A FUNCTIONAL ANALYSIS OF THE RNA POLYMERASE II LARGE SUBUNIT CARBOXY-TERMINAL DOMAIN

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SYNOPSIS

Following the confirmation of DNA as the genetic material in the 1950's, work focussed on how this information is translated into the functional components from which cells are composed. The controlled transcription of specific coding regions into RNA lead to the discovery of a variety of RNA polymerases, which were later shown to differ based on the purpose of the RNA which they produce. Eucaryotic cells possess three distinct RNA polymerases for production of the three major catagories of cellular RNA: Pol I (28 S-, 18 S- and 5.8 S-ribosomal RNA); Pol II (messenger RNA); Pol III (5 S-ribosomal RNA; transfer RNA). However, only Pol II transcripts (mRNA) are further translated into proteins.

The results of the human genome project suggest the presence of approximately 30,000 genes, whose coding regions occupy less than 5 % of the 6 x 10^9 DNA base pairs from which it consists. Not all genes are expressed: the pattern of gene expression differs between cell types, and changes in response to cellular signals. The mechanism by which this is controlled requires the presence of gene-proximal sequences (promoters and enhancers), and proteins that specifically bind to them (transcription factors) in order to recruit the RNA polymerase.

Unlike the other RNA polymerases, RNA Pol II is extensively modified by a variety of enzymes that can influence its initiation, elongation and the processing of pre-mRNAs, in a promoter specific manner. Although the enzymatic core of all three mammalian RNA polymerases is almost identical, large differences are seen with their external surfaces, no more apparent than that of the C-terminal domain (CTD) of the RNA polymerase II large subunit: a 378 amino acid structure that is absent in all other polymerases.

The CTD of RNA polymerase II consists of 52 repeats of a heptapeptide consensus sequence, tyrosine - serine - proline – threonine – serine – proline - serine (YSPTSPS), an unusual sequence in that it contains a high percentage of phosphorylation sites (Payne and Dahmus, 1993). Nearly 20 years after its identification, the exact role of CTD still remains enigmatic: CTD is required for transcription initiation, RNA elongation, and the recruitment of important factors for mRNA capping, splicing, and 3'-processing. Exactly how CTD fulfils these functions has been complicated by conflicting *in vitro* and *in vivo* data: the CTD appears unimportant for *in vitro* transcription. This observation, and an

increased understanding of the role of chromatin in transcription regulation made the development of good *in vivo* systems a necessity. The development of a tetracycline-inducible, α–amanatin-resistant RNA polymerase II expression system by this laboratory was such a development: for the first time, an exogenously expressed mutant RNA polymerase II large subunit could be tested *in vivo*, on the endogenous, chromatin template. This system was initially used to study the importance of CTD length, based on the differences seen between different organisms. During this time, a plethora of research appeared regarding the specific phosphorylation of the CTD by various kinases, and the factors recruited as a result. Chromatin immunoprecipitation experiments by the Buratowski laboratory imply position-specific changes in phosphorylation, resulting in the sequential recruitment of factors for pre-mRNA processing during the transition from early to late elongation phases. The kinases (CDKs 7, 8 and 9) involved in these processes are probably involved in the transcription of every gene. Despite expressing some preference for consensus or non-consensus CTD repeats *in vitro*, the patterns of phosphorylation produced *in vivo* and what factors they specifically recruit remains speculation.

The discovery that other enzymes act on the CTD in a non-general, stimulus-specific way is cause for much interest. What signal is conveyed to the transcription machinery and for what purpose? Through this work I have tried to elucidate the purpose of the last CTD repeat of the RNA Pol II large subunit, a sequence known to be the target of the kinases CKII, c-Abl and c-Arg. At present, this is the only known specific site of interaction for CTD kinases. Contrary to earlier speculation, no effect on transcriptional elongation could be seen when this domain was removed, suggesting that these interactions serve some other function. The removal of the last repeat had no effect on the induction of specific gene transcription in response to ionising radiation, indicating that the phosphorylation of the CTD by c-Abl is not important for this function. Analysis of CKII site point mutants confirmed previous suggestions that the greater number of acidic amino acids surrounding serine 13 of the last repeat make it the preferential site for CKII phosphorylation. Mutation of the CKII phosphorylation sites within this domain had no notable effect on any aspects of function tested, indicating that this modification may be redundant, or of little importance. However, complete removal of this domain, or severe mutation thereof, resulted in the proteolytic degradation of the large

subunit to the CTD-less, Pol IIb form – a form previously only seen *in vitro.* This form is probably inactive *in vivo,* suggesting that the last CTD repeat might be involved in a mechanism by which the activity of RNA polymerase II is regulated through its specific degradation.

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LIST OF ABBREVIATIONS

´	minute
´´	second
A	adenosine
Amp	ampicillin
APS	ammonium peroxodisulphate
ATM	ataxia telangiectasia mutated
ATP	adenosine triphosphate
bp	base pair
BL	Burkitt's lymphoma
BSA	bovine serum albumin
C	cytosine
cDNA	complementary DNA
CDK	cyclin-dependent kinase
CKII	caesin kinase II / protein kinase CKII
CTD	carboxy terminal domain of RBP1
CTP	cytosine triphosphate
DEPC	diethyl pyrocarbonate
DNA	2'-deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	3'-deoxyribonucleoside-5'-triphosphate
DMSO	dimethyl sulphoxide
D. melanogaster	*Drosophila melanogaster*
DPM	degradations per minute
DTT	dithiothreitol
EBV	Epstein Barr Virus
EBNA	Epstein-Barr virus nuclear antigen
E. coli	*Escherichia coli*
EDTA	ethylene diamine tetra-acetic acid
EGFP	enhanced green fluorescent protein
EGTA	ethylene glycol-0,0'-bis (2-aminoethyl)-N,N,N',N'-tetra-acetic acid
ERK	extracellular-regulated kinase (MAPK)
FCS	foetal calf serum
G	guanosine
GTP	guanosine triphosphate
h	hour
HA	haemagglutinin
HIV	human immunodeficiency virus
Hsp	heat shock protein
Ig	immunoglobulin
IP	immunoprecipitation
IR	ionising radiation (γ-radiation)
JNK	c-Jun N-terminal kinase
kb	kilo base pair
kD	kilo Dalton
LCL	lymphoblastic cell line
LMP1	latent membrane protein 1
LS	large subunit
MAPK	mitogen-activated protein kinase
MCS	multiple cloning site
MOPS	3-(N-morpholino)propansulphoic acid
mRNA	messenger RNA
NELF	negative elongation factor
NFkB	nuclear factor kappa binding protein
neo	neomycin / G418
NTP	nucleotide triphosphate
OD	optical density
oriP	origin of replication
pA	polyadenosine
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction

PIC	pre-initiation complex
Pol I/II/III	DNA dependent RNA-polymerase I / III / III
PMSF	phenylmethyl-sulphonyl fluoride
Rb	retinoblastoma
RNA	ribonucleic acid
RNase	ribonuclease
RT	room temperature
RT-PCR	reverse transcription-PCR
RPB1	RNA polymerase B: the large subunit of RNA polymerase II
rpm	revolutions per minute
S. cerevisiae	*Saccharomyces cerevisiae*
SDS	sodium dodecylsulphate
SRB	suppressor of RNA polymerase B
SSC	sodium chloride-sodium citrate buffer
T	thymidine
TAE	Tris-acetate-EDTA buffer
TAT	transcription factor required for transcription of the HIV LTR
TBE	Tris-borate-EDTA buffer
TBP	TATA-box binding protein
Tc	tetracycline
TE	Tris-chloride / EDTA (10:1)
TEMED	N,N,N',N'-tetramethylethylenediamine
TFIIA-F	the general transcription factors of RNA pol II
tTA	tetracycline-responsive transcriptional activator
TTP	thymidine triphosphate
U	units
UTP	uridine triphosphate
UV	ultraviolet
VP16	viral protein 16 from the herpes simplex virus
v/v	percentage volume to volume
w/v	percentage weight to volume
wt	wild type

1. Introduction

1.0 Overview

The tight regulation of gene expression is essential to all cellular organisms in order to respond to changing environments, and for the regulation of their growth, development, proliferation, and even death in more complex organisms. Gene expression starts with the copying of coding deoxyribonucleic acid (DNA) sequences into a ribonucleic acid (RNA) transcript (Transcription), which itself is then used as the template for protein synthesis (Translation). The DNA dependent RNA-polymerases, responsible for transcription of RNA, are composed of multiple protein subunits that assemble to form a so-called 'transcription complex'. The components of these complexes provide not only the enzymatic function for RNA synthesis, but regulate the ability of the enzyme to initiate, elongate and terminate transcription of a template.

A major difference between the genomes of higher organisms to that of procaryotes is the packaging of DNA in to chromatin. Not only does chromatin serve to structurally organise the DNA in the nucleus, but also functions as a general mechanism of gene repression. Only when this repression is removed can transcription occur. Transcription by all polymerases is thus dependent on the presence of accessory factors that may activate or repress gene expression through the modulation of chromatin structure.

1.1 The regulation of gene expression through chromatin

Eucaryotic transcriptional control can be divided into two modes of regulation: at the level of chromatin and through the recruitment and activation of RNA polymerase II. Although similarities to procaryotes exist, the large size of eucaryotic genomes requires its packaging in order to prevent it occupying many times the cell volume. Since not all genes are required for every cell type, only those required are in an accessible form of chromatin. This in turn requires a more complex transcription apparatus to transcribe through it.

The nuclear DNA is complexed with histone and non-histone proteins. The DNA double helix is stored in beads, called nucleosomes, which consist of two molecules each of the histone proteins H2A, H2B, H3 and H4. Nucleosomes form on approximately every 200 base pairs of DNA, with 146 base pairs wrapped around each nucleosome, and the rest forming a linker to the next. This first packaging step already reduces the DNA molecule to one-third of its original size. Histone H1 forms contacts with both nucleosomes and DNA, optimising the exit path of DNA from a nucleosome to improve stacking, and thereby producing a 30 nm nucleosome fibre. This type of packaging accounts for the majority of DNA in a typical mammalian cell (euchromatin), with about one tenth being in a more highly packaged, inaccessible form (heterochromatin).

The binding of nucleosomes to DNA is not sequence specific, but due to steric constraints, expresses a preference for A-T rich regions, leading to irregular spacing. More importantly, nucleosome positioning is also regulated by the binding of proteins to DNA regions. This results in the exact positioning of nucleosomes along certain stretches of DNA, and influences the access of other proteins to these regions. The promoter regions of most genes have a defined nuclear structure, that is modulated to influence the recruitment and initiation of transcription complexes.

1.1.1 Chromatin remodelling

In order for transcription factors and RNA polymerase II to bind to promoters, the nucleosome structure must first be altered to permit access. Specialised proteins can bind this packaged DNA, and using the energy of ATP hydrolysis, may change the structure of nucleosomes temporarily to allow their movement. The remodelled state probably results from movement of the H2A-H2B dimers in the nucleosome core (Kireeva et al., 2002), since the H3-H4 tetramer is very stable and therefore difficult to rearrange. Two families of multiprotein remodelling complexes have so far been identified: SWI/SNF and ISWI (imitation switch). They distinguish themselves from one another through their helicase/ATPase components: SWI/SNF family members perturb the nucleosome core-particle structure, whereas ISWI complexes function to shift nucleosomes to different locations on the DNA. Nevertheless, both complexes contain common subunits, but how their specificity and activity

is regulated is still not known. Incubation of nucleosome-bound DNA with these complexes creates 'nuclease-hypersensitive sites' due to the formation of nucleosome-free regions. Such sites have been found to contain the enhancers and promoters of transcriptionally active genes, exposure of which is an important step early in transcription activation, explaining why the SWI/SNF complex is one of the first co-activators to be recruited to a locus initiating transcription (Cosma et al., 1999). Importantly, remodelling complexes may also function to repress transcription through the formation of inaccessible chromatin structures.

1.1.2 Modification of histone tails alters the properties of nucleosomes

The study of transcriptional activators has revealed one of their major functions to be the recruitment of enzyme 'co-activators', that often contain catalytic activities towards histones. Histone tails are subject to several modifications that influence the ability of their containing nucleosomes to form stable higher chromatin structures, for example, the 30 nm chromatin fibre. Acetylation of the positively charged lysine residues in histone tails by histone acetyl-transferase (HAT) activities may influence the ability of histones to neutralise the charges on DNA, thereby reducing the stability of such higher structures. The recruitment to promoter regions of HAT-containing co-activators, such as p300 and CBP (cAMP-responsive-element-binding (CREB)-binding protein), opens nucleosome-bound templates to allow the access of other remodelling factors. Histone tails are subject to other modifications, for example, methylation and ubiquitinylation. Such modifications may function to specifically recruit regulatory and structural proteins to modulate the compaction of chromatin. In telomeric DNA, under acetylation of histone H4 tails has been shown to recruit complexes of Sir proteins (Sir2 is itself a histone deacetylase) to assist the condensation required for the formation of heterochromatin.

The architecture of promoters is therefore governed by an interplay of positive (HATs) and negative (HDACs; methylases) factors, which in turn influence the accessibility of the general transcription machinery, and thus the initiation of transcription.

1.2 RNA polymerase II

The eucaryotic RNA polymerase II consists of a 12 subunit, > 0.5MD complex with two magnesium ions in its active site (Cramer et al., 2001). The two largest subunits, RPB1 (approx. 220 kDa) and RBP2 (approx. 140 kDa), form the catalytic domain through which the DNA-RNA assembly from ribonucleotides takes place. These two subunits exhibit structural and functional homology to the ß and ß' subunits of the *E. coli* RNA polymerase. The elucidation of the crystal structure of RNA polymerase II (Cramer et al., 2000; Cramer et al., 2001) has revealed that the enzymatic core structure of bacterial and mammalian polymerases is highly conserved through evolution. Sequence comparison of RPB1 and RPB2 to ß and ß' revealed homology in 8 of 9 core domains within their structure. The linking regions between these domains may have evolved to allow enhanced functions of the polymerase complex. Conservation is also seen between the three eucaryotic polymerases: Pol II contains five subunits that are also present in Pols I and III (Bushnell et al., 2002).

The large subunit of RNA polymerase II (RBP1) differs strikingly from other eucaryotic polymerases in its possession of a unique C-terminal domain (CTD). The CTD of eucaryotic RBP1 consists of repeats of the consensus hepta-peptide sequence YSPTSPS (Corden et al., 1985). The consensus sequence is highly conserved across organisms, but the number of repeats appears to have increased through evolution (Fig. 1.) (Stiller and Hall, 2002); the more complex an organism, the greater the number of repeats: *Plasmodium* has 17, *Saccharomyces* 26 or 27, *Drosophila* ≈45, and mice and humans 52 repeats (Allison et al., 1988).

Deletions of more than half of the repeats in the yeast, or mouse CTD interferes with cell viability. Mice homozygous for a deletion of 13 repeats are smaller then wild type littermates and have a high rate of neonatal lethality, suggesting that the CTD is important in growth regulation during mammalian development (Litingtung et al., 1999). Tissue culture experiments to test the importance of CTD length in mammalian cells have demonstrated that a truncation to 31 repeats, although capable of transcription of *hsp70A* and c-*fos* genes (Meininghaus et al., 2000; Meininghaus and Eick, 1999), is growth limiting and cannot replace the endogenous RPB1 with respect to long-term survival. A truncation to five repeats exhibits a global defect in transcription

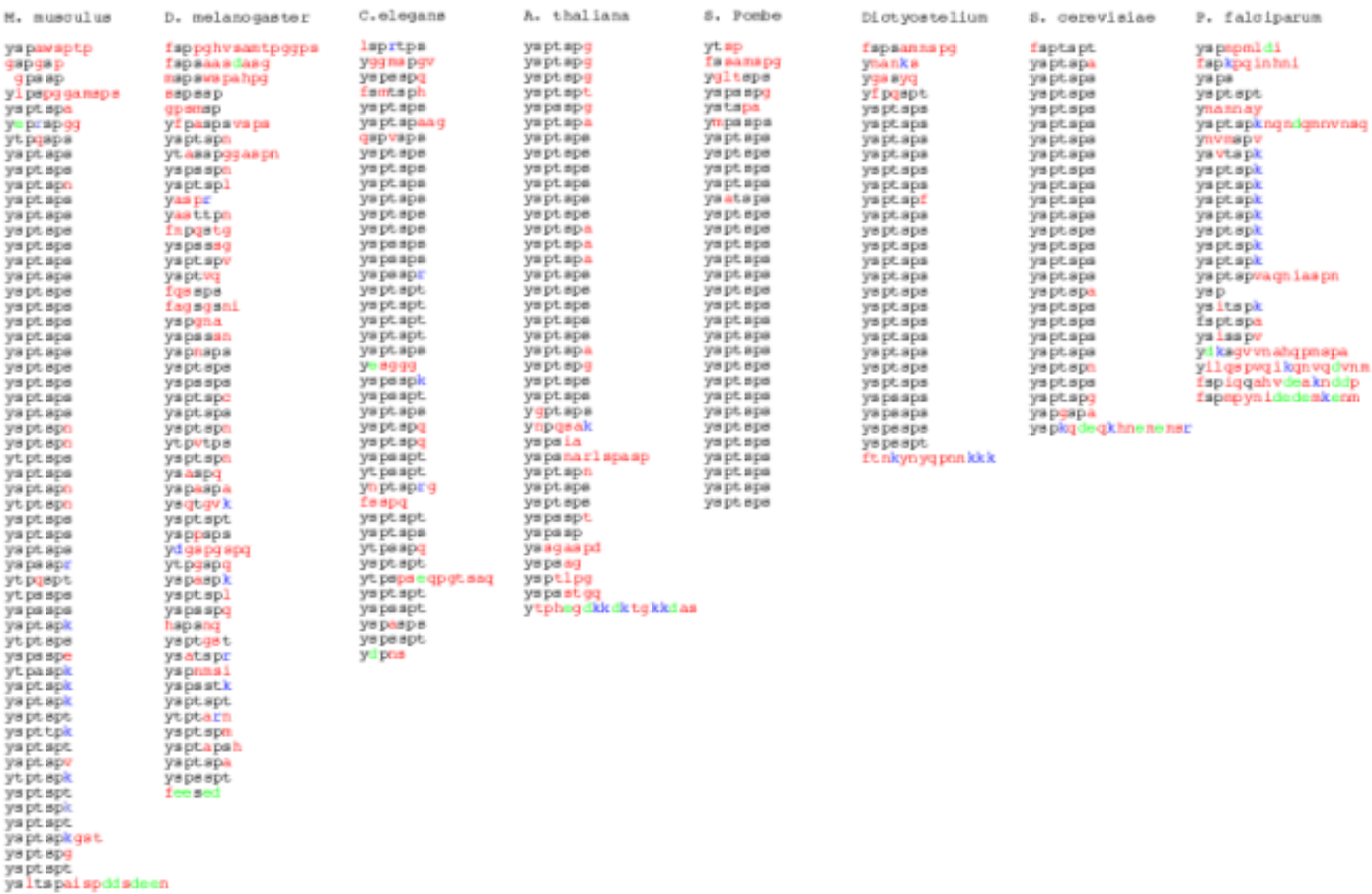


Figure 1. **A comparison of the composition of RNA polymerase II CTDs across species.** Residues that differ from the CTD consensus sequence YSPTSPS are highlighted by colour (red, green, or blue). Acidic residues are labelled green; basic, blue; neutral, red.

on a native template, and is thus unable to support life (Meininghaus et al., 2000).

The CTD becomes heavily phosphorylated at a specific phase of the transcription cycle (Cadena and Dahmus, 1987; Dahmus, 1994), the number of phosphorylation sites exceeding 50 (Payne and Dahmus, 1993). The phosphorylation status of the CTD has been shown to be essential for regulation of gene expression. Only the non-phosphorylated (IIA) form of Pol II can participate in the formation of a pre-initiation complex (PIC), while CTD phosphorylation is essential for transcriptional elongation (the IIO form) (Cadena and Dahmus, 1987; Payne et al., 1989). Stress induced phosphorylation of non-DNA-bound Pol II by kinases, such as ERK, may function to down regulate transcription by preventing the formation of new PICs (Bonnet et al., 1999; Dubois et al., 1994). Recently, it has been shown that phosphorylation of serines 2 and 5 (YS^2PTS^5PS) differs dependent upon the position of the RNAPII complex (Komarnitsky et al., 2000). A bias toward serine-5 phosphorylation is observed in the CTD of polymerases in promoter regions, while more serine-2 phosphorylation is observed during transcription of coding regions. Such phosphorylation events create specific phospho-amino acid motifs allowing selective recruitment of accessory proteins to the CTD in a kinase dependent manner. The CTD of the elongating form of Pol II *in vivo* contains an estimated 50 phosphorylated amino acids, of which the majority being serine (Hengartner et al., 1998). The phospho-CTD binding partners so far described exhibit dependence for phospho-serine; roles for phospho-threonine and phospho-tyrosine are yet to be described. The structure of the CTD may be further modified through isomerisation of proline residues from *cis*- to *trans*- conformations by the parvulin family isomerase Pin1 (Albert et al., 1999), and cyclophilin SRcyp / CASP10. These proteins have been implicated in controlling access to the phospho-CTD, of co-factors involved in 3' pre-mRNA-end formation, and the ubiquitination of RPB1 (Carty et al., 2000; Chang et al., 2000; Cho et al., 1997; Goldstrohm et al., 2001; Lindstrom and Hartzog, 2001; Morris and Greenleaf, 2000; Morris et al., 1999). The hypophosphorylated form of RPB1 (Pol IIa) has also been found to undergo glycosylation, through the addition of O-linked N-acetyl glucosamine residues (O-GlcNac) to the CTD, the purpose of which is not known (Kelly et al., 1993)

Figure 2. The amino acid sequence of the final non-consensus repeat of the RNA polymerase II (RPB1) subunit. In some organisms, an unusual domain is found at the C-terminal end of the CTD, in mammals refered to as the 52nd, or ´last´ CTD repeat. Residues conforming to the CTD consensus sequence (YSPTSPS) are labelled black; acidic residues, green; basic residues, blue; neutral residues, red.

1.3 The last repeat of the RNA polymerase II CTD

The repeat sequence of the CTD increasingly deviates from the consensus sequence (YSPTSPS) with proximity to the carboxy-terminus. The evolution of longer CTDs, and non-consensus repeats have been speculated to attribute specific functions to different regions of the CTD (Fong and Bentley, 2001). A comparison of different organisms reveals the development of divergent C-terminal sequences of unknown purpose, often referred to as the last repeat (Fig. 2). These sequences not only differ in their amino acid composition, but also length: the last repeat of *Plasmodium falciparum* CTD consists of 68 amino acids; *Caenorhabditis elegans,* 5 amino acids; and *Saccharomyces pombe* ends in a simple consensus repeat. Despite the great diversity seen between species, it is interesting to note the presence of similar acidic amino acids in the last repeat of both *Drosophila melanogaster* and mammalian CTDs.

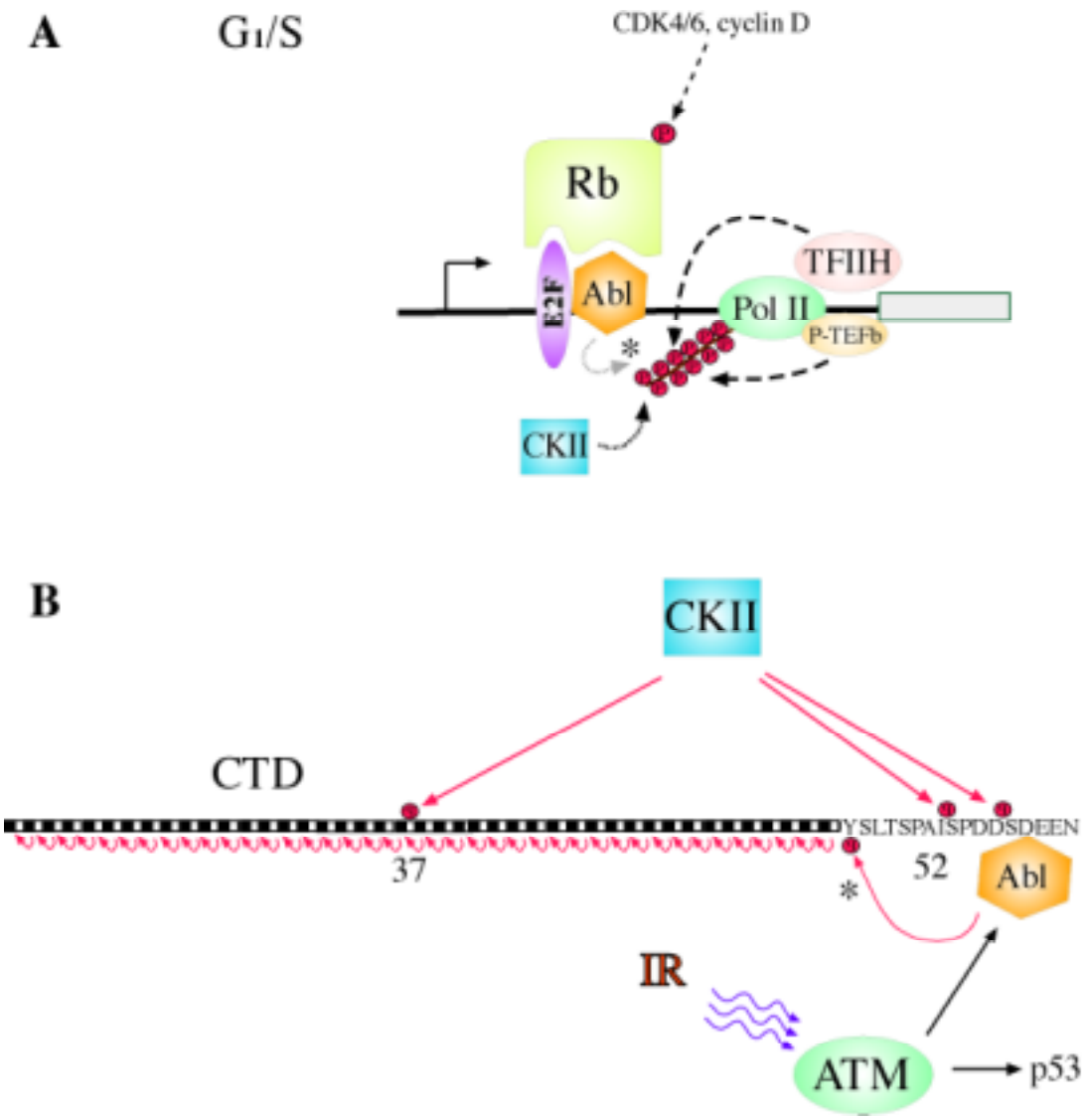


Figure 3. Signalling to the last 17 amino acids of the mammalian RNA polymerase II CTD. (A) A simplified model of potential kinase activities at an E2F-1 regulated promoter. Active Rb binds and inhibits both E2F-1 and c-Abl. Following deactivation of Rb by CDK4/6 at the G1/S phase transition, E2F-1 may stimulate initiation and elongation of RNA polymerase II. The initiation-associated kinase of the TFIIH general transcription factor (CDK7/ cyclin H), and the kinase of the elongation factor P-TEFb (CDK9/ cyclin T), may differentially phosphorylate serines at positions 2 and 5 in each repeat of the CTD. (B) The ATM kinase activates c-Abl and p53 in response to a specific type of DNA damage caused by ionising radiation (IR). The c-Abl tyrosine kinase, recruited to the promoter by Rb can bind and phosphorylate tyrosines in the CTD, but only following activation by ATM (*). c-Abl recognises a motif lying within the 52nd repeat that it requires for binding to, and efficient phosphorylation of the CTD. The CTD contains three consensus phosphorylation sites for the casein kinase II (CKII): one contained in repeat 37; two in the final 17 amino acids of CTD which constitute repeat 52.

Between mammals, the sequence is 100% conserved, suggesting it may have evolved to serve an important new function in higher organisms. Indeed, the mammalian final repeat contains a total of 17 amino acids, forming what constitutes two potential casein kinase II (CK II) recognition sites (Bregman et al., 2000), and is essential for recognition by the tyrosine kinases c-Abl and c-Arg (Baskaran et al., 1997a; Baskaran et al., 1999). The purpose of these interactions is not known, but a consideration of current knowledge of the CTD, and its phosphorylation by specific kinases may assist our understanding. It is possible that individual promoters may be regulated by specific CTD kinases. Figure 3 depicts a simplified view of the kinases present at an E2F-1 regulated promoter: in addition to transcription-associated cyclin-dependent kinases, the presence of E2F-1 indirectly recruits c-Abl through its interaction with Rb.

1.4 Initiation of transcription

Changes in the chromatin structure of certain control regions influences the access of activator proteins to stimulate the recruitment of a pre-initiation complex to a gene promoter region. These elements may be located many kilobases away, and may assert a positive ('enhancer'), or negative ('silencer') influence on transcription of a gene locus (Seipel et al., 1992).

