Identification and Functional Analysis of Epstein-Barr Virus Nuclear Antigen 2 (EBNA2) Target Genes

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LIST OF ABBREVIATIONS

A	adenosine
AIDS	acquired immune deficiency syndrome
BL	Burkitt's lymphoma
bp	base pair
BrdU	5-bromo-2'-deoxy-uridine
BSA	bovine serum albumine
C	cytosine
CD	cluster of differentiation
Cdk4	cycline-dependent kinase 4
cDNA	complementary DNA
ChIP	chromatin immunoprecipitation
CHX	cycloheximide
Ci	Curie
CMV	cytomegalovirus
CR2	complement receptor type 2
cRNA	complementary RNA
CTP	cytosine triphosphate
dCTP	deoxycitosine triphosphate
DEPC	diethyl pyrocarbonate
DIG	digoxigenin-dUTP
DNA	2'-deoxyribonucleic acid
dNTP	3'-deoxyribonucleoside-5'triphosphate
DPM	degradations per minute
dsRNA	double-stranded RNA
DTT	dithiotreitol
dUTP	deoxyuridine triphosphate
E.coli	*Escherichia coli*
e.g.	for example *(*lat. *exempli gratia*)
EBER	EBV-encoded RNA
EBNA	Epstein-Barr nuclear antigen
EBV	Epstein-Barr virus
EDTA	ethylene diamine tetra-acetic acid
ELISA	enzyme linked immunosorbent assay
EMSA	electromobility shift assay
ER/EBNA2	oestrogen receptor/Epstein-Barr nuclear antigen 2
EST	expressed sequence tag
FCS	foetal calf serum
Fig.	figure
G	guanidine
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GC	germinal centre
GM-CSF	granulocyte-macrophage colony-stimulating factor
h	hour
HAT	histone acetyltransferase
HD	Hodgkin's disease

HDAC	histone deacetylase complex
HHV4	human herpesvirus 4
HIV	human immunodeficiency virus
Hsp90	heat shock protein 90
i.e.	that is (lat. *id est*)
Ig	immunoglobulin
IL	interleukin
IPTG	isopropyl β-D-thiogalactoside
Kb	kilo base pair
LB-medium	Luria-Bertani-medium
LCL	lymphoblastoid cell line
LMP	latent membrane protein
LP	leader protein
MACS	MicroBeads assisted cell sorting
MAPK	mitogen-activated protein kinase
MHC	major histocompatibility complex
mm	mismatch mutation
MOPS	3-(N-morpholino) propansulphonic acid
mRNA	messenger RNA
NFAT	nuclear factor of activated T cells
NF-κB	nuclear factor kappa binding protein
NGFR	nerve growth factor receptor
NTP	3'-ribonucleoside-5'triphosphate
Oct	octamer binding protein
ORF	open reading frame
PBS	phosphate buffered saline
PCAF	p300/CBP associating factor
PCR	polymerase chain reaction
PI	propidium iodide
PMA	phorbol-12-myristate 13-acetate
PTLD	post-transplant lymphoproliferative disease
RBP-Jκ	recombination signal-binding protein Jκ
RE	random expectation
RNA	2'-ribonucleic acid
rRNA	ribosomal RNA
RT-PCR	reverse transcriptase-PCR
SDS	sodium dodecylsulphate
siRNA	short interfering RNA
snRNA	small nuclear RNA
SRBC	sheep red blood cells
SSC	sodium chloride-sodium citrate buffer
STAGA	SPT3-TAF_{II}31-GCN5L acetylase
SV40	Simian virus 40
T	tymidine
TAF_{II}	TBP-associated factor II
TBP	TATA-binding protein
Tet	tetracycline
TF	transcription factor
TFTC	TBP-free TAF_{II} complex
TNF	tumor necrosis factor

TR	terminal repeat
TRRAP	transformation/transcription domain-associated protein
TSS	transcription start site
U	uridine

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1.0 Introduction

1.1 Structure of the Epstein-Barr virus genome

Epstein-Barr virus (EBV), or human herpesvirus 4 (HHV4), belongs to the herpesvirus family. The EBV genome is a linear double-stranded DNA molecule of 172 kb flanked by terminal repeats (TR). Upon infection of the host cell the linear genome undergoes circularisation (Fig. 1) and, as a multicopy episome, is replicated during each cell division by the host DNA polymerase together with the host chromosomes. The EBV genome encodes over 85 open reading frames (ORFs) (Kieff and Liebowitz, 2001).

1.2 EBV infection of the B cells *in vitro*

In vivo, EBV infects naive B cells (Thorley-Lawson and Babcock, 1999), but *in vitro* it will infect any B cell, driving it out of the resting state into continual proliferation resulting in the outgrowth of immortal lymphoblastoid cells. The mechanism of EBV infection is mostly studied *in vitro*. There are two reasons for that. First, EBV infects exclusively humans and non-human primate mammals. This very narrow host range represents an obstacle in the study of EBV infection in *in vivo* animal models. Second, the frequency of infected cells in the EBV positive individual is very low (0.5-50 per million, depending on the individual) (Khan et al., 1996). The infection of B cells is mediated through interaction of viral gp350/220 with the cellular CD21/CR2, the physiological receptor for the complement factor C3d (Fingeroth et al., 1984). Following CD21 binding, the viral envelope fuses with the host cell membrane and triggers host cell activation. A nucleocapsid is then transported to the nuclear boundary and subsequently degraded. The linear viral DNA is released into the nucleus. This process is followed by the initial transcription of EBNA2 and EBNA-LP (Kieff and Liebowitz, 2001). These two proteins are essentially required for the initial B cell growth transformation, but their activity is modulated by the subsequent expression of Epstein-Barr virus nuclear antigens 3A, 3B and 3C (EBNA3A, 3B and

3C, respectively) which are also essential for the process of B cell immortalisation. A second event is the circularisation of the viral genome, for which the host cell DNA repair machinery is used (Sixbey and Pagano, 1985, Hurley and Thorley-Lawson, 1988). After circularisation of the genome, a full spectrum of latent proteins is expressed: nuclear antigens EBNA1, 2, 3A, 3B, 3C and LP, latent membrane proteins 1 and 2 (LMP1 and 2, respectively) (Fig.1) and small non-polyadenylated RNAs EBER1 and 2. Cells proliferate in the so called "growth programme". Throughout the immortalisation process some of the infected cells enter a proliferative crisis and die due to the shortening of telomeres. Only those clones which are able to stabilise their telomeres by activating telomerase continue to proliferate indefinitely, i.e., become immortal (Counter et al., 1994). Although critical, the telomerase activity is not sufficient for the immortalisation process. Unknown genetic events are required for final immortalisation.

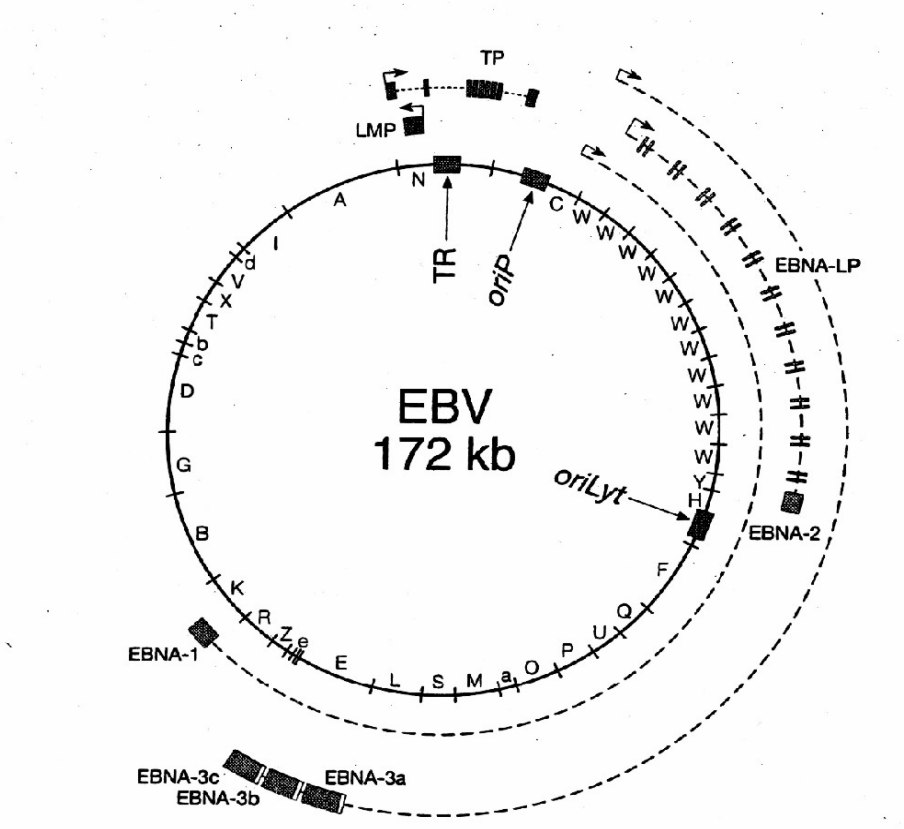


Figure 1.

The episomal EBV genome.

A simplified circular map of the 172 kb EBV episome showing the relative locations of individual EBNA2, EBNA-LP, EBNA3A, EBNA3B, EBNA3C, EBNA1, LMP (LMP1) and TP (LMP2A) genes. The positions of the origin of replication during latency (oriP) and the origin of replication active in the lytic cycle (oriLyt) are also marked. TR shows the fused terminal repeats.

(taken from Bornkamm and Hammerschmidt, 2001)

1.3 Biology of EBV infection *in vivo*

The common features of all gammaherpesviruses are their lymphotropism, their ability to establish life-long latent infection of their host cell and induction of proliferation of the latently infected cells (reviewed in Roizman, 2001).

1.3.1 Viral persistence and reactivation

Recently, information about the mechanism of EBV persistence *in vivo* has begun to accumulate by combining the cell separation methods with very sensitive techniques, like a single cell PCR.

Initially, it has been believed that the sites of primary EBV infection are oropharyngeal epithelial cells. However, there are increasing evidences that the virus requires a B cell compartment for persistence in the host. First, individuals with the heritable disorder X-linked agammaglobulinemia lack mature B cells and harbour no EBV in their blood or throat washings. Also, they do not have an EBV specific memory cytotoxic T cell response. Both findings illustrate that B cells are targets of primary EBV infection and are required for the establishment of viral persistence (Faulkner et al., 1999).

B-cell activation requires signals from T_H cells, antigen presenting cells and regulatory cytokines. Activated B cells either differentiate into low-affinity plasma cells or enter the adjacent follicle to initiate the germinal centre (GC) reaction (reviewed in MacLennan, 1994 and Kelsoe, 1996). There, in a concert with the antigen-specific GC T cells and follicular dendritic cells, B cells mutate their immunoglobulin genes. This specialised hypermutation machinery is activated within centroblasts, the highly proliferating cells. Centroblasts give rise to less proliferative centrocytes which are subjected to the negative selection. Following multiple rounds of mutation and selection, centrocytes terminally differentiate into memory B cells

or high-affinity plasma cells. B cell receptor (BCR) signalling is required for GC B cell differentiation. A further signal is delivered by CD40 as a result of interaction with CD40 ligand on activated T_H cells.

EBV mimics the process of normal B cell activation and differentiation through the coordinated expression of eight latent proteins, which are under the direction of a master transcription factor EBNA2 (Ling et al., 1994). This transcription pattern allows a rapid polyclonal expansion (therefore called "a growth programme") of infected B cells as lymphoblasts which proliferate and subsequently differentiate through a GC-type reaction. Finally, they enter a peripheral B cell pool as resting memory cells latently carrying virus for life-long (Fig. 2) (Rickinson and Lane, 2000, Thorley-Lawson, 2001). During the period from infection up to the establishment of a life-long persistence the viral transcription pattern gradually changes depending on the location and differentiation stage of the infected B cell. With respect to the latent viral gene expression three transcriptional programmes can be differentiated: (i) a growth programme in the initial stage where all latent genes and EBER1 and 2 are expressed, (ii) a default programme following the shutting off of EBNA2, 3A, 3B, 3C and LP and (iii) a latency programme, a programme in which EBV resides in the latent state in memory B cells and where no latent gene is expressed except for LMP2A in some cases (Fig. 2). So far it is still unknown which mechanism shuts off viral latent genes in this process, but it probably involves signals originating in the environment of the germinal centre follicles.

Taken together, in contrast to *in vitro* infection where cells use the "growth programme" and proliferate, *in vivo* EBV exhibits two, diametrically opposite, behaviours (reviewed in Thorley-Lawson, 2001). On one hand, EBV infects a naive B cell and drives the activation and proliferation of that cell. On the other hand, the virus can reside quiescently in resting memory cells and thus establishes persistence.

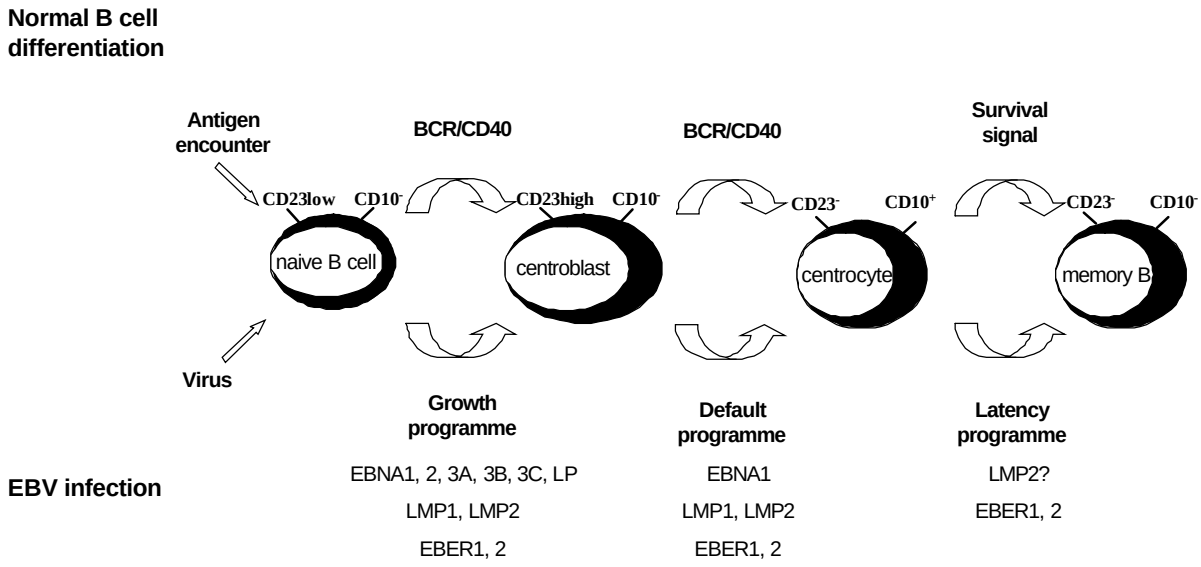


Figure 2.

EBV exploits the normal pathway of B cell differentiation.

The comparison between EBV and antigen driven B cell activation and differentiation into memory cells is illustrated. Signals which drive this process are shown in the upper part of the figure for antigen activated B cells and in the lower part of the figure for EBV infected B cells.

(modified from Thorley-Lawson and Babcock, 1999)

1.4 Pathogenicity of EBV

EBV infects more than 95% of the World's adult population. Following primary infection the individual remains a life-long carrier of the virus. The majority of primary infections occurs in early childhood and are generally subclinical. However, when primary infection is delayed until adolescence or adulthood it may cause infectious mononucleosis (IM), a self-limiting lymphoproliferative disorder characterised by an increased number of EBV infected B cells in peripheral blood with a heterogeneous EBV gene expression pattern at the single cell level. Persistent infection is characterised by a stable number of latently infected B cells in the blood (0.5-50 per million, depending on the individual) (Khan et al., 1996). In spite of these facts the majority of the individuals who are EBV-positive will never develop any kind of lymphoproliferative disease caused by or associated with EBV. The viral proteins expressed in the growth programme, except of EBNA1, are highly immunogenic and the cells expressing those proteins are immediately diminished by the immune response. Thus, only cells expressing the latency programme survive

immunosurveillance, but they are not able to proliferate. However, in the absence of effective immunosurveillance or due to additional genetic changes, the EBV infected cells may express growth or default programme leading to various lymphoproliferative diseases.

Burkitt's lymphomas (BL) and Hodgkin's disease (HD) arise in immunocompetent individuals. The exact role which the virus plays in the etiology and pathogenesis of these malignancies is still controversial. About 95% of endemic BL in Central Africa and 20-50% of HD are EBV positive. In Europe and USA the association of BL with EBV is only 15-20% (Magrath et al., 1992). In most BLs the only latent gene expressed is EBNA1 (Rowe et al., 1986), a protein which cannot be presented by the MHC class I due to the specific block of proteasomal degradation (Levitskaya et al., 1997). Recently, a subset of BLs that show atypical patterns of viral gene expression characterised by the use of the Wp promoter has been described (Kelly et al., 2002). These cells express EBNA1, -3A, -3B, 3C and truncated –LP. However, EBNA2 is deleted in the EBV genome residing in these cells. An important feature of all BLs is the presence of the *t*(8;14) chromosomal translocation which juxtaposes the c-myc oncogene to the Ig heavy chain locus on chromosome 14, resulting in overexpression of c-myc (Zech et al., 1976). The HD cells express EBNA1, LMP1 and LMP2A (default programme) which play an active role in development of HD along with the oncogenic changes which occur during tumor development (Kuppers and Rajewsky, 1998).

EBV is also associated with some non-B cell malignancies like extranodal T-cell and NK cell lymphomas, nodal T-cell lymphomas, nasopharyngeal carcinoma (NPC), gastric carcinoma and some others (reviewed in Middeldorp et al., 2003) indicating the capacity of EBV to infect non-B cells as well.

The lymphoproliferative disorders that arise due to immunosuppression following transplant surgery are collectively known as a post-transplant lymphoproliferative disorder (PTLD). The majority of EBV-positive PTLD cases exhibit an unrestricted pattern of the latent viral gene expression (EBNA1, -2, -3A, -3B, -3C, -LP, LMP1 and LMP2) governed by EBNA2. The proliferation of these cells represents the early stage of the infected B cells (before shutting off the EBNA2 and EBNA3s), and resembles a "growth programme" seen in LCLs *in vitro*. After the restoration of the immune system these lymphomas perish spontaneously (Rooney et al., 1995)

implicating that they are primarily driven by EBV. Similar disorders occur in some of the inherited immunodeficiencies and in patients with AIDS (Miller, 2001).

1.5. Epstein-Barr virus nuclear antigen 2 (EBNA2)

EBNA2 and EBNA-LP are the first viral genes expressed upon infection of B cells by EBV. The inability of P3HR1, an EBV strain carrying a deletion of the EBNA2 gene, to immortalise B cells indicates a crucial role of this protein in the transformation process (Hammerschmidt and Sugden, 1989, Cohen et al, 1989). EBNA2 is a 487 amino acid protein which acts as a transcriptional activator to regulate the pattern of EBV latency gene expression in B cells, contributes to the changes in surface expression of B cell activation antigens and modifies cellular gene expression resulting in the stimulation of G_0 to G_1 cell cycle progression (Sinclair et al., 1994, Kempkes et al., 1995a). EBNA2 response elements are described for the viral promoters of the LMP2A (Zimber-Strobl et al., 1993) and LMP1 genes and the C promoter (Ling et al., 1993, Laux et al., 1994a). This element has been identified to bind a protein called RBP-Jκ, also known as CBF-1 (Ling et al., 1993, Henkel et al., 1994, Grossman et al., 1994, Waltzer et al., 1994, Zimber-Strobl et al., 1994). EBNA2 does not bind to DNA directly but is (probably as an oligomer (Harada et al., 2001)), tethered to DNA through the interaction with RBP-Jκ in order to activate transcription. RBP-Jκ is a ubiquitous cellular factor that binds a consensus DNA sequence GTGGGAA. RBP-Jκ is also targeted by the intracellular domain of the cell-surface receptor Notch (NotchIC) which is generated upon ligand-induced protease cleavage (reviewed in Artavanis-Tsakonas et al., 1999 and Lai, 2000). RBP-Jκ acts as a transcriptional repressor by carrying a histone deacetylase-containing complex to the promoter (Tun et al., 1994, Hsieh and Hayward, 1995). EBNA2 and Notch activate the RBP-Jκ bound promoters by competing with the co-repressor complex, masking a repression domain of RBP-Jκ (Hsieh and Hayward, 1995) and assembling the positively activating transcription complex (Zhou et al., 2000a, Zhou et al., 200b). Thus, EBNA2 mimics an activated Notch pathway (Fig. 3). In spite of similarities between EBNA2 and NotchIC, there are some peculiarities in the function of EBNA2 since activated Notch was unable to completely substitute for EBNA2 either in the

induction or maintenance of B cell proliferation (Gordadze et al., 2000, Höfelmayr et al., 2001).

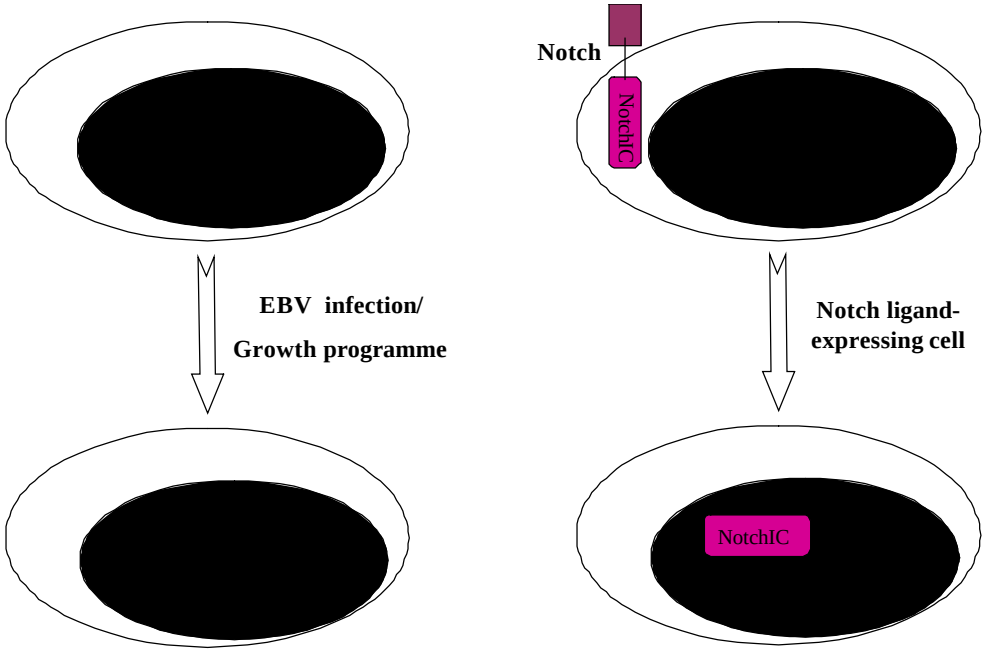


Figure 3.

Similarity of the mechanism of transcriptional activation by the EBV protein EBNA2 and the cellular protein Notch.

RBP-Jκ binds to the consensus sequence GTGGGAA and blocks the transcription by recruiting the co-repressor complex. EBNA2 and NotchIC binding displaces the co-repressor complex, thus masking the RBP-Jκ repression domain and recruiting co-activator complex to positively up-regulate transcription.

The RBP-Jκ-binding domain in EBNA2 spans amino acids 316 and 326 (Ling et al., 1993, Yalamanchili et al., 1994). This region is absolutely necessary for successful immortalisation by EBV (Cohen et al., 1991) although EBNA2 lacking this region is still able to activate the viral LMP1 promoter (Sjöblom et al., 1995, Yalamanchili 1994). Thus, the EBNA2 responsive element of the viral promoters is more complex. A PU.1 protein has been identified as an additional factor involved in transactivation by EBNA2 in the LMP1 promoter (Laux et al., 1994b, Johanssen et al., 1995). EBNA2 assembles with the transcription machinery, i.e. TFIIB, TAF40, TFIIH, p100 and RPA70 (Tong et al., 1995a, b, c) and the histone acetyltransferases p300, CBP and PCAF (Wang et al., 2000). Furthermore, it interacts with hSNF5/Ini1, a member of the family of chromatin remodelling proteins (Wu et al., 1996). A SKIP protein has been found to be required for the activation of RBP-Jκ-repressed promoters by

EBNA2 (Zhou et al., 2000b). Recently, a potential role of EBNA2 in blockage of apoptosis mediated through Nur77, a protein that induces cytochrome c release, has been suggested (Lee et al., 2002).

1.5.1 Viral target genes of EBNA2

EBNA2 is induced as one of the first viral genes upon EBV infection and it can regulate the expression of other latent viral genes. The viral promoters activated by EBNA2 have been studied intensively. The latency W promoter (Wp) is used initially after infection and the switch to the EBV latency C promoter (Cp) coincides with the appearance of transcripts for the other members of the EBNA-family: EBNA1, EBNA3A, 3B and 3C. This process is regulated by EBNA2 (Woisetschlager et al,. 1990, Rooney et al., 1992).

The viral C promoter and the promoters of two latent membrane proteins, LMP1 and LMP2, are up-regulated by EBNA2 (Wang et al., 1990a, Abbot et al., 1990, Fahraeus et al., 1990, Tsang et al., 1991, Zimber-Strobl et al., 1991, Jin and Speck, 1992, Fahraeus et al., 1993, Zimber-Strobl et al., 1993). As described above, EBNA2 binds to DNA indirectly through the cellular protein RBP-Jκ. At least one RBP-Jκ site has been mapped in each EBNA2 responsive element of these viral genes (Zimber-Strobl et al., 1993, Ling et al., 1993, Laux et al., 1994). Taken together, EBNA2 regulates the expression of all latent genes. Among them, LMP1 is a constitutively active transmembrane receptor which activates simultaneously several cellular pathways: NF-κB (Huen et al., 1995, Mitchell et al., 1995), p38MAPK (Eliopolus et al, 1999, Vockerodt et al., 2001) and JNK1/AP1 (Kieser et al., 1997, Kieser et al., 1999) and STAT (Gires et al., 1990).

1.5.2 Cellular target genes of EBNA2

So far, several cellular genes have been identified as directly regulated by EBNA2: a chemokine receptor CCR7/EBI1/BLR2 (Burgstahler et al., 1995), the cellular proto-oncogene c-myc (Kaiser et al., 1999) and a member of the AP-1/ATF super-family BATF (Johansen et al., 2003). The increase in the expression of the cell surface

molecules CD21 and CD23 by EBV is also partially attributable to the action of EBNA2 (Cordier et al., 1990). So far no RBP-Jκ binding site could be identified in the promoters of CCR7, c-myc, BATF or CD21. In contrast several RBP-Jκ sites have been identified in the promoter of CD23 (Hubmann et al., 2002). Therefore, the assumption that RBP-Jκ site in the promoter region plays an important role in the gene transactivation by EBNA2 does not hold true for all EBNA2 cellular targets. Interestingly enough, Makar et al., 1998 found that the binding of RBP-Jκ in the intron 1 region of CD21 causes gene silencing. The CD21 promoter has also been described as an NF-κB regulated promoter activated by LMP1 (Wang et al., 1990b). Thus, EBNA2 and LMP1 might synergistically regulate CD21 expression by derepression and activation. A similar mechanism was described for the CD23 gene. The induction of CD23 upon EBV infection has been attributed to the action of LMP1 (Calender et al., 1987), but the full expression in some lymphoblastoid cell lines was observed only if both EBNA2 and LMP1 were present (Wang et al., 1990b). Taken together, it seems that EBNA2 opens a cascade of transcriptional events by initiating the transcription of primary target genes. The cascade gets broader by the action of genes like LMP1 or c-myc encoding for transcriptional effectors. These transcriptional effectors then, either synergistically with EBNA2 or individually, activate numerous cellular genes which are involved in the process of B cells immortalisation.

1.5.3 Biological systems used for the identification of primary EBNA2 target genes

In order to be able to identify early events upon EBNA2 induction, dependent strictly on EBNA2, the EREB2-5 cell line has been developed by Kempkes et al., 1995a. EREB2-5 cells are a conditional system developed by the co-infection of P3HR1 EBV and mini-EBV expressing EBNA2 as a chimeric fusion protein with the hormone binding domain of the oestrogen receptor (ER/EBNA2). As EBNA2 is deleted in the P3HR1 virus, the proliferation of EREB2-5 lymphoblastoid cells is strictly oestrogen-dependent. In the absence of oestrogen about half of the cells enter a quiescent non-proliferative state whereas the others die by apoptosis. Growth arrest is reversible and

cells enter the cell cycle post-oestrogen addition due to a sequential accumulation and modification of cell cycle regulating proteins (Fig. 4, upper part).

The EREB2-5 cellular system had served to characterise the c-myc gene as a primary target gene of EBNA2 (Kaiser et al., 1999). c-myc and LMP1 are induced in the EREB2-5 cells as quickly as twenty minutes post-oestrogen stimulation so that a distinction between target genes which are induced directly through the EBNA2 activation mechanism and those which are induced indirectly through c-Myc or LMP1 is virtually impossible.

One important criterion for a primary EBNA2 target gene is its transcriptional activation in the presence of the protein synthesis inhibitors. An additional advantage of this cellular system is that ER/EBNA2 is not regulated transcriptionally. Independently of whether the protein synthesis inhibitor is added to the culture medium or not, EBNA2 is present in the cell. That allows the monitoring of only those primary events upon EBNA2 activation by oestrogen which do not require *de novo* protein synthesis. However, the protein synthesis inhibitor cycloheximide may lead to the induction and /or stabilisation of mRNA. This EBNA2-independent activity of cycloheximide very often represents an obstacle in the identification of primary target genes by this approach.

The cell lines P493-6 and BL41-K3 can be used to overcome this problem.

P493-6 cell line was described by Pajic et al., 2001. It was developed starting from the EREB2-5 cells by stably transfecting a tetracycline (Tet) regulated c-myc gene which can be switched on by the withdrawal of Tet or switched off by addition of Tet. These cells therefore can grow in two different modes: as typical LCLs driven by EBNA2 or as germinal centre-like cells with the proliferation driven by c-Myc (Fig. 4, lower part). This system makes it possible to distinguish genes induced by c-Myc in contrast to those which are induced exclusively by EBV.

BL41-K3 cells are EBV-negative Burkitt's lymphoma cells stably transfected with an ER/EBNA2 expressing vector (Kempkes et al., 1995b). Due to the translocation of c-myc to the Igμ locus in these cells these cells proliferate largely and independently of EBNA2.

EBNA2 not only induces the transcription of genes, but it also acts as a repressor of genes as it in the case of the Igμ gene (Jochner et al., 1996).The immediate repression of Igμ by EBNA2 down regulates c-Myc translocated to the Igμ locus. Therefore

BL41-K3 cells cease to proliferate and are growth suppressed soon after oestrogen is added to the culture medium.

The BL41-K3 cellular system provides the opportunity of monitoring very early events following EBNA2 activation in the absence of other viral proteins and c-myc.

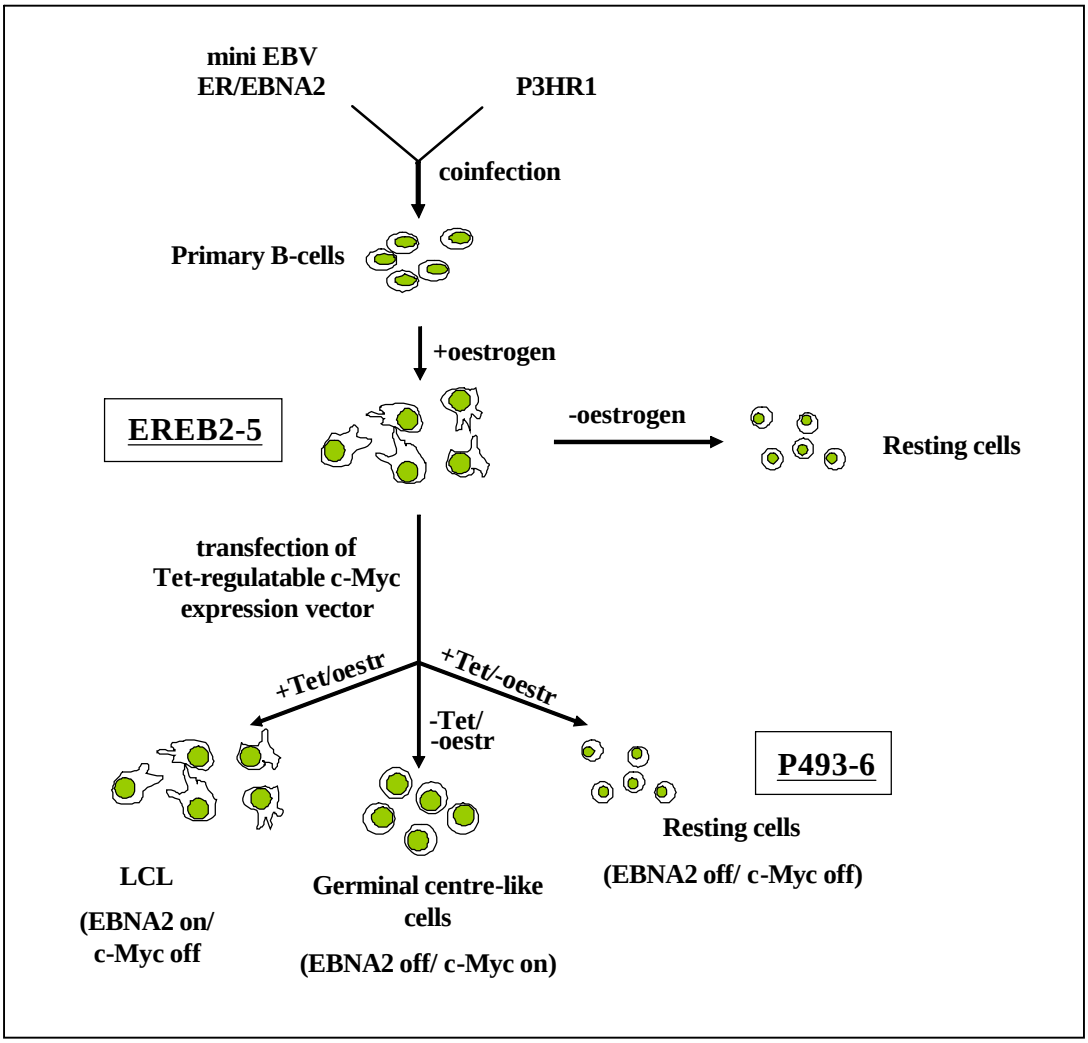


Figure 4.

Scheme of EREB2-5 and P493-6 cells.

EREB2-5 cells were generated from primary B cells co-infected with a mini-EBV carrying the oestrogen receptor domain fused to the EBNA2 gene (ER/EBNA2) and the EBNA2-lacking P3HR1 EBV strain (Kempkes et al., 1995a). ER/EBNA2 is active in the presence of oestrogen. Cells proliferate and show the phenotype of a typical lymphoblastoid cell line (LCL). In the absence of oestrogen Hsp90 binds to the oestrogen receptor domain (Jensen et al., 1972) preventing conformational changes and translocation into the nucleus. Cells do not proliferate, resembling resting B cells. Additional stable transfection of EREB2-5 cells with a vector containing tetracycline (Tet) regulated c-Myc gene yielded the cell line P493-6. These cells behave as the parental EREB2-5 LCL when growing under Tet/oestrogen conditions (Myc off/EBNA2 on). If they grow in the plain complete culture medium (Myc on/EBNA2 off) they behave like germinal-centre cells. The state of resting cells is achieved by addition of Tet to the cell culture medium (Myc off/EBNA2 off).

1.6. The goal of this project

EBNA2 is one of the first viral gene products expressed post-EBV infection. It plays an essential role in the immortalisation process by initiating and maintaining the phenotype of the immortalised B cells *in vitro.* In addition, EBNA2 expression is a characteristic feature of EBV positive B cell lymphomas, which can arise in immunocompromised patients. EBNA2 acts as a transactivator of viral and cellular genes. Primary EBNA2 target genes initiate a cascade of secondary events, which subsequently promote cell cycle entry and proliferation. The mechanism by which EBNA2 activates cellular target genes directly is complex and only partially understood.

The primary goal of the study described here was to compare these target genes in order to define *cis*-regulatory elements reflecting common signal transduction pathways shared by these genes.

The second goal of the project was identification of the target genes, which are induced specifically in those situations in which EBV drives the proliferation of B cells and subsequently to develop techniques in order to analyse the function of the corresponding gene products in the context of EBV immortalised B cells.

2.0 Results

2.1 Identification of EBNA2 target genes

2.1.1 Experimental systems for the identification of EBNA2 target genes

Recent technical and analytical advances have greatly facilitated the simultaneous analysis of expression of thousands of genes using DNA microarrays. Since it is known that the established techniques differ in sensitivity and these variations can even be RNA specific due to the different labelling techniques, three different DNA microarray techniques have been applied for the analysis of EBNA2 target genes. These were: (i) an ExpressCode™ DNA microarray developed by GPC Biotec GmbH, Germany; (ii) a DNA microarray called "lymphochip" developed by L. Staudt, National Institutes of Health, USA and (iii) a GeneChip® developed by Affymetrix Inc, USA. Basic features, similarities and diversities of each of these techniques will be presented in Fig. 6 (a more detailed description of each method is placed in the chapter *Methods*). Differences of these three methods strongly influence the results as it will be described later in this chapter.

Proliferation of the lymphoblastoid cell line EREB2-5 (described in detail in the *Introduction*, section 1.5.1) is strictly dependent on the presence of oestrogen in the culture medium. Cells deprived of oestrogen become growth arrested, but can re-enter the cell cycle after the addition of oestrogen to the medium. These cells are therefore a convenient system to study and identify early events after ER/EBNA2 activation. As described in the previous section EBNA2 activation initiates the transcription of a cascade of primary and secondary target genes. In addition, primary EBNA2 target genes might be co-regulated by the LMP1 protein. Already two hours post-oestrogen induction, LMP1 and c-myc are expressed. Thus, secondary screens are required in order to dissect EBNA2 primary and secondary induced events.

In this work the level of mRNA isolated from EREB2-5 across the three cell cycle stages has been compared: (i) for the growth arrested cells in G0/G1; (ii) for cells that

have just entered G1 phase (2 hours post-oestrogen activation of ER/EBNA2) and (iii) cycling cells proliferating at least 48 hours post-oestrogen stimulation.

2.1.2 Screen I: The analysis of results from the ExpressCode™ DNA microarray

Screen I involved the hybridization of the ExpressCode™ DNA microarrays in collaboration with GPC Biotech AG, Germany with radioactively labelled cDNA probes derived from the RNA populations of EREB2-5 cells harvested at different times after oestrogen stimulation. All hybridisations were performed in four replicates for each cDNA probe.

The ExpressCode™ filters had been spotted with 9600 PCR products, which represented the inserts of a plasmid library. This plasmid library had been generated by the accumulation of cDNAs selected from non-redundant inserts by the oligonucleotide fingerprinting mehod (Meier-Ewert et al., 1998) by GPC Biotech AG, Germany. Since these PCR products had not been mapped to single genes, an unknown fraction of the PCR products could correspond to identical uni gene clusters.

ER/EB2-5 cells were activated by the addition of oestrogen to the cell culture and samples were taken at various time points before and after S phase was initiated. Thus cells were stimulated with oestrogen for 1, 2, 3, 4, 5, 6, 8, 9, 12 and 16 hours or the cells were proliferating for at least 48 hours post-oestrogen stimulation. Clones from the cDNA library which represented spots which showed induction were sequenced in order to identify the genes they represent. Expression of known EBNA2 target genes e.g. myc, cdk4, IgM, LMP1, cyclin D2 and CCR7 was monitored and could be confirmed. Additionally, 163 genes were identified to be novel genes potentially regulated by EBNA2. Induced genes were clustered according to the expression profiles as similar to the marker genes c-myc or CCR7, which are typical primary target genes of EBNA2. Cdk4, a typical secondary target gene of EBNA2, or tubulin alpha, a gene which gradually increases following a long term kinetic, served as further model genes (Fig.5). As shown in Fig. 5A and B the two primary target genes of EBNA2, c-myc and CCR7, fall into two different groups. They differ mainly in the slope of the curve during the first 2 hours post-induction. In contrast to c-myc and CCR7, the secondary target gene cdk4 was induced with a significant delay of 3 to 4

hours (Fig. 5C). The last group of RNAs accumulated steadily over time and was considered as a group of genes, the induction of which might coincide with growth of the cell and entry into the cell cycle (Fig. 5D). Thus they are not directly activated by EBNA2 either.

A specific feature of the genes belonging to the group exhibiting profiles similar to c-myc is the very low base line expression level which upon induction, raised up to 1000 fold. The basal expression of CCR7 was higher and the increase of expression post-induction less pronounced. Since c-myc and CCR7 were both found to be induced after two hours, this two hours time point was considered to be the earliest time at which a majority of early EBNA2 target can be identified.

