comparison of intracellular localization of Crm1-GFP, eIF-5A-GFP and 11.5.1-GFP in HeLa cells 24h after transfection, in presence and absence of leptomycin B is shown in Fig. 3.26.

HeLa cells showed a nuclear and cytoplasmic localization of Crm1-GFP, eIF-5A-GFP and 11.5.1-GFP with a predominant nuclear accumulation. No differences in the cellular distribution of all of these proteins were observed after treatment with leptomycin B.

Fig. 3.26 Localization of cellular Rev interacting proteins. (A) HeLa cells were transfected with 1µg of pcCrm1-sg143, pceIF-5A-sg143 and pc11.5.1-sg143 expression plasmids. Leptomycin B (LMB) was added at the concentration of 10nM 24h after transfection, and images of living cells were taken 2h later.

3.5.4.3. Influence of protein and RNA synthesis on nuclear import and export of Risp

To analyze whether the blocking of new protein-synthesis could influence the nuclear import or export of Risp-GFP, the translation inhibitor cycloheximide was used. Approximately 24h after transfection with pcRisp-sg143 or pcPKI-sg143 plasmid (1µg), HeLa cells were incubated for 2h in medium supplemented with cycloheximide alone (25µg/ml), or with leptomycin B (10nM) after a 30 min pretreatment with cycloheximide (25µg/ml). The results are shown in the Fig. 3.27.

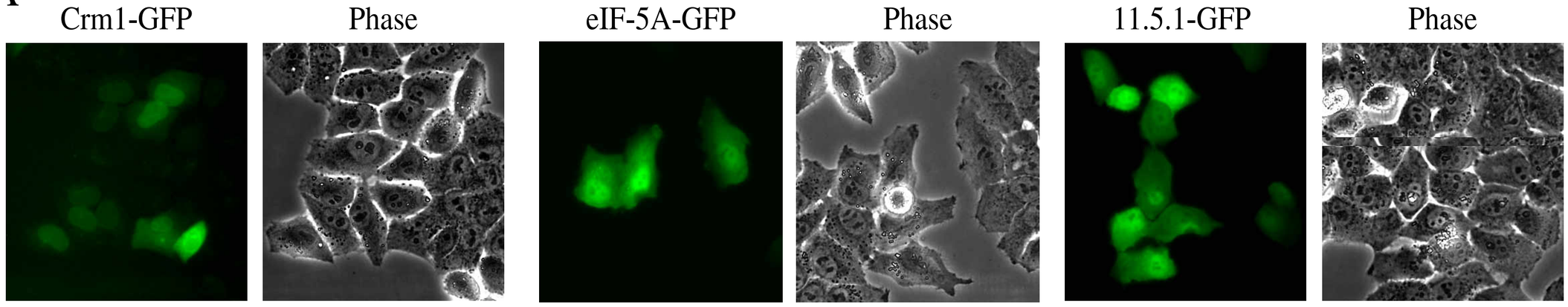
Blocking new protein synthesis by cycloheximide had no effect on the cytoplasmic localization and the nuclear import of Risp-GFP or PKI-GFP. In fact, the combination of leptomycin B and cycloheximide induced the same nuclear accumulation of Risp-GFP as observed in the absence of cycloheximide.

Fig. 3.27 Localization of Risp-GFP in the presence of cycloheximide. HeLa cells were transfected with 1µg of pcRisp-sg143 or pcPKI-sg143 expression plasmids. Cycloheximide (25µg/ml) and leptomycin B (LMB, 10nM) were added in the culture medium 24h after transfection.

It is known that the treatment of Rev-expressing cells with transcription inhibitors leads to an accumulation of Rev in the cytoplasm (Meyer and Malim, 1994).
The effect of transcription inhibitors on the localization and shuttling property of Risp was therefore evaluated.
Approximately 24h after transfection with pcRisp-sg143 or pCsRev-sg143 (used as a control), HeLa cells were incubated for 2h in medium supplemented with the transcription inhibitor actinomycin D (5µg/ml), which at this concentration is known

Fig. 3.26

A



B

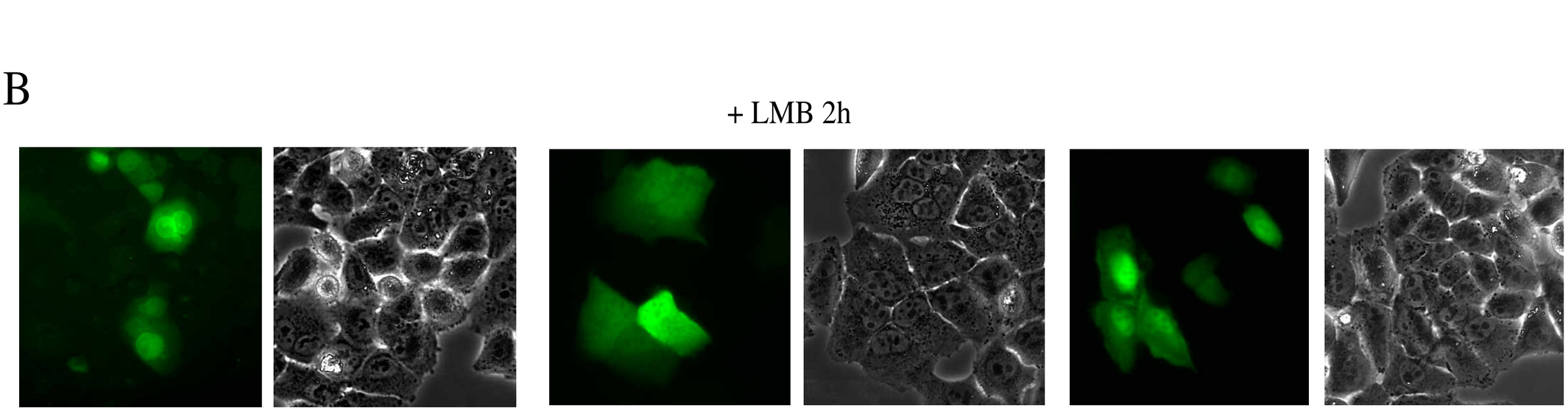
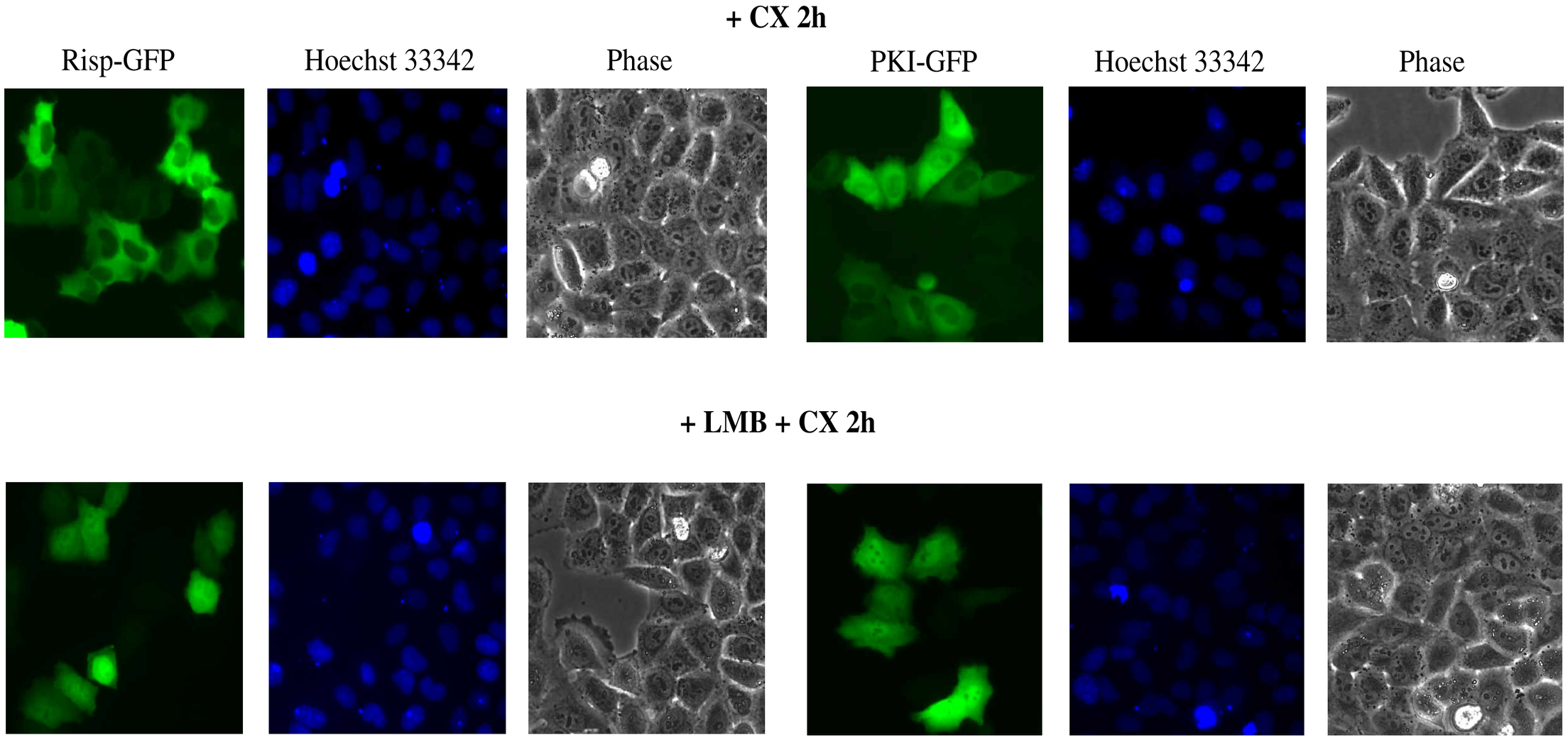


Fig. 3.27


+ CX 2h
Risp-GFP
Hoechst 33342
Phase
PKI-GFP
Hoechst 33342
Phase
+ LMB + CX 2h

to inhibit all three RNA polymerases. Additionally, to exclude new synthesis of Risp or Rev proteins, the translation inhibitor cycloheximide was added to the cell cultures 30 min prior to the inhibition of transcription and was maintained in the medium together with actinomycin D.

As previously noted treatment with actynomycin D and cycloheximide resulted in a significant accumulation of Rev in the cytoplasm, which was not observed by treatment of cells with cycloheximide alone (data not shown).
On the contrary, treatment with actynomycin D and cycloheximide did not alter the cytoplasmic localization of Risp-GFP (Fig. 3.28).

Moreover, to analyze the effect of transcription inhibition on the shuttling properties of Risp-GFP and Rev-GFP, the export-inhibiting drug leptomycin B (10nM) was added to the cells previously treated for 2h with actinomycin D.

In the presence of actinomycin D and leptomycin B, Risp-GFP and Rev-GFP accumulated in the nuclei, indicating that the inhibition of transcription did not alter the nuclear import of both proteins (Fig. 3.28).

Fig. 3.28 Localization of Risp-GFP in the presence of actinomycin D. HeLa cells were transfected with 1µg of pCsRev-sg143 or pcRisp-sg143 expression plasmid respectively. Actinomycin D (AD, 5µg/ml) was added 24h after transfection to cells pre-treated for 30' with cycloheximide (CX, 25µg/ml). Two hours later, leptomycin B (LMB, 10nM) was added in the culture medium for 1h.

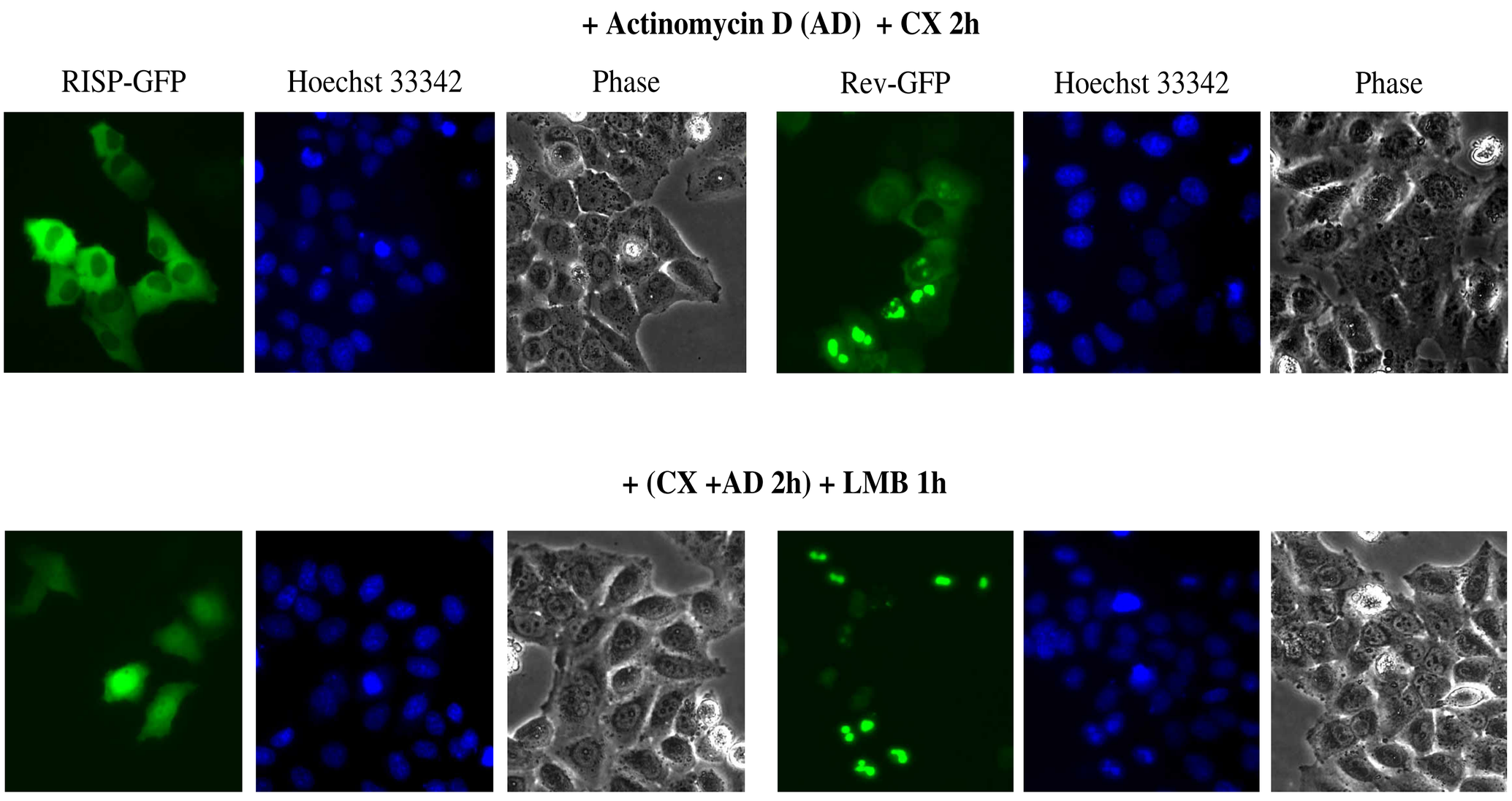
3.5.4.4. Nuclear transport signals in Risp: Identification of regions containing a NES and a nucleolus localization signal (NOS)

Nuclear localization and nuclear export signals are important to mediate nuclear import and export pathways. Because it could be shown that Risp-GFP can shuttle between the nucleus and the cytoplasm using Crm1 as an export receptor, the identification of NLS and NES sequences in Risp was attempted.

3.5.4.4.1. Localization of several pcIg-plasmids containing different regions of Risp

To localize the domains of Risp involved in the nuclear import and export of the protein, full-length or parts of the *risp* cDNA were fused to the C-terminus of a fusion protein cassette, containing the constant domains CH2 and CH3 of the human immunoglobulin G1 (IgG), into the pcIg vector (see Fig. 2.1).
The seven cDNAs (5'MluI/3'NotI) encoding Risp protein and its N-terminal or C-terminal peptides formerly inserted into the yeast two hybrid vector pJG4-6 were recloned into the unique cloning sites MluI/NotI of the pcIg vector.

Fig. 3.28


+ Actinomycin D (AD) + CX 2h
RISP-GFP
Hoechst 33342
Phase
Rev-GFP
Hoechst 33342
Phase
+ (CX +AD 2h) + LMB 1h

After confirmation by sequence analysis, each correct plasmid was kept for further use (Fig. 3.31).
First, HeLa cells were transfected with the pcIg-Risp and the pcIg expression plasmids (1µg). Twenty-four hours post-transfection, the localization of IgG alone or fused to Risp protein was analyzed by indirect immunofluorescence using a Cy3-conjugated goat anti-human IgG antibody.

As previously observed in cells transfected with pcRisp-sg143, the predominantly cytoplasmic distribution of Risp-GFP was observed also in steady-state localization of Risp fused to the constant region CH2-CH3 of the human IgG (cytoplasmic fluorescence 84%). Moreover, in one experiment, the addition of leptomycin B to cells transfected with pcIg-Risp, led to an increase the nuclear fluorescence of IgG-Risp (cytoplasmic fluorescence 63%) (Fig.3.29).

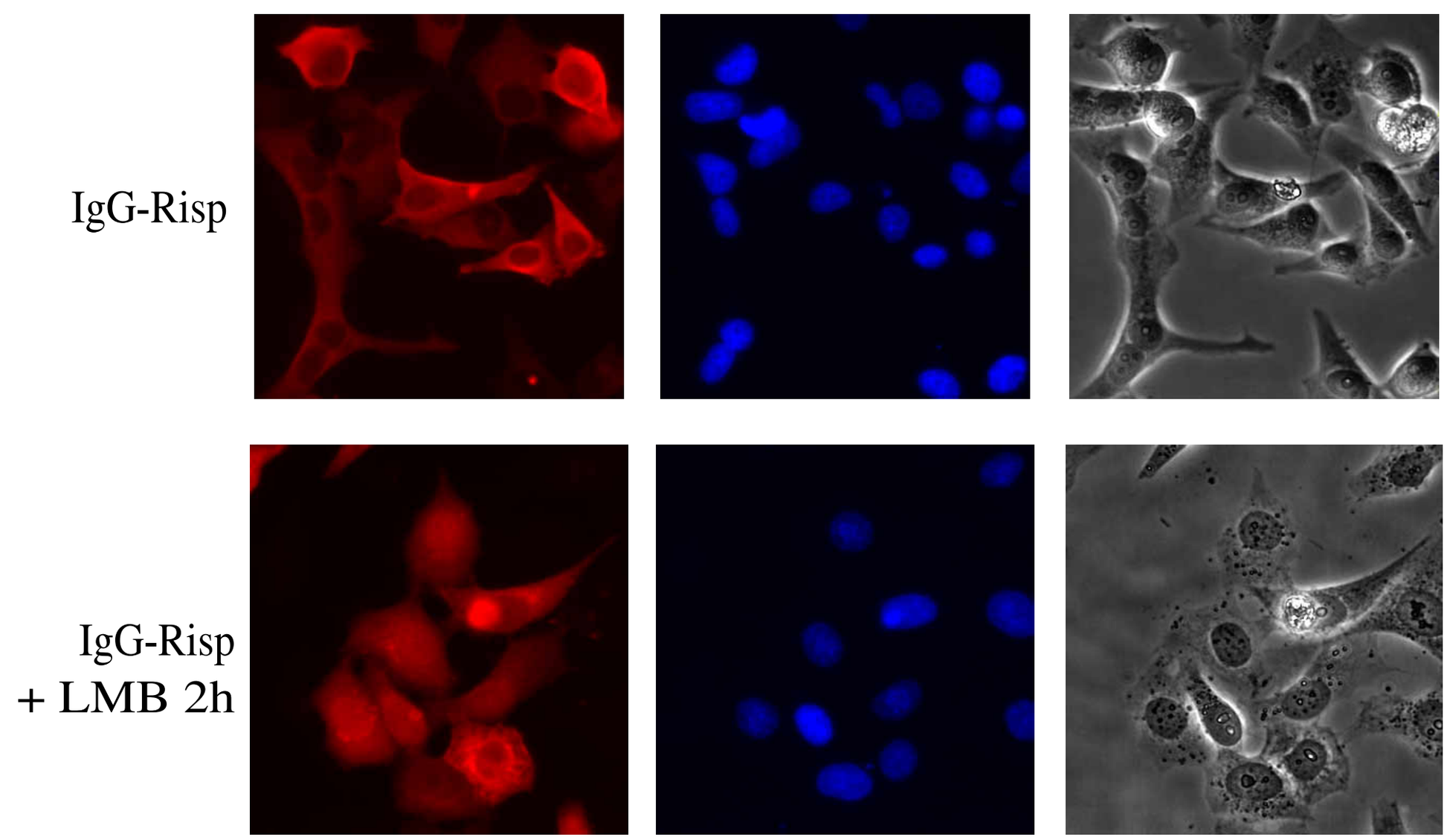
Fig. 3.29 Localization of IgG-Risp. HeLa cells were transfected with 1µg of pcIg-Risp expression plasmid. Leptomycin B (LMB) was added at the concentration of 10nM 24h after transfection. Cells were fixed, permeabilized and analyzed by indirect immunofluorescence microscopy using a Cy3-conjugated goat anti-human IgG (1:200). (B) Quantification of the proportion of cytoplasmic fluorescence in HeLa cells expressing IgG-Risp before and after LMB treatment. Symbols indicate values for single cells and bars mean values for each protein.