Unlike procaryote RNA polymerase, mammalian RNA polymerase II is by itself unable to initiate and complete the transcription of a gene. Additional proteins, called the general transcription factors (GTFs), are first required to bind a promoter before the recruitment of Pol II. These proteins are ‚general' because they are required by all promoters used by Pol II. The text book model of PIC assembly starts with promoter binding of TFIID, followed by TFIIA, TFIIB, TFIIE, TFIIH and the remaining GTFs, including Pol II. Not surprisingly, the promoters of class II eucaryotic genes contain elements to direct the binding of GTFs. The transcription start site usually contains a pyrimidine-rich initiator element (INR), with the consensus sequence $(Py)_2A_{+1}{}^{A}/_{T}(Py)_2$, that binds the GTF, TFIID. Approximately 25-30 bp upstream from INR lies the TATA box (Consensus sequence TATA ${}^{A}/_{T}$A ${}^{A}/_{T}$), an element recognised and bound by the TBP (TATA-binding protein) subunit of TFIID. Binding of TFIID to the TATA box causes an ≈80° kink in the promoter DNA thereby enabling the binding of TFIIB to the adjacent BRE element (Consensus: ${}^{G}/_{C}{}^{G}/_{C}{}^{G}/_{A}$ CGCC), which lies 35

bp upstream from INR. Binding of TFIIB stabilises the binding of TFIID to DNA, forming the interface for recruitment of the Pol II/TFIIF complex. Unlike most other transcription factors TFIID also binds a sequence in the transcribed region downstream of INR: the DPE element (Consensus: $^{A}/_{G}G^{A}/_{T}CGTG$).

TFIID is composed of at least eight TBP-associated factors (TAFs) which probably function in the specific recognition of promoters, and activator-stimulated transcription (Komarnitsky et al., 1999). Interestingly, a TAF-containing complex (TFTC) produced by depleting TBP from TFIID, still permits basal and activated transcription of TATA-containing and TATA-less promoters. Several TAFs exhibit sequence and structural homology to histones and their properties of DNA binding may, alike the bacterial sigma factor, assist recognition of specific promoter sequences. As mentioned, only non-phosphorylated Pol II (Pol IIA) can form initiation complexes. Dephosphorylation is probably achieved through a CTD-specific phosphatase FCP1, which associates with, and is stimulated by TFIIF.

PIC formation results in the ATP-dependent melting of DNA in the region of the transcription start site. The mammalian TFIIH complex consists of nine subunits and is the only GTF possessing ATP-dependent enzymatic activities (Dvir et al., 1996). TFIIH contains two helicases encoded by the *Xeroderma pigmentosum* complementation group B (XPB) and D (XPD) genes, and the CTD- and cyclin-dependent kinase cdk7/cyclin H (Moreland et al., 1999). Initiation is completed following the completion of the first phosphodiester bonds of the RNA transcript (Kugel and Goodrich, 2002).

Since this model of assembly was based on *in vitro* data, the exact order in which GTFs actually assemble on promoters *in vivo* is not certain. However, studies in yeast suggest that the GTFs and Pol II exist in a pre-assembled form called a 'holoenzyme', containing TFIIB, TFIIF, TFIIH and the SRB (Suppressor of RNA polymerase B)/Mediator protein (Koleske and Young, 1995). The holoenzyme model suggests initiation is not regulated through the assembly of the individual PIC components, rather the rate with which the activators recruit a holoenzyme to a promoter. Transcriptional activator proteins bind to TFIIB, TFIID and TFIIH, and genetic evidence implicates these interactions in gene activation *in vivo*. However, transcriptional activators fail to stimulate transcription reconstituted with Pol II and the GTFs *in vitro*. The reaction requires the addition of a protein complex called 'Mediator', which provides the interface between activator and holoenzyme (Mittler et al., 2001). Mediator is

thought to integrate and transduce positive and negative regulatory information from enhancers and operators to promoters (Kornberg, 2001).

1.4.1 The Mediator complex

Mediator was originally isolated as a 20-subunit complex from yeast, and corresponding complexes have since been found in mice and human cells. Two-thirds of the Mediator subunits are products of genes previously identified in screens in yeast for mutations that affect transcription control, demonstrating the importance of Mediator for *in vivo* regulation (Myers et al., 1999; Myers and Kornberg, 2000). The Mediator subunits can be divided in to three groups: the products of the five SRB genes, characterised as interacting with the RPB1 CTD; the products of four genes identified as global suppressors, and six members of a new protein family, termed Med, implicated in transcriptional activation. Mediator is only associated with initiation complexes (Pokholok et al., 2002). Reconstitution experiments *in vitro* have shown mediator to stimulate the phosphorylation of CTD by the TFIIH kinase 30- to 50-fold. Since Mediator binds only hypophosphorylated CTD, this function may serve to release itself from the initiation complex once the signal for elongation is received. This is supported by evidence for the recycling of Mediator to initiation complexes and the absence of Mediator from elongating polymerases (Pokholok et al., 2002; Svejstrup et al., 1997). The transition to elongation was subsequently shown to involve the replacement of Mediator with an elongation complex termed 'Elongator' (Otero et al., 1999).

1.5 Transcription elongation

The exact mechanisms of transition from the initiation phase to the elongation phase are confused by conflicting data from *in vitro* and *in vivo* systems. However, the process can be broken down in to five defined steps: PIC formation, initiation, escape commitment, promoter escape, and transcript elongation. It is proposed that following synthesis of a 4-nt RNA, a conformational change occurs in the ternary transcription complex, rendering it insensitive to otherwise inhibitory single stranded RNAs, and thus escape committed (Kugel and Goodrich, 2002). Promoter escape is accomplished after

the synthesis of a 10-15-nucleotide-long transcript, leading to the release of Pol II from initiation factors and the promoter (Conaway et al., 2000). Early Pol II elongation complexes containing transcripts of less than approximately nine nucleotides in length are unstable, and are prone to abortive transcription (Fiedler and Timmers, 2001).

Elongation complexes lack basal factors, with the exception of TFIIF, which in addition to being required for initiation (Dvir et al., 2001) is also an elongation factor, and acts during the early stages of elongation to decrease the frequency of abortive transcription. It has been proposed that TFIIF functions to stabilise the tight interaction of the PIC with promoter DNA and facilitates its unwinding during the formation of the open complex (Yan et al., 1999).

1.5.1 General elongation factors

A variety of positive and negative elongation factors exist, which may direct the elongation, arrest, or abortion of transcription complexes. The tendency of early Pol II elongation complexes *in vitro* to arrest can be overcome by the GTFs, TFIIE and TFIIF, in a reaction that requires the ATP-dependent XPB DNA helicase activity of TFIIH (Dvir et al., 2001; Moreland et al., 1999). Unlike a classical DNA helicase, XPB induces opening by twisting promoter DNA (Fiedler and Timmers, 2001). This may function to assist the release of TFIIF-DNA contacts, that although essential for initiation, may impede elongation (Conaway et al., 2000).

It is suggested that RNA polymerase traverses a DNA template either by I) the continuous addition of nucleotides, one after the other (monotonous), or more probable, II) in a discontinuous manner, through phases of expansion and contraction ('inchworming') (Chamberlin, 1992; Nudler et al., 1994). Structural comparisons of bacterial and eucaryotic RNA polymerases has allowed speculation as to the paths taken by the DNA, NTPs and RNA product within the complex. A highly conserved structure, the 'bridge helix', traverses the active site of both polymerases, but is straight in Pol II and bent in the bacterial structure. The bridge helix forms contacts with the end of the DNA-RNA hybrid in elongation complexes, and is speculated to provide elasticity to the translocating complex, while firmly maintaining nucleic acid-protein interaction (Cramer et al., 2001). Addition of a nucleotide to the growing RNA strand provides energy for the distortion of the complex, which is only reversed

upon forward translocation to the next position, or through a reverse translocation at pause- or termination-sites. Elongation blocks imposed by inhibitory structures, or proteins bound to the template cause transient stalling of elongation complexes, which in some cases may convert to stable transcriptional arrest. Arrest leads to backtracking of the polymerase and removal of the 3'-end of the nascent RNA from the active site, through the NTP access pore of the 'cleft' domain (pore1) in to the funnel (Cramer et al., 2001), where it is subject to degradation by Pol II's proof-reading endonuclease activity. The transcription elongation factor TFIIS/SII has been shown to reactivate such complexes by stimulating exonucleolytic cleavage of the nascent RNA, thereby realigning the 3'-end in register with the catalytic centre (Reviewed in: Pal and Luse, 2002; Sijbrandi et al., 2002; Wind and Reines, 2000). TFIIS probably binds via funnel in the proximity of pore1, and may induce allosteric changes in the catalytic centre, to stimulate Pol IIs exonucleolytic activity upon DNA:RNA mismatch, or backtracking of arrested complexes (Powell et al., 1996; Sijbrandi et al., 2002).

1.5.2 The Elongator complex

Elongating RNA polymerase II is associated with the multi-subunit complex Elongator, whose stable interaction is dependent on hyperphosphorylation of the Pol II CTD (Otero et al., 1999). Elongator was identified in yeast as consisting of the products of the genes *ELP1-ELP6* (Fellows et al., 2000; Winkler et al., 2001). Purified Elongator can exist in two forms: an unstable, six-subunit complex, the so-called 'holo-Elongator', which possesses HAT activity directed against histones H3 and H4 (Winkler et al., 2002), and a three-subunit core form consisting of Elp1-3, which lacks HAT activity, despite the presence of the Elp3 HAT (Hawkes et al., 2002). This anomaly was recently elucidated by the finding that the three smallest Elongator subunits - Elp4, Elp5 and Elp6 – are required for the HAT activity of Elp3 (Winkler et al., 2002). Deletion of *ELP* genes in yeast cells results in a variety of phenotypes consistent with a role for the factor in transcription elongation. Importantly, mutations that destroy the HAT activity of Elp3 *in vitro*, also produce *elp* phenotypes *in vivo*, suggesting that the HAT activity of Elongator is essential for its function (Winkler et al., 2001; Wittschieben et al., 2000). Deletion of ELP3 is synthetically lethal with deletion of the tail of histone

H4, or of the Rpb9 subunit of RNA polymerase II and confers a severe growth defect with the gene encoding the Gcn5 subunit of the SAGA complex (Van Mullem et al., 2002; Winkler et al., 2002). It is to be noted however, that despite the growing evidence for Elongator function, recent work in yeast has been unable to detect Elongator associated with Pol II under any conditions, and revealed that the majority of Elongator is cytoplasmic (Pokholok et al., 2002).

1.6 Control of elongation

The transition to elongation has been shown to involve an interplay of positive and negative elongation factors. A wealth of data suggests that this transition *in vivo* is dependent on the phosphorylation of the Pol II CTD. The phosphorylation status of CTD is dynamic throughout the transcription cycle: the phosphorylation patterns created by CTD kinases function to specifically recruit and displace factors for elongation and pre-mRNA processing at different stages of transcription (Komarnitsky et al., 2000).

1.6.1 Cyclin-dependent kinases regulate *in vivo* transcription

The specific pattern of phosphorylation of serines-2 and -5 (Y**S**PT**S**PS) of each repeat has been shown to change with the transition from initiation to elongation (Komarnitsky et al., 2000). A plethora of recent publications has identified many factors that associate with specific phosphorylated or non-phosphorylated forms of CTD, but the regulation of this differential phosphorylation, and the kinases responsible is not clear. The CTD kinases of the initiation-associated general transcription factor TFIIH (CDK7/cyclin H), and elongation factor P-TEFb (CDK9/cyclins T1, T2 and K) have been shown *in vitro* to preferentially phosphorylate serine-5, but this does not discount the possibility that when augmented by other factors, these kinases may be directed towards serine-2 *in vivo* (Reviewed in Oelgeschlager, 2002). Indeed, the putative homologue of P-TEFb, the CtkI subunit of the *S. cerevisiae* CTD kinase CTDK-I, specifically directs its kinase activity towards serine-2 (Cho et al., 2001).

The ability of these transcription-associated CTD kinases to dynamically regulate the interactions of Pol II with elongation and processing factors has

brought to question the purpose of other CTD-modifying enzymes that act only under certain conditions.

1.6.2 The inhibition of transcription through CTD phosphorylation

Since only hypophosphorylated Pol II CTD (IIA) may stably initiate at promoters, CTD phosphorylation prior to this event prevents initiation. The CDK8/cyclin C subunits of the Mediator complex (SRB10/SRB11 in yeast) may inhibit transcription through the phosphorylation of cyclin H of the CAK/TFIIH complex. CDK8/cyclin C also phosphorylates CTD, with preference for consensus repeats. The cyclin-dependent kinase, $p34^{CDC2}$, that triggers entry into mitosis also stimulates the pre-initiation hyperphosphorylation of Pol II CTD, TFIID and CDK7 to inhibit transcription (Reviewed in Bregman et al., 2000).

The p42, p44 ERK 1/2 kinases have also been shown to directly phosphorylate the CTD, resulting in the accumulation of a novel form of RPB1, called IIm, that migrates at a speed between IIo and IIa (Bonnet et al., 1999). *In vitro* studies suggest ERK 1/2 targets serine-5 of the CTD repeats, with no apparent preference for consensus or non-consensus repeats (Trigon et al., 1998). ERK 1/2 phosphorylation of CTD is induced in response to stressful stimuli such as heat or osmotic shock, oxidative stress and ionising radiation (Bonnet et al., 1999). Additionally, ERK 1/2 activity prior to fertilisation inhibits transcription, and possibly leads to sequestration of the hyperphosphorylated (IIM) Pol II form (Reviewed in Oelgeschlager, 2002). It is not known whether these modifications may also function to target specific factors to the polymerase complex.

1.6.3 CTD phosphorylation displaces negative elongation factors

The hypophosphorylated Pol II associates with the negative elongation factors DSIF (DRB sensitivity inducing factor) and NELF (Negative elongation factor) (Yamaguchi et al., 1999). The positive Pol II elongation factor P-TEFb, composed of CDK9 and one of its cyclins (cyclin T1, T2a, T2b or K) (Peng et al., 1998), acts following promoter clearance to phosphorylate itself, the Pol II CTD, TFIIF and the SPT5 subunit of DSIF (Majello and Napolitano, 2001). Activation of P-TEFb counteracts the inhibition by DSIF (Wada et al., 1998) and NELF to

stimulate elongation (Reviewed in Oelgeschlager, 2002). Transcriptional elongation is blocked by potent inhibitors of P-TEFb such as 5,6 dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) and Flavopiridol suggesting it to be a major elongation kinase (Barboric et al., 2001). This is supported by the finding that many transcription factors such as NFkB (Barboric et al., 2001), CIITA (Kanazawa et al., 2000), and HIV Tat (Wei et al., 1998) function to recruit P-TEFb to promoters and thereby stimulate transcription elongation.

1.7 CTD modifying enzymes of unknown function

A variety of enzymes have been identified that can modulate the structure of the CTD, however, the purpose of such modifications is not yet clear. The response to DNA damage resulting from a double-stranded break activates the DNA-dependent protein kinase (DNA-PK) family members Ataxia-telangiectasia-mutated (ATM) and DNA-PK. The DNA-PK, but not ATM has been shown to directly phosphorylate CTD (Peterson et al., 1995). ATM activates the c-Abl tyrosine kinase, which via a specific domain, the CTD-ID, binds to, and phosphorylates the CTD (Fig. 2B) (Baskaran et al., 1996). It has been speculated that these activities function to modulate the expression of specific genes in response to DNA damage and in HIV transcription elongation (Baskaran et al., 1993; Baskaran et al., 1999).

Another cyclin-dependent kinase, PITSLRE (now called CDK11) and cyclin L, have been shown to bind the hyperphosphorylated form of the CTD, as well as to the Elongator subunit ELL2. CDK11 was co-purified with hyperphosphorylated CTD and another CTD kinase activity that was shown to be casein kinase II (CKII). However, this interaction has led to CDK11/cyclin L being incorrectly identified as a CTD kinase (Kidd V.J., personal communication).

The Pin1 peptidyl-prolyl, *cis-trans* isomerase (PPIase) has been shown to bind hyperphosphorylated CTD and may alter its conformation (Morris et al., 1999). Recent results suggest this isomerisation is important for the efficient dephosphorylation of the CTD by the FCP1 phosphatase (Kops et al., 2002). Pin1 is thought to play a role in mitosis control, through the binding and isomerisation of cell cycle regulatory proteins but has more recently been linked to chromatin remodelling complexes and the general transcription machinery (Wu et al., 2000). Interestingly, Pin1 also interacts with the CK2α subunit of

CKII and inhibits its ability to phosphorylate topoisomerase IIa (Messenger et al., 2002).

1.7.1 CKII phosphorylation of the CTD

Despite its early discovery, the precise role of CKII still remains elusive (Allende and Allende, 1995). Nuclear CKII activity is required for cell cycle progression in the G1 phase of cell cycle, and is implicated in the responses to genotoxic and other stresses. CKII interacts with, and phosphorylates many factors associated with the regulation of growth and apoptosis: p53 (Keller et al., 2001); the p65 subunit of NF-κB (Wang et al., 2000), and its inhibitor IκB (McElhinny et al., 1996); the caspase 8-inhibiting protein ARC (Li et al., 2002); c-Myc induces growth, but apoptosis in the absence of growth factors (Luscher et al., 1989).

The consensus sequence for CKII phosphorylation is (S/T)XX(D/E) (Pinna, 1990) and is found once in the non-consensus repeat 37 and twice in the final repeat of the Pol II CTD (Fig. 2B). It has been shown that CKII phosphorylates CTD (Dahmus, 1981), but not a fragment containing just consensus repeats (Bregman et al., 2000). Stoichiometric analysis suggests only one of these sites becomes phosphorylated *in vivo,* likely the most C-terminal serine (position 1966; serine 13) of the last CTD repeat, given the preference of CKII for sites surrounded by acidic residues (Kuenzel et al., 1987; Payne et al., 1989). Results from one study suggest the final repeat to be continuously in the phosphorylated state (Palancade B. & Bensaude O. personal communication), and that this phosphorylation is not necessarily CKII dependent, given that only Pol IIa, and not Pol IIo is a CKII substrate (Cadena and Dahmus, 1987; Payne et al., 1989).

1.7.2 Phosphorylation of tyrosine in the CTD

Some CTD tyrosine phosphorylation can be detected in cell lines lacking c-Abl, suggesting the presence of other CTD-tyrosine kinases (Baskaran et al., 1993). Indeed, the product of the Abl-related gene, c-Arg also contains a CTD-ID domain, and can also phosphorylate the Pol II CTD. However, unlike c-Abl it is not known what signal induces phosphorylation of CTD by c-Arg (Baskaran et al., 1997a). To understand the action of c-Abl, it is important to

understand the context in which it is activated: c-Abl requires its non-specific DNA binding domain for high stoichiometric phosphorylation of the CTD, but since its DNA binding domain is non-specific, c-Abl is probably recruited to DNA by its interacting partners. Nuclear c-Abl can only be strongly activated in response to DNA damage during the S/G2 phases of cell cycle, when RB is inactive (Wang and Ki, 2001). The cell cycle regulation of c-Abl is implemented by the RB protein (Welch and Wang, 1993), which binds and inhibits both E2F-1 and c-Abl until it becomes deactivated by cyclin-dependent kinases or caspases (Wang and Ki, 2001). This predicts a scenario where c-Abl is specifically recruited to E2F-1-regulated promoters, enabling it to easily phosphorylate the Pol II CTD, and other transcription factors in response to a specific type of cellular stress, in a cell cycle dependent manner (Depicted in Fig. 3).

1.8 The RNA polymerase II CTD and the processing of pre-mRNA

The correct processing of pre-mRNA has long been shown to be a pre-requisite for efficient transcription (Edery and Sonenberg, 1985; Hart et al., 1985; Jove and Manley, 1982). That these different processes require RNA polymerase II suggested an intimate connection between the transcription complex and the RNA processing machinery (Hirose and Manley, 2000). Of the three known RNA polymerases in eucaryotic cells, only Pol II transcripts undergo splicing of introns, 5´end capping, and polyadenylation. A major distinction between RNA polymerase II and other polymerases is the presence of the CTD. Interestingly, the size of CTD apparently increases with complexity of an organism, which may also correlate to their requirement for pre-mRNA processing.

mRNA capping

Messenger RNA production *in vivo* occurs co-transcriptionally and is exclusive to Pol II transcripts. The CTD of Pol II is required for the capping, splicing and polyadenylation of pre-mRNAs *in vivo.* The 5´ capping of mRNA occurs shortly after transcription initiation, when the nascent RNA is approximately 25 bases long (Coppola et al., 1983). Phosphorylation of the CTD occurs soon after initiation and is essential for recruitment of the capping enzyme complex. The capping enzyme binds CTD heptapeptides

phosphorylated at either serine-2 or serine-5, but only serine-5 phosphorylation stimulates guanyltransferase activity. Capping is performed by a series of three enzymatic activities: RNA triphosphatase removes the γ-phosphate of the first nucleotide of the nascent RNA; RNA guanyltransferase transfers GMP to the resulting diphosphate end; RNA (guanine-7-) methyltransferase then adds a methyl group to the N7 position of the cap guanine, to form the m7G(5´)ppp(5)N cap.

Splicing

Although splicing reactions can be reconstituted *in vitro* using pre-transcribed RNA and splicing competent cell extracts, the localisation and orchestration of splicing events *in vivo* appears to require CTD. Components of the spliceosome, a large macromolecular complex of snRNPs and members of the serine/arginine-rich (SR) protein family, can be found localised to active sites of transcription (Zhang et al., 1994). The hyperphosphorylated form of RNA polymerase II (IIO) has been found associated with splicing factors, and only this form has been detected in active spliceosomes. Co-localisation of splicing factors and Pol II *in vivo* is not observed in cells expressing a polymerase with a truncated CTD (Misteli and Spector, 1999). The Pol IIO form co-immunoprecipitates with splicing factors, including SR proteins, further supporting a role for CTD in the coordination of splicing events. Additionally, several phospho-CTD binding proteins identified so far, also contain the SR and RRM domains that are characteristic of many splicing factors (Morris and Greenleaf, 2000).

3´-cleavage and polyadenylation

The polyadenylation of the 3´-end of nearly all eucaryotic pre-mRNA appears to be a requirement for the correct termination and the precise definition of the 3-end. 3´-end formation is a two step process that is tightly coupled *in vivo.* First, endonucleolytic cleavage of the pre-mRNA occurs, 10 to 30 nucleotides downstream of the sequence motif, AAUAAA, followed by extension of the upstream fragment by poly(A) polymerase (PAP). At least 12 different proteins are involved in this process *in vivo*, forming four multi-subunit proteins, cleavage polyadenylation specificity factor (CPSF), cleavage stimulation factor (CstF), and two cleavage factors (CF I; CF II) (Hirose and Manley, 2000). CPSF and CstF function to recognise and define the poly(A) site

and its surrounding region while CF I and CF II are required for the cleavage reaction. The CTD of RNA polymerase II appears to be essential for this reaction *in vivo.* Both CPSF and CstF have been shown to bind CTD, however, unlike capping and splicing factors, the phosphorylation status of the CTD does not affect their binding. Although CTD may act as a scaffold for these factors, it appears that the recruitment of these factors may occur through the initiation complex: the general transcription factor TFIID purifies with CPSF (Dantonel 1997); a transfer of CPSF from TFIID to Pol II during initiation is seen in reconstituted transcription assays. An extensive analysis of the involvement of CTD in this reaction by (Ryan et al., 2002) further supports its role as a scaffold. Their data suggest the principle determinant of CTD activity in 3´-cleavage is its length. The non-consensus repeats 30-37 of the eucaryotic CTD may play a slightly more important role than other heptads in CTD cleavage activity, but are not essential since a full length CTD composed of only consensus repeats still has 80 % activity compared to *wt*CTD.

1.9 Termination of transcription

The termination of transcription is dependent on the presence of a functional poly(A) signal (Proudfoot, 1989). The poly(A) signal not only directs 3´-end processing, but also the termination of transcription. The mechanism by which this occurs is still cause for speculation. Models suggested to explain the coupling of 3´-end processing to termination fall in to two categories: (i) a cleavage-dependent ('torpedo') models where cleavage of the transcript at the poly(A) site initiates termination by promoting degradation of the 3´-product still attached to the elongating polymerase, and (ii) cleavage-independent ('anti-terminator') models, in which recognition of the poly(A) site invokes some conformational change in the transcription complex that makes it termination competent (Proudfoot, 1989).

Although the poly(A) site signal is a definite requirement for termination, 3´-end processing still occurs some distance downstream of the point at which it was transcribed (Tran et al., 2001). A model has thus been proposed in which the poly(A) site is held to the CTD via interaction with the CPSF and CstF factors (Dichtl et al., 2002), and the non-coding, nascent RNA is looped out between the CTD-associated RNA processing factors and the Pol II

RNA exit channel (Cramer et al., 2001; Proudfoot et al., 2002). The cleavage of the emerging RNA is suggested to produce an entry site for 5´-3´exonucleases to degrade the downstream cleavage product. The exonuclease may then ´catch up´ with the elongating polymerase, eventually disturbing the RNA:DNA hybrid and causing termination. Pause sites present downstream of the poly(A) site, originally thought to be termination sites, are non-essential but may enhance poly(A) site recognition and cleavage by slowing the elongation rate of Pol II (Proudfoot et al., 2002).

1.10 Transcription and the DNA damage response

DNA damage is a major problem for every organism: genetic damage prevents a cell from correctly reading and replicating its genome. Damage may result from environmental agents, such as high frequency, electromagnetic radiation (ionising radiation: IR) and reactive chemicals, as well as from metabolic by-products such as reactive oxygen intermediates. Damage may threaten survival of a cell, or lead to mutations that are then passed to the next generation.

Such mutations have certainly resulted in survival advantages, through the modification of coding or controlling regions in the DNA. On the other hand, these mutations may lead to deregulation of certain processes, for example, proliferation, which in multicellular organisms results in cancer. To maintain genomic stability, cells have developed a variety of mechanisms to detect and respond to DNA damage, involving complex signalling pathways that coordinate repair and cell fate decisions.

Although general mechanisms exist, it has become clear in recent years that specific pathways exist for the sensing and relaying of DNA damage signals, dependent on the specific type of DNA damage (Abraham, 2001). One of the consequences of DNA damage is a change in the regulation of gene transcription. Despite an observed general reduction in transcription, the transcription of some genes involved in cell cycle control and apoptosis, is up-regulated.

1.10.1 The p53 tumour suppressor

The tumour suppressor protein and transcription factor, p53, is activated following exposure to DNA-damaging agents, by a post-translational mechanism that confers its stability. p53 tetramises and binds to promoters, activating the transcription of its target genes, which include p21, mdm2, BAX and GADD45 (detailed list: (Zhao et al., 2000) (Reviewed in: Levine, 1997; Vogelstein et al., 2000). Under certain conditions p53 induces cell cycle arrest, and under others programmed cell death (apoptosis). The mechanism by which p53 decides cell fate is not entirely understood, but an allosteric model has been proposed, where p53 binds with a greater affinity to promoters of cell cycle arrest genes (for example, p21), and to the promoters of apoptosis inducing genes with a lower affinity. This model implies that low-level expression of p53 induces cell cycle arrest, whereas a high level of p53 expression (resulting from serious damage) may induce apoptosis (Chen et al., 1996; Hupp and Lane, 1994a; Hupp and Lane, 1994b; Ludwig et al., 1996). p53 has a very short half-life as a result of its continuous degradation by the ubiquitin-proteasome machinery (Maki et al., 1996). This degradation is mediated by the Mdm2 E3 ubiquitin ligase, which binds to the transactivation domain of p53 (Honda et al., 1997; Honda and Yasuda, 1999). Interestingly, transcription of the *mdm2* gene is strongly activated by p53, creating a negative auto-regulatory feedback loop (Barak et al., 1993; Momand et al., 2000).

Stabilisation is achieved by blocking the interaction of p53 with Mdm2 through post-translational mechanisms. The ARF tumour suppressor regulates p53 by directly blocking the E3 ligase activity of Mdm2 and transporting it to the nucleolus, thereby preventing its interaction with p53 (Honda et al., 1997; Honda and Yasuda, 1999; Tao and Levine, 1999a; Tao and Levine, 1999b; Weber et al., 1999). p53 is target for a number of modifying enzymes, including kinases and acetylases. Phosphorylation of p53 on serine 20 has been shown to increase its stability by disrupting its interaction with Mdm2 (Shieh et al., 2000), and serine 15 phosphorylation enhances its transactivation potential through recruitment of the HAT, p300 (Dumaz and Meek, 1999; Lambert et al., 1998). The preferential phosphorylation of certain residues by the different kinases is suggested to modify the function of p53, and one modification may permit further modification by another enzyme (Dumaz et al., 1999). Different types of cellular stress activate distinct protein kinases and acetylases, which modify p53

at different residues and might partly explain why the p53 transcriptional response is dependent on the type of cellular stress (Zhao et al., 2000).

1.10.2 RNA polymerase II in the detection and repair of damage

The cellular response to DNA damage has been extensively researched, however, the mechanisms by which damage is detected still remain enigmatic. The time required for the induction of a DNA damage response is extremely short, suggesting an extremely sensitive detection mechanism. Since transcription complexes are constantly present on the DNA it has been suggested they may have a dual role in both the detection and repair of DNA damage. Indeed, the basal transcription factor TFIIH, required for initiation and early transcription elongation of RNA polymerase II, contains the XPB and XPD proteins, which play an essential role in nucleotide excision repair (Schaeffer et al., 1993; Svejstrup et al., 1995).