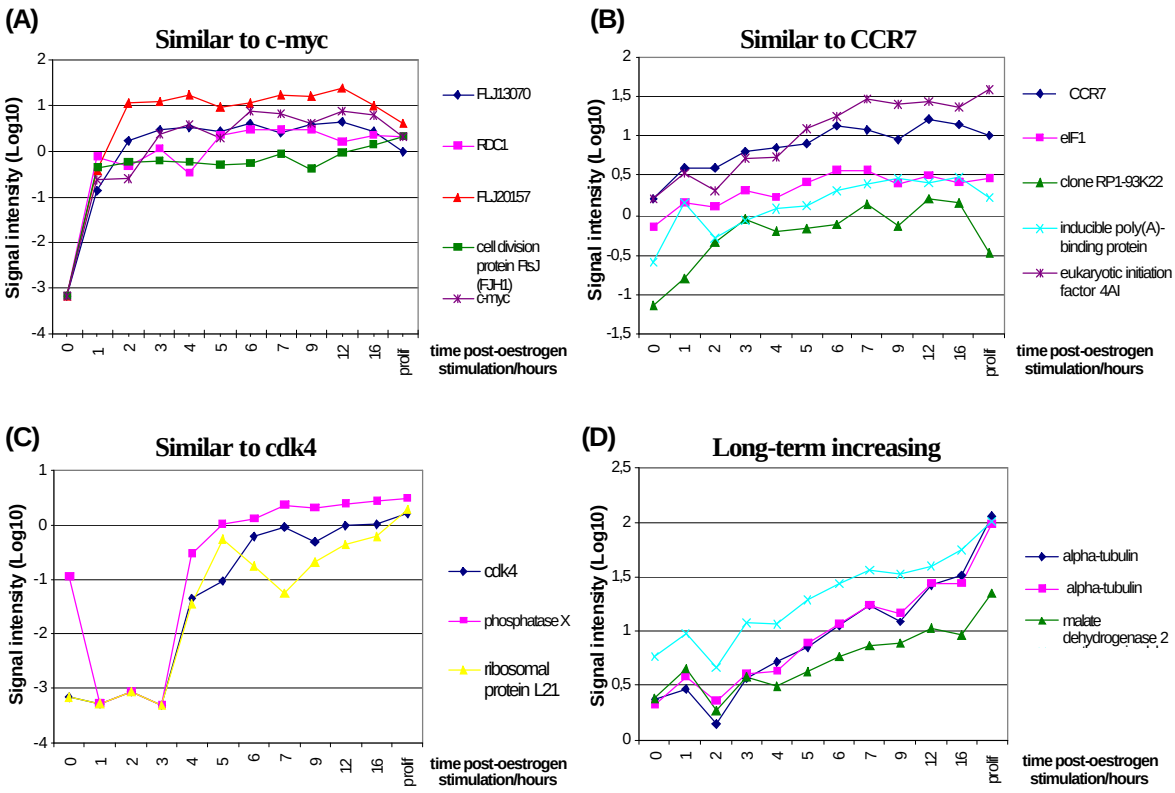


Figure 5.

The examples of expression profile clusters for genes induced on the ExpressCode™ microrray.

Genes induced in EREB2-5 cells post-oestrogen stimulation were clustered into four clusters according to the similarity of their expression profile with four marker genes: c-myc (A) or CCR7 (B), cdk4 (C) and tubulin-alpha (D). Only a few examples from each cluster are shown. The complete data can be found on a CD enclosed (“*ExpessCode raw data and profiles”*).

52 genes, induced at least twofold within first two hours post EBNA2 activation, were considered to be potential primary target genes. The list of genes and the fold induction values are presented in the section *Supplementary material*, Table 1. A further group of 128 genes was identified as potentially EBNA2 directly induced genes in proliferating EREB2-5 (the list of genes and the fold induction values are presented in the section *Supplementary material*, Table 2).

2.1.3 Screen II: The analysis of results generated by "lymphochip" technology

Lymphochip has been designed by L. Staudt (NIH, USA). Data analysed herein were obtained as a generous gift from M. Schlee (GSF, Munich), who performed the screening. Lymphochip was designed by selecting genes that are preferentially expressed in lymphoid cells and genes with known or suspected roles in processes important in immunology or cancer. The total number of genes analysed was 12,500, but most genes were represented more then once on the chip. Fluorescent cDNA probes, labelled with the Cy5 dye, were prepared from mRNA of growth arrested EREB2-5 cells and EREB2-5 cells stimulated for one, two or three hours with oestrogen. A reference cDNA probe prepared from a pool of mRNAs isolated from nine different lymphoma cell lines was labelled with Cy3 dye. Each Cy5-labelled experimental cDNA probe was combined with the Cy3-labelled reference probe and the mixture was hybridized to the microarray consisting of the PCR products synthesized on the glass substrate. The fluorescent ratio Cy5/Cy3 was quantified. The experiment was done only once and the genes which did not show increased abundance at all three time points after induction were disregarded for further analysis. There were 94 genes up-regulated twofold after two hours post-oestrogen activation. The list of genes and the fold induction values are presented in the section *Supplementary material*, Table 3.

2.1.4 Screen III: The analysis of results generated by GeneChip® technology

The GeneChip® screen was performed in collaboration with Jörg Mages (Institute of Medical Microbiology, Munich) and Reinhard Hoffmann (Max von Pettenkofer

Institute, Munich). The GeneChip® Human Genome U133 Set is comprised of two microarrays (HG-U133A and HG-U133B) containing over 1,000,000 unique oligonucleotide features covering more than 39,000 transcript variants which in turn represent more than 33,000 well-substantiated human genes. The HG-U133A array represents the majority of the known genes, while the HG-U133B contains mostly ESTs. As the final goal of this work was to study promoters of EBNA2 directly regulated genes only the analysis of the HG-U133A array representing 15 000 genes was performed. The RNAs which were analysed derived from growth arrested EREB2-5 cells (0 hr), EREB2-5 cells stimulated for 2 hours with oestrogen to induce EBNA2 activation and proliferating EREB2-5 cells. Every experiment was repeated four times and the gene which was induced 1.4-fold or more in at least two independent experiments was considered as an EBNA2 induced gene. The 1.4-fold induction was set-up as a threshold for induced vs. non-induced genes since this was the induction level measured for the c-myc gene. There were 205 genes induced after two hours. The list of genes and the fold induction values are listed in the section *Supplementary material*, Table 4. 3228 genes were induced at the stage of proliferating cells compared to growth arrested cells.

2.1.5 The overlapping gene pool identified in the ExpressCode™ DNA microarray, lymphochip and GeneChip®

The total number of genes identified two hours after activation of EBNA2 in EREB2-5 was 351 genes (52 ExpressCode™ microarray, 94 lymphochip, 205 GeneChip®). This is however a too large number of genes for further evaluation and study. Also, it is not very likely that this number of genes is indeed under direct regulation of EBNA2. In order to avoid further analysis of false positives, a subset of genes which was found by more then one technique was further evaluated.

There were only three genes, c-myc, RDC1 and CCR7, found to be induced by all three experimental systems two hours post-stimulation (Table 1). Also, there were only 14 induced genes common to the lymphochip and GeneChip® experiments (Table 2). 40 genes were found to be induced in proliferating cells in comparison to resting cells by the ExpressCode™ and GeneChip® method (Table 3). These 40 genes also comprised c-myc, RDC1 and CD83.

This was rather unexpected since most genes found by the ExpressCode™ and lymphochip screens were represented on GeneChip® U133A. Only 13 genes found on the ExpressCode™ array and 7 genes found on the lymphochip array were represented on GeneChip® U133B and thus could not be tested. In Fig. 6 the three techniques are outlined and the major results are summarised.

Table 1.

Overlapping pool of EBNA2 induced genes identified by the ExpressCode™ DNA microarray and GeneChip® at two hours post-induction.

GPC clone number	Probe set [a]	Accession number	Gene
CLONE 21B16	202431	NM_002467	c-myc
CLONE 12H13	204440	NM_004233	CD83
CLONE 11K11	212977	AI8170041	RDC1

[a] Unique Affymetrix identifier

Table 2.

Overlapping pool of EBNA2 induced genes identified by lymphochip and GeneChip® at two hours post-induction.

Lymphochip clone number	Probe set [a]	Accession number	Gene
19381, 29723, 28401, 15841,	202431	NM_002467	c-myc
24762	201694	NM_001964	EGR-1
27686, 17185	209457, 204794	NM_004418	DUSP2
17066, 27545, 16610	204440	NM_004233	CD83
26475	205114	NM_002983	MIP-1 alpha
28377, 16671	212240	M61906	PI3K
16089, 28639	205681	NM_004049	bfl-1
26409	204103	NM_002984	MIP-1 beta
19277, 28049	206181	NM_003037	SLAM
27463	204533	NM_001565	IP-10
27536	221576	BC000529	PLAB
27970	214567	NM_003175	Lymphotactin
15889	212977	AI8170041	RDC1
28383, 17364, 25237, 35090, 27987	205544	NM_001877	CD21

[a] Unique Affymetrix identifier

Table 3.

Overlapping pool of EBNA2 induced genes in the ExpressCode™ DNA microarray and GeneChip® at the stage of cells proliferating for at least 48 hours in the presence of oestrogen.

GPC clone	Probe Set[a]	Accession number	Gene
CLONE-11A7	217932	BC000241	30S ribosomal protein S7 homolog
CLONE-11E6	217932	BC000241	30S ribosomal protein S7 homolog, clone MGC:711
CLONE-11K11	212977	HSU67784	orphan G protein-coupled receptor (RDC1)
CLONE-12A22	202021;211956; 212130; 212225	NM_005801	translation initiation factor 1(eIF1(A121/SUI1))
CLONE-12B24	218356; 222130	NM_013393	cell division protein FtsJ (FJH1)
CLONE-12E17	201157;201158; 201159	NM_021079	N-myristoyltransferase 1 (NMT1)
CLONE-12H13	204440	XM_004500	CD83, mRNA
CLONE-12K22	209118; 212639; 213646; 211058;201090; 211750	AF141347	alpha-tubulin
CLONE-12M13	220587	AF195883	G protein beta subunit
CLONE-12N2	208420; 208830; 208831	NM_003170	SUPT6H
CLONE-13L11	209118; 212639; 213646; 211058;201090; 211750	AF141347	alpha-tubulin
CLONE-14K13	209036; 213333	NM_005918	malate dehydrogenase 2, NAD (mitochondrial) (MDH2)
CLONE-15K19	200830	HSU12596	TNF type 1 receptor assoc.pr.(TRAP2)
CLONE-15K5	216232; 212139	HSU88836	clone 738, translational activator GCN1
CLONE-15L16	200772; 200773; 211921	NM_002823	prothymosin alpha
CLONE-15N14	200658;200659	NM_002634	prohibitin (PHB)
CLONE-16E18	202471; 214333	HSU40272	NAD+-specific isocitrate dehydrogenase gamma subunit
CLONE-16F3	201530; 211787	NM_001416	eukayotic translation initiation factor 4A (EIF4A1)
CLONE-16I9	201625; 201626; 201627	NM_005542	insulin induced gene 1 (INSIG1)
CLONE-16M14	204328; 214958	NM_007267	expressed in activated T/LAK lymphocytes (LAK-4P)
CLONE-17D12	200772; 200773; 211921	NM_002823	prothymosin alpha
CLONE-17G5	200772; 200773; 211921	NM_002823	prothymosin alpha
CLONE-17J22	201531	HUMGOS24B	GOS24
CLONE-18L6	212223; 212221	HSM801060	cDNA DKFZp434G012
CLONE-20G22	213113; 210692	HSM802470	cDNA DKFZp762A227
CLONE-20L19	212181	AF191654	NUDT4
CLONE-20L2	200877	XM_002285	chaperonin containing TCP1, subunit 4
CLONE-21B16	202431	HSMYCC	c-myc
CLONE-21F17	204192	HSCD37	CD37
CLONE-21N4	201516	NM_003132	spermidine synthetase
CLONE-3D16	208420; 208830; 208831	HSU46691	putative chromatin structure regulator (SUPT6H)
CLONE-3F23	200783; 217714	X53305,	p18 (stathmin)
CLONE-3K5	214095; 214096; 214437	NM_005412	serine hydroxymethyltransferase 2
CLONE-3L14	201586; 201585	X70944	PTB-associated splicing factor
CLONE-4C16	218130	HSU66243	p38gamma MAP Kinase mRNA
CLONE-4N23	206303; 206302; 212181; 212182, 212183	NM_019094	NUDT4
CLONE-5A5	204999	AF305687	transcription factor ATFx
CLONE-6B15	203470; 203471	NM_002664	pleckstrin (PLEK)
CLONE-8H4	204998; 204999; 217389	NM_012068	ATF5
CLONE-9J10	209953	HSU43077	CDC37

[a] Unique Affymetrix identifier.

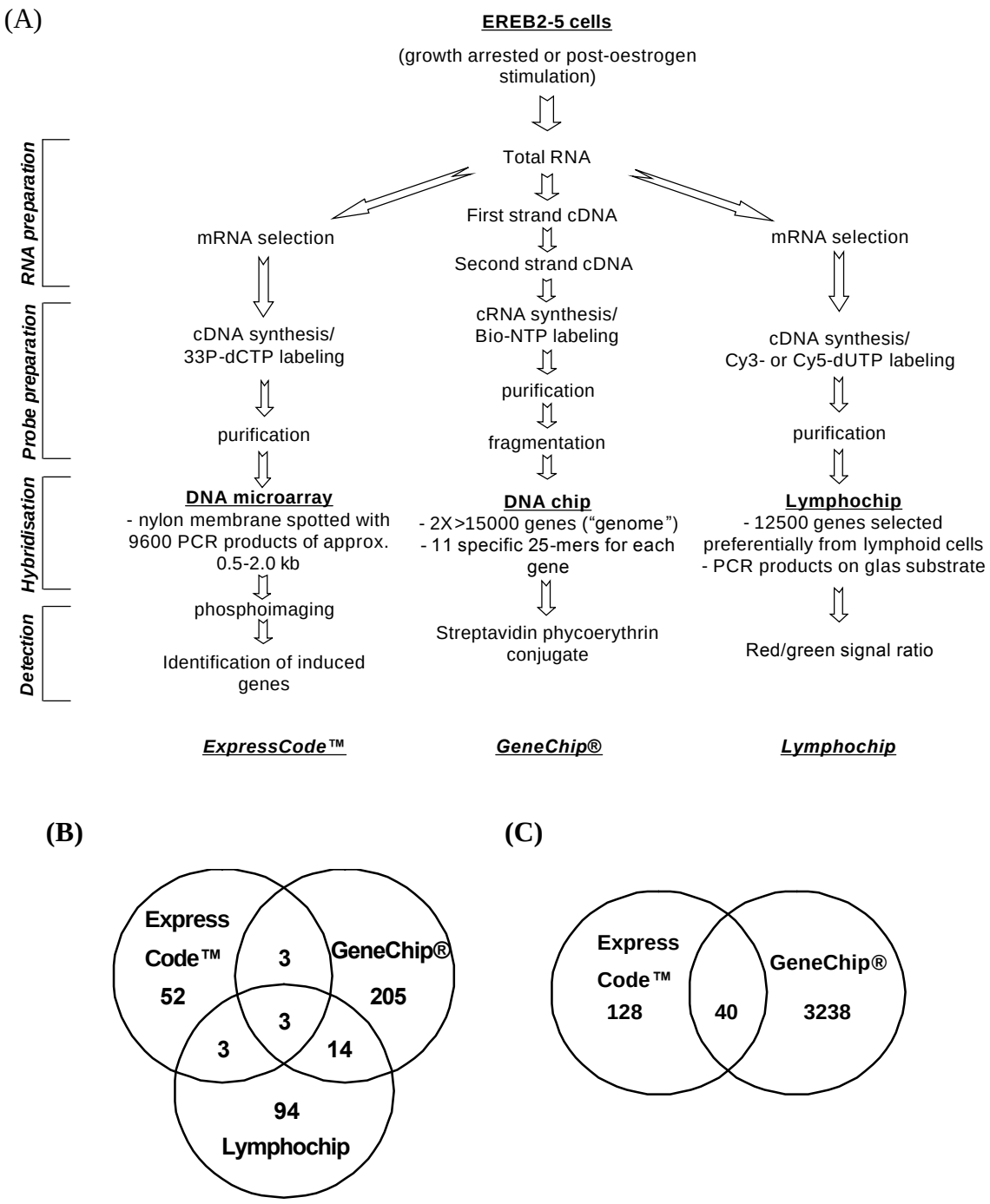


Figure 6.

Scheme of three different DNA microarray methods.

(A) Basic features of the DNA microarray methods presented in order to point out the fundamental differences between these methods. (B) Overlapping pool of EBNA2 induced genes in EREB2-5 cells identified by the ExpressCode™ DNA microarray, lymphochip and GeneChip® at two hours post-induction. (C) Overlapping pool of EBNA2 induced genes identified by the ExpressCode™ DNA microarray and GeneChip® in the proliferating EREB2-5 cells. (see text for details)

On a single gene level the results for given gene varied significantly. On the ExpressCode™ DNA microarray the abundance of the c-myc mRNA shows a very strong increase (109.3 fold). On the lymphochip the increase is already 10-fold lower than on the ExpressCode™ microarray and ranges from 7.4 to 10.4 for different clones on the array. The GeneChip® exhibits on the other hand a quite low increase of mRNA with only 1.4, 2.2, 2.5 or 5.0-fold. It is noteworthy to say that prior each experiment the quality of mRNA was controlled and verified by the Northern blot analysis for the c-myc gene. The induction observed in Northern blot explicitly indicates a fast and strong induction of c-myc in EREB2-5 at the given time points. Thus, the differential outcome of the individual experiments cannot be explained by varying RNA qualities, but it rather reflects the differential sensitivities of the different techniques.

2.1.6 Screen IV: Evaluation of the potential EBNA2 target genes by the nuclear run-on experiments

EBNA2 acts as a transcriptional activator. The nuclear run-on experiment permits a direct analysis of transcriptional activity for a given gene. The basic mechanism of this technique is the labelling and measuring of a nascent RNA associated with the transcription complex. Therefore, the increase or decrease in the transcription rate of the gene of a particular interest can be measured.

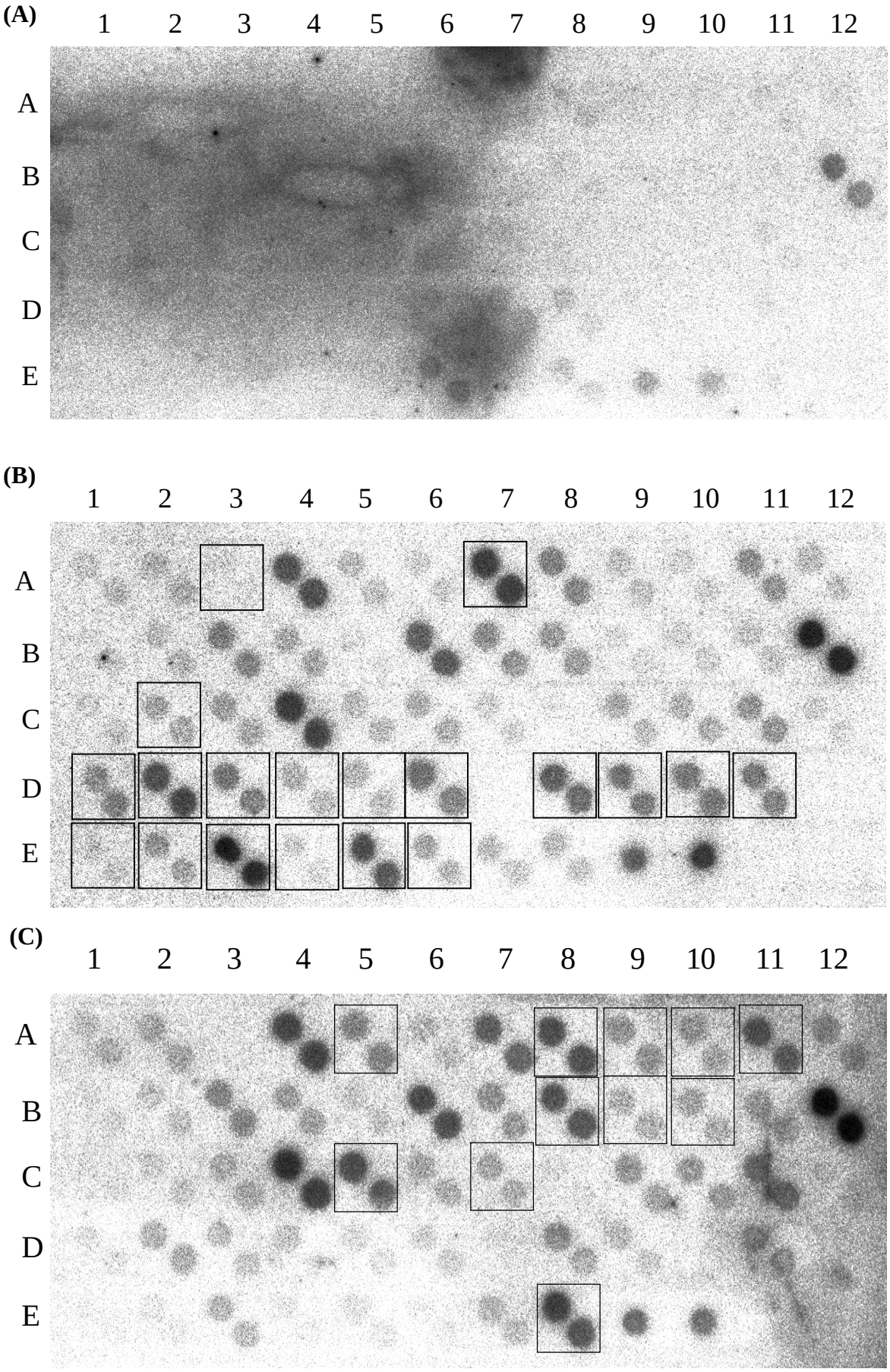
Here the nuclear run-on experiment was used to discriminate genes induced by the c-myc growth programme as opposed to the EBV/EBNA2 growth programme. For that purpose P493-6 cells were used. The P493-6 cell line is a B cell line expressing a conditional c-myc gene (described in more detail in *Introduction*, chapter 1.5.1). In the presence of tetracycline, c-myc is switched off and cells are growth arrested in the G0/G1 phase of the cell cycle. c-Myc can be re-induced by removal of tetracycline which induces the cell cycle entry. These cells can also behave like the parental EREB2-5 cells in the presence of tetracycline and the addition/deprivation of oestrogen.

We compared the run-on profile of nuclei from the P493-6 cells induced for 8 hours with oestrogen and tetracycline (cell proliferation driven by EBNA2) and of nuclei from the P493-6 cells depleted from tetracycline and grown in the plain complete

medium for 8 hours (cell proliferation driven by myc). The purpose of this experiment was to identify the genes which are preferentially activated in either the c-myc or the EBNA2/EBV driven growth programme. Nuclear run-on has the advantage of monitoring the transcriptional activity in the contrast to previous experiments where the steady state RNA levels were observed. Run-on RNAs were labelled with ^{32}P-CTP and hybridised to "self-made" DNA arrays. A "self-made" DNA array was generated by spotting PCR products. 14 genes induced in both lymphochip and GeneChip® at 2 hours post-stimulation with oestrogen plus 40 genes induced in the proliferating cells analysed with the ExpressCode™ microarray and GeneChip® were represented on the "self-made" DNA array. The genes LMP1, LMP2A, GAPDH and IgM were included as positive controls. The positions of the genes are described in Table 4. Signals were detected and quantified after 24 hours by phosphoimaging (Fig. 7).

Table 4.
The list and positions of probes in "self-made" DNA arrays used for nuclear run-on.

Row	Column	Gene	Accesion number
A	1	30S ribosomal protein S7 homolog	BC000241
	2	30S ribosomal protein S7 homolog	BC000241
	3	RDC1	HSU67784
	4	eIF1	NM_005801
	5	cell division protein FtsJ	NM_013393
	6	N-myristoyltransferase 1 (NMT1)	NM_021079
	7	CD83	XM_004500
	8	alpha-tubulin	AF141347
	9	G protein beta subunit	AF195883
	10	SUPT6H	NM_003170
	11	alpha-tubulin	AF141347
	12	MDH2	NM_005918
B	1	TRAP2	HSU12596
	2	GCN1	HSU88836
	3	prothymosin alpha	NM_002823
	4	prohibitin (PHB)	NM_002634
	5	isocitrate dehydrogenase	HSU40272
	6	EIF4A1	NM_001416
	7	INSIG1	NM_005542
	8	LAK-4P	NM_007267
	9	prothymosin alpha	NM_002823
	10	prothymosin alpha	NM_002823
	11	GOS24	HUMGOS24B
	12	DKFZp434G012	HSM801060
C	1	DKFZp762A227	HSM802470
	2	NUDT4	NM_019094
	3	TCP1	XM_002285
	4	c-myc	HSMYCC
	5	CD37	HSCD37
	6	spermidine synthetase	NM_003132
	7	SUPT6H	NM_003170
	8	p18 (stathmin)	X53305,
	9	serine hydroxymethyltransferase 2	NM_005412
	10	PTB-associated splicing factor	X70944
	11	p38gamma MAPK	HSU66243
	12	NUDT4	NM_019094
D	1	ATFx	AF305687
	2	PLEK	NM_002664
	3	ATF5	NM_012068
	4	CDC37	HSU43077
	5	MIP-1 alpha	NM_002983
	6	SLAM	NM_003037
	7	PI3K	M61906
	8	DUSP2	NM_004418
	9	Bfl-1	NM_004049
	10	MIP-1 beta	NM_002984
	11	EGR-1	NM_001964
	12	PLAB	BC000529
E	1	IP-10	NM_001565
	2	lymphotactin	NM_003175
	3	CD21	NM_001877
	4	LMP1	V001555
	5	LMP2A	V001555
	6	CCR7	NM_001838
	7	GAPDH	BE561479
	8	IgM	BC001872
	9/10	EBNA2	V001555



(Figure 7. continues)

(D)

Gene	Experiment 1				Experiment 2			
	Arrested cells	EBV growth	c-Myc growth	EBV / c-myc	Arrested cells	EBV growth	c-Myc growth	EBV / c-myc
RDC1	1	73,3	16,6	4,4	1	12	8	1,5
CD83	1	2210	944,8	2,3	1	127,4	60,2	2,1
NUDT4	1	163,6	6,4	25,6	1	272	201	1,4
ATFx	1	202,5	18,8	10,8	1	76,8	83	0,9
PLEK	1	575,8	13,3	43,3	1	126,1	85,9	1,5
ATF5	1	826	151	5,5	1	43	43,1	1
CDC37	1	197,8	55,8	3,5	1	152	46,8	3,2
MIP-1 α	1	114	29,1	3,9	1	176,5	77	2,3
SLAM	1	638	82	7,8	1	13,4	2	6,7
DUSP2	1	57,7	26,9	2,1	1	156	31	5
Bfl-1	1	101	6,9	14,6	1	717	107	6,7
MIP-1 β	1	406,3	16,7	24,3	1	823	101	8,1
EGR-1	1	437,4	165,8	2,6	1	248,3	171,7	1,4
PLAB	1	176,4	31,1	5,7	1	397	295	1,3
IP-10	1	262,7	56,3	4,7	1	183	204	0,9
CD21	1	794,7	29,7	26,8	1	461,5	148,5	3,1
LMP1	1	12,5	4,2	3	1	442,5	156,5	2,8
LMP2A	1	208,5	13,5	15,4	1	995	239	4,2
CCR7	1	5,3	2,3	2,3	1	150,5	71,5	2,1

(E)

Gene	Experiment 1				Experiment 2			
	Arrested cells	EBV growth	c-Myc growth	c-Myc/ EBV	Arrested cells	EBV growth	c-Myc growth	c-Myc/ EBV
Ftsj	1	115,7	203,4	1,8	1	137,3	141,8	1,0
tubulin	1	21,8	87,7	4,0	1	37,4	59	1,6
G protein	1	91,3	641	7,0	1	5,7	137,7	24,2
SUPT6H	1	13,2	489,3	37,1	1	6	156,5	26,1
tubulin	1	27,1	172,6	6,4	1	27,3	64,5	2,4
LAK-4P	1	108	666,8	6,2	1	50,2	59	1,2
prothymosin	1	6,1	77,4	12,7	1	13	117,1	9,0
prothymosin	1	21,3	291,8	13,7	1	12,1	114,3	9,4
CD37	1	59,6	1045,9	17,5	1	90,5	114,5	1,3
SUPT6H	1	35,7	141,8	4,0	1	30,3	55,8	1,8

(Figure 7. continues)

(F)

Gene	Experiment 1				Experiment 2			
	Arrested cells	EBV growth	c-Myc growth	EBV/ c-Myc	Arrested cells	EBV growth	c-Myc growth	EBV/ c-Myc
30S ribosomal pr	1	447,5	511	0,9	1	30,8	29,5	1
30S ribosomal pr	1	779,5	822,5	0,9	1	42	47,5	0,9
eIF1	1	1631,6	2168,2	0,8	1	58,3	74,9	0,8
NMT1	1	121,3	131,9	0,9	1	2,3	2	1,2
MDH2	1	595	894,8	0,7	1	3,6	4,2	0,9
TRAP2	1	175,6	147,6	1,2	1	16	14	1,1
GCN1	1	514,5	458,5	1,1	1	15	15,5	1
prothymosin	1	851,3	823	1	1	9	7,3	1,2
prohibitin	1	339,3	476	0,7	1	32,5	19,5	1,7
isocitrate DH	1	36,1	40,8	0,9	1	2,3	2,3	1
eIF4A1	1	1087,8	1974,8	0,6	1	59,4	62,6	0,9
INSIG1	1	278	348	0,8	1	37,5	34,5	1,1
GOS24	1	249	196	1,3	1	4,6	4	1,2
DKFZp434G012	1	15,8	17,1	0,9	1	34,6	29,1	1,2
DKFZp762A227	1	27,3	22,7	1,2	1	30	26	1,2
TCP1	1	207,2	249,3	0,8	1	49	56	0,9
c-myc	1	2226,8	3753,3	0,6	1	167,3	140,3	1,2
spermidine synt.	1	192,2	198,2	1	1	36,3	26,3	1,4
p18	1	35,7	141,8	0,3	1	30,3	30,8	1
Ser HMT2	1	11	10	1,1	1	8,4	7,6	1,1
PTB	1	171	223,3	0,8	1	36	34,5	1
p38 MAPK	1	184,1	232,5	0,8	1	56	64	0,9
NUDT4	1	30	34,5	0,9	1	0,7	0,6	1,2
PI3K	1	2,7	1,9	1,4	1	0,3	0,3	1
lymphotactin	1	41,9	31,4	1,3	1	1	1	1
GAPDH	1	13,7	16,6	0,8	1	13,1	14,7	0,9

Figure 7.

Nuclear run-on expression of potential EBNA2 target genes in P493-6 cells.

P493-6 cells were growth arrested (A) or grown in either EBV/EBNA2 (B) or myc (C) growth programme for 8 hrs. Run-on RNAs of isolated nuclei were labelled with ^{32}P-CTP and subsequently hybridised to "self-made" DNA arrays (each spot contained 400 ng of PCR product and was diagonally spotted twice, genes and positions are listed in Table 4). Phosphoimages of the membranes after 24 hrs of exposure are presented. Genes which were differentially expressed on the blot hybridised with nuclear run-on RNA from EBNA2 on/myc off state and EBNA2 off/myc on state are squared on the blots (B) and (C), respectively. Signals were quantified and the phosphoimage values of two experiments are shown as fold induction of two growth programs compared to an un-induced state and as the ratio of induction between two programmes. (D), (E) and (F) summarise respectively genes induced predominantly induced in EBV/EBNA2 growth programme, genes predominantly induced in the c-Myc growth programme and genes which were induced approximately at the same level in both growth programmes.

Table 5.
EBV induced genes identified by nuclear run-on.

position	gene
A3	RDC1
A7	CD83
C2	NUDT4
D1	ATFx*
D2	PLEK
D3	ATF5*
D4	CDC37
D5	MIP-1 alpha
D6	SLAM
D8	DUSP2
D9	bfl-1
D10	MIP-1 beta
D11	EGR-1
E1	IP-10
E2	PLAB
E3	CD21
E4	LMP1
E5	LMP2A
E6	CCR7

Table 6.
c-Myc induced genes identified by nuclear run-on.

position	Gene
A5	Ftsj
A8	Alpha tubulin
A9	G protein beta subunit
A10	SUPTH6
A11	Alpha tubulin
B8	LAK-4P
B9	Prothymosin alpha
B10	Prothymosin alpha
C5	CD37
C7	SUPTH6
E8	IgM

The control genes spotted onto membrane were LMP1 and LMP2A (viral EBNA2 target genes), c-myc and CCR7 (cellular EBNA2 target genes), IgM (a gene repressed by EBNA2) and GAPDH (a house keeping gene). All control genes show at least in one experiment to be induced more than twofold (LMP1, LMP2A, c-myc, CCR7), repressed (IgM) or without change in expression (GAPDH) by the EBV/EBNA2 growth programme.

Any gene induced at least twofold in one programme compared to the other programme was considered to be predominantly expressed by that programme. Under these conditions, the nuclear run-on analysis revealed a list of 19 genes (Figure 7. and Table 5) with increased transcriptional rates for an EBV/EBNA2 growth programme. Two of those genes are control viral genes LMP1 and LMP2A. Eleven genes (RDC1, CD83, MIP-1 alpha, SLAM, DUSP2, Bfl-1, MIP-1 beta, EGR-1, PLAB, IP-10 and CD21) originated from the comparison between the lymphochip and the GeneChip® at 2 hours post-induction. Further five genes (NUDT4, ATFX/ATF5*, PLEK, CDC37

*ATFx and ATF5 might represent the same gene since the sequences in the data bank partially overlap.

and CCR7) came from the comparison of gene induction in the proliferating cells on the ExpressCode™ DNA microarray and GeneChip®. All genes show no inconsistencies in two experiments except NUDT4. NUDT4 was spotted twice on the DNA array, each time from the different clones. Nuclear run-on showed that one clone was induced in one experiment, but not in another. The second clone does not show induction. However, as the criterion for gene to be considered as induced in one of the programmes was to be induced more than twofold in at least one experiment, NUDT4 was analysed further. In the c-Myc growth programme eight genes have been identified as potential c-Myc target genes (Figure 7. and Table 6): cell division protein FtsJ, alpha tubulin, G-protein, SUPTH6, LAK-4P, prothymosin alpha, CD37 and IgM. Of those genes, prothymosin alpha has been already identified as putative c-Myc target gene (Deng, 1999). As expected, the c-myc gene signal was strongly increased in both growth programmes, since c-myc is a primary EBNA2 target gene and it is also highly expressed in cells grown in the absence of Tet (c-Myc growth programme).

Finally, 42 % of the genes on the membrane (21 genes out of 50) were induced by both growth programmes to the same intensity. 10 % of genes on the membrane (5 genes out of 50) were not detectable.

2.1.7 Evaluation of the potential target genes by the Northern blot analysis

Candidate EBNA2 target genes were further evaluated by the Northern blot analysis in order to verify induction as well as to discriminate potential pathways causing induction. For these analyses only a subselection of target genes was chosen which scored by the following two criteria: (i) induced by EBNA2 within 2 hours in at least two independent screens and (ii) induced by the EBV/EBNA2 growth programme in the nuclear run-on analysis. The change of mRNA abundance was monitored in three different cell lines (described in *Introduction,* chapter 1.5.1, and earlier in *Results*): EREB2-5, P493-6 and BL41-K3.

The total RNA was isolated from the EREB2-5 cell at time points 0 and 6 hours post-oestrogen stimulation. Furthermore, the protein synthesis inhibitor cycloheximide was added to the cell culture medium of EREB2-5 cells in order to distinguish EBNA2

directly regulated target genes from those indirectly regulated by EBNA2. Cycloheximide was added to the medium with or without oestrogen and cells were harvested 6 hours later. Very often, however, cycloheximide addition leads to induction or stabilisation of mRNA for some genes and it is not possible to say whether they are induced in the absence of *de novo* protein synthesis or they require additional stimulus. Therefore two additional cell lines were included. The P493-6 cell line can be grown in two diverse modes: the c-Myc growth programme or the EBV/EBNA2 growth programme. Cells were grown in one of the growing conditions and 8 hours later cells were harvested and the total RNA was isolated.

BL41-K3 is an EBV-negative Burkitt's lymphoma cell line ectopically expressing ER/EBNA2. Thus, in this cell line the function of EBNA2 can be studied in the absence of other viral proteins. However, a special feature of the cell line BL41-K3 is the fact that c-myc is down regulated by ER/EBNA2. Thus, c-myc target genes will be down regulated by EBNA2 under these specific conditions.

Northern blot analysis revealed a classification of genes into five distinct groups according to the pattern of expression (Table 7): group I - genes with induction directly regulated by EBNA2 in the absence of *de novo* protein synthesis; group II - genes potentially regulated directly by EBNA2 in cooperation with another cellular or viral protein; group III - genes induced by EBNA2, but which are induced by cycloheximide as well, group IV - genes induced by EBNA2/EBV, but are not induced in the absence of other viral proteins and group V - c-Myc induced genes. All results will be described in the following sections.

Table 7.

EBNA 2 induced genes classified into five distinct groups according to the expression pattern in EREB2-5, P493-6 and BL41-K3 cells.

The intensity of the signals is presented with +++ for very strong signal to + as moderate signal. Very low signal was denoted as +/-, and no signal with -.

		EREB2 -5				P493 -6			BL41 -K3	
	Gene	Ø	oestr	CHX	oestr/CHX	Ø	oestr	tet	Ø	oestr
Group I	c-myc	-	+++	+	+++	-	++	+++	+	-
Group II	RDC1	-	+++	+	+++	-	-	-	-	++
	CCR7	-	+++	+	+++	-	++	-	-/+	++
Group III	bfl -1	-	+++	-	+	-	+++	-	-	-/+
	MIP -1 beta	-	+	-	-/+	-	++	-	-	+
	CD21	-	+++	++	++	-	+++	-/+	-/+	+++
	CD83	-	+++	++	++	-	+++	-/+	-/+	+++
	PLEK	-	++	++	++	-	+++	+	+	+++
	EGR -1	-	++	++	++	-	++	-	-	-
Group IV	DUSP2	-	+++	++	++	-	+++	-	-	-
	SLAM	-	++	+	+	-	+++	-	-	-
	lymphotactin	-	+++	-	-/+	-	-	-	-	-
	IP-10	-	++	++	++	-	++	-/+	+	+
	MIP -1 alpha	-	+	-	-/+	-	+++	-	+	+
	PLAB	-	+	+	+	-	++	-	+	+
Group V	PI3K	-	+	+/-	++	-	+	-	+	+
	ATF5	-	++	++	++	-	++	-	+	+
	NUDT4	-	++	+	+	-	+++	+	++	+
	CDC37	-	+++	++	++	-	+++	++	++	+

2.1.7.1 Group I: EBNA2 direct target genes

Northern blot analysis of the genes c-myc, RDC1 and CCR7 (Fig. 8) revealed that they are primary target genes of EBNA2. They are induced in EREB2-5 cells upon activation of EBNA2 by oestrogen (Fig. 8A). A low level of induction was demonstrated for these genes in cells treated with cycloheximide only, but when the cells were stimulated with oestrogen in the presence of the protein synthesis inhibitor cycloheximide a significant super-induction was observed. That firmly indicates that EBNA2 *per se* is enough to induce the transcription of this gene. Furthermore, RDC1 and CCR7 are also induced in the BL41-K3 cells in the absence of viral proteins (Fig.8 C). The CCR7 gene is clearly induced by EBNA2 in the P493-6 cells as well (Fig 8B). In contrast, RDC1, although induced in EREB2-5 by EBNA2, it is not induced in P493-6. This result cannot be explained as P493-6 should behave essentially like EREB2-5 cells when growing in the EBV/EBNA2 programme.