To identify a NLS or a NES in Risp protein responsible for its shuttling properties, HeLa cells were transfected with the different expression plasmids pcIg-(2-38), pcIg-(2-73), pcIg-(2-133), pcIg-(39-171), pcIg-(74-171) or pcIg-(134-171) (1µg). Twenty-four hours post-transfection, the localization of the different N-terminal and C-terminal segments of Risp protein was analyzed by indirect immunofluorescence using anti-human IgG or anti-Risp.

As shown in Fig. 3.30, HeLa cells expressing segments of Risp up to residue 73 or from residues 134-171 showed fluorescence in both nucleus and cytoplasm compartments, as observed for IgG alone. On the contrary, peptides containing the region of Risp between amino acids 74-133 showed a high accumulation in the cytoplasm.
These data suggest that the region between 74 and 133 of Risp is required for nuclear export of Risp, while the region responsible for nuclear localization signal has not been identified yet.
Moreover, although the antibodies anti-Risp #1, #4 and #7 recognized Risp in different regions in the 3´part of the protein, similar results were obtained using the anti-Risp and the anti-IgG antibodies .

Fig. 3.29

A


anti-human IgG
Hoechst 33342
Phase
IgG-Risp
IgG-Risp
+ LMB 2h

B

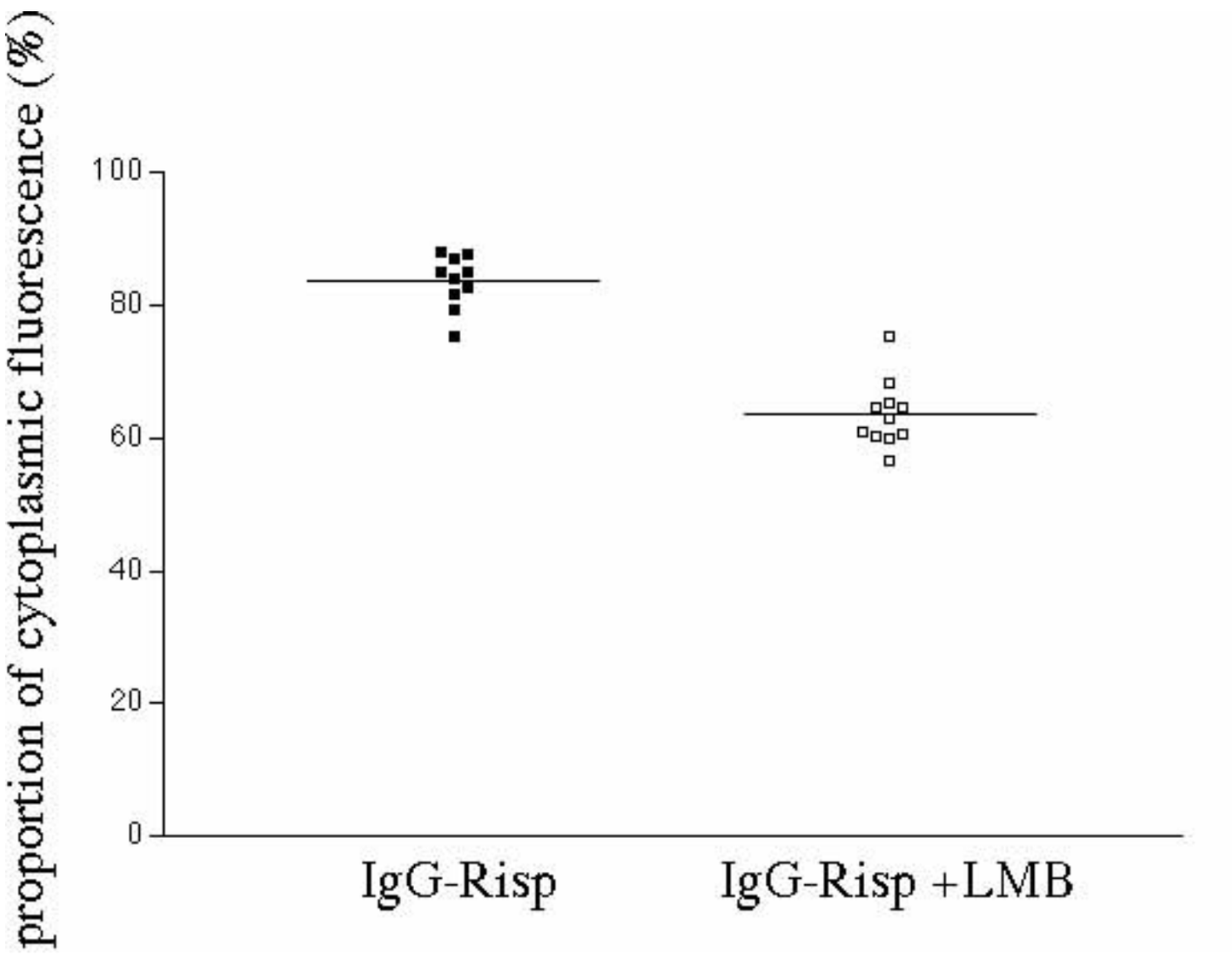

proportion of cytoplasmic fluorescence (%)
100
80
60
40
20
0
IgG-Risp
IgG-Risp +LMB

Fig. 3.30

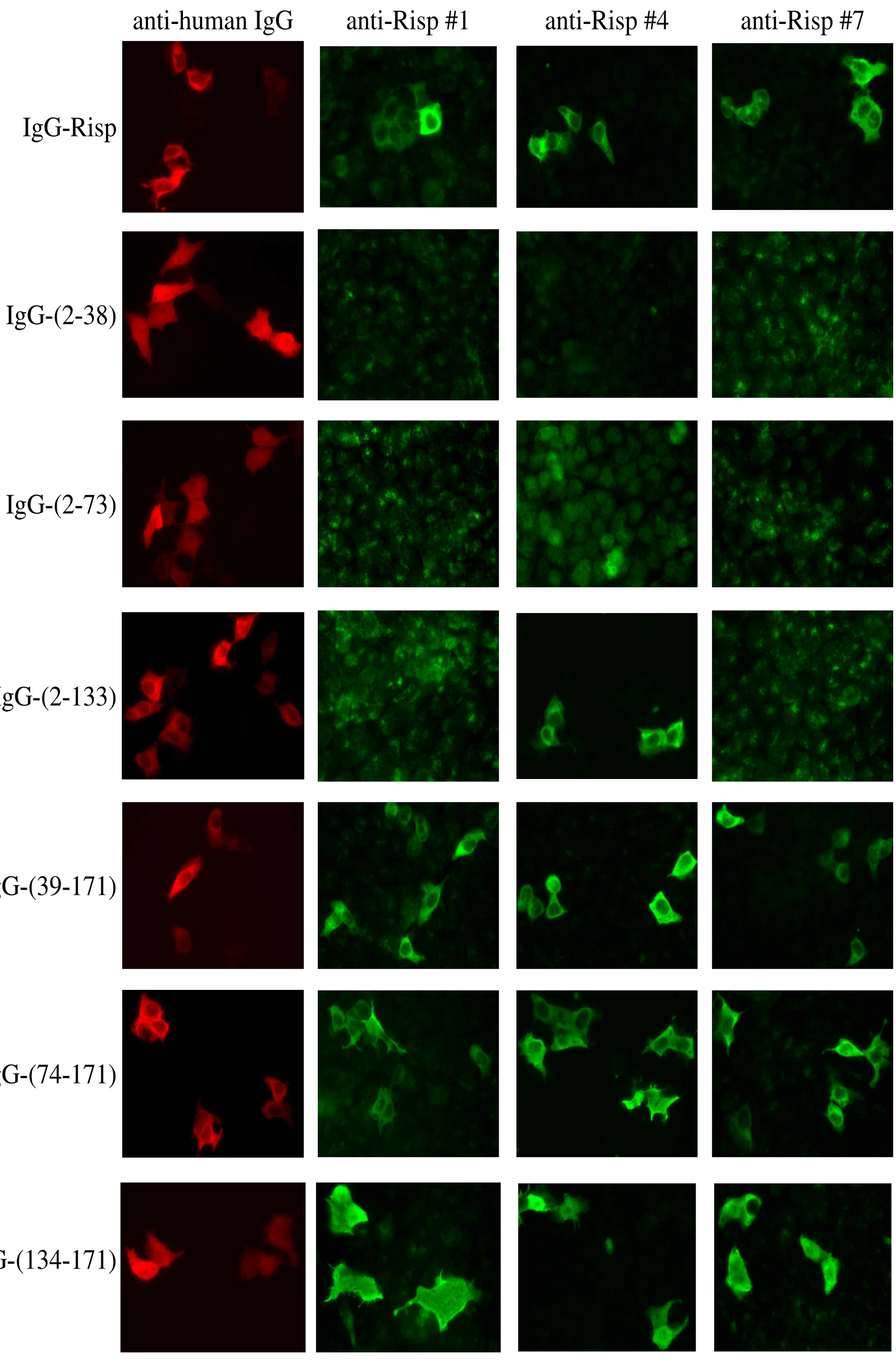

anti-human IgG
anti-Risp #1
anti-Risp #4
anti-Risp #7
IgG-Risp
IgG-(2-38)
IgG-(2-73)
IgG-(2-133)
IgG-(39-171)
IgG-(74-171)
IgG-(134-171)

Fig. 3.30 Localization of several IgG-segments of Risp protein. HeLa cells were transfected with 1µg of pcIg or pcIg-Risp expression plasmid respectively [pcIg-(2-38), pcIg-(2-73), pcIg-(2-133), pcIg-(39-171), pcIg-(74-171) or pcIg-(134-171)]. Twenty-four hours post-transfection, cells were fixed, permeabilized and analyzed by indirect immunofluorescence microscopy using a Cy3-conjugated goat anti-human IgG (1:200) or the rat monoclonal anti-Risp #1, #4 and #7 antibodies (1:100), followed by a FITC-conjugated goat anti-rat IgG (1:50).

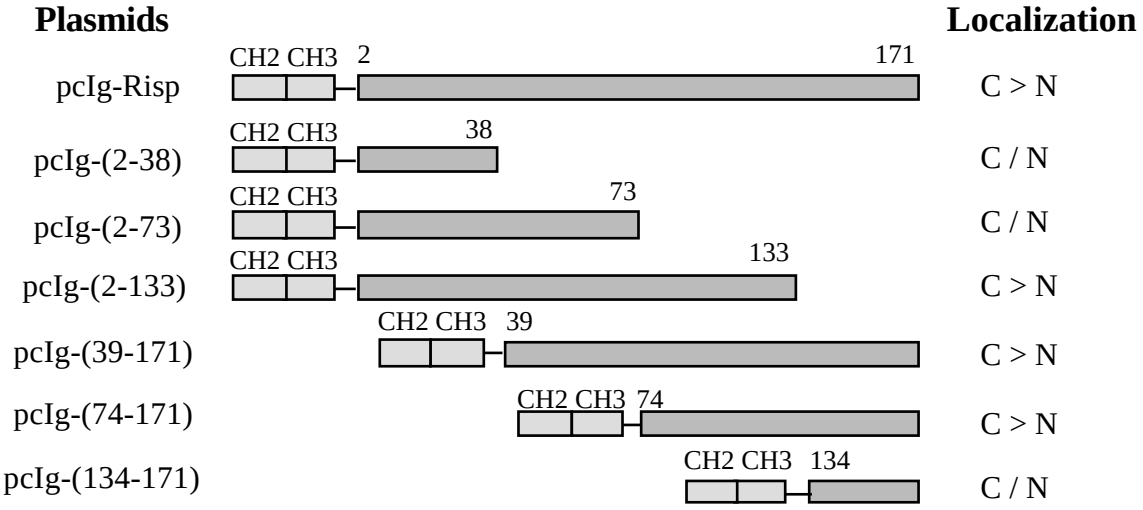


Fig. 3.31 Schematic diagram of the constructed IgG-segments of Risp protein and summary of localization analysis.

3.5.4.4.2. Cellular microinjection of Risp peptides

The analysis of Risp primary amino acid sequence suggested that Risp sequence does not contain “classical” signals known to mediate nucleo-cytoplasmic transport.

However, the intracellular localization analysis of different segments of Risp suggested the presence of a NES in the region comprising the amino acids 73-133. A more thorough analysis of this sequence showed at position 88 a short cluster (LESNLFDDNIDIFADL) richer of leucine and isoleucine residues. Therefore, to investigate whether this sequence could function as NES, a peptide consisting of the residues 86-105 was synthesized and cross-linked to Alexa fluor 488-labeled BSA (see 2.5.12. and Fig. 3.32). At 68 kDa, BSA is too large to passively diffuse through the nuclear pores. Therefore, BSA is a heterologous protein commonly used as a linker to peptides, with candidate nuclear transport signals. Linkage of peptide with NLS or NES has been shown to mediate the nuclear import or export of the chimera fusion protein (Fischer et al., 1995).

In addition, the presence of a lysine-rich cluster (KPKEKSKKKVEAK) at position 106 that could possibly function as NLS was previously detected. Therefore, a second peptide consisting of the residues 106-118 was synthesized and cross-linked to Alexa fluor 488-labeled BSA (Fig. 3.32).

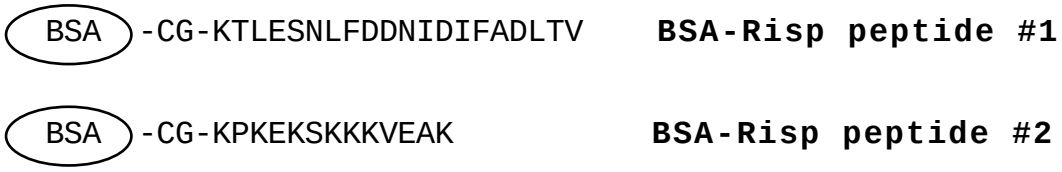


Fig. 3.32 Schematic description of the structure of Risp peptides conjugates to BSA used in this study. BSA-Risp peptide #1 consists of Alexa fluor 488-BSA cross-linked to peptides encompassing the residues 86-105 of Risp protein with suspected NES. BSA-Risp peptide #2 consists of Alexa fluor 488-BSA cross-linked to peptides encompassing the residues 106-118 of Risp protein with suspected NLS. One glycine residue was additionally used as hinge region to allow correct folding of both protein and peptides.

To follow the intracellular trafficking of the Risp peptides, each purified Alexa fluor 488-labeled BSA conjugate (~1 mg/ml) was microinjected into the nuclei or into the cytoplasm of HeLa cells along with Alexa fluor 568-labeled BSA (~1 mg/ml).
First, the nuclear export ability of the Risp peptide #1 was tested.

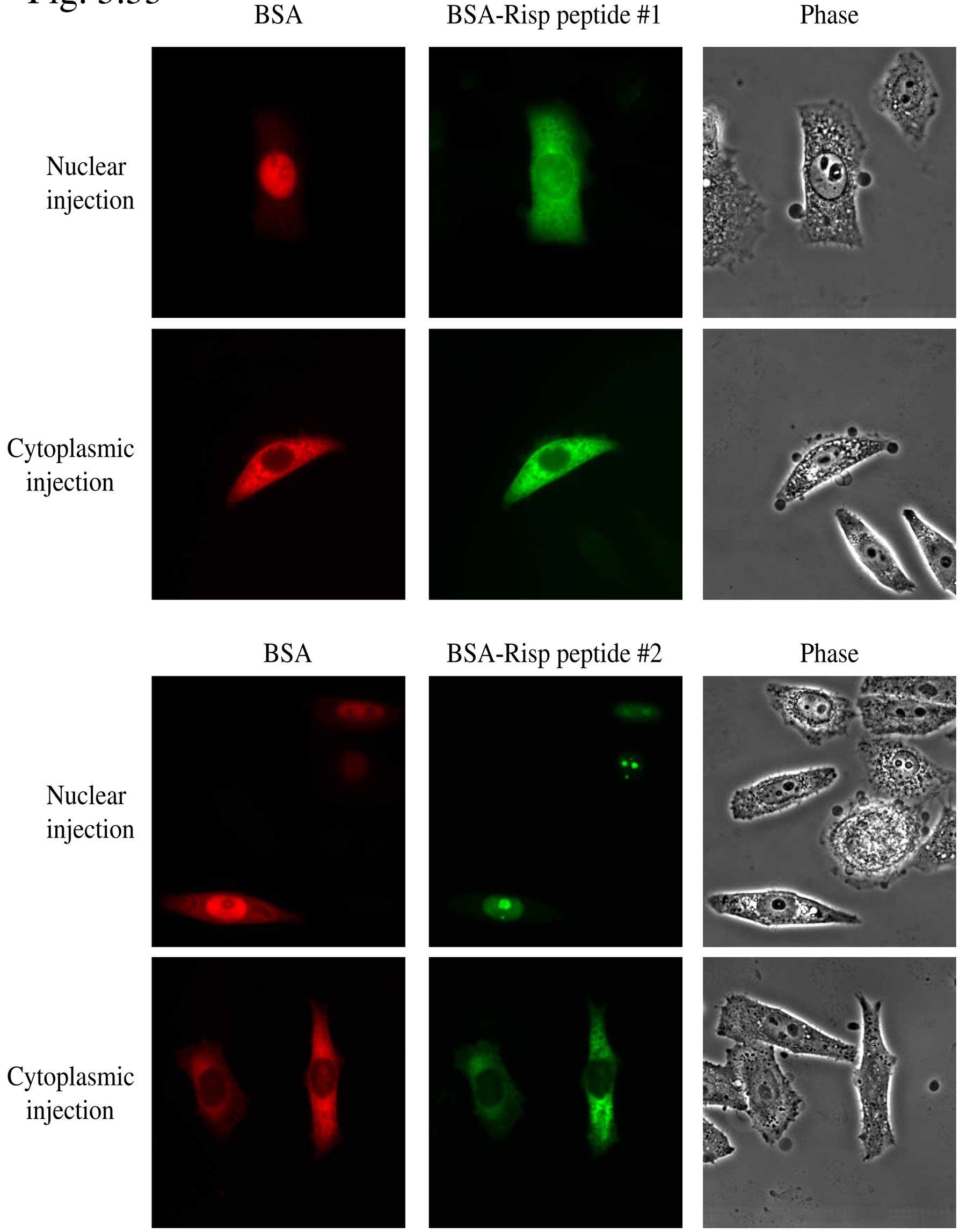
Two hours after nuclear microinjection of a mixture of BSA-Risp peptide #1 and BSA, unconjugated BSA remained exclusively in the nucleus and the conjugated BSA-Risp #1 was exported out of the nucleus into the cytoplasm (Fig. 3.33). On the contrary, 2h after cytoplasmic microinjection of a mixture of BSA-Risp #1 and BSA, both compounds stayed in the cytoplasm.
Therefore this experiment indicated the presence of a NES in the Risp protein between amino acids 86 and 105, which is able to mediate nuclear export of a BSA-conjugate.

To investigate whether the Risp peptide #2 could induce nuclear import of the BSA-conjugate via an active pathway, cytoplasmic and nuclear microinjections of a mixture of BSA-Risp peptide #2 and unconjugated BSA were performed.

Two hours after cytoplasmic microinjection, both unconjugated BSA and BSA-Risp peptide #2 stayed in the cytoplasm (Fig. 3.33), indicating that in the Risp region contained in BSA-Risp #2 peptide does not contain a NLS. On the contrary, the fluorescent-labeled BSA-Risp #2 conjugate was imported into the nucleoli (Fig. 3.33) when microinjected into the nucleus. Interestingly when the mixture of BSA-Risp #2 and BSA was microinjected in the same cell in both nuclear and cytoplasmic compartments, only the BSA-Risp #2 conjugate entered the nucleus and accumulated in the nucleoli, indicating the presence of a nucleolar localization signal (NOS) in Risp not associated with a NLS.

Fig. 3.33 Nuclear import and export of fluorescent dye labeled BSA conjugate to Risp peptides. HeLa cells were co-microinjected into the nucleus or into the cytoplasm with the indicated Alexa fluor 488-BSA-Risp peptides (BSA-Risp peptide #1 or BSA-Risp peptide #2) and the unconjugated Alexa fluor 568-BSA (BSA, injection control). To allow sufficient time for import, cells were incubated for 2h at 37 C, fixed and analyzed by fluorescence microscopy.

Fig. 3.33


BSA
BSA-Risp peptide #1
Phase
Nuclear injection
Cytoplasmic injection
BSA
BSA-Risp peptide #2
Phase
Nuclear injection
Cytoplasmic injection

3.5.4.4.3. Cellular microinjection of Risp-GST protein

To confirm the indications that Risp is a shuttle protein, also two microinjection experiments using the purified Risp-GST protein were performed. To follow the intracellular trafficking of this fusion protein, HeLa cells were microinjected in the nuclei and in the cytoplasm with a mixture of Risp-GST (~ 1mg/ml) along with Alexa fluor 568-labeled BSA (~1 mg/ml). After 2h the cells were fixed and stained with an antibody against GST or Risp, followed by a FITC-conjugated second antibody.

Two hours after nuclear microinjection Risp-GST was exported out from the nuclear injection site into the cytoplasm, while the unconjugated BSA remained in the nucleus, (Fig. 3.34).

On the contrary, 2h after cytoplasmic microinjection of a mixture of Risp-GST and BSA, both compounds stayed in the cytoplasm.

Moreover, to test the ability of Risp-GST to be imported into the nucleus, cytoplasmic microinjections of cells pre-treated with leptomycin B (10nM, 2h) were performed in a preliminary experiment. Unfortunately, the experiment did not work, because also BSA as the negative control diffused in and out of the nuclei, indicating that the cells were damaged. Therefore additional experiments should be performed.