It has been demonstrated that transcription inhibition serves to trigger the induction of p53 (Ljungman, 1999; Ljungman and Hanawalt, 1996; McKay et al., 1998), and prolonged inhibition of mRNA synthesis may induce apoptosis (McKay et al., 2000; McKay et al., 1998). Experiments by Ljungman and co-workers compared the induction of p53 in response to inhibitors of RNA polymerase II CTD phosphorylation, and that induced by DNA damaging agents. Both resulted in accumulation of p53, however, only agents that inhibited the elongation phase of transcription (UV, actinomycin D) resulted in phosphorylation of p53 at serine 15, and acetylation at lysine 382 (Ljungman et al., 2001; Ljungman et al., 1999). This suggests that blockage of the transcription elongation phase results in the activation of different signalling pathways.

An elongating RNA polymerase II will stall at DNA lesions and recruit the transcription-coupled repair (TCR) apparatus (de Laat et al., 1999). Stalling of RNA polymerase II also activates other mechanisms involved in the response to genotoxic stress, including induction of p53. Interestingly, there appears to be different mechanisms for detecting, and responding to different types of genotoxic insult. UV irradiation (254nm) induces bulky DNA lesions (Friedberg, 1995) and gamma/ionising irradiation (IR) induces double strand breaks. Stabilisation of p53, a nuclear event, occurs after both treatments, but two distinct signalling pathways are activated. UV also induces membrane-associated signalling proteins that lead to the activation of the transcription

factors AP1 and NFkB (Devary et al., 1992; Liu et al., 1996). The response to IR however, requires the ataxia telangiectasia mutated (ATM) protein, a member of the phosphoinositide 3-kinase related kinase family (PIKK), that play critical roles in early signal transmission through cell cycle checkpoints (Reviewed in (Abraham, 2001). ATM accelerates the response and repair of such damage. Ataxia-telangiectasia (A-T) patients are extremely sensitive to IR, and ATM -/- cells exhibit a delayed p53 response following IR exposure (Kastan et al., 1992). How a double stranded break is recognised is not yet known, but many data suggest the Rad group of checkpoint proteins to be key players (O'Connell et al., 2000). It is not clear how the protein kinase activity of ATM is activated. One possibility is that reactive oxygen species (ROS), or DNA double-strand breaks produced following a genotoxic insult like IR exposure, may result in a post-translational modification of ATM that stimulates its kinase activity.

ATM has been identified in complexes containing a broad range of proteins with clear links to cell cycle, DNA repair and human chromosomal instability syndromes. The elucidation of ATM substrates provides clues to its function. ATM forms part of the BRCA1 (breast cancer susceptibility antigen 1)-associated genome surveillance complex (BASC), along with the mismatch repair proteins, MLH1, MSH2 and MSH6, the Mre11-Rad50-NBS1 complex, that is involved in the recombinatorial repair of DNA double strand breaks, and the Bloom's syndrome helicase (BLM). The phosphorylation of complex components by ATM, and other checkpoint kinases (for example, hChk2), is thought to co-ordinate the recruitment and activation of the repair machinery (Abraham, 2001). The roles of other substrates is, however, not so apparent.

1.11 ATM and the regulation of cell cycle

ATM has been shown to be involved in cell cycle regulation at the G_1/S checkpoint, through p53-mediated induction of the cyclin-dependent kinase (CDK) inhibitor p21. Cell cycle progression in to S-phase requires the inactivation of the retinoblastoma protein (Rb), which is normally achieved through its phosphorylation by cyclin-D-CDK4/6. Rb belongs to the family of so-called pocket proteins, which suppress E2F family proteins through binding of their transactivation motifs. The E2F proteins are S-phase specific transcription factors, whose activity allows DNA synthesis to proceed

(Reviewed in Helin, 1998). Hypo-phosphorylation of Rb blocks the transition from G_1 to S-phase. Induction of the p53 response by ATM can thus lead to cell-cycle arrest.

Interestingly, the Rb protein also binds, via its C-pocket, a portion of the nuclear-localised, non-receptor tyrosine kinase, c-Abl (Welch and Wang, 1993), which is also an ATM substrate. Rb binds the ATP-binding lobe of c-Abl, thereby ensuring it may only be activated during S-phase (Wang, 1993; Wang and Ki, 2001), or removal of Rb by another mechanism (Park et al., 2000). This suggests a mechanism by which ATM may orchestrate phosphorylation of substrates, in a cell cycle dependent manner. Importantly, since E2F sites are not present in the promoter region of every gene (Helin, 1998; Muller and Helin, 2000), this interaction serves to recruit the c-Abl kinase to specific regions of DNA, where it may among other functions, modulate the activity of certain transcription factors (Gong et al., 1999).

1.11.1 The c-Abl tyrosine kinase

The *c-abl*, and related *c-arg* (*abl*-related gene) gene products contain large N-terminal domains, homologous to those of the c-Src family of protein tyrosine kinases. However, in contrast, both proteins contain unique, large, C-terminal domains of no homology to Src and relatively little to each other. The C-terminal domain of Abl is key to its function. Extensive mutational analysis has revealed an array of interaction motifs for regulatory proteins, three DNA-binding domains (Kipreos and Wang, 1990; Kipreos and Wang, 1992; Van Etten et al., 1994), F-and G-actin binding domains (Van Etten et al., 1994), three nuclear-localisation signals (Van Etten et al., 1989; Wen et al., 1996) and one nuclear-export signal (Reviewed in Van Etten, 1999). The structural attributes of c-Abl, and its ability to shuttle between cytoplasmic and nuclear locations suggest a 'multi-tasking' function, as it functions both as an integrator and effector of signalling pathways (Kharbanda et al., 1998). This is supported by the phenotype of mice where either *c-abl* was knocked out, or replaced by an allele coding a C-terminal truncation with intact kinase activity. Such mice are born runted, have defects in osteoblast maturation, exhibit abnormal eyes, frequent rectal prolapse, and defective spermatogenesis, resulting in shortened survival. (Hardin et al., 1995; Hardin et al., 1996; Li et al., 2000; Schwartzberg et al., 1990; Schwartzberg et al., 1991; Wong et al., 1995).

The localisation of c-Abl has been shown to have a profound effect on its function. Nuclear c-Abl is cell cycle regulated, through sequestration by Rb, and can be activated in S-phase following DNA damage (Kharbanda et al., 1995). In contrast, cytoplasmic c-Abl is not affected by cell cycle or DNA damage, but instead responds to changes in cell adhesion (Lewis et al., 1996).

The *c-abl* gene was first identified due to its homology to the transforming gene of the Abelson murine leukaemia virus (v-*abl*) (Abelson and Rabstein, 1970; Goff et al., 1980), and has since been identified in several leukaemia-associated fusion proteins, most notably the t(9;22) Philadelphia chromosome, that results from aberrant chromosome translocations. Importantly, all the transforming Abl proteins are exclusively cytoplasmic, and transformation of fibroblasts is enhanced through the membrane-anchoring, myristylation of Abl's N-terminus. Conversely, activation of nuclear c-Abl is associated with growth arrest and apoptosis. This difference in nuclear and cytoplasmic function, was elegantly demonstrated by Wang *et al.*, who forcibly localised active Abl kinase to the cytoplasm or nucleus, with drastically different consequences (Wang, 2000).

Until recently, the nuclear mechanisms of c-Abl induced growth arrest and apoptosis were completely enigmatic. c-Abl was shown to require its SH2 and kinase (SH1) domains to mediate its cytostatic and cytotoxic effects, indicating that interaction with one, or more of its substrates is required (Goga et al., 1995; Wen et al., 1996). Activated c-Abl has been reported to phosphorylate, and down-regulate components of the DNA repair machinery, including Rad51 (Yuan et al., 1998), and DNA-PK (Kharbanda et al., 1997), however, the relevance of this is not understood since c-abl -/- cells exhibit no defined cell cycle or repair defects (Liu et al., 1996). Other substrates include transcription factors: c-Abl can complex with the transcription factors CREB and E2F-1 to modulate *c-myc* transcription (Birchenall-Roberts et al., 1997); in S-phase c-Abl can phosphorylate the CTD of RNA polymerase II, a step which may affect gene-specific initiation, elongation of transcription, and pre-mRNA processing (Baskaran et al., 1997a; Baskaran et al., 1996; Baskaran et al., 1993; Baskaran et al., 1999; Oelgeschlager, 2002)

Increasing evidence implicates activated nuclear c-Abl in pro-apoptosis signalling. c-Abl was found to phosphorylate and stabilise the p73 protein, a p53-family member involved in cell fate decisions (Gong et al., 1999) (For review see Yang et al., 2002), and in the inhibition of the anti-apoptotic

transcription factor NFkB (Kawai et al., 2002).

1.11.2 The role of c-Abl in transcription

The DNA binding domain of c-Abl is regulated by the cdc2 kinase. During early mitosis, cdc2 hyperphosphorylates c-Abl on multiple threonines and serines, including serines 852 and 883 of the DNA-binding domain, thereby inhibiting its DNA binding (Kipreos and Wang, 1992). The DNA binding domain of c-Abl can recognise a specific consensus sequence in DNA, similar to those bound by HMG-like proteins such as LEF-1 and SRY, which bind preferentially to 5'-AACAAAG, and 5'-$^{A}/_{T}$AACAA$^{A}/_{T}$ respectively (David-Cordonnier et al., 1998a; David-Cordonnier et al., 1998b). The Abl DNA binding motif has a high proline content, and does not bear homology to any known binding motif (David-Cordonnier et al., 1998a). It was initially described as binding the palindromic EP element of the HBV enhancer (Dikstein et al., 1996; Dikstein et al., 1992), but this could not be confirmed by other groups (Arcinas et al., 1994; Miao and Wang, 1996). The role of this DNA binding capacity has still not been established.

Since c-Abl has been shown to interact with transcription factors, its DNA binding may function to position it correctly within the transcription complex, thereby facilitating its phosphorylation of the RNA polymerase II CTD and other substrates (Baskaran et al., 1997a; Baskaran et al., 1993; Baskaran et al., 1999; Birchenall-Roberts et al., 1995; Birchenall-Roberts et al., 1997; David-Cordonnier et al., 1998b; Yuan et al., 1999). Recent work by the Wang lab confirms this theory: c-Abl phosphorylates p300/CBP in response to doxorubicin induced DNA damage, thereby stimulating their activities. Inhibition of acetyltransferase activity was shown to prevent Abl-induced apoptosis, implicating activation of nuclear acetyltransferases as a requirement for DNA damage-induced apoptosis (Costanzo et al., 2002).

1.12 The purpose of this work

RNA polymerase II has been shown to be both a sensor and effector of DNA damage: the repair of DNA damage in actively transcribed regions is much faster than that in inactive regions (Svejstrup, 2002). The differential phosphorylation of the Pol II CTD by specific, transcription-associated kinases has been shown to affect transcription initiation, elongation and the processing of RNA. The last repeat of the CTD contains sites of interaction for the kinases c-Abl, c-Arg and CKII – enzymes normally associated with proliferation and the response to DNA damage. Unlike other known CTD kinases, these interactions might not occur universally and may thus have gene specific effects, either by acting to affect the transcription complex directly, or by controlling the recruitment of other factors to the polymerase. The specific nature of these interactions necessitates an *in vivo* system for their study. The goal of this project is therefore to establish an *in vivo* system to investigate the effect on global cellular transcription, of removal, or mutation of the last CTD repeat.

2. Results

2.0 A system for the conditional expression of Pol II CTD mutants *in vivo*

To determine the importance of the mammalian CTD 'last repeat', a vector containing the full length, haemagglutinin-(HA)-tagged, mouse RPB1 gene was adapted to permit easier manipulation of the RPB1 CTD coding sequence. The introduction of a multiple cloning site (MCS) immediately before the stop codon enables extension or truncation of the CTD, however, its own presence results in a 21 bp insertion encoding a further seven amino acids.

Given the repetitive nature of the CTD, the possibilities for manipulation within its sequence are limited. Fortunately, some non-consensus repeats like repeat 49 contain unique restriction enzyme sites. Figure 4. depicts the changes in the amino acid sequence of three CTD mutants, LS*49+52, LS*49M+52 and LS*49+50, that were produced by truncation of the sequence to 49 repeats followed by the addition of sequence for either the endogenous repeat 50, or 52. The mutant LS*49M+52 truncates the non-consensus repeat 49 to the consensus repeat length of seven amino acids. Since the importance of repeat 49 is not known, this mutant will control that any effects seen are resulting from mutation of the last repeat, and not a consequence of disturbing the region around this non-consensus repeat.

An additional mutant was produced by cloning of the cDNA for the enhanced, green fluorescent protein (EGFP) in to the MCS (Fig. 4B). In addition to producing a control for expression and localisation, the EGFP domain may also impede interactions with the last repeat. 24h following induction, the EGFP labelled polymerase is seen distributed throughout the entire cell (Fig. 5). Further culture in the presence of α-amanatin may assist its nuclear importation by inducing the degradation of the endogenous Pol II – 48h later almost all EGFP label is nuclear, concentrated in speckles and absent from the nucleolus (data not shown).

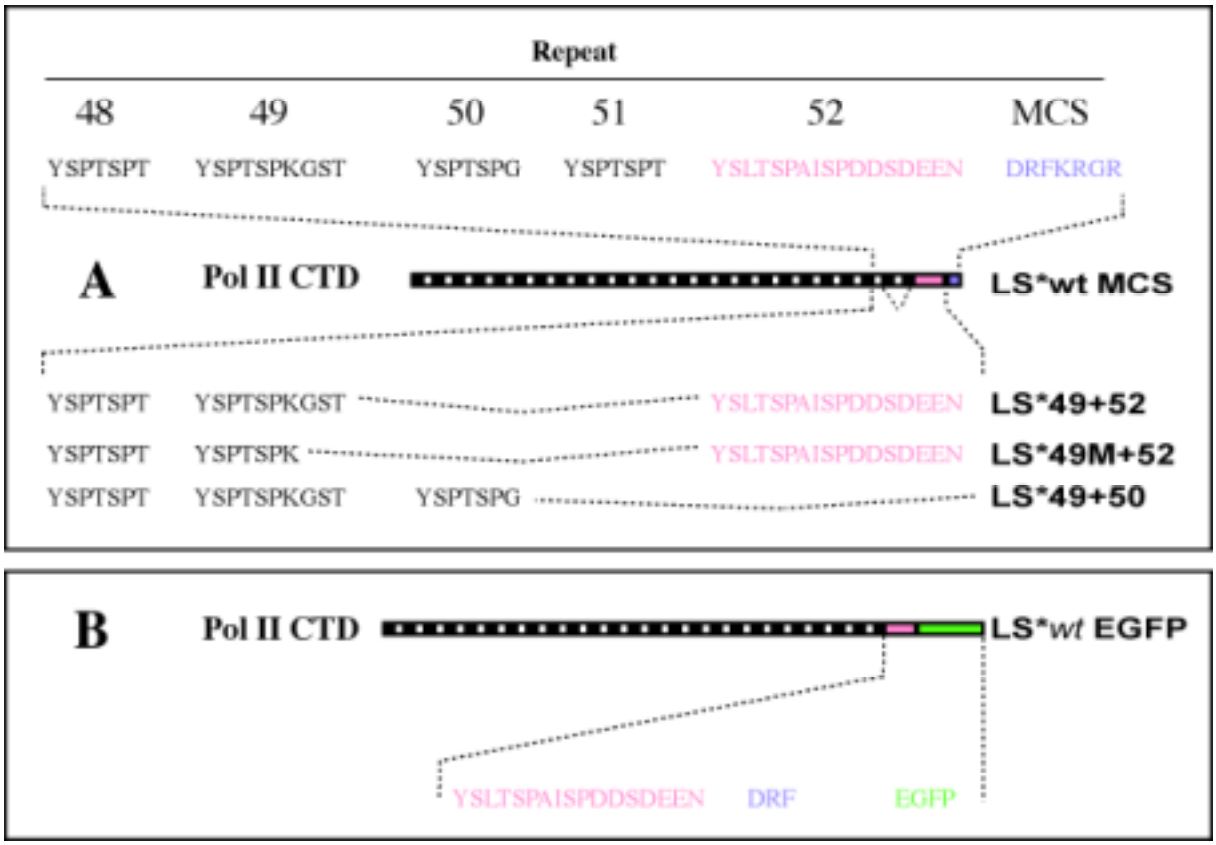


Figure 4. Overview of the amino acid composition of CTD mutants. (A) The multiple cloning site (MCS) region of the mutant LS**wt*MCS produces an extension of seven amino acids to the wild type last repeat. Unique restriction enzyme sites in the DNA coding sequence for the non-consensus repeat 49 and in the MCS region allow this region to be removed and replaced. Mutants were produced ending in either the 52nd (last-) or 50 repeats. An additional mutant, ending in repeat 52, but lacking the non-consensus, three amino acid extension of repeat 49 (designated 49M) was also produced. **(B)** The MCS allows insertion of other sequences to produce fusion proteins, such as with the EGFP localisation tag.

The Pol II LS (*Rpb1)* sequence used contains a point mutation (Asn793Asp) conferring α-amanatin resistance (*) (Bartolomei and Corden, 1987) to our recombinantly expressed Pol II LS (Pol II LS*). By growing cells in the presence of α-amanatin for 24 hours, the endogenous Pol II is effectively, chemically ´knocked-out´, thereby allowing the properties of our mutant polymerases to be examined *in vivo.*

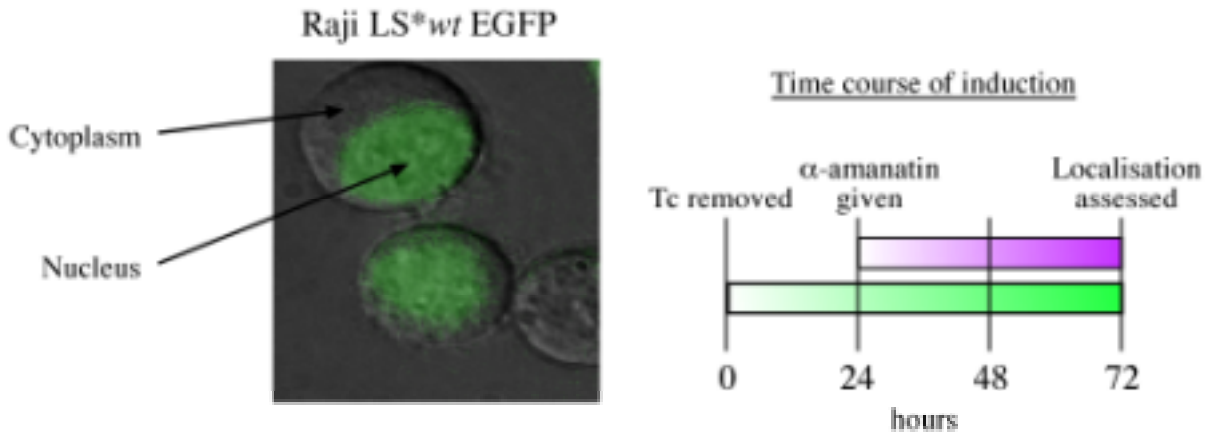


Figure 5. Localisation of exogenously expressed Pol II LS*EGFP in Raji cells.. α-amanatin [2µg/ml] was applied to cell cultures, 24h following the removal of Tc. Cells were then cultured for a further 48h to allow stabilisation of the expression and distribution of the EGFP tagged Pol II before analysis by UV microscopy.

2.1 Establishment and characterisation of cell lines conditionally expressing Pol II CTD-mutants

The vector used, ´LS*mock´, contains several important features for the stable transfer and conditional expression of our mutants into cell lines (Fig. 6). Many transfection systems involve the random integration of vector DNA in to the host genome. This creates problems associated with so-called 'position effects': endogenous regulatory elements in the region of integration may provide unwanted positive, or negative effects on the transcription of introduced sequences. Our vector overcomes this effect by utilising the replication origin of the Epstein-Barr virus (EBV-oriP). This confers the advantage that it is episomally maintained, and does not integrate into the genome of human cells expressing the EBV-nuclear antigen 1 (EBNA1). Episomes replicate like extra chromosomes, simultaneously with the genomic DNA. The lack of position effects removes the need to produce and test many different cell lines for every different vector, allowing cell lines instead to be produced as a ´batch-culture´.

Conditional expression of RPB1 is achieved through control of its promoter by a tetracycline-response element (TRE): the *E. coli* tetracycline- (Tc)-regulated operator (*tetO7*) combined with a minimal portion of the EBV-latent membrane protein 1 (LMP1) promoter. The LS*mock vector also contains, and constitutively expresses a fusion protein (TcTA) of the *E. coli* Tc-repressor with

the transactivator (TA) domain of the herpes virus protein, VP16. The TcTA used in this system binds its operator only in the absence of Tc, thus, removal of Tc results in its binding to, and activation of the TRE (Gossen and Bujard, 1992).

The vector constructs LS*mock (empty vector), LS**wt*MCS, LS**wt*EGFP, LS*49+52 and LS*49+50 were transfected into the Burkitt's Lymphoma cell line, Raji and selected in the presence of Tc and G418 over several weeks to establish stable, polyclonal cell lines.

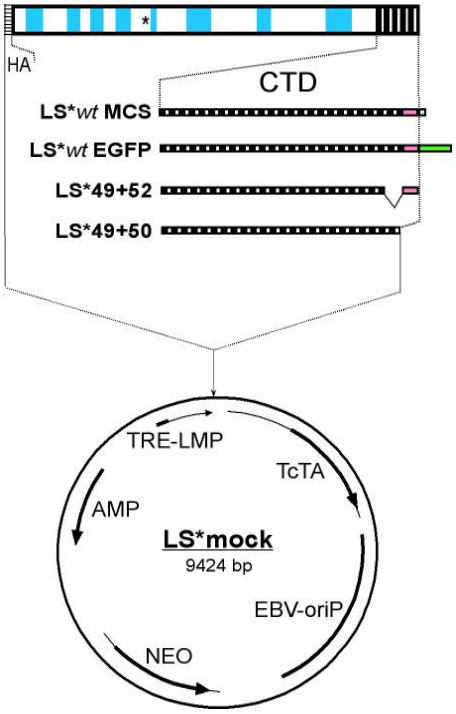


Figure 6. Schematic representation of the expression vector. The mouse α–Amanatin-resistant RPB1, and CTD mutants thereof were cloned in to the vector LS*mock. Key features: HA, haemagglutinin tag; H, position of the point mutation that confers α–Amanatin resistance; CTD, carboxy-terminal domain; EBV-oriP, a replication origin of the Epstein-Barr Virus (EBV) for episomal replication of the plasmid; TcTA, tetracycline (Tc)-regulated transactivator that binds to the *tetO7*-sequence of the Tc-response element (TRE) contained in the EBV minimal promoter of the latent membrane protein 2A (LMP) in the absence of tetracycline; NEO, neomycin (G418)-resistance gene; AMP, ampicillin-resistance gene

2.1.1 Removal of the last CTD repeat induces its IIb form

The tight regulation of expression through Tc is demonstrated in figure 7A. Stably transfected cell lines were grown in the presence or absence of Tc for 24 h before protein extraction and Western blot analysis. The HA-tagged,

recombinant polymerases are only detected in the absence of Tc. The hyper- (IIo) and hypo- (IIa)-phosphorylated forms of Pol II LS* are visible for each mutant. The truncated mutant LS*49+52 appears like LS**wt* (data not shown). The LS**wt*EGFP mutant migrates at a slower rate due to its greater size. The addition of EGFP appears to have little effect on the proportions of the IIa and IIo forms. In contrast however, the mutant lacking the endogenous last repeat (LS*49+50) exhibits reduced levels of the IIo form, almost proportional to the appearance of a truncated form, Pol IIb (Christmann and Dahmus, 1981). This suggests that the IIo form of this mutant might be preferentially degraded.

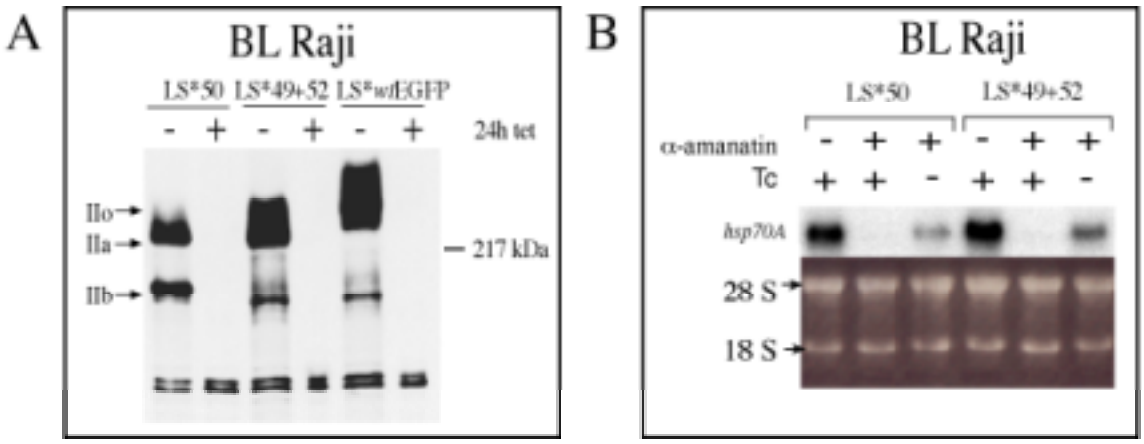


Figure 7. Analysis of tetracycline-regulated recombinant Pol II LS* expression and function. **(A)** Tetracycline (Tc)-regulatable expression of stably transfected Pol II LS* mutants in the Burkitt's lymphoma cell line, Raji. The recombinant proteins have been identified using the high affinity, anti-HA antibody 3F10. **(B)** Raji cell lines were grown in the presence or absence of Tc for 24h, after which, α-amanatin [2μg/ml] was added to the indicated samples and incubated a further 24h. Cells were exposed to a heat shock for 2h at 43°C before harvesting of their RNA. 10μg of total RNA from each sample was probed for levels of *hsp70*A RNA using a complementary radioactive probe. The 28S and 18S RNA bands, stained with ethidium bromide, provide a control for equal loading.

The α-amanatin-resistant phenotype of our mutants allows them to functionally replace the endogenous Pol II when grown in the presence of α-amanatin (Meininghaus et al., 2000). Despite the high affinity of α-amanatin for Pol II ($K_d \approx 10^9$M), the inhibition and the degradation of Pol II LS is very slow. The limiting step appears to be the take up of α-amanatin by cells. For this reason, an incubation time of 24 h is required before mutant function can be analysed.

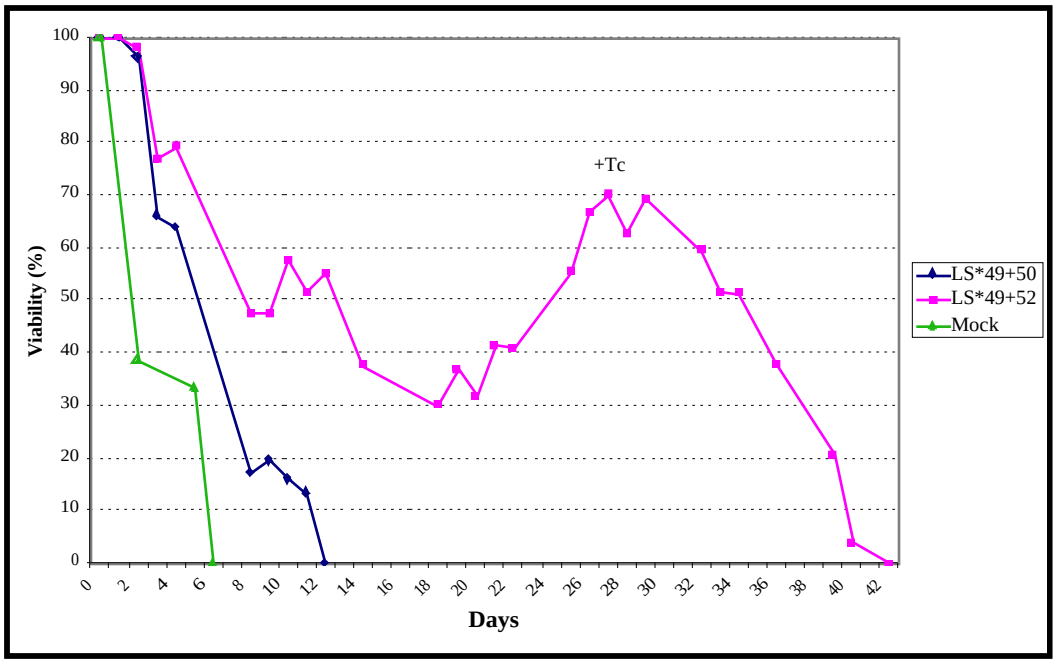
2.1.2 A mutant lacking the last repeat exhibits a reduced ability to transcribe the *hsp70A* gene, and cannot sustain cell viability

The heat-shock inducible, *hsp70A* gene, was used to assess the mutants for their ability to transcribe a chromatin template (Fig. 7B). In the presence of Tc alone, the endogenous Pol II produces a strong induction of *hsp70A* RNA in response to heat shock. In the presence of α-amanatin and Tc, no signal is observed since no α-amanatin resistant polymerase is present. In the presence of α-amanatin and absence of Tc, both mutants transcribe *hsp70A* in response to heat shock. The signal seen for both polymerases is weaker compared to that of the endogenous Pol II. Additionally, the signal produced by LS*49+50 was seen to be consistently weaker than that produced by LS*49+52. This may be a result of the large proportion of this mutant converted to the inactive IIb form.