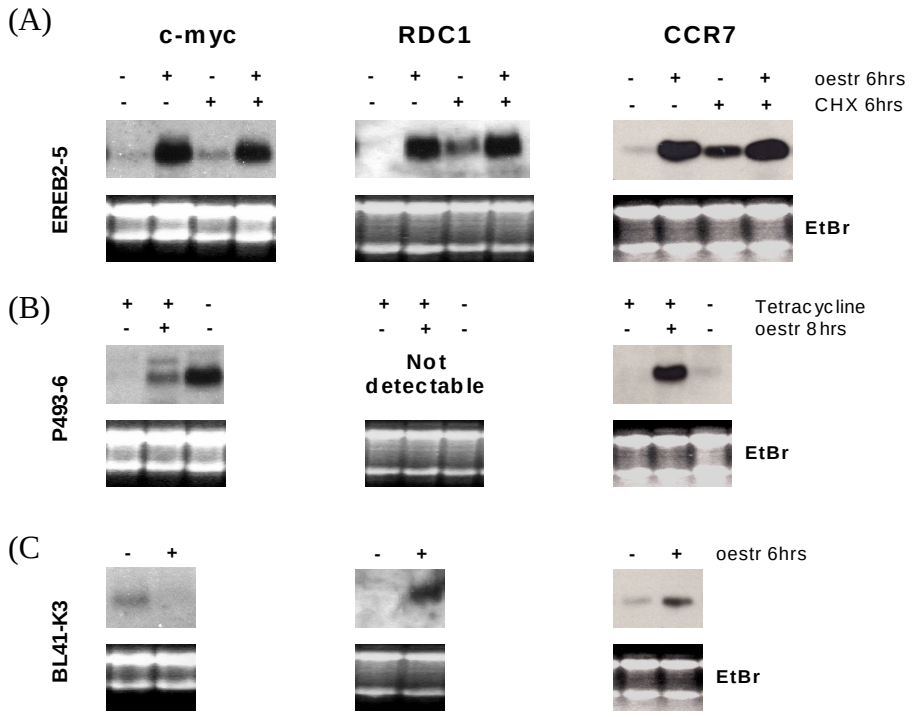


Figure 8.

EBNA2 direct target genes.

The EREB2-5 (A), P493-6 (B) and BL41-K3 (C) cells were grown under conditions and harvested at time points as indicated. Total RNA was isolated and 10 µg were loaded on an 1%-denaturing agarose gel and electrophoresis was performed. RNA was blotted to the nylon membrane overnight and subsequently the membrane was hybridised with DIG-labelled PCR probes for c-myc, RDC1 and CCR7. Ethidium bromide staining of rRNA is shown as a loading control for each lane.

2.1.7.2 Group II: EBNA2 directly regulated genes which require an additional action of cellular or viral genes for induction

The genes bfl-1 and MIP-1 beta were classified as potential EBNA2 direct target genes. As shown in Fig. 9A they are induced in the EREB2-5 cell by EBNA2, are not induced with cycloheximide only, but are induced, although weakly, by oestrogen in the presence of cycloheximide. Also in BL41-K3 (Fig. 9C) EBNA2 per se is enough to up-regulate those genes, although never to the same amount as in EREB2-5 cells, indicating that EBNA2 *per se* is sufficient for weak induction, but some other cellular or viral protein, which is missing in BL41-K3, is required for a strong induction observed in EREB2-5. However this protein is not c-Myc since myc induction in P493-6 (Fig. 9B) does not correlate with bfl-1 or MIP-1 beta gene induction.

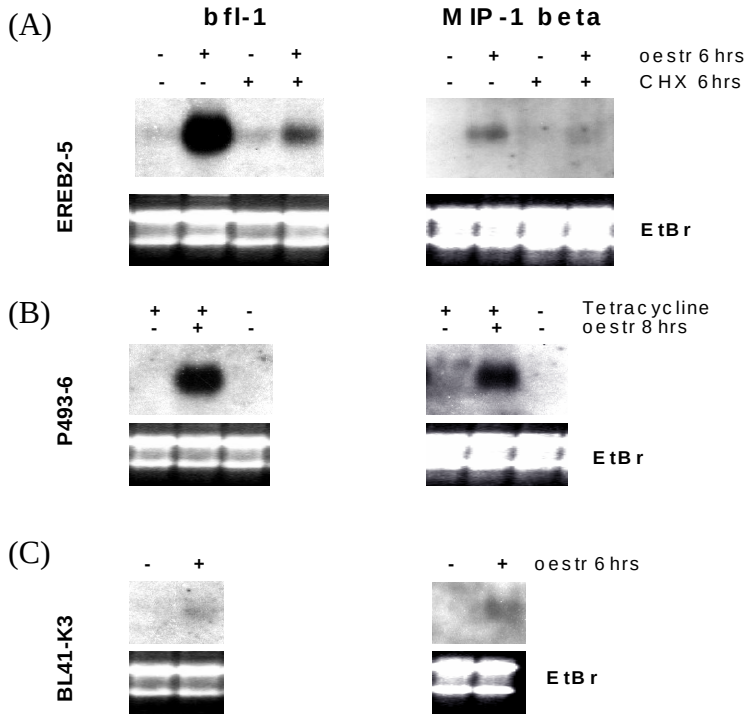


Figure 9.

Potential EBNA2 direct target genes.

The EREB2-5 (A), P493-6 (B) and BL41-K3 (C) cells were grown under conditions and harvested at time points as indicated. Total RNA was isolated and 10 µg were loaded on an 1%-denaturing agarose gel and electrophoresis was performed. RNA was transferred to the nylon membrane and subsequently the membrane was hybridised with DIG-labelled PCR probes for Bfl-1 and MIP-1 beta. Ethidium bromide staining of rRNA is shown as a loading control.

2.1.7.3 Group III: EBNA2 induced genes which are also induced by the protein synthesis inhibitor cycloheximide

CD21, CD83 and PLEK are strongly induced by EBNA2, but the induction in the presence of the protein synthesis inhibitor is approximately at the same level regardless whether EBNA2 is activated or not (Fig. 10A). Therefore, the direct effect of EBNA2 cannot be studied easily and it is not clear whether EBNA2 induces these genes directly or through some indirect mechanism. The induction in the P493-6 cells (Fig. 10B) shows that c-Myc can, induce a very low amount of mRNA of these genes. However, if c-Myc would be the key factor for induction of any of these genes, their mRNA would be down-regulated in BL41-K3 cells due to the down-regulation of c-myc in these cells, but instead, they are strongly up-regulated by EBNA2, indicating that these genes can be expressed in the absence of c-Myc and do not require viral proteins for induction (Fig. 10C). This suggests that CD21, CD83 and PLEK might be direct target genes and their mRNA is induced or stabilised in the presence of cycloheximide.

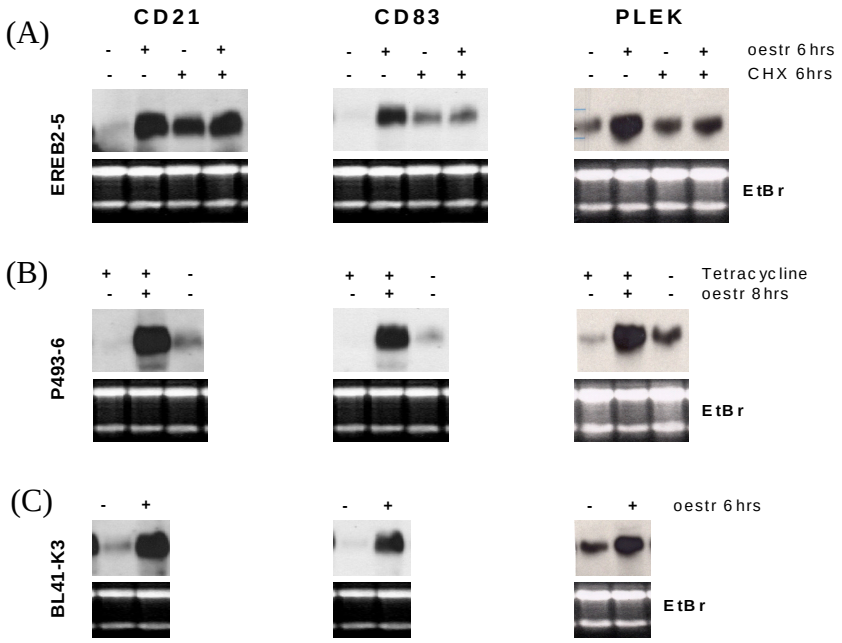


Figure 10.

Genes induced by both EBNA2 and cycloheximide.

The EREB2-5 (A), P493-6 (B) and BL41-K3 (C) cells were grown under conditions and harvested at time points as indicated. Total RNA was isolated and 10 µg were loaded on an 1%-denaturing agarose gel and electrophoresis was performed. RNA was blotted to the nylon membrane overnight and subsequently the membrane was hybridised with DIG-labelled PCR probes for CD21, CD83 and PLEK. Ethidium bromide staining of rRNA is shown as a loading control.

2.1.7.4 Group IV: genes which require viral proteins in addition to EBNA2 for induction

The following nine genes EGR-1, DUSP2, SLAM, lymphotactin, IP-10, MIP-1 alpha, PLAB, PI3K and ATF5 were induced by EBNA2 in EREB2-5 cells. In some cases cycloheximide induced these genes to varying levels (Fig. 11A). None of these genes was induced by c-Myc in P493-6 (Fig. 11 B), but all were induced by EBNA2. Lymphotactin is not expressed in P493-6 cells grown in the EBV/EBNA2 growth programme which should be cellular equivalent of EREB2-5 cells. Expression in BL41-K3 (Fig. 11C) was either not detectable at all, like for EGR-1, DUSP2, SLAM and lymphotactin, or there was no up-regulation by EBNA2, as in the case of IP-10, MIP-1 alpha, PLAB, PI3K and ATF5. This finding indicates that viral proteins play a crucial role in the up-regulation of these genes, and EBNA2 is not directly responsible for their up-regulation. Therefore, these genes were excluded from the following promoter analysis which focussed on potential primary EBNA2 target genes.

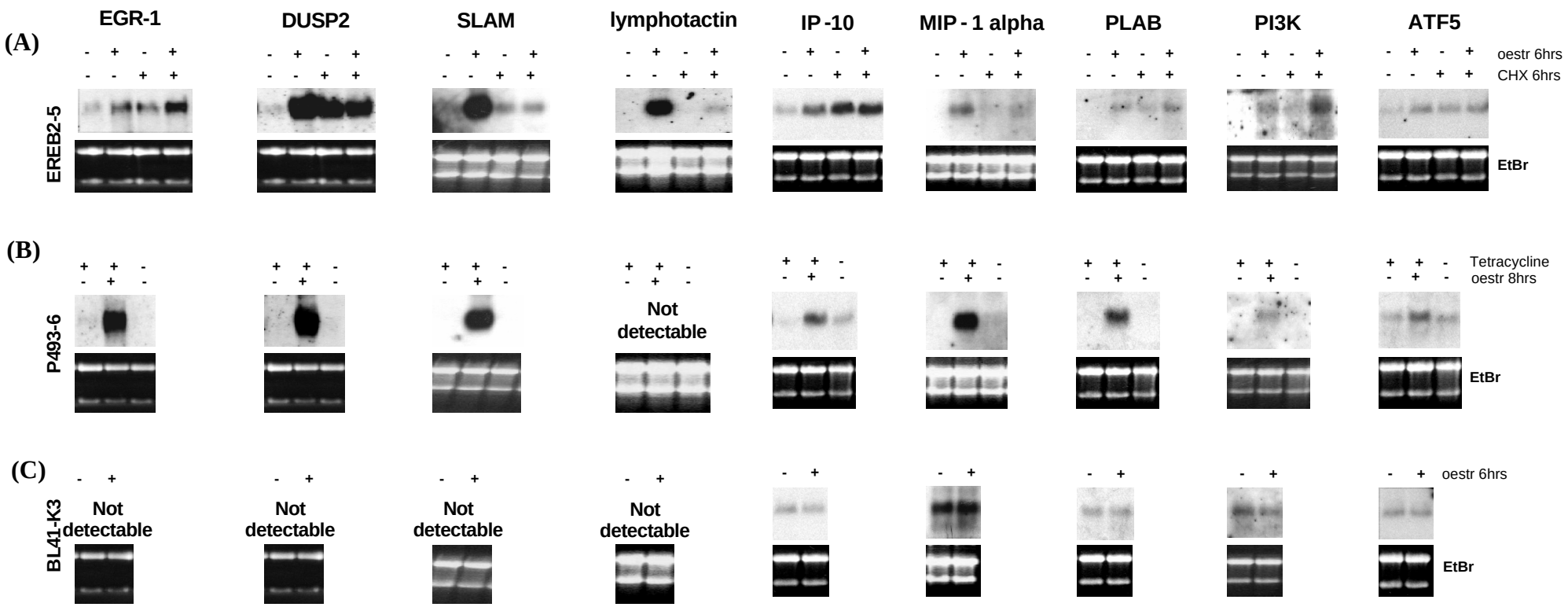


Figure 11.

Genes which require the contribution of viral proteins in addition to EBNA2 for induction.

The EREB2-5 (A), P493-6 (B) and BL41-K3 (C) cells were grown under conditions and harvested at time points as indicated. Total RNA was isolated and 10 µg were loaded on an 1%-denaturing agarose gel and electrophoresis was performed. RNA was blotted on the nylon membrane overnight and subsequently the membrane was hybridised with DIG-labelled PCR probes for EGR-1, DUSP2, SLAM, lymphotactin, IP-10, MIP-1 alpha, PLAB, PI3K and ATF5. Ethidium bromide staining of rRNA is shown as a loading control.

2.1.7.5 Group V: c-Myc regulated genes

CDC37 and NUDT4 were up-regulated in EREB2-5 (Fig. 12A), but also in both EBV/EBNA2 and c-myc growth programmes in P493-6 cells (Fig. 12B). c-myc is down-regulated in BL41-K3 cells because it is translocated to the Igμ locus which is transcriptionally repressed by EBNA2. In these cells CDC37 and NUDT4 were also down-regulated (Fig. 12C). The up-regulation in the c-myc driven P493-6 cells and down-regulation in BL41-K3 cells reveal genes which are c-Myc target genes and are not interesting for promoter analysis of this project. These genes had not been identified as c-Myc target genes before. In spite of very stringent criteria for choosing potential EBNA2 target genes, these two genes were proven to be c-Myc regulated.

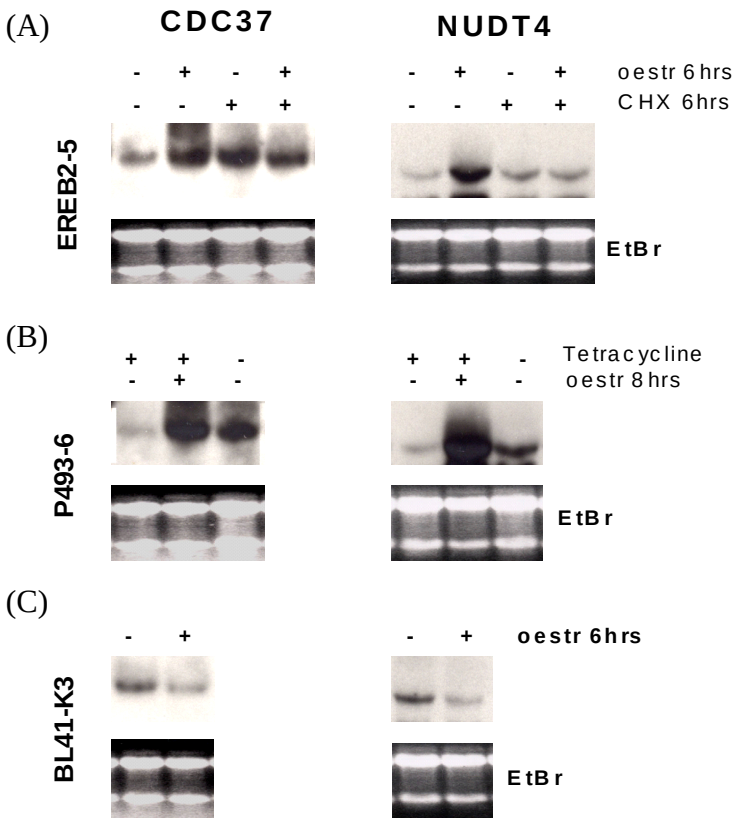


Figure 12.

Genes regulated by c-Myc.

The EREB2-5 (A), P493-6 (B) and BL41-K3 (C) cells were grown in conditions and harvested at time points as indicated. Total RNA was isolated and 10 µg were loaded on an 1%-denaturing agarose gel and electrophoresis was performed. RNA was blotted to the nylon membrane overnight and subsequently membrane was hybridised with DIG-labelled PCR probes for CDC37 and NUDT4. Ethidium bromide staining of rRNA is shown as a loading control.

2.1.8 Comparative analyses of promoter organisation *in silico*

Transcription factor (TF) binding sites common to all or a subset of target genes served as a starting point for the systematic experimental analysis of EBNA2 signalling pathway(s). A comparison of the putative promoters of the potential EBNA2 target genes c-myc, CCR7, RDC1, bfl-1, MIP-1 beta, CD21, CD83 and PLEK was performed in order to define a potential common denominator of promoters of these genes.
All promoter modelling and subsequent database searches were carried out by using the GEMS Launcher 1.0 software, Genomatix Software GmbH, Germany (www.genomatix.de).

2.1.8.1 The identification of potential TF binding sites in the promoters of potential EBNA2 target genes by the MatInspector programme

As there is no clear-cut defined 5'-boundary for promoters, the region situated -1000 upstream of the transcription start site (TSS) was analysed for each gene. The promoter function is not coupled to fixed stretches of sequence homology, but rather to highly variable elements representing individual TF binding sites that act as a recognition site for their cognate protein. The majority of the known TFs recognize short DNA stretches of about 10-15 nucleotides in length which show different degrees of internal variation. The MatInspector programme is a tool that utilizes a large library of predefined matrix descriptions for TF binding sites to locate the matching sequences in genes (Quandt et al., 1995). There are two criteria by which the quality and relevance of the matched TF binding sites are determined: core and matrix similarity. Core similarity denotes the degree of divergence of a sequence present in an analysed promoter from the defined core sequence. A matrix is represented by a two-dimensional table of numbers that reflects the nucleotide preferences for the individual positions of the aligned transcription factor binding sites and is ideally derived from a set of functionally characterised binding sites of a given transcription factor (Quandt et al., 1995). For all analyses described here the core similarity was set up to 1.00 and the matrix similarity was defined as optimized. Numerous potential *cis*-acting elements within the promoter region of the analysed

genes were identified by using the MatInspector programme (Table 8). Three elements were found as a common feature in all eight analysed promoters. These were TATA-box elements and the binding sites for NFAT1 and Oct-1 transcription factors. The random expectation (RE) is the number of matches per 1000 bp of a random DNA sequence. For the NFAT1 binding site the RE value is 2.06 and thus is relatively high. In contrast, the RE value for the Oct-1 binding sites is 0.09. As shown in Fig.13 both, the NFAT1 and Oct-1 binding sites, form clusters in some putative promoter regions. While the appearance of the NFAT1 binding sites could be a simply by chance, the Oct-1 binding site appears much more often than randomly expected.

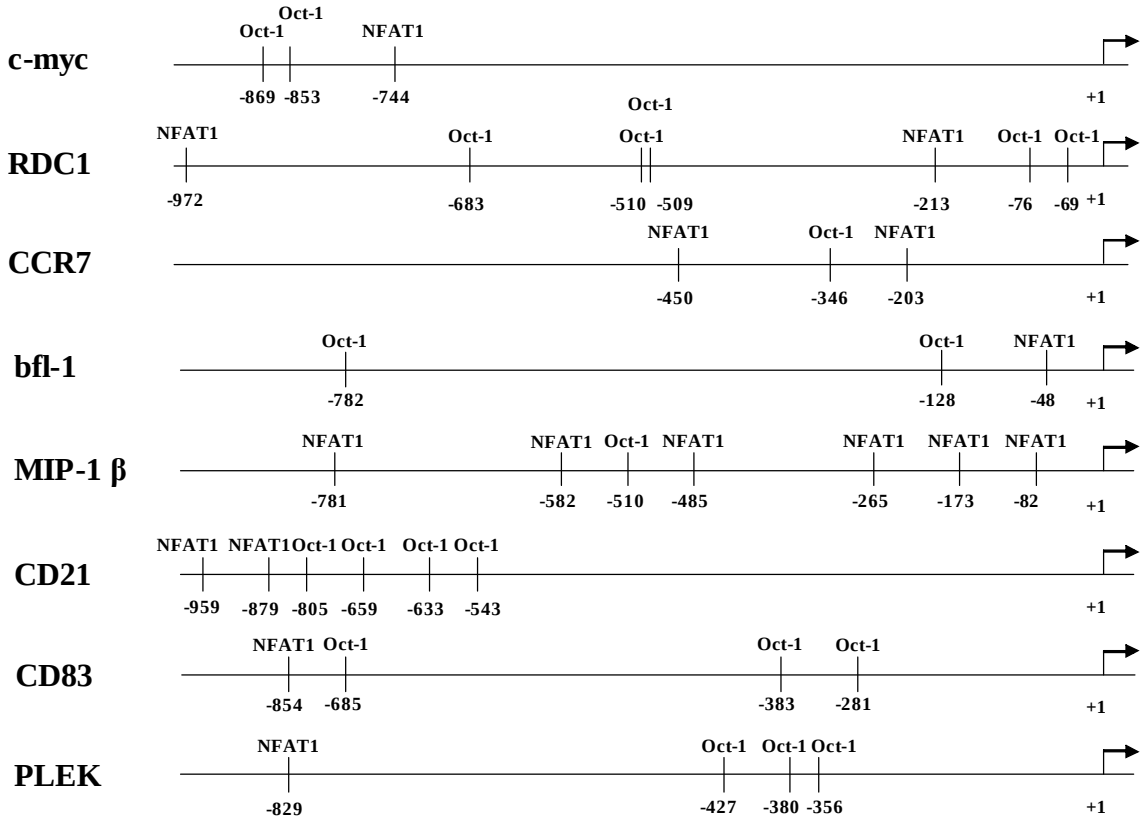


Figure 13.

Schematic diagram of NFAT1 and Oct-1 binding sites in the putative promoter region of the genes belonging to Group I, II and III.

The putative promoter region (-1000 bp region upstream from TSS) was screened for potential NFAT1 and Oct-1 binding sites with the MatInspector programme (Genomatix Software GmbH, Germany). Positions of each binding site as well as TSS are indicated.

Nuclear factors of activated T cells (NFAT) are transcription factors that have been implicated in the regulation of genes involved in the immune response of the T cells, B cells, NK cells, macrophages, mast cells and eosinophils (Tsai et al., 1996, Rao et al., 1997). The importance of the octamer binding protein-1 (Oct-1) for the B cell specific transcriptional regulation has also been well established (Staudt et al., 1986, Wirth et al., 1987). Taken together these two transcriptional factors are highly active in B cells and might have a role in the regulation of these genes by EBNA2 as it will be discussed.

Table 8.

Number of potential TF binding sites in the putative promoters (-1000 bp region upstream of the TSS) of genes potentially regulated by EBNA2. The TF binding sites were identified by the MatInspector programme.

Gene	Number of identified TF binding sites
c-myc	**88**
CCR7	**99**
RDC1	**90**
bfl-1	**78**
MIP-1 beta	**133**
CD21	**104**
CD83	**124**
PLEK	**82**

As described before, the EBNA2 induced genes have been assigned to five groups. Of these 5 groups, group I, II and III were shown to be regulated by EBNA2, while group IV and V genes are activated by viral genes or c-Myc. The differential induction patterns of group I-III genes indicated that different molecular mechanisms might underline the induction processes. In order to search for molecular mechanisms common to single groups, the number of the TF binding sites common to each group was analysed. The results of this analysis are summarised in Table 9.

Apart from TATA-box element, and binding sites for NFAT1 and Oct-1, which are mentioned before as common to all groups, some binding sites were found within single groups only, while others were common to more than one group (Table 9). The binding sites for ECAT, EVI1, GATA and PBXC appear in all genes of groups I and II. The binding sites for FKHD and HNF1 are common to all genes in groups I and

III, and the TF binding sites for CREB, MYT1 and NF-κB are common to genes in groups II and III.

Table 9.

The list of common TF binding sites identified within each group of genes up-regulated by EBNA2. Identification was performed by using the MatInspector programme.

Group	Common TF binding sites
Group I	
c-myc	**BARB, ECAT, EVI1, FKHD, GATA,HNF1,**
CCR7	**NFAT1, OCT1, PBXC, PIT1, TBPF**
RDC1	
Group II	**AREB, CREB, ECAT, EVI1, GATA, HMTB,**
bfl-1	**IKRS, MYT1, NFAT1, NFKB, OCT1, OCTB,**
MIP-1 beta	**PAX5, PBXC, PCAT, RCAT, TALE, TBPF**
Group III	
CD21	**CART, CLOX, CREB, FKHD, HNF1, MEF2,**
CD83	**MYT1, MZF1, NFAT, NFKB, OCT1, PAX2,**
PLEK	**PDX1, SORY, TBPF**

2.1.8.2 Characterisation of higher levels of promoter organisation

Co-regulated genes have been frequently shown to exhibit similarities with regard to their promoter organisation at higher level. At this level, transcription factor binding sites form promoter modules which act in a coordinated way (either synergistically or antagonistically). The contributing elements are arranged within a defined distance and sequential order (Firulli and Olson., 1997, Klingenhoff et al., 2002). In the promoter module, both sequential order and distance can be crucial for function. The ModelInspector programme was used to identify potential promoter modules within EBNA2 regulated promoters of genes identified in this study. ModelInspector utilizes either a library of predefined and experimentally verified modules or modules generated by the programme FastM. Our analysis revealed several modules for each gene (Table 10) consisting of two elements each. Exception was the c-myc promoter since the promoter module could not be identified. The relevance and quality of a given module is represented by the model score and quality assessment. The model score is a value that describes the similarity of the found module with a defined one. It is a sum of the scores of all individual elements of the model and is given in % of the maximum possible score of a model match. Quality assessment is a random

expectation of module appearance in a human sequence per 1000 bp. For modules identified within the analysed promoters the quality assessment values show that these modules do not appear by chance. Nevertheless, model scores were relatively low indicating that found modules are not highly authentic. Furthermore, no promoter module common to all promoters could be identified. A single module consisting of an ETS family protein and an AP1 family protein was found in the promoters of the RDC1 and the CCR7 genes. However, the ETS matrix family members in these two modules differ: CETS1P54 in CCR7 and ELK1 in RDC1.

In summary, on the level of high order promoter organisation, the EBNA 2 target genes do not show a common module pattern.

Table 10.

Promoter modules identified in the promoters of primary EBNA2 taget genes. Modules have been identified by using the ModelInspector programme. The promoter modules common to two genes are underlined.

Gene	Module name	Quality assessment	Position (bp) relative to TSS	Model Score
c-myc	No module	-	-	-
CCR7	ETSF-AP1F	0.544	-152 to -182	91.3 %
	MEF2-AP1F	FM*	-620 to -656	97.3 %
	MEF2-T3R	FM*	-620 to -717	93.0 %
	GATA-AP1F	0.061	-767 to -817	99.2 %
	AP1FAP1F	0.185	-817 to -705	97.8 %
	IKRS-AP2F	0.295	-844 to -813	90.3 %
	HAML-ETSF	0.097	-881 to -901	90.3 %
	ETSF-AP1F	0.073	-901 to -793	97.9 %
RDC1	SP1F-ETSF	0.170	-367 to -561	91.2 %
	XBBF-AP1F	0.031	-472 to -438	92.2 %
	ETSF-AP1F	0.554	-751 to -780	91.5 %
bfl-1	CEBP-GATA	0.132	-33 to -62	96.2 %
	SP1-ETSF	0.178	-422 to -441	90.3 %
	NFAT-AP1F	0.354	-957 to -933	91.1 %
MIP-1	MYOD-TBPF	0.139	-250 to – 228	90.4 %
	BRAC-NKXH	0.085	-727 to -701	97.0 %
CD21	NFKB-AP1F	0.323	-467 to -438	91.2 %
	NF1F-EBOX	0.201	-802 to -828	94.0 %
	MYOD-MYOD	0.201	-944 to -909	93.7%
CD83	STAT-CEBP	0.022	-200 to -119	96.2 %
	HOXF-HOXF	0.205	-324 to -298	91.5 %
	MEF2-AP1F	0.208	-648 to -688	97.0 %
PLEK	HAML-SMAD	0.130	-437 to -524	90.0 %

*** Generated by the FrameWorker programme (Genomatix Softaware GmbH).**

2.1.9 Identification of putative bindings sites for RBP-Jκ, PU.1, NF-κ and c-Myc in EBNA2 target genes

The promoter analysis of EBNA2 induced genes had not revealed a common pattern, apart from the presence of the NFAT1 and Oct-1 site common to all promoters. As described in the introduction, RBP-Jκ binding sites have been found in all viral promoters activated by EBNA2. In contrast, the cellular promoters of EBNA2 target genes are far less well characterised. Recently, an intronic silencer has been defined in the EBNA2 and Notch target gene CD21 (Makar et al., 1998). Subsequently, it was shown that RBP-Jκ binding to this intronic silencer is crucial for its repressive function (Makar et al., 1998, Ulgiati and Holers, 2001). Thus, the search for potential *cis*-regulatory elements was extended towards the intron 1 region of these genes. This search thus involved DNA fragments ranging between 1.2 and 15 kb in size. Since already by random expectation numerous TF binding sites will be found on gene fragments of this site, the analysis was limited to a subset of TF sites including RBP-Jκ, PU.1, NF-κB and c-Myc.

PU.1 was included as studies of the LMP1 promoter had revealed a second, RBP-Jκ distinct sequence-specific, DNA-binding protein and transcriptional activator PU.1 important for this promoter (Laux et al., 1994b, Johannsen et al., 1995). Both, LMP1 and c-myc, are not only EBNA2 target genes, but are also potential partners of EBNA2 which might cooperate with EBNA2 and thus enhance EBNA2 functions.

In the next step, the putative promoter region covering 1000 bp upstream of the transcriptional start (TSS), exon 1 and intron 1 of the genes from group I, II and III were inspected for the RBP-Jκ, PU.1, NF-κB and c-Myc binding sites. A random expectation (RE) values for these TFs are: 0.84 (RBP-Jκ), 0.22 (PU.1), 0.88 (NF-κB) and 0.92 (c-Myc). While the RE value for PU.1 is low (once per 4545 bp), others have a high random occurrence rate. Thus, the occurrence of a site within a relatively large sequence can be expected at random and the interpretation of the results can only be preliminary.

The search was done by the MatInspector programme (Genomatix Software GmbH, Germany). Results are presented schematically in Fig. 13. Interestingly enough, none of the analysed genes, except bfl-1, was found to contain an RBP-Jκ binding site in the putative promoter region (-1000 region), but they all had at least one RBP-Jκ binding site in intron 1. An exception was CD83, which has a very short intron 1 of

only 101 bp. Even a more extensive search over 15 kb sequence did not reveal any RBP-Jκ binding site in the CD83 gene. A PU.1 binding site appeared in all inspected genes except in CD83 and MIP-1 beta. An E-box was found in either the putative promoter region or in the intron 1 region of the genes CCR7, bfl-1, CD21 and PLEK. The biological relevance of these sites is questionable since it has been shown earlier in this work that c-myc does not induce any of these genes. An NF-κB binding site has been identified in all genes except in CCR7. Interestingly, the NF-κB binding site in c-myc and RDC1 (group I) was found outside the putative promoter region, while in other genes it was found within the promoter.

A potential interaction of these TFs within the promoter and/or intronic region and the importance of that interaction still remain to be addressed experimentally.

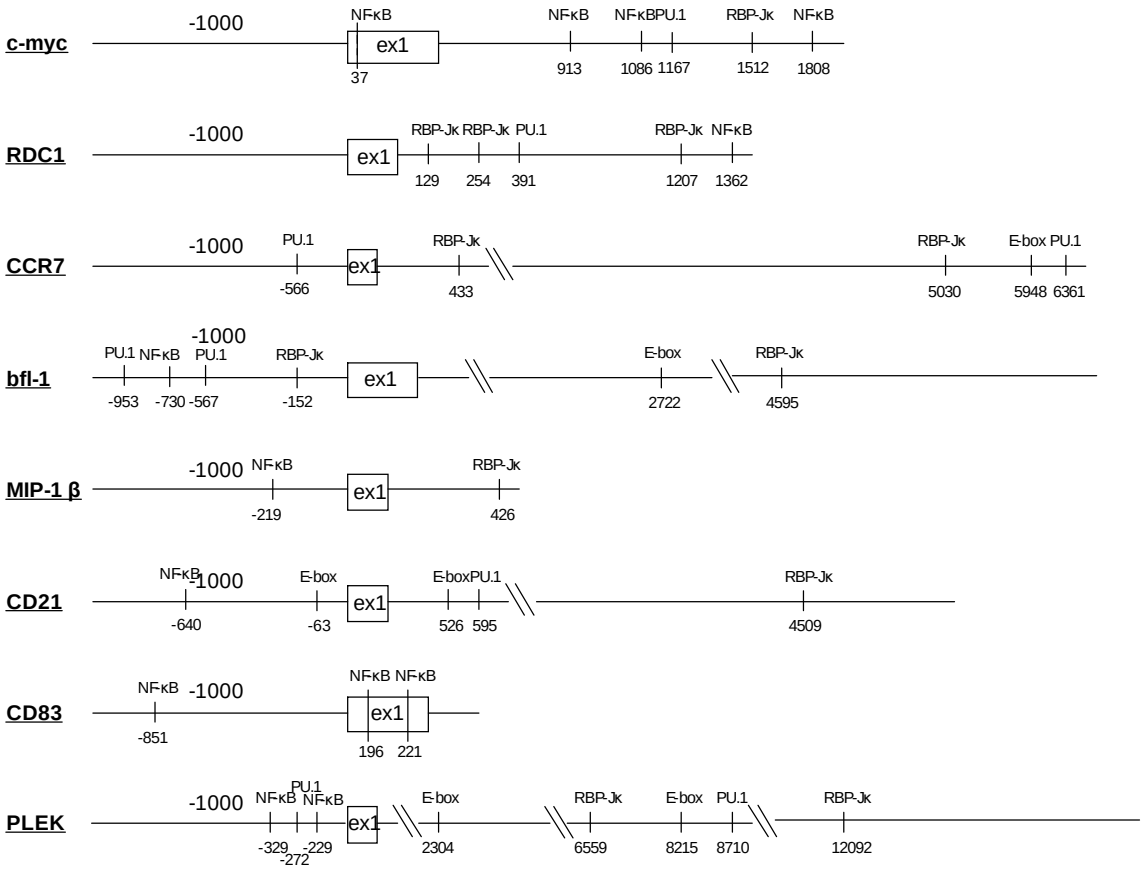


Figure 14.

Scheme of RBP-Jκ, PU.1, NF-κB and c-Myc binding sites in the putative promoter and the intron region of the genes belonging to Group I, II and III.

The putative promoter (-1000 bp), exon 1 and intron 1 regions were screened for the potential RBP-Jκ, PU.1, NF-κB and c-Myc binding sites. They were identified by the MatInspector programme, Genomatix Software GmbH, Germany.

2.2 Functional analysis of the TRRAP gene in the context of EBV immortalisation

The second goal of this project was the identification and functional analysis of a gene which is specifically induced in the context of EBV driven proliferation.
In the ExpressCode™ DNA microarray system described in chapter 2.1.2 TRRAP was found as an up-regulated gene after the stimulation of EREB2-5 cells with oestrogen. The mechanism of TRRAP activation as well as its function in the context of EBV immortalization was studied in the following part of this work since TRRAP is a relevant element in processes such as cellular transformation (McMahon et al., 1998), cell cycle check points (Vassilev et al., 1998, Herceg et al., 2001) and the DNA damage response (Brand et al., 2001, Martinez et al., 2001, Ikura et al., 2000).
TRRAP was first identified by its direct interaction with the transactivation domain of c-Myc and it was clearly shown that it is an essential cofactor for c-Myc and E1A-mediated oncogenic transformation (McMahon et al., 1998). Later it was demonstrated that TRRAP is a component of four multiprotein histone acetyltransferase (HAT) complexes: TIP60 (Ikura et al., 2000), PCAF (Vassilev et al., 1998), STAGA (Martinez et al., 2001) and TFTC (Brand at al., 1999). A role for TRRAP-HAT complexes in mediating transcriptional activation by c-Myc is suggested by several pieces of evidence: TRRAP is recruited to certain c-Myc target genes during Myc-dependent activation *in vivo* and induces a Myc-dependent increase in histone H3 and H4 hyperacetylation at the target promoters/regulatory DNA regions. The role of TRRAP itself in this process is however not known. So far it seems not to have any active catalytical function, but it is rather a scaffold element of the HAT complex (McMahon et al., 1998).

2.2.1 Induction of TRRAP by EBNA2 requires *de novo* protein synthesis

The fact that TRRAP has an essential role in the transformation process by c-Myc and E1A led to the presumption that the induction of TRRAP by EBV infection could play an important role for the EBV induced immortalisation. In order to confirm the induction of TRRAP, demonstrated previously by the ExpressCode™ DNA microarray, growth arrested EREB2-5 cells were stimulated by oestrogen during a kinetic similar to the one used in the microarray screen. The RNA abundance was monitored during a time course of 24 hours. cDNA was quantified by real time PCR following reverse transcription (RT) or the Northern blot analysis (Fig. 13A). The level of TRRAP mRNA increases relatively slowly with a significant leap 6 hours after EBNA2 had been activated. The maximal peak of approximately 25-fold induction is reached 12 hours post-oestrogen stimulation. Well known EBNA2 direct target genes, c-myc and LMP1, reach the maximal intensity approximately three hours earlier. This kind of induction kinetic therefore indicates that TRRAP is one of the secondary EBNA2 target genes.

To address this question, the abundance of TRRAP mRNA was monitored in the presence of the protein synthesis inhibitor cycloheximide (Fig. 13B). Growth arrested EREB2-5 cells were cultured for six hours in medium containing oestrogen, cycloheximide or both cycloheximide and oestrogen. The TRRAP mRNA expression was quantified by real time RT-PCR. Cells stimulated with oestrogen for 6 hours induced TRRAP approximately 3-fold. Cells treated with cycloheximide only did not raise the TRRAP mRNA abundance above the basal level of the growth arrested cells. The activation of EBNA2 in the presence of cycloheximide failed to significantly induce TRRAP mRNA. This doubtlessly shows the existence of another (or more then one) protein downstream of EBNA2 in the cascade governed by EBNA2 which is required to activate TRRAP induction.

These data confirmed TRRAP induction observed in the ExpressCode™ DNA microarray. Further, they show that TRRAP is a secondary target gene of EBNA2 not induced in the absence of *de novo* protein synthesis.

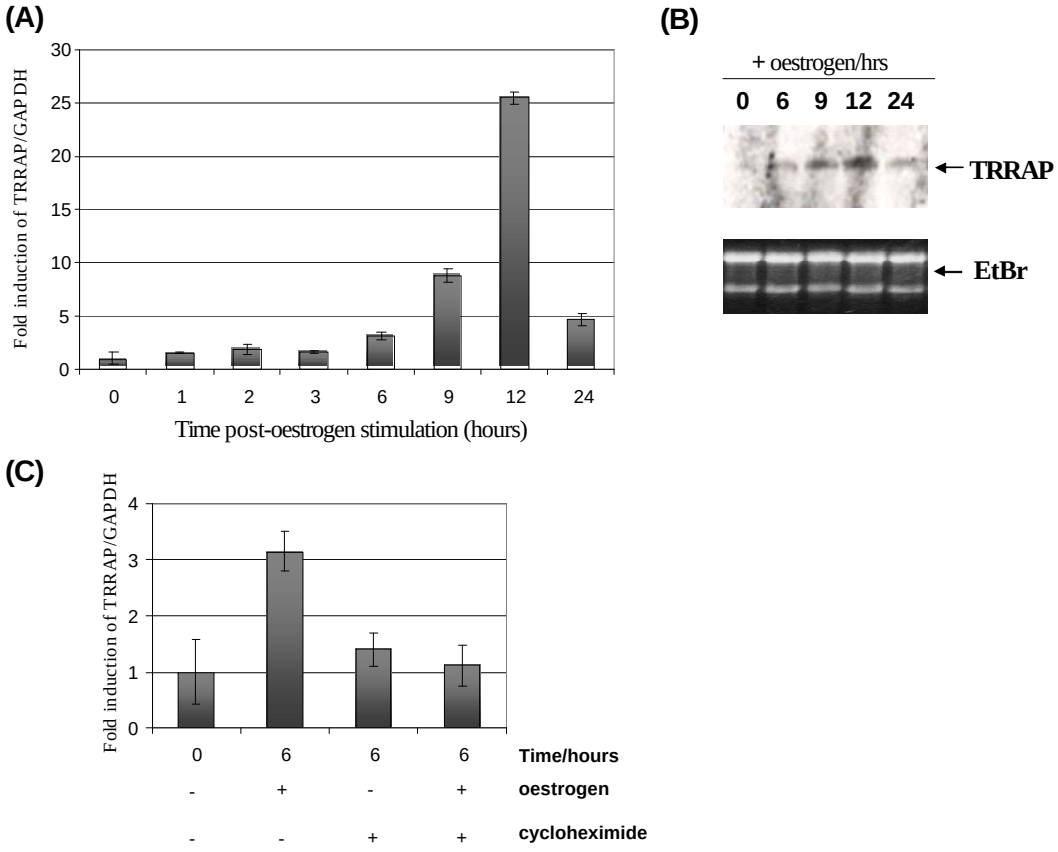


Figure 15.