Fig. 3.34 Nuclear export of Risp-GST fusion protein. HeLa cells were co-microinjected into the nucleus or into the cytoplasm with the purified Risp-GST protein and the unconjugated Alexa fluor 568-BSA (BSA, injection control). Cells were incubated for 2h at 37˚C, fixed and permeabilized. The injected Risp-GST fusion protein was visualized by immunostaining using a rat monoclonal antibody anti-GST (1:100) or a rat monoclonal antibody anti-Risp #4 (1:100), followed by a DTAF-conjugated goat anti-rat IgG (1:50).

3.5.4.5. Influence of the presence of Rev on Risp localization

To investigate whether the presence of Rev could induce a change in the localization of Risp-GFP and vice versa, HeLa cells were transfected with the pcRisp-sg143 in the absence or presence of pBsRev or pcRev expression plasmid. As control the plasmids pL3Tat (see plasmids) and pcPKI-sg143 were used.

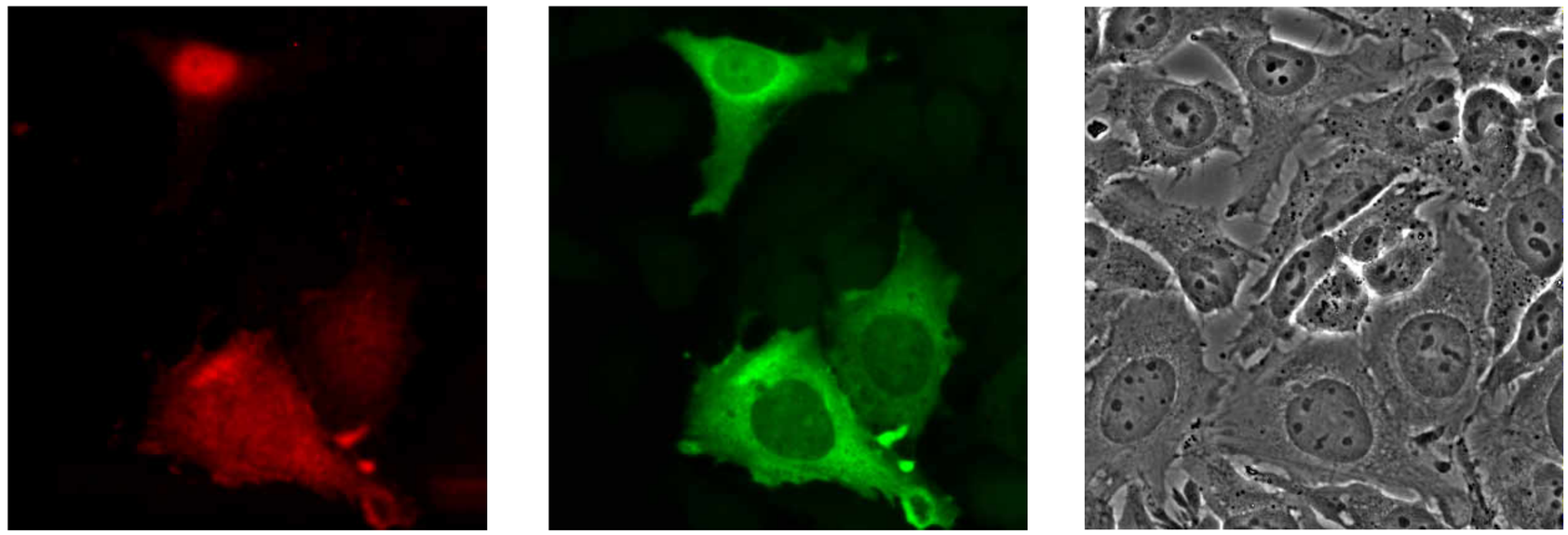
The intracellular localization of Risp-GFP alone and in the presence of the Rev- and/or Tat- expression plasmid was observed and quantified. In addition, the localization of PKI-GFP was compared with and without Rev. Statistical analysis of differences between data sets was determined by calculating two-tail P values using the Mann-Whitney U test.

Fig. 3.34

Nuclear injection

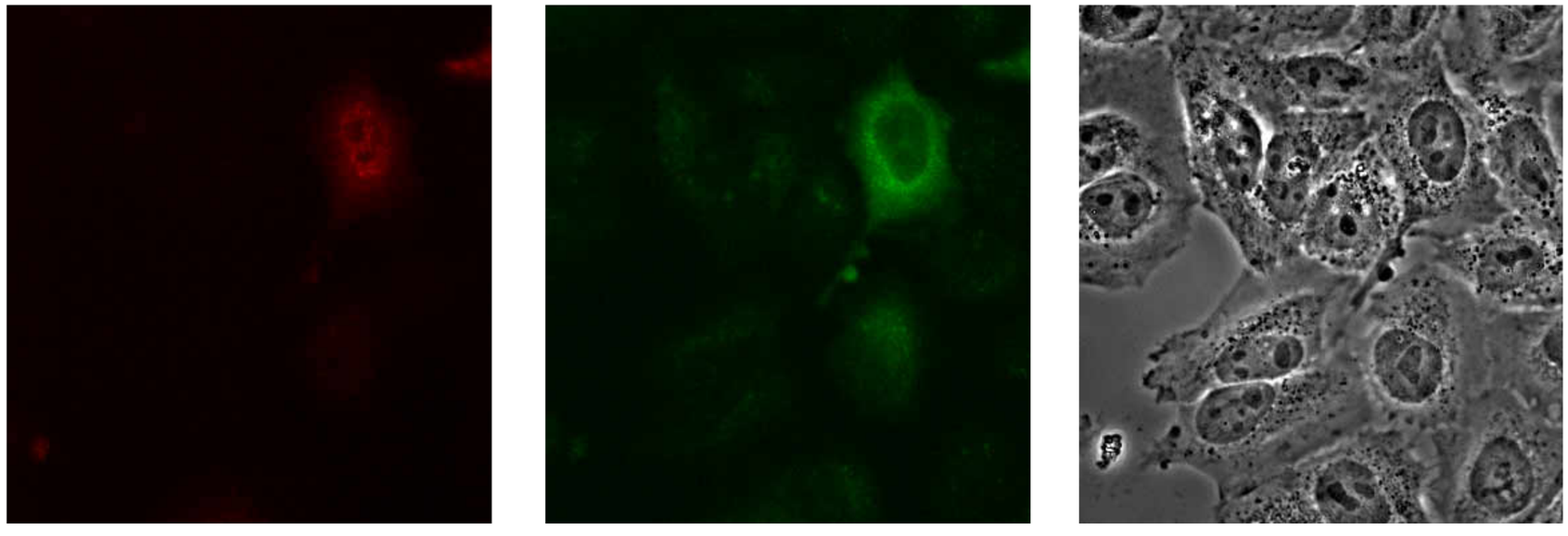
Alexa fluor 568 | anti-GST | Phase

BSA+ Risp-GST



anti-Risp #4

BSA+ Risp-GST



The presence of Rev protein, independently of which expression plasmid was used, induced a statistical significant increase in nuclear fluorescence of Risp-GFP 5% with pcRev and 15% with pBsRev in comparison to Risp-GFP alone. On the contrary, the presence of Tat did not induce any significant change in the typical cytoplasmic fluorescence of Risp-GFP, and the cytoplasmic localization of PKI-GFP did not significantly change by the presence of Rev (Fig. 3.35).

To confirm that the increased nuclear localization of Risp-GFP was really due to the presence of Rev protein in the cell, immunofluorescence was performed using rabbit antibodies against Rev. Since with the anti-Rev used distinguishing between Rev protein and coexpressed Risp-GFP was not observed, a clear result was not obtained.

Fig. 3.35 Localization of Risp-GFP in the presence of Rev. (A) HeLa cells were transfected with pcRisp-sg143 or pcPKI-sg143 plasmid (1µg) in presence of pcRev, pBsRev and pL3Tat or pL3Tat expression plasmid (1µg). (C) Quantification of the proportion of cytoplasmic fluorescence was performed. Symbols indicate values for single cells and horizontal bars indicate mean values. Statistical significance of differences between Risp-GFP/PKI-GFP cytoplasmic data sets and the Risp-GFP/PKI-GFP cytoplasmic data sets obtained in the presence of Rev or Tat determined by calculating two-tail P values using the Mann-Whitney U test are shown.

3.5.4.6. Localization of Risp-GFP in astrocytes

Rev has been described to be a nuclear shuttle protein that accumulates predominantly in the nucleoli of Rev-expressing cells. Nevertheless in a recent study, carried out in the group directed by Dr. Brack-Werner, an unusual and strong cytoplasmic localization of Rev-GFP was shown associated with a diminished function of Rev in various human astrocytoma/glioblastoma cell lines and in primary human fetal astrocytes (Ludwig et al., 1999). Astrocytes constitute the first example of a human cell type with an impaired Rev-response and a strong steady-state cytoplasmic accumulation of Rev. Since Risp is a Rev-interacting protein, I also compared intracellular distribution of Risp in astrocytes and Rev-permissive control cells (HeLa). Fig. 3.36 shows a comparison of intracellular localization of Risp-GFP 24h after transfection in two astrocytoma cell lines (85HG66 and U138), in primary astrocytes (H4/96) and in HeLa cells.

HeLa cells transfected with pcsRisp-sg143 plasmid showed a predominantly cytoplasmic distribution of Risp-GFP, whereas astrocytoma cell lines and primary astrocytes showed a non-homogeneous cellular localization of the fusion protein. Depending on the cellular confluence on the plate, a variable number of astrocyte cells (10-60%) showed accumulation of Risp-GFP in both cytoplasmic and nuclear compartments.

Fig. 3.35

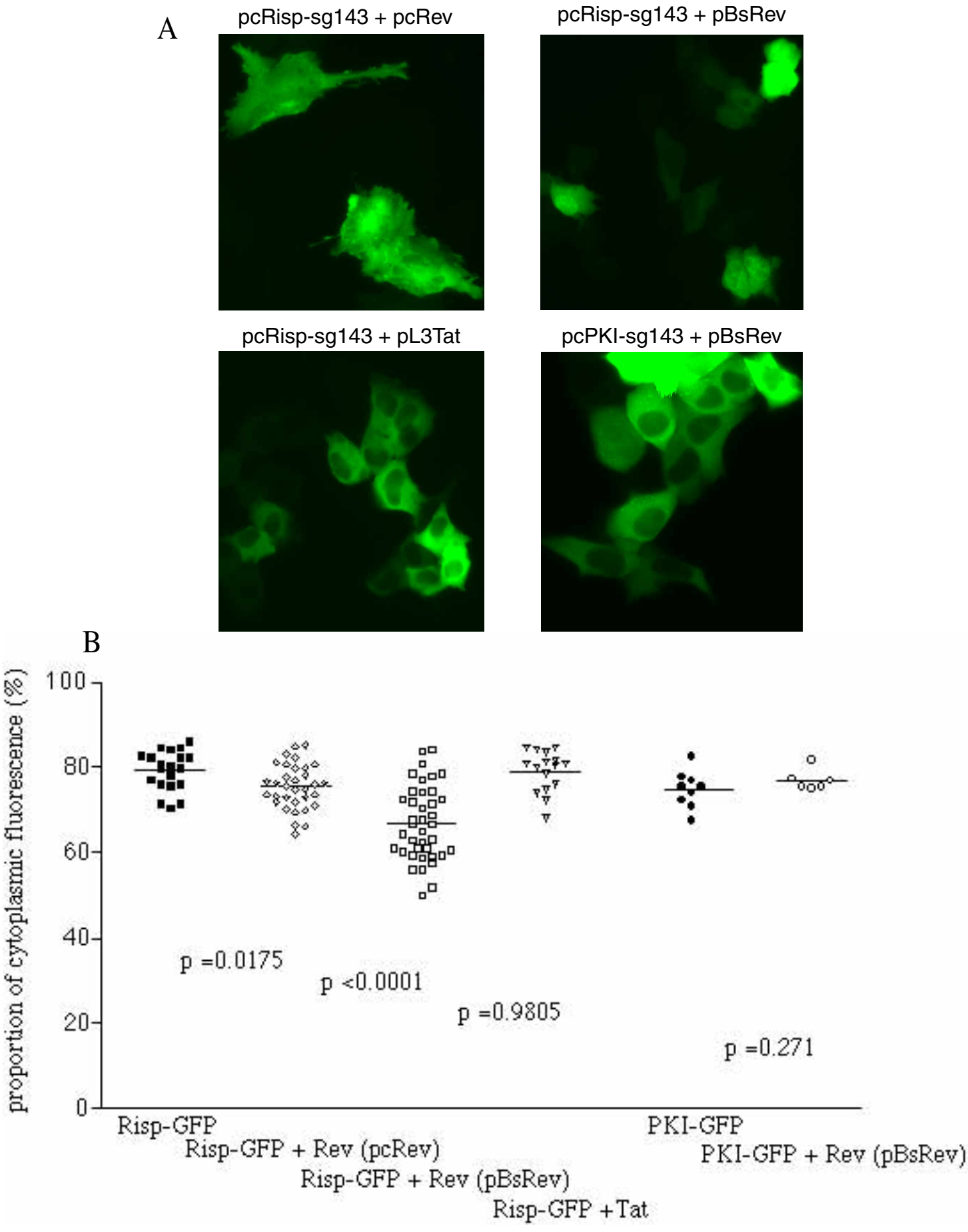

A
pcRisp-sg143 + pcRev
pcRisp-sg143 + pBsRev
pcRisp-sg143 + pL3Tat
pcPKI-sg143 + pBsRev
B
proportion of cytoplasmic fluorescence (%)
100
80
60
40
20
0
p =0.0175
p <0.0001
p =0.9805
p =0.271
Risp-GFP
Risp-GFP + Rev (pcRev)
Risp-GFP + Rev (pBsRev)
Risp-GFP +Tat
PKI-GFP
PKI-GFP + Rev (pBsRev)

Fig. 3.36

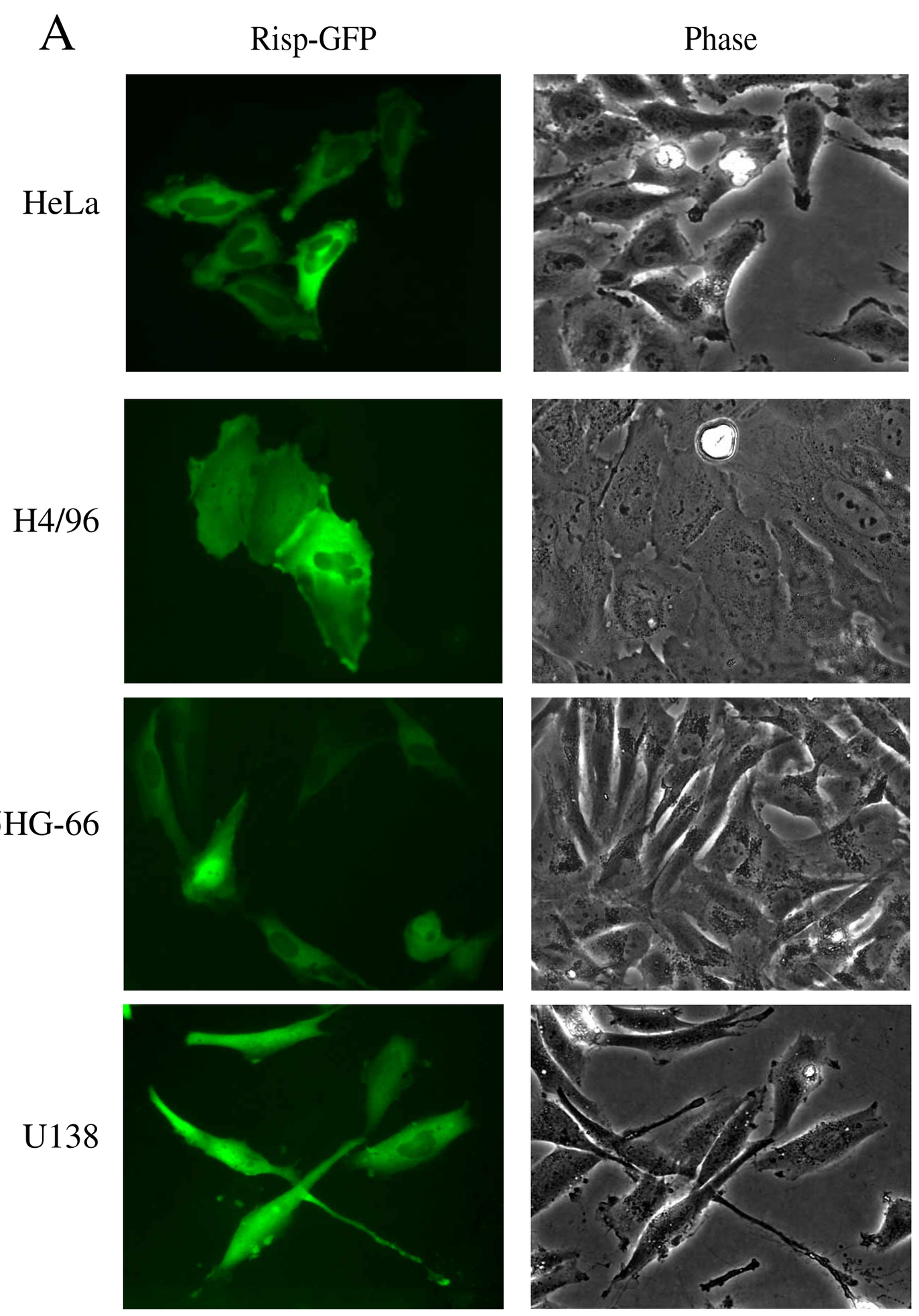

A
Risp-GFP
Phase
HeLa
H4/96
85HG-66
U138
B
Nuclear fluorescence intensity
50
40
30
20
10
0
Risp-GFP
GFP
Rev-GFP
HeLa
H4/96
85HG-66
U138
HeLa
HeLa

Fig. 3.36 Localization of Risp-GFP in astrocytes. (A) HeLa, 85HG66, U138 and primary astrocyte cells (H4/96) were transfected with pcRisp-sg143 or pcPKI-sg143 plasmid (1µg). (C) Quantification of nuclear fluorescence intensity was performed. Symbols indicate values for single cells and bars indicate mean values.

In summary, the results of four experiments showed that Risp-GFP was able to inhibit the SLIIB-CAT expression induced by Tat-Rev at different levels according to its concentration (Fig. 3.20). Risp was able to reduce the SLIIB-CAT activation, presumably by competition to the binding of Rev with its RNA target, however an inconsistent inhibition not related to Risp was also observed. In addition, despite the overexpression of GFP or Rab proteins, which did not influence the HIV-1 Gag production, cells expressing Risp-GFP showed distinctly diminished Rev-dependent stimulation of Gag production. To confirm the specificity of the reduction of Rev-SLIIB binding and HIV-1Gag production by Risp, more experiments have to be performed to allow a statistical analysis of the data.

Qualitative and quantitative analysis of the intracellular localization of Risp in HeLa cells showed that Risp-GFP is a shuttle protein that can enter and exit the nucleus and accumulates in the cytoplasm, indicating the presence of a strong nuclear export signal (NES). The identification of Risp NES was confirmed by localization analysis of different transfected segments of Risp and by cytoplasmic accumulation of nuclear microinjected BSA-fusion protein containing a peptide from the C-terminal part of Risp. The inhibition of the Crm1-mediated nuclear export, induced by the presence of leptomycin B, showed that the nuclear export of Risp is Crm1-dependent.

A different cellular distribution of Risp-GFP in astrocytes with respect to the typical cytoplasmic accumulation in HeLa cells was observed. Depending on the cellular confluence on the plate, a variable number of astrocyte cells (10-60%) showed accumulation of Risp-GFP in both cytoplasmic and nuclear compartments.

3.6. Identification of an additional potential NLS in Rev

An increasing number of NLSs and NESs have been identified in the last years. Recently it has been shown that a cellular RNA-binding protein, Sam68 (Src-associated protein in mitosis), represents a functional homologue of the HIV-1 Rev protein (Reddy et al., 1999). In addition, it was published that Sam68 contains a NLS with non-apparent homology to any other known nuclear localization signal (Ishidate et al., 1997). However, the sequence responsible for the nuclear localization was found to contain a motif, PPXXR, which is conserved in various RNA-binding proteins.

To determine whether Risp or Rev contains a similar PPXXR motif, the amino acid sequence of Risp and Rev were examined. This new motif could not be found in the Risp sequence.
On the contrary, analysis of the HIV-1 Rev amino acid sequence revealed that this viral homologue of Sam68 contains the PPXXR motif inside its activation domain (LQL**PP**LE**R**LTLD). Therefore, the possibility that Rev could contain an additional potential NLS in the activation domain was investigated.

3.6.1. Localization of RevM5 and RevM5-GFP in HeLa cells

First, the intracellular localization of RevM5, a mutant of Rev in the arginine-rich NLS (Malim et al., 1989), was analyzed.
For this purpose, a new construct linking the GFP to RevM5 was created (pcRevM5-sg143, see plasmids). *RevM5* gene was amplified by PCR from the pcRevM5 plasmid (see plasmids) using the primers 23957 and 23958 (see Table 2.1), and inserted into the unique NheI restriction site in the pFred143 vector. After sequence analysis, the confirmed correct plasmid containing the *revM5* insert in sense and in frame with the *GFP* gene was kept for the further experiments.
HeLa cells were transfected with the pcRevM5 or pcRevM5-sg143 plasmids (1µg) and as a control with wt pcRev and pCsRev-sg143 (1µg). Twenty four hours post-transfection, the localization of the proteins was analyzed by indirect immunofluorescence using antibodies against Rev, or in living cells by the direct fluorescence of the GFP fusion proteins. The intracellular distribution of the proteins was measured by quantifying the fluorescence in nuclear and cytoplasmic compartments. Statistical significance of observed differences were analyzed by using the Mann-Whitney U test.