Pol II LS* mutants able to support all essential Pol II functions should be able to support growth and proliferation of cells in the presence of α-amanatin. Previous work has shown that cells expressing Pol II LS**wt* under α-amanatin first undergo a selection, perhaps to become accustomed to expression levels and the degradation of the endogenous polymerase.

To compare the abilities of LS*49+50, LS*49+52 and LS**wt*EGFP to that of LS**wt*MCS, cells were induced to express their mutants 24 h before the addition of 2mg / ml α-amanatin (Day 0). The viability of cells was assessed regularly over a period of 40 days, during which time cultures were split and medium exchanged as necessary, to maintain the viable cell number at $\approx 5 \times 10^5$ cells / ml (Fig. 8). The cell line, LS*mock, expresses no polymerase, and thus possesses no α-amanatin-resistance, was included as a control. Inhibition of Pol II has been shown to induce stabilisation of mRNA, which may account for the unexpectedly long time for non-resistant cultures to reach 0 % viability (Meininghaus M., personal communication). The mutants LS**wt*MCS, LS*49+52 and LS**wt*EGFP all sustain the viability of Raji cell lines, which, following the initial crisis phase returns to between 80 and 90 percent.

A



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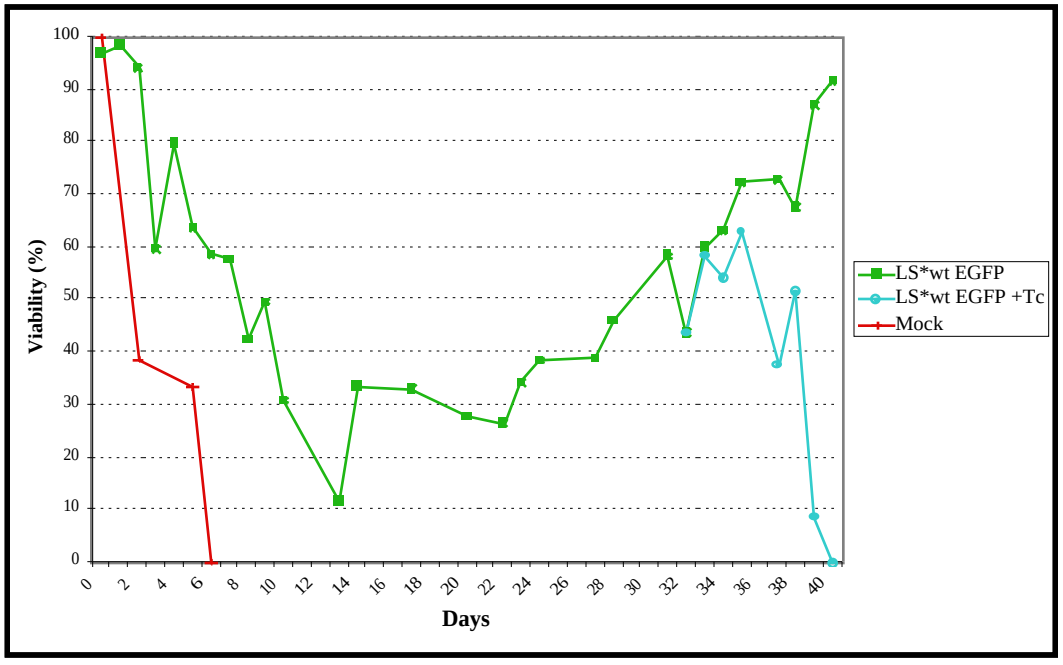

Figure 8. Viability of Raji cell lines in the presence of α-amanatin. (A) Stably transfected cell lines were grown in tetracycline (Tc)-free medium to induce the expression of the Pol II LS* CTD mutants. 24h after induction, α-amanatin was given to the medium [2µg/ml]. A control cell line containing an empty expression vector, `Mock´, was included. Tc was reapplied to LS*49+52 following recovery at day 27. (B) Comparison of cell lines Mock and LS**wt* EGFP resistance to α-amanatin, and the effect of Tc re-addition at day 31. These data are representative of several repeated experiments.

The viability experiment shown in figure 8B is a representative example of the results from several studies. As shown for the Raji LS**wt*EGFP cell line, re-addition of Tc (day 33) represses expression of the mutant Pol II LS*, thereby removing resistance to α-amanatin and resulting in the loss of viability.

2.2 Advanced mutational analysis of the last repeat

The mutants tested in the previous experiments indicate that elements contained in the last repeat of Pol II CTD are important for its stability and cell viability. This element is apparently undisturbed by the addition of a small, random amino acid sequence (LS**wt*MCS), or a much larger domain (LS**wt*EGFP).

The last repeat has been identified as the essential recognition site for the tyrosine kinases c-Abl and c-Arg (Baskaran et al., 1997a; Baskaran et al., 1996), and contains two consensus phosphorylation sites for CKII. A series of further mutants were therefore produced, to better identify the important elements within the last repeat (Fig. 9). Two mutants were produced with one point mutation, resulting in the change of a serine to an alanine in either one of the two CKII sites (LS*49+52 S9A; LS*49+52 S13A), and also a double point mutant where both sites were mutated (LS*49+52 S9/13A). To disturb c-Abl interaction, a nonsense mutant was produced where the amino acid sequence of the last repeat was randomly reconstituted, while still preserving two CKII sites (LS*49+NS52). Additionally, a mutant was produced, containing repeat 50, plus a ten amino acid sequence known to be the c-Abl recognition motif in ATM (LS*49+50ATM). Whether this short motif can indeed interact with c-Abl in this context is not known, but nevertheless it provides another useful control: alike the *wt* last repeat, a non-consensus repeat (YSPTSPG) is flanked by an additional ten amino acids (DPAPNPPHFP). This mutant also contains one potential CKII site.

Figure 9. Overview of RNA polymerase II CTD mutants. Expression vectors were produced for the above CTD mutants. With the exception of LS**wt* MCS, all mutants contain just 49 of the original repeats, plus the wild-type final 17 amino acids, or mutant thereof. The addition of a multiple cloning site to the wild type (*wt*) sequence results in the addition of seven extra amino acids. Green boxes signify potential caesin kinase II (CKII) sites, based on the consensus recognition sequence S/TxxD/E. Residues labelled red denote point mutations from the original sequence. Mutant LS*49+NS52 contains the *wt* final 17 amino acids in a scrambled (non-specific) order. Mutant LS*49+50ATM contains repeat 50 fused with the c-Abl interaction motif of ATM.

2.3 Characterisation of last repeat mutants in different Burkitt's Lymphoma cell lines

Stable cell lines were produced for each mutant in Raji, and as a control, the mutants LS*49+50, LS*49+52 and LS*49+NS52 were also stably transfected in to two other EBV+ Burkitt's lymphoma cell lines, BL29 and Elijah. Expression analysis in these cell lines (Fig. 10A) reveals a similar pattern to that seen in Raji (Fig. 10B): mutants where the final repeat is absent, or severely disturbed (LS*49+50; LS*49+NS52), result in the appearance of the truncated Pol IIb form, thus demonstrating that this phenomenon is not cell line specific. Unfortunately, due to their extreme sensitivity to stress, production of these cell lines was difficult, and those stable lines achieved could not be used beyond simple expression studies because of their sensitivity to α-amanatin.

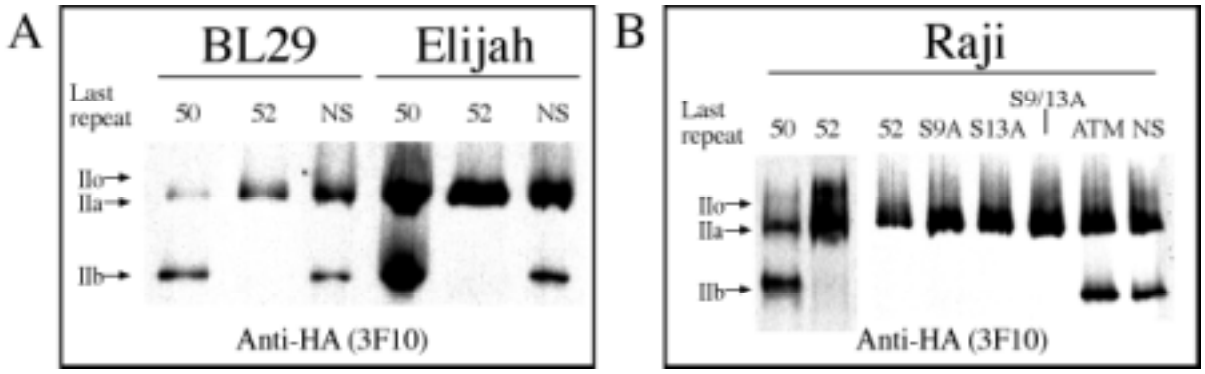

Figure 10. Expression of LS* CTD mutants in Burkitt's lymphoma (BL) cell lines. (A) Expression of Pol II LS* mutants containing or lacking the *wt* CTD final repeat in the stably transfected EBV+, BL cell lines, BL29 and Elijah. (B) Expression of Pol II LS* mutants containing *wt*, point-mutated, or scrambled final repeat in the EBV+, BL cell line, Raji.

2.4 Further characterisation of last repeat mutants in the Raji cell line

2.4.1 Disruption of the last repeat, but not the disruption of its CKII consensus sites, results in the appearance of the IIb form

Stable Raji cell lines were achieved for all the mutants. No apparent difference in expression pattern is seen between mutants with an intact last repeat and those with CKII site mutations (Fig. 10B). This suggests that these mutations are not important for induction of the IIb form. Some motif present in the *wt* last repeat, and absent in the mutants LS*49+50, LS*49+52 and LS*49+NS52, must protect the RNA Pol II LS from destruction. The Pol IIb form was originally thought to be a novel form Pol II that lacks activity (Christmann and Dahmus, 1981; Corden et al., 1985). However, it is noteworthy that the IIb form results from one single cut, suggesting a specific protease, rather than a general proteasome activity to be responsible. The existence and role of this form *in vivo* has not yet been described.

2.4.2 Only mutants containing the last repeat become heavily tyrosine phosphorylated following IR

Raji cell lines expressing various CTD mutants were exposed to IR, a stimulus known to induce the phosphorylation of tyrosines in the CTD (Baskaran et al., 1993), by the c-Abl tyrosine kinase (Baskaran et al., 1996). Since this interaction requires the Pol II LS last CTD repeat (Baskaran et al., 1999), removal of this domain should prevent an increase in CTD tyrosine phosphorylation in response to IR. Since the levels of phospho-tyrosine *in vivo* are low in comparison to other phospho-amino acids, cells must be incubated in the presence of phosphatase inhibitors one hour prior to irradiation to assist detection. Immunoprecipitation of nuclear extracts was performed to purify the HA-tagged Pol II LS mutants, and reduce the phospho-tyrosine background to enable visualisation of specific CTD tyrosine phosphorylation.

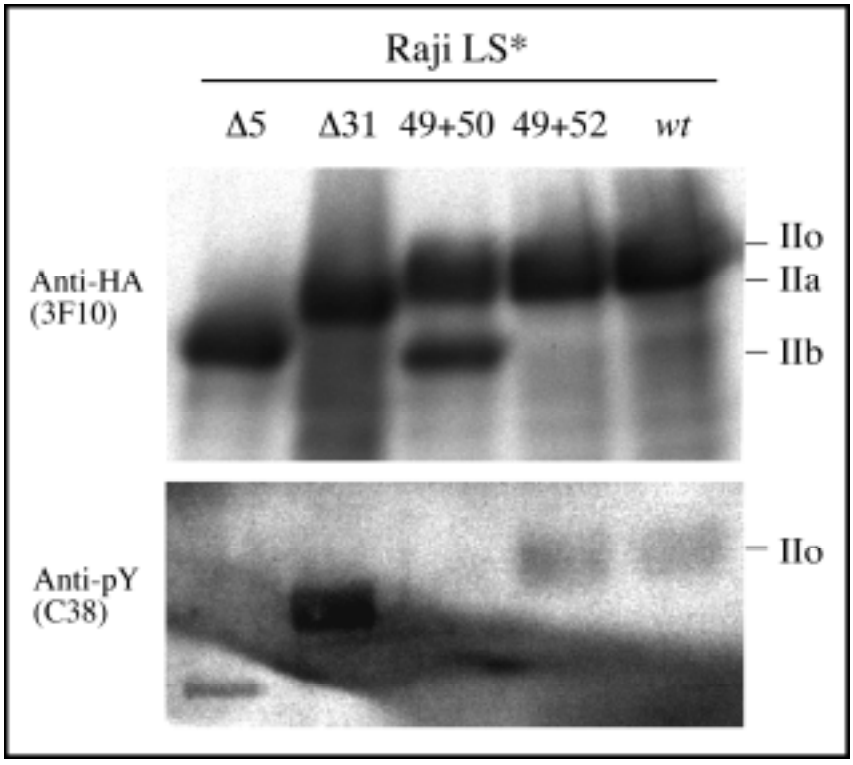

Figure 11. Tyrosine phosphorylation of Pol II LS* CTD mutants following IR. Cells expressing Pol II LS* CTD mutants were irradiated with 8 Gy IR in a Gammacell, then harvested 1h later. Immunoprecipitation was performed using the anti-HA antibody, 3F10. Western blots were stained with the anti-pY antibody, C38, then stripped and re-probed with the anti-HA antibody 3F10 to confirm the presence of the proteins.

The panel of mutants compared differ in their number of CTD repeats (Fig. 11: from left to right: 5,31,50,50 and 52 repeats). However, LS*49+50 is the only mutant not containing the last CTD repeat. The amount of tyrosine phosphorylation seen for some mutants is greater than others, and does not appear proportional to the number of repeats present. This may be a result of disproportionate loading, as seen for LS*Δ31, or incomplete inhibition of phosphatases in some samples. The same blot was re-probed with anti-HA antibody to control loading: all mutants appear loaded in sufficient quantity. In confirmation of earlier data (Baskaran et al., 1999), the mutant lacking the last CTD repeat, LS*49+50, was the only not to demonstrate increased tyrosine phosphorylation in response to IR. The length of number of CTD repeats appears irrelevant: tyrosine phosphorylation in response to IR being dependent only on the presence of the last CTD repeat. Additionally, since the Pol II LS* is N-terminally tagged, the IIb form is also efficiently precipitated, and migrates at a similar, or slightly faster rate than Pol II LS*Δ5. This suggests that the site of proteolytic cleavage that yields the IIb form to be in close proximity to the start of the CTD.

2.4.3 The appearance of the IIb form is not inhibited by a panel of protease inhibitors

To investigate what protease may be responsible for the production of the IIb form, Tc was removed from the Raji LS*49+50 cell line to induce expression. After four hours, specific protease inhibitors were applied to the cell cultures before harvesting 7 and 18 hours later. Figure 12 shows the effects of the different inhibitors on the formation of Pol IIb. AAF-CMK is a specific inhibitor of the tripeptidyl-peptidase II (TPII) enzyme that is up-regulated in BL cells (Gavioli et al., 2001). Proteasome inhibitors lactacystin and MG-132 have been shown to inhibit the degradation of Pol II following UV irradiation (Ratner et al., 1998). Proteases involved in the regulation of cell proliferation, differentiation and apoptosis were tested: ALLM (calpain inhibitor II), calpeptin, and calpain inhibitor III are inhibitors of the calpain family of cysteine proteases (Perrin and Huttenlocher, 2002); Caspase 8 has been shown to proteolytically cleave Pol II LS (Lu et al., 2002), an inhibitor of which was also included. Earlier work in yeast demonstrated that treatment of samples with the serine protease inhibitor phenylmethyl-sulfonyl fluoride (PMSF) completely

inhibited the conversion of Pol B_{22o} (Pol IIa) to Pol B_{180} (Pol IIb) (Dezelee et al., 1976). A cocktail of protease inhibitors (Roche) directed against serine, cysteine and metalloproteases, as well as calpains were also tested. In addition to protease inhibitors, inhibitors of signalling pathways known to signal to the Pol II CTD were also tested: SKF-86002 inhibits osmotic stress and UV–induced apoptosis by blocking p38 MAP kinase activation. None of the inhibitors used in this study appeared to be able to inhibit formation of the IIb form.

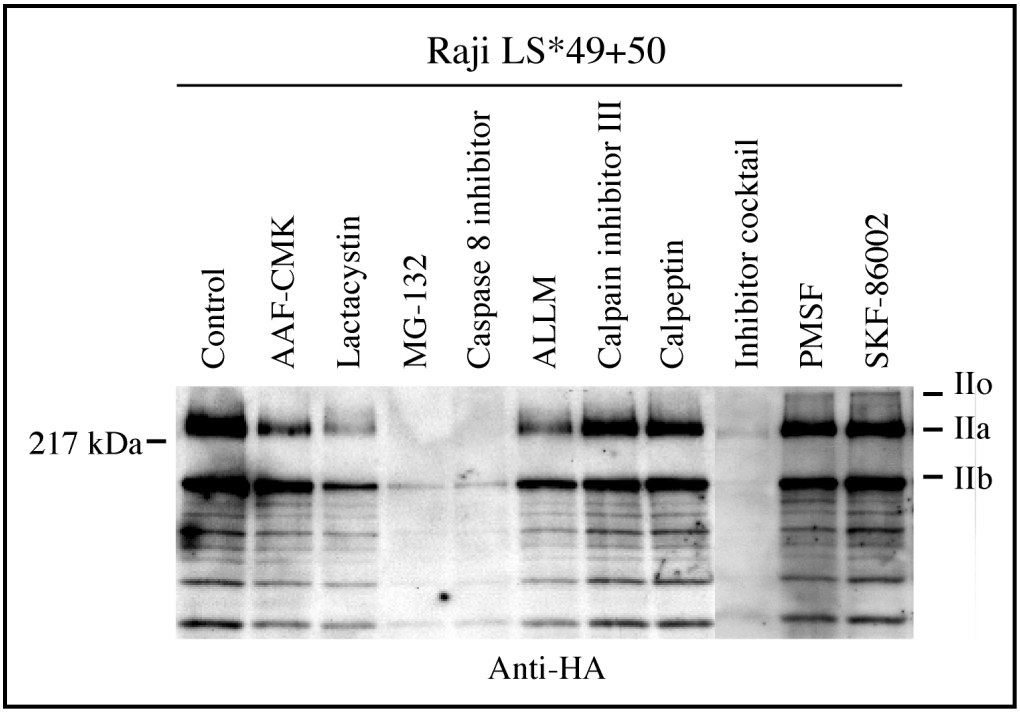

Figure 12. The effect of inhibitors on Pol IIB formation. Expression of the recombinant Pol LS*50 mutant was induced by removal of tetracycline. 2h after induction the following inhibitors were applied at the indicated concentrations: STI571, 20µM; AAF-CMK, 100µM; ALLM, 500µM; Calpeptin, 100µM; Calpain inhibitor III, 500µM; Caspase 8 inhibitor I, 50µM. Cells were harvested after 18h. Proteins were identified using the high-affinity, anti-HA antibody 3F10.

The levels of IIa and IIb forms appear equal in all samples tested, except in the sample treated with inhibitor cocktail, where no IIb form can be seen. The low level of IIo form seen is to be expected, given that some time is required for nuclear localisation, where the CTD phosphorylation required to produce this form takes place. The weak signals seen for samples treated with MG-132, Caspase 8 inhibitor, or the inhibitor cocktail may result from the general toxic effect these inhibitors have on cellular processes. It is possible that

some inhibitors are no longer active at the time point used (For example, PMSF), however, the appearance of the Pol IIb form could not be inhibited even at ten times the concentrations used here (data not shown).

2.4.4 Cell viability is severely affected by removal of the last repeat, but not by its disruption, or mutation of its CKII sites

Raji cell lines expressing Pol II LS**wt* have been previously shown to tolerate growth in α-amanatin (Meininghaus et al., 2000). To test the ability of last repeat mutants to support growth and viability, their expression was induced over a period of 24 h, before further culture in the presence of α-amanatin (Fig. 13). Cell number and viability were regularly recorded over a period of more than 80 days. As controls, two well characterised mutants, LS*Δ5 and LS*Δ31, possessing only 5 or 31 CTD repeats, respectively (Meininghaus et al., 2000), were also included in the analysis. A similar pattern is seen for all cell lines containing functional polymerases: viability drops steeply during the first 15 days of growth in the presence of α-amanatin, followed by a crisis phase of little growth, and recovery after day 30.

The presence of α-amanatin appears to induce the stabilisation of cellular mRNA by an as yet, unknown mechanism (Meininghaus et al., 2000). This may explain how in the first few days post-α-amanatin, a cell line lacking a fully functional polymerase (LS*Δ31) is still able to grow. This phenomenon may also explain the exponential growth seen for all other cell lines until day 8 (Fig. 13B). Cellular stress due to the over- or under-expression of the exogenous polymerases, or titration of transcription factors by the Tc-transactivator (TcTA) and Pol II LS* itself may lead to sub-optimal gene expression. The life span of stabilised mRNAs is not known, and it is tempting to speculate that the crisis phase that ensues until around day 30, results from a shortage of essential mRNAs. During this period, where little cumulative growth is observed, the cells are effectively selected for those adapted to their new conditions. The increase in viability shortly before this point (day 25) results more likely from the disappearance of dead cells than an increase in cell number (Fig. 13A).

As previously shown (Meininghaus et al., 2000), the transcription-defective LS*Δ5 mutant rapidly loses viability in the presence of α-amanatin, exhibiting a kinetic similar to that of a control cell line containing empty vector (LS*mock). Cell lines LS*Δ31 and LS*49+50, sustain cell viability for a period

approximately three times longer than LS*Δ5, but do not survive the crisis phase and instead exhibit a sharp drop in cell number following day 8, suggesting that they are incapable of replacing all the functions of the endogenous Pol II LS. Of all the last repeat mutants, only LS*49+50 is not viable: for all other mutants almost 90% viability is regained around day 50. It is theoretically possible that the recovery seen results from the endogenous polymerase gaining α-amanatin resistance. However, the inhibition of exogenous Pol II LS* expression by re-application of Tc at day 51, resulted in α-amanatin sensitivity and cell death. In rare cases, re-addition of Tc failed to have an effect on growth and viability. Analysis of these cell lines revealed that the exogenous, α-amanatin-resistant polymerase was still expressed: control through Tc had been lost (data not shown). Two mutants containing the *wt* last repeat, LS*49+52 and LS*49M+52 (non-consensus repeat 49 is truncated to consensus length), survived longer than the other mutants following Tc re-addition. This may indicate that last repeat mutants do in some way differ from those with a *wt* last repeat.

The inability of the LS*49+50 mutant to sustain viability would not be surprising if it were constantly being degraded. However, mutants LS*49+NS52 and LS*49+50ATM also exhibit induction of the IIb form. Apart from the slightly reduced length of its last repeat, it is not clear what differs LS*49+50 from these other mutants. If it is indeed the case, that the crisis phase observed results from insufficient mRNA production, mutation of the last repeat may affect transcription or pre-mRNA processing. Since the last repeat is the target of CKII and c-Abl, it may be the disturbance of these signal pathways that is responsible for this phenotype. However, the CKII mutants exhibit no apparent phenotype, so it is more likely that interaction of c-Abl or an as yet, unidentified protein with the last repeat is required.

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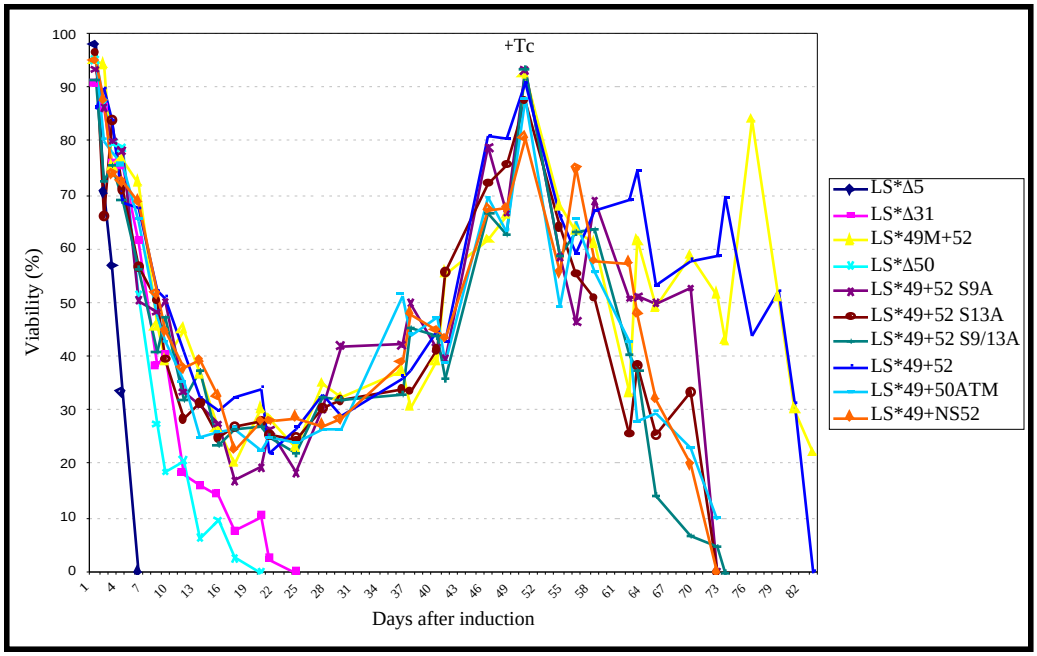


B

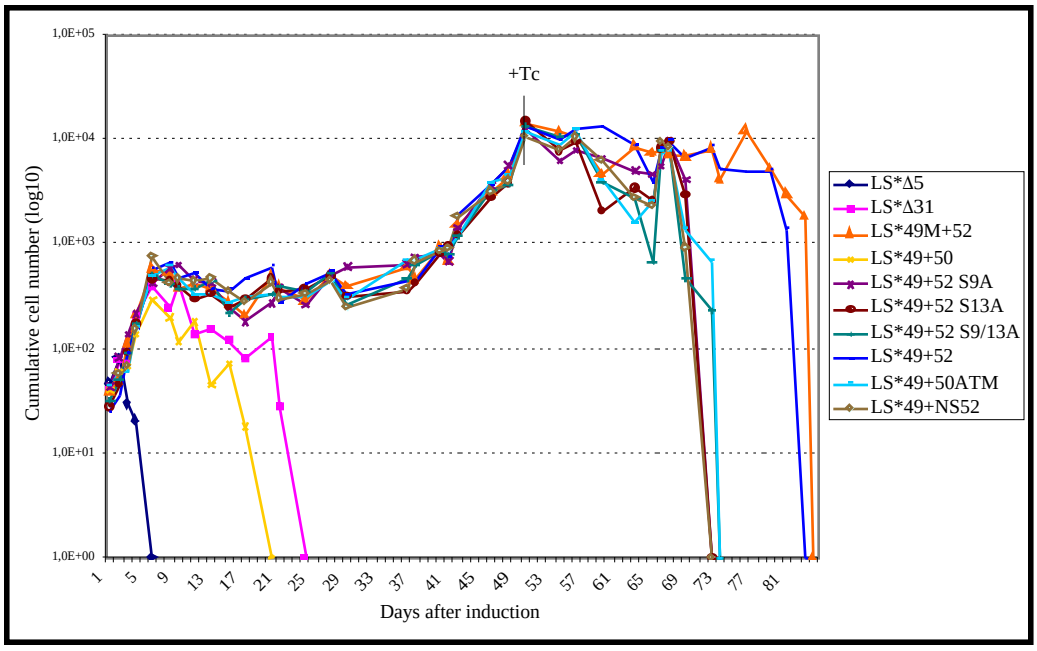


Figure 13. Growth and viability of Raji cell lines expressing Pol II LS*CTD mutants in the presence of α-amanatin. Stably transfected Raji cells were induced to express Pol II LS*CTD mutants by removal of tetracycline (Tc) from their growth medium. 24h after induction, α-amanatin was given to the medium [2mg/ml]. Tc was reapplied to cultures exhibiting recovery at day 51. Viability and cumulative cell growth were assessed regularly over a period of 84 days.

2.4.5 Mutation of the last CTD repeat does not affect the specific transcription of genes

Since c-Abl might only be recruited to some promoters, its influence on transcription could be gene specific. It has long been postulated that c-Abl may influence transcription elongation (Baskaran et al., 1997a; Baskaran et al., 1999; Bregman et al., 2000; Oelgeschlager, 2002). To examine this possibility a DNA array was used to assess the transcription of 1176 genes. Due to the mRNA stabilisation caused by α-amanatin treatment, the classic RT-PCR-based method for gene expression analysis is not applicable. This problem is overcome by instead using the nuclear run-on technique to measure the polymerase density on actively transcribed genes (Eick and Bornkamm, 1986; Greenberg and Ziff, 1984). Figure 14 shows the results of such an experiment. The cell lines LS*49+52 and LS*49+50 were analysed and two fields, designated A and B, are compared from the same array.

Despite repetition of this experiment several times, no reproducible differences in gene expression between the two polymerases were seen. The only striking difference is the reduced signal intensity seen for the LS*49+50 mutant. A similar difference was seen earlier in the ability of the two mutants to transcribe the *hsp*70A gene (Fig. 7B). The reason for this apparent weakness is not clear, but may be a result of the degradation of Pol LS*49+50 to the inactive IIb form. That the transcription of any single gene was unaffected may not be surprising: if the kinases that act through the final repeat are only active under certain circumstances, differences would not be seen in the absence of stimulus; if these kinases work in synergy with other transcription factors for their effect, no effect would be seen if they were not present. Nuclei from non-induced cell lines (+Tc), or a cell line expressing no α-amanatin resistant Pol II (LS*mock), failed to demonstrate any specific Pol II transcription, indicating that the transcription seen results from the α-amanatin resistant Pol II LS mutants (data not shown).