TRRAP induction in the EREB2-5 cells.

EREB2-5 cells were transiently deprived from oestrogen and 72 hours later were stimulated by the addition of oestrogen (1µM) (A and B) and/or cycloheximide (50 µg/ml) (C). Total RNA was isolated at the given time points. 2 µg of RNA were subjected to reverse transcription. cDNA was quantified using real time PCR with TRRAP and GAPDH specific primers (A and C). Raw data were normalised versus GAPDH mRNA level and fold induction is shown. For the Northern blot analysis (B) 10 µg of total RNA were loaded on a 0.6 %-denaturing agarose gel and blotted for 48 hours to the nylon membrane. Membrane was subsequently hybridised with a DIG-labelled TRRAP specific probe. Ethidium bromide staining of rRNA is shown as a loading control for each lane.

2.2.2 Potential links between EBNA2 and TRRAP: LMP1 and c-myc

Since EBNA2 did not transactivate TRRAP directly the potential link between these two genes had been searched. LMP1 and c-myc are the primary EBNA2 target genes (Kaiser et al., 1999). Both are able to activate the transcription of other secondary genes. Therefore, LMP1, further viral genes and c-Myc were tested as potential mediators in TRRAP induction.

2.2.2.1 The latent membrane protein 1 (LMP1), a viral EBNA2 target gene, does not induce TRRAP

LMP1 mimics a constitutively activated receptor of the tumour necrosis factor receptor family (Gires et al., 1997, Eliopoulus et al., 1998) and shares functional similarities to CD40 signalling (Zimber-Strobl et al., 1996, Kilger et al., 1998, Uchida et al., 1999). It activates four signal transduction pathways: NF-κB (Huen et al., 1995, Mitchell et al., 1995), p38 MAPK (Eliopolus et al, 1999, Vockerodt et al., 2001), STAT (Gires et al., 1990) and JNK1/AP1 (Kieser et al., 1997, Kieser et al., 1999). In order to address whether LMP1 was responsible for TRRAP induction, two complementary cell lines, conditional for LMP1 expression were used: 1852.4 and 1194-3.

Mini-EBV contains all EBV elements necessary for B cell immortalisation. In 1852.4 cells (Kilger et al., 1998) the LMP1 gene is substituted with an LMP1 gene under the control of a conditional, artificial promoter which contains 10 copies of the tet operator (tetO) permitting a selective repression or activation of LMP1 transcription depending on the absence or the presence of tetracycline in the culture medium, respectively. However, this modification does not affect the expression of other viral genes. Cells which do not express LMP1 or express a very low level of LMP1 upon tetracycline deprivation cease to proliferate, but survive in a resting state. Upon re-induction of LMP1 by addition of tetracycline, B cells return to normal proliferation. Published data (Kilger et al., 1998) indicate that it is not possible to obtain a state of cells where LMP1 is absolutely shut off, but is expressed at considerably lower level than in normal EBV-immortalised cell. In order to prevent expression of LMP1 by residual tetracycline in foetal calf serum, which is a common constituent of the complete culture medium, a tetracycline-free approved foetal calf serum was used. By doing so, LMP1 mRNA could not be detected even after longer exposure (Fig. 14A). Thus it can be presumed that LMP1 was not expressed under these specific culture conditions. To test whether TRRAP induction in these cells depends on LMP1, cells were cultured in the presence of tetracycline or in the tetracycline-free medium for three days. TRRAP mRNA abundance was measured by real time RT-PCR. As shown in Fig. 14B there is no difference in TRRAP expression regardless whether LMP1 is induced or not. These result shows that LMP1 is not sufficient to super-induce TRRAP.

1194-3 cells were established (Zimber-Strobl et al., 1996) by infection of B cells with a mini-EBV carrying the ER/EBNA2 chimeric gene and a constitutive SV40-promoter driven LMP1 gene. Thus, in the 1194-3 cells LMP1 is expressed in the absence of functional EBNA2. The TRRAP mRNA level was analysed in the presence or absence of oestrogen. These data show that the TRRAP mRNA abundance increases over time after ER/EBNA2 activation in a similar manner as detected in EREB2-5 cells. This finding indicates that LMP1 expression is not sufficient to maintain TRRAP at increased level in the absence of EBNA2.

Taken together, this data show that TRRAP induction is not caused by LMP1.

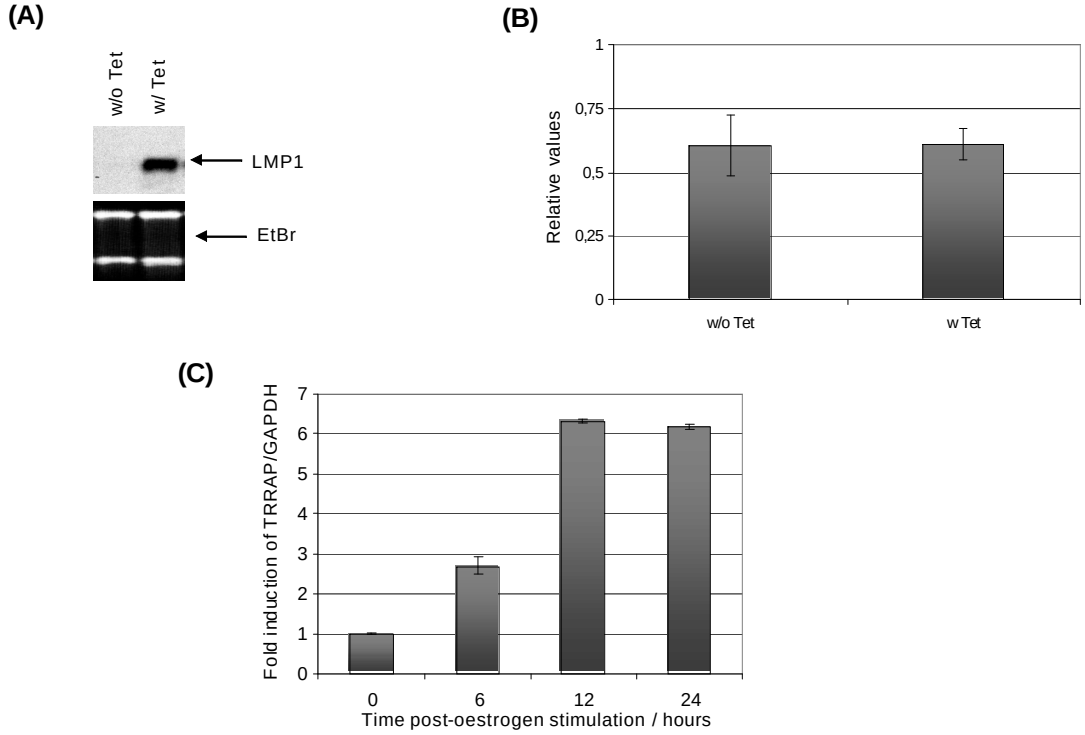


Figure 16.

TRRAP is not induced by LMP1.

In two complementary cell lines it has been shown that LMP1 has no influence on TRRAP induction. (A and B) 1852.4 cells were cultured in the presence (w) or absence (w/o) of tetracycline (Tet) regulated LMP1 for three days. (A) 10 µg of total RNA were used for the Northern blot analysis and probed with DIG-labelled LMP1 specific probe. The data show that LMP1 expression is tightly controlled by Tet. The loading of samples was controlled by ethidium bromide (EtBr) staining. (B) 2 µg of total RNA used in (A) were subjected to real time RT-PCR for the quantification of TRRAP mRNA using TRRAP specific primers. Raw data were normalised versus GAPDH expression and are presented. (C) The cell line 1194-3 constitutively expresses LMP1 and ER/EBNA2. Oestrogen deprived cells were growth arrested for 3 days and then stimulated with oestrogen for the indicated time. Total RNA was isolated and 2 µg of RNA were subjected to real time RT-PCR with TRRAP specific primers. Raw data were normalised versus GAPDH expression and fold induction is shown.

2.2.2.2 Viral genes are dispensable for TRRAP induction

Apart from transactivating cellular genes, EBNA2 activates the transcription of the LMP1 and LMP2 genes and initiates the transcription from the C promoter of the viral EBNA3 genes. In the previous section it was shown that LMP1 on its own cannot induce TRRAP expression. Next examined was the impact of viral genes on TRRAP induction by EBNA2. The B cell lymphomas BL41 and BJAB transfected with a mini-EBV plasmid carrying the chimeric ER/EBNA2 gene were used in order to gain cell lines expressing EBNA2 only (BL41-K3 and BJAB-K3) (Kempkes et al., 1995b, Kempkes et al., 1996). In addition, the P3HR1 convertants of BL41 and BJAB transfected with ER/EBNA2 construct (BL41-P3-9A and BJAB-P3-B6) (Kempkes et al., 1995b, Kempkes et al., 1996) were used to analyse the induction of TRRAP in the genetic background of virus. EBNA2 was fused to the oestrogen receptor domain and hence was functionally dependent on the presence of oestrogen in the medium. Cells were stimulated with oestrogen and harvested at time points indicated in Fig. 15. It is shown that in BL41-K3 and BJAB-K3 cells EBNA2 per se is already sufficient to induce TRRAP up to 3-4-fold (Fig. 15A and B). Latent viral proteins amplify TRRAP mRNA abundance up to two fold above the level seen in BL41-K3 and BJAB-K3 (Fig. 15C and D).

This finding indicates that viral genes are dispensable for TRRAP induction although they facilitate, in an unknown manner, TRRAP induction.

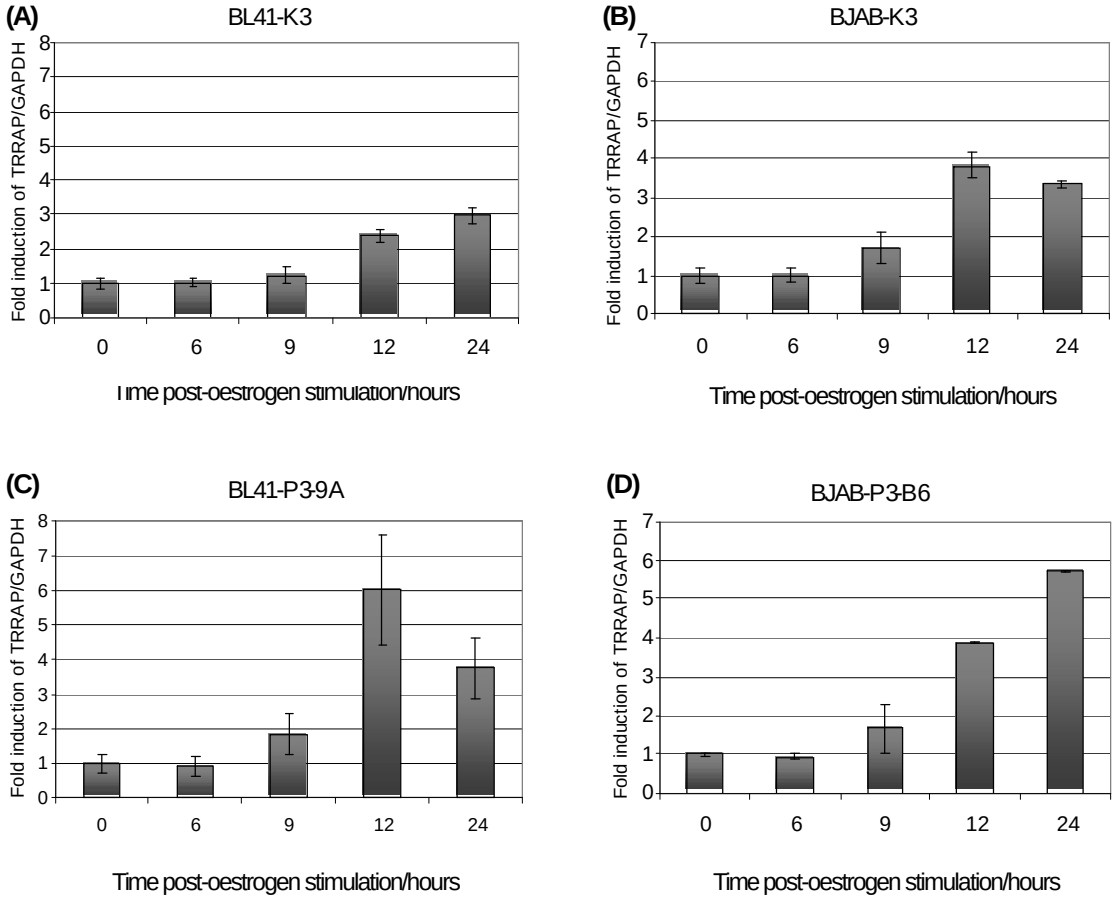


Figure 17.

Viral genes are dispensable for TRRAP induction, but enhance the rate of induction.

BL41-K3 (A), BL41-P3-9A (C), BJAB-K3 (B) and BJAB-P3-B6 (D) cells were stimulated with oestrogen (1 µM) and harvested at the indicated time points post-oestrogen stimulation. Total RNA was isolated and 2 µg were subjected to real time RT-PCR analysis using TRRAP and GAPDH specific primers in order to determine the TRRAP mRNA level. Raw real time PCR data were normalised versus GAPDH mRNA level and fold induction is presented.

2.2.2.3 c-Myc, a cellular EBNA2 target, does not induce TRRAP

c-Myc is a transcription factor which acts as a key inducer of genes involved in cellular growth and proliferation, transformation and apoptosis (reviewed in Dang et al., 1999). It has been shown that c-myc is a primary target gene of EBNA2 (Kaiser et al., 1999). Therefore, next to be tested was whether c-Myc acts as a link between EBNA2 and TRRAP induction. In order to address this question, TRRAP induction in the P493-6 cell line was monitored.

The B-cell line P493-6 (described in Pajic et al., 2000) is an EREB2-5 transfectant conditionally expressing c-myc gene which can be switched off and on depending on the presence or the absence of tetracycline, respectively. As described earlier, the P493-6 cell line can grow in two phenotypically different programmes: (i) c-myc driven growth programme in the absence of both oestrogen and tetracycline (EBNA2 off/c-myc on) and (ii) EBV/EBNA2-driven growth programme in the presence of oestrogen and tetracycline (EBNA2 on/c-myc off). In order to achieve a growth arrested state of the cells a tetracycline was added to the culture medium for the time period of three days. To re-activate the cells the tetracycline was removed from the cell culture and they were either stimulated with oestrogen and tetracycline (EBNA2 on/c-myc off) or cultured in complete medium (EBNA2 off/c-myc on).

Since P493-6 cell line had been established, the time required for the entrance of the cells from the arrested state into the S phase depending on the two different growth programmes had never been defined. EBNA2 and c-Myc are activated through different mechanisms in this cell line. EBNA2 is fused to the oestrogen receptor (ER) domain. In the absence of oestrogen in the medium, the ER/EBNA2 fusion protein is bound to Hsp90 which prevents a proper conformation of this fusion protein and dwells it in the cytoplasm. Upon addition of oestrogen to the cell culture, the fusion protein readily undergoes conformational changes, enters the nucleus and subsequently acts as a transactivator. In contrast to EBNA2, which is constantly present as a protein either in the cytoplasm or in the nucleus, c-myc is transcribed not before the addition of tetracycline. Thus, the EBV/EBNA2 driven growth programme is one step ahead of the c-Myc driven growth programme.

To be sure which time points can be compared during TRRAP induction by the EBV/EBNA2 and c-Myc growth programmes, a BrdU incorporation assay was performed as a measure of cell entry into the S-phase. Fig. 16 shows that the cells grown in the c-Myc programme initiate DNA synthesis 18 hours post-Tet stimulation. That is approximately 6 hours later than it is the case for cells grown in the EBNA2 programme. Since the S phase entry occurs with a significant delay in the c-Myc driven P493-6 cells, all further experiments were performed in the time course of a complete cell cycle.

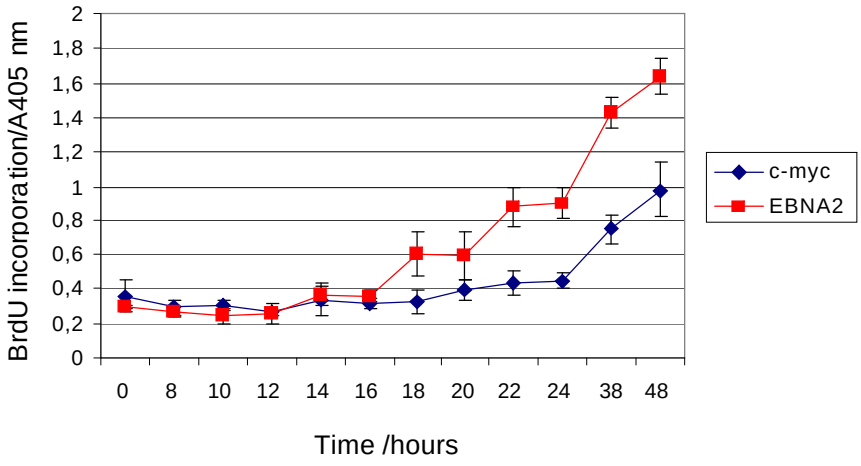


Figure 18.

ER/EBNA2 initiates DNA synthesis more rapidly than Tet-regulated c-myc.

Proliferating P493-6 cells were growth arrested by addition of tetracycline for 72 hours. Cells were washed with PBS/10% FCS three times in order to remove traces of tetracycline and seeded in either free medium or in medium supplemented with oestrogen and tetracycline. The BrdU incorporation assay was used in order to determine the initiation of DNA synthesis for two growth programmes: c-Myc (♦) or EBV/EBNA2 (■). Cells were seeded in triplicates in 96-well plate at a density of $5x10^4$ cells per well and incubated with BrdU for four hours. After incubation with peroxidase-labeled anti-BrdU antibody and the addition of the ABTS-substrate the extinction was measured at 405 nm. Three independent experiments were done. Data from one representative experiment are shown.

The TRRAP mRNA abundance at different time points after re-activation of P493-6 cells was quantified by real-time PCR (Fig. 17). Cells proliferating in the EBV/EBNA2 driven growth programme increase the level of TRRAP up to 5 fold with the peak at 12 hours, thus TRRAP accumulates before S phase entry. In comparison, cells proliferating in the c-Myc driven programme do not change the level of TRRAP during the whole time course even though time points later than 24 hours were tested. Differential expression of TRRAP is maintained in the proliferating P493-6 cells driven by both growth programmes as confirmed by the Western blot analysis (Fig. 17B). These results clearly show that c-Myc does not induce TRRAP, while EBNA2 does.

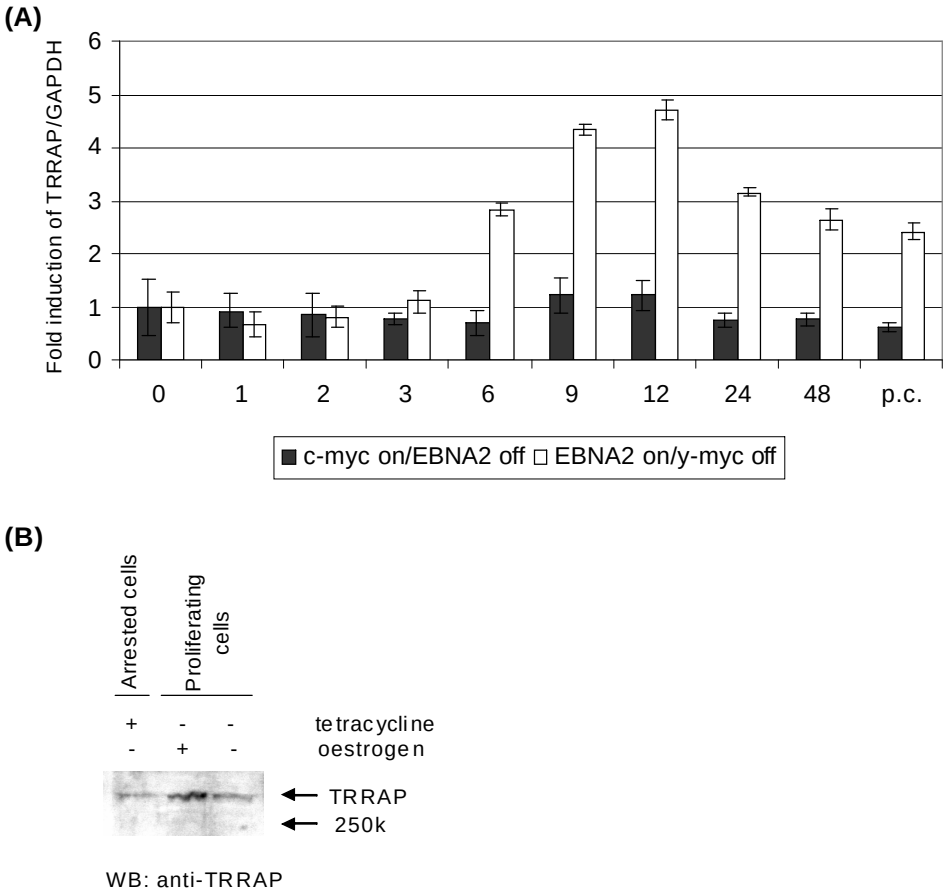


Figure 19.

c-Myc does not induce TRRAP.

P493-6 cells were growth arrested by addition of tetracycline for 72 hours and then washed with PBS/10% FCS three times to remove traces of tetracycline. Cells were seeded in either free medium or in medium supplemented with oestrogen and tetracycline and harvested at indicated time points. (A) Total RNA was isolated and 2 µg were subjected to reverse transcription. TRRAP and GAPDH mRNA abundance was quantified in real time RT-PCR with TRRAP and GAPDH specific primers. Raw data for TRRAP were normalised versus GAPDH and fold induction is presented. (B) Western blot analysis of TRRAP expression in proliferating P493-6 cells grown in either of the two growth programmes. Proteins were extracted with buffer containing 1 % NP-40 detergent and 80 µg of proteins was separated on 8% SDS/PAGE. The immunostaining of TRRAP was performed using primary rabitt anti-TRRAP monoclonal antibody (Serotec, UK). As secondary antibody an anti-rabitt IgG HRP-conjugated antibody was used (Promega, USA).

2.2.3 TRRAP is induced in primary B cells upon EBV infection, but not in B cells stimulated with CD40L and IL-4

All data so far presented were obtained examining different cell lines in which EBNA2 had been activated by oestrogen. Thus, it could not be excluded that TRRAP induction was somehow linked to oestrogen in the cell culture medium. To test whether TRRAP induction is an event that occurs under the physiological conditions of EBV infection, and is neither an artefact of cell lines kept in culture for longer period or cell proliferation itself, nor that TRRAP was induced by the oestrogen receptor domain stimulated by oestrogen, the following experiments were performed.

Primary B cells were infected with B95.8 EBV and TRRAP induction was compared to B cells activated with CD40 ligand (CD40L) plus interleukin-4 (IL-4). Cross-linking of CD40 with immobilised CD40 specific antibodies or fibroblast feeder cells expressing CD40L (CD40L cells) induces B cells proliferation *in vitro*. The addition of IL-4 or IL-13 allows the generation of factor-dependent long-term normal human B cells lines (reviewed in Banchereau et al., 1994).

Human tonsillar B cells were separated from the rest of tonsillar cells by rosseting with sheep red blood cells. The final cell suspension consisting of more than 96% CD19+ B cells was cultured on irradiated CD40L cells (Garrone et al., 1995) in the presence of IL-4 (100 U/ml). In parallel, the same number of cells was infected with B95.8 EBV. Cells without any stimuli were cultured as a negative control.

PCR following the reverse transcription of total RNA isolated from the cultured primary B cells shows that the outgrowth of primary B cells was not caused by the EBV already residing in the B cells prior cultivation (shown in Fig. 18 by amplification of LMP1 genomic DNA and mRNA). Only cells infected with EBV show the induction of LMP1 on day 3 post-infection. The LMP1 genomic DNA was readily detected already 24 hours post-infection. Cells cultured with either no stimuli or stimulated with CD40L/IL-4 clearly show absence of LMP1 expression once more confirming the negative EBV status of the cultured cells.

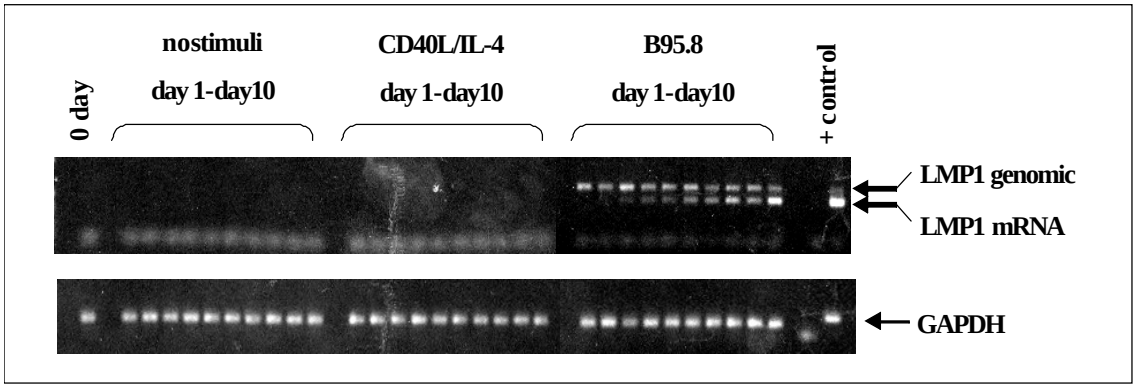


Figure 20.

EBV status of the cultured primary B cells.

Total RNA was isolated from human tonsillar B cells stimulated with either B95.8 EBV, CD40L/IL-4 or cultured with no stimuli for ten days as well as from cells prior stimulation (day 0). 2 µg of total RNA were used to make cDNA. PCR with LMP1 specific primers gave two fragments (upper panel): a larger one (625 bp) which corresponds to the amplified genomic fragment and a smaller one (246 bp) which corresponds to the amplified cDNA fragment of LMP1. GAPDH was amplified as an input control (lower panel).

As expected, cells were able to incorporate ^{3}H-thymidine and proliferate (Fig. 19A and B, respectively) if stimulated with CD40L/IL-4 or infected with the virus. In contrast, cells kept in culture without stimuli were detained from dying until day 5, but then they died extensively. CD40L/IL-4 stimulation resulted in fast incorporation of ^{3}H-thymidine in the first 48 hours followed by the outgrowth of B cell clones with an increased cell size. The level of ^{3}H-thymidine incorporation then declined and from day 5 onwards was kept at the same level. Since the cells were constantly kept on the CD40L feeder layer and stimulated with IL-4, this decrease of ^{3}H-thymidine incorporation is probably due to the discontinuance of the synchronous cell culture. In comparison to that cell number and ^{3}H-thymidine uptake in cells infected with B95.8 EBV was delayed for approximately three days (Fig. 19A and B, respectively), but then it attained the level of CD40L/IL-4 stimulated cells. This delay can be explained by the different mechanisms of B cell activation. CD40L and IL-4 are readily accessible to B cells and cause the immediate activation of the signal transduction machinery. In contrast to that, EBV infection is a multi-step process which subsequently leads to B cell transformation.

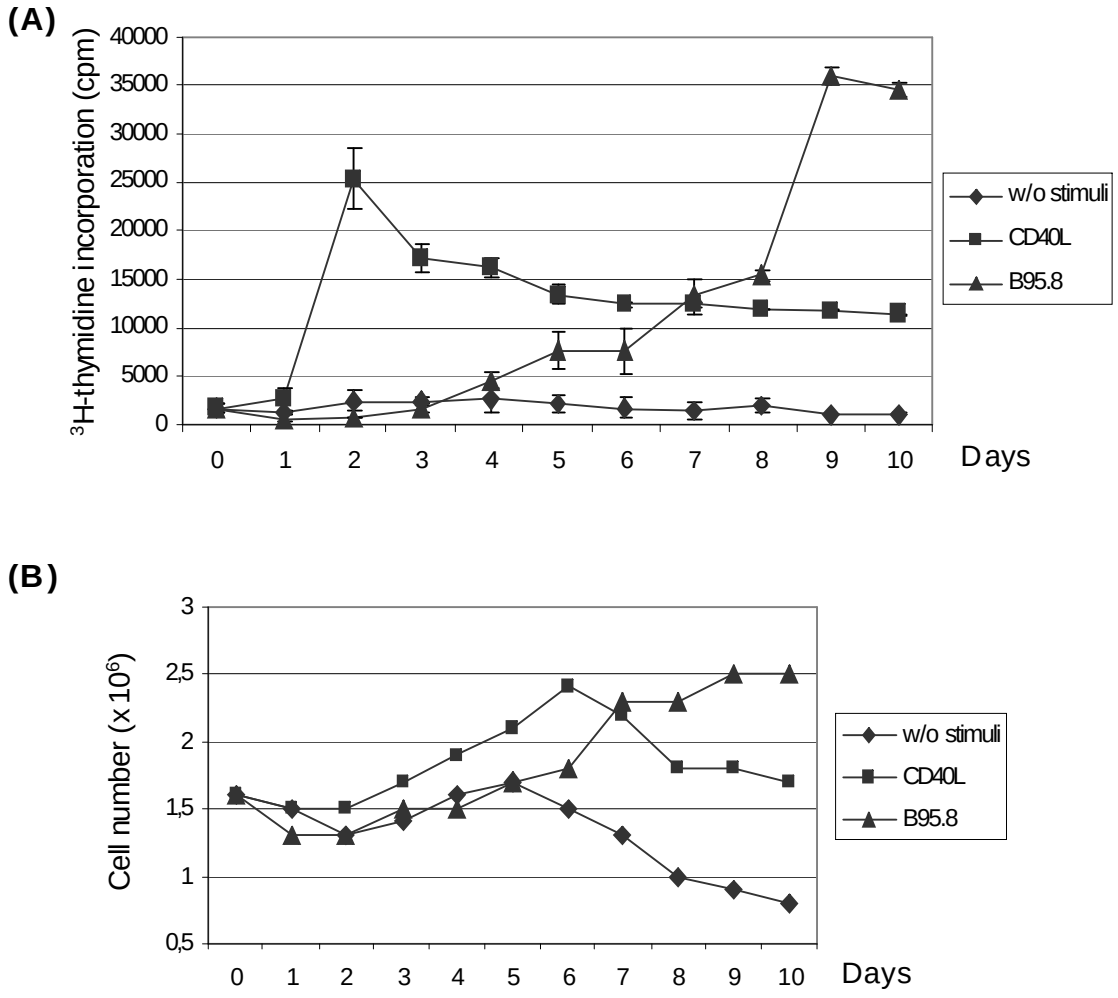


Figure 21.

Growth characteristics of primary B cells stimulated with different stimuli.

Purified B cells (>96% CD19+) were cultured at a density of 2,5x10^6 cells/well in 6-well plates for up to 10 days with different stimuli: no stimuli (♦), cells grown on irradiated CD40L cells plus IL-4 (100 U/ml) (■) or infected with B95.8 EBV (▲). (A) Cells (1.5 x 10^5/well in 96-well plates) were pulsed with ^{3}H-thymidine every 24 hours for 4 hours and the incorporation of ^{3}H-thymidine was measured. (B) Cells were counted every 24 hours. Dead cells were excluded by tryphan blue exclusion and the number of viable cells is shown.

TRRAP induction was monitored in the cultured B cells in the time intervals of 24 hours during the course of ten days (Fig. 20A). The TRRAP transcripts were detected under all conditions, but the abundance was selectively increased in the EBV infected cells. In contrast, in cells without any stimuli or in CD40L/IL-4 stimulated cells the TRRAP mRNA abundance did not change beyond the initial basal level. To control

the efficiency of B cell activation by CD40/IL-4, c-myc induction was monitored by real time RT-PCR (Fig. 20B). The c-myc transcript was induced in both cells stimulated with CD40L/IL-4 and infected with B95.8, but at different time points. B cells stimulated with CD40L/IL-4 strongly induce c-myc after 5 days while EBV infected B cells reach the same level of c-myc expression 8 days after infection. A delay of three days in c-myc induction in B95.8 infected B cells coincides with the delay in the kinetic of ^{3}H-thymidine incorporation. This delay in c-myc induction in EBV infected B cells happens perhaps due to the mechanism of activation discussed above. c-myc induction was absent in the cells cultured without external stimuli.

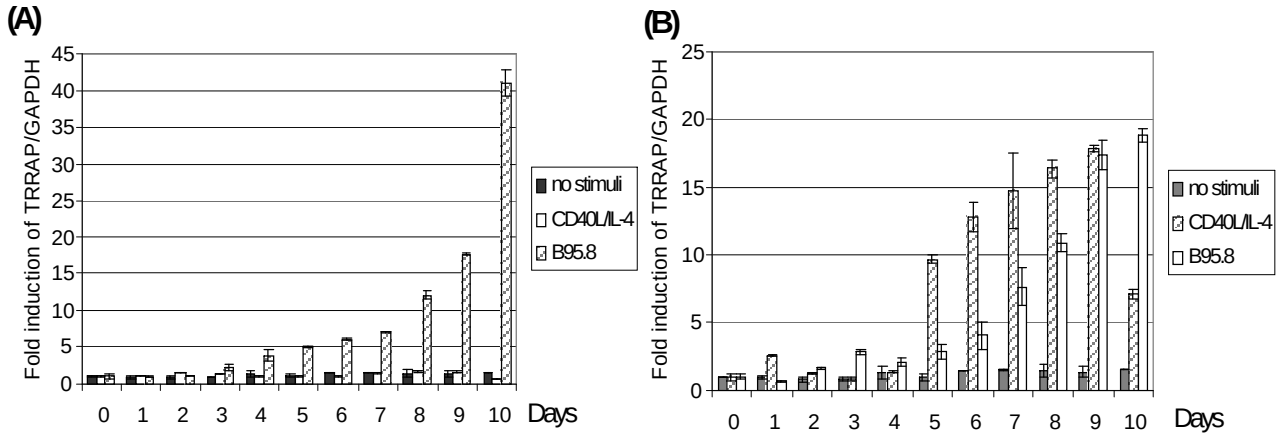


Figure 22.

B-cells infected with B95.8 EBV induce both TRRAP and c-myc, but if stimulated with CD40L/IL-4 only c-myc is induced.

The purified tonsillar B cells (>96% CD19+) were cultured in 6-well plates (2.5 x 10^6 cells/well, 1 x 10^6 cells/ml). Every 24 hours cells were harvested. The total RNA was isolated and 2 µg were subjected to reverse transcription. TRRAP (A) and c-myc (B) mRNA level was quantified using real time PCR with TRRAP and c-myc specific primers, respectively. Raw data from were normalised with 28 S rRNA quantified in the Northern blot analysis.

In summary, TRRAP was detected in all cells irrespective of the growth state. However, the induction of TRRAP above basal level was observed only in the context of EBV/EBNA2 driven proliferation and precedes or coincides with S phase entry.

2.2.4 A vector system for expression of TRRAP specific short interfering RNA

To test whether the TRRAP protein is rate limiting for the viability of EBV immortalised B cells, TRRAP gene expression was inhibited in 721 EBV-positive LCL by employing a vector-based expression system to produce endogenous, functional short interfering RNA (siRNA) molecules.

The introduction of double-stranded siRNA has proven to be a powerful tool to suppress (knock-down) the expression of a given gene through a process known as RNA interference (RNAi) (Sharp, 1999, Elbashir et al., 2001). Nowadays, there are several ways of introducing an siRNA for a targeted gene into the cell. While some adherent cell cultures can be transfected with efficiencies reaching almost 100 % of cells, transfection efficiencies in suspension cultures typically do not exceed 50 % of the cells. In general, the transfection efficiencies of LCLs are in the range of 5-15 %. In order to monitor the biological effect of negative regulation of a factor within a heterogeneous population of transfected and untransfected cells an efficient selection procedure is required. Therefore, a vector system (a gift from B. Jungnickel) carrying siRNA sequence specific for the targeted gene under the transcriptional control of the U6 promoter and subsequently the selection of transfected cells according to the nerve growth factor receptor (NGFR) expressed at the cell surface was used (Fig. 21A).

The U6 RNA polymerase III promoter appeared to be a convenient tool for an siRNA expression because of the following reasons. It produces a small RNA lacking a polyadenosine tail. It has a well-defined start of transcription and a termination signal consisting of five thymidines in a row. Most importantly, the cleavage of the transcript at the termination site is after the second uridine yielding a transcript resembling the ends of synthetic siRNAs, which also contain two 3’ overhanging U nucleotides (Fig. 21B). These 3’ overhangs seems to be essential for functionality of siRNA (Elbashir et al., 2001).

Immediately downstream from the U6 promoter a 53-nt oligo was inserted (Fig 21C). Within this 53-nt oligo, the 19-nt target sequence derived from the TRRAP transcript (position 282-300) was included in both sense and antisense orientation, separated by an 8-nt spacer sequence. A 19-nt target sequence design is critical and was done by the software tool provided on the web page www.oligoengine.com. The design strategy of this programme includes some basic rules for siRNA design such as: at lest 30 % of sequence should be G or C, the sequence must not contain a stretch of four or

more A or T (this would lead to premature termination of the transcript) and the sequence should be positioned not closer then 100 bases downstream from the start codon. A selected siRNA sequence was also submitted to a BLAST search against the human genome sequence to ensure that only one gene of the human genome was targeted. An alternative sequence was designed by introducing a single nucleotide mismatch in the middle of the targeting sequence (Fig. 21C). It was shown that a single nucleotide mismatch abrogates the ability to suppress gene expression (Brummelkamp et al., 2002). This construct along with the vector containing only NGFR was used as negative control in the following experiments. The resulting transcript from the U6 promoter followed by the insert sequence is predicted to fold back on itself to form the 19-base pair stem-loop structure (Fig. 21B). This stem-loop precursor is a substrate for the enzyme Dicer, which cleaves the hairpin to generate the short dsRNA which function as siRNA.

NGFR cloned in this vector expresses a truncated NGFR protein containing only a transmembrane part while the cytoplasmic part is missing. Thus, this form of NGFR is not able to activate signalling and does not have any biological effect on the cell expressing it. NGFR was used solely to permit the selection of transfected cells from those which were not transfeced. The NFGR specific monoclonal antibody recognizes the transmembrane part of NGFR and was used for positive selection on magnetic beads.

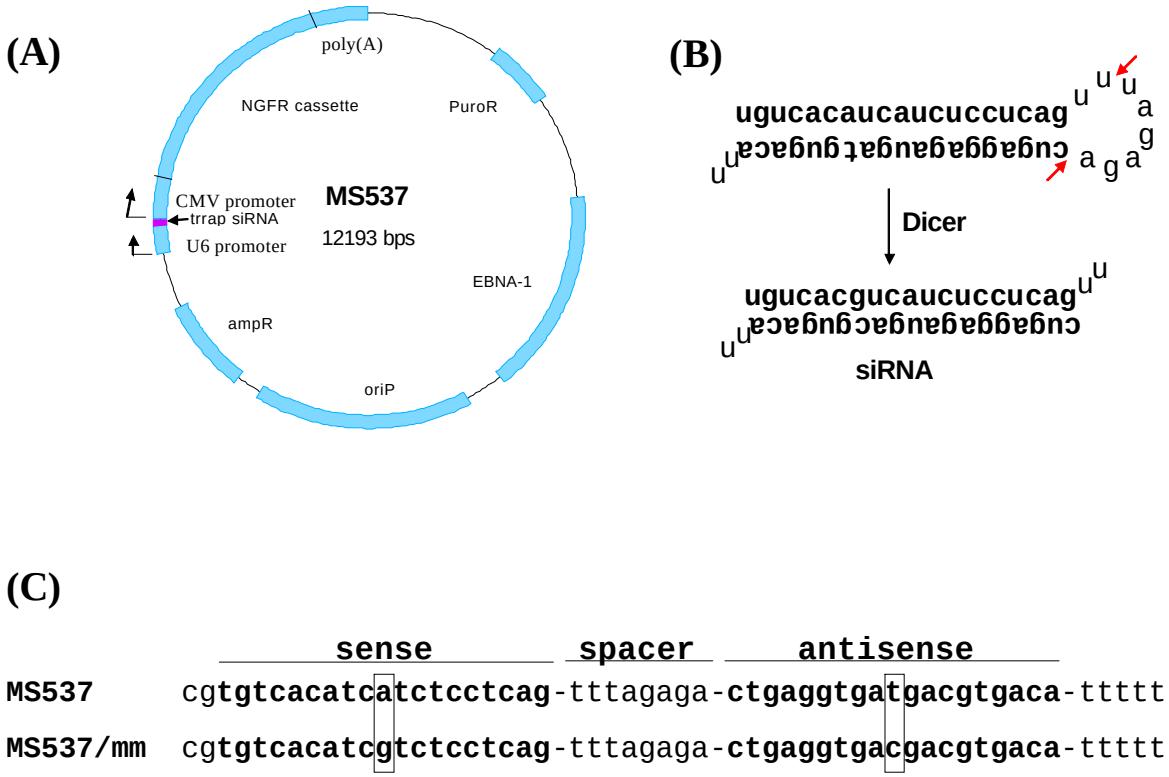


Figure 23.