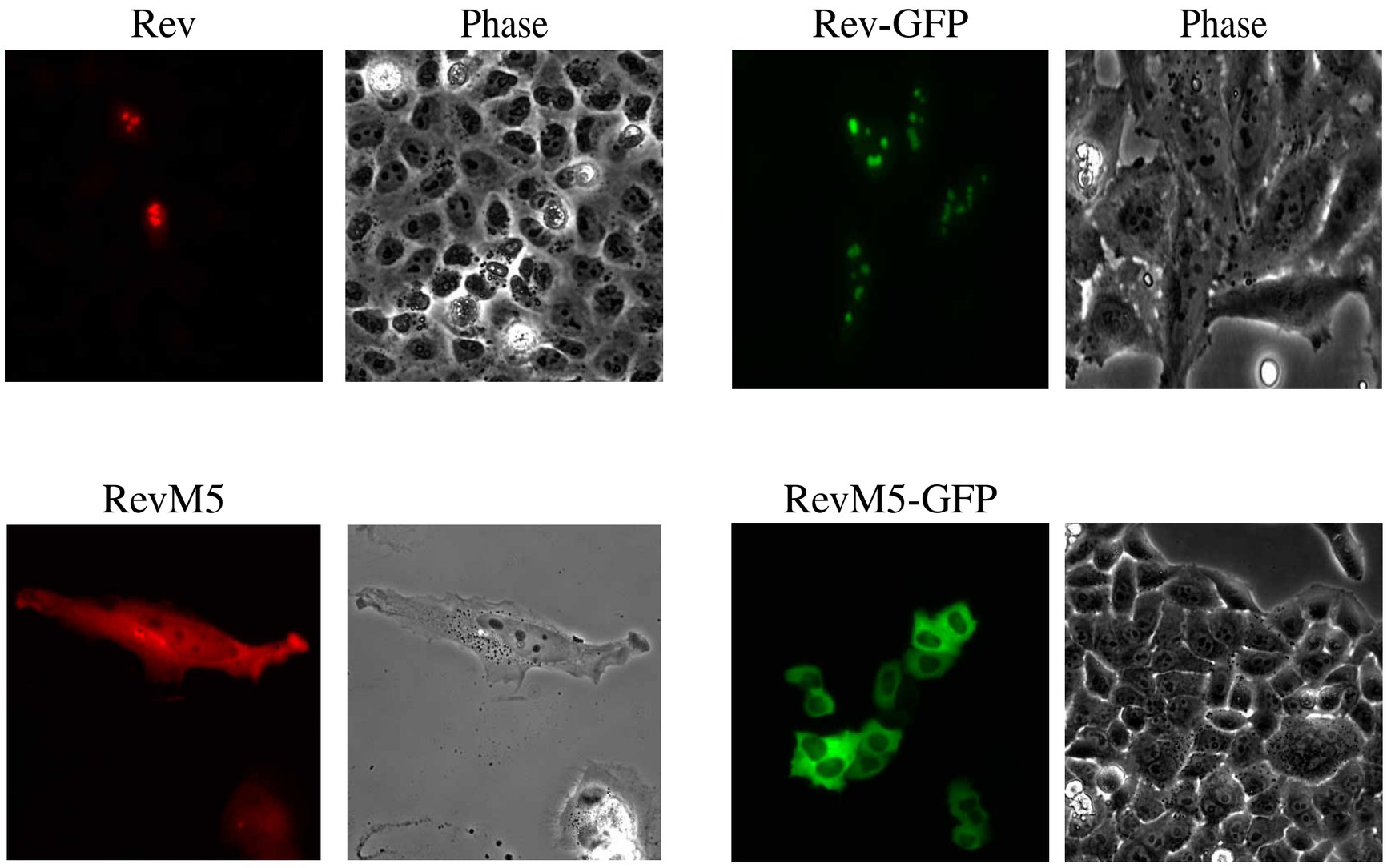
As previously described for RevM5 (Malim et al., 1989), *RevM5 (cytoplasmic fluorescence 70%) and RevM5-GFP ((cytoplasmic fluorescence 81%) accumulated to a higher extent in the cytoplasm than wt Rev (cytoplasmic fluorescence 26%) and Rev-GFP (cytoplasmic fluorescence 16%) (Fig. 3.37).*

Moreover, to analyze the ability of RevM5/RevM5-GFP to enter the nucleus, the export-inhibiting drug leptomycin B (10nM) was added for 2h to the cells previously transfected (24h) with the pcRevM5 or pcRevM5-sg143 plasmid.

As shown in Fig. 3.37, RevM5 and RevM5-GFP, although containing a mutation in the NLS region, were able to enter the nucleus and accumulate there in the presence of leptomycin B (cytoplasmic fluorescence 55% and 53% respectively). Therefore, the presence of an additional and functional NLS in Rev would be possible.

Fig. 3.37

A


Rev
Phase
Rev-GFP
Phase
RevM5
RevM5-GFP
RevM5
+ LMB
RevM5-GFP
+ LMB

B

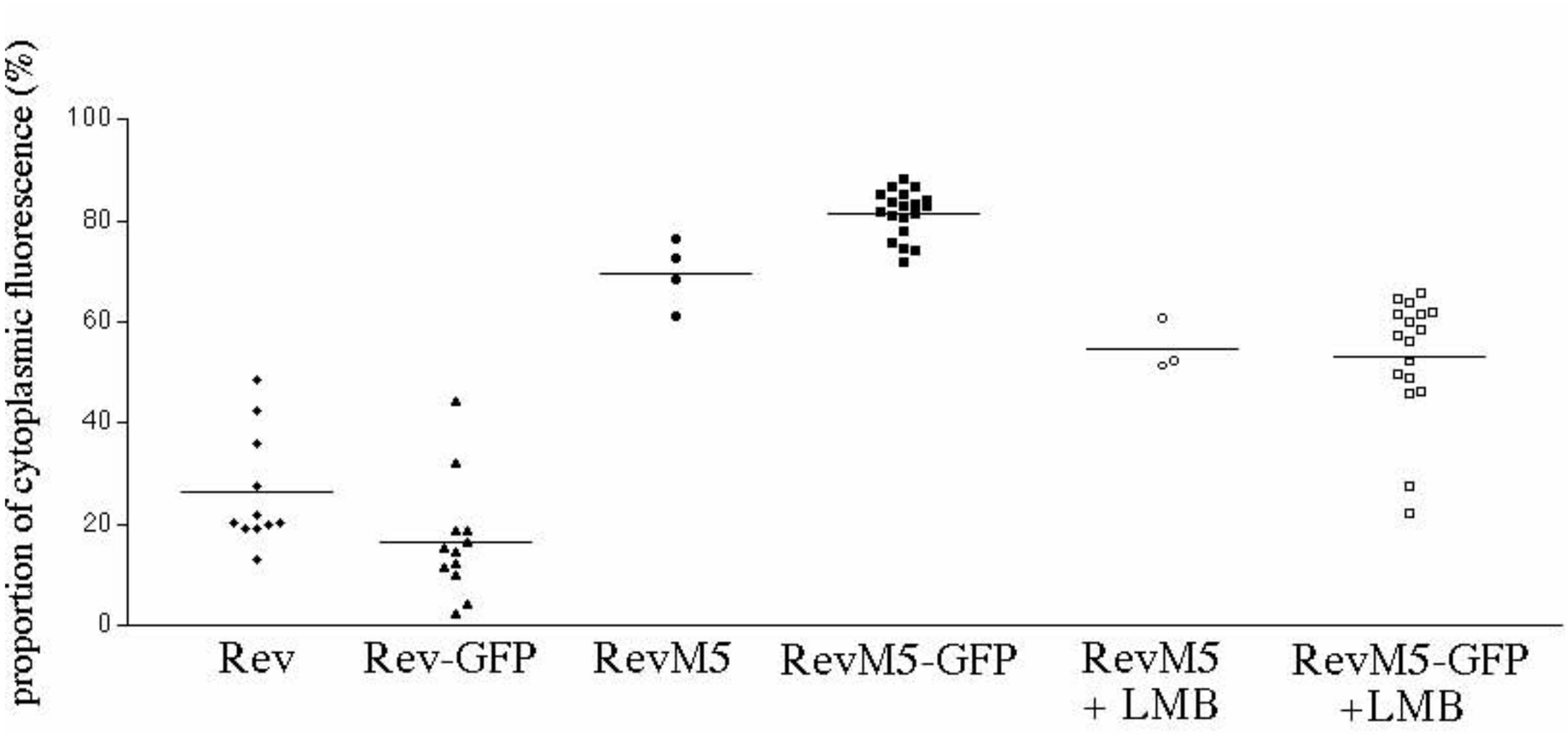

proportion of cytoplasmic fluorescence (%)
100
80
60
40
20
0
Rev
Rev-GFP
RevM5
RevM5-GFP
RevM5
+ LMB
RevM5-GFP
+LMB

Fig. 3.37 Localization of RevM5 and RevM5-GFP. HeLa cells were transfected with 1µg of pcRev, pCsRev-sg143, pcRevM5 or pcRevM5-sg143 expression plasmid respectively. Leptomycin B (LMB) was added at the concentration of 10nM 24h after transfection. Cells were fixed and permeabilized. Cells transfected with pcRev and pcRevM5 plasmid were analyzed by indirect immunofluorescence microscopy using a rabbit polyclonal anti-Rev antibody (1:100), followed by Cy3-conjugated goat anti-rabbit IgG. Phase indicates phase-contrast image. (B) Quantification of the proportion of cytoplasmic fluorescence in HeLa cells before and after LMB treatment. Symbols indicate values for single cells and bars mean values for each protein.

3.6.2. Cellular microinjection of Rev activation domain peptides

To analyze whether the activation domain of Rev, containing the signal PPXXR, could also function as a nuclear localization signal, several peptides consisting of the Rev NES (residues 73-84) were generated and cross-linked to Alexa fluor 488-labeled BSA. Two fusion-peptides containing the activation domain of the wt Rev and of the export-deficient mutant RevM10 (Malim et al., 1989) were generated (BSA-RevNES and BSA-RevM10NES, Fig. 3.38). In addition, it has been published that in the Sam68 motif PPXXR replacement of the arginine by alanine diminished the nuclear accumulation activity of Sam68 (Ishidate et al., 1997). Therefore, two additional peptides with a specific mutation (R->A) in the activation domain of the wt Rev and of the mutant RevM10 were generated (BSA-RevNES-A and BSA-RevM10NES-A, Fig. 3.38).

To follow the intracellular trafficking of the Rev NES peptides, each purified conjugate (~1 mg/ml) was microinjected into the nuclei or into the cytoplasm of HeLa cells along with Alexa fluor 568-labeled BSA (~1 mg/ml). The co-microinjection of unconjugated fluorescent labeled BSA was used to assess the site of injection and the post-injection integrity of the nucleus.

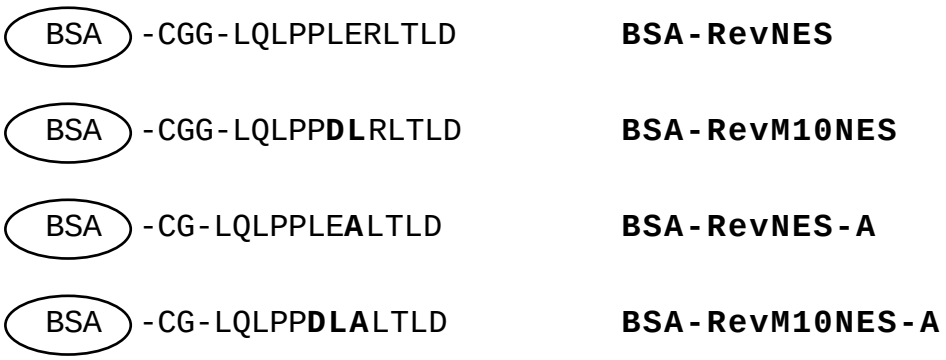


Fig. 3.38 Schematic description of the structure of Rev NES peptides conjugates to BSA used in this study. BSA-RevNES and BSA-RevM10NES consist of BSA cross-linked to peptides encompassing the activation domain of wt Rev and of export-deficient mutant RevM10 (^{78}LE->DL)(Malim et al., 1989). The peptides of BSA-RevNES-A and BSA-RevM10NES-A harbor a single amino acid substitution (^{80}R->A). The

mutated amino acids are expressed in bold. The BSA conjugated to the peptides was previously labeled with Alexa fluor 488. One or two glycine residues were additionally used as hinge region to allow correct folding of both protein and peptides.

First, the nuclear export ability of the intact activation domain of Rev was tested.

As expected, 2h after nuclear microinjection of a mixture of BSA-RevNES and BSA, unconjugated BSA remained exclusively in the nucleus and the conjugated BSA-RevNES was exported out of the nucleus into the cytoplasm (Fig. 3.39). On the other hand, 2h after nuclear microinjection of a mixture of BSA-RevM10NES and BSA, both unconjugated BSA and the export-deficient mutant NES conjugated to BSA stayed in the nucleus.

To investigate whether the nuclear export of our BSA-RevNES conjugate could be inhibited by leptomycin B, cells were pretreated with (10nM) 2h before nuclear microinjection.

As expected, leptomycin B completely abolished the nuclear export of the wt activation domain of Rev-BSA conjugate (Fig. 3.39).

To investigate whether the Rev NES could induce a nuclear import of BSA-conjugate via an active pathway, microinjections of BSA-RevNES peptides into the cytoplasm were performed.

Two hours after cytoplasmic microinjection of a mixture of BSA-RevNES and BSA in cells pretreated with leptomycin B (10nM) for 2h, BSA alone stayed in the cytoplasm whereas the BSA-RevNES conjugate was imported into the nucleus (Fig. 3.40). Not all conjugated BSA-RevNES entered the nucleus and did not enter the nucleoli, indicating that the signal and the mechanism of entry of this additional Rev NLS is different from the Rev arginine-rich NLS, which has been shown to mediate also nucleolar localization (Cochrane et al., 1990).

Similar results were obtained by the cytoplasmic microinjection of the export-deficient mutant RevM10 NES, indicating the presence of a nuclear import signal in the activation domain of Rev. Two hours after cytoplasmic microinjection of a mixture of BSA-RevM10NES and BSA, unconjugated BSA remained exclusively in the cytoplasm and the conjugated BSA-RevM10NES passed through the nuclear membrane, and accumulated in the nucleus (Fig. 3.40).

Finally, to investigate whether the exchange of the arginine against alanine in the activation domain of wt Rev and of RevM10 could induce a change in nuclear export or import of BSA-NES-conjugates, nuclear and cytoplasmic microinjections of BSA-RevNES-A and BSA-RevM10NES-A peptides were performed.

Fig. 3.39

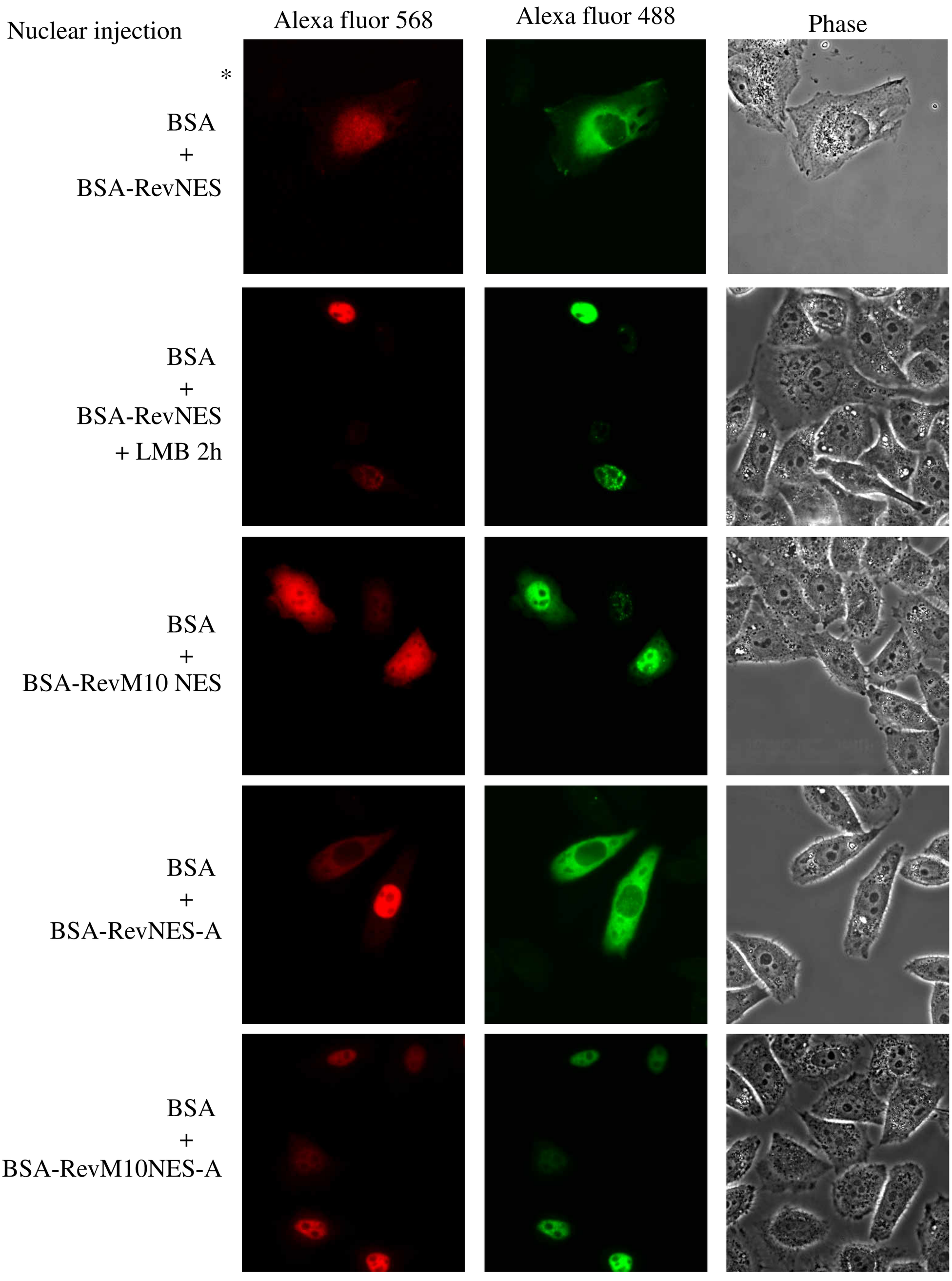
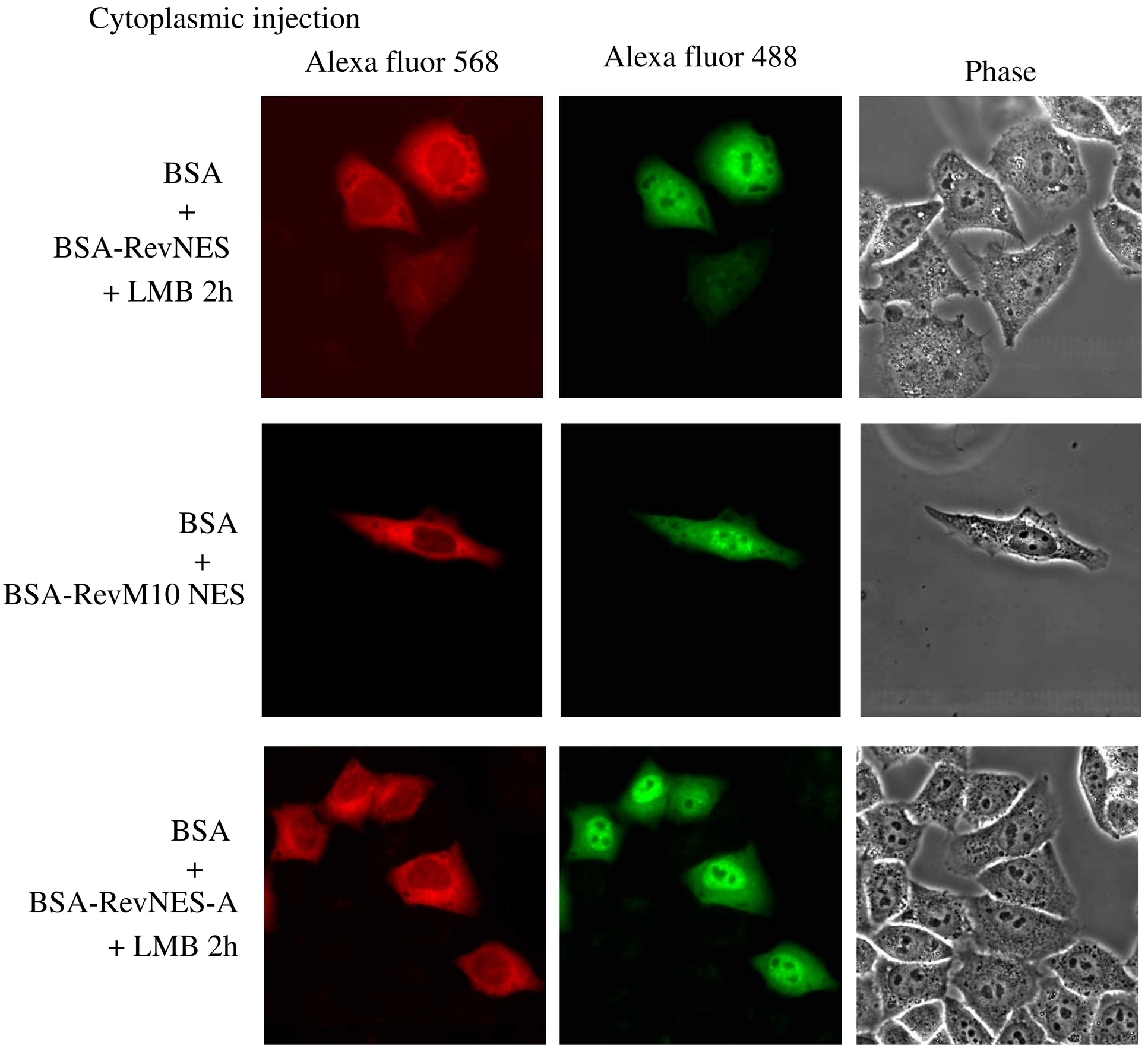

Nuclear injection
Alexa fluor 568
Alexa fluor 488
Phase
*
BSA + BSA-RevNES
BSA + BSA-RevNES + LMB 2h
BSA + BSA-RevM10 NES
BSA + BSA-RevNES-A
BSA + BSA-RevM10NES-A

Fig. 3.40


Cytoplasmic injection
Alexa fluor 568
Alexa fluor 488
Phase
BSA + BSA-RevNES + LMB 2h
BSA + BSA-RevM10 NES
BSA + BSA-RevNES-A + LMB 2h
BSA + BSA-RevM10NES-A

After nuclear microinjection, BSA-RevM10NES-A conjugate was not exported from the nucleus into the cytoplasm, as observed with the BSA-RevM10NES conjugate and BSA-RevNES-A was exported out of the nucleus like BSA-RevNES (Fig. 3.39). This indicates that the single alanine exchange did not alter the nuclear export ability of the Rev NES.