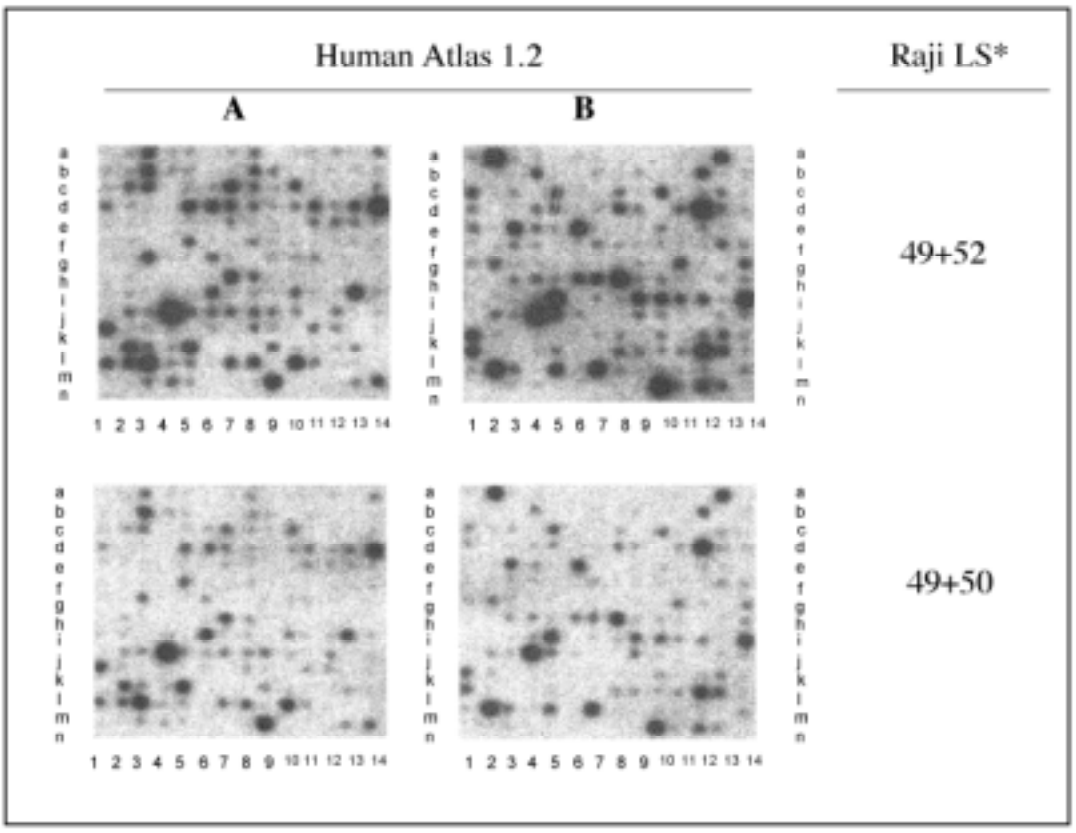


Figure 14. Comparison of global run-on transcription by Pol II LS* last CTD repeat mutants. Raji cells expressing mutants containing (LS*49+52), or lacking the *wt* CTD last repeat (LS*49+50) were grown for 24h in the presence of [2μg/ml] α-amanatin before isolation of nuclei. Run-on reactions were performed and the labelled RNAs hybridised to Clontech Human Atlas 2.1 DNA arrays. Regions **A** and **B** correspond to two representative fields from the same array, for each mutant.

2.4.6 IR-induced tyrosine phosphorylation of the CTD does not affect the specific transcription of genes

c-Abl is activated by the ATM protein in response to IR (Baskaran et al., 1997b). ATM normally also induces the tumour suppressor p53 (Lavin and Khanna, 1999), however, in Raji cells p53 is disabled through mutation (Farrell et al., 1991). Thus, any changes in transcription seen following irradiation of Raji cells, should be a direct result of c-Abl interaction with the CTD. The highest level of CTD tyrosine phosphorylation is seen approximately 1 hour post-irradiation (Baskaran et al., 1993; Baskaran et al., 1999), at which point the nuclear run-on analyses of nuclei from the two cell lines were performed. Figure 15 indicates the actively transcribed genes in the cell lines LS*49+50 and

LS*49+52, before and 1 hour after exposure to IR. Two representative fields, D and E, from each array are shown for each cell line under each condition. Again, the average signal intensity is weaker for LS*49+50 compared to LS*49+52. However, no reproducible difference could be seen in the activation of specific genes, either between mutants, or in response to IR.

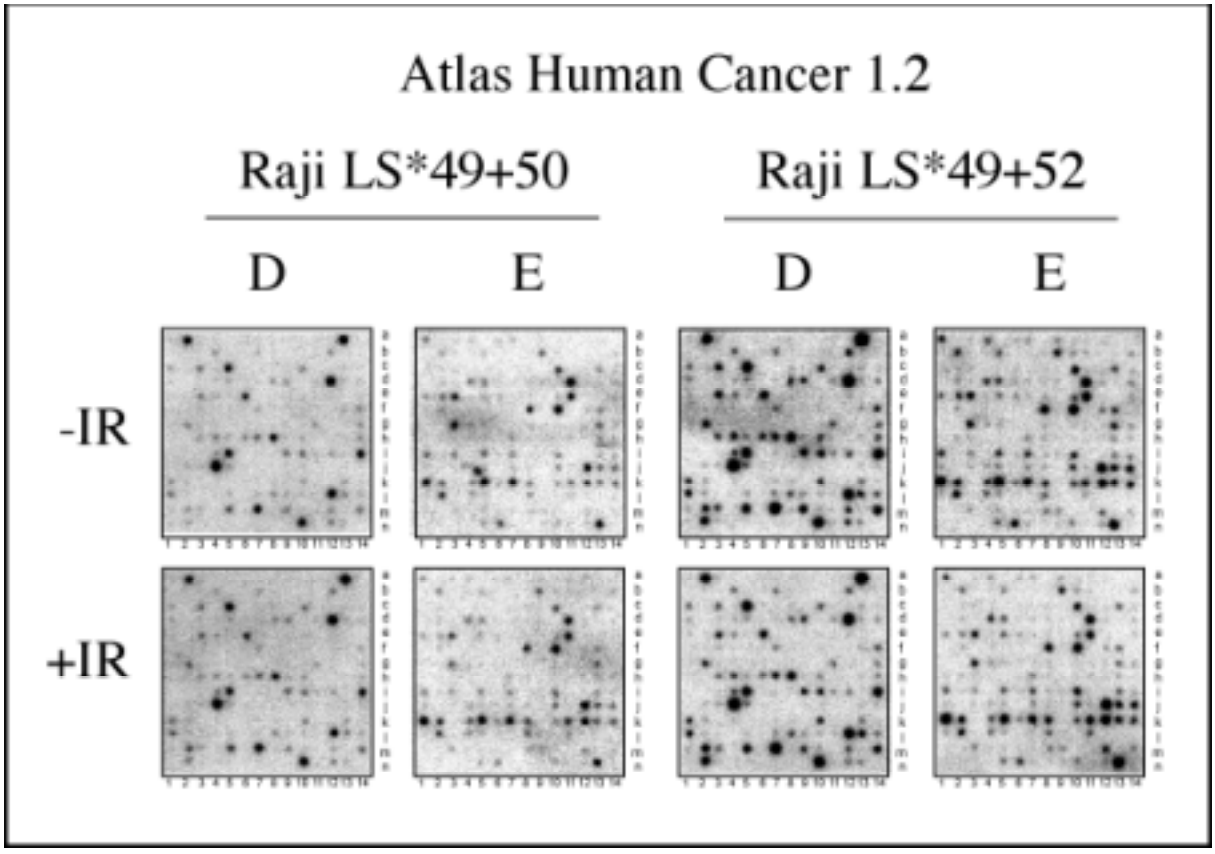


Figure 15. The effect of IR on active gene transcription in Raji cells. Following 24h induction of their respective recombinant Pol II LS* mutants, cells were grown for a further 24h in the presence of [2μg/ml] α-amanatin before exposure to 12 Gy IR, and isolation of nuclei 1 hour later. Run-on reactions were performed and the labelled RNAs hybridised to Clontech Atlas Human Cancer 2.1 DNA arrays. Regions **D** and **E** correspond to two representative fields from the same array, for each mutant. The expression pattern of irradiated cells (+IR) is compared to a non-irradiated control (-IR).

2.5 Testing of cell lines for a response to IR

In order to ask questions about specific signalling pathways, it is important to be asking questions in the correct system. Since both CKII and c-Abl are implicated in the response to DNA damage, the response in the cell line to be used should be intact. Since a loss of CKII activity would be lethal

(Reviewed in Allende and Allende, 1995), we can assume this enzyme functions normally in these cells. The Raji cell line, however, was first described in 1964 and has certainly evolved during its time in tissue culture. By its nature, a Burkitt's lymphoma (BL) cell line with translocated c-myc t(8;14), the internal apoptotic machinery has been compromised to permit its survival. A mutation in the tumour suppressor p53 (Farrell et al., 1991; Stenger et al., 1994) removes its ability to tetramise, resulting in loss of this response: p53 activated cell cycle arrest and apoptosis does not occur following DNA damage.

Since the nature of our expression vector relies on the Epstein-Barr virus nuclear antigen one (EBNA1) for its replication and maintenance, we are limited to cell lines expressing this protein. A common method for producing cell lines from primary B cells is to infect, and thereby immortalise isolated cells with EBV. The resulting cell lines, referred to as lymphoblastoid cell lines (LCLs), have the advantage that, despite the influence of the viral programme, most cellular systems and the genome remain intact. For this reason, a panel of LCLs were tested for their ability to stabilise p53 following exposure to 8 Gy IR. Cellular p53 levels were compared before and after irradiation (Fig. 16A). Cell lines 1.11, 1.26 and 1087 showed little increase in p53 levels following irradiation and were thus discounted. Strong inductions were seen in the LCLs GB.4.C3, 1.25, Kinert and Rosi. Further tests showed the Rosi cell to be the best candidate due to the relative ease with which it was possible to produce stable cell lines. Unstimulated Rosi cells show little stabilisation of p53: a slower migrating band can be seen that is most probably the ubiquitinated form (Fig. 16B). One hour after irradiation a strong p53 induction can be seen, which is almost double the intensity at two hours post IR, and then maintained for at least a further two hours.

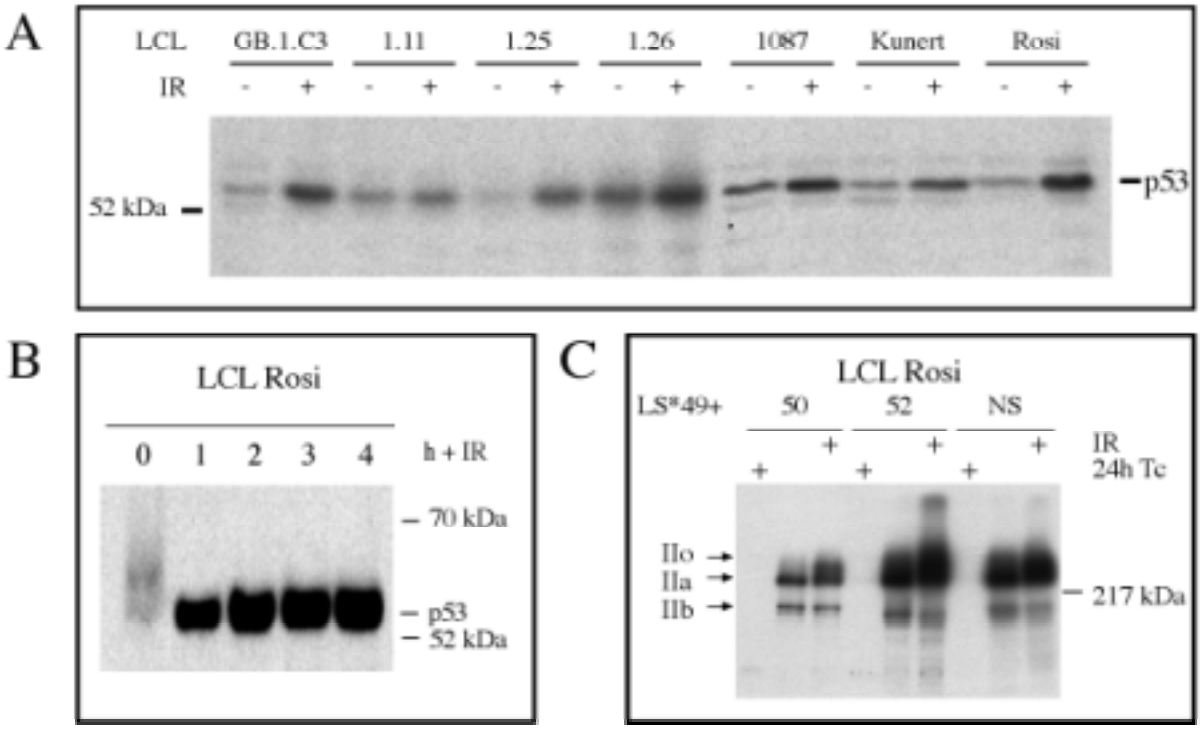


Figure 16. The effect of 8 Gy ionising radiation (IR) on lymphoblastoid cell lines. (A) Expression of the tumour suppressor p53, 1h following irradiation, in a panel of different LCLs. (B) Time course of p53 expression in the Rosi cell line. (C) Tetracycline regulatable expression of stably transfected Pol II LS*CTD mutants in the Rosi LCL and the effect of 8 Gy IR on the mobility of the Pol II LS*CTD mutants.

Stable Rosi cell lines of mutants LS*49+50, LS*49+52 and LS*49+NS52 were established. The expression profile is similar to that seen in Raji: the Pol IIb form only appears with mutants LS*49+50 and LS*49+NS52, but not with the mutant LS*49+52, which contains a *wt* last repeat (Fig. 16C). A band can be seen migrating slightly faster than the IIb form, and is an artefact of the lysis procedure resulting from incomplete inhibition of proteases. It is this band that is visible with the LS*49+52 mutant, and not IIb. Comparison of the different forms present for each mutant reveals a similar pattern to that seen in the Raji cell lines. Again, little of LS*49+50 mutant appears to be in the hyperphosphorylated (IIo) form as compared to LS*49+52, but interestingly this is not the case for the LS*49+NS52 mutant, that expresses a similar ratio of forms to that of LS*49+52. IR exposure causes a shift to the IIo form for all mutants, probably as a result of increased stress kinase activity (ERK/JNK), and the transcription of stress-activated genes. Since c-Abl is activated by IR,

increased phosphorylation should only be seen in mutants containing a *wt* last repeat: mutants LS*49+50 and LS*49+NS52 should no longer interact and become phosphorylated by this kinase. This may explain the small discrepancy in the amount of the IIo form between mutants LS*49+52 and LS*49+NS52.

2.6 Characterisation of CTD last repeat mutants in the Rosi LCL

Stable Rosi cell lines were produced for all the mutants. The same initial tests were performed as with the previously described cell lines. Figure 17 shows their expression pattern 24h following induction. Again, a pattern similar to that seen in Raji can be seen: the Pol IIb form is only seen for mutants LS*49+50, LS49+50ATM and LS*49+NS52. The shortfall in Pol IIo form detected results partly from the extraction buffer (RIPA) used, which fails to extract all nuclear Pol II, and partly that the distribution of the expressed polymerases is largely cytoplasmic at the time point of extraction (Based on the time course of distribution of an EGFP-labelled Pol II LS). It can be seen that the level of expression is not equal in all cell lines. This may be due to incomplete removal of tetracycline in this experiment.

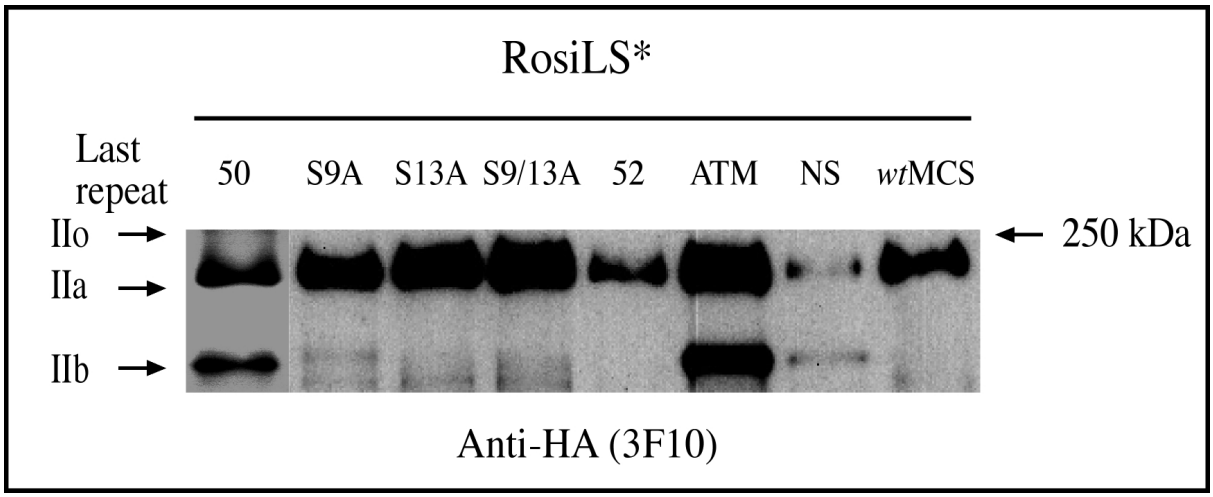


Figure 17. Expression patterns of last CTD repeat mutants in the Rosi LCL. Cells were induced to express their respective Pol LS* mutants by removal of tetracycline. 18 h later, cell extracts were prepared using RIPA buffer, and mutants visualised following SDS-PAGE/ western blotting using the 3F10 anti-HA antibody.

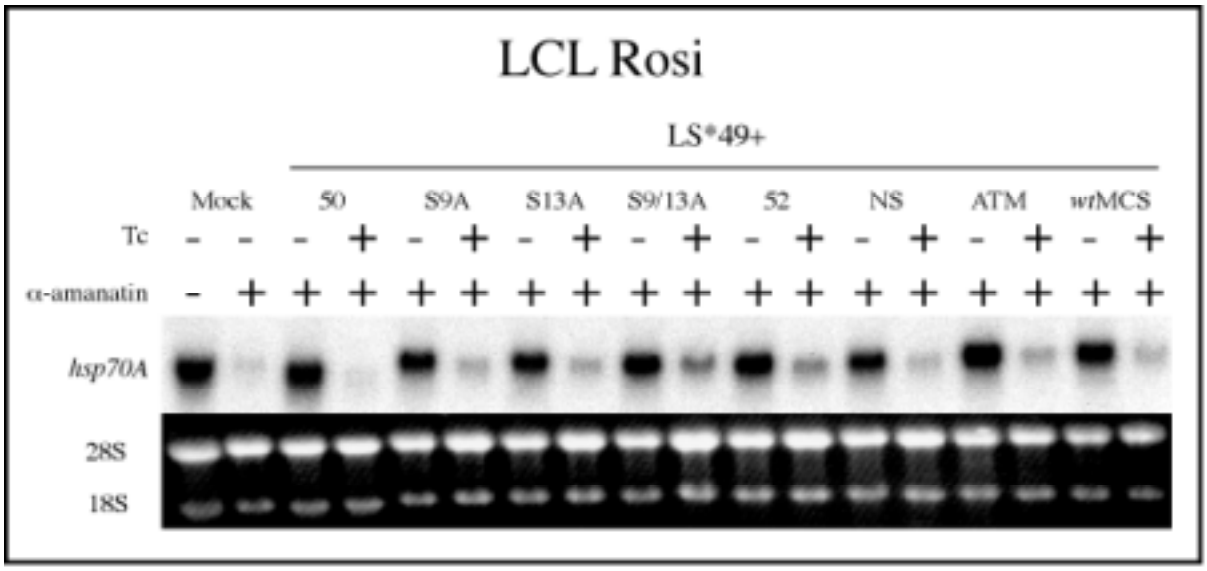


Figure 18. The ability of last CTD repeat mutants to induce the *hsp70A* gene in response to heat shock. Rosi cell lines were grown in the presence or absence of Tc for 24h, after which, α-amanatin [2mg/ml] was added to the indicated samples and incubated a further 24h. Cells were exposed to a heat shock for 2h at 43°C before harvesting of their RNA. 10mg of total RNA from each sample was probed for levels of *hsp70*A RNA using a complementary radioactive probe. The 28S and 18S RNA bands, stained with ethidium bromide, provide a control for equal loading.

The relative ability of these mutants to transcribe the *hsp70A* gene was also assessed (Fig. 18). In the presence of α-amanatin, only cell lines grown in the absence of Tc show an induction of the *hsp70A* gene in response to heat shock, suggesting all our mutants can functionally replace the endogenous Pol II LS in the transcription of this gene. The signal seen for all cell lines is similar to that of a control cell line, 'Mock' which only expresses the endogenous polymerase. The faint background bands seen for some mutants in the presence of Tc may result from leakiness in the expression system, or technical error, and are negligible in comparison to the induction seen.

2.6.1 Cell viability is not severely affected by removal of the last repeat in the Rosi cell line

Analysis of the ability of these mutant polymerases to sustain viability in the presence of α-amanatin (Fig. 19) contrasts the results seen in Raji (Fig. 13): all cells show an increased resistance to α-amanatin. Experiments in Raji repeatedly show death of the control 'Mock' cell line around day 7 plus α-amanatin, whereas death is seen for all cells around day 9 in Rosi. As in Raji, the first 7 days are characterised by a major drop in viability for all cell lines. However, the crisis phase that ensues is more acute in Rosi cell lines, with almost full viability attained around 25 days, compared to almost 50 days for Raji cell lines. Most interestingly, the LS*49+50 mutant does not exhibit a lethal phenotype in Rosi. This mutant initially shows a weaker recovery than other mutants, but is able to restore and sustain viability to over 80%. This difference is perhaps inherent to the cell lines: Raji, alike most BL cell lines, is more sensitive to stress than LCLs.

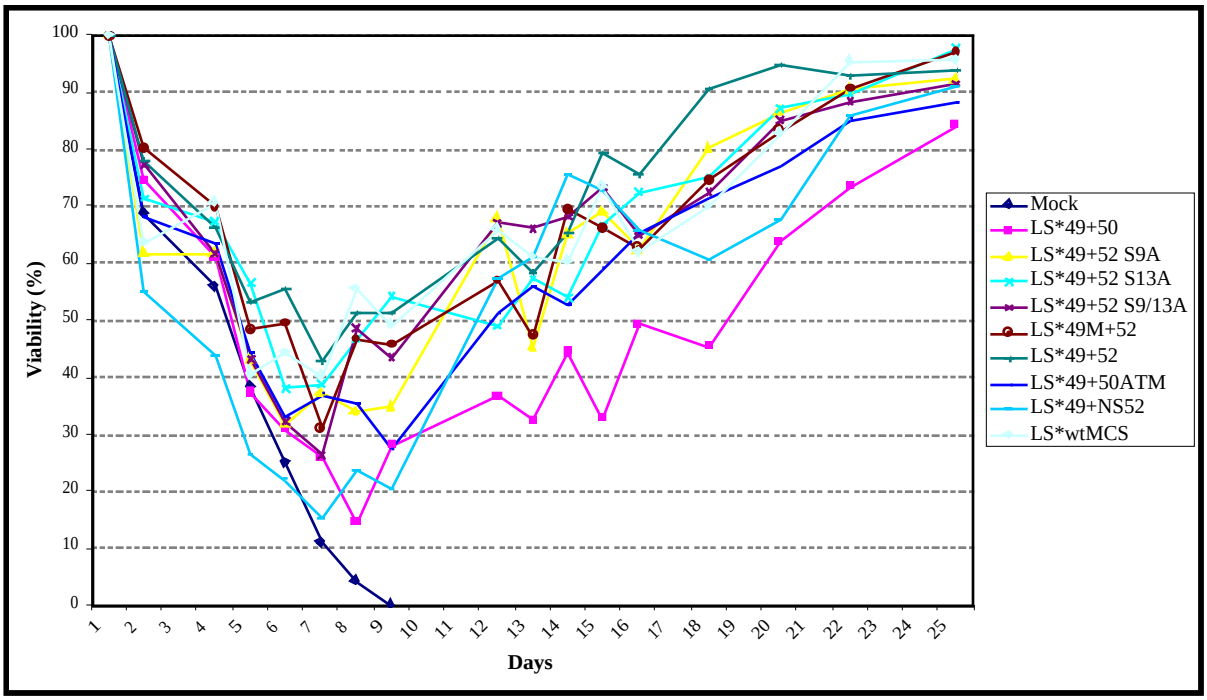


Figure 19. Growth and viability of Rosi cell lines expressing Pol II LS*CTD mutants in the presence of α-amanatin. Stably transfected Rosi cells were induced to express Pol II LS*CTD mutants by removal of tetracycline (Tc) from their growth medium. 24h after induction, α–amanatin was given to the medium [2mg/ml]. Viability and cumulative cell growth were assessed regularly over a period of 26 days.

2.6.2 Mutation of the final repeat reduces the tyrosine phosphorylation of Pol II LS in response to IR

If our mutations really affect the tyrosine phosphorylation of CTD by c-Abl, in response to DNA damage, then the level of phosphorylation on some mutants should be reduced compared to those containing a *wt* last repeat. Cells expressing the previously untested CKII and 'last repeat' Pol II LS* mutants were exposed to IR before harvesting one hour later, a time point where the most tyrosine phosphorylation is to be seen (Baskaran et al., 1993; Baskaran et al., 1999). In order to detect the phospho-tyrosine content of the expressed mutant, immunoprecipitation (IP) is first required to remove the background signal from other cellular proteins.

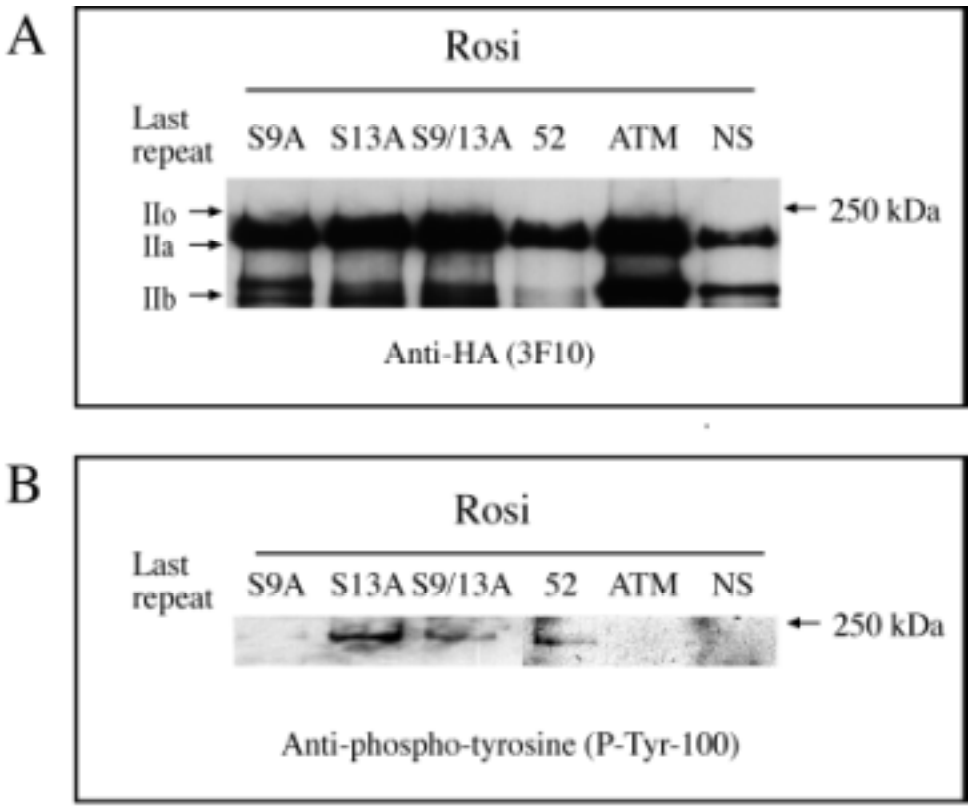


Figure 20. Tyrosine phosphorylation of Pol II LS* CTD mutants following IR. Cells expressing Pol II LS* CTD mutants were irradiated with 12 Gy IR, then harvested 1h later. Immunoprecipitation (IP) was performed using the anti-HA antibody, 3F10. Samples were separated by SDS-PAGE and Western blots thereof were stained with either **(A)** anti-HA (3F10) mAb, to control IP yield, or **(B)** the anti-pY antibody, pY100.

Western analysis of the immunoprecipitates reveals successful IP of the mutants (Fig. 20A), as visualised using antibody against the HA-tag. The IIb form is also immunoprecipitated, since the HA tag is N-terminal. IIb is only produced in cells expressing mutants LS*49+50ATM, LS*49+NS52. A band migrating slightly faster than IIb can be seen for some mutants, but is a degradation product other than IIb, which may result from incomplete inhibition of proteases during IP.