Schematic structure of the components of the siRNA expression vector system.

(A) Schematic presentation of the anti-TRRAP siRNA expression vector. (PuroR=puromycin resistance gene, EBNA1=Epstein-Barr nuclear antigen 1, oriP=EBNA1-dependent origin of replication, ampR=ampicilin resistance gene). (B) The stem-loop precursor transcript is quickly cleaved in the cell by the cellular protein Dicer to produce functional siRNA. The Dicer cleavage sites are marked by arrows. (C) Sequence of the 53-nt oligo insert containing the sense and antisense target sequence for TRRAP mRNA separated by the 8-nt spacer sequence. MS537 represents the functional oligo while MS537/mm represents a sequence which is a non-functional siRNA due a single mismatch mutation (mm) in the central part of the 19-nt sequence (squared nucleotides).

2.2.5 Transfection and selection of 721 cells expressing siRNA specific for TRRAP

Cells were transfected with pMS537 (TRRAP specific siRNA expression vector, shown in Fig. 21A), pMS537/mm (TRRAP specific siRNA expression vector with the mismatch mutation (mm) in the central part of siRNA sequence as indicated in Fig. 21C) or pBJ3 (empty vector containing NGFR cassette only). Twelve hours post-transfection cells were further analysed. A scheme of the procedure is presented in Fig. 22.

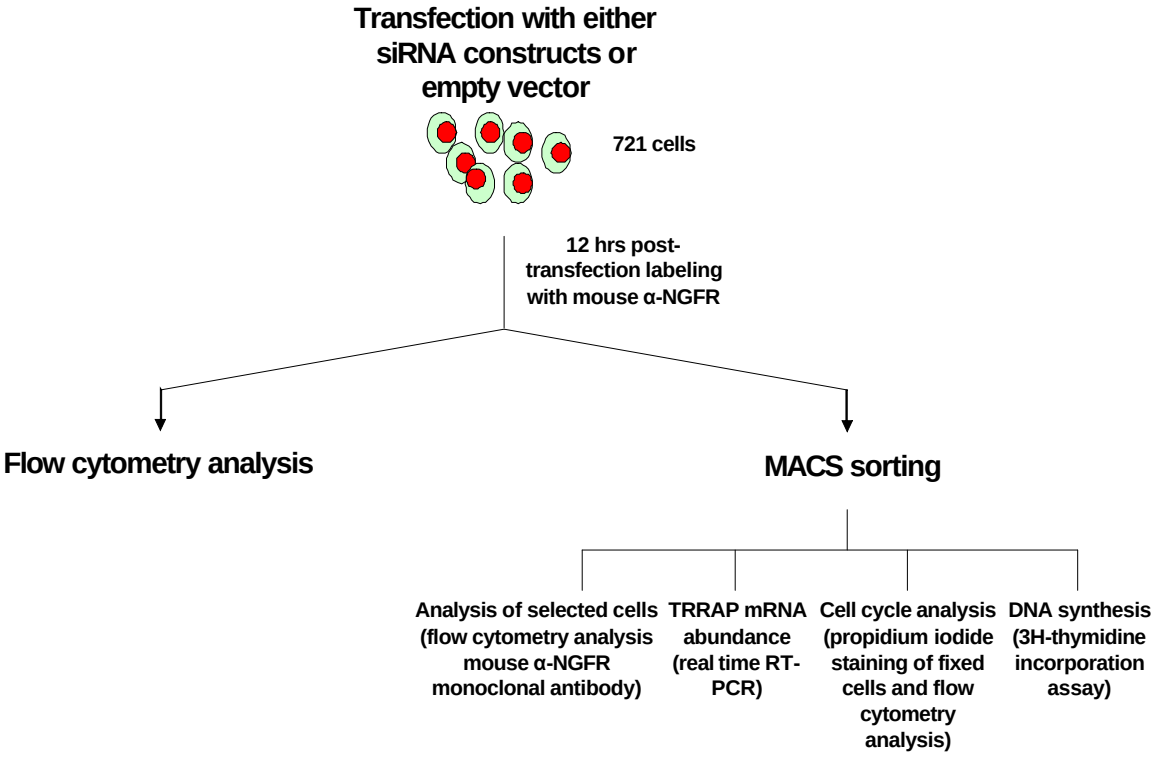


Figure 24.

Scheme of analyses done with the purpose to analyse a significance of TRRAP in EBV-positive 721 cells (see text below for details).

Cells were analysed by flow cytometry in order to determine the proportion of NGFR positive cells. Subsequently, the cells were subjected to MicroBeads assisted cell sorting (MACS). Cells were labelled with monoclonal mouse anti-NGFR antibody and positively selected by magnetic beads coated with goat anti-mouse IgG antibodies. The purity of selected cells was analysed by flow cytometry with the NGFR antibodies. The rest of the cells were used for the isolation of total RNA, the analysis of cell cycle by propidium iodide staining or for the analysis of the cell competence to synthesize DNA.

A striking finding of the flow cytometry analysis performed 12 hours post-transfection was that transfection with the construct carrying a TRRAP specific siRNA sequence (pMS537) resulted in only 0.33 % of cells positive for NGFR. In contrast, cells transfected with either a mutated anti-TRRAP construct (pMS537/mm) or an empty vector (pBJ3) showed a significantly higher proportion of NGFR-positive cells, 5.4 and 4.8 %, respectively. Another striking result was obtained by the analysis of sorted NGFR-positive cells. Approximately 45 % of selected NGFR positive cells transfected with pMS537 were shown to be dead by tryphan blue exclusion. For cells transfected with pMS537/mm or pBJ3 by the same method only 1-2 % of the NGFR positive cells scored as being dead in the tryphan blue assay. This indicates that the low number of cells in the NGFR positive fraction of MS537 transfected cells was not due to the irregularities during transfection process or the unspecific toxicity of the vector, but rather due to a cell death which was an ongoing process in these cells.
The total RNA was isolated from both NGFR-positive and NGFR-negative fractions of the cells after cell sorting. The level of TRRAP mRNA was monitored by semi-quantitative RT-PCR (Fig. 23). It is clearly shown that TRRAP mRNA is expressed in all NGFR-negative cells and also in NGFR-positive cells carrying pMS537/mm or an empty vector pBJ3. In contrast, only very low amounts of TRRAP cDNA were amplified in the NGFR-positive cells carrying pMS537 construct. This clearly demonstrates that a period as short as 12 hours is sufficient for the activation of transcription of TRRAP specific siRNA and subsequent down-regulation of TRRAP mRNA.

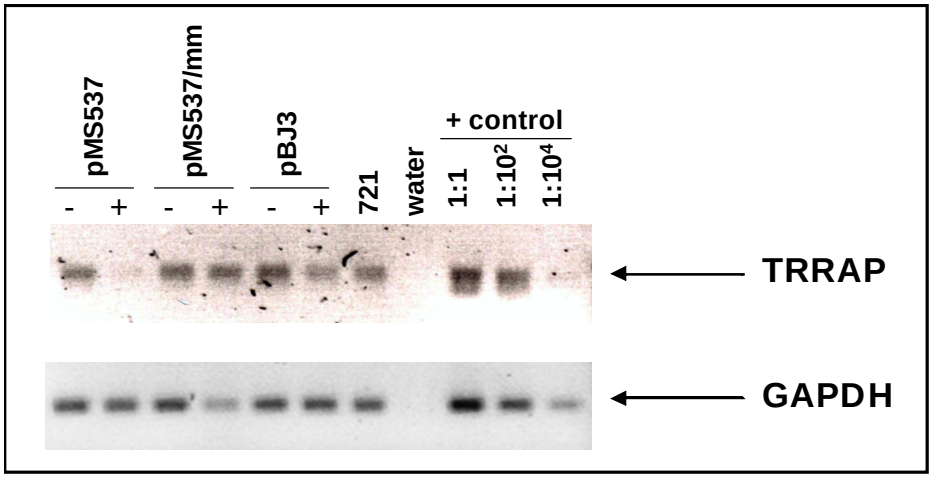


Figure 25.

TRRAP mRNA is knocked down in cells transfected with the specific siRNA.

The 721 cells transfected with pMS537 (anti-TRRAP siRNA construct), pMS537/mm (anti-TRRAP siRNA construct with missense mutation) or pBJ3 (empty vector with NGFR cassette) were labelled with the monoclonal mouse anti-NGFR antibody and sorted by anti-mouse IgG magnetic beads. RNA isolated from the NGFR-negative (-) and NGFR-positive (+) fractions after sorting and non-transfected 721 cells was subjected to reverse transcription and subsequently to PCR with TRRAP specific primers (upper panel). PCR products were loaded on the 1.5 %-agarose gel. Water was used instead of cDNA as negative control and previously PCR amplified TRRAP cDNA as positive control. The equal amount of RNA used for reverse transcription is controlled by PCR with GAPDH specific primers (lower panel).

In summary, the TRRAP mRNA abundance was significantly knocked-down. This process is immediately accompanied by cell death of a major proportion of the cultured cells.

2.2.6 DNA replication and cell cycle stage distribution of TRRAP siRNA expressing 721 cells

NGFR-positive cells transfected with pMS537, pMS537/mm and pBJ3 were tested for DNA replication by the ^{3}H-thymidine incorporation assay. Fig. 24 demonstrates that pMS537 transfected cells do not incorporate ^{3}H-thymidine significantly. In contrast, pMS537/mm and pBJ3 transfected cells behave very similar to untransfected proliferating 721 cells. In fact, MS537/mm and pBJ3 transfected cells incorporate

about 5-10% less ^{3}H-thymidine than control cells. The minor decrease of ^{3}H-thymidine incorporation could be the consequence of either transfection or sorting process.

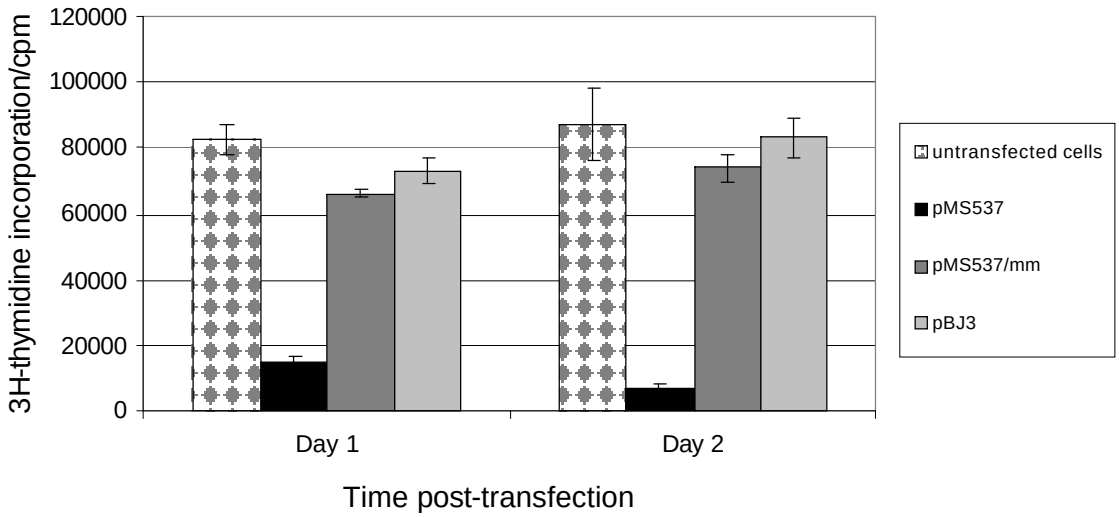


Figure 26.

TRRAP is rate limiting for DNA replication in 721 cells.

721 cells were transfected with pMS537 (anti-TRRAP siRNA construct), pMS537/mm (anti-TRRAP siRNA construct with missense mutation) or pBJ3 (empty vector with NGFR cassette). Twelve hours post-transfection cells were labelled with a mouse anti-NGFR antibody and sorted on anti-mouse IgG magnetic beads. Twelve and 36 hours after sorting 3 x 10^{4} viable cells were pulsed for 4 hours with 3H-thymidine and the incorporation was measured. Untransfected, proliferating 721 cells were used as a measure of normal DNA synthesis.

For cell cycle analysis NGFR positive cells were ethanol fixed and the DNA cell content was determined by flow cytometry after propidium iodide staining (Fig 25). Apoptotic cells were quantified as the percentage of cells with hypodiploid DNA content. Fig 25 shows that apoptosis was more pronounced in pMS537 transfected cells (39.17 %) compared with cells which were transfected with pMS537/mm, pBJ3 or untransfected cells which thus have normal levels of TRRAP (18.91, 14.91 or 17.22%, respectively). There was also a clear decline in the number of G1 phase cells in TRRAP knocked-down cells. However, the proportions of S phase and G2/M phase cells were essentially the same for all tested cell samples. Since it was shown before that pMS537 transfected cells incorporate ^{3}H-thymidine only at a very low level, it is unlikely that the cells identified by this assay indeed are in the replicative phase of

cell cycle DNA. In contrast, they might reflect a population of cells which either ceased to replicate DNA or apoptosed in G2/M phase.

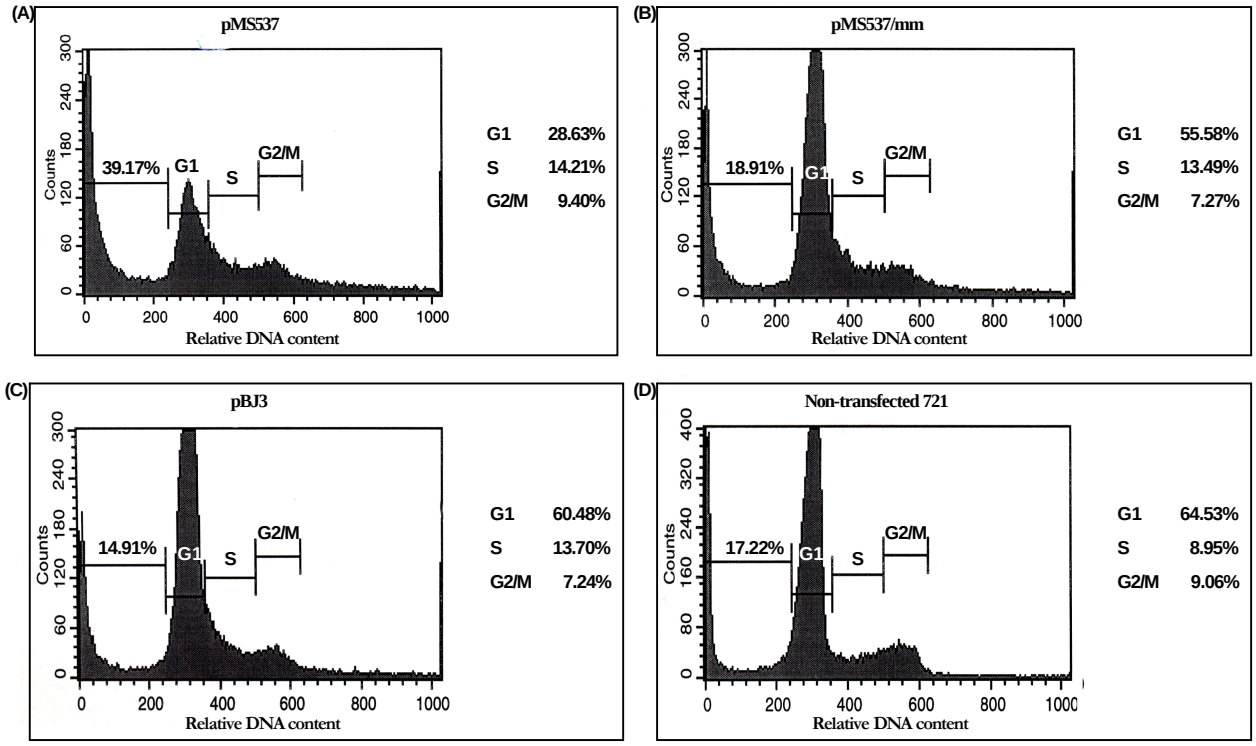


Figure 27.

TRRAP knocked-down cells show pronounced apoptosis and decrease in the G1 phase cell number.

The 721 cells transfected with pMS537 (TRRAP specific siRNA construct), pMS537/mm (TRRAP specific siRNA construct with missense mutation) or pBJ3 (empty vector with NGFR cassette) were labelled with mouse anti-NGFR antibodies and sorted on anti-mouse IgG magnetic beads. Sorted cells and untransfected 721 cells were immediately ethanol fixed and subsequently stained with propidium iodide and analysed by flow cytometry. The cell cycle profiles were generated by plotting cell number (Y-axis) against DNA content (X-axis). The regions mark the populations with sub G1 DNA content, cells with G1 DNA content, cells in the apparent S or G2/M phases of cell cycle.

These data strongly suggest that TRRAP expression is intimately involved in the cell cycle regulation and EBV immortalised B cells require TRRAP function for viability and cell cycle progression.

3.0. Discussion

Molecular and genetic analyses indicate that EBNA2 is essential for EBV-mediated B cell immortalisation (Hammerschmidt and Sugden, 1989, Cohen et al., 1989). The present study was undertaken to elucidate the mechanism of transactivation of cellular genes by EBNA2, a process which cannot be separated from the transformation function of this gene (Cohen et al., 1991). During the first part of this work, a comprehensive screen was performed aiming at the identification of EBNA2/EBV target genes activated in EBV immortalized B-cells. Three different techniques, differing in various technical aspects, were used to monitor changes in steady state RNA levels upon EBNA2 activation. In addition, a subset of genes was screened for the activation of run-on transcription in order to monitor transcriptional activation more directly. During the second part of this study, a single gene TRRAP, shown to be induced by the EBV/EBNA2 growth programme, was chosen for further functional analysis.

3.1 A comprehensive screen for EBNA2 target genes

Three different cellular systems were used in order to identify the target genes, and thus to define viral and cellular pathways which contribute to target gene activation.
EREB2-5, a lymphoblastoid cell line, is strictly dependent on the presence of oestrogen in the cell culture medium. Upon activation by oestrogen, EBNA2 induces a cascade of primary and secondary viral and cellular target genes, which initiate a physiological growth programme similar to B-cell activation. The advantage of the EREB2-5 system is that very early events can be monitored. In addition, the activation of EBNA2 can be performed in the absence of *de novo* protein synthesis, thus primary and secondary target genes can be distinguished by adding the protein synthesis inhibitor cycloheximide to the culture medium. It was important to identify a set of primary EBNA2 target genes, since only this set can be expected to be induced by similar mechanisms and thus, would reveal common cis-acting regulatory elements. However, since it is known that cycloheximide *per se* can also influence the steady state levels of RNA, the cycloheximide approach could not be used during the initial screening process, but rather later in the individual Northern blot experiments.

The P493-6 cell line is an EREB2-5 derivative, expressing a tetracycline regulated c-myc gene. P493-6 can switch between two growth programmes in which proliferation is either driven by either the c-Myc protein or the EBV/EBNA2 pathway. Thus, this cell line can be used to identify target genes specifically activated by the EBV/EBNA2 pathway. Target genes belonging to this pathway, irrespective of the activating mechanisms involved, might support the proliferation of EBV immortalized B-cells and therefore are interesting candidates for therapeutic intervention in the EBV associated malignancies.

BL41-K3 cells are stable transfectants of the EBV negative Burkitt's lymphoma cell line BL41 and express a conditional ER/EBNA2 protein. This cell line served as a tool in order to identify the subset of the EBNA2/EBV target genes which can be activated by EBNA2 in the absence of other viral proteins. In contrast to the experiments done with EREB2-5 in the presence of cycloheximide, in the BL41-K3 experiments *de novo* protein synthesis is permitted. Thus target genes identified in this system can either be secondary target genes induced by primary cellular target genes or primary target genes induced by EBNA2 in cooperation with cellular proteins.

As described in the *Results* section, three independent screens, performed in order to identify EBNA2 activated genes, differed in several technical features: (i) the nature of the arrayed material, (ii) the handling and labelling of one or more probes which are hybridised to the microarray and (iii) the detection system as well as algorithms used for data analysis. As summarised in Fig. 5 (*Results*, chapter 2.1.5) these three methods show a lot of diversities in every aspect mentioned above.

A comparison of all data revealed a surprisingly divergent set of EBNA2 induced genes in EREB2-5 cells two hours post-oestrogen stimulation. By criteria that any gene that is induce at least twofold (ExpressCode™ and lymphochip) or 1.4-fold (GeneChip®) is considered as an up-regulated gene, a relatively high number of genes were identified: on ExpressCode™ a total number of 52 genes, on lymphochip 94 genes and in GeneChip® 205 genes were found to be up-regulated. Surprisingly, only three genes were found to be up-regulated in all three screens: c-myc, RDC1 and CD83. The subsequent re-evaluation of target genes by the Northern blot analysis or nuclear run-on revealed that the rate of false positives within selected target gene is low. These results indicate that the limited set of genes found induced in all three settings was not due to the fact that false positive results had been generated. The

differences were rather due to variations in template dependent sensitivity towards different labelling techniques as well as variations and, the dynamic range and the detection limits of the different techniques. To demonstrate this, the relative induction levels of c-myc, RDC1 and CCR7 in the different screens are summarised in Table 11. At lest for these three genes the ExpressCode™ system appeared to be the most sensitive, followed by lymphochip and GeneChip®.

Table 11.

Sensitivity of different DNA microarray techniques.

The relative induction rates of the c-myc, RDC1 and CD83 RNA are shown for ExpressCode™, lymphochip and GeneChip at 2 hours post-oestrogen stimulation in EREB2-5. If the gene was present more then once on the microarray all values are shown. The GeneChip® experiments were done four times and all four values are shown

Gene	ExpressCode™	lymphochip	GeneChip®
c-myc	**109.3**	**10.4/7.4**	**1.4/2.2/2.1/5.0**
RDC1	**722.3**	**2.2**	**2.7/8.7/2.4/2.8**
CD83	**238.0**	**2.1**	**3.3/2.2/1.3/1.3**

3.2 Evaluation of the DNA microarray data by the nuclear run-on and Northern blot analyses

Since the molecular probes for the genes identified by the ExpressCode™ were available, a “self-made” array was generated. This “self-made” arrays contained PCR products representing 40 up-regulated genes in the proliferating cells tested on both ExpressCode™ and GeneChip®. In addition, PCR products for 14 genes up-regulated in lymphochip and Gene Chip® were generated by the amplification of gene fragments cloned into the plasmid vectors, and were spotted on the membrane. A secondary screen using a nuclear run-on technique was performed for the P493-6 cells growing in two different growth programmes. By this experiment, the target genes were sorted into EBV dependent and independent pathways.

Since all genes which were spotted onto these arrays were candidate or well known EBNA2 target genes derived from the previous screens, it would be expected that all of them generate a signal. This was not the case. Even signals of the well characterized target gene LMP1, known to be transcriptionally activated by EBNA2

were weak, indicating that the efficiency of the nuclear run-on technique can vary significantly depending on the individual gene analyzed.

Genes which seemed to belong to the EBNA2 pathway were further evaluated by the Northern blot analysis. In order to sort these genes into primary and secondary EBNA2 target genes, three cell lines were used: EREB2-5 in the presence and absence of *de novo* protein synthesis, P493-6 and BL41-K3. The results of the Northern blot analyses suggested that some of the genes are not primary EBNA2 target genes. One group, which encompasses the genes EGR-1, DUSP2, SLAM, lymphotactin, IP-10, MIP-1 alpha, PLAB, PI3K and ATF5, showed no change in gene expression in BL41-K3 thus, indicating the requirement of another cellular or viral protein for the induction of these genes. These genes were considered as secondary EBNA2 target genes and were not subjected to promoter analysis. Furthermore, two novel c-Myc target genes have been identified. CDC37 and NUDT4 were identified as genes induced downstream of c-Myc and thus were also excluded from any further promoter analysis. The rest of the genes formed three groups according to the results of the Northern blot analyses.

Group I contained the genes c-myc, CCR7 and RDC1 since these genes are induced in the absence of *de novo* protein synthesis. This indicates that these genes are primary EBNA2 target genes. The transcription factor and proto-oncogene c-myc and the chemokine receptor, a member of the G-protein-coupled receptor family, CCR7 had been shown to be primary target genes of EBNA2 (Kaiser et al., 1999, Burgstahler et al., 1995, respectively).

RDC1 is a novel primary EBNA2 target gene. It is an orphan G-protein coupled receptor. The data presented herein convincingly show a strong induction of RDC1 in EREB2-5 and BL41-K3. P493-6 cells failed to induce RDC1 according to the Northern blot analysis. Since the P493-6 cells are an EREB2-5 transfectants, there is no obvious explanation for this finding. The product of this gene is expressed in various cell types. RDC1 can act as a co-receptor for HIV (Shimizu et al., 2000), but its cellular function is not known.

Group II included two genes: bfl-1 and MIP-1 alpha. The bfl-1 protein is a member of the Bcl-2 gene family which comprises anti- and pro-apoptotic regulators. The protein encoded by this gene is able to reduce the release of pro-apoptotic cytochrome c from mitochondria and it blocks caspase activation. This gene is a direct transcription target of NF-κB (Grumont et al., 1999, Edelstein et al., 2003) in response to inflammatory

extracellular signals, such as GM-CSF, CD40, PMA, TNF and IL-1, which suggests a cytoprotective function essential for lymphocyte activation as well as cell survival. It was also shown by D'Souza et al., 2000 that the EBV protein LMP1 can transcriptionally up-regulate bfl-1. A second member of group II, the macrophage inflammatory protein 1 beta (MIP-1 beta) directly participates in the activation and directional migration of lymphocytes and monocytes to sites of inflammation. It is up-regulated in human T cells by the induction of the NF-κB pathway (Guo et al., 2002, Guo et al., 2003). Given that the induction of these genes is regulated by the NF-κB elements, which are among other factors activated by the EBV protein LMP1, it becomes clear why the induction of these genes is decreased in the absence of viral proteins in comparison to an EBV/EBNA2 situation.

Group III is formed by the genes CD21, CD83 and PLEK. CD21 is a human complement receptor type 2 (CR2). Additionally, it is the receptor for EBV and mediates EBV infection. The regulation of expression of this gene has been extensively studied (Makar et al., 1998, Ulgiati and Holers, 2001) and it will be discussed later in the context of promoter analysis. CD83 is a cell surface glycoprotein, expressed mainly on dendritic cells. It had been shown that the induction of this gene is strictly dependent on the NF-κB element (Berchtold et al., 2002). PLEK encodes a protein called pleckstrin, a major substrate of protein kinase C in blood platelets (Abrams et al., 1995) which is involved in the reorganisation of the actin skeleton (Ma and Abrams, 1999). It contains a pleckstrin homology domain (PH) which is commonly found in eukaryotic signalling molecules. The transcriptional regulation of this gene has not been described to date. All three genes are strongly induced by EBNA2. They require neither viral genes nor c-Myc to be induced. However, it is not possible to claim that they are directly regulated by EBNA2 as their mRNA is induced and/or stabilised by cycloheximide. Thus a comparison of the induction level between the activated and non-activated EBNA2 state in the presence of the protein synthesis inhibitor could not be indicative.

Although the results described above provide strong evidences that all genes belonging to group I, II or III are directly activated by EBNA2, the mechanism by which EBNA2 does so could only be indirectly addressed. Very recently, a direct biochemical approach towards the identification of target genes of a given transcription factor has been developed. This methodology identifies transcription factors bound to the chromatin of a given gene *in vivo* and is called chromatin

immunoprecipitation (ChIP). Briefly, cells are lysed, chromatin associated transcription factors and DNA are chemically crosslinked and protein/DNA complexes are immunoprecipitated. DNA fragments bound to the immunoprecipitate are quantified by PCR. This technique can now be used to identify genomic regions to which EBNA2 is bound and thus provide a direct biochemical approach by which EBNA2 target genes can be identified (Weinman et al., 2001).

3.3 A search for potential *cis*-acting elements relevant for EBNA2 function within the promoters of EBNA2 target genes

In order to define a common denominator of EBNA2 pathways, the putative promoter region (-1000 region upstream from the transcription start site) of candidate EBNA2 target genes was inspected by using complex software tools (Genomatix Software GmbH, Germany) containing a large predefined library of individual consensus transcription factor binding sites and modules.

Numerous potential *cis*-acting elements were identified. Only three of them were found in the putative promoters of all analysed genes: a TATA-box element and binding sites for Oct-1 and NFAT1.

Although EBNA2 binds weakly to the TATA-binding protein (TBP) it interacts with TAF40 through its acidic activation domain creating a potential link between EBNA2 and TBP (Tong et al., 1995a). The TATA-box-dependence of EBNA2 responsive genes had been shown in experiments by Wu et al., 2000, which demonstrated that the deletion of TATA-box element from the episomal promoters abolishes transcription.

The octamer binding protein 1(Oct-1) is a member of a POU family of homeodomain proteins which regulate the transcription of snRNA and histone H2B genes (LaBella et al., 1988, Murphy et al., 1989). The active form of Oct-1 was also observed in B cells where it acts as a regulator of Ig gene transcription during B cell development (Shah et al., 1997). The combinatorial action of the Oct-1 site and the PU.1 autoregulatory site has been shown to contribute to the activity of the PU.1 promoter itself (Chen et al., 1996). A direct interaction of the EBNA2 protein with the POU domain proteins has been described by Sjöblom et al., 1995 in the context of the LMP1 promoter. However, Sjöblom et al., 1995 excluded Oct-1 and Oct-2 as a

candidate POU-box proteins in their experiments. Thus, a direct interaction of EBNA2 and Oct-1 has never been demonstrated.

Nuclear factor of activated T cells (NFAT) transcription factors play critical roles in gene transcription during the immune response. CD40 activates the cyclosporine-sensitive, calcium regulated phosphatase calcineurin, which dephosphorylates cytoplasmic NFATs and enables them to translocate to the nucleus and thereby assist in the gene transcription (Flanagan et al., 1991). NFAT1 is required for the normal homeostasis and the differentiation of B and T cells (Peng et al., 2001). It has been shown that the NFAT1 site plays a critical role in the induction of TNF alpha by calcium ionophore (Tsai et al., 1996).

In addition to the individual roles of Oct-1 and NFAT1 as effectors during B cell development and activation, a functional synergy of these two transcription factors has been demonstrated by Zabel et al., 2002. They performed the comparative analysis of mouse and human intron 1 of the CD21 gene and identified seven areas of significant sequence homology which are located at or near sites previously identified as important for cell specific CD21 gene expression. Sequence competition and Ab supershift analyses utilizing nuclear extracts from T and B cells identified the primary constituents as Oct-1, YY1 and NFAT4, and potentially other NFAT members as well. In that complex Oct-1 and YY1 were proposed to act as regulatory elements which repress transcription in the CD21 non-expressing cells by recruiting HDAC to the regulatory elements. Association of NFATs with that complex is directly linked to the increased expression of CD21. The treatment of CD21-expressing cells with cyclosporine A, a potent and specific inhibitor of NFAT function, resulted in the specific loss of CD21 transcription (Zabel et al., 2002).

The question regarding the physiologic role of the Oct-1 and NFAT1 transcription factors in EBNA2 transactivation remains unanswered, but it will be addressed in the future work.

Within each of three groups of genes directly activated by EBNA2, there were a number of common transcription factor binding sites identified. However, the attempt to identify module common to all promoters analysed failed and only one module consisting of two different ETS family members and AP1 was identified in the promoters of RDC1 and CCR7.

3.4 A potential role of binding sites for RBP-Jκ, PU.1, NF-κB and c-Myc in promoter and intron 1 of primary EBNA2 target genes

Since there is an increasing evidence that the intronic regions might participate in the regulation of transcriptional gene regulation, the search for *cis*-regulatory elements within the EBNA2 target genes was extended beyond the promoter regions and the intron 1 region was included in all further analyses. The analysis was limited to TF binding sites for RBP-Jκ, PU.1, NF-κB and c-Myc, which had been shown to bind to intronic regions also (Henkel and Brown, 1994, Nikolajczyk et al., 1996, van Dijk et al., 1998, Makar et al., 1998, Sica et al., 1997, Jones et al., 1997, Horikawa et al., 2002, Ge at la., 2003)

A functional significance of RBP-Jκ and PU.1 sites in transactivation by EBNA2 has been previously described for the viral LMP1, LMP2A and C promoters (Ling et al., 1993, Henkel et al., 1994, Grossman et al., 1994, Waltzer et al., 1994, Zimber-Strobl et al., 1994, Laux et al., 1994, Johannsen et al., 1995). However, neither RBP-Jκ nor PU.1 alone could convey EBNA2 responsiveness to the LMP1 promoter (Ling et al., 1993, Johannsen et al., 1995). As published data show, four out of eight genes identified as EBNA2 target genes in this work require a functional NF-κB element to be efficiently transcribed. In the context of EBV infection, NF-κB has been found to be one of the major activators of transcription. During the latent growth programme, LMP1, which is an EBNA2 regulated viral gene (Kaiser et al., 1999), activates also an NF-κB signalling pathway in order to mediate the up-regulation of target genes (Hammarskjold and Simurda, 1992, Laherty et al., 1992, D'Souza et al., 2000, Dudziak et al., 2003). A well defined primary EBNA2 target gene c-myc encodes a transcription factor which, as a next link in the cascade, can also be a potential co-activator in the gene transactivation process by EBNA2.

As shown herein, RBP-Jκ binding site was only found in the putative promoter of the bfl-1 gene. The most striking result was that all analysed genes had at least one RBP-Jκ binding site in intron 1. An exception was CD83, which has a very short intron 1 of only 101 bp. A more extensive search ranging over 15 kb downstream of the TSS did not identify an RBP-Jκ binding site. Studies of Makar et al., 1998 have pointed out a critical function of RBP-Jκ as an intronic silencer of the CD21 gene. The CD21 silencer must interact with the CD21-proximal promoter for function (Ulgiati and Holers, 2001). At this point it is not known, whether the RBP-Jκ binding sites within

the intronic regions of EBNA2 target genes are functional. As a first step in order to test for a potential functional relevance of these RBP-Jκ recognition sites it should be to test whether RBP-Jκ can bind to these sites in gel retardation assays or by ChIP. For gel retardation assays the protein/DNA complexes are assembled *in vitro* and anaylsed by gel electrophoresis. In contrast, the ChIP experiments visualize binding sites for a given transcription factor *in vivo*. The next step would be to functionally inactivate the RBP-Jκ binding site in the context of the gene and ectopically express this mutagenized gene fragment in B cells. This approach had been chosen by Makar et al. (2001). They could show that inactivation of the RBP-Jκ site within the intron of the CD21 gene activates a reporter gene. Technically, this experiment was difficult since the stable transfectants of the mutagenized gene fragments had to be established in order to see a significant change in activities. Thus, the intronic silencing most likely requires a proper chromatin assembly which cannot form after the transient transfection of the gene fragment into the cell. In order to test, whether the intronic RBP-Jκ binding sites of EBNA2 target genes are relevant for EBNA2 induction, the genomic loci should be mutagenized and reconstituted on episomal vectors, which can stably replicate and associate with ordered chromatin structures in nuclei of transduced cells.

The c-Myc, RDC1 and CCR7 mRNA were detected as primary EBNA2 target genes induced by EBNA2 in the absence of *de novo* protein synthesis. Thus, in addition to EBNA2 there are minimal requirements for transcription factors in the induction of these genes. Analysed regions of the RDC1 and c-myc genes contain NF-κB binding site. The relevance of the NF-κB binding site in the activation of B cells by c-myc has been demonstrated by Grumont et al., 2002. What would activate NF-κB pathway in the absence of *de novo* protein synthesis or is there an alternative regulatory mechanism which activates c-myc transcription, and possibly RDC1, in the EBV infected B cells is still an issue to be resolved.

A direct role of the NF-κB signalling pathway has been reported in the regulation of the expression of bfl-1 (D'Souza et al., 2000, Edelstein et al., 2003), MIP-1 beta (Guo et al., 2002, Guo et al., 2003), CD21 (Tolnay M et al., 2002) and CD83 (Berchtold et al., 2002, Dudziak et al., 2003). bfl-1 was found to be transcriptionally regulated by LMP1 (D'Souza et al., 2000, Dirmeier, 2002). The requirement for co-expression of EBNA2 and LMP1 explains a very low induction of this gene in BL41-K3 cell where

no viral protein is present. A similar mechanism is probably involved in the regulation of MIP-1 beta gene expression.

Recently, it was found that CD83 and PLEK are LMP1 regulated genes (Dudziak et al., 2003 and Dirmeier, 2002, respectively). In the work presented here, it is clear that, even in the absence of any of the viral proteins, these genes are highly induced. These genes could, therefore, have two independent mechanisms of regulation or this controversy can be explained by the usage of different *in vitro* systems.

According to what is known about the regulation of the CD21 gene and the importance of RBP-Jκ site in intron 1, it is suggested that EBNA2 binding to RBP-Jκ has an important role in the activation of transcription. However, the minimal requirement for other factors in this process is still not clear.

From the expression analysis of these eight genes in the BL41-K3 cells it is evident that neither of them was induced in c-Myc-dependent manner indicating that, although present, E-box is not rate limiting for the up-regulation of these genes.

This study identified potential binding sites for either PU.1 or NF-κB in the promoters of CCR7, MIP-1 beta, CD21, CD83 and PLEK. In addition, NFAT1 and Oct-1 binding sites are present in all promoters. Thus, PU.1, NF-κB NFAT1 and Oct-1 are candidate factors which might cooperate with protein/DNA complexes bound to intronic regions in order to regulate EBNA2 target genes.

Given that these transcription factors are expressed in B cells that undergo immortalisation induced by EBV infection, there are several *in vitro* or *in vivo* approaches that could be used to confirm the binding and relevance of these proteins for EBNA2 function.

As discussed earlier, the binding of a transcription factor to its potential TF binding site within a specific gene fragment can be shown by gel retardation assays or chromatin immunoprecipitation. The functional relevance of a transcription factor binding site within a promoter region is routinely checked by the transient transfection of promoter reporter constructs of a given gene fragment. The interesting finding of *in silico* analysis of the EBNA2 target genes was the occurrence of the potential Oct-1 binding site found in all promoters. Although a direct EBNA2/Oct-1 interaction has been previously excluded by Sjöblom et al., 1995, it would be worth testing, if EBNA2 can functionally interact with Oct1.

In some cases, as discussed for intronic silencing before, stable transfectants have to be established in order to allow the necessary chromatin assembly for functional

analysis. Since all but one EBNA2 target genes might be regulated by its promoter regions as well as intronic silencing mechanisms, again episomal vectors carrying the corresponding gene fragments should be stably expressed in the cells. Within these plasmids the binding sites for the transcription factors PU.1, NF-κB, NFAT1 and Oct-1 should be mutagenized and their function for basal activity of the genes as well as activation by EBNA2 should be tested.

In addition, the function of the transcription factors could be tested by specific inhibition of their function or expression. For NF-κB and NFAT1 specific inhibitors, which block protein function are avaiable. A very attractive alternative would be to specifically destroy the transcripts for PU.1, NF-κB, NFAT1 and Oct-1 by siRNA and then test whether the transactivation by EBNA2 is abolished.

3.5 A hypothetical model for EBNA2 function

Taken together, the results of this study suggest a novel working hypothesis for the mechanism by which EBNA2 activates cellular target genes (Fig. 26). While the viral promoters activated by EBNA2 are characterised by RBP-Jκ binding sites, the majority of cellular genes activated by EBNA2 lack an RBP-Jκ binding site in their promoter region. In contrast, a potential RBP-Jκ binding site was identified in the intronic region of the majority of genes analysed in this study. The binding of EBNA2 to RBP-Jκ within intronic regions could cause alterations in chromatin structure. Thus, EBNA2 would facilitate chromatin accessibility to transcription factors, resulting in the relief of repression rather than by directly recruiting the transcription machinery. The second characteristic feature of all target promoters was a cluster of NFAT1 and Oct-1 binding sites. So far, a direct binding of EBNA2 to either of these two proteins has not been demonstrated. For genes belonging to group I, the requirement for additional factors is minimal, since transcriptional activation of these genes is successful even in the absence of *de novo* protein synthesis. A simple two step mechanism as illustrated in Fig. 26 (left part) might apply to this process. Nevertheless, a potential NF-κB binding site is shared by all promoters belonging to groups II and III. For several genes belonging to these two groups, an experimental data had been published showing that NF-κB can activate these genes via its recognition site within the promoter. Unfortunately, as discussed earlier, the relative

contribution of *de novo* protein synthesis to group III gene activation cannot be accessed for experimental reasons. This study suggest that the high level induction of group II and group III genes by EBNA2 might require NF-κB activation and therefore require *de novo* protein synthesis. Activation of cellular target genes by EBNA2 is performed by two synergistically and potentially sequentially acting mechanisms. The relief of repression due to changes in the chromatin state caused by EBNA2 are followed by the action of transcription factors, which might, although not necessarily, interact with EBNA2 directly.