As it is shown in Fig. 3.40 both activation domains of wt Rev and of export-deficient mutant RevM10 harboring the alanine mutation kept the nuclear entry ability when injected in the cytoplasm. Nevertheless, a minor nuclear accumulation of the BSA-RevM10NES-A conjugate in comparison to the BSA-RevM10NES was observed, indicating that the alanine mutation could alter the nuclear import ability of the Rev NES especially if mutated in its nuclear export function.

Although many cells microinjected with RevM10 NES-A peptides showed a minor nuclear accumulation as observed with the RevM10 NES peptides, higher number of microinjection experiments are required. In fact, a kinetic analysis, as well as a quantification and a statistical analysis should be performed.

Fig. 3.39 Nuclear export of fluorescent labeled BSA conjugate to Rev NES peptides. Untreated or LMB pre-treated (2h) HeLa or 85HG66 (*) cells were co-microinjected into the nucleus with the indicated Alexa fluor 488-BSA-RevNES peptides (BSA-RevNES, BSA-RevM10NES, BSA-RevNES-A, BSA-RevM10NES-A) and the unconjugated Alexa fluor 568-BSA (BSA, injection control). To allow sufficient time for export, cells were incubated for 2h at 37 C, fixed and analyzed by fluorescence microscopy.

Fig. 3.40 Nuclear import of fluorescent labeled BSA conjugate to Rev NES peptides. Untreated or LMB pre-treated (2h) HeLa cells were co-microinjected into the cytoplasm with the indicated Alexa fluor 488-BSA-RevNES peptides (BSA-RevNES, BSA-RevM10NES, BSA-RevNES-A, BSA-RevM10NES-A) and the unconjugated Alexa fluor 568-BSA (BSA, injection control). To allow sufficient time for import, cells ells were incubated for 2h at 37 C, fixed and analyzed by fluorescence microscopy.

In summary, in this part of the study it has also been shown that a sequence in the activation domain of the Rev protein can function as an additional nuclear localization signal. The sequence is characterized by a motif PPXXR, that was already described being important for nuclear accumulation of Sam-68, a nuclear protein proposed to be the corresponding human cellular factor for the HIV-1 Rev protein.

4. Discussion

4.1. Identification of new HIV-1 Rev-interacting proteins by yeast two-hybrid selection

The screening of a Jurkat T cell cDNA library, using the yeast two-hybrid selection and HIV-1Rev as a bait protein, led to the isolation of two new Rev-interacting cDNA-encoded proteins (11.5.1 and 16.4.1). The computer analysis of available sequence databases revealed a 100% identity of the 11.5.1 cDNA with the C-terminal part of a previously reported human nuclear single strand DNA binding protein (Sakura et al., 1988). A partial sequence identity of 16.4.1 cDNA was observed with two previously identified human genes with unknown function: a cDNA clone from the EST database (ref) and the 3'end of a large brain-derived cDNA clone (Nagase et al., 1998).

The subsequent study of the 16.4.1 clone led to the characterization of one fragment of a larger protein, consisting of 171 amino acids that interacted specifically with Rev. Because of its ability to shuttle between the nucleus and the cytoplasm it was termed Risp (Rev-interacting shuttle protein).

The yeast two-hybrid system has revealed a number of useful applications (Allen et al., 1995), including the capacity to isolate novel genes encoding proteins that associate with a known protein of interest. Previous studies have already successfully used Rev or its activation domain in the yeast two-hybrid selection to identify Rev-interacting proteins (table 2.3) (Bogerd et al., 1995; Farjot et al., 1999; Fritz and Green, 1996; Fritz et al., 1995; Luo et al., 1994; Neville et al., 1997; Stutz et al., 1995). The two-hybrid system is able to detect *in vivo* binding that is often undetectable *in vitro* by methods such as immunoprecipitation. In fact, the two-hybrid system gives very sensitive results. First of all, the hybrid-proteins are generally over-expressed by strong promoters present in high-copy-number plasmids, which favor a complex formation. In addition, the stability of the hybrid-protein complex is probably enhanced by the interaction of the activation domain with proteins from the transcriptional initiation complex, which also associate with the promoter DNA. Therefore, the overall stability of the complex on DNA further increases the sensitivity of detection of the bait-prey protein interaction.

However, the two main obstacles preventing the success of the yeast two-hybrid system are the activation of transcription of the reporter gene by the bait protein itself and the failure to express the bait properly. In our case, the Rev protein could be proven to be a suitable bait, since it was not able to activate the transcription of the reporter genes LEU2 and lacZ without the presence of the B42 activation domain.

Moreover, the expression of the Rev protein fused to the binding domain LexA was demonstrated by western blot analysis using a Rev-specific antibody.
Another major problem of the yeast two-hybrid system is the risk to detect "false positive" interactors. In fact, some proteins contain regions that have low affinities for many different proteins and therefore may form stable complexes with several bait proteins, which result in an unspecific detection.
To limit the number of "false positive" interactions, a dual screen selection based on the use of two distinct reporter genes, lacZ and LEU2 was performed. Moreover, for additional controls the screening was performed in presence of the testing bait (Rev sense) and two unspecific baits (Rev antisense and Lexcd2). While the lacZ expression provided a detectable blue color phenotype on plates containing Xgal, the LEU2 expression allowed a nutritional selection on plates lacking leucine. It is known that the requirement for the expression of two reporter genes driven by different promoters significantly reduces the number of false-positive signals, since separate transcriptional events must be maintained at distinct chromosomal loci.
The screening of a Jurkat T cell cDNA library led us to sequence 6 yeast clones: two were assumed to be clear Rev-interacting (11.5.1 and 16.4.1) and 4 "putative" Rev-interacting clones (6.2.1, 11.2.1, 15.2.2 and 16.3.15) (see tables 3.5 and 3.6).
The sequence analysis and the subsequent BLAST search with available sequence databases suggested that 3 out of the 4 "putative" clones were "false positive". In fact they were very similar (85%) or 100% identical to proteins generally known as common false positive in the yeast two-hybrid system (i.e. ribosomal proteins, zinc finger containing proteins or heat shock proteins) (Hengen, 1997). The "putative" clone 16.3.15 was 100% identical with a HLA-DR associated protein (924bp) and the phosphatase 2A inhibitor I2PP2a (833 bp).
I2PP2a is a truncated form of SET, a largely nuclear protein that is fused to nucleoporin Nup214 in acute non-lymphocytic myeloid leukemia (Li et al., 1996). However, even though this clone could be of certain interest - Nup214 is one of the nucleoporins known to interact with Rev (Zolotukhin and Felber, 1999)- in this work the other two Rev-interacting clones were further characterized for several reasons. First, the yeast clone 16.3.10 (representing the same cDNA-encoded protein of 16.3.15) showed clearly unspecific binding to Rev. Second, the two clones 11.5.1 and 16.4.1 showed a certain specific binding to Rev. Third, the absence of homology of the clone 16.4.1 to any proteins with known function.
It is worth noting that in our screen none of the known Rev-interacting proteins were detected, maybe because of the insufficient number of analyzed clones.

4.2. Computer sequence analysis and proposal for Risp cellular function

The DNA and the amino acid sequences of the clones 11.5.1 and 16.4.1 were analyzed in detail with several computer programs. Regardless which program was used, FASTA, BLAST or BEAUTY, the 11.5.1 cDNA-encoded protein was always related to DNA binding proteins, and was reported to be 100% identical to the human nuclear single strand DNA binding protein dbpB (Sakura et al., 1988). On the contrary, from the beginning of the study until now, the sequence of the 16.4.1 clone did not show any significant percentage of identity to proteins with known function. Partial identity (92%) was first observed with Soares fetal heart NbHH19W cDNA clone (EST database; Fig. 3.9). Subsequently, high identity (99%) was observed with the C-terminal part of a large brain cDNA clone for the KIAA0592 protein (PRI database, (Nagase et al., 1998)) and of two other human cDNA clones related to the Nagase protein (accession number CAB45765 and CAB43380, PRI database, submitted 1999) (Fig. 3.2). However, a minor degree of homology appeared with some RNA-/DNA-binding proteins, such as the human fibrillarin protein, methyl-CPG-binding protein 2, histone H2B, the Xenopus Laevis histone H5, the Arabidopsis Thaliana RNA Helicase and the Saccharomyces Cerevisiae U1 small nuclear ribonucleoprotein A. In addition, partial homology was observed with two rat shuttle proteins such as Nopp140 and nucleolin C23, and with other unrelated proteins, such as the human structural protein cylicin and the mouse microtubule associated protein 1B (Table 3.9).

Among the group of the RNA-/DNA-binding proteins similar to the clone 16.4.1, fibrillarin is a component of a nucleolar small nuclear ribonucleoprotein particle and is thought to participate in the first steps of pre-ribosomal RNA processing. It is associated with the U3, U8 and U13 small nuclear rRNAs (Jansen et al., 1991). The nuclear methyl-CPG-binding protein 2 (MECP-2 protein) is proposed to be involved in the regulation of transcription and alteration of chromatin structure. Histones are chromosomal proteins remarkably conserved across the gamut of eukaryotic organisms. They form the unit around which DNA is coiled in the nucleosomes. Nucleosomes contain always an octamer-complex of two units, consisting in histones H2A, H2B, H3 and H4. The histone H5 has the same function as H1, being necessary for the condensation and stabilization of nucleosome chains into higher order structures, and it replaces histone H1 in certain cells. Nucleolin C23 is the major nucleolar protein of growing eukaryotic cells that shuttles between the nucleolus and the cytoplasm (Borer et al., 1989). It is associated with intranucleolar chromatin and pre-ribosomal particles. It induces chromatin decondensation by binding to histone H1 and is thought to play a role in pre-rRNA transcription and ribosome assembly

(Bourbon et al., 1988; Medina et al., 1995). Recently it was proposed to play also a role in poliovirus genome amplification, since it appeared to strongly interact with the poliovirus 3' noncoding region (Waggoner and Sarnow, 1998), a nucleotide region involved in the synthesis of viral negative-stranded RNA (Johnson, 1995). The principal role of the U1 small nuclear ribonucleoprotein A is to support to fold or maintain U1 RNA in an active configuration. It is the first sn-RNP to interact with pre-mRNA (Liao et al., 1993).

The sequence similarity of Risp to each of the previously mentioned proteins is very minimal and therefore suggests low significance. However, a more general analysis of the proteins could include Risp protein - or the larger protein containing Risp - as member of a family of proteins with RNA-/DNA-binding function.

Therefore it is possible to assume that Risp itself - or the larger protein containing Risp - could actively be involved in one of the processes regarding the chromatine metabolism or/and the pre-RNA processing.

In addition, some of these proteins, such as Nopp140 and nucleolin C23, proposed to be similar to Risp, are nucleocytoplasmic shuttling proteins and accumulate in the nucleoli. In this study it could be proved that also Risp is a shuttle protein, however, it accumulates in the cytoplasm, and when present in the nucleus, it never accumulates in the nucleoli, although Risp contains a stretch of basic amino acids with nucleolar import activity (Fig. 3.33). It will be very important to clarify whether Risp is a part of a larger protein and subsequently analyze its intracellular localization and its cellular function.

A PROSITE pattern search was performed - this program is able to recognize known functional motifs in the query sequence. The results suggested several possible post-translational modifications in both the cDNA-derived protein sequences of 11.5.1 and Risp. The analysis of the 11.5.1 clone sequence suggested consensus sequences targeting one N-glycosylation, one amidation and three phosphorylation sites. Regarding the Risp protein, one N-glycosylation and ten phosphorylation sites were proposed. However, these results are only computer predictions and they need experimental confirmations.

Nevertheless, it is interesting to note that among the 14 seryl residues in the Risp sequence, 5 matched with the consensus sequence for casein kinase II (CK-II), a kinase involved in the phosphorylation of HIV-1 Rev and rat Nopp140. In fact, CK-II together with the mitogen-activated protein kinase (MAPK) has been suggested to be potential candidate for Rev phosphorylation *in vivo*, based on their capacity to phosphorylate Rev *in vitro* (Critchfield et al., 1997; Ohtsuki et al., 1998; Yang and Gabuzda, 1999). The nucleolar phosphoprotein Nopp140, a shuttle protein proposed

to possess a weak homology to Risp, is characterized by the presence of a 10-fold repeated motif of highly conserved acidic serine clusters containing an abundance of phosphorylation consensus sites for CK-II (Meier and Blobel, 1992). Therefore, it would be important to determine using phosphorylation experiments if also the Risp protein - or the larger protein containing Risp - is phosphorylated *in vivo* and if CK-II is involved in this process. Moreover, the relevance of the phosphorylation with respect to the protein function should be also established. For instance it is known that Rev is phosphorylated *in vivo* at different serine residues (Cochrane et al., 1989; Hauber et al., 1988; Meggio et al., 1996). However, the mutation of some of those serines did not change Rev trans-activation in transfection experiments, suggesting that the phosphorylation may not be essential for Rev function (Cochrane et al., 1989; Malim et al., 1989). To predict additional sorting and localization signals in the amino acid sequences of 11.5.1 and Risp proteins, a PSORT II search was performed. Using this program the presence of putative cleavage sites, transmembrane segments and nuclear localization signals was investigated. Performing the search on the amino acid sequence of 11.5.1 clone, the presence of three arginine-rich consensus NLS with a very high NLS score (1.87) was detected. These results confirmed those obtained by the BLOCKS search, which suggested the presence of an arginine-rich region, bearing weak similarity with the NLS of both HIV-1 Rev and Tat, in the 11.5.1 clone. On the contrary, the analysis of the Risp sequence indicated the absence of any classical signal but suggested the presence of a low score lysine-rich cluster with potential NLS function. However, the results achieved by these programs should be taken with the appropriate precautions, considering the limited number of signal sequences available in the database. Furthermore, these programs cannot provide all the information on the functionality of a protein. When the PSORT II search was performed on the Rev protein as a control, its arginine NLS signal was sorted, although the NLS score was negative (-0.16) and no RNA-binding motif was identified (data not shown).

4.2.1. Identification of a new specific motif common to cellular Rev-interacting factors

Possible similarities of the sequences of the new Rev-interacting factors 11.5.1 and Risp with the sequences of already known Rev-interacting factors were investigated with local alignment and DIALIGN, programs able to identify similar regions within large unrelated sequences. Interestingly, each Rev-interacting protein, previously proposed to support Rev function in the nucleus, such as Crm1, eIF5-A, hRab/Rip, yRip1p and p32, showed a high percentage of identity with a same N-terminal region of Risp. Based on these results a consensus motif consisting of 12 amino acids was

proposed for these Rev-interacting factors (Fig. 3.7): P (AELNR) (KHILST) (KCRST) T N (PD) F (GLQ) (LST) (LIN) (EAGQS). The consensus motif is based on occurrence of all indicated amino acids in each protein domain with one mismatch allowed (brackets indicate the less conserved residues).

This motif by computer analysis seems to be highly specific. Scanning it against all available sequence databanks (with a total number of 407576 sequences analyzed), the only proteins identified to contain that motif were the above mentioned Rev-interacting proteins plus any hypothetical protein containing the Risp sequence. Moreover, query patterns containing random modifications of one or more amino acids in the consensus sequence led to the sort of the Rev-interacting proteins plus a variable number of different proteins, with a minimum of 5 Rev-unrelated factors (Table 3.15).

Nevertheless, among all the Rev-interacting proteins, the cytoplasmic import receptor importin β, the nucleolar protein B23, the two recently identified Rev-interacting nucleoporins Nup98 and NLP-1, and Rev itself were not present in this consensus motif. Therefore, from each of these proteins the sequence with highest similarity with the 16.4.1 motif sequence (PAKKTNPFPLLE) has been used to generate new motifs allowing alignment of each one of these additional proteins (Fig. 3.8). The subsequent screening analysis among all the sequences available in the database, using the Nup98- or Rev-modified motif led to the identification of the Rev-interacting proteins only. In contrast, the screening among the database of the importin β-, NLP-1- or B23-modified motif led to the identification of the Rev-interacting proteins and the addition of one unrelated protein (importin β, NLP-1) and three unrelated proteins (B23)(Fig. 3.8).

Therefore, these results provide for the first time the description of a specific and common motif shared between Rev-interacting factors. Further, only Risp, and not the 11.5.1 cDNA-encoded protein, shares this specific motif. Finally, among all the Rev-interacting factors two subgroups could be distinguished which share the motif with different degree of specificity (Fig. 4.1). Although in every Rev-interacting factor a region containing a sequence, which could be associated to the consensus motif, was identified, a particular group of cellular proteins, mostly involved in the Rev/RRE nuclear export, showed to be highly specific with respect to the motif. In fact, applying the shared motif to the subsequent screening analysis among all the sequences available in the database led to the identification of only those Rev-interacting proteins. In contrast, the screening analysis using modified motifs related to the second group of proteins led to the identification of a few additional (1-3) unrelated proteins beside the Rev-interacting proteins (Fig. 3.8).

Experimental data are currently missing to propose a functional significance of the Rev-interacting proteins-motif; in fact, this was generated based on amino acid sequence similarity and computer analysis. Moreover, a limit of the computer analysis is the difficult interpretation of the different degrees of specificity. To investigate, whether a specificity window for the Rev-interacting proteins-motif may exist, Rev-unrelated proteins such as GFAP or fibronectin were also tested in the motif alignment. The subsequent screening of those modified motifs led to a selection of 5 and 13 additional proteins respectively in addition to the Rev-interacting proteins and GFAP or fibronectin (data not shown). This suggests that the threshold for non specificity could start with a number of additional proteins larger than 4. Even if the discovered motif shared between all the Rev-interacting proteins might be highly specific for those proteins, the significance of the few (1-3) additional proteins selected, using the modified motif from the second group of the Rev-interacting proteins, remains to be interpreted.

Presently, it is unknown whether the hypothetical 31.3 kDa protein (NPSA sptr||Q9X6Z7) and/or the putative lipoprotein periplasmic GNA2132 (appeared as additional proteins in the Importin-β and NLP-1 modified motifs respectively) are non Rev-related proteins or on the contrary potential new Rev-interacting factors. In addition, it is worth to note that among the proteins sharing the Rev-interacting proteins-motif Sam68 is included in the group of proteins with lower specificity. Sam68 represents a cellular functional homologous of HIV-1 Rev (Reddy et al., 1999). It can specifically bind to RRE *in vitro* and *in vivo* and can partially substitute for as well as synergize with Rev in RRE-mediated gene expression and virus replication, although a direct binding of Rev-Sam68 was not shown. Screening among the database of the Sam-68-modified motif led to the identification of additional unrelated proteins like the aforementioned putative lipoprotein periplasmic GNA2132 and the adenyl cyclase. Therefore, it would be very interesting to investigate whether Rev binds to one of these “additional” proteins.

However, until experimental data regarding the significance of the Rev-interacting motif are available, a possible role in mediating the Rev function is speculative.

It is difficult to attribute a direct Rev-binding function to the motif domain. Although the motif sequence in the proteins hRab/Rip, yRip1p or in Rev is part of the Rev-binding region (in Rev is contained in the first multimerization domain), this was not the case in proteins such as p32 (Tange et al., 1996) and Risp itself. In fact, the use of several segments of Risp showed that its N-terminal region, containing the motif sequence (22-33aa) was not essential for the Rev-binding in the yeast two-hybrid system. Yeast transformed with the plasmid PJG4-6-(38-171), harboring a mutant of

Risp missing that region, bound Rev as well as the wt Risp. In addition, the location of the Rev-binding region in factors such as eIF-5A, Crm1, importin β or Nup98 is not yet identified and therefore, it is impossible to determine whether the motif sequence is included in the region of these proteins responsible for the Rev-binding.