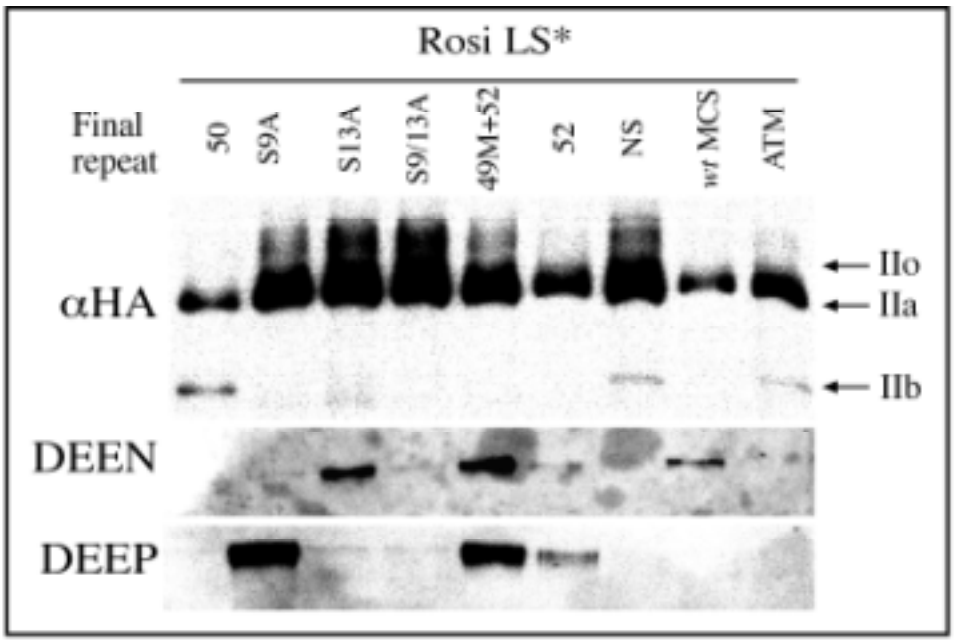
Immunoprecipitated material from irradiated cells was probed with anti-phospho-tyrosine antibody to determine the tyrosine phosphorylation induced. The levels of tyrosine phosphorylation detected were overall very weak for all mutants tested, leading to a low signal/ background ratio. The blocking procedures for the anti-HA antibody produced an unacceptable level of background when used with the anti-phospho-tyrosine antibody, and *vice-versa*, thereby preventing stripping and re-probing of the same blots. Following testing of several commercially available antibodies, the best results were obtained using the P-Tyr-100 antibody. This antibody was raised against a synthetic peptide, and has been shown to recognise a synthetically phosphorylated CTD peptide (manufacturer's claim). However, it is not clear how the recognition by this antibody, and other anti-phospho-amino acid antibodies, is affected by other changes in the surrounding sequence (for example, phosphorylation of other residues). Despite the weak signals obtained, it could be shown that only mutants containing an intact CTD last repeat become tyrosine-phosphorylated following exposure to IR (Fig. 20B). Signals could be seen for CKII mutants LS*49+52 -S9A, -S13A, and -S9/13A, indicating that these mutations do not interfere with c-Abl interaction.

2.7 CKII phosphorylation of the CTD

Through point mutation of the CKII consensus sites in the Pol II LS final repeat we hoped to destroy this interaction *in vivo.* CKII has been shown only to phosphorylate fragments containing the C-terminal, largely non-consensus region of CTD, and not N-terminal consensus fragments (Kuenzel et al., 1987). It is thus still not clear whether the consensus CKII sites are actually real targets. Work on the CTD last repeat by the Bensaude group resulted in the production of rabbit polyclonal antibodies to address this question. Two synthetic peptides of the last repeat were produced for immunisation: one

phosphorylated on every serine, the other possessing no phosphorylated amino acids. The resulting serum was shown in their own tests to specifically recognise phosphorylated, and non-phosphorylated forms of Pol II LS, however the CKII consensus sites used could not be determined. To address this question, these antibodies were tested for their ability to recognise our last repeat mutants. Rosi cells were induced to express the Pol II LS mutants for 24h before addition of α-amanatin for a further 24h, to remove the background of the endogenous Pol II LS. Western blots using either polyclonal antibody against the phosphorylated (DEEP1), or dephosphorylated (DEEN4) peptide are shown in figure 21A, compared to the loading control using anti-HA antibody. The mutants lacking the last repeat (LS*49+50, LS*49+50ATM and LS*49+NS52) or both CKII sites (LS*49+52 S9/13A), show no response to either antibody as expected. However, those containing the final repeat show a mixed reaction: LS*49M+52 and LS*49+52 react to both antibodies; LS**wt*MCS to just DEEN4. This result is interesting, since the Bensaude lab failed to identify an *in vivo* situation where the DEEN4 antibody recognised the endogenous Pol II LS. This may be due to a structural artefact of our mutants, or that as a result of over expression, not all Pol II LS* has been able to become phosphorylated. The absence of reactivity to the DEEP1 antibody seen with LS**wt*MCS may be explainable after considering the CKII point mutants: Pol II LS*49+52 S9A only reacts with DEEP1, while Pol II LS*49+52 S13A only reacts with DEEN4. This suggests that only serine 13 of the last repeat is a CKII target. The extra seven amino acids in the LS**wt*MCS last repeat may interfere with its structure, or prevent the interaction with CKII. Figure 21B shows a summary of the reactivity of the antibodies which each of the mutants and the epitopes present. However, it is important to note that these antibodies are polyclonal mixtures raised against a *wt* last repeat peptide, and that there reactivity to synthetic point mutants has not been characterised. For the purposes of this experiment we assume structural similarity of the point-mutated sequence to that of *wt*.

A



B

Cell Line	Epitope present		Antibody	
	S9	**S13**	**DEEN4**	**DEEP1**
49+50				
49+S9A		+		+
49+S13A	+		+	
49+S9/13A				
49M+52	+	+	+	+
49+52	+	+	+	+
49+NS				
49+*wt* MCS	+	+	+	
49+ATM				

Figure 21. Phosphorylation specific antibodies determine the level of phosphorylation at the final repeat CKII sites *in vivo*. (**A**) Expression of Pol II LS* CTD mutants in stably transfected Rosi cell lines. Cells were harvested 48h after induction of expression, and incubation with α-Amanatin for the last 24h. Expression levels were determined by staining Western blots with the high-affinity anti-HA antibody 3F10. Polyclonal antibodies raised against a repeat 52 peptide, phosphorylated at positions 5, 9 and 13 (DEEP), were used to determine the level of phosphorylation resulting from CKII activity. Polyclonal antibodies raised against an equivalent, non-phosphorylated peptide (DEEN), were used as a control. (**B**) The table reflects the potential CKII target epitopes present in each CTD mutant, and their recognition by the phosphorylation-specific antibodies.

2.8 Analysis of differences in gene expression between mutants

Unlike Raji, the Rosi cell line appears to have an intact p53 and ATM response. This can be seen at the level of transcription by the up regulation and down regulation of individual gene transcription at various time points following stimulus. Nuclear run-on analysis of Rosi cells one hour following exposure to 12 Gy IR reveals the up-regulation of known p53 targets, in accordance with data already published (Zhao et al., 2000).

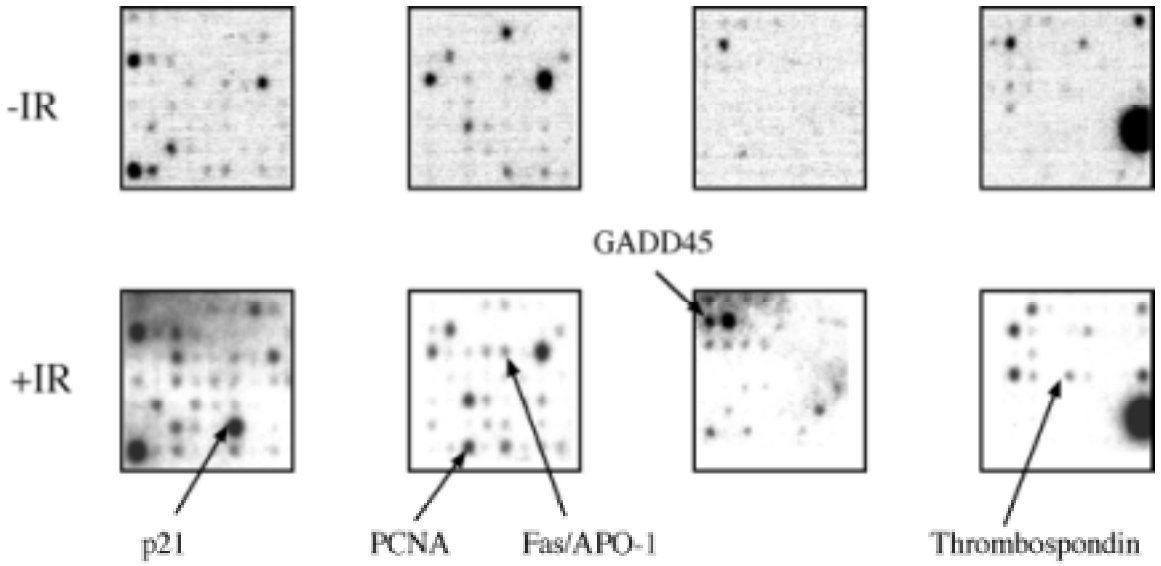


Figure 22. The effect of ionising radiation on active gene transcription in Rosi cells expressing a Pol II LS* mutant. Expression of the LS*49+52 mutant was induced for 24h before the addition of 2mg/ml α-amanatin. Following a further 24h incubation, a portion of cells were exposed to 8 Gy IR and nuclei harvested 1h later. For each treatment, the labelled RNA from $2x10^7$ nuclei was hybridised to an Atlas human cancer 1.2 array (CLONTECH). Identical fields from arrays hybridised with either RNA from irradiated cells (+IR) or that of non-irradiated cells (-IR) are compared. p53 target genes induced following IR are labelled.

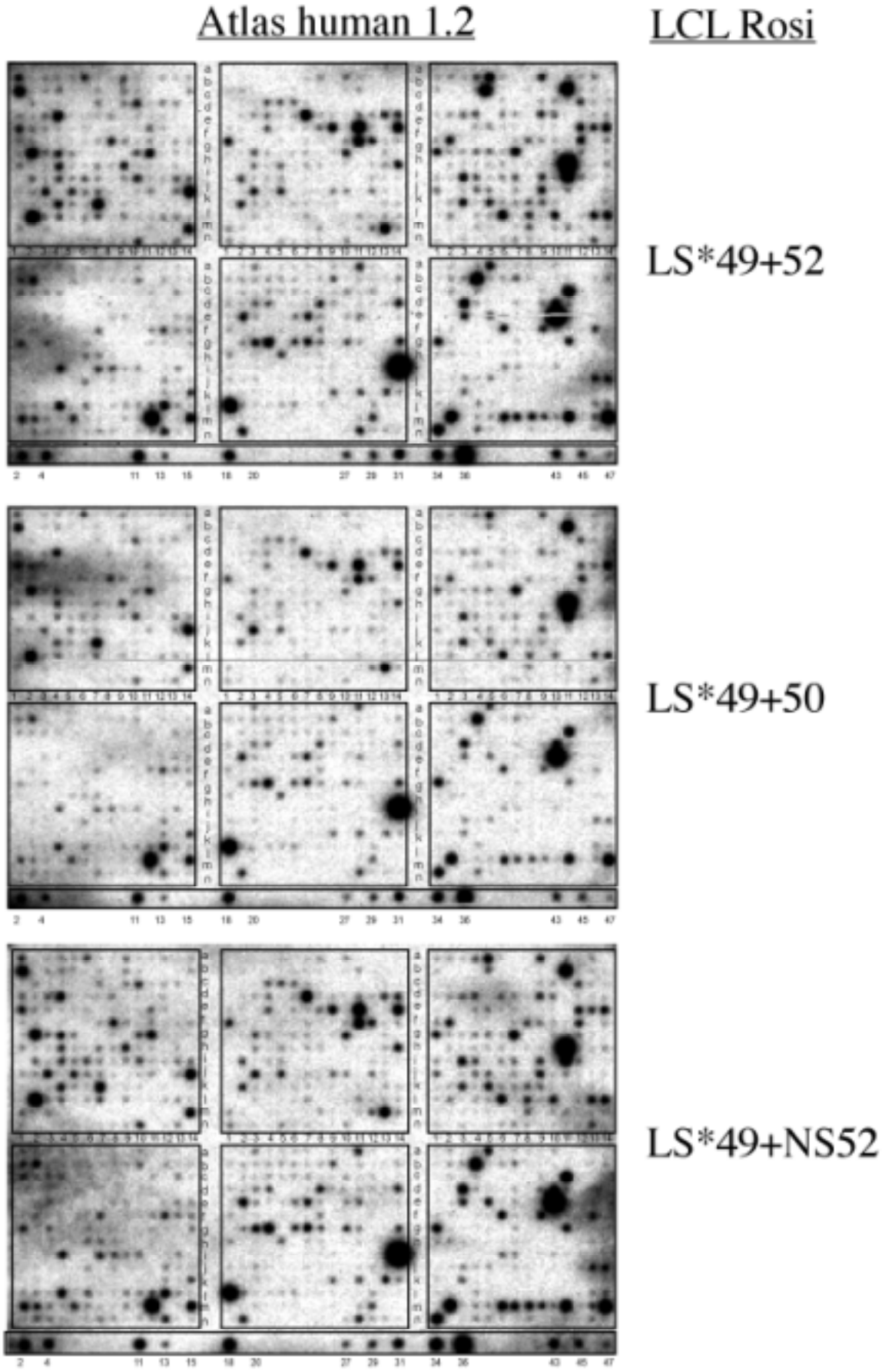
Comparison of active transcription by two mutants lacking the CTD last repeat (LS*49+50; LS*49+NS52) to that of a mutant where it is present, reveals no reproducible difference in the specific transcription of genes (data not shown). Run-on analysis of active transcription 1 hour post-IR reveals the induction of genes known to be induced by p53 in response to IR (Fig. 22). The cyclin-dependent kinase inhibitor, *p21/Waf1/Cip1* is strongly induced, as is the proliferating cell nuclear antigen gene (*PCNA*). The gene for the Fas/APO-

1/CD95 membrane receptor, the cell cycle regulator GADD45, and the angiogenesis regulator thrombospondin were also significantly up-regulated. This type of array is designed as an initial screen for gene targets and is not designed for accurate quantification, thus the changes in gene expression noted here are purely qualitative (For quantification of gene expression in response to IR see (Zhao et al., 2000).

Comparison of mutants revealed no reproducible difference in the level of transcription of single gene (Fig. 23). The only notable difference between mutants is the overall, slightly weakened signal for all genes transcribed by the LS*49+50 mutant, as also previously seen for the same mutant in the Raji cell line (Fig. 4). A control region at the base of each array allows the comparison of specific gene expression with that of 'house-keeping' genes, three genomic DNA probes (Positions 2,18, and 34), and a fragment of the vector pUC19 (Position 36). The episomal vectors contained in these cell lines also contain fragments of pUC19, that are transcribed by both RNA polymerase II, and III due to the presence of their respective promoters within the vector backbone. Since these activities are not inhibited by α-amanatin, and are constant between cell lines, this spot serves to control that RNA from approximately the same number of nuclei is being compared. At least 5 probes on the Atlas 1.2 array contain *Alu*-sequence elements and thus have cross reactivity with RNA Pol III transcripts, leading to their increased intensity.

(overleaf)

Figure 23. Run-on analysis of polymerase density on 1176 genes. The Pol II mutants LS*49+50, LS*49+52 and LS*49+NS52 were induced for 24h before the addition of 2mg/ml α-amanatin. Following a further 24h incubation, cells were exposed to 8 Gy IR and nuclei harvested 1h later. For each cell line, the labelled RNA from $2x10^7$ nuclei was hybridised to an Atlas human 1.2 array (CLONTECH).


Atlas human 1.2
LCL Rosi
LS*49+52
LS*49+50
LS*49+NS52

3.0 Discussion

The aim of this project was to examine the function of the final repeat of the RNA polymerase II large subunit CTD. The CTD has been shown to be essential at almost every stage of *in vivo* transcription: initiation, elongation, termination and pre-mRNA processing. The CTDs of different organisms vary in the number of repeats of a consensus sequence YSPTSPS. In mammals, the number of repeats is 52, however, not all repeats conform to the consensus sequence. It has long been postulated that such ´non-consensus´ repeats may have evolved to fulfil more specific functions. The majority of work to examine this hypothesis has relied on reconstituted, *in vitro* transcription systems or transiently transfected reporter constructs. Additionally, synthesised polypeptides representing consensus-, and non-consensus repeats have been used to assess their ability to catalyse the different stages of pre-mRNA processing (Fong and Bentley, 2001). Although valid, these techniques fail to account for the effects of chromatin and the controlled recruitment of factors to transcription complexes. The last repeat of the Pol II LS CTD is the site of interaction for two kinases known to play roles in the response to DNA damage; a response that due to the number of unknown parameters, would be difficult to reproduce *in vitro.* For this reason a system was used that allowed the study of Pol II LS CTD mutants *in vivo.* This system requires that all mutants be based on a Pol II coding sequence containing a point mutation that confers resistance to the RNA polymerase inhibitor, α-amanatin. The endogenous polymerase is effectively, chemically ´knocked-out´ when cells are grown in the presence of α-amanatin, enabling the ability of the mutants to replace the function of the endogenous polymerase to be examined.

The α-amanatin system:

The toxin produced by the 'death cap' mushroom (*Amanita phalloides*), α-amanatin, is a cyclic peptide that binds with high affinity to the Pol II LS. Binding of α-amanatin to the elongating Pol II complex reduces the rate of translocation from several thousand to only a few nucleotides per minute (Chafin et al., 1995; Rudd and Luse, 1996). Binding of α-amanatin still permits the formation of the phosphodiester bond between ribonucleotides, but inhibits the following translocation step, and therefore elongation (de

Mercoyrol et al., 1989; Vaisius and Wieland, 1982). The efficacy of this inhibition in cell culture experiments appears dependent on the permeability of the cell to α-amanatin: an incubation time of 24h is required for complete inhibition of the endogenous Pol II in cultured Raji cells (Meininghaus et al., 2000), whereas inhibition occurs in minutes following treatment of isolated nuclei with the same concentration.

Structural analysis has revealed that the site of α-amanatin binding lies beneath the ´bridge helix´, which extends across the cleft between the two largest Pol II subunits, Rpb1 and Rpb2, within a ´funnel´-shaped cavity in the Pol II structure (Bushnell et al., 2002). A number of different point mutations conferring α-amanatin resistance have been described, most mapping to this site, which lies in the region ´F´ of the Pol II large subunit (*M. musculus*, (Bartolomei and Corden, 1987); *R. rattus*, (Crerar et al., 1983); *D. melanogaster*, (Chen et al., 1993); *C. elegans*, (Rogalski et al., 1990). The α-amanatin resistant Pol II mutant C4 from *D. melanogaster* exhibits a reduced elongations rate *in vitro* (Coulter and Greenleaf, 1985), and mutations in the same region of the Pol III large subunit also affect elongation (Thuillier et al., 1996; Thuillier et al., 1995), suggesting the region where α-amanatin binds is involved in transcription elongation. These findings are consistent with the finding that the α-amanatin binding site is too far from the active site to interfere with nucleotide entry or RNA synthesis (Bushnell et al., 2002).

The resulting block in Pol II elongation caused by α-amanatin creates a problem for the cell in that it creates a physical block on the gene that it was transcribing. It is therefore not surprising that treatment of cells with α-amanatin induces the degradation of the Pol II LS (Nguyen et al., 1996). Treatment of cells with agents that cause DNA lesions (e.g. UV radiation or cisplatin) also results in a transcription block, and degradation of Pol II LS. In both cases degradation occurs through the ubiquitin proteasome pathway (Ratner et al., 1998). The E3 ubiquitin-protein ligase, Rsp5, is the most likely candidate for this activity. Rsp5 has been shown to interact with the CTD of Pol II via its WW domain (Chang and Kornberg, 2000), and mediates the ubiquitination of lysines in the Pol II LS, non-CTD region (Ratner et al., 1998). That Rsp5 interacts directly with the CTD suggests that a modification of the CTD in response to the stalling of a polymerase could control its recruitment to the Pol II complex (Chang et al., 2000). Treatment of cells with the transcriptional and CTD kinase inhibitor 5,6-dichloro-1-β-D-

ribofuranosylbenzimidazole (DRB) blocks the ubiquitination and degradation of Pol II LS following UV treatment, implicating one of these kinases in the control of this response (Luo et al., 2001).

3.1 Removal of the last CTD repeat induces the degradation of Pol II LS to the IIb form

In order to study the role of the last repeat in transcription, various α-amanatin resistant mutants were produced, and expression vectors thereof stably introduced in to several cell lines. Expression of the Pol II LS mutants is controlled through a tetracycline-inducible system: in the presence of tetracycline, expression is inhibited (Tc-off). Following the establishment of stable cell lines, the effectiveness of this system to regulate expression was controlled. In the absence of Tc, expression of the Pol II LS mutants could be observed. All the mutants produced (with the exception of Pol II EGFP) exhibit a similar expression profile to that of the endogenous mammalian Pol II LS, which typically migrates with a weight of between approximately 214-240 kDa relative to the degree to which it is phosphorylated (Corden et al., 1985). However, an additional form of Pol II LS was seen to appear in mutants where the last repeat is absent, or severely disrupted. This form migrates faster than the other forms, IIa and IIo, and with a rate suggestive of a molecular weight of approximately 180 kDa. In Raji cells, a decrease in the amount of polymerase in the IIo form occurs concomitant with the appearance of this form, suggesting a conversion of one form to the other. These observations are characteristics of the IIb form of RNA polymerase II (Chambon et al., 1972).

The IIB form was originally described as one of three subspecies of RNA polymerase II: IIO, IIA and IIB (Kedinger and Chambon, 1972; Schwartz et al., 1974). These forms differ in their ability to be phosphorylated (Dahmus, 1981), and in the apparent molecular weight of their largest subunit, designated IIo (240 kDa), IIa (214 kDa) and IIb (180 kDa), for forms IIO, IIA and IIB, respectively (Dezelee et al., 1976; Roeder et al., 1976). The IIo and IIa forms are identical in sequence, but differ from each other by the degree to which they are phosphorylated. Pol IIo arises from the phosphorylation of multiple sites within the CTD of Pol IIa (Cadena and Dahmus, 1987; Kim and Dahmus, 1988), and the Pol IIb form results from the proteolytic cleavage of the CTD by an unknown protease (Allison et al., 1988; Corden et al., 1985).

Work by Dahmus and colleagues has extensively characterised these forms, and revealed the importance of the CTD in the regulation of transcription (reviewed in Dahmus, 1996). The discovery that the transition from initiation to elongation phases occurs concomitant with the conversion of Pol IIa to the IIo form (Payne et al., 1989) not only supported other groups' data identifying IIo as the transcriptionally active form (Bartholomew et al., 1986; Cadena and Dahmus, 1987), but also led to the dismissal of Pol IIb being a relevant form *in vivo*. With improved resources for the preparation of samples, notably the use of protease inhibitors, the Pol IIb form was rarely further seen to any extent in tissue extracts of proteins (Kim and Dahmus, 1986; Payne et al., 1989). After this point, no further research was performed on the IIb form, since it appeared to be an artefact found only *in vitro*. The *in vivo* proteolytic degradation of mutants lacking the last CTD repeat, to the IIb form, suggests this may now not be the case. An examination of earlier work may therefore assist our understanding of the properties of this form.

Initial comparisons of the abilities of the three, purified Pol II subspecies to transcribe the major late promoter of adenovirus-2 revealed that only the IIA and IIO forms are capable of selective transcription *in vitro*. However, all three forms were able to transcribe calf thymus DNA, which is known to contain nicks, and thus permits non-selective transcription (Dahmus and Kedinger, 1983). In these experiments, monoclonal antibody raised against the CTD was able to block selective transcription from several promoters, but had no effect on non-selective transcription. These findings are consistent with the requirement of the CTD for transcription initiation *in vivo*. Later, work by the same group using a polymerase-dependent transcription system, reconstituted from partially purified transcription factors, demonstrated that actually all three forms could transcribe the major late promoter of adenovirus-2 (Kim and Dahmus, 1989). However, this work suggests that the IIA and IIB forms are transcriptionally more active than IIO – a finding not supported by current research. One of the major problems of reconstituted systems is knowing what factors need to be present, and the physiological concentrations at which they are should be present; reactions may occur *in vitro*, which would otherwise not *in vivo* and *vice-versa*. In another study, a mutant of the Pol II large subunit was produced, possessing just five CTD repeats. This mutant is able to initiate selective transcription in vitro, and on a transiently-transfected template *in vivo*, but is unable to transcribe a chromatin template (Meininghaus et al., 2000;

Meininghaus and Eick, 1999). Comparison of the same truncated mutant with the Pol IIb form produced by ´last repeat´ mutants revealed that they are of approximately the same size. Based on these findings, it is unlikely that the Pol IIB form is active in *in vivo* transcription.

3.2 Removal of the last CTD repeat affects the viability and proliferation of the Raji Burkitt's lymphoma cell line

It is possible that the ability of α-amanatin to inhibit RNA Pol II elongation takes advantage of an as yet, undiscovered, endogenous mechanism for transcriptional regulation. Some studies of α-amanatin resistant RNA Pol II large subunits suggest that these polymerases may not behave exactly as the endogenous Pol II large subunit: Pol II large subunits with a reduced affinity for α-amanatin have been shown to confer developmental defects in *D. melanogaster* (Chen et al., 1993), induce pleiotropic effects during the differentiation of rat myoblasts (Crerar et al., 1983), and may demonstrate a reduced elongation rate compared to wild type polymerase (Coulter and Greenleaf, 1985). Such defects have not yet been described for the α-amanatin resistant Pol II LS used in this work, which has already been shown to successfully replace the function of the endogenous Pol II LS *in vivo* (Meininghaus et al., 2000).

Cell lines expressing Pol II LS* CTD mutants were tested for their ability to grow in the presence of α-amanatin. Expression of an α-amanatin resistant, wild type Pol II LS (LS**wt*MCS), or a mutant thereof containing the last repeat (LS*49+52), transferred α-amanatin resistance to the cell line. Cells continue to proliferate during the first nine days post-α-amanatin, before entering a 'crisis phase' of reduced proliferation lasting between 20 and 30 days. The inhibition of transcription through α-amanatin appears to induce the stabilisation of cellular mRNA by an as yet, unknown mechanism (Kuo et al., 1995; Meininghaus et al., 2000; Yamaizumi and Sugano, 1994). This may explain the continued exponential growth seen in the first few days post-α-amanatin. Cellular stress due to the over- or under-expression of the exogenous polymerase, or titration of transcription factors by the Tc-transactivator (TcTA) and Pol II LS* itself, may lead to sub-optimal gene expression. Although the life span of stabilised mRNAs is not known, it is tempting to speculate that the

crisis phase results from a shortage of essential mRNAs. During this period, where little cumulative growth is observed, the cells are effectively selected for those adapted to their new conditions. In contrast, a control cell line expressing no polymerase (LS*mock), or an α-amanatin resistant mutant possessing just 5 CTD repeats (LS*Δ5), exhibit no resistance to α-amanatin. Presumably, these cells undergo apoptosis, as is described for another transformed cell line (HELA) following α-amanatin treatment (Koumenis and Giaccia, 1997). Another control mutant tested contains a CTD truncated to 31 repeats (LS*Δ31) (Meininghaus et al., 2000). This mutant supports growth and viability for the first nine days, alike all the other mutants tested. However, this mutant fails to recover from the crisis phase encountered thereafter, indicating that this mutant can fulfil some, but not all the functions of the endogenous polymerase. Interestingly, a mutant lacking the last repeat (LS*49+50) exhibits a phenotype similar to LS*Δ31, whereas a mutant of equivalent repeat number (RajiLS*49+52) can sustain viability and proliferation. This is a surprising result, since a genetically modified mouse possessing a polymerase with just 39 repeats is viable (Litingtung et al., 1999). It should be noted, however, that the mutant used in that work, alike the majority of existing CTD deletion mutants, still contained the last CTD repeat. The repetitive nature of the CTD sequence creates problems for its genetic manipulation. Most mutants of differing CTD length characterised in detail to date were invariably produced by internal deletion, and thus retain the last repeat. No reproducible difference was seen between the CKII point-mutants produced, suggesting that the phosphorylation of the last repeat by CKII does not affect growth and proliferation. It is also interesting to note that a mutant containing a fusion of the CTD to the enhanced, green fluorescent protein (LS**wt*EGFP) behaves no differently than a *wild type* mutant, despite EGFP potentially blocking access of other factors to the last repeat, or interfering with the CTD itself.

3.3 Removal of the last CTD repeat only slightly affects the viability and proliferation of the Rosi LCL

Repetition of the growth and survival assay in a different cell line, Rosi – a lymphoblastoid cell line, did not produce the same result as in Raji – a Burkitt's lymphoma cell line. In the Rosi cell line, the LS*49+50 mutant is able to sustain

growth and viability in the presence of α-amanatin. This mutant exhibits a weaker recovery compared to other mutants, but still survives α-amanatin selection. What could explain this difference? Since the cell lines are polyclonal, and the expression vectors do not integrate, this difference is unlikely to be a result of an inferior clone, rather the cell lines instead.