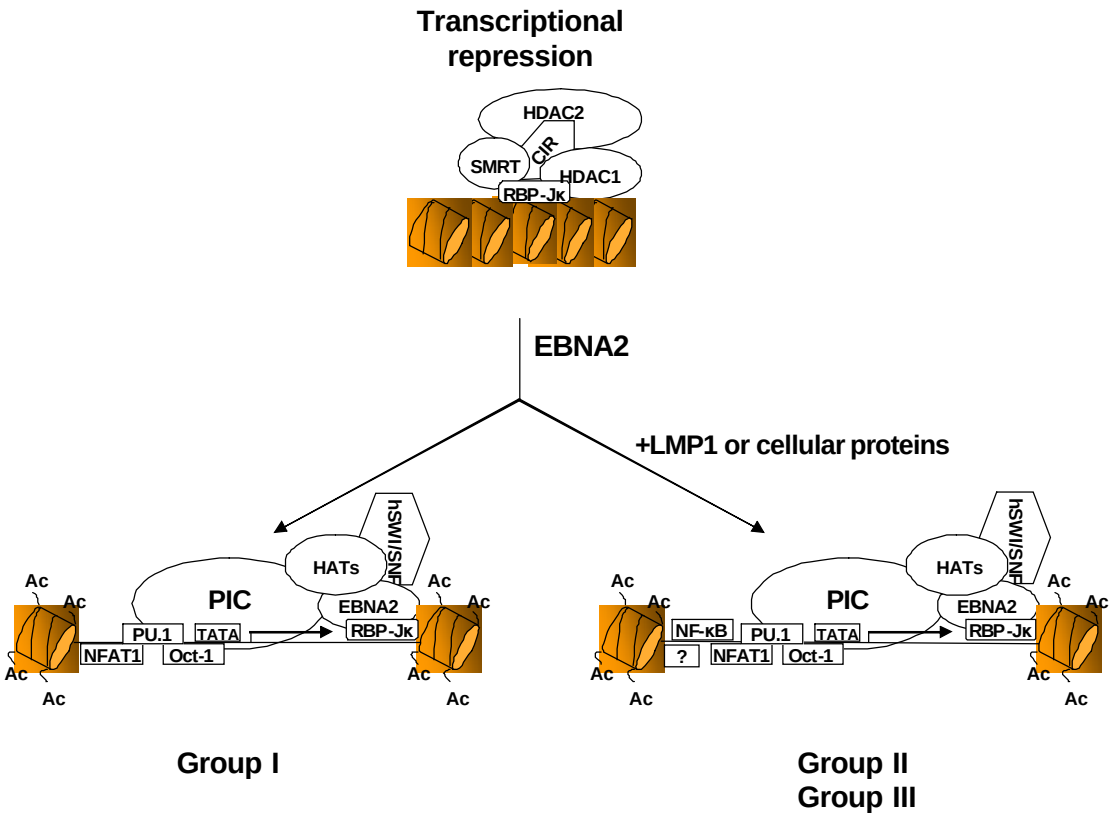


Figure 28.

A hypothetical model of transcriptional regulation by EBNA2.

Diagram outlines potential steps in the pathway from transcriptionally inactive promoter to the activation of transcription. The EBNA2-responsive promoter is tightly packed in chromatin what is mediated by RBP-Jκ and co-repressor complex bound to RBP-Jκ. EBNA2 displaces the co-repressor complex and binds to RBP-Jκ. Simultaneously, it binds co-activators responsible for chromatin modifications and remodelling. An open chromatin structure is accessible for the assembly of pre-initiation complex (PIC) and the binding of other transcription factors (TFs). In the case of c-Myc, CCR7 and RDC (i.e. group I) the requirement for other TFs is minimal and transcription is at a high level even in the absence of *de novo* protein synthesis. Genes belonging to groups II and III need additional expression of some viral or cellular gene to acquire full induction.

3.6 Induction of TRRAP is a characteristic feature of the EBV/EBNA2 controlled growth programme

3.6.1 The TRRAP protein

The TRRAP gene has been identified relatively recently (McMahon et al., 1998, Vassilev et al., 1998) as a 400 kDa cofactor for c-Myc and E1A mediated transformation. The assembly of DNA into nucleosomes and higher order chromatin represents a repressive block to transcription that must be overcome during transcriptional activation. One mechanism of alleviating chromatin repression involves the acetylation of histone tails. TRRAP is a common constituent of four mammalian multiprotein histone acetyltransferase (HAT) complexes: TFTC, STAGA, PCAF and TIP60 (Brand at al., 1999, Martinez et al., 2001, Vassilev et al., 1998, Ikura et al., 2000, respectively). These complexes acetylate not only histones, but also other proteins. So far it has been shown that TRRAP interacts with c-Myc and E2F and this interaction is necessary for the function and acetylation of these transcription factors (McMahon et al., 1998, McMahon, data published on the www.wistar.upenn.edu), as well as for p53 (Ard et al., 2002, McMahon, data published on the www.wistar.upenn.edu). According to sequence homology TRRAP belongs to the ATM protein superfamily which includes the proteins ATM, ATR, FRAP and DNA-PK. The members of this protein family are involved in cell cycle check point signalling causing cell cycle arrest, apoptosis or DNA repair. However, unlike other members of the ATM superfamily, TRRAP is not a protein kinase as judged from the lack of essential amino acids in the kinase motif. It might play a role in DNA damage/repair response as TFTC, STAGA and TIP60 HAT complexes which contain TRRAP are involved in these processes (Brand et al., 2001, Martinez et al., 2001, Ikura et al., 2000, respectively). *In vivo* evidence for TRRAP being essential for DNA repair, mitotic checkpoints and normal cell cycle progression was provided by Herceg et al., 2001. They showed that loss of TRRAP causes early embryonic lethality, block in cell proliferation accompanied by aberrant mitotic exit, abnormal nuclear morphology, cytokinesis failure and endoreduplication.

The primary sequence of TRRAP has no homology with the proteins that have been implicated in gene regulation and hence offers little insight into its precise function.

TRRAP has no apparent DNA-binding motif so it is unlikely that it binds to DNA in the absence or other factors. Extensive searches for motifs that might predict a function of TRRAP, such as acetylation, deacetylation or methylation, have proven negative to date. The lack of obvious motifs conferring biochemical activity and its unusually large size suggest that TRRAP may serve as a scaffold element for the assembly of multiprotein complexes (McMahon et al., 1998).

3.6.2 TRRAP is super-induced in EBV/EBNA2 driven proliferation

In the ExpressCode™ DNA microarray experiment described in this work, TRRAP was shown to be induced by EBNA2. EBNA2 plays a crucial role in the B cell transformation process (Cohen et al., 1989, Hammerschmidt et al., 1989) as a transactivator of both cellular and viral genes (Fahraeus et al., 1990, Zimber-Strobl et al., 1993, Ling et al., 1994, Kaiser et al., 1999). Referring to a functional necessity of TRRAP for the oncogenic transformation by c-Myc and E1A, it was of great interest to explore the mechanism and the role of TRRAP induction by EBNA2.

As a first step, the results derived from the microarray experiment were confirmed by real time PCR. EBNA2 activation mediated a strong induction of TRRAP, but failed to do so in the presence of the protein synthesis inhibitor cycloheximide. Thus, TRRAP is a secondary EBNA2 target gene.

Therefore, in the work herein a potential link between EBNA2 and TRRAP induction was searched.

The viral primary EBNA2 target gene LMP1 up-regulates a broad spectrum of genes (recently described by Dirmeier, 2002) by activating the NF-κB (Huen et al., 1995, Mitchell et al., 1995), AP-1 (Fos/Jun pathway) (Kieser et al., 1997, Kieser et al., 1999), STAT (Gires et al., 1990) and p38 MAPK (Eliopolus et al, 1999, Vockerodt et al., 2001) signalling pathways. TRRAP induction was analysed in two complementary cell lines 1852.4 (Kilger et al., 1998) and 1194-3 (Zimber-Strobl et al., 1996). The 1852.4 cells conditionally express LMP1 and constitutively express EBNA2. The cell line 1194-3 constitutively expresses LMP1, but can regulate EBNA2 function also. In both cases LMP1 expression is independent of EBNA2. Thus the effect of LMP1 on TRRAP induction could be analysed independently of EBNA2 function. The results show that: (i) LMP1 expression was not able to super-induce TRRAP above the level

induced by EBNA2 (showed in 1852.4 cells) or (ii) LMP1 *per se* was not enough to induce TRRAP in the absence of EBNA2 (1193-4 cells). This indicates that none of the LMP1 induced pathways is sufficient to activate TRRAP induction although there were STAT-1 and NF-κB binding sites found by *in silico* analysis in the putative promoter of TRRAP at positions -434 and -165, respectively (data not shown).

The involvement of other viral proteins in the induction of TRRAP by EBNA2 was tested in the cell lines BL41-K3 (BL41-ER/EBNA2) and BJAB-K3 (BJAB-ER/EBNA2) and the corresponding P3HR1 convertants (BL41-P3-9A and BJAB-P3-B6). EBNA2 *per se* was already sufficient for TRRAP induction. Viral genes, although dispensable, may additionally increase induction up to twofold. Nevertheless, LMP1 can immediately be excluded since it fails to induce TRRAP in 1852.4 cells. LMP2A has been shown to activate the phosphoinositide 3-kinase (PI3K) pathway (Swart et al., 2000) and therefore could be a potential candidate.

In summary, viral proteins facilitate TRRAP induction, but a cellular protein downstream of EBNA2 initiates the induction of TRRAP. Also, a synergistic action of cellular and viral genes in this process cannot be excluded.

Since TRRAP induction coincided with the activation of cellular proliferation in all systems tested, it was hypothesised that TRRAP induction might be associated with the cell cycle entry or cell growth. The transcription factor c-Myc, activated by EBNA2, was a candidate protein, which could mediate TRRAP induction in the context of cell cycle entry. c-Myc activates a diverse group of genes as a part of a heterodimeric complex together with its partner protein Max (Dang, 1999, Schuhmacher et al., 2001). The c-Myc-Max heterodimers are capable of binding a specific DNA sequence CACGTG, called E-box (Blackwood et al., 1991). In order to distinguish the effect of EBNA2 and c-Myc on TRRAP induction, P493-6 cells were used (explained in more detail in *Introduction*). These cells proliferate in two distinct programs: EBV/EBNA2 growth programme or c-Myc growth programme. Here, it was evident that EBNA2 is able to induce TRRAP, but c-Myc was not able to do so.

Germinal centre (GC) centroblasts strongly express c-myc and proliferate (Martinez-Valdez et al., 1996). As shown herein, primary B cells stimulated with CD40L/IL-4 also strongly express c-myc and proliferate vigorously. However, TRRAP mRNA abundance does not increase above basal level as it does in the primary B cells infected with B95.8 EBV. These results indicate that c-Myc has no influence on

TRRAP induction and also that TRRAP induction is not a phenomenon associated with cell proliferation *per se.*

Since all TRRAP induction studies had been done in established cell lines, based on the ER/EBNA2 system, a secondary test system was used in order to ensure that TRRAP induction by EBV is of general relevance. To this end, the expression of TRRAP upon EBV infection was confirmed to CD40 ligation in the presence of IL-4. Again, the induction of TRRAP was exclusively seen in EBV stimulated cells, confirming that the EBV growth programme specifically activates TRRAP.

In order to gain further insights into the mechanism by which EBV might activate TRRAP, the putative promoter region of the TRRAP gene was submitted to an *in silico* analysis. This *in silico* analysis revealed potential binding sites for numerous transcription factors (data not shown). This analysis will be helpful in the mutational analysis of the TRRAP promoter by promoter reporter assay. Transient transfection assays should define elements in the promoter which are important for TRRAP regulation by EBV/EBNA2 growth programme.

3.6.3 Functional analysis of TRRAP induction in the context of EBV infection

The next issue studied in this work was whether the TRRAP protein was a rate limiting for the growth and proliferation of EBV-infected B cells. To answer this question the TRRAP expression was knocked down in EBV immortalised B cells by the RNAi technology. The RNAi technology is sequence-specific, post-transcriptional gene silencing initiated by double-stranded RNA (dsRNA) that is homologous in sequence to the silenced gene (Fire, 1999, Hammond et al., 2001, Tuschl, 2001). To down-regulate the TRRAP protein expression, a small interfering RNA (siRNA) expression vector-based system which produces functional siRNA molecule was used. The 721 cells were cells of choice as they are LCL which can be transfected with acceptable efficiencies. 721 cells transfected with the TRRAP specific siRNA expression vector show the reduction of the TRRAP transcript and pronounced cells death as soon as 12 hours post-transfection. This effect is accompanied with a decreased number of cells in the G1 phase of cell cycle, while the numbers of cells in S or G2/M phase were not significantly modified compared with the non-transfected

cells. However, cells transfected with TRRAP specific siRNA show a reduced DNA synthesis, indicating that the cells in the S and G2 phase are rather cells in the early stage of apoptosis before DNA fragmentation and thus are not recognised as sub-G1 cells. Herceg et al., 2001 reported on TRRAP requirement for the mitotic checkpoint and the normal cell cycle progression. Work presented here confirms that finding.

Recently, it has been reported that siRNA can have non-specific effects. Sledz et al., 2003 showed the activation of the interferon system by synthetic siRNA. To test for the gene specific effect of anti-TRRAP siRNA, cells were transfected with the mutated TRRAP specific siRNA expression vector or empty vector as a control. These cells behave in a very similar manner to the non-transfected cells with negligible differences which are probably due to the fact that these cells were robustly treated during transfection and the selection process. These data clearly show that a general toxicity of vector or the activation of the interferon response due to a dsRNA can be ruled out as a cause of the biological effect described in this work.

In summary, these results indicate that TRRAP is intimately related and necessary for processes involved in proliferation and survival of LCLs.

The role of TRRAP in LCLs has not been elucidated, but according to the known facts about TRRAP there are several possible suggestions. As described above, TRRAP is a part of the HAT complexes which are the co-activators of the transcription machinery and are involved in the DNA repair process. Therefore TRRAP plays an important role in the regulation of transcription, cell cycle progression and cell viability. There is a need of TRRAP for cell cycle progression and survival of EBV-immortalised B cells since the latent EBV-driven proliferation of B cells requires constant and enormous gene activation accompanied by the enhancement of genetic instability (Gualandi et al., 2001). The effect of TRRAP knock-down in LCL resulting in cell death was not surprising as a similar effect was observed earlier in mouse embryonic fibroblasts (Herceg et al., 2001). However, the role of the elevated levels of TRRAP during B cell immortalisation by EBV remains unresolved. In contrast, the same is not needed for B cells activation by CD40L/IL-4. It would be of great interest to see whether lymphoblastoid cells would be viable if the level of TRRAP would be kept at the basal level.

Although EBV transformed LCLs are generally believed to be immortalised, a series of studies (Counter et al., 1994, reviewed in Sugimoto et al., 1999, Okubo et al., 2001) provide strong evidence that EBV infected B cells are mortal and have non-

malignant properties, except for a small proportion of cells that acquire additional genetic changes as well as constitutive telomerase activity. Telomere shorting with each cell division in the absence of telomere maintenance mechanism is suggested to function as an intrinsic clock that counts cell division and eventually causes permanent cell growth arrest in human cells (Chiu and Harley, 1997, Meyerson, 2000). Activation of telomerase is observed in ~90% of human cancers, but not in most normal somatic cells (Kim et al., 1994, Chiu and Harley, 1997, Meyerson, 2000). Biochemical and genetic studies have established an association between telomere maintenance and extended lifespan mediated through expression of human telomerase reverse transcriptase (hTERT) (Sharma et al., 2003). Telomerase is a reverse transcriptase that synthesises telomeric DNA thereby compensating for telomere loss that occurs with each replication cycle. hTERT is silent in almost all somatic cells (Greider, 1999) but is up-regulated in the vast majority of human cancer cells. c-Myc was shown to be the only transcription factor capable of interacting with the hTERT promoter and inducing its expression from the chromosomal locus (Wang et al., 1998). It was found that activation of a silent hTERT gene in exponentially growing primary human fibroblasts requires TRRAP recruitment and is accompanied by both H3 and H4 acetylation. Importantly, c-Myc mutants that lose their ability to interact with TRRAP fail to activate hTERT in primary cells (Nikiforov et al., 2002). Induction of c-Myc and TRRAP which are required for hTERT expression, and thus for maintenance of the constant telomere length, could be the prerequisite, though not sufficient, for the immortalisation of EBV infected B cells.

All studies addressing the function of TRRAP in EBV immortalised B cells were performed by transiently overexpressing TRRAP specific siRNA. In that system, high level expressing cells were selectively enriched and biologically investigated. Thus, TRRAP expression was most likely knocked-down below basal level seen in EBV-negative situation. For the future it would be more interesting to generate cellular systems, which would allow the titrating of siRNA expression and eventually testing whether TRRAP expression above basal level is required in B cells immortalized by EBV. Such cellular systems could be established by expressing TRRAP specific siRNAs as conditional systems.

4.0 Materials

4.1 Bacterial and eukaryotic cell culture reagents

Invitrogen, UK

Bacto-tryptone, bacto-yeast extract, bacto-agar, Versene, Penicillin/Streptomycin, L-glutamine

SIGMA-Aldrich, USA

Ampicillin, tryphan blue, β-oestradiol, tetracycline, cycloheximide, propidium iodide

Amersham Bioscience, Sweden

Ficoll-Paque™ PLUS

Biochrom KG, Germany

Foetal calf serum

PAA Diagnostics, Austria

RPMI 1649 (1x)

PeqLab, Germany

IPTG, X-gal

Promega, USA

Human recombinant IL-4

Clontech, USA

Tet system approved foetal calf serum

OXOID GmbH, Germany

Sheep blood

4.2 Molecular biology reagents

Roche Diagnostics GmbH, Germany

RNase, dNTP, NTP, oligo$(dT)_{15}$, proteinase K, Complete protease inhibitor cocktail, hexanucleotide mix

Invitrogen, UK

SuperScript II, *E.coli* DNA ligase, *E.coli* DNA polymerase, RNase H, T4 DNA polymerase

Promega, USA

M-MLV reverse transcriptase, Rnasin

PeqLab, Germany

Taq DNA polymerse

New England Biolabs, USA

Restriction endonucleases, alkaline shrimp phosphatase, T4 DNA ligase, DNA ladders

Amersham Bioscience, Sweden

Full range Rainbow protein marker

SIGMA-Aldrich, USA

Salmon testes shared DNA

Merck KGaA, Germany

Ethidium bromide, Coomasie Brilliant Blue

4.3 Antibodies

SEROTEC, UK

Rabbit anti-TRRAP

SIGMA-Aldrich, USA

Anti-mouse IgG-FITC conjugate

Promega, USA

Anti-rabbit IgG HRP conjugated

Roche Diagnostics GmbH, Germany

Mouse anti-DIG antibody AP conjugated

Elisabeth Kremmer, GSF, Germany

Mouse anti-NGFR HB8737

Dianova, Norway

Human anti-CD19-PE/CD3-FITC

4.4 Radioactive isotopes

Amersham Pharmacia Biotech, Germany

[α-^{32}P] dCTP (3000Ci/mmol, 10 mCi/ml)

[methyl-^{3}H]Thymidine (1 mCi/ml)

4.5 Disposables and kits

Invitrogen, UK

Agarose

Roche Diagnostics GmbH, Germany

DIG DNA Labeling mix, LightCycler-FastStart DNA Master SYBR Green I, LightCycler capillaries, CSPD, BrdU ELISA

Qiagen, Germany

QIAquick Gel Extraction kit, Rneasy Midi, Rneasy Mini

Clontech, USA

ExpressHyb solution

Amersham Bioscience, Sweden

Hybond-N+, NICK-columns, ECL™ kit

Affymetrix Inc., USA

GeneChip Human Genome U133A

Millipore, USA

Microcon-YM50 columns, Immobilion PVDF membrane

Enzo Diagnostics Inc., USA

RNA Transcript Labelling Kit

Whatman Ltd., USA

3MM-paper

Bio-Rad Laboratories, UK

Electroporation cuvettes 4 mm

Packard Bioscience GmbH, Germany

Uni-Filter 96 GF/C

Renner GmbH, Germany

0.8 µm filter

Sartorius, Germany

0.45 µm filter, 0.2 µm filter

Machery Nagel, Germany

NucleoSpin Plasmid kit

Genomed, Germany

JET STAR kit

Miltenyi Biotec, USA

MACS separator, MACS goat anti-mouse IgG microbeads, MACS LS separation columns

Eastman Kodak Company, USA

Biomax MR-1 X-ray film

Plastic laboratory wares were purchased from *NUNC GmbH, Germany, Greiner GmbH, Germany, Beckton-Dickinson GmbH, Germany and Eppendorf, Germany.*

4.6 Chemical reagents

Chemical reagents were purchased from *Merck KgaA, Germany, SIGMA-Aldrich, USA and ICN, USA.*

4.7 Laboratory equipments

Bio-Rad Laboratories, USA

Electroporator

PeqLab, Germany

Gel electrophoresis units (DNA, RNA), UV-transiluminator

Amersham Bioscience, Sweden

Gel electrophoresis units (proteins) ″Mighty Small″

Bioscan Inv., USA

Radioisorope counter QC-4000

Biometra GmbH, Germany

Spectrophotometer

Atomic Energy of Canada Ltd., Canada

Gammacell 40

SIGMA Centrifuges GmbH, Germany

6K10 centrifuge, 2K15 centrifuge

Roche Diagnostics GmbH, Germany

LyghtCycler

Heraeus Instruments, Germany

Fume hood work bench, eukaryotic cell incubator

Raytest GmbH, Germany

FUJI FILM BAS-1800 phosphoimager

Packard Bioscience GmbH, Germany

Micro Plate Scintilation Counter

ThermoLifeSciences, Germany

-80 °C freezer

Perkin Elmer, Germany

PCR System 9600

Beckton-Dickinson GmbH

Flow cytometer FACSan

Eppendorf, Germany

5415D centrifuge, Thermomixer 5436

MWG Biotech, Germany

Hybaid hybridisation oven

SAVANT, USA

SpeedVac Concentrator

Sorvall Instruments, USA

RC5C centrifuge

Eastman Kodak Company, USA

X-ray film developer

New Brunswick Scientific, USA

C25 bacteria shaker

Sterling Diagnostic Imaging, USA

Quanta Rapid exposure cassette

4.8 Bacteria

DH5α	*E.coli* strain, purchased from *Invitrogen, UK*.

4.9 Eukaryotic cell lines

EREB2-5	a human, EBV-positive lymphoblastoid cell line, oestrogen dependant (Kempkes et al., 1995a)
P493-6	a stable transfectant of EREB2-5 with tetracycline-regulated myc expression construct (Pajic et al., 2001)
BL41-K3	a human, EBV-negative Burkitt's lymphoma cell line BL41 stably transfected with the ER/EBNA2 construct (Kempkes et al., 1995b)
BL41-P3-9A	a human, EBV-positive Burkitt's lymphoma cell line BL41-P3HR1 stably transfected with the ER/EBNA2 construct (Kempkes et al., 1995b)
BJAB-K3	a human, EBV-negative lymphoblastoid cell line BJAB stably transfected with the ER/EBNA2 construct (Kempkes et al., 1995b)
BJAB-P3-B6	a human, EBV-positive lymphoblastoid cell line BJAB-P3HR1 stably transfected with the ER/EBNA2 construct (Kempkes et al., 1995b)
1852.4	a human, EBV-positive lymphoblastoid cell line infected with a mini-EBV carrying the Tet-inducible LMP1 gene (Kilger et al., 1998)
1194-3	a human, EBV-positive lymphoblastoid cell line infected with a mini-EBV carrying an oestrogen-dependent EBNA2 and a constitutive LMP1 gene (Zimber-Strobl et al., 1996)
721	a human, EBV-positive lymphoblastioid cell line generated by infection of human B cells by B95.8
B95.8	a marmoset, EBV-positive B cell line (Sample et al., 1990)
L-CD40L	a murine fibroblast cell line Ltk stably transfected with the human CD40 ligand gene (Garrone et al., 1995)

4.10 Oligonucleotides

All oligonucleotides were manufactured by *Metabion GmbH, Germany*.

Primers used in real-time PCR:

TRRAP cDNA:

MS119	5’ GGCCTGGAAGGTGAACATGTACCG 3’
MS120	5’ GCATGAACCCCAAGCATAGCGTTA 3’

c-myc cDNA:

MS331	5’ ATCATCATCCAGGACTGTATGTGG 3’
MS332	5’ CTCTTTTCCACAGAAACAACATCG 3’

LMP1 cDNA:

MS335	5’ GGTGTTCATCACTGTGTCGTTGTC 3’
MS336	5’ GCTACTGTTTTGGCTGTACATCGT 3’

GAPDH cDNA:

MS151	5’ AGAACATCATCCCTGCCTCTACTG 3’
MS152	5’ TGTCGCTGTTGAAGTCAGAGGAGA 3’

Primers used for the sequencing and DIG-labelling of inserts cloned into the pUC19 vector:

M13 for	5’ GCTGGCGAAAGGGGGATGTG 3’
M13 rev	5’ CACTTTATGCTTCCGGCTCG 3’

Oligonucleotide used in the procedure of probe labelling for hybridisation of GeneChip®:

MSaffy dT	5’GGCCAGTGAATTGTAATACGACTCAC TATAGGGAGGCGG$(T)_{18}$ 3’

siRNA oligonucleotides:

MS537 5’ CG TGTCACGTCATCTCCTCAG TTTAGAGA CTGAGGAGATGACGTGACA TTTTT GAATTCATTATT 3’

MS537/mm 5’ CG TGTCACGTCGTCTCCTCAG TTTAGAGA CTGAGGAGACGACGTGACA TTTTT GAATTCATTATT 3’

Primers for the amplification and DIG-labelling of cDNA fragments:

bfl-1

MS524 bfl-1 for 5’ ATTATTGAATTCACCAGCCTCCATGTATCA 3’

MS524 bfl-1 rev 5’ ATTATTGAATTCCATCCAGCCAGATTTAGG 3’

MIP-1 beta

MS524 mip-1 b for 5’ ATTATTGAATTCAGCTTCTGAGTTCTGCAG 3’

MS524 mip-1 b rev 5’ ATTATTGAATTCCCCCATAGGACACTTATC 3’

PLAB

MS524 plab for 5’ ATTATTGAATTCAAGATTCGAACACCGACC 3’

MS524 plab rev 5’ ATTATTGAATTCGTCATCATAGGTCTGGAG 3’

PI3K

MS524 pi3k for 5’ ATTATTGAATTCTACAGAACACAGAGCTCC 3’

MS524 pi3k rev 5’ ATTATTGAATTTCCATACCGTTGTTGGCTAC 3’

SLAM

MS524 slam for 5’ ATTATTGAATTCAATTCAGACAGCCTCTGC 3’

MS524 slam rev 5’ ATTATTGAATTCTGGACTTGGGCATAGATC 3’

DUSP2

MS524 dusp2 for 5’ ATTATTGAATTCCTTTGCCCTACCTGTTCCT 3’

MS524 dusp2 rev 5’ ATTATTGAATTCAAGGCTCACAGACAGACA 3’

Lymphotactin

MS524 lymph for 5’ ATTATTGAATTCATGAGACTTCTCATCCTG 3’

MS524 lymph rev 5’ ATTATTGAATTCGTCACAGCTGTATTGGTC 3’

EGR-1

MS524 egr-1 for 5’ ATTATTGAATTCCTATACTGGCCGCTTTTC 3’

MS524 egr-1 rev 5’ ATTATTGAATTCCTTCCACAGATGTCGCAG 3’

IP-10

MS524 ip-10 for 5’ ATTATTGAATTCTACCTCTCTCTAGAACCG 3’

MS524 ip-10 rev 5’ ATTATTGAATTCTTCCAGGAGAGTACCTCA 3’

MIP-1 alpha

MS524 mip-1 a for 5’ ATTATTGAATTCAGAATCATGCAGGTCTCC 3’

MS524 mip-1 a rev 5’ ATTATTGAATTCGCATCCGATACACATTTG 3’

4.11 Plasmids used during this work

pUC19:

a small, high copy number *E.coli* plasmid.

pBJ2:

an expression vector containing the U6 promoter, EBNA1 ORF, the EBV origin of replication and the resistance genes for ampicillin and puromycin (a gift from Berit Jungnickel, GSF, Germany).

pBJ3:

an expression vector containing the U6 promoter, an NGFR cassette, EBNA1 ORF, the EBV origin of replication and the resistance genes for ampicillin and puromycin (a gift from Berit Jungnickel, GSF, Germany).

p432:
a pUC19 derived plasmid containing a fragment of the LMP1 gene (a gift from Wolfgang Hammerschmidt).

pBT34/2:
a pUC19 derived plasmid containing an ORF of the IgM gene (a gift from Bettina Kempkes).

The following plasmids were derived from the linker cloning of the cDNA libraries into pUC19 (obtained from GPC Biotec AG, Germany). They were either amplified or DIG-labelled with the M13 for and M13 rev primers. Subsequently, they were either spotted onto "self-made" arrays or used as probes for the Northern blot analysis, respectively

Clone 11A7	contains a fragment of the gene for 30S ribosomal protein
Clone 11E6	contains a fragment of the gene for 30S ribosomal protein
Clone 11K11	contains a fragment of the RDC1 gene
Clone 12A22	contains a fragment of the eIF1 gene
Clone 12B24	contains a fragment of the FJH1gene
Clone 12E17	contains a fragment of the NMT1gene
Clone 12H13	contains a fragment of the CD83gene
Clone 12K22	contains a fragment of the tubulin alpha gene
Clone 12M13	contains a fragment of the gene for G protein beta subunit
Clone 12N2	contains a fragment of the SUPTH6 gene
Clone 13L11	contains a fragment of the tubulin alpha gene
Clone 14K13	contains a fragment of the MDH2 gene
Clone 15K19	contains a fragment of the TRAP2 gene
Clone 15K5	contains a fragment of the GCN1 gene
Clone 15L16	contains a fragment of the prothymosin alpha gene
Clone 15N14	contains a fragment of the prohibitin gene
Clone 16E18	contains a fragment of the isocitrate dehydrogenase gene
Clone 16F3	contains a fragment of the eIF4A gene
Clone 16I9	contains a fragment of the INSIG1 gene
Clone 16M14	contains a fragment of the LAK-4P gene

Clone 17D12	contains a fragment of the prothymosin alpha gene
Clone 17G5	contains a fragment of the prothymosin alpha gene
Clone 17J22	contains a fragment of the GOS24 gene
Clone 18L6	contains a fragment of the DKFZp434G012 cDNA
Clone 20G22	contains a fragment of the DKFZp762A227 cDNA
Clone 20L19	contains a fragment of the NUDT4 gene
Clone 20L2	contains a fragment of the TCP1 gene
Clone 21B16	contains a fragment of the c-myc gene
Clone 21F17	contains a fragment of the CD37 gene
Clone 21N4	contains a fragment of the spermidine synthetase gene
Clone 3D16	contains a fragment of the SUPTH6 gene
Clone 3F23	contains a fragment of the p18 gene
Clone 3K5	contains a fragment of the Ser hydroxymethyltransferase 2 gene
Clone 3L14	contains a fragment of the PTB-associated splicing factor gene
Clone 4C16	contains a fragment of the p38 MAPK gene
Clone 4N23	contains a fragment of the NUDT4 gene
Clone 5A5	contains a fragment of the ATFx gene
Clone 6B15	contains a fragment of the PLEK gene
Clone 8H4	contains a fragment of the ATF5 gene
Clone 9J10	contains a fragment of the CDC37 gene

4.12 Plasmids generated for this work

pMS122:
a pUC19 derived plasmid containing an ORF of the CCR7 gene.

pMS123:
a pUC19 derived plasmid containing an ORF of the CD21 gene.

pMS540/1
a pUC19 derived plasmid containing a fragment of the MIP-1 alpha gene amplified with the pair of primers MS524 mip-1 a for/MS524 mip-1a rev.

pMS540/2
a pUC19 derived plasmid containing a fragment of the SLAM gene amplified with the pair of primers MS524 slam for/MS524 slam rev.

pMS540/3
a pUC19 derived plasmid containing a fragment of the PI3K gene amplified with the pair of primers MS524 pi3k for/MS524 pi3k rev.

pMS540/4
a pUC19 derived plasmid containing a fragment of the DUSP2 gene amplified with the pair of primers MS524 dusp2 for/MS524 dusp2 rev.

pMS540/5
a pUC19 derived plasmid containing a fragment of the bfl-1 gene amplified with the pair of primers MS524 bfl-1 for/MS524 bfl-1 rev.

pMS540/6
a pUC19 derived plasmid containing a fragment of the MIP-1 beta gene amplified with the pair of primers MS524 mip-1 b for/MS524 mip-1 b rev.

pMS540/7
a pUC19 derived plasmid containing a fragment of the EGR-1 gene amplified with the pair of primers MS524 egr-1 for/MS524 egr-1 rev.

pMS540/8
a pUC19 derived plasmid containing a fragment of the lymphotactin gene amplified with the pair of primers MS524 lymph for/MS524 lymph rev.

pMS540/9
a pUC19 derived plasmid containing a fragment of the IP-10 gene amplified with the pair of primers MS524 ip-10 for/MS524 ip-10 rev.

pMS540/10
a pUC19 derived plasmid containing a fragment of the PLAB gene amplified with the pair of primers MS524 plab for/MS524 plab rev.

pMS500/1
a plasmid generated by the insertion of the MS537 oligonucleotide in the *PmlI/EcoRI* linearised pBJ2.

pMS500/2
a plasmid generated by the insertion of the MS537/mm oligonucleotide in the *PmlI/EcoRI* linearised pBJ2.

pMS537
a plasmid generated by the insertion of the NGFR expression cassette in the *XhoI/BamHI* linearised pMS500/1.

pMS537/mm
a plasmid generated by the insertion of the NGFR expression cassette in the *XhoI/BamHI* linearised pMS500/2.

4.13 WWW provided software

BLASTN programme	www.ncbi.nlm.nih.gov/blast
GEMS Launcher	www.genomatix.de
The design of PCR primers	www.alces.med.umn.edu/rawprimer.html
The design of the siRNA sequences	www.oligoengine.com

5.0 Methods

5.1 Bacterial cell culture

5.1.1. Cultivation and maintenance of bacteria

Bacteria were cultured in the liquid LB-medium at 37 °C with agitation, usually overnight (approximately 16 hours). The volume of the culture was adjusted according to downstream application. If a single colony was required, bacteria were streaked out on the solid LB-agar plates and kept at 37 °C overnight.

Transformed bacteria were selected for the antibiotic resistance of the transformed plasmid through the addition of specific antibiotic required. If bacteria were transformed with a plasmid containing the *lacZ* gene (e.g. pUC19) they were grown on LB-agar plates containing isopropyl β-D-thiogalactosidase (IPTG) and X-gal (a synthetic analogue of lactose). This process facilitated the distinction of transformants containing a plasmid that carries an insert in *lacZ* (white colonies, indicating non-functional β-galactosidase) versus transformants with an empty plasmid (blue colonies, indicating functional β-galactosidase).

LB-medium:	20 mM $MgSO_4$; 10 mM KCl; 1 % Bacto-Tryptone; 0.5 % Bacto-yeast extract; 0.5 % NaCl
LB-agar:	20 mM $MgSO_4$; 10 mM KCl; 1 % Bacto-Tryptone; 0.5 % Bacto-yeast extract; 0.5 % NaCl; 1.2 % Bacto-agar
Ampicilin:	100 µg/ml (for liquid LB-medium) 50 µg/ml (for LB-agar plates)
IPTG:	40 µl of the 100 mM stock solution spread evenly on LB-agar plate
X-Gal:	40 µl of the 40 mg/ml stock solution (in dimethylformamide) spread evenly on LB-agar plate

5.1.2 Preparation of competent *E.coli*

The following procedure yields competent cultures of *E.coli* that can be transformed at frequencies >5 x 10^8 transformed colonies per microgram of supercoiled plasmid DNA.

Bacteria were streaked on the surface of LB-agar plate directly from a frozen stock and incubated at 37 °C overnight. A single bacterial colony was transferred into 5 ml of liquid LB-medium and grown at 37 °C overnight. The fresh overnight culture was diluted 1:100 in LB-medium and grown at 37 °C with shaking to an OD_{595}=0.15- 0.2. Bacteria were kept on ice for 10 min and then centrifuged at 1 100 x g for 15 min at 4 °C. Pellet was gently resuspended in 1/25 volume of the initial volume of culture of ice-cold TFB I. Suspension was kept on ice for 10 min and centrifuged at 1 100 x g for 15 min at 4 °C. Pellet was resuspended in 1/8 V of the initial volume of culture of ice-cold TFB II. Suspension was kept on ice for 10 min and 0.5 ml aliquots were stored at -80 °C.

TFB I: 30 mM KAc; 50 mM $MnCl_2$; 100 mM KCl (or RbCl); 10 mM $CaCl_2$; 15 % glycerol

TFB II: 10 mM MOPS; 10 mM $CaCl_2$; 10 mM KCl (or RbCl); 15 % glycerol

5.1.3 Introduction of plasmid DNA into bacteria

For the transformation of 100 µl of competent bacteria a ligation mixture or 100 ng of plasmid DNA were used. Suspension of plasmid and bacteria was incubated on ice for 30 min, heat shocked at 42 °C for 1.5 min and re-incubated on ice for 2 min. Bacterial suspension was kept at room temperature for 10 minutes. 0.9 ml of LB medium was added to the bacteria and they were incubated at 37 °C for 1 to 2 hours to acquire the antibiotic resistance encoded by the transformed plasmid. Following transformation, bacteria were centrifuged at 1 100 x g. Supernatant was decanted and pellet was resuspended in 100 µl of the fresh LB-medium. Bacteria were plated on LB-agar plate containing the selective antibiotic and incubated at 37 °C overnight.

5.1.4 Isolation of plasmid DNA

Essentially, all three methods used for the isolation of plasmid DNA are based on the SDS/alkaline lysis method (Sambrook et al., 1989), but are modified according to the yield and the purity of the plasmid DNA preparation required for the specific application.

5.1.4.1 Small-scale preparation of plasmid DNA

For small-scale preparation of plasmid DNA two methods were used.
If there were no special requirements for a high quality of the isolated plasmid DNA (e.g. digestions following cloning procedure), the following method was used. Single colonies obtained from LB-agar plates were inoculated into 5 ml LB-medium containing selective antibiotic and kept at 37 °C overnight with agitation. 1.5 ml of the overnight culture was pelleted and the supernatant was removed. Pellet was resuspended in 200 µl of Solution I until suspension was homogeneous. The equal volume of Solution II was added. Mixture was gently mixed by turning the tube upside-down and this mixture was kept at room temperature for 5 min. Solution III was subsequently added for neutralisation and the mixture was gently mixed. This mixture was centrifuged at 4 °C for 10 min at >4 600 x g. The 1 volume of isopropanol was added to the supernatant and re-centrifuged at 4 °C for 30 min at >4 600 x g. The precipitated plasmid DNA formed a visible white pellet. Pellet was washed twice with 300 µl of 70 % ethanol, air-dried and dissolved in 50 µl of water.
For a small-scale preparation of a highly pure plasmid DNA (for sequencing, cloning etc.) a NucleoSpin Plasmid kit was used according to the manual. Briefly, the bacterial pellet was obtained as described above. The homogeneous bacterial suspension containing RNase was lysed by adding lysis solution and neutralised by addition of the neutralisation solution. Mixture was centrifuged at 4 °C for 10 min at >4 600 x g. Supernatant containing DNA was loaded onto a silica membrane packed in a column. After several centrifugation steps in which a DNA bound to a column was washed and precipitated, the elution step was done by applying 50 µl of water to a column and centrifuging.

Solution I: 50 mM Tris-HCL, pH 8.0; 10 mM EDTA
Solution II: 200 mM NaOH; 1 % (w/v) SDS
Solution III: 3.1 M K-acetate, pH 5.5

5.1.4.2 Large-scale preparation of plasmid DNA

If large amounts of a highly pure plasmid DNA were required (for cloning, transfection etc.) a JET STAR plasmid purification system based on an anion exchange separation was used according to instructions supplied by manufacturer. Briefly, a bacterial culture was prepared by inoculating 1 ml of 5 ml overnight suspension culture in 400 ml of LB-medium with the selective antibiotic. Following overnight incubation at 37 °C with agitation the bacteria were centrifuged at 1 100 x g for 15 min. Pellet was resuspended in solution containing RNase and lysed in 1 V of SDS/NaOH solution for 5 min. Mixture was neutralised by adding 1 V of neutralising solution containing K-acetate. Suspension was centrifuged and the supernatant was applied to a previously equilibrated column. A column was allowed to empty by gravity flow and washed. Elution has been done with a solution containing Tris-HCl, pH 8.5. DNA was precipitated with 0.7 V of isopropanol and centrifuged at 4 °C and > 4 600 x g for 30 min. The plasmid DNA pellet was washed with 70 % ethanol and re-centrifuged. Air-dried pellet was dissolved in 200 µl of water.