Transcription factors and many other proteins including Rev itself contain one binding and one activation domain both essential for the function of the protein. There is a certain possibility that also the Rev-interacting proteins contain besides a Rev-binding domain a Rev-modulatory domain that harbors that consensus motif. In some proteins such as the nucleoporin Rab/Rip or Rev itself the binding and the modulatory domains are very proximal to each other, whereas in other proteins such as Risp or p32 the two functional domains are located in different regions.

It is interesting to note that among the Rev-interacting proteins shown to be highly specific with respect to the motif (Fig. 4.1), with the exception of p32, which it is not known to bind Crm1, all the others, including Risp, use the Crm1 export receptor for their nuclear export. Therefore, it cannot be ruled out that this motif is related to the Rev/RRE nuclear translocation and to Crm1 function. In fact, proteins such as importin β and Sam68 that are proposed to use distinct nuclear export pathways belong to the group of the Rev-interacting proteins that share this motif with less specificity.

Therefore, the role and the relevance of the consensus motif present in Risp and in the other Rev-interacting factors should be addressed in detail in future studies. Mutational analysis and deletions of the domain in Risp and/or Crm1 and in Rev itself, will be established to analyze whether the function of Rev could be modified.

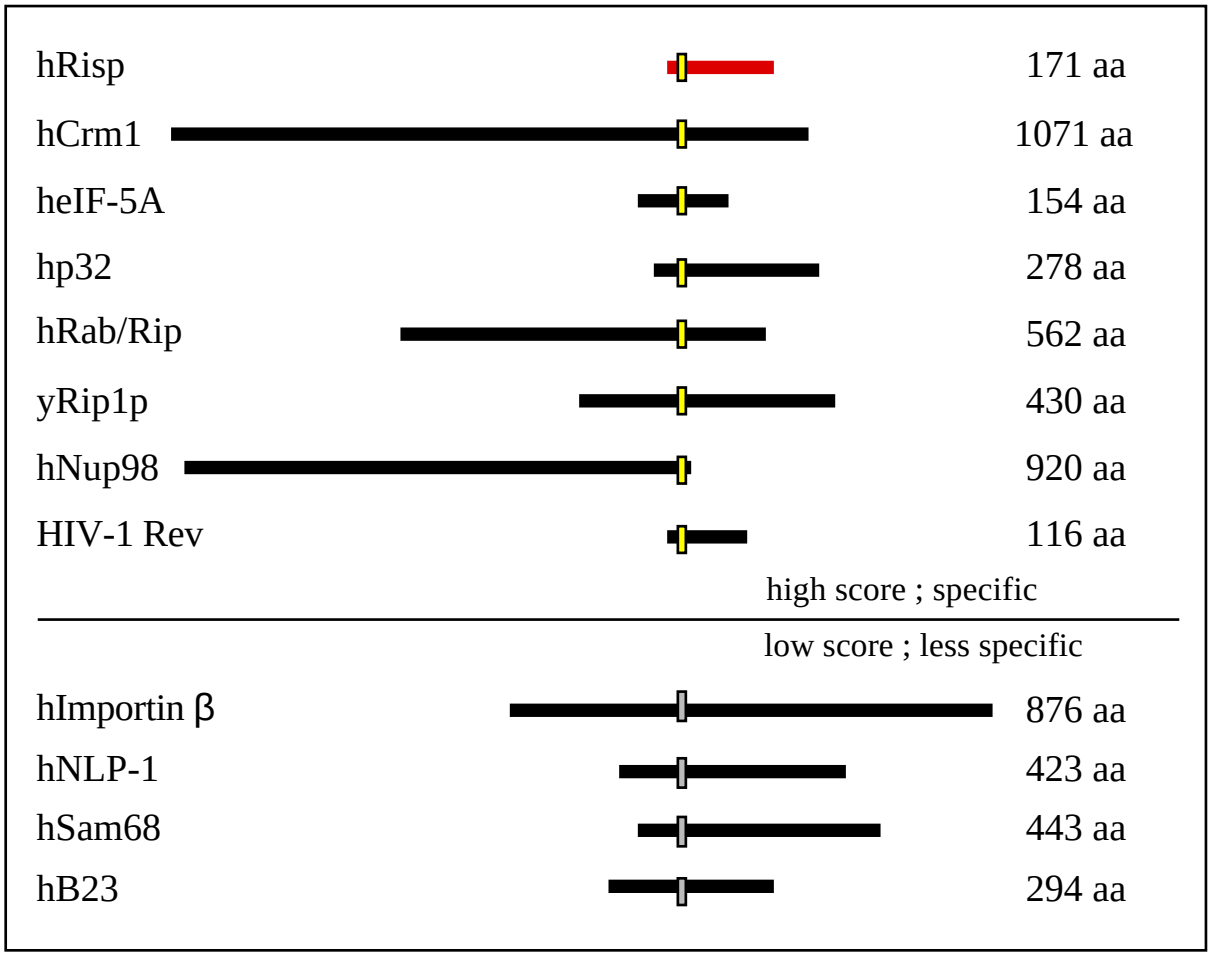


Fig. 4.1 Detection of a common 12 amino acids motif in the Rev-interacting proteins. The location of each domain of the Rev-interacting proteins that showed high percentage of identity with the Risp sequence PAKKTNPFPLLE is represented in yellow. A consensus motif for those Rev-interacting factors was designed and showed high specificity. The location of a domain of other Rev-interacting or Rev-related proteins (Sam68) that showed a less high percentage of identity with the Risp sequence PAKKTNPFPLLE is represented in gray. Modifications of the motif allowing the presence of one of these additional proteins created less specific motifs.

4.3. Risp expression

Since this common and specific motif was found in several known host Rev-interacting proteins and the newly isolated Risp, further studies on the characterization of this protein have been undertaken.

An important issue to investigate was the cellular expression of Risp, therefore an analysis on the DNA-, RNA- and protein level was performed.

The Southern Blot analysis using a commercial blot, which contains genomic DNAs derived from 9 eukaryotic species indicated that the Risp gene is very common among different species (Fig. 3.10). However, the different size of the bands observed indicated that homologous genes with sequence divergence may occur in different species.

Two northern blot experiments showed a main, however weak, mRNA band around the size of 4.6-5 kb, indicating that the *risp* probe recognized a larger mRNA than the expected *risp* of 513 bp. This supports the hypothesis that Risp could be part of a larger protein as the predicted for KIAA0592 (4623 bp; 1353 aa) (Nagase et al., 1998). However, in one experiment, where different conditions were used to probe and wash the RNA-filter, an additional band slightly lower than the 18S rRNA (~1600 bp) was observed. This band was present only in the RNA obtained from cells transfected with Rev and not from non-transfected cells. Since this is a result from only one experiment, it has to be confirmed by further experiments. It is possible that, as suggested from the submitters of the sequence of the clone CAB43380 in the database, an mRNA splice variant encoding a smaller protein in addition to the protein KIAA0592 might exist. Interestingly, it is also possible that the expression of this splice variant could be upregulated by the presence of Rev.

An analysis of the Risp protein expression was performed with immunofluorescence and western blot analysis using HeLa and some astrocytoma cell lines. Several tested monoclonal anti-Risp antibodies were able to recognize only exogenous expression of Risp fusion proteins (IgG-Risp, Risp-GFP or Tat-Risp). No specific staining was observed in untransfected cells, suggesting that the available monoclonal anti-Risp antibodies are not sensitive to recognize the constitutive expression of the protein. Possible explanations for this are that the protein itself is expressed at very low levels and/or the sensitivity of the antibodies is not adequate, or the epitope recognized by the anti-Risp antibody is masked in the full-length protein. Moreover, as mentioned above in the paragraph 3.3.3 a weak band around 220 kDa was observed in some cell lysates. This weak signal was initially interpreted as being the larger protein KIAA0592 constitutively expressed. However, in other western blot experiments, the same anti-Risp antibodies recognized similar bands around 220 kDa in several samples containing the purified GST-Risp protein diluted in buffer (Michael Krappmann, (Table 3.16), suggesting that this large band could represent also only unspecific antibody background.

Therefore, the production and use of additional new specific anti-KIAA0592 antibodies or antibodies against the full length protein will be necessary to analyze the intracellular expression and localization of the endogenous protein and to determine its exact size.

4.4. Characterization of the domains responsible for Rev-Risp binding

One of the advantages that the two-hybrid system offers is that the residues required for the physical interaction of a given pair of proteins can be easily mapped by generating deletion hybrid constructs in one or both of the genes of interest. This approach was utilized to delineate the regions responsible for the Risp-Rev interaction. Several segments of Risp and several mutants of Rev were generated, fused to the corresponding yeast vectors and then used in the interaction trap.

The data suggest that the binding domain in Risp is located between amino acids 39-133, although, the minimal sequence of Risp or Rev required for the Risp-Rev interaction has not been defined. As already mentioned, the N-terminal region of Risp harboring the motif common for the Rev-interacting factors (22-33aa) is neither essential nor sufficient for the Rev-binding (Fig. 3.13). Concerning the Risp-binding region of Rev the results presented here suggest that it is situated in a region overlapping the domain of RNA binding/NLS (34-50aa) and the second multimerization region (52-60aa) (Fig. 2.4; Fig. 3.15). Two Rev mutants, the RevM5, that affects the RNA binding region and the NLS, and the RevSLT40, that affects the second multimerization region, were unable to interact with the Risp fusion protein. On the contrary, two Rev mutants affecting the first multimerization region (RevM4) and the effector domain (RevM10BL) were able to bind Risp (Fig. 3.15). When Rev itself was used as control in the binding assay with wt Rev and all the Rev mutants, it was able to bind to wt Rev, RevM4 and RevM10BL. On the contrary, like Risp, wt Rev was not able to bind to the mutants RevM5 and RevSLT40 (data not shown, Ceccherini-Silberstein and Chris Bickel). The fact that in the two-hybrid system Rev was not able to bind to RevSLT40, a mutant in one of the two multimerization domains, but it was able to bind to RevM10BL, a mutant that does not affect the Rev multimerization, is not surprising. The fact that Rev was able to bind RevM4 mutant is very interesting and will therefore be discussed below in more detail.

An important and often neglected aspect of the biological Rev functions is its ability to form multimers. It has been suggested that this multimerization event is responsible for a certain threshold level of intracellular Rev proteins, which must be overcome in order to establish a productive HIV-1 infection, thereby regulating viral latency (Malim and Cullen, 1991; Pomerantz et al., 1990). Rev has been reported to form homomultimeric complexes *in vivo* and *in vitro* even in the absence of RRE RNA (Kjems and Askjaer, 2000). The Rev sequences involved in the multimerization process have been mapped using different assays and random mutational analysis of the *rev* gene and often yielded opposing results. It is generally agreed that the residues flanking the basic domain, which is responsible for RNA binding, and the

NLS are controlling the Rev-Rev interactions (Fig. 2.4). Nevertheless, some reports show that mutations in the NES also influence multimerization of Rev *in vivo* (Bogerd and Greene, 1993; Madore et al., 1994). Little is known on the precise localization of the domains responsible for the interactions and how many Rev molecules form a biologically active complex on the RRE since as many as 12 Rev molecules have been shown to bind to the RRE *in vitro* (Kjems and Askjaer, 2000).

Originally RevM4 was proposed to be a prototype multimerization-deficient Rev mutant because of its failure to multimerize in an *in vitro* RNA gel retardation analysis (Malim and Cullen, 1991). However subsequent studies showed that RevM4 was able to multimerize about 40% as effectively as wild-type Rev in two *in vivo* assays, while RevSLT40 appeared to be completely unable to form functional multimeric complexes (Madore et al., 1994; Thomas et al., 1998). In agreement with these previous *in vivo* assays, using the yeast two-hybrid system we were able to confirm that the mutant RevM4, unlike the RevSLT40, is able to bind the Rev protein. One explanation for the discrepancy often observed between the *in vitro* and *in vivo* assays could be that the interaction and multimerization of mutant proteins is quantitative in the *in vitro* assay, while the *in vivo* assay reflects the ability of a mutant protein to interact with its wt protein in the cellular context.

Therefore, it is possible, that RevM4-Rev binding would occur in the yeast-two-hybrid system, however RevM4 could not multimerize efficiently as its wt protein. The minimal amount of interaction would allow to recruit the B42 activation domain and the LexA binding domain on the transcription machinery and activate the transcription of the yeast reporters. On the contrary, in previous data as well as data presented here, the mutant RevSLT40 appears to be inactive in binding Rev and unable to form functional multimeric complexes. Therefore, it can be considered as a real prototype multimerization-deficient Rev mutant.

Moreover, we have shown that the mutant RevSLT40 was also unable to bind the Risp protein. Therefore, it is possible that either a crucial Rev-/Risp-binding site is located in the region harboring the SLT40 mutation (59-60aa), or that the mutation in this region perturbs the overall structure of Rev thus resulting unfavorable the binding to Rev and Risp proteins.

On the other hand, the fact, that we observed no binding of RevM5 to Risp and Rev, points to the limitations of this system

Other investigators have shown that RevM5, a mutant in the RNA-binding and NLS domain, could bind and multimerize efficiently *in vivo* with wt Rev when fused to Tat (Madore et al., 1994; Thomas et al., 1998). In our system, the yeast pEG202 plasmid (see 2.2.6.3.1.), used for the bait expression, does not contain an additional NLS like in the Tat-fusion plasmid. Therefore, the absence of the strong functional NLS in

RevM5 protein could be responsible for limiting the nuclear concentration of RevM5, which is required for the binding with the Rev prey protein. It is also possible that Risp was not able to bind the RevM5 mutant, not necessarily because the Risp-binding region in Rev could be located in the RNA-binding/NLS domain, but because not sufficient RevM5 protein was available for Rev binding in the nucleus. It will be interesting to increase the nuclear expression of the bait RevM5 protein and to clarify if Risp could bind Rev in its RNA binding domain. Therefore, the pJK202 plasmid, which contains LexA fused to the SV40 NLS, will be used in repeating experiments employing the yeast-binding selection.

To confirm the functional significance of the interaction between Risp and Rev observed in yeast in human cells, an *in vivo* mammalian-binding assay was performed. Because of the unknown location of the Risp-binding site in the Rev protein the Rev-SLIIB-binding assay was performed as described in Fig. 3.16. This assay has been already successfully used to prove the binding between the cellular cofactor Rab (fused to Tat) and Rev (Bogerd et al., 1995) and the multimerization between RevM5 (fused to Tat) and several Rev mutants (Thomas et al., 1998). In addition, both the original RNA binding assay (Tiley et al., 1992) and the multimerization assay (Madore et al., 1994; Thomas et al., 1998) were already established in this laboratory (van Empel, 2000) and many plasmids were available. In light of the results obtained with the Rev mutants in the yeast two-hybrid, this assay turned out to be not the best suited one. In fact, the binding of Rev protein to a Rev-interacting cofactor fused to Tat in this assay, depends also on the binding of Rev to the SLIIB RNA. Therefore, in the context of proteins interacting with Rev at its RNA-binding domain, a binding competition could occur that would limit the reliability of the assay.
In the case of Risp protein, Tat-Risp did not activate the SLIIB-CAT expression in the presence of Rev like Tat-Rab did. Two possible explanations have been initially considered: 1) Risp could bind to Rev in its RNA binding domain. Therefore, Risp prevents the binding of the Rev protein to its SLIIB RNA target and the recruitment of Tat-Risp on the HIV-1 LTR promoter element thus limiting the CAT expression.
2) In this cellular context Risp is not able to bind the Rev protein, and does not activate the expression of SLIIB-CAT. Hence, the possibility that Risp could have represented an artifact of the yeast two-hybrid system was considered. Nevertheless, the identification of a common motif shared between several Rev-interacting factors in Risp protein supports the idea that Risp could represent a specific new Rev-interacting protein. To confirm, that Risp is in fact a Rev-binding protein, and that it

could bind to the RNA binding domain of Rev, a Tat-Rev/SLIIB-binding inhibition assay was performed in presence of a Risp expression plasmid.

The results of four experiments showed that Risp-GFP was able to inhibit the SLIIB-CAT expression induced by Tat-Rev at different levels according to its concentration (Fig. 3.20). The overexpression of two control proteins, which should not compete in the Rev-SLIIB binding (such as Rab, which binds Rev in its activation domain, or GFP, which is not a Rev-binding protein) inhibited the Tat-Rev induced CAT activations in some experiments, even though at lower levels than Risp. Risp was able to reduce the SLIIB-CAT activation, presumably by competing against the binding of Rev with its RNA target. However, an inconsistent inhibition not related to Risp was observed as well. This unspecific reduction effect was observed using larger amounts of plasmid DNAs, especially with the GFP constructs. In addition, it is worth to note that when the fold-activations are expressed by the median values instead of the average values, the Risp inhibition is highly specific (Fig. 3.20). The overexpression of Risp-GFP reduced the CAT activation for about 67%, the overexpression of GFP for 29% and the overexpression of Rab for 23%.

A part of the unspecific reduction could be caused by a competition between the different promoters with each other. In fact, the plasmids contained different promoters (CMV promoter directed the expression of Risp-GFP, Rab, GFP and Tat-Rev; HIV-LTR promoter directed the expression of the CAT reporter and murine leukemia virus-related RFB-LTR the luciferase gene). It was impossible to use always the same DNA-plasmid preparations for all the transfections. The different quality of the plasmids could have interfered in some experiments with the efficiency of the transfection itself. In support of this idea, it should be considered, that among the different experiments, in cells transfected with the pSLIIBCAT indicator plasmid and in absence of the inhibitors, a wide variation of CAT activation levels induced by Tat-Rev was observed. The induction levels varied between 15-fold and 187-fold (see Table 3.19). The luciferase expression also varied between the cells transfected with different plasmid mixtures (2-10 fold) and between the different experiments (2-20 fold).

Therefore, due to the possible competition of the promoters and/or the different transfection efficiencies, it could not be excluded that in some experiments the expression of some proteins did not reach optimal levels and functions. This consideration would suggest, that the inhibitory effects observed are not only caused by protein interactions and may also explain the inhibitory effect by the GFP on the HIV-1 LTR driven CAT expression seen in one single experiment (Fig. 3.21). In fact, the presence of GFP, also at low levels of the expression plasmid (0.5µg), reduced the Tat-dependent trans-activation of the HIV-1 LTR for more than 50%, whereas the

presence of Risp-GFP (until 1µg of plasmid) reduced the LTR-CAT activation by Tat for around 11%. Even if these data are obtained by a single experiment, nevertheless it strongly supports the presence of a highly unspecific reduction independent of the protein interactions.

Unfortunately, the limited number of the experiments excludes a statistical analysis of the available data. On one hand more experiments should be performed to firmly establish whether the observed reduction of the SLIIB-CAT inductions by Risp-GFP is caused by the Rev-Risp protein interaction (i.e. is specific). On the other hand, these results show that in binding-competition experiments, where the intracellular concentration of the ligand and the competitor should be essentially precise and controlled, a transfection system complex as the one used in this study is not well suited.

Moreover, Risp-GFP should be fused to a strong NLS, such as the SV40 NLS, to increase the nuclear concentration of Risp-GFP. In fact, another possible explanation of the low reduction observed in the SLIIB-CAT inhibition by Risp, could be that the levels of Risp-GFP in the nucleus are too limited to ensure a successful binding to Rev. Even if Risp-GFP could shuttle between the cytoplasmic and the nuclear compartments, its main location is in the cytoplasm, due to its strong NES.

Presumably a different method should be used to confirm that the Risp-Rev interaction is real. In addition, the clarification of the region involved in this interaction could be helpful. Currently, Chris Bickel, in the group of Dr. Brack-Werner, is trying to set up a mammalian two hybrid system, already successfully used to confirm the binding of Rev to Rab and to NLP-1 (Bogerd et al., 1995; Farjot et al., 1999). A further possibility, within the use of the two hybrid technology (mammalian or yeast), could be to test the ability of hybrid proteins to associate, when the bait protein (Rev) is switched to the activation domain and the library-derived protein (Risp) is fused to the DNA-binding domain. In fact, observed interaction between reciprocal hybrids is generally considered as a strong indication of true physical association. An alternative approach could be to use *in vitro* detection by biochemical methods such as co-immunoprecipitation or *in vitro* binding. However, this may cause a problem of sensitivity. Physical methods, such as the above-mentioned, generally rely on a low rate of dissociation since complexes must survive several washing steps, which decrease the signal, but are necessary to prove specificity. In addition, protein-protein interactions may require other proteins for stability, therefore it is not always possible to prove *in vivo* binding of proteins with *in vitro* assays.

Finally, another potential protein-hybrid technology that could be applied to intracellular events, could be the applying of the autofluorescent GFP reporter

protein. A potential use of this reporter is based on fluorescence resonance energy transfer (FRET), which can be detected, when two fluorochromes are brought into close proximity. FRET is measured in real time to measure distances up to 100Å (Pollok and Heim, 1999). GFP has been already modified to produce fluorochromes with different absorption and excitation spectra. Therefore, it may be possible to generate a fusion of Risp and Rev to GFP-modified fluorochromes and Risp-Rev interaction could either be detected by cell sorting or observed microscopically in living cells.