A notable difference between mutants expressed in the Rosi LCL and the various BL cell lines was the relative proportions of the Pol IIb form to that of IIa and IIo. It was the general impression during this work that less IIb form was generated in the Rosi LCL than in the BL cell lines tested. An increased degradation of Pol II LS to its likely, inactive IIb form, may account for the overall global reduction in transcription seen, and loss of viability in BL cell lines.

Burkitt's lymphoma cells are very sensitive to sub-optimal growth conditions and undergo apoptosis induced by oxidative stress when seeded at low cell density (Brielmeier et al., 1998; Falk et al., 1993). LCLs on the other hand are comparatively robust (Bornkamm GW, personal communication). Both the Raji BL and Rosi LCL are infected with Epstein-Barr virus (EBV), which remains latent under normal tissue culture conditions. The Rosi LCL is dependent on the EBV programme for its immortality, whereas the Raji BL (Pulvertaft, 1964) contains a t(8;14) chromosomal translocation, placing the *c-myc* gene under the control of the immunoglobulin enhancer, leading to its uncontrolled expression (Spencer and Groudine, 1991).

In EBV-immortalised cells, only a limited number of EBV genes are expressed, including those required for episome maintenance (EBNA1), proliferation (EBNA2) and survival (LMP1). EBNA2 is a viral transactivator which activates gene expression, including that of *c-myc*, through its interaction with the transcription factors RBPjK and PU.1 (Reviewed in Bornkamm and Hammerschmidt, 2001). Paradoxically, c-Myc over expression has the effect of reducing the expression of these viral genes (Staege et al., 2002), perhaps explaining why BL cells are more susceptible to apoptosis. The ability of the LS*49+50 mutant to confer α-amanatin resistance might therefore depend on the sensitivity of a cell line to oxidative stress.

Interestingly, both c-Abl and c-Arg are activated following oxidative stress, and induce apoptosis (Cao et al., 2001; Sun et al., 2000). The activity of both nuclear and cytoplasmic c-Abl is inhibited through binding of the *pag* gene product, PAG (also known as macrophage 23-kD stress protein: MSP23), to its

regulatory SH3 domain. Oxidation of PAG may remove its inhibitory effect, leading to increased Abl activity. The *pag* gene is constitutively expressed in most human tissues, but its expression is increased in tissues with a high rate of proliferation, or in response to oxidative stress (Prosperi et al., 1998). It is possible that phosphorylation of RNA Pol II by c-Abl confers protection against oxidative stress.

3.4 Removal of the last CTD repeat does not affect the specific transcription of genes.

The removal of the Abl-Pol II interaction may be expected to affect the transcription of some genes. Indeed, c-Abl has been shown to influence transcriptional elongation at the HIV-1 promoter (Baskaran et al., 1999). However, analysis of active transcription by Pol II mutants containing or lacking the last CTD repeat revealed no specific alteration in the transcription of 1176 genes. The only notable difference seen was a general weaker signal for all class II genes transcribed by a mutant lacking this region. Since the last CTD repeat is target for kinases involved in the response to DNA damage it has been postulated that this repeat may be important for their signalling to the transcription machinery (Baskaran et al., 1999; Bregman et al., 2000; Oelgeschlager, 2002). The c-Abl kinase is activated following DNA damage by the ATM kinase (Baskaran et al., 1997b). ATM is activated by a specific type of DNA damage caused by chemical agents such as cisplatin, or ionising radiation (IR) that induce DNA double-strand breaks (Durocher and Jackson, 2001). Both the activation of c-Abl and stabilisation of p53 occur within one hour of such an insult. IR induced tyrosine phosphorylation of the RNA Pol II CTD is maximal at this time point (Baskaran et al., 1993).

Since both c-Abl and p53 could affect the activation of gene transcription, stable cell lines with intact ATM signalling were compared on a *wt* (Rosi) or mutant (Raji) p53 background. This enables the transcriptional effects of c-Abl to be distinguished from those of p53. In the presence of α-amanatin, CTD tyrosine phosphorylation was only seen in mutants containing the CTD last repeat, following irradiation. In Raji cells, no effect on active gene transcription was seen at this time point following IR exposure. This is an important finding, since it would suggest that contrary to speculation, activation of c-Abl alone

does not noticeably affect transcription elongation. Reproduction of the same experiment in Rosi cells revealed the induction of genes known to be induced by p53 following IR (Zhao et al., 2000). However, no difference in the induction of these genes could be seen between mutants containing or lacking the last CTD repeat. This suggests that tyrosine phosphorylation of the Pol II CTD by c-Abl neither directly or indirectly influences transcription elongation.

So far, only signalling to the Pol II CTD has been discussed, however, it is also possible that, as in the response to transcription blockage, Pol II itself induces the response to DNA damage (Ratner et al., 1998). It is thus possible that the last CTD repeat not only participates in signal transduction to Pol II, but also away from it. These data suggest that the last CTD repeat, and therefore its interacting partners c-Abl and CKII, are not important for the induction of the DNA damage response.

3.5 Mutation of the last CTD repeat CKII sites does not specifically affect the stability, or transcriptional ability of Pol II LS.

In the functional tests performed in this study, the Pol II LS*CTD mutants containing point mutations in either one (S9A; S13A), or two (S9/13A) of the potential CKII sites in the last repeat failed to behave differently from non-mutated controls. This suggests that these sites are not essential for the function of Pol II in the cell lines used. The Pol IIb form was induced by mutants lacking the last CTD repeat, but not by mutants containing point-mutated CKII sites, indicating that phosphorylation at these sites does not affect Pol II LS stability. However, these data were produced in tissue culture, and cannot discount that this phosphorylation serves an important function *in vivo.*

3.6 Only one CKII site within the last repeat is phosphorylated *in vivo*

Work performed in co-operation with the group of Bensaude failed to find a circumstance when CKII does not phosphorylate the last CTD repeat. Polyclonal antibodies produced for that study were raised against

phosphorylated (DEEP1), or non-phosphorylated (DEEN4) last CTD repeat. Analysis of CKII point mutants with these same antibodies revealed more specific information about the CKII phosphorylation of the last repeat. Only mutants containing Ser13 were recognised by the DEEP1 antibodies, and those lacking Ser13 were recognised by DEEN4, suggesting this to be the only site of CKII phosphorylation. Some *wt* mutants exhibited reactivity to both antibodies, but this may be an artefact of over expression, since the *wt,* endogenous Pol II LS reacts only with DEEP1. CKII phosphorylates serines and threonines immersed in acidic sequences within proteins and peptides. The minimum requirements for phosphorylation are depicted by the sequence S/T XX D/E, where X is any non-basic amino acid (Reviewed in (Allende and Allende, 1995)). In naturally occurring CKII substrates, the phosphorylated threonine or serine is surrounded by a cluster of acidic residues. This might explain why Ser13 is preferentially phosphorylated compared to Ser9.

3.7 The role of the last CTD repeat in the processing of mRNA and the response to DNA damage

The specific phosphorylation of the CTD regulates Pol II initiation and elongation *in vivo* through the orchestrated recruitment and displacement of positive and negative elongation factors (Majello and Napolitano, 2001). How these processes are regulated may partly be explained in that the CTD kinases discovered so far, exhibit preferences for the different amino acids present in the consensus repeat YSPTSPS. This suggests a mechanism by which the pattern of phosphorylation may be altered to create or destroy sites of protein-protein interaction. In addition, the presence of non-consensus repeats provides for further specificity, enabling factors to be recruited to specific regions of the CTD. Work by the group of Bentley suggests that this may indeed be the case, showing that consensus and non-consensus regions of the mammalian CTD possess different properties for the processing of pre-mRNA. The first 27 repeats of the CTD conform largely to the consensus sequence and support 5' capping, whereas the non-consensus repeats 27-52 support 5'-capping, splicing and 3' processing of RNA (Fong and Bentley, 2001).

The majority of CTD kinases target serine residues (for review see Kobor and Greenblatt, 2002). The change from phosphorylation at serine-5 of the CTD consensus repeat at promoter proximal regions, to serine-2 during elongation

and termination, has been shown to correlate with the change in composition, and activity of complexes present in these regions (Komarnitsky et al., 2000). The activity of the capping enzyme complex is dependent on serine-5 phosphorylation (Rodriguez et al., 2000; Schroeder et al., 2000), whereas the recruitment and activation of splicing factors requires serine-2 phosphorylation (Licatalosi et al., 2002). Based upon these observations, many laboratories have performed screens for proteins recognising different phosphorylated forms of CTD, the outcome of which being the identification of novel and known factors implicated in elongation, repression and pre-mRNA processing (reviewed in (Howe, 2002)). It is therefore possible that the phosphorylation of the last repeat by CKII, or the phosphorylation of CTD tyrosines by c-Abl and c-Arg regulates the recruitment to Pol II, of factors involved in pre-mRNA processing, or the DNA damage response.

Tyrosine-1 of the CTD is the only residue to be completely conserved between all repeats, and has been shown to be essential for viability in yeast (West and Corden, 1995). The phosphorylation of this residue may therefore be expected to affect some function of the CTD, and has been shown to overcome the requirement of Tat for the activation of the HIV promoter (Baskaran et al., 1999). However, in the experiments performed during the course of this work, no effect on the transcription of any specific genes has been seen using mutants no longer able to interact with the only currently described CTD tyrosine kinases. A role for tyrosine-1 phosphorylation in the regulation of pre-mRNA processing can not be ruled out: by recruiting specific splicing and 3'processing factors to the CTD, the preferential production of certain splice variants could be achieved in response to cellular stress. Alternatively, tyrosine-1 phosphorylation may control the presence of factors that target the degradation of the polymerase. We have shown here that mutants no longer able to interact with the only known CTD tyrosine kinases appear sensitive to degradation to the Pol IIB form. Elongation blocks caused by DNA lesions have been shown to target the destruction of RNA Pol II through the recruitment of the Rsp5 ubiquitin ligase to the CTD (Beaudenon et al., 1999; Huibregtse et al., 1997). Tyrosine phosphorylation of the CTD may thus play a role in regulating the stability of the Pol II large subunit, perhaps through the negative regulation of factors that target its digestion. However, this theory is not convincing, given that inhibitors of c-Abl and c-Arg tyrosine kinases fail to induce the Pol IIb form.

3.8 Outlook

The orchestration of transcriptional-, and co-transcriptional processes through phosphorylation of the Pol II CTD was undoubtedly an evolutionary development of major significance in higher eucaryotes. It can therefore be assumed, that pathways resulting in the modification of this domain have some influence on these processes. During this work I tried to identify genes that depend on signalling through the last repeat of Pol II LS for their regulation (activation of transcription). Despite much literature suggestive of the opposite, this does not appear to be the case. However, this does not exclude the possibility that signalling through the last repeat may regulate gene transcription in another way, for example, through pre-mRNA processing. Some preliminary tests were performed during the course of this work, but to little avail. Since the potential effects could be gene specific, the problem arises of knowing which genes are affected: using the chromatin IP (ChIP) technique in combination with a DNA array composed of many different promoter regions, it may be possible to identify promoters to which the c-Abl and CKII kinases are recruited.

The major finding of this work was the discovery that *in vivo* expression of a mutant lacking the last CTD repeat, resulted in the appearance of the Pol IIb form. This is the first time the Pol IIb form has been identified *in vivo*, and suggests that the last CTD repeat may regulate its formation. Since the Pol IIb form is probably inactive, degradation to this form may provide a novel mechanism for transcriptional control. Initial experiments to identify the protease responsible, or agents that induce its activation have proved unsuccessful. Further experiments are required to identify this protease, and other interacting partners of the last CTD repeat.

4. Materials

4.1 Suppliers

4.1.1 Chemical reagents

α-amanatin	*Roche Diagnostics, GmbH, Mannheim*
Acrylamide	*Carl Roth GmbH & Co., Karlsruhe*
Agarose	*Invitrogen GmbH, Karlsruhe*
Bacto-agar	*Difco Laboratories, Michigan, USA*
Bacto-yeast extract	"
Bacto-tryptone	"
DNA ladders	*Promega Corp., Wisconsin, USA*
dNTPs	"
Foetal calf serum	*Biochrom KG, Berlin*
L-glutamine	*Invitrogen GmbH, Karlsruhe*
Neomycin / G418	*Promega Corp., Wisconsin, USA*
NTPS	*Roche Diagnostics, GmbH, Mannheim*
Penicillin-streptomycin	*Invitrogen GmbH, Karlsruhe*
Protein molecular weight markers	*Bio-Rad laboratoriesGmbH, München*
RMPI-1640 medium	*Invitrogen GmbH, Karlsruhe*
Sephadex-G50, DNA grade	*Amersham Pharmacia Biotech, Freiburg*

4.1.2 Radioactive Isotopes

Radioactively labelled reagents were used before the expiration of their first half-life.

[α-^{32}P] dCTP (3000Ci / mmol, 10mCi / ml)	*Amersham Pharmacia Biotech, Freiburg*
[α-^{32}P] dCTP (800Ci / mmol, 10mCi / ml)	"

4.1.3 Enzymes

Alkaline phosphatase	*Promega Corp., Wisconsin, USA*
DNAse I, RNAse-free (10U / µl)	*Roche Diagnostics, GmbH, Mannheim*
Klenow fragment (1U / µl)	*Promega Corp., Wisconsin, USA*

Proteinase K (20U / µl)	*Promega Corp., Wisconsin, USA*
Pfu DNA polymerase	"
Restriction endonucleases	*Promega Corp., Wisconsin, USA*
RNAse A (50U / µl)	*Roche Diagnostics, GmbH, Mannheim*
RED-Taq DNA polymerase	*Sigma-Aldrich GmbH, Deisenhofen*
T4-polynucleotide kinase (10U / µl)	*Promega Corp., Wisconsin, USA*
T4-DNA ligase (400U / µl)	"

4.1.4 Antibodies

Primary antibodies:

3F10:
A rat monoclonal antibody raised against an epitope contained in the haemagglutinin polypeptide of the human influenza virus (*Roche Diagnostics, GmbH, Mannheim)*

DEEN4:
A rabbit polyclonal antibody raised against the peptide TSPAISPDDSDEEN. (*A gift from Olivier Bensaude, Paris, France)*

DEEP1:
A rabbit polyclonal antibody raised against the peptide T**S**$^{-P}$PAI**S**$^{-P}$PDD**S**$^{-P}$DEEN. (*A gift from Olivier Bensaude, Paris, France)*

Pol 3 / 3:
A mouse monoclonal antibody that recognises an evolutionary-conserved epitope in the Pol II large subunit (non-CTD region). *Originally produced by E.K. Bautz, University of Heidelberg. Received as a gift from Olivier Bensaude, Paris, France*

PY-100:
A monoclonal antibody (IgG1) derived from mice immunised with a synthetic phospho-tyrosine peptide. *NEB/Cell Signalling Technology, Frankfurt am Main*

2C8:
A mouse monoclonal antibody (IgG1) raised against a synthetic phosphotyrosine peptide. *Nanotools, Teningen*

Secondary antibodies:

Goat anti-rat HRP conjugate:	*Santa Cruz Biotech, California, USA*
Anti-mouse HRP conjugate	*Promega Corp., Wisconsin, USA*

4.1.5 Disposables and Kits

3MM-paper	*Whatman Ltd., Kentucky, USA*
Agar plates	*Greiner GmbH, Frickenhausen*
Atlas Human 1.2 cDNA Arrays	*CLONTECH,*
Cell culture plasticware	*Greiner GmbH, Frickenhausen*
General laboratoryware	*GLW GmbH, Würzburg*
DNA Midi / Maxi kits	*Qiagen GmbH, Hilden*
ECL™ kit	*AmershamPharmacia Biotech, Freiburg*
Electroporation cuvettes, 2mm, 4mm	*Peqlab, Erlangen*
Hybond N+ nylon membrane	*AmershamPharmacia Biotech, Freiburg*
Immobilion P, PVDF membrane	*Millipore GmbH, Eschborn*
Oligonucleotides	*Metabion GnbH, München*
Parafilm	*Dynatech, Denkendorf*
Polypropylene tubes 15 ml, 50 ml	*Beckton-Dickinson GmbH, Heidelberg*
Reaction viles 1.5 ml, 2 ml	*Eppendorf-Netheler-Hinz, Hamburg*
Rediprime DNA labelling kit	*AmershamPharmacia Biotech, Freiburg*
Sterile filters 0.22 µm, 0.45 µm	*Millipore GmbH, Eschborn*
Tri-fast RNA extraction kit	*Peqlab, Erlangen*
X-ray film (XAR5, *BIOMAX-MS*)	*Eastman Kodak Co, New York, USA*

4.1.6 Laboratory equipment

-80°C freezer	*Colora Messtechnik GmbH, Lorch*
Oven	*Heraeus Sepatech GmbH, Osterode*

Bacteria incubator	*Heraeus Sepatech GmbH, Osterode*
Bacteria shaker (Series 25)	*New Brunswick ScientificCo., NJ, USA*
Centrifuge, *Sepatech Varifuge 3.2 RS*	*Hereaus Sepatech GmbH, Osterode*
Centrifuge, *Sigma 2K15*	*Sigma Centrifuges GmbH, Taufkirchen*
Centrifuge, *Sorval RC5C and rotors GS3, HS4, and SS34*	*DuPont GmbH, Bad Homburg*
DNA thermal cycler	*Therma-Hybaid GmbH*
Electroporator (eucaryotic cells)	*Bio-Rad laboratories GmbH, München*
Electroporator (bacteria)	*Peqlab, Erlangen*
Flow hood work bench	*Bio-Flow technik, Meckenheim*
Fuchs-Rosenthal cell chamber	*GLW GmbH, Würzburg*
Gammacell 40	*Atomic Energy of Canada L.t.d.*
Gel electrophoresis units (nucleic acids)	*Bio-Rad laboratories GmbH, München*
Gel electrophoresis (protein), TE22 *'Mighty Small'* series	*AmershamPharmacia Biotech, Freiburg*
Hybridisations oven, MKII	*MWG Biotech GmbH, Ebersberg*
Light microscope, *Axiovert* 135	*Carl Zeiss Jena GmbH, Göttingen*
Mammalian cell incubator, *Hereaus-6000*	*Hereaus Sepatech GmbH, Osterode*
pH-meter, *Multi-Calimatic 763*	*Knick*
Radioisotope counter QC-4000	*Bioscan Inc., Washington D.C., USA*
Speed-vac, concentrator SVC100	*Savant Instruments Inc., NY, USA*
Spectrophotometer DU-64	*Beckman Instruments GmbH, München*
Thermomixer 5436	*Eppendorf-Netheler-Hinz, Hamburg*
Transformer (power pac 300)	*Bio-Rad Laboratories GmbH, München*
UV transilluminator TL-33	*UVP Inc., Calafornia, USA*
Vortexer, *Vortex Genie 2*	*Bender & Hobein GmbH, Ismaning*
Water purification system	*Millipore GmbH, Eschborn*

4.2 Materials for cloning

4.2.1 Oligonucleotides

All sequences are listed with the standard 5'-3' orientation. All oligonucleotides were manufactured by *Metabion GmbH, Martinsried*

Primers for MM128-MCS:

wt‡
5'CTCCTGCTGACGCACCTGTTCT 3'

d31**fl**
5'GTCCCCAAACTCACCCTGAA 3'

RDC17-2
5'GACCGGTTTAAACGCGGCCGCTGAGCGAACAGGGCGAAGAGCTGG3'

RDC17-3
5'CGCCCTGTTCGCTCAGCGGCCGCGTTTAAACCGGTCGTTCTCCTC3'

Primers for RDC135*wt* (LSwt*EGFP):**

EGFP Fwd.:
5'AACGACCGGTTTGTGAGCAAGGGCGAGGAGCTGTTCACC 3'

EGFP Rev.:
5'AAAAGGAAAGCGGCCGCGTCACTTGTACAGCTCGTCCATGCCGAG3'

RX2 primers:

RX2 49+50 Fwd.:
5'CAAGGGCTCCACCTACTCTCCCACTTCTCCTGGCTGAGA3'

RX2 49+50 Rev.:
5'CCGGTCTCAGCCAGGAGAAGTGGGAGAGTAGGTGGAGCC3'

RX2 49+52 Fwd.:
5'CAAGGGCTCCACCTACAGCCTCACCAGCCCAGCCATCAGCCCAGATGAC
AGCGATGAGGAGAACTGAGA3'

RX2 49+52 Rev.:
5'CCGGTCTCAGTTCTCCTCATCGCTGTCATCTGGGCTGATGGCTGGGCTGG
TGAGGCTGTAGGTGGAGCC3'

RX2 49+NS52 Fwd.:
5’CAAGGGCTCCACCCCCGACAACCCCGACTCCTCCACCGACACCTCCGAG
TCCACATACGAGCTGTGAG3’

RX2 49+NS52 Rev.:
5’CCGGCTCACCGCTCGTATGTGGACCCGGAGGTGTCGGTGGAGGAGTCGG
GGTTGTCGGGGGTGGAGC3’

RX2 49+52 S9A Fwd.:
5’CAAGGGCTCCACCTAGCAGCCTCACCAGCCCAGCCATCGCCCCAGATGA
CAGCAGTGAGGAGAACTGAGC3’

RX2 49+52 S9A Rev.:
5’CCGGGCTCAGTTCTCCTCATCGTCATCTGGGGCGATGGCTGGGCTGGTGA
GGCTGTAGGTGGAGCC3’

RX2 49+52 S13A Fwd.:
5’CAAGGGCTCCACCTACAGCCTCACCAGCCCAGCCATCAGCCCAGATGAC
GCCGATGAGGAGAAACTGAGC3’

RX2 49+52 S13A Rev.:
5’CCGGGCTCAGTTCTCCTCATCGGCGTCATCTGGGTCAGTGGCTGGGCTGG
TGACGCTGTAGGTGGAGCC3’

RX2 49+52 S9/13A Fwd.:
5’CAAGGGCTCCACCTACAGCCTCACCAGCCCAGCCATCGCCCCAGATGAC
GCCGATGAGGAGAACTGAGC3’

RX2 49+52 S9/13A Rev.:
5’CCGGGCTCAGTTCTCCT6CATCGGCGTCATCTGGGGCGATGGCTGGGCTG
GTGAGGCTGTAGGTGGAGCC3’

RX2 49M+52 Fwd.:
5’CAAGTACAGCCTCACCAGCCCAGCCATCAGCCCCAGATGACAGCGATGA
GGAGAACTGAGA3’

RX2 49M+52 Rev.:
5’CCGGTCTCAGTTCTCCTCATCGCTGTCATCTGGGCTGATGGCTGGGCTGG
TGAGGCTGTA3’

RX2 49+50 ATM Fwd.:
5’CAAGGGCTCCACCTACTCTCCCACTTCTCCTGGCGACCCAGCCCCAAACC
CACCACACTTCCCAACGAG3’

RX2 49+50 ATM Rev.:
5’CCGGCTCATGGGGAAGTGTGGTGGGTTTGGGGCTGGGTCGCCAGGAGAA
GTAGGAGAGTAGGTGGAGCC3’

4.2.2 Existing plasmids used during this work

pUC19:
A small, high copy number *E. coli* plasmid containing portions of pBR322, M13mp19 and a 54 bp MCS (Yanisch-Perron et al., 1985). GenBank Accession #: X02514

pEGFP-C1 (CLONTECH):
An expression vector containing the modified (enhanced) cDNA sequence encoding the green fluorescent protein from the jellyfish *Aequoria victoria*. GenBank Accession #: U55763

MM126-10 (LS*MOCK):
A tetracycline-regulated expression vector (Tc-off), containing the resistance genes for neomycin / G418 and ampicillin. This vector contains the EBV origin of replication, and is maintained episomally in EBNA1-positive cell lines (Meininghaus et al., 2000).

MM172-2 / 1:
A vector based on MM126-10, that contains the genomic sequence for the N-terminal part of the α-amanatin resistant, Pol II LS gene (A gift from Mark Meininghaus, GSF, München).

MM128:
A vector based on MM126-10, that contains the genomic sequence of the C-terminal part of the α-amanatin resistant, Pol II LS gene (A gift from Mark Meininghaus, GSF, München).

4.2.3 Plasmids produced for this work

MM128-MCS:
A vector based on MM128, that is modified to include a multiple cloning site (*Age* I, *Pme* I, *Not* I) before the STOP codon of the genomic sequence for the C-terminal part of the α-amanatin resistant, Pol II LS gene. The primer pairs *wt*> & RDC17-2, and RDC17-3 & d31**fl** were used to amplify two fragments from the plasmid MM128. An overlap PCR protocol using the products from each of

these reactions and primers *wt*‡ and d31, was used to produce a fusion product of them both. The primers 17-2 and 17-3 contain overlap sequences (encoding the MCS) to enable this. The overlap PCR product was digested with the enzymes *Ngo* MIV, and *Cla* I, and ligated into the ??bp vector backbone of MM128 following its digestion with the same enzymes.

pUC19-CTD:
A sub-cloning vector containing the coding sequence of the last exon of the α-amanatin resistant, Pol II LS gene. The primers *wt*‡ and d31 were used to amplify a 1600 bp fragment from vector MM128-MCS, which was then ligated blunt-ended, to pUC19 linearised with digestion with *Hinc*II.

RDC100*wt* (LS***wt*MCS):
A tetracycline-regulated expression vector containing the full length, α-amanatin resistant, Pol II LS gene, with a multiple cloning site inserted before its STOP codon. The ~ 14.5 kbp *Sfi* I - *Apa* LI digest product of the vector MM128-MCS was ligated to the ~ 12 kbp *Sfi* I - *Sex* AI digest product of the vector MM172-2 / 1.

RDC135*wt* (LS***wt*EGFP):
A tetracycline-regulated expression vector containing the full length, α-amanatin resistant, Pol II LS gene, with the coding sequence of EGFP inserted in frame, before its STOP codon. Primers EGFP *Fwd.*, and EGFP *Rev*. were used to amplify a 715 bp fragment from the plasmid pEGFP-C1. The *Not* I – *Age* I - digested PCR product was ligated to the ~ 26 kbp product of vector RDC100*wt* following digestion with the same enzymes.

RX2 vectors (CTD49+: 50; 52, NS; 52 S9A; 52 S13A; 52 S9 / 13A; 50 ATM):
Vectors containing last exon mutants of the α-amanatin resistant, Pol II LS gene. Mutation of the last CTD repeat was achieved by ligation of the 4274 bp pUC19-CTD *Sty* I - *Age* I digestion product to synthesized, complementary oligonucleotides incorporating the modified CTD sequences, and corresponding overhangs for *Sty* I and *Age* I (see RX2 primers). The primer sequence was designed to destroy the *Age* I site following annealing.

RX3 vectors (CTD49+: 50; 52, NS; 52 S9A; 52 S13A; 52 S9/13A; 50 ATM):
A vector based on MM128-MCS, that contains mutations in the genomic sequence for the last CTD repeat, encoded within the C-terminal part of the mouse, α-amanatin resistant, Pol II LS gene. The ~ 10 kbp product of MM128-MCS digestion with *Ngo* MIV and *Cla* I, was ligated to the ~1600 bp product of RX2 digestion with Xmn I, *Ngo* MIV and *Cla* I.

RX4 vectors (CTD49+: 50; 52, NS; 52 S9A; 52 S13A; 52 S9/13A; 50 ATM):
A tetracycline-regulated expression vector containing last CTD repeat mutants of the mouse, full-length, α-amanatin resistant, Pol II LS gene. The ~ 13 kbp product of RX3 digestion with *Nhe* I, *Not* I and *Eco105* I was ligated to the ~ 13 kbp product of RC100*wt* with *Not* I, *Nhe* I and *Sex* AI.

4.2.4 Bacteria

DH5α: *E. coli* strain purchased from *Invitrogen GmbH, Karlsruhe.* Used for the routine cloning of plasmid DNA smaller than 5 kbp.

DH10B: *E. coli* strain purchased from *Invitrogen GmbH, Karlsruhe.* Used for the cloning of plasmid DNA larger than 5 kbp

4.2.5 Probes for Northern-blot anaylsis

For the *hsp70A*-gene:
A 2.4 kb *Eco*RI-fragment from pUCHsp70A (*Stressgene*)

4.3 Eucaryotic cell lines

4.3.1 Basic cell lines

Raji:
A human, EBV-positive Burkitt's lymphoma cell line (endemic Burkitt's lymphoma) containing a t(8;14) translocation (Pulvertaft, 1964).