5.2 Eukaryotic cell culture

5.2.1 Cultivation of eukaryotic cells in culture

Eukaryotic cells were cultured in either suspension or as adherent cells in complete medium at 37 °C in a 6 % CO_2 atmosphere. Cells in suspension were usually split every third day 1:3 with the fresh complete medium so that the final density of the cells was 5 x 10^5cell/ml. Some cells used in this work required additional supplements in order to grow: oestrogen or tetracycline. In some experiments cells were grown in the presence of the protein synthesis inhibitor cycloheximide.

Adherent cells CD40L-L were treated with 1 ml of Versene solution per approx. 15 cm^2 of the growing surface. Cells were detached from the flask surface with the splash of complete medium and washed from Versene in 5 V of complete medium at 310 x g for 6 min. These cells were split 1:6 every third day.
The living cells were distinguished from the dead cells by tryphan-blue exclusion. Cell suspension was diluted 1:1 with 0.4 % tryphan-blue solution and counted in the improved Neubauer chamber. Dead cells were identified by blue staining, which is caused by the incapability of dead cells to exclude the dye.

Complete medium:	RPMI 1640-medium; 100 U/ml penicillin; 100 µg/ml streptomycin; 2 mM L-glutamine; 10 % foetal calf serum (FCS)
Medium with oestrogen:	1 µM β-oestradiol in complete medium
Medium with tetracycline:	1 µg/ml tetracycline in complete medium
Medium with cycloheximide:	50 µg/ml cycloheximide in complete medium

5.2.1.1 Activation of the ER/EBNA2 fusion protein by oestrogen

Cells were grown in complete medium supplemented with oestrogen up to density of 1 x 10^6 cells/ml and then harvested. Pellet was washed three times in PBS supplemented with 10 % FCS. Cells were then counted and seeded at density 5 x 10^5 cells/ml in the plain complete medium. Oestrogen was re-added to the culture 72 hours later and cells were further treated according to the experimental conditions.

PBS, pH 7.3: 137 mM NaCl; 2.7mM KCl; 4.3 mM Na_2HPO_4 x 7 H_2O; 1.4 mM KH_2PO_4

5.2.2 Transfections of 721 lymphoblastoid cells

Recombinant plasmids are introduced into eukaryotic cells by electroporation. 721 cells are lymphoblastoid cells and were grown up to a density of 1 x 10^6 cells/ml. Cells were harvested, washed once in PBS and resuspended at density 4 x 10^7 cells/ml. 1 x 10^7 cells was added to sufficient amount of vector and electroporated in the 4 mm electroporation cuvette at 220 V/950 µF. Immediately after electroporation

the cells were resuspended in 1 ml of complete medium and transferred into 9 ml of the fresh complete medium. Cells were cultured under the standard conditions.

5.2.3 *Ficoll-Paque* cell separation

Ficoll-Paque™ PLUS (Pharmacia, USA) was used for simple and rapid lymphocyte separation from cell debris, dead cells and other types of cells. Suspension of cells was layered onto a Ficoll-Paque™ PLUS in 1:1 ratio and centrifuged at room temperature for 30 min at 400 x g with a slow acceleration and deceleration. The interphase containing lymphocytes was collected and washed with PBS three times at 380 x g, 350 x g and 310 x g, respectively. The final cell suspension was resuspended in an appropriate volume of complete medium.

5.2.4 Cell sorting on magnetic microbeads

MACS colloidal super-paramagnetic microbeads conjugated to goat anti-mouse IgG $F(ab)_2$ fragments (Miltenyibiotec, USA) were used for the positive selection of cells labelled with primary mouse IgG antibodies according to the instruction of the manufacturer. Before separation, a cell suspension was subjected to density gradient centrifugation using Ficoll-Paque™ PLUS to remove the dead cells and cell debris. Cells were labelled with primary mouse IgG antibody. Subsequently, cells were incubated with with goat anti-mouse IgG coupled microbeads and separated on a column which was placed in the magnetic field of a MACS separator. Cells adhering to magnetic beads were retained in the column while the unlabeled cells ran through. Thus, unlabelled cells were depleted from the labelled cells. After removal of the column from the magnetic field, the magnetically retained cells were eluted as a positively selected cell fraction.

5.2.5 Indirect immunofluorescence labelling

Usually, 1 x 10^6 cells were washed once with PBS and resuspended in10 µl of FACS buffer containing a sufficient amount of the primary antibody. After 30 min on ice, cells were washed twice at 310 x g, resuspended in 10 µl of FACS buffer containing a sufficient amount of the secondary antibody and incubated for another 30 min on ice. The secondary antibody was a fluorochrome-labelled antibody directed towards the primary antibody. Finally, cells were washed twice with FACS buffer and resuspended in 1 ml of FACS buffer and analysed by flow cytometry.

FACS buffer: PBS with 0.1 % sodium azide; 2 % FCS

5.2.6 Cell fixation and DNA staining for cell cycle analysis

This method of DNA staining utilizes ethanol to fix the cells and permeabilize the membrane, which allows the propidium iodide dye (PI) to enter the cells. Cells were pelleted and resuspended in 500 µl of PBS and chilled on ice for 10 min. The cold cell suspension was rapidly mixed with the 1 volume of ice-cold absolute ethanol and kept on ice for 15 min. Subsequently, cells were centrifuged for 3 min at 310 x g, supernatant was carefully removed and the RNase solution was added followed by incubation at 37 °C for 15 min. The PI solution was added and the cells were allowed to stand at room temperature at least 30 min. Cells were analysed on the flow cytometer using the FL3 channel detection (620 nm).

RNase solution: 20 µg/ml RNase in PBS
Propidium iodide (PI) solution: 50 µg/ml propidium iodide (PI) in PBS

5.2.7 Isolation of primary B cells

Primary B cells were isolated from the human tonsils and separated from the rest of tonsillar cells by the method of sheep red blood cells (SRBC) rosetting. Tissue was minced, then teased into a single cell suspension through the net and layered onto

Ficoll-Paque™ PLUS cushion. The lymphocyte fraction was isolated as described above. The lymphocyte suspension was diluted to 1 x 10^7 cells/ml and mixed with 1:50 volume of sheep blood and incubated at 37 °C for 10 min. The cell suspension was transferred on ice and incubated for another 50 min. Subsequently, the cell suspension was again layered onto a Ficoll-Paque™ PLUS cushion followed by centrifugation. The interphase contained >95 % B cells while the SRBC-T cell rosettes were clumped at the bottom. The purity of the cell populations before and after separation was confirmed by flow cytometry using the CD19-specific phycoerythrin conjugated and CD3-specific fluorescein-isothiocyanate conjugated monclonal antibodies.

5.2.8 EBV infection of B cells

EBV was obtained from B95.8 cells. Cells were grown in complete medium to the density of 1 x 10^6 cells/ml and were kept in culture for another five days until medium was completely yellow. Cells and cellular debris were removed from the virus by centrifugation at 310 x g followed by filtration through a 0.8-µm filter. Primary B cells were resuspended in the viral supernatant. Cells were cultured under the standard conditions. After 24 h, half of the medium was replaced by the fresh medium.

5.2.9 Cultivation of primary B cells on the CD40L monolayer

CD40L cells grow as adherent cells. At confluence >80% these cells were irradiated with 6 000 rad and washed with PBS. Primary B-cells, isolated as described above, were resuspended in complete medium containing 100 U/ml of the human recombinant IL-4 and seeded on the CD40L monolayer at density 1 x 10^6 cells/ml.

5.2.10 DNA synthesis and cell proliferation assays

Methods which measure the DNA synthesis rate were used for the determination of S phase entry, cellular proliferation, viability and activation.

5.2.10.1 BrdU Enzyme Linked Immunosorbent Assay

5-bromo-2'-deoxy-uridine (BrdU) incorporates into DNA during DNA synthesis. The amount of incorporated BrdU can be easily quantified using monoclonal antibody against BrdU by enzyme linked immunosorbent assay (ELISA). The experiment was done according to the instructions of the kit used herein. Briefly, cells were cultured in triplicate in 96-well plate in the presence of BrdU for 4 hours. Cells were spun down at 310 x g for 10 min in a centrifuge with a rotor device for microtiter plates. Supernatant was removed and the cells were fixed in fixative at -20 °C for 30 min. Subsequently, the fixative was removed and cells were treated with nuclease at 37 °C for 30 min. Cells were washed, the anti-BrdU antibody conjugated with peroxidase was added and incubated at 37 °C for 30 min, cells were washed again and a peroxidase substrate was added. The incubation of the reaction with a substrate was determined by the coloured reaction of the control cells and ranged between 20 and 45 minutes. Extinction of the samples was measured in a microtiter plate reader at 465 nm. Culture medium alone and cells incubated with the peroxidase-labelled anti-BrdU antibody in the absence of BrdU were used as controls for non-specific binding.

Fixative: 70 % ethanol; 0.5 M HCl

5.2.10.2 ^{3}H-thymidine incorporation assay

As an alternative to the BrdU ELISA the incorporation of radioactive ^{3}H-thymidine into DNA was performed when a high sensitivity was required. Cells were cultured in triplicate cultures in 96-well plates and pulsed for 4 h with 0.5 µCi of ^{3}H-thymidine per 150 µl of culture. Cells were lysed with water and the cell content was harvested on Uni-Filterplates-96 which immobilises DNA. Plates were dried and taped at the bottom. 25 µl of the scintillation fluid was added to each sample. Plates was taped at the top and measured in a Micro Plate Scintilation Counter.

5.3 Molecular biology techniques

5.3.1 Polymerase chain reaction

Polymerase chain reaction (PCR) is an in vitro method for the enzymatical synthesis of defined sequences of DNA. It uses sequence-specific oligonucleotide primers that hybridize to complementary strands and flank the target DNA sequence which is to be amplified. The elongation of the primers is catalysed by DNA polymerase. In this work, reactions were performed in either 100 or 50 µl volume. In the case of multiple reactions a master mix containing all components was prepared and divided in the separate PCR tubes. The amount of template was variable and depended on DNA used as a template: for the genomic DNA 100 ng was used, for plasmid DNA 20 ng and for cDNA 3 µl of reverse transcription reaction. The amplification was performed in a PCR System 9600 cycler machine. A standard PCR consisted of initial denaturation performed at 93 °C for 3 min, followed by the multiple rounds of reactions (30-40 cycles) with the conditions as follows: denaturation at 93 °C for 1 min, primer annealing at temperature usually 3-6 °C lower then the Tm of primers for 30 s and elongation at 72 °C for 1 to 2 min depending on the size of the amplified product. The final step of every PCR was final elongation at 72 °C for 7 min.

PCR Master Mix: 1 x PCR buffer (including 0.15 mM $MgCl_2$); dNTP mix (0.1 mM of each dNTP); 0.25 mM $MgCl_2$; 0.1 µM primer A; 0.1 µM primer B; 5 U Taq DNA polymerse; variable amount of template; water up to final volume of 50 or 100 µl

5.3.2 Agarose gel electrophoresis and purification of DNA fragments

Electrophoresis through agarose gel is a standard method used to separate, identify and purify DNA fragments. Agarose gel was prepared by melting a sufficient amount of agarose powder in 1 x electrophoresis buffer. Buffers used for electrophoresis were either TAE or TBE. An 1 % agarose gel is usually of sufficient density to separate fragments from 0.5 to 5 kb. For smaller fragments of DNA, an agarose gel of higher

density (1.5-2 %) should be used. For larger DNA, an agarose gel of lower density (0.3-0.7 %) is useful. Voltage used for electrophoresis is adjusted according to the size of gel and is usually 5 V per 1 cm of the gel length. Ethidium bromide, a fluorescent dye that intercalates between the stacked base pairs, is used to visualise DNA in the gel on the source of UV light. The size of DNA fragment is determined by comparison with the standardised DNA ladder containing dsDNA fragments in the same range as expected ones.

PCR products were purified by electrophoresis and isolated from agarose gel with a QIAquick Gel Extraction Kit according to manual instructions. Briefly, a band of expected size was excised from gel with a clean scalpel and 3 volume of the QB solution (a solution provided by kit, the composition unknown) was added. Tube containing the excised fragment was kept on the 50 °C for 10 min for agarose to melt. Suspension containing the melted agarose was applied to the column and the several washing and precipitation steps have been performed. As the final step, DNA was eluted from the column with 50 µl of water. PCR products purified in this way can be subjected to sequencing or used for cloning.

TAE: 40 mM Tris-acetate, 1 mM EDTA
TBE: 45 mM Tris-borate, 1 mM EDTA

5.3.3 Strategies for cloning in plasmid vectors

All cloning procedures in this work were done as a straightforward ligation of inserts carrying protruding termini identical to termini in the linearised plasmid vector (Sambrook et al., 1989). Plasmid DNA is cleaved with one or alternatively two restriction enzymes and the 5'-phosphate groups were removed with alkaline phosphatase to prevent self ligation and the circularisation of plasmid DNA in the following ligation step. Insert DNA fragments were designed to carry sites for the identical restriction enzymes used for linearization of the plasmid vector. DNA fragments were obtained either as oligonucleotides or the products of PCR. After the digest step both, plasmid and insert were purified by the agarose gel electrophoresis. Ligation reaction was set up in 10 to 20 µl volume. The molecular ratio of vector to insert was 1:3. Ligation was performed at 16 °C overnight in the presence of 40 U of

T4 DNA ligase. 100 µl of competent bacteria was transformed with the total ligation reaction and plated on the selective LB-agar plates. Single colonies were inoculated for the miniprep isolation of plasmid DNA and the correct clones were verified according to the restriction fragment size in comparison to an empty vector.

5.4 Methods for the analysis of RNA

5.4.1 Isolation of the total RNA

The total RNA was isolated from animal cells growing in the cell suspension or from primary B cells. For that purpose, a Qiagen RNeasy kit was used according to manual instructions. For samples containing 5 x 10^6 cell to 1 x 10^8 cells the Midi version of this kit was used, for less cells the Mini version was used. Cells were spun down at 300 x g for 7 min and washed once with PBS. Cells were then lysed and homogenised under the highly denaturing conditions of guanidinium isothiocynate and β-mercaptoethanol, which immediately inactivate RNase to ensure the isolation of intact RNA. Ethanol was added to provide appropriate binding conditions and the sample was applied to a spin column where the total RNA bound and contaminants were washed away. After washing steps, RNA was eluted in 500 or 50 µl of DEPC-water, for Midi or Mini version, respectively. RNA was precipitated with 2 volumes of absolute ethanol and 0.1 volume Na-acetate at -20 °C for 2 h. The precipitated RNA was pelleted at 4 °C for 30 min. Pellet was dissolved in 20-100 µl of DEPC-water. The RNA concentration was determined by measuring the absorbance at wave length 260 nm.
The purified RNA was ready for any of the downstream application such as Northern blotting, cDNA synthesis, real-time PCR and poly A^+ RNA selection.

5.4.2 Northern blot analysis

One way to detect and/or quantitate the specific mRNA molecules in the preparations of the cellular RNA is the Northern blot analysis. It consists of the following steps:

labelling of the probe specific for the target mRNA, RNA formaldehyde agarose gel electrophoresis, blotting, hybridisation of the blot with the labelled probe and detection of the signals from the labelled probe.

5.4.2.1 Labelling of the probe

The specific probe was PCR-labelled with digoxigenin-dUTP. PCR was set up by adding all reagents (see below) to a template DNA. cDNA or plasmid DNA with a specific insert can serve as a template. Plasmid DNA used as a template gives higher yield and a purer probe, and was therefore used in this work. The PCR conditions were the following: initial denaturation at 95 °C for 2 min, 30 cycles of denaturation at 95 °C for 30 s, annealing at temperature depending on the primer design for 30 s, elongation at 72 °C for 40 s with the prolongation of 5 s for each subsequent cycle, finally, elongation at 72 °C for 10 min was performed as a last step. The size and the concentration of the DIG-labelled probe was determined by agarose gel electrophoresis.

PCR mix: 1 x buffer, 0.5 mM dNTP, 0.5 x DIG labelling mixture, 0.625 mM $MgCl_2$, 0.25 mM of each primer, 2.5 U Taq DNA polymerase, 40 ng DNA plasmid as template, water up to 20 µl

DIG labelling mix: 1 mM dATP, 1 mM dGTP, 1 mM dCTP, 0.65 mM dTTP, 0.35 mM DIG dUTP

5.4.2.2 RNA formaldehyde agarose gel electrophoresis

RNA was separated according to size by electrophoresis through a denaturing agarose gel containing formaldehyde. The electrophoresis chamber was cleaned with 0.1% SDS before pouring the gel. 1 % agarose gel containing formaldehyde was poured in the clean gel support and left to solidify for at least 20 min. The amount of RNA sufficient for the Northern blot analysis ranged between 5 and 10 µg. RNA was placed in the clean tube and subjected to speedvac evaporation. The dried RNA was dissolved in 20 µl of the denaturing buffer and denatured at 70 °C for 5 min. The

denatured RNA was loaded on the gel and electrophoresis was run at 40 V overnight in the 1 x MOPS running buffer.

1 % RNA agarose gel: 1 % agarose, 1 x MOPS, 6.4 % formaldehyde

RNA denaturing buffer: 1 x MOPS, 6.4 % formaldehyde, 5 % glycerol, 0.005 % ethidium bromide, 0.05 % bromphenol blue, water up to final volume

5.4.2.3 Transfer of the denatured RNA to the nylon membrane

After the gel electrophoresis ran far enough the equal loading of RNA was visualised by UV-light and documented by the Polaroid photography. The transfer of RNA from gel to the positively charged nylon membrane (Hybond-N+) was done overnight by the capillary elution in 20 x SSC. Ribosomal RNAs were used as size markers and the positions were marked on the membrane. Finally, membranes with the bound RNA were UV-crosslinked.

20 x SSC: 3 M NaCl, 0.3 M trisodium citrate, pH 7.0

5.4.2.4 Hybridisation and detection of the probe/target hybrids by chemiluminescence

Membrane was washed with 2 x SSC and prehybridised in Hyb-mix solution at 68 °C for 30 min. A DIG-labelled probe (10 ng/ml of Hyb-mix solution) was denaturated in boiling water bath for 10 min, cooled on ice and added to the pre-heated Hyb-mix solution. The prehybridisation solution was replaced by the Hyb-mix solution containing the probe. Hybridisation was performed at 68 °C overnight. The following day, the membrane was washed 3 x with Hyb-wash buffer at 65 °C. All subsequent incubations and washings were done at room temperature. Membrane was incubated in blocking solution for 60 min, then 30 min in Ab-blocking solution, washed 4 x with wash buffer, equilibrated 5 min with substrate buffer and finally incubated with CSPD-Ready-to-use substrate and exposed to the X-ray film. Time required for the detection of signals on the film ranged between 2 min to 16 hours.

Hyb-mix:	25 mM Na_2HPO_4, pH 7.2; 1 mM EDTA; 10 % SDS, 0.5 % blocking reagent
Hyb-wash buffer:	20 mM Na2HPO4, pH 7.2; 1 mM EDTA; 1 % SDS
Blocking solution:	0.5 % blocking reagent; 100 mM maleic acid, pH 8.0; 3 M NaCl; 0.3 % Tween 20
Ab-blocking solution:	anti-DIG-AP-antibody diluted 1:15 000 in blocking solution
Wash buffer:	100 mM maleic acid, pH 8.0; 3 M NaCl; 0.3 % Tween 20
Substrate buffer:	100 mM Tris HCl, pH 9.5; 100 mM NaCl; 50 mM $MgCl_2$

5.4.3 Reverse transcription

In order to quantify the specific mRNA by real-time PCR the mRNA had to be transcribed into cDNA by a process called reverse transcription (RT). 2 µg of total RNA were mixed with 1 µg of $(dT)_{15}$ primer in a total volume of 14 µl and heated at 70 °C for 5 min to denature the secondary structure. Tubes were placed on ice and 11 µl of the RT reaction mix was added. Reaction was incubated at 42 °C for 60 min.

RT reaction mix:	1 x M-MLV buffer; 0.625 mM of each dNTP; 200 U M-MLV reverse transcriptase; 25 U RNase inhibitor

5.4.4 Real time PCR

Real-time PCR is yet another technique for quantification of mRNA in a sample. It is based on the measuring of the increasing incorporation of the fluorescent SYBR Green I dye into dsDNA. It was performed on a LightCycler (Roche, Germany) by using LightCycler-FastStart DNA Master SYBR Green I ready-to-use kit according to the instruction manual with minor modifications. LC master mix was prepared for all samples and 18 µl aliquots were given to each capillary. Sample cDNA was diluted 1:20 and added to the LC master mix. Previously amplified PCR products of the same sequence were diluted in several subsequent 1:100 dilutions and used as relative standards for quantification. Capillaries were sealed and centrifuged at 700 x g for 5 s. Each real-time PCR started with initial denaturation at 95 °C for 10 min. 55 cycles followed, each containing the following conditions: heating up to 95 °C, annealing at 65 °C for 10 s and amplification at 72 °C. The amplification time depended on the

sequence length and it was calculated according to the DNA polymerase amplification rate which is 25 bp in 1 s. The authenticity of amplicon was verified by melting curve made by slowly increasing the temperature from 65 °C up to 95 °C with a ramp time 0.15 °C/s. Additionally, PCR products can be subjected to agarose gel electrophoresis to verify the PCR quality. Finally, the reaction was cooled to 40 °C.

LC master mix: 13.4 µl water; 1.6 µl $MgCl_2$ (25 mM); 1 µl of each primer (10 mM); 1 µl MasterSYBR Green I

5.4.5 Nuclear run-on

Nuclear run-on was performed essentially as described by Eick and Bornkamm, 1986. It consists of several steps including the isolation of nuclei, run-on reaction, hybridisation, wash and the detection of signals.

5.4.5.1 Isolation of nuclei

Nuclei were isolated from 1 x 10^8 cells per one run-on reaction. Cells were spun down at 310 x g for 7 min at 4 °C and washed once with the ice-cold PBS. Cells were resuspended in 20 ml lysis-buffer and incubated for 5 min on ice. The nuclei suspension was spun down at 472 x g for 10 min at 4 °C and the supernatant was carefully removed. Nuclei were resuspended in 100 µl of storage buffer and snap-frozen in liquid nitrogen followed by storage at -80 °C.

Lysis buffer: 10 mM NaCl; 10 mM $MgCl_2$; 10 mM Tris-HCl, pH 7.5; 0.5 % NP40

Storage buffer: 40 % glycerol; 5 mM $MgCl_2$; 0.1 mM EDTA; 50 mM Tris, pH 8.3

5.4.5.2 Nuclear run-on

The nuclei suspension was defrosted on ice and 100 µl of 2 x Run-on buffer and 100 µCi of ^{32}P-CTP were added. Mixture was incubated at 28 °C for 15 min. DNA was

degraded by the two rounds of DNaseI treatment at room temperature for 10 min. Proteins were degraded by adding 20 µl of proteinase K/SDS solution and incubation at 40 °C for 60 min. The reaction products were purified by size exclusion columns (NICK-Columns, Pharmacia). The radioactive nuclide incorporation should range between 50 and 80 million DPM/sample. The run-on probes were hybridised to "self-made" DNA arrays.

4 x Run-on buffer: 600 mM KCl; 10 mM $MgCl_2$; 1 mM ATP; 1 mM GTP; 1 mM UTP; 20 mM Tris, pH 8.0; 2.4 % sarcosyl

Proteinase K/SDS: 3 V proteinase K (10 mg/ml); 1 V SDS (10 %)

5.4.5.3 Hybridisation and washing of „self-made" DNA arrays

„Self-made" arrays were prepared by spotting the PCR products of genes of interest onto the nylon membrane. Membrane was dried and DNA was denatured by soaking the membrane into denaturising solution for 1 min, neutralised by neutralisation solution for 1 min, washed with 2 x SSC and finally crosslinked by UV. Each membrane was equilibrated with an Express-Hyb solution (Clontech, Sao Paolo, USA) containing 0.1 mg/ ml denatured shared salmon testes DNA at 68 °C for 30 min. The labelled RNAs were denatured at 95 °C for 5 min, added to a preheated Express-Hyb and together they were applied to the membrane. Membrane was hybridised at 68 °C for 48 h, and then washed at 45 °C twice with solution I and twice with solution II. Solution III was used to wash away SDS and then the membrane was washed with solution III/Rnase at room temperature for 20 min. Subsequently, the membrane was washed twice with solution II and exposed to the phosphoimager plate.

Denaturing solution: 0.5 M NaOH; 1.5 M NaCl

Neutralisation solution: 0.5 M Tris, pH 7.4; 1.5 M NaCl

Solution I: 2 x SSC; 1 % SDS

Solution II: 0.1 x SSC; 0.5 % SDS

Solution III: 2 x SSC; 1mM EDTA

Solution III/RNase: 2 x SSC; 1 mM EDTA; 2 µg/ml RNase A

5.4.6 DNA microarray techniques

5.4.6.1 ExpressCode™ DNA microarray

The ExpressCode™ DNA microarray was developed by GPC Biotec AG, Germany, and hybridisation and analysis were done in collaboration.

The 9 600 PCR products of three different cDNA libraries were arrayed at high densities on the 8x12 cm nylon membrane and hybridised with the ^{32}P-labelled DNA generated by reverse transcription from different RNA populations. Briefly, 200 ng of mRNA was reverse transcribed with random hexamers as primers in the presence of the master mix at 42 °C for 1 h. Samples were purified on a SpinColumns (Roche) and added to Hybsolution. Hybsolution with the probe was applied to UV crosslinked membranes and hybridised at 50 °C for 40 h. Membranes were washed twice with wash solution I and twice with wash solution II. Each washing step was performed at 65 °C for 15 min. The expression pattern on the membranes was analysed by phosphoimager. A statistical confidence derived from the four replicates of each experiment. Clones which show induction were sequenced and identified by BLASTN programme.

Master mix:	1 x First Strand Synthesis Buffer; 50 µCi ^{32}P-dCTP; 0.6 mM dATP; 0.6 mM dTTP; 0.6 mM dGTP; 4 µM dCTP; 100 mM DTT; 100 U RNase inhibitor; 200 U SuperScript reverse transcriptase
Hybsolution:	0.7 % SDS; 50 % formamide; 5 x SSC; 50 mM Na_2HPO_4; 2 % milk powder
Wash solution I:	1 % SDS; 2 x SSC
Wash solution II:	0.5 % SDS; 0.1 x SSC

5.4.6.2 GeneChip® DNA microarray

The hybridisation and data analysis of the GeneChip® DNA microarray (Affymetrix Inc., USA) were done in collaboration with Jörg Mages (Institute of Medical Microbiology, Munich) and Reinhard Hoffmann (Max von Pettenkofer Institute, Munich). GeneChip® Human Genome U133A was used in this work. It contains >15000 genes on an array of 1.28 x 1.28 cm.

RNA was isolated from the different cell populations. Quality control of all RNAs was performed by the measurement of OD260/280 ratio (>1.6) and gel electrophoresis. 5-20 µg of total RNA were subjected to a first strand cDNA synthesis with 100 µmol of oligo-dT-T7 primer in the presence of the First Strand master mix at 42 °C for 1 h. Second strand cDNA was synthesised in the presence of Second Strand master mix at 16 °C for 2 h. Subsequently, the mixture was incubated with 10 U of T4 DNA polymerase at 16 °C for 5 min to fill the protruding 3' tails. RNA was digested with RNase at 37 °C for 30 min and 25 µg of proteinase K was used to degrade proteins at 37 °C for 30 min. cDNA was extracted with phenol/chloroform/isoamylalcohol (50:49:1) three times. The final volume was reduced by Microcon-YM50 Columns (Millipore, USA). cRNA was made from cDNA by *in vitro* transcription using RNA Transcript Labelling kit (Enzo Diagnostics, Inc., USA) according to the manual instructions and purified with the RNeasy kit (Qiagen, Germany). The final step in the preparation of probes was fragmentation of 15 µg of cRNA with 5 x fragmentation buffer at the 95 °C for 20 min. cRNA was added to Hybridisation Cocktail and heated at 95 °C for 5 min. The probe array cartridge was filled up with Hybridisation Cocktail and hybridised for 16 h. Following hybridisation, chip was washed in Affymerix FluidicsStation 400 with the non-stringent and stringent buffer. The fluorescent signals were detected by washing the chip with Stain solutions I, II and III. Chip was scanned and the data were analysed.

First Strand Master mix: 100 U RNase inhibitor; 1 x First Strand buffer; 10 mM DTT; 0.5 mM each dNTP; 200 U Superscript II reverse transcriptase

Second Strand Master mix: 1 x Second Strand buffer; 0.2 mM each dNTP, 10 U *E. coli* DNA ligase; 40 U *E.coli* DNA polymerase I; 2 U RNase H; water up to final volume

5 x fragmentation buffer: 200 mM Tris-acetate, pH 8.1; 500 mM KOAc; 150 mM MgOAc

Hybridisation Cocktail: 50 pM control oligonucleotide B2; 1X eukaryotic hybridisation controls (*bioB, bioC, bioD, cre*); 0.1 mg/ml herring sperm DNA; 0.5 mg/ml BSA; 1 hybridisation buffer, water up to final volume

Non-stringent buffer: 75 mM MES sodium salt; 27.5 mM MES free acid monohydrate; 0.1 M NaCl; 0.01 % Tween 20

Stringent buffer: 0.9 M NaCl; 60 mM NaH_2PO_4; 6 mM EDTA; 0.01 % Tween 20

Stain solution I: 1 mg/ml streptavidin in Stain buffer

Stain solution II: 10 mg/ml anti-streptavidin goat antibody; 0.5 mg/ml biotinylated anti-streptavidin goat antybody in Stain buffer

Stain solution III:	1 mg /ml Streptavidin-Phycoerytrin (SAPE) in Stain buffer
Stain buffer:	150 mM MES sodium salt; 200 mM MES free acid monohydrate; 1.85 M NaCl; 0.1 % Tween 20

5.5 Methods for the analysis of proteins

5.5.1 Preparation of protein extracts

Protein extracts were usually made from 1 x 10^7 cells. Cells were harvested and washed in PBS at 310 x g for 6 min at room temperature. They were resuspended in 100 µl of single-detergent lysis buffer (Sambrook et al., 1989) containing DTT and protease inhibitor. Suspension was incubated on ice for 30 min. Proteins were separated from the cellular debris by centrifugation at 12 000 x g for 30 min at 4 °C. Supernatant containing proteins was separated into a clean tube and kept on ice or stored at -80 °C. The protein concentration was determined using Bradford method and measured at OD_{595} with the BSA dilutions as standards.

Single-detergent lysis buffer:	50 mM Tris-HCl, pH 8.8; 150 mM NaCl; 0.02 % Na-azide; 1 % NP-40; 2 mM DTT; 1 x Complete protease inhibitor cocktail solution (Roche, Germany)
Bradford solution:	100 mg/l Coomassie brilliant blue, 5 % absolute ethanol, 8.5 % phosphoric acid, water up to final volume

5.5.2 Western blot analysis

The sufficient amount of proteins (between 10 and 80 µg) was denatured by the addition of loading buffer and heating at 95 °C for 10 min. The protein suspension was separated on an 8 % denaturing SDS-polyacrylamide gel electrophoresis in conjunction with a 5 % polyacrylamide stacking gel. Gel electrophoresis was performed at 30 mA in 1 x running buffer. The stained marker standards were separated simultaneously on the same gel and were used for the estimation of

molecular weight. Proteins were blotted to a PVDF membrane using electroblotting at 400 mA in blotting buffer for 60 min. Following the transfer, membrane was incubated with blocking buffer for 1 h at room temperature in order to reduce the unspecific protein-binding capacity of the membrane. The primary antibody was diluted in a fresh blocking buffer which was added to the membrane. Membrane was incubated for 30 min and then washed 4 x with washing buffer. The peroxidase conjugated secondary antibody was diluted also in a fresh blocking buffer and applied to the membrane. Following 30 min of incubation with the secondary antibody, the membrane was washed 4 x with washing buffer. The ECL solution was used as a substrate for the peroxidase and the protein-antibody complex were detected by the chemoluminescence reaction by exposure to the X-ray film.

4 x loading buffer:	60 mM Tris, pH 6.8; 4 % SDS; 4 % glycerol; 0.05 % bromphenol blue; 4 µM DTT; 0.2 µM EDTA
8 % polyacrylamide gel:	8 % acrylamide mix (37 %); 375 mM Tris, pH 8.8; 0.1 % SDS; 0.1 % ammonium persulfate; 0.6 µl/ml TEMED, water up to final volume
5 % stacking gel:	5 % acrylamide mix (37 %); 125 mM Tris, pH 6.8; 0.1 % SDS; 0.1 % ammonium persulfate; 1 µl/ml TEMED; water up to final volume
Running buffer:	25 mM Tris-base; 200 mM glycin; 0.1 % SDS
Blotting buffer:	25 mM Tris-base; 0.2 M glycin; 0.1 % SDS; 20 % methanol
Blocking buffer:	5 % milk powder; 0.5 M Tris, pH 7.5; 150 mM NaCl
Washing buffer:	0.05 % Tween 20 in PBS

6.0 Summary

EBNA2 is one of the first viral proteins expressed post-EBV infection and is absolutely required for the process of B cell immortalisation. EBNA2 transactivates other viral genes, but it also enhances or represses the transcription of many cellular genes. These primary viral and cellular EBNA2 target genes initiate a cascade of secondary events which finally cause the immortalisation of infected B cell.

To elucidate the process of B cell immortalization by EBV it is essential to understand the mechanism by which EBNA2 activates its target genes. In the first part of this study, the cellular EBNA2 target genes were identified and the regulatory regions of primary EBNA2 target genes were analysed in order to gain more insights into the complex EBNA2 signalling pathways.

EREB2-5 is a lymphoblastoid cell line conditional for EBNA2. It provides a tool for monitoring very early events following EBNA2 activation. Three different DNA microarray techniques were used to identify novel cellular EBNA2 target genes. Genes identified by these DNA microarray techniques were additionally verified by the nuclear run-on and Northern blot analyses using cellular systems which permit the discrimination of primary EBNA2 genes among numerous genes up-regulated upon EBNA2 activation. c-myc, CD21 and CCR7 had been identified earlier as primary EBNA2 targets and herein they were confirmed. RDC1, bfl-1, MIP-1 beta, CD83 and PLEK were identified as novel primary EBNA2 target genes. In order to identify common *cis*-regulatory elements of the EBNA2 signalling pathway, an *in silico* analysis was performed on the promoter and intron 1 region of these genes. The *in silico* analyses of primary EBNA2 target genes identified potential binding sites for several transcription factors which appear in all or almost all genes: (i) the putative binding site for NFAT1 and Oct-1 were identified in the promoters of all target genes indicating potential novel transcription factors which might contribute to the EBNA2 signalling; (ii) RBP-Jκ binding sites was identified in the intron 1 region of all genes except for CD83 suggesting that EBNA2 does not only recruit transcriptional machinery to promoters, but might also induce a chromatin modification and remodelling resulting in an open chromatin accessible to transcriptional machinery; (iii) PU.1, a previously identified transcription factor important for EBNA2 transactivation was found in the majority of genes and (iv) NF-κB was also identified

in the majority of primary EBNA2 target genes. The viral protein LMP1, co-expressed with EBNA2 in EBV immortalised B cells, acts as a potent activator of the NF-κB pathway. Thus, the presence of the NF-κB binding sites within EBNA2 target genes could result in a convergence or a crosstalk of LMP1 and EBNA2 signalling pathways.

The second part of this work characterizes the function of TRRAP, which was found to be specifically induced by the EBV infection of primary B cells in this study. In contrast, B cell activation by crosslinking CD40 on the surface of primary B cells in the presence of IL-4 failed to induce TRRAP expression. TRRAP activation was dependent on EBNA2, but it required the activity of the additional cellular proteins. Activation of c-Myc in the absence of EBNA2 could not induce TRRAP. Further viral proteins enhanced TRRAP induction by EBNA2, but could not replace EBNA2 function. Thus, elevated TRRAP levels could be defined as a characteristic feature of EBV immortalized cells.

The TRRAP gene was chosen for further functional studies, since it is known that TRRAP function is crucial for the transformation processes driven by c-Myc or the adenoviral E1A protein. Thus, it was of great interest to test whether TRRAP is relevant for the immortalisation process driven by EBV. In order to address a potential function of TRRAP in this process, short interfering RNAs (siRNAs) specific for the TRRAP transcript were expressed in EBV immortalised B cells. Expression of siRNA specific for TRRAP repressed TRRAP expression below the detection limits. Within 12 hours the transfected cells ceased to proliferate and apoptosed indicating that TRRAP expression is crucial for the viability of EBV immortalized B cells.

7.0 References

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8.0 Supplementary material

(complete data from each screen can be found on a CD enclosed herewith)

Table 1.