4.5. Influence on HIV-1 Gag production in the presence of Risp-GFP

Additional experiments to test Rev function in the presence of Risp-GFP were performed by analysis of the production of the Rev-dependent structural viral proteins. Results from three independent experiments are presented in Fig. 3.22.

Despite the overexpression of GFP or Rab proteins, which did not influence the HIV-1 Gag production, cells expressing Risp-GFP showed a distinctly diminished Rev-dependent stimulation of Gag production. The overexpression of Risp-GFP reduced the Rev-inductions by 30% (1µg plasmid) up to 70% (2µg plasmid). However, the limited number of experiments excludes the possibility of a statistical analysis of the present data. Therefore, further experiments should be warranted.

Nevertheless, these preliminary data suggest that Risp, as a Rev-interacting protein, is able to inhibit the Rev-trans-activation while Risp did not interfere with Tat in a Tat-trans-activation assay (Fig. 3.21)

It is possible to speculate that the diminished stimulation of Gag synthesis in the presence of Risp is caused by an inhibitory effect of this protein in the function of Rev. Even if it is not yet possible to conclude, that Risp binds Rev in its RNA-binding domain, the binding in its II multimerization domain could be sufficient to explain the inhibitory effect of Risp to Rev function.

The ability of HIV-1 to persist, even when attacked with multiple drugs, imposes to continue the search for novel strategies in HIV therapy. Several anti-Rev strategies have been proposed and tested (Gilboa and Smith, 1994; Lee et al., 1994; Pomerantz and Trono, 1995). The development of effector molecules that could interfere with the Rev-RRE interaction and/or Rev multimerization (Kjems and Askjaer, 2000) is considered at this moment as a promising approach, regarding Rev targeted therapy. It might be worth to employ a mutational analysis of the Rev-binding region in the Risp protein, in an attempt to isolate more potent Rev-inhibitory mutants of Risp which act by interfering with the RRE- and/or Rev-Rev interactions. Moreover, the fact that the constitutive overexpression of Risp in HeLa and U87 astrocyte cells is

not toxic, is promising. The mutation in the Rev-binding region should not change the toxicity of the protein.

4.6. Nucleocytoplasmic shuttling properties of Risp: identification of a region containing a NES

Qualitative and quantitative analysis of the intracellular localization of Risp showed that Risp-GFP is a shuttling protein that can enter and exit the nucleus and accumulates in the cytoplasm. The cytoplasmic accumulation at steady-state is similar to that observed previously with proteins subjected to nuclear export, like PKI (Wen et al., 1995), suggesting that Risp itself could contain a strong NES or may interact with a protein containing a NES. In addition, Risp accumulated in the nucleus in the presence of the Crm1 inhibitor leptomycin B, indicating that the nuclear export of Risp-GFP is Crm1-dependent.

The identification of Risp NES was confirmed by localization analysis of different transfected segments of Risp (Fig. 3.30 and 3.31) and by cytoplasmic accumulation of nuclear microinjected BSA-fusion protein, containing a peptide from the C-terminal part of Risp (residues 86-105, BSA-Risp peptide #2) (Fig. 3.33). Previous studies used cellular microinjection of fusion proteins into the nucleus or cytoplasm as experimental approach to identify signals or regions mediating protein nuclear export or import. Fischer and coworkers demonstrated, using nuclear microinjection of peptides comprising the activation domain of Rev cross-linked to BSA, that the Rev activation domain constitutes a nuclear export signal that directs RRE-containing viral RNAs export pathway (Fischer et al., 1995). Wen and coworkers also could demonstrate that the activation domain of Rev has an intrinsic export capacity, in that it mediates export of nuclear injected GST-Rev activation domain conjugate (Wen et al., 1995).

The export receptor Crm1 is reported to mediate export of several viral and cellular proteins involved in different biological functions (Fornerod et al., 1997; Gorlich and Kutay, 1999; Kjems and Askjaer, 2000). Generally, these proteins are characterized by different NES structures, although each contains a leucine-rich cluster. Using sequence alignment and *in vivo* selection studies, a leucine-rich NES consensus sequence was generated containing four characteristically spaced hydrophobic residues “L X_{2-3} L X_{2-3} L X L” (where X denotes any residue) (Bogerd et al., 1996; Kjems and Askjaer, 2000). Although leucine residues are most common, other hydrophobic residues (such as F, I or M) are found, suggesting that this consensus is not highly stringent.

Interestingly, an analysis of Risp primary amino acid sequence with the available computer programs suggested that Risp sequence does not contain “classical”

signals known to mediate nucleo-cytoplasmic transport. However, a more thorough analysis of the sequence showed at position 88 a short cluster richer of leucine and isoleucine residues (**L**ESN**L**FDDN**I**D**I**FAD**L**), motivating the choice of using these residues to synthesize a Risp peptide and to test it for nuclear export (BSA-Risp peptide #2).

The discovery of a new Crm1-dependent NES in Risp with no obvious relation to other leucine-rich NESs, strongly support the necessity to get more detailed information on how Crm1 recognizes the apparently different NES structures. One possibility is that different adapters could be involved in recognition of those NESs. Recently the existence of different specific nuclear export pathways for leucine-rich NES-containing proteins was suggested, one represented by the nuclear export of the viral RNA export factors Rev and Rex, and one by the export pathway accessed by PKI (Elfgang et al., 1999). In the proposed model, Crm1 is a general export receptor that mediates together with RanGTP the translocation of all nuclear export cargoes containing leucine-rich NESs through the NPC, whereas eIF-5A is specifically required for the nuclear export of the retroviral Rev and Rex RNA transport factors. EIF-5A was shown previously to interact functionally with the Rev- and Rex- NES (Katahira et al., 1995; Ruhl et al., 1993), and distinct mutants have been described to inhibit the nuclear export of Rev protein and thereby HIV-1 replication (Bevec et al., 1996; Junker et al., 1996). Elfgang et al confirmed that the inhibition of eIF-5A by antibodies, blocked the nuclear export of Rev and Rex, whereas the nucleocytoplasmic translocation of the PKI NES was unaffected (Elfgang et al., 1999). Regarding the Risp protein, it could be assumed, that its NES also belongs to the classical leucine-rich NESs (with a little modification of the consensus "L X_{2-3} L X_{2-4} I/L X I/L"). In any case it will be essential to determine, whether the leucine or isoleucine positions are crucial for the nuclear export of Risp as it was performed for the identification of amino acids in the Rev NES which direct its export activity.

The study of the export activity of Risp NES mutant peptides and Risp mutant protein with alterations in one or more of the leucine or isoleucine hydrophobic positions is necessary.

Moreover, analyses should be performed to understand whether these mutants may also affect the nuclear export of the Rev protein. The cellular function of Risp and the significance of the binding to Rev are still undefined. It cannot be excluded that since both proteins are using the same export receptor Crm1, they also could compete at the level of the nucleocytoplasmic translocation. Alternatively, Risp might be an additional Rev-interacting protein, involved in the Rev nuclear export. However, in both cases it will be interesting to analyze whether eIF-5A may play a role in the

nuclear export of Risp. Hence, tests using anti-eIF5A antibodies should demonstrate whether the inhibition of eIF-5A could also block the nucleocytoplasmic translocation of Risp NES peptides or Risp protein.

Proposing Risp as a new nucleocytoplasmic shuttling protein implies, that in addition to its identified Crm1-dependent NES, also a NLS should be present in the protein to mediate its nuclear entry. The presence of a lysine-rich cluster (KPKEKSKKKVEAK) in Risp at position 106, with low score of identity with the classical SV40 NLS, that could possibly act as NLS, motivated to test this region in microinjection experiments as BSA-Risp peptide conjugate (BSA-Risp peptide #2). However, a nuclear entry of cytoplasmic injected BSA-Risp peptide #2 was not observed, while when microinjected into the nucleus, it was imported into the nucleoli (Fig. 3.33). Interestingly, when the mixture of BSA-Risp peptide #2 and BSA was microinjected in the same cell in both nuclear and cytoplasmic compartments, only the BSA-Risp peptide #2 conjugate entered the nucleus and accumulated in the nucleoli, indicating the presence of a nucleolar localization signal in that basic region of Risp.
According to the result, that this region was not sufficient to mediate the nuclear import of the BSA conjugate, it cannot be excluded that a larger sequence containing the lysine-rich cluster would be necessary to trigger nuclear import of Risp. Moreover, the proximity of the NES and the putative NLS (Fig. 4.2) allow the speculation that Risp could be one of the proteins containing nucleocytoplasmic shuttling signals (NSSs), which are transport signals that can direct nuclear import as well as nuclear export (Michael, 2000). Therefore, further experiments with Risp mutants in its NES and/or putative NLS could help to clarify the importance of this region in the function of Risp.
It should be taken into account that Risp is most probably only a part of a larger protein. One or more functional NLSs could be also located in another part of the larger protein. In support of this idea a PSORT II analysis with the clone K1AA0592 identified, with a total score of 1.92, 6 different possible NLSs in the sequence, which all contain lysine and some also arginine residues (Fig. 4.2). Therefore, the possibility, that the nuclear accumulation of Risp-GFP or IgG-Risp in the presence of leptomycin B might be rather caused by passive diffusion than by active transport, has to be considered.
Recently a study of Rev-trafficking in astrocytes indicated the presence of an inhibitory activity in the cytoplasm of astrocytes, which prevented efficient nuclear uptake of Rev during its nucleocytoplasmic shuttling (Neumann et al., 2001). These results support the hypothesis of the existence of factor(s), that inhibit Rev function in astrocytes. Since a different cellular distribution of Risp-GFP in astrocytes with

respect to HeLa cells was observed, it remains very interesting to analyze the constitutive expression of Risp (or the larger protein containing it) in astrocytes. May be that Risp in these cells could interfere with the Rev function in a different way than HIV-1 permissive cells.

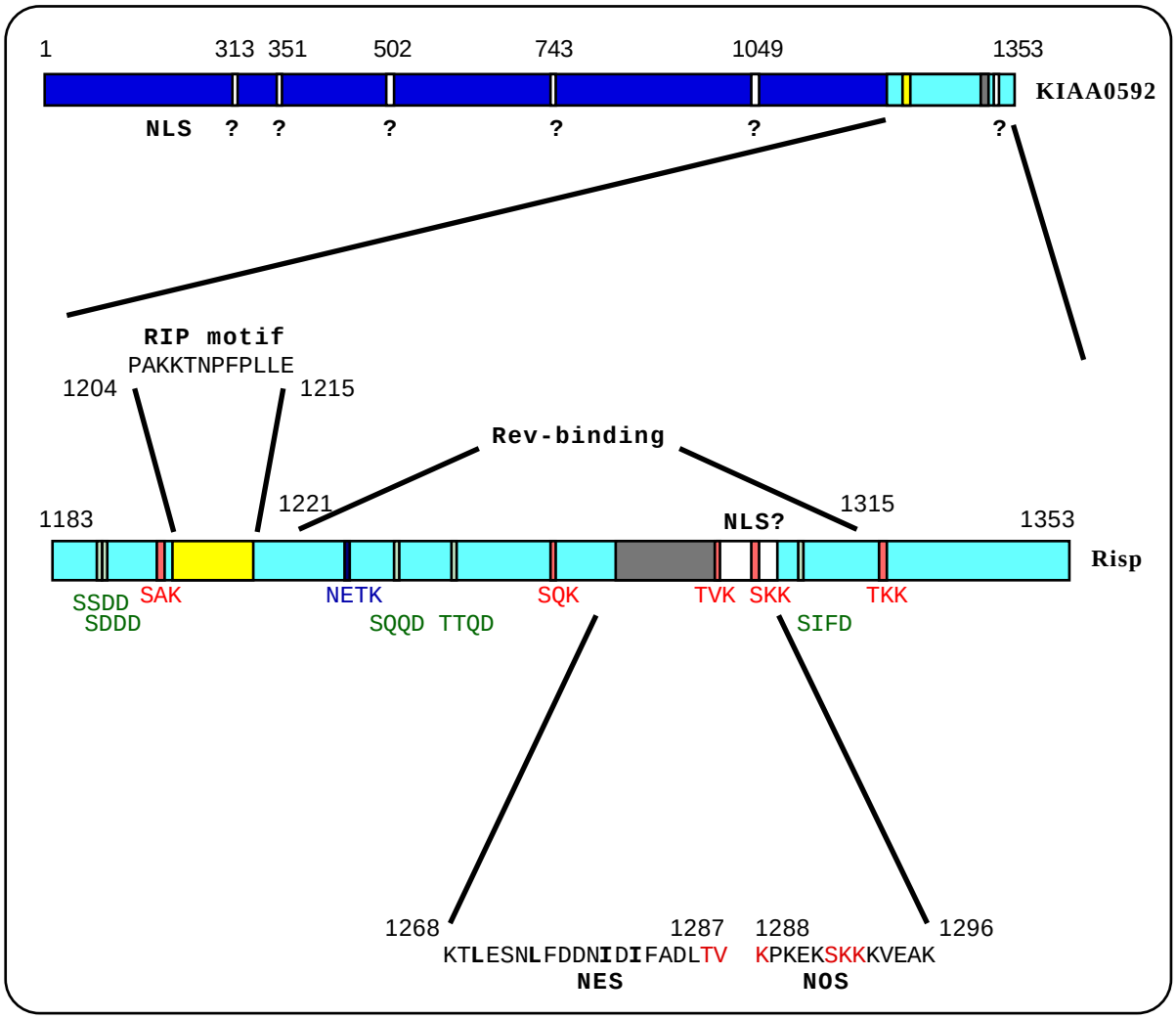


Fig. 4.2 K1AA0592 and Risp protein structure proposal. The location of Risp within the larger protein KIAA0592 is represented in light blue. The 6 KIAA0592 putative NLSs proposed with the PSORT II analysis are shown with the question marks. Regarding Risp (171 aa), the domain containing the Rev-interacting proteins (RIP)-motif is shown in the N-terminus. In the C-terminus the proximal leucine-isoleucine-rich stretch constituting the NES and the basic region with NOS and possibly NLS function are represented. The minimal sequence for the Rev-binding is not yet identified, however we assume that the Rev-binding domain is located between amino acids 39-133 (1221-1315). Moreover, the consensus sequences, recognized by the PROSITE pattern search, targeting 1 Asn-glycosylation (NETK), 5 phosphorylation sites for casein kinase II (S/T.K) and 5 phosphorylation sites for protein kinase C (S/T..D) are shown as possible post-translational modifications in Risp.

4.7. Nucleocytoplasmic shuttling properties of Rev: identification of a new additional NLS in the Rev protein

Although Rev has been described to be a nuclear protein that accumulates predominantly in the nucleoli of Rev-expressing cells, a part of the Rev molecules continuously shuttles between the nucleus and the cytoplasm (Meyer and Malim, 1994). Generally, small proteins with sizes below 40-60kDa can enter the nucleus by passive diffusion. However, in spite of Rev being significantly smaller than this limit (18 kDa), nucleocytoplasmic traffic of Rev appears to be actively controlled.

In this study, the identification of a new region with intrinsic nuclear import activity in the Rev protein has also been shown. Interestingly, analysis of the HIV-1 Rev amino acid sequence revealed the presence of a particular motif, the PPXXR motif, inside its activation domain (LQL**PP**LE**R**LTLD). This motif is conserved in various RNA-binding proteins and was proposed to be important to mediate nuclear translocation of the cellular functional homologous of HIV-1 Rev Sam68 (Ishidate et al., 1997). The possibility, that the Rev activation domain harboring this motif could be important in mediating nuclear import of Rev and that it could represent an additional NLS, has been hypothesized and investigated.

First, the intracellular localization of RevM5, a mutant of Rev in the arginine-rich NLS (Malim et al., 1989), was analyzed. It is known that the arginine-rich region of the Rev protein (aa 38-46), located within the core of the RNA binding domain, mediates both the Rev nuclear import and nucleolar targeting (Cochrane et al., 1990; Kubota et al., 1989; Malim et al., 1989; Perkins et al., 1989). As it was previously described for RevM5 (Malim et al., 1989), HeLa cells transfected with the pcRevM5 or pcRevM5-sg143 plasmids showed much higher levels of cytoplasmic accumulation of the Rev mutant protein (cytoplasmic fluorescence > 70%) than wt Rev or Rev-GFP (cytoplasmic fluorescence < 26%) (Fig. 3.37). However, in this study it has been shown, that in the presence of the export-inhibiting drug leptomycin B RevM5 and RevM5-GFP were able to enter the nucleus and accumulate there, suggesting that, even if mutated in its arginine-rich NLS, Rev is able to shuttle between the cytoplasm and the nucleus. The possibility, that the nuclear translocation of RevM5 was caused by an active transport and not by passive diffusion, was confirmed by the observation that the nuclear accumulation was energy dependent. In fact, recent results from our group (Severine Demart, data not shown) indicated that in the presence of leptomycin B RevM5-GFP only accumulated in the nuclei of cells incubated at 37°C (nuclear fluorescence 48%). At 4°C only weak nuclear translocation could be observed probably caused by passive diffusion (nuclear fluorescence 35%).

These results indicate that Rev may contain other signals mediating nuclear import in addition to its arginine-rich NLS. To analyze, whether the activation domain of Rev, containing the signal PPXXR, could act also as a nuclear localization signal and mediate the nuclear import of Rev independently from the arginine-rich NLS, several peptides consisting of the Rev activation domain (residues 73-84) were cross-linked to fluorescence labeled BSA (Fig. 3.38). In this work different BSA-Rev NES peptides, with either wt Rev or export-deficient mutant RevM10 activation domain were used to investigate the nuclear export. The peptides were also microinjected into the cytoplasm and their nuclear import activity was investigated.
Nuclear accumulation of cytoplasmic microinjected BSA-Rev NES peptides in cells pretreated with the export-inhibiting drug leptomycin B clearly showed the ability of the activation domain to induce nuclear import of BSA-conjugate (Fig. 3.40).
These results suggest that the leucine-rich domain of Rev harbors signals able to mediate shuttling between the nuclear and cytoplasmic compartments, rather than exclusively conferring nuclear export. This impression is supported by another recent study, which showed nuclear entry *in vivo* and nuclear accumulation *in vitro* of beta-galactosidase fusion protein containing the Rev NES, when microinjected in the cytoplasm (Efthymiadis et al., 1998). However, Efthymiadis et al. observed, that NESs of both Rev and PKI drove nuclear entry of the large heterologous protein β-galactosidase. The absence of the motif PPXXR in the PKI NES lets suppose, that if this motif is important for nuclear import activity of the Rev NES, a different signal should be responsible for import activity of the PKI NES.

A point mutation changing the arginine in the PPXXR motif to alanine was described to diminish the nuclear accumulation activity of a Sam68 NLS peptide, suggesting the importance of this residue in nuclear translocation. Hence it was investigated, whether the exchange of the arginine by an alanine in the activation domain of Rev could also induce a change in its intrinsic nuclear import activity. As expected, the alanine exchange did not alter the nuclear export ability of the Rev NES.
As it is shown in Fig. 3.40 both activation domains of wt Rev and of export-deficient mutant RevM10 harboring the alanine mutation kept the nuclear entry ability when injected into the cytoplasm. Nevertheless, at the same time post-injection a minor nuclear accumulation of the BSA-RevM10NES-A conjugate in comparison to the BSA-RevM10NES was observed. This indicates that the alanine mutation could alter the nuclear import ability of the Rev activation domain if already mutated in its nuclear export function.

Although the PPXXR motif in Sam68 is thought to be important in mediating nuclear entry, an additional mutational analysis of the Rev NES should be performed to address the significance of the PPXXR motif in nuclear import of Rev.

The activation domain of Rev has been mapped to amino acids 73-83 on the basis of mutational analyses (Kjems and Askjaer, 2000). Mutation of any of the three leucines (Leu 78, Leu 81 or Leu 83) in this element not only abolished Rev function by blocking its nuclear export, but also inhibited the activity of co-expressed wild type Rev protein, a phenomenon known as trans-dominant inhibition.

In this study, we showed that the mutations of Leu 78 and Glu 79 to Asp 78 and Leu 79 (in RevM10) abolished the Rev nuclear export, whereas they did not abolish the nuclear import of the activation domain. However a weak reduction of nuclear import of the RevM10 NES with respect to the Rev wt NES was observed and this increased in presence of the Arg 80 mutation.

In a recent study, where the toleration of diverse amino acid substitutions in the Rev NES was investigated, the single mutational exchange of the arginine in the PPXXR motif with the hydrophobic alanine reduced Rev function by 50% (Zhang and Dayton, 1998). According to what is known so far, the impairment of Rev function related to the Arg mutation, could depend also on inhibition of Rev nuclear import via its activation domain.

However, the highly similar concentration of leucine residues in the PKI (**L**A**L**K**L**AG**L**DI) and Rev (**L**Q**L**PP**L**ER**L**T**L**) NES sequences (essential for the nuclear export) and the absence of the PPXXR motif in PKI NES could also indicate, that this motif itself is not important to confer nuclear import in Rev. Whereas the alternating presence of hydrophobic leucine and hydrophilic residues (acidic, basic or uncharged) in both NESs is the essential requirement to transform both domains in target signal for the transport receptors.

It is interesting to note that until now no Rev mutants exist, which show only disabled import activity. Since the arginine-rich NLS is located within the RNA binding domain, mutants in this region of course exhibited impairment in nuclear entry and in RNA binding, which resulted in a clear abrogation of Rev function (Kubota et al., 1992; Malim et al., 1989). Even though the existence of two NLS in the Rev protein is confirmed, it is not yet known ,which cellular or viral element controls their function.

Interestingly not all the cytoplasmic injected BSA-RevNES conjugated entered the nucleus and furthermore the BSA-RevNES did not accumulate in the nucleoli The signal and the mechanism of entry of this additional Rev NLS might be different from the Rev arginine-rich NLS, which has been shown to mediate also nucleolar

localization of transfected fusion protein (Cochrane et al., 1990). However, since no microinjection experiments with BSA-Rev NLS have been performed so far, it is difficult compare the two NLSs directly.

Since the arginine-rich NLS is very basic, while the NES is very hydrophobic, these two motifs target very likely different sites to achieve nuclear translocation. Despite information on the identification of an additional NLS in the activation domain of Rev, the import pathway through which this signal acts has not yet been investigated, therefore only hypothesis on known pathways could be proposed and discussed.

It is known that nuclear import of proteins with classical lysine-rich NLS is conferred by their NLS-dependent interaction with the importin α/importin β heterodimer transport receptors. Arginine-rich NLSs, of both Tat and Rev (that are very similar to each other) directly interact with importin β but not importin α *in vitro*. Importin β is both necessary and sufficient for the nuclear import *in vitro* assays suggesting a function independent of importin α (Truant and Cullen, 1999). Already Henderson and Percipalle (Henderson and Percipalle, 1997) have reported that the Rev NLS can bind directly to importin β and that this interaction is inhibited by added importin α, thus suggesting that importin α and Rev compete for overlapping binding sites on importin β. It is interesting to note that these authors also reported a Rev-importin α interaction. However, since it was not dependent on a functional Rev NLS it was therefore regarded to be non specific. Nevertheless, with the discovery of a nuclear import activity in the Rev NES it could not be excluded that the activation domain itself may also bind specifically to importin α. It may be that the nuclear translocation of the Rev protein via its activation domain could be dependent on importin α. However Efthymiadis and coworkers were not able to prove a direct binding between the Rev and PKI NESs with the importin α/β-NLS-binding subunits using an ELISA-based binding assay (Efthymiadis et al., 1998). They proposed, that exportins, such as Crm1, which are known to mediate export of NES-containing proteins could themselves facilitate nuclear import under certain conditions. However, in this study it was shown that the Crm1-binding leptomycin B inhibited nuclear export but not nuclear import of BSA-Rev NES conjugate. Moreover, the mutations of Leu 78 and Glu 79 (RevM10) abolished the Rev nuclear export but not the nuclear import of the NES. Since it is known that RevM10 in the yeast two-hybrid is not able to bind Crm1 (Neville et al., 1997) it is conceivable that other transport receptors different from Crm1 may be involved in the binding of the Rev activation domain and mediate its nuclear import.

In summary, the Rev activation domain might be considered as a nucleocytoplasmic shuttling domain containing a nucleocytoplasmic shuttling signal (NSS) rather than a

"simple" NES. Several proteins involved in mRNA nuclear export are known to contain NSSs, which are transport signals, that can direct both nuclear import and nuclear export (Michael, 2000). Analysis of NSS has demonstrated, that subcellular trafficking of mRNA-binding proteins occurs by a mechanism fundamentally different from that of other shuttling proteins. Therefore, the import receptor recognizing the Rev NSS could be one of those which are mediating the import of other NSS-containing proteins. Until now only transportin 1, a distant relative of the importin β, was discovered to mediate the import of two NSS-containing proteins hnRNP A1 and Tap (Bonifaci et al., 1997; Pollard et al., 1996; Truant et al., 1999), whereas the import receptor for the other proteins is still unknown (Michael, 2000).
In conclusion, these results demonstrate, how, for a small protein such as Rev, which could in principle cross the nuclear pores free by passive diffusion, the nucleocytoplasmic shuttling, which is essential for its key regulatory roles, is highly controlled by different targeting signals and cellular transport mechanisms. Therefore, HIV-1 Rev still represents an excellent system to study and increase the knowledge on the signal-mediated transport across the nuclear envelope.

5. Concluding Remarks

In this work a new Rev-interacting protein has been identified using the yeast two-hybrid system. The protein was termed Risp (Rev-interacting shuttle protein), because it shuttles between the nuclear and the cytoplasmic compartments.
The Risp gene is widely expressed in human cells and conserved among various species, most probably as part of a larger gene. High identity (99%) with the C-terminal part of a large brain cDNA clone for KIAA0592 protein (Nagase et al., 1998) has been found, whereas no high sequence homology has been associated with any protein with known function. However, a weak and partial homology appeared with several RNA-/DNA-binding and shuttle proteins. This might indicate that Risp protein - or the larger protein containing it - could be a member of a new family of nucleocytoplasmic shuttle proteins with RNA-/DNA-binding function.

In HeLa cells Risp-GFP localizes in both nuclear and cytoplasmic compartments, but clearly accumulates in the cytoplasm, indicating the presence of a strong nuclear export signal (NES). The identification of Risp NES was confirmed by localization analysis of different transfected segments of Risp and by cytoplasmic accumulation of nuclear microinjected BSA-fusion protein containing a peptide from the C-terminal part of Risp. The inhibition of the Crm1-mediated nuclear export, induced by the presence of leptomycin B, showed that the nuclear export of Risp is Crm1-dependent.

As it is rich in leucine and isoleucine residues, the Risp NES might be related with other leucine-rich NESs. To determine if these hydrophobic amino acids are also crucial for the nuclear export of Risp, the export activity of Risp NES mutant peptides and Risp mutant protein with alterations in one or more of the leucine and isoleucine positions has to be analyzed.

Risp has been shown to share a specific motif with other Rev-interacting proteins. This consists of 12 amino acids [P (AELNR) (KHILST) (KCRST) T N (PD) F (GLQ) (LST) (LIN) (EAGQS)] termed RIP motif. This is the first indication for the presence of a specific and common motif shared between Rev-interacting factors, including Rev itself. A region containing a sequence matching to the consensus motif was identified in every Rev-interacting protein. Nevertheless, a particular group of cellular proteins, mostly involved in the Rev/RRE nuclear export, showed to be highly specific versus the motif. No correlation was found between the motif and the Rev-binding ability, in fact the region of Risp harboring the RIP motif was neither essential nor sufficient for the Rev-binding in the yeast two hybrid system.
Therefore, the role and the relevance of the consensus motif present in Risp and in the other Rev-interacting factors should be in detail investigated in future studies. For example mutational analysis and deletions of the domain in the Risp protein will be established to analyze whether the function of Rev could be modified.

Although, it is not yet clear which is the minimal sequence part required for the Risp-Rev interaction, we can assume that the Risp-binding region in Rev should be located in a region overlapping the basic domain deputed to RNA binding/NLS (34-50aa) and the second multimerization region (52-60aa). In the basic domain already other Rev-interacting proteins such as B23, p32, and importin β are supposed to bind Rev, whereas other proteins involved directly and indirectly to the Rev nuclear export, such as Crm1, eIF-5A, hRab/rip, are known to bind Rev in its activation domain (Table 2.3 and Fig. 5.1).

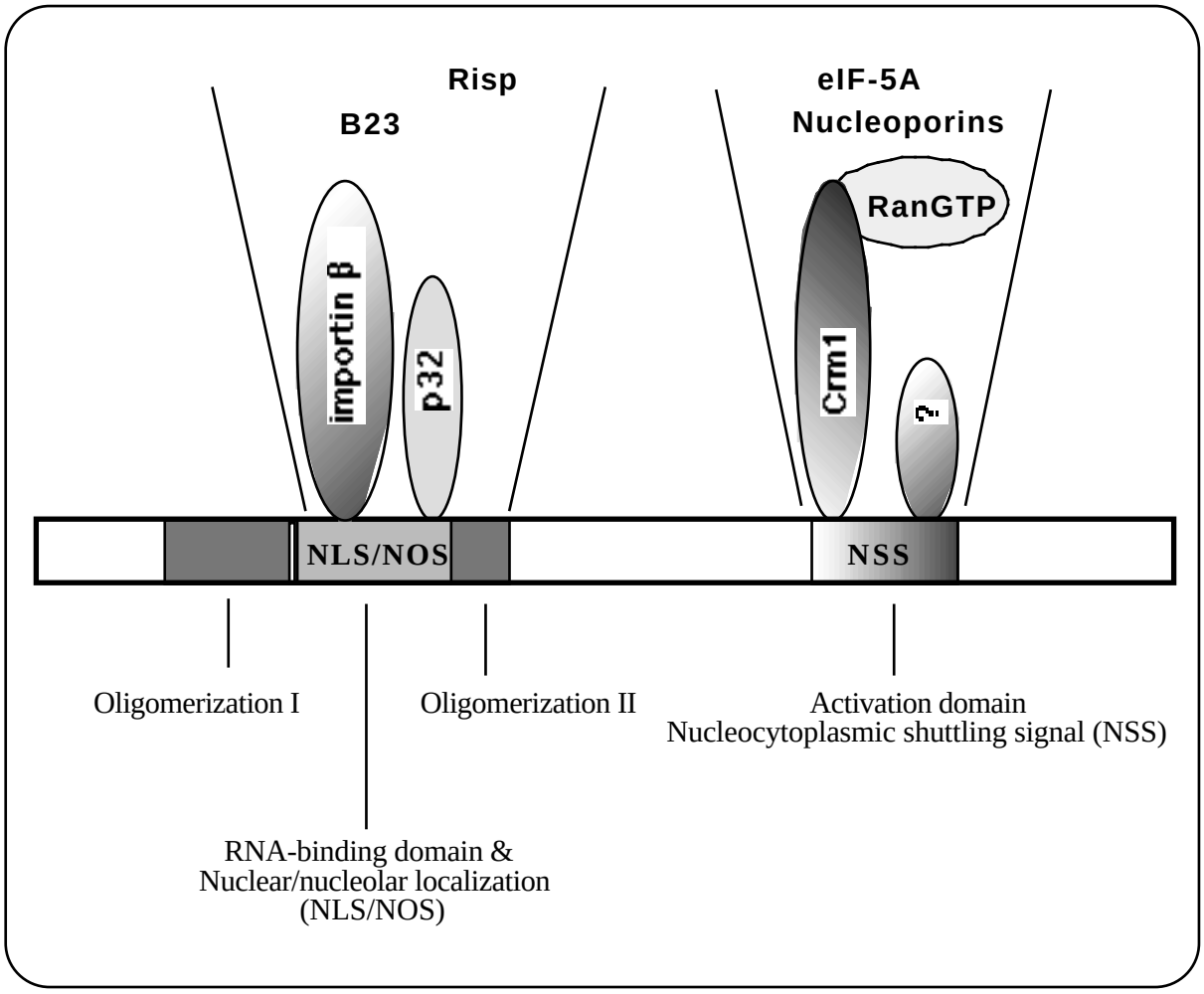


Fig. 5.1 Rev and cellular interacting factors. It is known that Rev is composed of several domains harboring distinct binding sites for cellular proteins. Here an updated scheme of the primary Rev structure is proposed. The leucine-rich activation domain in the C-terminus (aa 73-84) contains a nucleocytoplasmic shuttling signal (NSS) mediating the Rev nuclear export and nuclear import. In the nucleus, Rev NSS binds directly together with RanGTP to the export receptor Crm1 to form a stable complex Rev/Crm1/RanGTP able to dock and translocate the nuclear pore complex. Several nucleoporins (hRip/Rab, yRip1p1/Nup42, Nup98, Nup153, Nup214, NLP-1) and eIF-5A, identified as Rev NES-interacting proteins, are involved to mediate the nuclear export of Rev/Crm1/RanGTP complex. The interacting import protein (**?**) mediating the Rev import via the NSS domain is not yet discovered. The arginine-rich stretch located toward the N-terminus (aa 34-50) serves as a RNA-binding domain as well as a nuclear/nucleolar localization signal (NLS/NOS). This is, in turn, closely flanked on both sides by residues that mediate Rev multimerization (aa 12-29 and 52-60). Importin β and p32, have been proposed to bind directly with the Rev basic domain. In the cytoplasm, the import receptor importin β recognizes the REV NLS and thus mediates the Rev nuclear entry. The interaction of Rev with the protein p32, normally associated with the cellular splicing factor SF2/ASF, may participate in the removal of splicing factors from intron-containing RNAs and permit a nuclear accumulation of RNAs substrate for Rev-mediated nuclear export. A binding between the nucleolar phosphoprotein B23 and Rev has been also observed. However, the accumulation of both proteins in the nucleolus may reflect the ability of the interaction, while no B23 role in supporting Rev function has been demonstrated. Finally, in this study, we showed the presence of a new Rev-interacting protein, Risp, that binds Rev in its RNA-binding and the II multimerization domain.

Preliminary experiments suggested that Risp, as a Rev-interacting protein, is able to inhibit Rev-trans-activation, while Risp does not interfere with Tat in a Tat-trans-activation assay. In fact, the over-expression of Risp-GFP was able to reduce the production of the Rev-dependent structural viral protein p24gag up to 70% and fairly specifically the SLIIB-CAT expression in a Tat-Rev/SLIIB-binding assay. Therefore, it is possible that the binding of Risp to Rev in its RNA-binding and multimerization domain may compete with the binding of Rev to its SLIIB RNA target and to other Rev molecules inhibiting thereby Rev function.
Since Risp most probably is only a part of a larger protein, it is also important to isolate its full-length cDNA and repeat the Rev-trans-activation studies. Currently, Chris Bickel, in the group of Dr. Brack-Werner, is trying to enlarge to the 5' end of the full-length cDNA. It will be of high interest to determine whether the original protein containing Risp is able to interfere with the Rev function and thus to limit HIV-1 replication.

Moreover, since both Risp and Rev are using the same export receptor Crm1, an additional role of Risp could also be proposed at the level of the nucleocytoplasmic translocation of Rev. The direct binding of Risp to Crm1 could compete with the Rev-Crm1binding and therefore reduce Rev nuclear export. On the other hand, Risp bound to Rev in its RNA-binding and multimerization domain may accelerate the nuclear export of Rev, providing also its NES, and therefore facilitating the association with Crm1and RanGTP to form an active export complex in the absence of HIV-1 RNA.

Of course, most of the implications reported here are still speculative, requiring future experimental work to obtain a better understanding of the function(s) of Risp and its interactions with Rev.

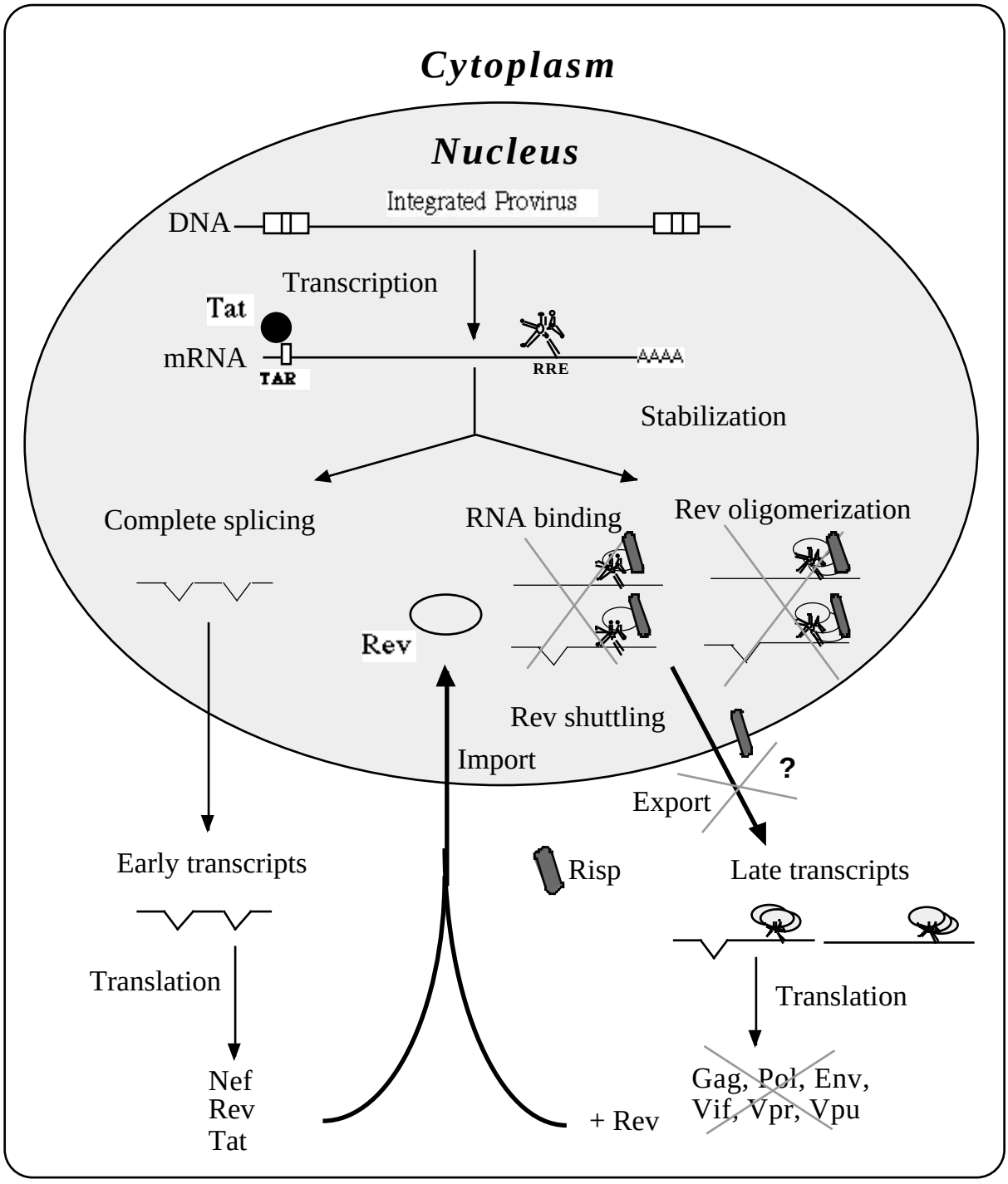


Fig. 5.2 Possible interference on the Rev functions by Risp. The interaction of Risp with Rev in its RNA-binding and multimerization domain may compete with the binding of Rev to the unspliced and single spliced mRNAs and to other Rev molecules. The binding of Risp to the export receptor Crm1 may result in competition with the Crm1-Rev binding at the level of the nucleocytoplasmic translocation. Therefore, the transport out of the nucleus and the following translation of the viral mRNAs responsible for the production of the viral enzymes and structural proteins might be inhibited by the presence of Risp.

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Summary

The aim of this study was to characterize the role of Rev-interacting cellular proteins in controlling the function of Rev in the host cell. The HIV-1 protein Rev plays an essential role in the temporal regulation of the virus gene expression by stimulating the expression of viral structural proteins. Rev enhances the nucleocytoplasmic transport and the translation of unspliced and single spliced viral mRNAs by binding with high affinity to a specific target element on the HIV-RNA. It was assumed that interaction with cellular factors is essential for Rev function. At the onset of this study, only a few potential cofactors were known with no clearly defined functional relevance. So we decided to search for new Rev-interacting factors using the yeast two-hybrid system.

In this work a new Rev-interacting protein has been identified, by screening a Jurkat T cell cDNA library. The protein was termed Risp (Rev-interacting shuttle protein), because it shuttles between the nuclear and the cytoplasmic compartments.
The Risp gene is widely expressed in human cells and conserved among various species, most probably as part of a larger gene. High amino acid homology (99%) with the C-terminal part of a large brain cDNA clone for KIAA0592 protein has been found, whereas no obvious homology to proteins with known function was observed. However, a weak and partial similarity appeared with several RNA-/DNA-binding and shuttle proteins. This might indicate that the Risp protein - or the larger protein containing it - could be a member of a new family of nucleocytoplasmic shuttle proteins with RNA-/DNA-binding function.
Next, the intracellular localization and shuttling of Risp was investigated. In HeLa cells Risp-GFP localized in both nuclear and cytoplasmic compartments, but clearly accumulated in the cytoplasm, indicating the presence of a strong nuclear export signal (NES). The identification of a NES sequence was confirmed by deletion analysis of Risp and by nuclear microinjection of BSA-fusion proteins conjugated to peptides from the C-terminal part of Risp.
Treatment with leptomycin B, a drug which has been shown to specifically block Crm1 (exportin) mediated export, resulted in nuclear accumulation of Risp-GFP, showing that the nuclear export of Risp, like that of Rev, is Crm1-dependent.
Using bioinformatic tools able to detect weak homologies with high specificity, sequence comparisons between Risp and all currently known Rev interacting factors were performed. This analysis for the first time revealed a common motif shared between Rev and Rev-interacting cellular factors, termed RIP. The region of Risp harboring the RIP motif was neither essential nor sufficient for the Rev-binding in the yeast two hybrid system, suggesting no direct correlation between RIP and the Rev-binding ability.
Preliminary experiments suggested, that Risp, as a Rev-interacting protein, is able to inhibit Rev-trans-activation, while Risp does not interfere with Tat in a Tat-trans-activation assay. The over-expression of Risp-GFP reduced the production of the Rev-dependent structural viral protein p24gag up to 70%.
In addition a previously unrecognized sequence motif in the activation domain of Rev with intrinsic nuclear import activity was found and tested in transfection and microinjection assays. This motif (“PPXXR”) is conserved in various RNA-binding proteins and was proposed to mediate nuclear translocation of the cellular functional homologue of HIV-1 Rev Sam68.

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