EBNA2 induced genes identified by the ExpressCode™ DNA microarray two hours after activation of EBNA2. Genes are shown that were up-regulated 2 hours post-oestrogen stimulation in EREB2-5 cells. Up-regulated genes have been considered those which showed at least 2-fold induction. (p.c. denotes up-regulation in the proliferating EREB2-5 cells)

GPC clone	Accession number	Gene	Fold induction		
			0 hr	2 hrs	p.c.
CLONE-11E6	BC000241	30S ribosomal protein S7 homolog	1	2	3,1
CLONE-12I17	HSU33818	inducible poly(A)-binding protein	1	2,1	6,4
CLONE-13M13	AF099100	WD-repeat protein 6 (WDR6)	1	2,2	4,5
CLONE-12G7	NM_014741	mRNA for KIAA0652 protein	1	2,4	24,6
CLONE-16J4	NM_007248	three prime repair exonuclease 1 (TREX1)	1	2,4	12,5
CLONE-12H9	NM_015388	DKFZP566C243 protein (DKFZP566C243)	1	2,5	13,3
CLONE-8L19	HS57A13	PAC 57A13	1	2,5	15,3
CLONE-10C13	AF037194	regulator of G protein signaling RGS14-variant	1	2,6	18,9
CLONE-16G19	NM_014889	metalloprotease 1 (pitrilysin family) (MP1)	1	2,6	12,9
CLONE-17L8	HSAJ2030	mRNA for putative progesterone binding protein	1	2,7	6,3
CLONE-2J4	AF240468	nicastrin	1	2,7	9,7
CLONE-11D24	NM_013366	anaphase-promoting complex 2 (APC2)	1	2,8	5
CLONE-9F16	HS211D12	cDNA: FLJ23435 fis	1	3	12,2
CLONE-7C13	NM_003685	KH-type splicing regulatory protein	1	3,1	30
CLONE-10L11	NM_001127	adaptor-related protein complex 1	1	3,7	5,4
CLONE-21B17	AK025157	FLJ21504 fis	1	3,8	10,2
CLONE-5E2	NM_001675	ATF4	1	4,6	24,5
CLONE-17B12	NM_019082	putative nucleolar RNA helicase (NOH61)	1	5,6	28,3
CLONE-12E11	HSDJ93K22	clone RP1-93K22	1	6,4	4,7
CLONE-11D9	NM_003851	cellular repressor of E1A-stimulated genes (CREG)	1	6,7	22,8
CLONE-12M13	AF195883	G protein beta subunit	1	8,3	45
CLONE-15E12	HUMLCAT	lecithin-cholesterol acyltransferase mRNA	1	9	2,9
CLONE-21B11	NM_007355	heat shock 90kD protein 1, beta (HSPCB)	1	9,7	217,4
CLONE-16O17	HUMGTPBP	GTP-binding protein	1	17,1	133,4
CLONE-18M22	AF055006	sec6 homolog	1	21,8	79,5
CLONE-13A17	HS57G9	clone CTA-57G9 on chromosome 22q12.1	1	27	12,9
CLONE-21A19	BE650475	mousecDNA clone UI-M-BZ1-bed-g-05-0-UI	1	38,9	20,8
CLONE-12J18	BF308884	clone IMAGE:4139956	1	91,2	168,8
CLONE-21E12	NM_017990	hypotethical protein FLJ10079	1	96,2	84,6
CLONE-20L19	AF191654	NUDT4	1	160,6	347
CLONE-8E18	HSA295637	URIM protein	1	164,9	1503,6
CLONE-12E17	NM_021079	N-myristoyltransferase 1 (NMT1)	1	170,7	512,8
CLONE-4L21	AK026673	cDNA: FLJ23020 fis	1	201,2	2740,8
CLONE-12H13	XM_004500	CD83	1	238	1354,7
CLONE-16I9	NM_005542	insulin induced gene 1 (INSIG1)	1	254,5	2110,1
CLONE-16C15	NM_004539	asparaginyl-tRNA synthetase	1	278	4747,7
CLONE-8H4	NM_012068	ATF5	1	283,4	6503,2
CLONE-8P22	AC007619	12p BAC RP11-253I19	1	333,1	351,7
CLONE-13O4	HUMDSITE1	D-site binding protein gene	1	354,1	505
CLONE-21J15	AF272833	misato	1	364,4	392,6
CLONE-4N23	NM_019094	NUDT4	1	395,7	970,5
CLONE-13G19	HSM800105	clone DKFZp564D116	1	412,1	2168,9
CLONE-15K19	HSU12596	TNF type 1 receptor assoc.pr.(TRAP2)	1	679,4	4199,5
CLONE-11K11	HSU67784	orphan G protein-coupled receptor (RDC1)	1	722,3	3193,1
CLONE-11H18	AV737985	cDNA clone:CBFBQB01	1	827,3	295,6
CLONE-12B24	NM_013393	cell division protein FtsJ (FJH1)	1	857,9	3088,7
CLONE-21B16	HSMYCC	c-myc oncogene	1	1092,6	4446,3
CLONE-14E20	HUMFAPTB1	TB1 gene	1	1161,9	2621,4
CLONE-10B2	AK023132	cDNA FLJ13070 fis	1	2533,6	1534,4
CLONE-16A19	AF128536	cytoplasmic phosphoprotein PACSIN2	1	12127	3126,5
CLONE-9A10	U73627	Chromosome 11 Cosmid cSRL186g7	1	15375,8	3536,3
CLONE-12A2	NM_017692	hypothetical protein FLJ20157	1	16817,7	6113

Table 2.
EBNA2 induced genes identified by the ExpressCode™ DNA microarray in proliferating EREB2-5 cells.
Genes are shown that were up-regulated in at least 48 hours post-oestrogen stimulation in EREB2-5 cells. Up-regulated genes have been considered those which showed at least 2-fold induction. (p.c. denotes up-regulation in the proliferating EREB2-5 cells)

GPC clone	Accession number	Gene	Fold induction		
			0 hr	2 hrs	p.c.
CLONE-4L15	AF286592	signaling molecule SPEC1 beta mRNA	1	0,4	2,1
CLONE-6J12	AC005255	chromosome 19, CIT-HSP-146e8	1	0,3	2,1
CLONE-2P22	HUMCTI	erythrocyte membrane protein mRNA	1	0,3	2,2
CLONE-7J10	NM_003557	phosphatidylinositol-4-phosphate 5-kinase, type I	1	0,2	2,5
CLONE-12A7	NM_001253	CDC5like (CDC5L)	1	0,4	2,6
CLONE-12G13	AF272833	misato	1	0,7	2,7
CLONE-10I3	NM_004953	eukaryotic translation initiation factor 4 gamma 1	1	0,8	2,8
CLONE-18C1	AK027160	cDNA: FLJ23507 fis, clone LNG03128	1	1,8	2,8
CLONE-15E12	HUMLCAT	lecithin-cholesterol acyltransferase mRNA	1	9	2,9
CLONE-11E6	BC000241	30S ribosomal protein S7 homolog, clone MGC:711	1	2	3,1
CLONE-11M13	AF218942	clone r56121 formin 2-like protein mRNA	1	0,7	3,1
CLONE-3B12	AF055010	clone 24561 unknown mRNA	1	0,5	3,1
CLONE-13I7	HUMSHPTP2A	phosphotyrosyl-protein phosphatase (SH-PTP2)	1	1,4	3,2
CLONE-16M2	NM_021639	hypothetical protein SP192	1	0,5	3,3
CLONE-12A22	NM_005801	translation initiation factor 1(eIF1(A121/SUI1))	1	1,7	3,9
CLONE-12N2	NM_003170	SUPT6H	1	0,9	4,1
CLONE-3D16	HSU46691	putative chromatin structure regulator (SUPT6H)	1	1,8	4,1
CLONE-3H2	AF159851	Rho GAP p190-A mRNA	1	0,4	4,1
CLONE-16I15	AF092565	splicing factor Prp8 mRNA	1	1	4,2
CLONE-8N11	XM_003286	sec61 homolog (HSEC61)	1	0,8	4,4
CLONE-9J10	HSU43077	CDC37 homolog	1	0,6	4,4
CLONE-13M13	AF099100	WD-repeat protein 6 (WDR6)	1	2,2	4,5
CLONE-3C15	L21208	TAP1	1	1	4,5
CLONE-10G24	NM_003496	TRRAP	1	1,2	4,6
CLONE-10I19	XM_008483	TRAF4	1	0,8	4,7
CLONE-11A7	BC000241	30S ribosomal protein S7 homolog	1	0,8	4,7
CLONE-12E11	HSDJ93K22	clone RP1-93K22	1	6,4	4,7
CLONE-21I8	NM_006145	heat shock 40kD protein 1	1	0,6	4,8
CLONE-21L10	D63477	mRNA for KIAA0143 gene	1	1	4,8
CLONE-11D24	NM_013366	anaphase-promoting complex 2 (APC2)	1	2,8	5
CLONE-6G5	AF082658	Era GTPase B protein (HERA-B)	1	1	5
CLONE-5B6	NM_006366	adenylyl cyclase-associated protein 2 (CAP2)	1	1,5	5,1
CLONE-8E6	HS57A13	PAC 57A13 between markers DXS6791-DXS8038	1	1,3	5,1
CLONE-3A10	X70218, S55208	phosphatase X	1	0,7	5,2
CLONE-10L11	NM_001127	adaptor-related protein complex 1	1	3,7	5,4
CLONE-8F7	AK024758	FLJ21105 fis	1	0,6	5,4
CLONE-17L8	HSAJ2030	mRNA for putative progesterone binding protein	1	2,7	6,3
CLONE-12I17	HSU33818	inducible poly(A)-binding protein	1	2,1	6,4
CLONE-13A19	NM_002631	phosphogluconate dehydrogenase (PGD)	1	1,5	6,4
CLONE-16E18	HSU40272	NAD+-specific isocitrate dehydrogenase gamma subunit	1	0,3	6,4
CLONE-6B15	NM_002664	pleckstrin (PLEK)	1	0,9	6,4
CLONE-11M2	HSU36188	clathrin assembly protein 50	1	0,9	6,5
CLONE-20F17	HUMIFNWRS	tryptophanyl tRNA synthetase	1	0,9	6,5
CLONE-21J4	HSM6	M6 antigen	1	0,7	7,1
CLONE-15K5	HSU88836	clone 738, translational activator GCN1	1	1	7,9
CLONE-14L2	AJ132637	ATP-dependent metalloprotease YMEIL	1	0,7	8,1
CLONE-5P5	NM_014287	pM5 protein, mRNA	1	0,6	8,2
CLONE-15H15	AC009329	clone RP11-264E12 on chromosome 7	1	2	8,9
CLONE-10E21	AF047469	arsenite translocating ATPase (ASNA1)	1	0,5	9

Table continues

Table 2. *continued*

GPC clone	Accession number	Gene	Fold induction 0 hr	2 hrs	p.c.
CLONE-14K13	NM_005918	malate dehydrogenase 2	1	0,8	9,3
CLONE-5A5	AF305687	transcription factor ATFx	1	0,9	9,3
CLONE-2J4	AF240468	nicastrin	1	2,7	9,7
CLONE-7D8	HSKHSRP4	KH type splicing regulatory protein	1	1,4	9,7
CLONE-8B15	AF150962	repressor of estrogen receptor activity (REA)	1	1,1	10
CLONE-7D1	NM_001320	casein kinase 2, beta polypeptide (CSNK2B)	1	0,6	10,1
CLONE-16A14	NM_012061	Ca<2+>dependent activator protein	1	0,8	10,2
CLONE-16G17	AK026171	HSPC235 mRNA	1	0,5	10,2
CLONE-21B17	AK025157	FLJ21504 fis	1	3,8	10,2
CLONE-6M11	NM_014734	KIAA0247 gene product	1	0,6	11,3
CLONE-9G24	AF202637	PP3111 mRNA	1	1,9	11,9
CLONE-9F16	HS211D12	cDNA: FLJ23435 fis	1	3	12,2
CLONE-11C19	HUMAPEA	apurinic/apyrimidinic endonuclease (HAP1h) mRNA	1	1,4	12,4
CLONE-16J4	NM_007248	three prime repair exonuclease 1 (TREX1)	1	2,4	12,5
CLONE-13A17	HS57G9	clone CTA-57G9 on chromosome 22q12.1	1	27	12,9
CLONE-16G19	NM_014889	metalloprotease 1 (pitrilysin family) (MP1)	1	2,6	12,9
CLONE-12H9	NM_015388	DKFZP566C243 protein (DKFZP566C243)	1	2,5	13,3
CLONE-21N4	NM_003132	spermidine synthetase	1	0,9	13,9
CLONE-8L19	HS57A13	PAC 57A13 between markers DXS6791-DXS8038	1	2,5	15,3
CLONE-17G5	NM_002823	prothymosin alpha	1	0,7	15,7
CLONE-15L16	NM_002823	prothymosin alpha	1	0,8	17,3
CLONE-16F3	NM_001416	eukayotic translation initiation factor 4A (EIF4A1)	1	1,1	17,6
CLONE-20L2	XM_002285	chaperonin containing TCP1, subunit 4	1	1	18
CLONE-2G4	X70218, 255208	phosphatase X	1	1	18,8
CLONE-10C13	AF037194	regulator of G protein signaling RGS14-variant	1	2,6	18,9
CLONE-15N14	NM_002634	prohibitin (PHB)	1	1,3	19
CLONE-5M13	D87742	KIAA0268, partial sequence	1	0,6	20,6
CLONE-21A19	BE650475	mousecDNA clone UI-M-BZ1-bed-g-05-0-UI	1	38,9	20,8
CLONE-11D9	NM_003851	cellular repressor of E1A-stimulated genes (CREG)	1	6,7	22,8
CLONE-13G15	HUM4AI	eukaryotic initiation factor 4AI	1	1,2	24
CLONE-17D12	NM_002823	prothymosin alpha	1	0,6	24
CLONE-5E2	NM_001675	ATF4	1	4,6	24,5
CLONE-12G7	NM_014741	KIAA0652 protein	1	2,4	24,6
CLONE-4B15	AB009282	cytochrome b5	1	1,1	25,8
CLONE-3K5	NM_005412	serine hydroxymethyltransferase 2	1	1,2	26
CLONE-3A18	X70218, S55208	phosphatase X	1	0	27,7
CLONE-17B12	NM_019082	putative nucleolar RNA helicase (NOH61)	1	5,6	28,3
CLONE-7C13	NM_003685	KH-type splicing regulatory protein	1	3,1	30
CLONE-9D11	NM_004718	cytochrome c oxidase subunit VIIa polypeptide 2 like	1	0,5	33,9
CLONE-4H15	AF054175	mitochondrial proteolipid 68MP homolog	1	1	38,9
CLONE-12M13	AF195883	G protein beta subunit	1	8,3	45
CLONE-13L11	AF141347	alpha-tubulin	1	1,1	45,4
CLONE-12K22	AF141347	alpha-tubulin	1	0,6	48,8
CLONE-18M22	AF055006	sec6 homolog	1	21,8	79,5
CLONE-21E12	NM_017990	hypotethical protein FLJ10079	1	96,2	84,6
CLONE-5H12	AF068235	barrier-to-autointegration factor	1	0,2	87
CLONE-16O17	HUMGTPBP	GTP-binding protein	1	17,1	133,4
CLONE-12J18	BF308884	clone IMAGE:4139956	1	91,2	168,8
CLONE-21B11	NM_007355	heat shock 90kD protein 1, beta (HSPCB)	1	9,7	217,4
CLONE-11H18	AV737985	cDNA clone:CBFBQB01	1	827,3	295,6

Table continues

Table 2. *continued*

GPC clone	Accession number	Gene	Fold induction		
			0 hr	2 hrs	p.c.
CLONE-20L19	AF191654	NUDT4	1	160,6	347
CLONE-8P22	AC007619	12p BAC RP11-253I19	1	333,1	351,7
CLONE-21J15	AF272833	misato	1	364,4	392,6
CLONE-13O4	HUMDSITE1	D-site binding protein gene	1	354,1	505
CLONE-12E17	NM_021079	N-myristoyltransferase 1 (NMT1)	1	170,7	512,8
CLONE-12D14	AF308297	breast cancer antigen NY-BR-81	1	1,3	743,1
CLONE-13P1	AC009329	clone RP11-264E12 on chromosome 7	1	1,3	800,2
CLONE-20L17	AF242729	HT022	1	1,3	872,6
CLONE-4N23	NM_019094	NUDT4	1	395,7	970,5
CLONE-3P2	AC003688	clone hRPC.4_G_17	1	1,3	1129,5
CLONE-14M10	AF298880	exportin 5	1	1,3	1213,7
CLONE-3L14	X70944	PTB-associated splicing factor	1	1,3	1311,7
CLONE-12H13	XM_004500	CD83	1	238	1354,7
CLONE-8E18	HSA295637	URIM protein	1	164,9	1503,6
CLONE-10B2	AK023132	cDNA FLJ13070 fis	1	2533,6	1534,4
CLONE-16I9	NM_005542	insulin induced gene 1 (INSIG1)	1	254,5	2110,1
CLONE-13G19	HSM800105	clone DKFZp564D116	1	412,1	2168,9
CLONE-14E20	HUMFAPTB1	TB1 gene	1	1161,9	2621,4
CLONE-4L21	AK026673	cDNA: FLJ23020 fis	1	201,2	2740,8
CLONE-12B24	NM_013393	cell division protein FtsJ (FJH1)	1	857,9	3088,7
CLONE-16A19	AF128536	cytoplasmic phosphoprotein PACSIN2	1	12127	3126,5
CLONE-11K11	HSU67784	orphan G protein-coupled receptor (RDC1)	1	722,3	3193,1
CLONE-9A10	U73627	Chromosome 11 Cosmid cSRL186g7	1	15375,8	3536,3
CLONE-15K19	HSU12596	TNF type 1 receptor assoc.pr.(TRAP2)	1	679,4	4199,5
CLONE-21B16	HSMYCC	c-myc oncogene	1	1092,6	4446,3
CLONE-16C15	NM_004539	asparaginyl-tRNA synthetase	1	278	4747,7
CLONE-12A2	NM_017692	hypothetical protein FLJ20157	1	16817,7	6113
CLONE-8H4	NM_012068	ATF5	1	283,4	6503,2
CLONE-3F23	X53305,	p18 (stathmin)	1	1,3	9014,6

Table 3.
EBNA2 induced genes identified by lymphochip two hours after activation of EBNA2 in EREB2-5 cells.
Genes are shown that were up-regulated 2 hours post-oestrogen stimulation in EREB2-5 cells. Up-regulated genes have been considered those which showed at least 2-fold induction.

Lymphochip identifier	Gene	Fold induction 0 hr	2hrs
27536	prostate differentiation factor	1	2,0
27463	IP-10	1	2,0
27785	HES-1	1	2,0
27785	HES-1	1	2,0
28049	SLAM	1	2,0
19388	CD9	1	2,0
24826	MAD2	1	2,1
28826	TCL-1A	1	1
16610	CD83	1	2,1
27987	CD21	1	2,1
29312	osteopontin	1	2,1
36979	dystrophin	1	2,1
19277	SLAM	1	2,2
15889	RDC1	1	2,2
15889	RDC1	1	2,2
27970	Lymphotactin	1	2,2
35090	CD21	1	2,2
31282	MMSET type II	1	2,2
30433	Terminal Deoxynucleotide Transferase	1	2,2
26409	MIP-1 beta	1	2,3
19250	IRTA4	1	2,3
28639	Bfl-1	1	2,3
36733	transcription factor 19 (SC1)	1	2,3
16089	Bfl-1	1	2,3
16671	phosphoinositide-3-kinase p85 alpha	1	2,3
24827	Cyclin B1	1	2,3
31585	homolog of yeast MCM10	1	2,4
27240	MAD2 (mitotic arrest deficient, yeast, homolog)-like 1	1	2,4
30534	Eg5	1	2,5
26989	WEE1-like protein kinase	1	2,5
25237	CD21	1	2,5
30013	cyclin A2	1	2,5
28494	CHK1 (checkpoint, S.pombe) homolog	1	2,6
27294	MMP-7	1	2,6
37371	TOPK	1	2,6
24479	Myosin class I	1	2,6
26657	RGS13	1	2,7
26200	profilin 2	1	2,7
29320	deoxyribonuclease I-like 3	1	2,7
24348	unknown UG Hs.265592 ESTs	1	2,8

Table continues

Table 3. *continued*

Lymphochip identifier	Gene	Fold induction 0 hr	2hrs
28377	phosphoinositide-3-kinase p85 alpha	1	2,8
15997	CENP-E	1	2,8
27545	CD83	1	2,8
35428	deoxyribonuclease I-like 3	1	2,8
37613	DNA polymerase theta	1	2,9
26475	MIP-1 alpha	1	2,9
28100	N-Cadherin	1	3,0
36919	deoxyribonuclease I-like 3	1	3,0
17185	DUSP2	1	3,1
17066	CD83	1	3,1
29820	RA-regulated nuclear matrix-associated protein	1	3,2
38030	lipoma HMGIC fusion partner-like 2	1	3,4
17281	hyaluronan-mediated motility receptor (RHAMM)	1	3,5
27686	DUSP2	1	8
29448	PNAS-108	1	3,9
29647	WNT2	1	3,9
28746	IL-7 receptor alpha chain	1	4,0
17373	IL-7 receptor alpha chain	1	4,0
28336	Guanine nucleotide binding protein	1	4,1
24762	EGR-1	1	4,2
35345	similar to S68401 (cattle) glucose induced gene	1	4,3
17364	CD21	1	4,6
36627	annexin A1	1	4,7
26129	Brush-1	1	4,7
28961	CD49F	1	4,9
26353	Cyclin E2	1	5,0
27293	Mononcyte/neutrophil elastase inhibitor	1	5,1
32635	CD3Z	1	5,3
34948	T cell receptor delta chain	1	5,4
20212	EBV EBNA-1	1	5,5
36581	IgE constant region	1	5,6
28379	protein kinase, cAMP-dependent, regulatory, type II	1	6,0
16561	ARK2	1	6,1
33705	DHFR	1	6,1
28853	TALLA	1	6,6
28383	CD21	1	6,8
36602	CO-029	1	6,8
33323	sperm associated antigen	1	7,0
15841	c-myc	1	7,4
32171	CD10	1	7,6

Table continues

Table 3. *continued*

Lymphochip identifier	Gene	Fold induction 0 hr	2hrs
28401	c-myc	1	8,2
30157	CENP-F kinetochore protein	1	9,0
29723	c-myc	1	9,2
20210	EBV LMP1	1	9,8
24385	unknown UG Hs.291994 EST	1	9,9
19381	c-myc	1	10,4
34980	EBV LMP2A (TP1)	1	12,1
19385	SLAP	1	12,3
19385	SLAP	1	12,31
28959	EDG-1	1	12,5
34966	EBV LMP1	1	15,2
35901	PEP-19	1	17,0
29897	FLJ10517	1	20,6
30449	CD3Z	1	242,9

Table 4.
EBNA2 induced genes identified by GeneChip® two hours after activation of EBNA2 in EREB2-5 cells.
Genes are shown that were up-regulated 2 hours post-oestrogen stimulation in EREB2-5 cells. Genes have been considered to be up-regulated if they showed at least1.4-fold induction in at least 2 experiment from 4 experiments done (Ex1, Ex2, Ex3, Ex4). YES denotes induction more then 1.4-fold, NO denotes induction less then 1.4 fold.

Probe set[a]	Accesion number	Gene	Fold induction			
			Ex1	Ex2	Ex3	Ex4
41386_i_at	AB002344	KIAA0346 protein	0,26	1,46	2,56	1,39
200603_at	AL050038	protein kinase, cAMP-dependent, regulatory, type I	2,32	1,52	1,89	1,43
200851_s_at	NM_014761	KIAA0174 gene product	2,32	2,04	2,41	1,01
200930_s_at	AA156675	vinculin	0,24	9,63	1,38	1,7
201163_s_at	NM_001553	insulin-like growth factor binding protein 7	0,3	3,86	2,43	0,3
201566_x_at	D13891	inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	1,96	1,94	1,39	1,54
201610_at	AL578502	isoprenylcysteine carboxyl methyltransferase	3,47	2,4	1,1	0,17
202241_at	NM_025195	phosphoprotein regulated by mitogenic pathways	1,24	1,68	2,47	1,54
202679_at	NM_000271	Niemann-Pick disease, type C1	0,95	2,03	2,59	1,78
202727_s_at	NM_000416	interferon gamma receptor 1	0,79	1,5	1,64	0,7
202769_at	AW134535	Homo sapiens mRNA; cDNA DKFZp434B142	1,95	3,82	2,72	1,94
203227_s_at	NM_005981	sarcoma amplified sequence	2,21	1,56	2,45	1,07
203296_s_at	NM_000702	ATPase, Na+/K+ transporting, alpha 2 (+) polypeptide	0,3	3,52	4,56	0,49
203735_x_at	N35896	PTPRF interacting protein, binding protein 1 (liprin beta 1)	0,76	2,26	2,44	1,09
203900_at	NM_024547	KIAA0467 protein	0,38	2,37	1,59	0,62
204103_at	NM_002984	chemokine (C-C motif) ligand 4	0,06	1,09	1,24	0,57
204197_s_at	NM_004350	runt-related transcription factor 3	0,51	1,93	1,7	0,79
204397_at	NM_012155	echinoderm microtubule associated protein like 2	2,51	2,05	1,38	0,28
204440_at	NM_004233	CD83 antigen (activated B lymphocytes, immunoglobulin superfamily)	0,3	1,46	1,78	0,78
204518_s_at	NM_000943	peptidylprolyl isomerase C (cyclophilin C)	0,93	1,43	1,13	0,12
204579_at	NM_002011	fibroblast growth factor receptor 4	0,39	1,82	1,48	0,41
204896_s_at	NM_000958	prostaglandin E receptor 4 (subtype EP4)	0,98	1,16	1,56	0,12
204995_at	AL567411	cyclin-dependent kinase 5, regulatory subunit 1 (p35)	0,25	1,16	1,2	0,16
205027_s_at	NM_005204	mitogen-activated protein kinase kinase kinase 8	1,87	1,57	1,36	0,4
205285_s_at	AI633888	FYN binding protein (FYB-120/130)	5,23	9,78	1,19	0,12

Table continues

Table 4. *continued*

Probe set[a]	Accesion number	Gene	Fold induction			
			Ex1	Ex2	Ex3	Ex4
205419_at	NM_004951	Epstein-Barr virus induced gene 2	0,25	1,22	1,39	0,53
205572_at	NM_001147	angiopoietin 2	0,61	4,88	1,71	0,15
205639_at	NM_001637	acyloxyacyl hydrolase (neutrophil)	1,6	2,8	1,13	0,15
205814_at	NM_000840	glutamate receptor, metabotropic 3	1,9	3,12	1,41	0,61
206115_at	NM_004430	early growth response 3	0,14	1,18	1,23	0,25
206181_at	NM_003037	signaling lymphocytic activation molecule	0,39	1,58	2,03	0,92
206370_at	NM_002649	phosphoinositide-3-kinase, catalytic, gamma polypeptide	0,58	10,82	1,62	0,12
206459_s_at	AB045117	wingless-type MMTV integration site family, member 2B	0,31	1,49	1,2	0,21
206482_at	NM_005975	PTK6 protein tyrosine kinase 6	1,3	2,86	1,18	0,43
206521_s_at	NM_015859	general transcription factor IIA, 1, 19/37kDa	0,24	13,23	1,25	0,47
206778_at	NM_000496	crystallin, beta B2	0,98	1,65	1,62	0,46
207391_s_at	NM_003557	phosphatidylinositol-4-phosphate 5-kinase, type I, alpha	1,36	1,87	2,81	1,36
207944_at	NM_006188	oncomodulin	0,77	3,34	1,32	4,22
208064_s_at	NM_015879	sialyltransferase 8C	0,5	7,18	1,41	0,11
208133_at	NM_006081	MHC binding factor, beta	1,53	3,42	1,13	0,11
208153_s_at	NM_001447	FAT tumor suppressor homolog 2 (Drosophila)	0,31	2,54	1,69	0,76
208208_at	NM_003802	myosin, heavy polypeptide 13, skeletal muscle	6,27	1,81	1,31	0,44
208476_s_at	NM_018027	hypothetical protein FLJ10210	0,31	1,66	1,62	0,74
208582_s_at	NM_012148	double homeobox, 3	0,35	5,12	1,07	0,5
208651_x_at	M58664	CD24 antigen (small cell lung carcinoma cluster 4 antigen)	1,49	4,48	3,07	1,31
209573_s_at	AI349506	ESTs, No similarity	0,18	1,31	1,61	0,43
209574_s_at	AI349506	ESTs, No similarity	0,38	1,32	1,41	0,41
209722_s_at	L40378	serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 9	0,69	2,43	1,34	0,54
209771_x_at	AA761181	CD24 antigen (small cell lung carcinoma cluster 4 antigen)	3,51	3,22	2,48	1,45
209795_at	L07555	CD69 antigen (p60, early T-cell activation antigen)	0,24	1,07	1,47	0,32

Table continues

Table 4. *continued*

Probe set[a]	Accesion number	Gene	Fold induction			
			Ex1	Ex2	Ex3	Ex4
209819_at	AF241831	hyaluronan binding protein 4	6,43	1,94	1,31	0,12
210327_s_at	D13368	alanine-glyoxylate aminotransferase	0,1	1,35	1,13	0,2
210403_s_at	U12543	potassium inwardly-rectifying channel, subfamily J, member 1	1,46	4,69	1,42	0,59
210439_at	AB023135	inducible T-cell co-stimulator	0,11	1,06	1,19	0,07
210767_at	BC003112	neurofibromin 2 (bilateral acoustic neuroma)	0,58	1,64	1,11	0,04
210812_at	BC005259	X-ray repair complementing defective repair in Chinese hamster cells 4	0,98	1,84	1,46	0,2
211306_s_at	U56237	Fc fragment of IgA, receptor for	0,06	2,08	1,14	0,17
211315_s_at	AB012043	calcium channel, voltage-dependent, alpha 1G subunit	0,58	2,78	1,21	0,18
211396_at	U90941	Fc fragment of IgG, low affinity IIb, receptor for (CD32)	0,77	1,63	1,19	0,16
211559_s_at	L49506	cyclin G2	1,78	2,39	2,71	0,68
211564_s_at	BC003096	LIM domain protein	1,18	4,45	1,34	0,32
211619_s_at	M13077	alkaline phosphatase, placental (Regan isozyme)	0,81	4,86	1,07	0,65
211736_at	BC005914	Sp2 transcription factor	0,08	2,56	1,12	1,94
212838_at	AB023227	KIAA1010 protein	0,36	1,48	2,45	1,01
213116_at	AI191920	NIMA (never in mitosis gene a)-related kinase 3	1,64	2,38	1,12	0,45
213146_at	AA521267	KIAA0346 protein	0,34	1,64	1,28	4,66
213563_s_at	AA016035	tubulin, gamma complex associated protein 2	0,4	4,67	1,29	0,89
213931_at	AI819238	inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	0,36	1,46	1,75	0,87
213942_at	AL134303	EGF-like-domain, multiple 3	0,59	1,62	1,42	1,24
214029_at	AI435954	Unknown [Homo sapiens], mRNA sequence	0,04	1,65	1,13	0,16
214206_at	AI739480	sphingomyelin phosphodiesterase 2	0,37	4,07	1,25	0,12
214561_at	NM_024317	leukocyte immunoglobulin-like receptor	0,29	3,43	1,44	0,26
214595_at	AI332979	potassium voltage-gated channel	5,67	2,95	1,66	0,13
214778_at	AB011541	EGF-like-domain, multiple 4	0,56	1,45	1,17	0,07
214993_at	AF070642	Homo sapiens clone 24488 mRNA sequence	0,79	4,26	1,22	0,25

Table continues

Table 4. *continued*

Probe set[a]	Accesion number	Gene	Fold induction			
			Ex1	Ex2	Ex3	Ex4
215036_at	AI952772	immunoglobulin lambda locus	3,15	2,1	1,42	0,61
215296_at	AK027000	KIAA0451 gene product	1,31	6,71	1,28	0,27
215340_at	L05500	adenylate cyclase 1 (brain)	0,21	4,69	2,7	2,2
215495_s_at	AL117523	KIAA1053 protein	0,66	11,45	2,62	0,32
215664_s_at	X95425	EphA5	2,15	2,58	1,36	0,45
215804_at	Z27409	EphA1	0,67	3,85	1,28	0,2
215870_s_at	AL158172	clone RP1-169O23 on chromosome 1	1,72	11,75	1,26	0,35
215894_at	U31099	prostaglandin D2 receptor (DP)	1,43	1,87	1,13	0,28
215917_at	AL049037	syntaphilin	0,13	4,12	1,35	0,2
215992_s_at	AL117397	PDZ domain containing guanine nucleotide exchange factor(GEF)1	0,05	1,37	1,31	0,68
216010_x_at	D89324	EMBL: Homo sapiens DNA for alpha (1,3/1,4) fucosyltransferase	14,07	1,13	1,28	1,25
216119_s_at	AL109804	EMBL: Human DNA sequence from clone RP5-1009E24	0,54	1,94	1,36	0,57
216169_at	AK025430	hypothetical protein FLJ21777	11,19	5,84	1,48	0,37
216227_at	X81001	HCGII-7 protein	0,56	1,7	1,45	0,4
216379_x_at	AK000168	Homo sapiens cDNA FLJ20161 fis	3,45	3,03	2,75	1,43
216616_at	AL137428	Homo sapiens mRNA; cDNA DKFZp761N1323	1,92	2,98	1,48	0,27
216834_at	S59049	B cell activation gene [Homo sapiens], mRNA sequence	0,32	1,07	1,91	0,16
217071_s_at	AJ249275	EMBL: Homo sapiens partial MTHFR gene	0,8	9,52	2,08	0,11
217213_at	AB022847	Norepinephrine transporter isoform 2 [Homo sapiens], mRNA sequence	0,35	3,04	1,35	0,6
217228_s_at	AC003079	EMBL: Homo sapiens BAC clone GS1-303P24 f	0,32	3,51	1,12	0,07
217236_x_at	S74639	immunoglobulin heavy constant mu	0,97	7,08	1,57	0,6
217257_at	L37198	Huntington's disease candidate region mRNA fragment	1	9,6	1,46	0,22
217338_at	AB041269	keratin 19 pseudogene	1,15	1,92	1,68	0,17
217722_s_at	NM_016645	mesenchymal stem cell protein DSC92	1,89	1,63	1,57	0,49
217752_s_at	NM_018235	hypothetical protein FLJ10830	1,48	2,34	2,41	1,29

Table continues

Table 4. *continued*

Probe set[a]	Accesion number	Gene	Fold induction			
			Ex1	Ex2	Ex3	Ex4
218538_s_at	NM_020662	MRS2-like, magnesium homeostasis factor (S. cerevisiae)	0,95	1,76	1,43	0,51
219334_s_at	NM_022837	hypothetical protein FLJ22833	2,59	5,43	1,04	0,3
219386_s_at	NM_020125	B lymphocyte activator macrophage expressed	3,56	1,71	5,43	0,28
219526_at	NM_024644	hypothetical protein FLJ21802	0,42	1,36	1,63	0,6
219744_at	NM_022158	fructosamine-3-kinase	1,27	2,02	1,2	0,14
219856_at	NM_023938	hypothetical protein MGC2742	0,57	3,64	1,6	0,17
219934_s_at	NM_005420	sulfotransferase, estrogen-preferring	0,35	14,31	1,09	0,49
220330_s_at	NM_022136	SAM domain, SH3 domain and nuclear localisation signals, 1	0,56	1,38	2,12	0,84
220364_at	NM_019033	hypothetical protein FLJ11235	1,74	3,14	1,39	0,57
220434_at	NM_024876	hypothetical protein FLJ12229	5,18	2,36	1,62	0,12
220445_s_at	NM_004909	taxol resistance associated gene 3	0,9	3,58	1,18	0,38
220704_at	NM_018563	hypothetical protein PRO0758	1,15	3,27	1,23	0,08
220770_s_at	NM_022090	transposon-derived Buster3 transposase-like	2,16	5,51	1,07	0,56
221081_s_at	NM_024901	hypothetical protein FLJ22457	0,67	1,61	1,82	0,76
221116_at	NM_016566	pparl	3,75	2,81	1,18	1,17
221345_at	NM_005306	G protein-coupled receptor 43	0,51	1,58	2,37	1,14
221368_at	NM_005383	sialidase 2 (cytosolic sialidase)	0,3	1,73	1,16	0,1
221576_at	BC000529	prostate differentiation factor	0,04	6,19	1,36	0,31
222174_at	AK024087	Homo sapiens cDNA FLJ14025 fis	0,47	5,18	1,39	0,32
210427_x_at	BC001388	annexin A2	1,06	1,93	2,26	1,14
203851_at	NM_002178	insulin-like growth factor binding protein 6	0,36	2,82	2,4	0,22
205734_s_at	AI990465	lymphoid nuclear protein related to AF4	1,08	6,05	1,55	0,17
209037_s_at	AW182860	EH-domain containing 1	0,73	1,36	1,57	0,79
209457_at	U16996	dual specificity phosphatase 5	0,36	1,34	1,54	0,58
217446_x_at	AL080160	Homo sapiens mRNA; cDNA DKFZp434M054	0,49	1,5	1,43	0,37

Table continues

Table 4. *continued*

Probe set[a]	Accesion number	Gene	Fold induction			
			Ex1	Ex2	Ex3	Ex4
214567_s_at	NM_003175	chemokine (C motif) ligand 2	0,08	1,14	1,7	0,57
217028_at	AJ224869	EMBL: Homo sapiens CXCR4 gene encoding receptor CXCR4	0,87	1,24	1,39	0,3
217078_s_at	AJ010102	leukocyte membrane antigen	0,18	1,36	1,98	1,32
221552_at	BC001698	lipase protein	0,15	1,25	1,6	0,51
221679_s_at	AF225418	lipase protein	0,18	1,31	1,6	0,51
221808_at	NM_004251	RAB9A, member RAS oncogene family	0,94	1,54	1,53	0,63
AFFX-LysX-3_at	X17013	Bacillus subtilis lys gene for diaminopimelate decarboxylase	1,66	4,19	1,46	0,58
201008_s_at	NM_006472	thioredoxin interacting protein	5,52	2,59	3,01	1,59
201009_s_at	NM_006472	thioredoxin interacting protein	3,88	2,77	2,64	1,53
201010_s_at	NM_006472	thioredoxin interacting protein	2,9	3,02	2,58	1,46
201235_s_at	BG339064	BTG family, member 2	0,36	1,31	2,17	0,86
201236_s_at	NM_006763	BTG family, member 2	0,61	1,42	1,81	0,9
201368_at	AI356398	zinc finger protein 36, C3H type-like 2	0,95	1,55	1,6	0,56
201369_s_at	NM_006887	zinc finger protein 36, C3H type-like 2	0,58	1,48	1,57	0,57
201694_s_at	NM_001964	early growth response 1	0,17	1,19	1,62	0,46
201908_at	NM_004423	dishevelled, dsh homolog 3 (Drosophila)	1,52	2,02	2,07	1,5
201998_at	AI743792	sialyltransferase 1 (beta-galactoside alpha-2,6-sialytransferase)	0,37	1,65	1,72	0,83
202092_s_at	NM_012106	binder of Arl Two	1,32	2,29	2,42	1,2
202693_s_at	NM_004760	serine/threonine kinase 17a (apoptosis-inducing)	1,59	1,78	1,78	0,74
204057_at	AI073984	interferon consensus sequence binding protein 1	0,48	1,58	1,82	0,88
204748_at	NM_000963	prostaglandin-endoperoxide synthase 2	0,35	5,5	1,19	1,65
204794_at	NM_004418	dual specificity phosphatase 2	0,18	1,41	1,68	0,74
204951_at	NM_004310	ras homolog gene family, member H	0,82	1,47	1,69	0,77
205089_at	NM_003416	zinc finger protein 7 (KOX 4, clone HF.16)	1,22	2,62	1,62	0,62
206440_at	NM_004664	lin-7 homolog A (C. elegans)	1,14	3,23	1,47	0,82

Table continues

Table 4. *continued*

Probe set[a]	Accesion number	Gene	Fold induction			
			Ex1	Ex2	Ex3	Ex4
206638_at	NM_000867	5-hydroxytryptamine (serotonin) receptor 2B	3,95	2,61	2,27	0,97
206641_at	NM_001192	tumor necrosis factor receptor superfamily, member 17	1,72	2,21	2,2	1,28
206806_at	NM_004717	diacylglycerol kinase, iota	0,53	29,35	1,34	0,79
207302_at	NM_000231	sarcoglycan, gamma (35kDa dystrophin-associated glycoprotein)	0,56	5,44	1,51	0,56
207332_s_at	NM_003234	transferrin receptor (p90, CD71)	0,58	1,52	1,93	0,92
207571_x_at	NM_004848	basement membrane-induced gene	0,59	1,49	1,76	0,62
207768_at	NM_001965	early growth response 4	0,01	1,02	1,12	1,07
208173_at	NM_002176	interferon, beta 1, fibroblast	0,18	3	1,16	0,18
208296_x_at	NM_014350	TNF-induced protein	2,35	1,98	2,45	1,76
208924_at	AB024703	ring finger protein 11	4,86	1,7	2,6	1,66
210109_at	AF191492	brain and nasopharyngeal carcinoma susceptibility protein	0,99	13,41	1,5	0,24
210429_at	X63097	Rhesus blood group, D antigen	6,41	4,44	1,13	2,45
210448_s_at	U49396	purinergic receptor P2X, ligand-gated ion channel, 5	0,43	1,68	1,79	0,41
211341_at	L20433	POU domain, class 4, transcription factor 1	0,96	6,72	1,14	0,14
211676_s_at	AF056979	interferon gamma receptor 1	0,54	1,49	1,77	0,79
211701_s_at	AF349720	trophinin	0,94	3,17	3,3	0,45
211742_s_at	BC005926	ecotropic viral integration site 2B	3,18	1,89	2,41	1,15
211965_at	X79067	zinc finger protein 36, C3H type-like 1	0,48	1,4	1,88	1,05
212418_at	M82882	E74-like factor 1 (ets domain transcription factor)	1,62	1,7	2,23	0,75
214331_at	AI796813	advillin	0,55	23,5	1,12	0,11
214805_at	U79273	Human clone 23933 mRNA sequence	0,27	1,51	2,29	1,11
216801_at	AK026910	Homo sapiens cDNA: FLJ23257 fis, clone COL05579	0,49	2,99	1,7	0,56
217660_at	AW188214	Unknown (protein for IMAGE:4111094)	0,09	3,6	1,27	0,16
218640_s_at	NM_024613	phafin 2	2,58	2,67	2,23	1,45
219371_s_at	NM_016270	Kruppel-like factor 2 (lung)	1,39	1,67	1,7	0,88

Table continues

Table 4. *continued*

Probe set[a]	Accesion number	Gene	Fold induction Ex1	Ex2	Ex3	Ex4
219824_at	NM_012450	solute carrier family 13 (sodium/sulfate symporters), member 4	1,2	7,18	1,62	0,7
219841_at	NM_020661	activation-induced cytidine deaminase	3,29	2,79	1,98	0,93
220719_at	NM_025012	hypothetical protein FLJ13769	0,88	4,77	1,56	0,49
221566_s_at	AF043244	nucleolar protein 3 (apoptosis repressor with CARD domain)	0,91	5,61	1,31	0,45
222221_x_at	AY007161	EH-domain containing 1	0,34	1,5	1,88	0,79

[a]Unique Affymetrix identifier.

9.0 *Curriculum vitae*

Maja Šantak

Born 11th July, 1974 in Kutina, Croatia

School Education

1981-1989	Primary School ″Marijan Grozaj″, Ivanić-Grad, Croatia
1989-1993	Secondary School ″Ivan Švear″, Ivanić-Grad, Croatia

University Education

1993-1998	B.Sc. Molecular Biology, Department of Biology, Faculty of Natural Science The University of Zagreb, Croatia

Research Experience

1998-2000	Research Assistant Section of Molecular Biomedicine Department for Research and Development Institute of Immunology, Zagreb, Croatia

Doctoral Thesis

2000-2003	"Identification and Functional Analyses of Epstein-Barr Virus Nuclear Antigen 2 (EBNA2) Target Genes" GSF-Institute for Clinical Molecular Biology and Tumour Genetics, Munich, Germany PD Dr. Bettina Kempkes

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