BL29:

A human, EBV-positive Burkitt's lymphoma cell line

Elijah:

A human, EBV-positive Burkitt's lymphoma cell line

Rosi:

A human lymphoblastic cell line

L187:

A human lymphoblastic cell line

4.3.2 Stably transfected cell lines

Cell line	Name	Plasmid	Parent cell line	Resistance
MM233-2	Raji1LS*mock	MM126-10	Raji	*neo*
XR4-100	RosiLS*mock	"	Rosi	"
MM233-4	Raji1LS*Δ31	MM217b	Raji	"
MM233-6	Raji1LS*Δ5	MM203B2	Raji	"
RC100*wt*	RajiLS**wt*MCS	RDC100*wt*	Raji	"
XR4*wt*MCS	RosiLS**wt*MCS	"	Rosi	"
RC135wt	RajiLS**wt*EGFP	RDC135*wt*	Raji	"
XR4-101	RajiLS*49+50	RX4-50	Raji	"
XR4-201	RosiLS*49+50	"	Rosi	"
XR4-301	ElijahLS*49+50	"	Elijah	"
XR4-401	BL29LS*49+50	"	BL29	"
XR4-102	RajiLS*49+52	RX4-52	Raji	"
XR4-202	RosiLS*49+52	"	Rosi	"
XR4-302	ElijahLS*49+52	"	Elijah	"
XR4-402	BL29LS*49+52	"	BL29	"
XR4-103	RajiLS*49+NS52	RX4-NS	Raji	"
XR4-203	RosiLS*49+NS52	"	Rosi	"
XR4-203	RosiLS*49+NS52	"	Elijah	"
XR4-203	RosiLS*49+NS52	"	BL29	"
XR4-104	RajiLS*49+S9A	RX4-S9A	Raji	"
XR4-204	RosiLS*49+S9A	"	Rosi	"
XR4-105	RajiLS*49+S13A	RX4-S13A	Raji	"
XR4-205	RosiLS*49+S13A	"	Rosi	"
XR4-106	RajiLS*49+S9/13A	RX4-S9/13A	Raji	"
XR4-206	RosiLS*49+S9/13A	"	Rosi	"
XR4-107	RajiLS*49+50ATM	RX4-ATM	Raji	"
XR4-207	RosiLS*49+50ATM	"	Rosi	"
XR4-108	RajiLS*49M+52	RX4-49M	Raji	"
XR4-208	RosiLS*49M+52	"	Rosi	"

5. Methods

The standard protein and molecular biology techniques used during this work are described in detail in the following text: (Sambrook et al., 1989)

5.1 Bacterial cell culture

5.1.1 The maintenance and preparation of bacterial plasmids

Bacteria were cultured either on LB-agar plates in a bacterial incubator, or in liquid LB medium in a thermoshuttler at 37 °C. overnight. Liquid cultures were produced through infection of 200-400 ml LB medium with a single bacterial colony picked from an agar plate. Transformed bacteria were selected for the antibiotic resistance of the transformed plasmid through the addition of antibiotic (kanamycin or Ampicillin) to growth media: liquid culture medium, 100 µg / ml; agar plates, 50µg / ml.

LB-medium: 20 mM $MgSO_4$; 10 mM KCL; 1 % (w / v) Bacto-Tryptone; 0.5 % (w / v) Bacto-yeast extract; 0.5 % (w / v) NaCl

LB-agar: 20 mM $MgSO_4$; 10 mM KCl; 1 % (w / v) Bacto-Tryptone; 0.5 % (w / v) Bacto-yeast extract; 0.5 % (w / v) NaCl; 1.2 % (w / v) Bacto-agar

5.1.2 Preparation of competent bacteria

To increase the efficiency of plasmid DNA uptake (transformation), bacteria were treated with solutions of di-valent cations. An LB plate was first inoculated with a probe from a bacterial stock and grown overnight at 37 °C. A single colony was then used to inoculate 2.5 ml of LB medium, which was then incubated overnight in a loose-capped vessel, with shaking. The following day, the entire overnight culture was used to inoculate 250 ml of LB medium containing 20 mM $MgSO_4$. Bacteria were grown in a 1 dm^3 flask, with shaking, at 37 °C until the absorbance at 600 nm (A_{600}) reached 0.4-0.6 (approx. 5-6 h). Bacteria were pelleted at 4,5000 x *g*, 5 ' at 4°C. Medium was discarded and the bacteria re-suspended in 0.4 volume (of the original culture volume) of ice-cold TFB1. Bacteria were incubated a further 5 'on ice before centrifugation (as above), and re-suspension in 1/25 of the original culture volume of ice-cold

TFB2 and incubated a further 15-60 ‘ on ice. Aliquots of 200 µl bacteria were snap-frozen in liquid nitrogen and removed to storage at –80 °C.
NOTE: All vessels and pipettes must be pre-chilled: for best results work in a 4°C lab.

TFB1: 30 mM Potassium acetate; 10 mM $CaCl_2$; 50 mM $MnCl_2$; 100 mM RbCl; 15 % glycerol. Adjust to pH 5.8 with 1 M acetic acid; sterile filter (0.2 µm).

TFB2: 10 mM MOPS pH 6.5; 75 mM $CaCl_2$; 10 mM RbCl; 15 % glycerol. Adjust to pH 6.5 with 1 M KOH; sterile filter (0.2 µm).

5.1.3 Transformation of bacteria

For standard sub-cloning and production of large amounts of cloned DNA, the recombination-deficient *Escherichia Coli* strain, DH10B was used. For transformation, 1 ng to 0.1 µg of plasmid DNA or 10 µl of ligation mixture was added to 100 µl of competent cell suspension and incubated on ice for 30 ‘. Cells were then subjected to heat shock at 42 ^{o}C for 30 ‘’ before returning to ice for 2 ‘. 400 µl (4 volumes) of LB recovery medium was added to cells and incubated at 37 ^{o}C for 1 h for cells to express resistance genes conferred by the plasmid. For routine transformations, 100 µl of suspension was plated onto LB-agar plates containing ampicillin or kanamycin and grown at 37 ^{o}C for 16-18 h. Following transformation of ligation mixtures, bacteria were pelleted by pulsing in a microfuge at 10,000 x *g* before resuspending in 100 µl of LB media and plating onto LB-agar plates.

5.1.3 Miniprep of plasmid DNA

Single colonies obtained from transformation of bacteria following ligation reactions were used to inoculate 5 ml LB, and grown overnight. Using the alkaline lysis method (Birnboim, 1983), plasmid DNA was isolated from a bacteria culture. This method relies on the fact that high molecular weight linear chromosomal DNA will be denatured when cells are lysed at pH 12.0-12.6, whereas low molecular weight supercoiled plasmid DNA remains unaffected. Neutralisation of pH in the presence of high salt concentrations subsequently precipitates chromosomal DNA, which can then be separated from the mix. The following protocol was routinely used: 5 ml of LB media

containing appropriate antibiotic was inoculated with a single colony of transformed bacteria in a 20 ml loose-capped tube. The culture was incubated overnight at 37 ^{o}C with vigorous shaking. 1.5ml of culture was then decanted into a microfuge tube and centrifuged at maximum speed for 30 s in a microfuge. Medium was removed from the pellet by aspiration with a disposable pipette tip, leaving the cell pellet as dry as possible. The cell pellet was re-suspended in 90 µl of alkaline lysis solution I by vigorous vortexing, ensuring complete dispersal of bacterial cells. 180 µl of freshly prepared alkaline lysis solution II was added, and mixed gently by inverting 5 times before incubation at room temperature for 5 '. To precipitate, 135µl of alkaline lysis solution III was added, and then mixed by inverting, before centrifugation at maximum speed for 5 '. The resulting supernatant was transferred to a fresh microfuge tube and mixed with 1 ml of ethanol to precipitate plasmid DNA, and centrifuged at 10,000 x g for 5 '. Supernatant was thoroughly removed using a drawn out Pasteur pipette and pellets re-suspended in 100 µl of TE buffer. For screening of these crude preparations with restriction enzymes, master mixes of enzymes and appropriate restriction enzyme buffers (Promega) containing RNase A (pancreatic RNase, Sigma) were prepared, such that 10 µl of mix could be added to 10µl of miniprep to give 0.5-3 units of enzyme (depending on efficiency), 1x restriction enzyme buffer and 20 µg / ml RNase A. Digests were incubated at appropriate temperatures for 2 h before separation on agarose gels.

Solution I: 50 mM Tris-Cl; pH 8; 10 mM EDTA
Solution II: 0.2 M NaOH; 1 % SDS
Solution III: 3 M potassium acetate; 2 M glacial acetic acid

5.1.4 Maxi-prep of plasmid DNA

Large quantities of plasmids were purified using Qiagen Maxi-prep protocols based on a modified alkaline lysis procedure. Plasmid DNA is recovered by running the bacterial lysate through an anion exchange column under appropriate low-salt and pH conditions. Following washing, the DNA can be eluted by a high-salt buffer.

A single colony was picked from a freshly streaked selection plate and used to inoculate a starter culture of 2-8 ml of selective LB medium and

incubated for overnight at 37 °C on an orbital shaker. The starter culture was then diluted 1:500 into 100-300ml of selective LB medium and grown overnight at 37 °C on an orbital shaker. Cells were harvested by centrifugation at 6000 x g for 15 ' at 4 °C. Supernatant was discarded and pellets were re-suspended in 10 ml of chilled alkaline lysis solution I containing 100 µg / ml RNAase A. 10 ml of alkaline lysis solution II was added to the suspension and mixed gently by inverting. Cells were lysed for 5 ' at room temperature before stopping the reaction by addition of 10 ml alkaline lysis solution III and mixed again by inverting. Mixtures were incubated on ice for 20 ' to aid precipitation of cell debris, genomic DNA and SDS. The sample was then centrifuged at 20,000 x *g* for 1 h, 4 °C. The supernatant was then passed through a filter paper to remove any residual precipitate and applied to a Qiagen column equilibrated with 10 ml of equilibration buffer and washed twice with 30 ml wash buffer. The bound plasmid was eluted in 15 ml elution buffer and precipitated by adding 10.5 ml (0.7 volumes) of room temperature isopropanol, followed by centrifugation at 20,000 x *g* for 30 ' at 4 °C. Pellets were re-suspended in 400 µl TE and transferred to a microfuge tube.

Equilibration buffer: 50 mM MOPS, pH 7; 750 mM NaCl; 15 % ethanol
Wash buffer: 50 mM MOPS, pH 7; 1M NaCl; 15 % ethanol
Elution buffer: 50 mM Tris-Cl, pH 8.5; 1.25 M NaCl; 15 % ethanol

5.2 Eucaryotic cell culture

5.2.1 Cultivation of Burkitt's lymphoma and lymphoblastoid cell lines

Both cell types grow in suspension culture at 37 °C in a 5 % CO_2, water saturated atmosphere. Optimal growth is achieved at a density of 3 x 10^5 cells / ml, which may be approximately maintained by splitting cells 1:3 with fresh medium every 3 days.

Cell culture medium: RPMI 1640-mediium; 10 % FCS; 2 mM L-glutamine; 100 U / ml penicillin; 100 mg / ml streptomycin; 1 mM sodium pyruvate; 0.2 nM Na_2SeO_3

For some experiments described, the medium was supplemented with:

α-Amanatin: Stock: 1 mg α-amanatin / ml in H_2O. Working concentration 2 µg / ml

5.2.2 Assessment of cell number and viability

The number of living and dead cells in a given volume was calculated using a Fuchs-Rosenthal haemocytometer. 100 µl of cell suspension was mixed with an equal volume of 0.5 % (w / v in PBS) trypan blue solution. In contrast to the porous membrane of dead cells, the membrane integrity of living cells prevents the entry of pigment, thereby allowing the number of clear (living) and blue (dead) cells to be ascertained. Percentage viability was calculated using the formula: 100 x a / (a+b), where a= alive cell #, and b= dead cell #.

5.2.3 Storage of cell lines

Stocks of cell lines can be stored for several years in liquid nitrogen. Cells were split 1:1 with fresh medium one day before storage. Cells were pelleted (302 x *g*, 5 ‘), and medium discarded, before resuspension in storage medium (1 x 10^7 cells / ml) and transfer of 1 ml aliquots to 1.5 ml cryotubes. Stocks were first stored overnight at –80°C before transfer to storage facilities in liquid nitrogen.

To revive stocks from storage, cryotubes were thawed at room temperature for 3 ‘ before transfer to 30 ml RPMI 1640 medium. Cells and medium was mixed through inversion several times, before re-centrifugation (302 x *g*, 5 ‘), and re-suspension in 10 ml fresh culture medium containing 20 % FCS. For an improved outcome with some cell lines, a slight tilt of the culture flask allows living cells to collect together, thereby increasing cell-cell contact.

Storage medium: 10 % RPMI 1640; 10 % DMSO; 80 % FCS

5.2.4 Stable transfection of B-cells

For the production of stable cell lines, plasmid vectors were introduced into cell lines, and a selection performed utilising an antibiotic resistance gene expressed by the introduced vector. For best results, cells should be split 1:1 one day prior to transfection. 2 x 10^7 cells are required per transfection. Cells were collected by centrifugation (302 x *g*, 5 ‘), and re-suspended at a density of 2 x 10^7 cells / 250 µl in RPMI 1640 medium. Cells were transferred to a 4 mm

electroporation cuvette on ice, and mixed with 10 µg plasmid DNA. Electroporation was performed using a voltage of 250 V and capacitance charge of 960 µF. Immediately following electroporation, 1 ml FCS was added to the cuvette, before transfer of the cell / FCS mixture to 9 ml fresh culture medium in a flask. Tetracycline (0.1 µg / ml) must also be applied if the vectors transfected contain the tetracycline-regulated, Tc-off system. Two days following transfection, the appropriate selection reagent for the vectors used was applied to the cell culture medium. Selection typically requires 2 weeks to recover a viability of 95 %. This technique produces a polyclonal cell line. Monoclonal cell lines can be produced by isolation, and cultivation of single cells if desired.

Neomycin (G418): Stock: 100 mg / ml in PBS; Working concentration 1 mg / ml

Tetracycline: Stock: 100 µg / ml in H_2O (5 mg Tc must be dissolved first in 0.5 ml EtOH before addition of 50 ml H_20); Working concentration 0.1 µg / ml

5.2.5 Induction of Tc-regulated expression

Expression of a Tc-regulated gene is controlled by the presence or absence of Tc. The vectors used in this work contain the Tc-off system: gene expression is silenced in the presence of Tc. To induce gene expression, Tc was washed out of the cells by repeated centrifugation and re-suspension in wash medium (3 x in 50 ml / 2 x 10^7 cells). Cells were then transferred to 10 ml of culture medium (without selection) for further growth. Expression may be detected 4h following induction, but optimally at +18 h.

Wash medium: PBS; 1 % FCS

5.3 Molecular biology techniques

5.3.1 Digestion of DNA using restriction endonucleases

Restriction enzymes were used as described by the manufacturer. For the analysis of plasmid 'mini-prep' DNA, multiple digests were performed in a compatible buffer. For cloning purposes, DNA was precipitated between individual digests to control, and reduce the detrimental effects of over digestion.

5.3.2 Dephosphorylation of 5' ends

To prevent the re-ligation of linearised vector containing compatible ends, 5' dephosphorylation was performed. Following digestion, DNA fragments were incubated with 1 U calf intestinal alkaline phosphatase (CIP) for 1 h at 37°C., before separation on an agarose gel.

5.3.3 Filling in of DNA overhangs

To enable the ligation of non-compatible ends, overhanging (sticky) ends may be filled in to produce universally-compatible (blunt) ends. Linearised DNA was treated with Klenow DNA polymerase for 1 h at 37 °C, in 1 x Klenow buffer, as recommended by the manufacturer. The reaction was stopped via phenolisation or separation on an agarose gel.

5.3.4 Ligation of DNA fragments

Eqimolar amounts of DNA fragments (ca. 200 ng) were mixed together, and heated to 45 °C for 5 ', before transfer to ice. Ligase buffer and T4 DNA ligase were added, as recommended by the manufacturer, and incubated overnight at 16 °C. The entire ligation mixture was used for transformation.

5.3.5 Hybridisation of complementary oligonucleotides

For the production of linker DNA, 5' phosphorylated complementary oligonucleotides were hybridised together. Equimolar amounts of

oligonucleotide were mixed together and heated to 95 °C in a thermomixer for 10 ‘, and then allowed to slowly cool to RT.

5.3.6 Polymerase chain reaction

The standard polymerase chain reaction (PCR) was performed essentially as recommended by the manufacturer. For standard (analytical) applications, *Taq* polymerase was used, whereas the proof-reading DNA polymerase, *Pfu*, was used for cloning purposes. In brief, 20 ng of the DNA template to be amplified was mixed with 50 pmol of forward and reverse primers, 0.5 mM dNTPs (dATP, dGTP, dCTP, dTTP), and 2.5 U *Taq* DNA polymerase, in a volume of 50 µl 1 x polymerase buffer. PCR was performed in a thermocycler. Samples were first denatured at 95 °C for 5 ‘ before commencement of the amplification stage, and for 30 ‘’ between each cycle thereafter. The annealing temperature used was 2 °C lower than the calculated melting temperature for the primers used. The elongation temperature, and elongation time used was dependent on the DNA polymerase used. *Taq* polymerase has an optimal elongation rate of 1.5 kbp / min at 72 °C; *Pfu* polymerase has an optimal elongation rate of 500 bp / min at 73 °C. Following the final cycle, a further elongation step of 5 ‘ was performed to ensure complete synthesis.

5.3.7 Isolation of DNA for cloning and analysis

The DNA products of PCR and restriction enzyme digests were isolated, essentially as described (sambrook, maniatis). Purification of DNA from non-DNA contaminants was achieved through phenolisation, followed by ethanol precipitation. Purification, or analysis of a mixture of different-sized DNA was achieved via separation over a 1 x TAE 0.7 % agarose gel, containing ethidium bromide (0.5 µg / ml). For the purification of DNA, a low-melting type of agarose was used. A band of interest was removed from the gel and melted at 68 °C in a 1.5 ml reagent tube. An equal volume of phenol (pH 8) was then mixed with the melted gel, before centrifugation at 15000 x *g* to remove unwanted proteins. The resulting supernatant was repeatedly phenolised (2-3 x) before precipitation, and re-suspension in a suitable volume of TE pH 8.0.

5.4 Methods for the analysis of DNA and RNA

5.4.1 Radioactive labelling of DNA fragments

The multi-prime method (Feinberg and Vogelstein, 1983; Feinberg and Vogelstein, 1984) was used for the labelling of DNA fragments. 2.5-25 ng DNA was labelled using the *redi*prime kit (*Amersham Pharmacia, Freiburg)* and 50 µCi [α-^{32}P]-dCTP (3000 Ci/ mmol, 10 mCi/ ml), as recommended by the manufacturer. Labelled probe was separated from non-incorporated nucleotide label using a G50 sephadex column. The incorporation of label was measured using a bench top radioisotope counter. Labelled probes were denatured by heating to 95 °C, for 5 ‘, before use.

5.4.2 Isolation of total RNA

Large amounts of total RNA were extracted from cells using a guanidinium thiocyanate-phenol-chloroform based reagent (*TriFast*: Peqlab, Erlangen). 0.5-1 x 10^7 cells were harvested by centrifugation (302 x *g*) and re-suspended in 1 ml of *Trifast* reagent. RNA was extracted and precipitated, as recommended by the manufacturer, before re-suspension in a 50 µl of DEPC-treated water. To increase solubility, samples were frozen at –80 °for >30 ‘ to improve pellet disruption, before mixing at 55 °C using a thermomixer. To measure RNA concentration, 1 µl of sample was diluted in 199 µl Tris-Cl pH 7.5 and the absorbance at 260 nm measured using a spectrophotometer.

5.4.3 Northern-blot analysis

10 µg of total RNA was mixed with 20 µl of RNA loading buffer and denatured at 65 °C for 5 ‘. RNA was separated on a 1 % formaldehyde-agarose gel in 1 x MOPS buffer overnight with a voltage of 0.7 V/ cm. Following separation, a UV (254 nm) trans-illuminator was used to visualise the RNA loading of the gels, and photographed. RNA was transferred to a nylon membrane (Hybond N+) using 10x SSC solution. Baking of the membrane at 80°C for 1 h covalently linked the RNA to the membrane. RNA transfer can be visualised by UV illumination.

Before hybridisation of probe to the membrane, pre-hybridisation steps are required to reduce background. The membrane must first be washed at 65 °C in 1 % SDS / 0.1 x SSC for 1 h, followed by a further 1 h at 65 °C in Church hybridisation buffer. Hybridisation was performed at 65 °C overnight in 5 ml fresh Church hybridisation buffer containing 1 x 10^7 DPM of the radioactive probe (5.4.1). Membranes were then washed 3 times in 10 ml of a solution of 1 % SDS / 0.1 x SSC, at a temperature of between 40-65 °C, depending on the probe used. Membranes were then dried, and exposed to X-ray film overnight. If a gene is expressed at low-levels, the use of an enhancer screen may be required, in which case exposure must be performed at –80 °C.

20 x SSC: 3 M NaCl; 300 mM Tri-sodium citrate / NaOH pH 7.0

Church hybridisation buffer: 400 mM Na_2HPO_4; 200 mM NaH_2PO_4; pH 7.1; 7 % (w / v) SDS; 1 mM EDTA / NaOH pH 8.0

10 x MOPS: 0.4 M MOPS (free-acid); 100 mM Na-acetate; 10 mM EDTA / NaOH pH 8.0; pH 7.0 (adjusted with 10 N NaOH)

RNA loading buffer; 50 % (v / v) formamide; 2.2 M formaldehyde; 1 x MOPS buffer; 50 µg / ml ethidium bromide; 0.2 % (w / v) bromophenol blue

1 % Formaldehyde-agarose gel: 2.2 M formaldehyde; 1 x MOPS buffer; 1 % (w / v) agarose

5.4.4 Preparation of cell nuclei

The preparation of nuclei for nuclear run-on reactions was performed essentially as described (Eick and Bornkamm, 1986; Greenberg and Ziff, 1984). 1 x 10^8 cells from an exponentially growing culture were pelleted by centrifugation (302 x *g*, 4 °C), before washing in 40 ml ice-cold PBS, re-centrifugation, and re-suspension in 10 ml ice-cold lysis buffer. Following 15 ' incubation on ice, nuclei were pelleted by centrifugation (472 x *g*), before careful re-suspension in 1 ml storage buffer (2 x 10^7 nuclei / 100 µl). Nuclei were dispensed in 100 µl aliquots, which were then snap-frozen in liquid nitrogen, before storage at –80°C.

Lysis buffer: 10 mM Tris / HCl pH 7.5; 10 mM $MgCl_2$; 10 mM NaCl; 0.5 % (w / v) NP40

Storage buffer: 50 mM Tris / HCl pH 8.3; 5 mM $MgCl_2$; 0.1 mM EDTA / NaOH pH 8.0; 40 % (v / v) glycerol

5.4.5 Nuclear run-on

Nuclear run-on reactions were performed essentially as described (Eick and Bornkamm, 1986; Greenberg and Ziff, 1984). 100 µl (2 x 10^7 nuclei) of cell nuclei preparation (5.4.4) was thawed gently on ice. At this point, 4 µl 0.1 mg/ ml α-amanatin may be applied to the nuclei, followed by a further 10 ‘ incubation (optional). The run-on reaction is commenced with the addition of 100 µl of 2 x run-on buffer (with or without sarkosyl), containing 100 µCi [α-^{32}P]-CTP (800 Ci/ mmol, 10 mCi/ ml) to the nuclei. Reactions were incubated at 28 °C for 15 ‘, after which 50 U DNase 1 (RNase-free) was applied and the mixture incubated for a further 12 ‘ at RT. The DNase I treatment was repeated if sarkosyl was included in the run-on buffer. The nuclei were then lysed with 20 µl proteinase K/ SDS, and incubated for at least 1 hour at 37 °C. The nuclear transcripts were then separated from non-incorporated ribonucleotides using a sephadex G50 column, and collected in a 500 µl volume of TE buffer. The incorporation of label was measured using a bench top radioisotope counter, before denaturing for 5 ‘ at 65 °C and hybridisation to a CLONTECH Atlas cDNA membrane.

4 x run-on buffer: 600 mM KCl; 10 mM $MgCl_2$; 1 mM each NTP; 20 mM Tris/HCl pH 8.0; 2.4 % (w/v) sarkosyl (optional)

Proteinase K/ SDS: 2.5 mg/ ml proteinase K; 2.5 % (w/v) SDS

5.4.6 Preparation and washing of Atlas cDNA membranes

The Atlas cDNA membranes were prepared as directed by the manufacturer. In brief, membranes were activated before use by boiling in 0.5 % SDS/ TE pH 8.0 for 10 minutes prior to pre-hybridisation. Membranes were pre-hybridised with 1.5 mg sheared salmon-sperm DNA/ membrane, in 15 ml Church hybridisation buffer (5.4.3) at 65 °C for > 1 h (or overnight). Hybridisation was performed in 5 ml Church hybridisation buffer containing the radioactively labelled probe, and incubated with rotation for 48 h at 65°C.

Membranes were washed at 65 °C using wash buffers of different stringencies as follows: 2 x with wash buffer I; 2 x with wash buffer II; 1 x RNase digestion in wash buffer III, 15 ‘ at RT; 1 x in wash buffer II. Membranes were packed wet, in plastic film and exposed to sensitive X-ray film (*BioMaxMS, Eastman Kodak, New York, USA)* at –80 °C.

Wash buffer I: 0.1 x SSC; 1 % (w / v) SDS
Wash buffer II: 0.1 x SSC; 0.5 % SDS
Wash buffer III: 2 x SSC; 1 mM EDTA / NaOH pH 8.0; 2 µg / ml RNase A

5.5 Methods for the analysis of protein

5.5.1 Preparation of protein extracts

For the analysis of total cellular protein, SDS-PAGE loading buffer was used. 3 x 10^5 cells were pelleted by centrifugation (302 x *g*, 5 ‘) in a 1.5 ml reaction vessel. Cells were then re-suspended in 100 µl of SDS loading buffer. The viscous lysate was repeatedly drawn through a narrow pipette tip to shear genomic DNA, before denaturing by boiling at 95 °C for 5 ‘. After briefly cooling on ice, samples were centrifuged (15000 x *g*, 10 ‘) to clear insoluble contaminants. 10 µl (30,000 cells) of lysate was loaded per lane on an SDS-PAGE gel.

SDS-PAGE loading buffer: 200 mM Tris / HCl pH 6.8; 8 % (w / v) SDS; 40 % glycerol; 400 mM DTT; 0.2 mM EDTA / NaOH pH8.0; 0.2% (w / v) bromophenol blue

5.5.2 Immunoprecipitation

Treatment of cells with buffers of different stringencies allows the extraction of different cellular compartments.

Membrane and cytosolic fraction

2 x 10^7 cells were harvested by centrifugation (302 x *g*, 5 ‘), washed once in 20 ml PBS, re-centrifuged and re-suspended in 500 µl ice-cold NP40 lysis buffer. Cells were mixed gently and incubated 5 ‘ on ice before pelleting of the nuclei (472 x *g*, 5 ‘, 4 °C). The supernatant was carefully removed for immunoprecipitation, or storage at –80 °C. The remaining nuclei can be used for extraction of nuclear proteins.

Total (soluble and non-soluble) nuclear fraction

Nuclei produced by the previous method may be digested to release their non-soluble protein content (for example, chromatin-bound factors). Nuclei were re-suspended in 100 µl of Dahmus digest buffer (Baskaran et al., 1993) and incubated on ice for 30 ‘, before the addition of 500 µl RIPA extraction buffer. Nuclei were incubated on ice for 10 ‘ before clearing by centrifugation (15000 x *g*, 5 ‘, 4 °C). The supernatant was carefully removed for immunoprecipitation, or storage at –80 °C.

Soluble nuclear fraction

Nuclei produced by the previous method may be ‘bled’ to release their soluble protein content. Nuclei were re-suspended in 500 µl of a variation of high-stringency RIPA buffer and incubated on ice for 10 ‘, before clearing by centrifugation (15000 x *g*, 5 ‘, 4 °C). The supernatant was carefully removed for immunoprecipitation, or storage at –80 °C.

NP40 lysis buffer*:	10 mM NaCl; 10 mM $MgCl_2$; 10 mM Tris pH 7.5; 5 mM EDTA; 0.5 % NP40
Dahmus digest buffer*:	25 mM Tris / HCl pH 8.0; 2.5 mM Mg-acetate; 2 mM $CaCl_2$; 50 µM EDTA; 0.1 mM DTT; 12.5 % glycerol; 100 µg / ml each of DNase and RNase
RIPA buffer*:	50 mM Tris / HCl pH 7.0; 300 mM NaCl; 1 % Triton X100; 1 % Na-deoxycholate

***** Buffers were supplemented with: 1 mM PMSF; complete protease inhibitor cocktail (*Roche Diagnostics, Mannheim*); 1 mM Na-orthovanadate; 1 mM Na-molybdate; 10 mM NaF

Immunoprecipitation (IP) of HA-tagged proteins was performed using anti-HA (3F10) affinity matrix beads, as recommended by the manufacturer (*Roche Diagnostics, Mannheim*). In brief, beads were washed 3 x in RIPA buffer (50 µl slurry / IP), before re-suspension in fresh RIPA buffer (50 µl / IP) and dispensing to lysates. Lysate / bead mixtures were incubated on ice for 30 ‘, with gentle mixing by inversion (never vortex!) every 5 ‘. Lysate / bead mixtures were then inversion-mixed at 4 °C for 4 hours. Beads were collected by centrifugation, washed extensively with IP buffer, and the solubilised in SDS loading buffer at 95 °C for 10 ‘ before SDS PAGE analysis.

5.5.3 SDS-polyacrylamide gel electrophoresis

Denaturing (SDS) polyacrylamide gel electrophoresis (PAGE) was performed essentially as described (Laemmli, 1970). 30,000 cells, or 10 µg protein solubilised in SDS-PAGE loading buffer were denatured by boiling at 95°C before separation on an SDS-PAGE gel. Stained marker standards were run simultaneously on the same gel for the estimation of molecular weight. The acrylamide content of SDS-PAGE gels was varied according to the size of protein being examined (described in detail (Sambrook et al., 1989)) in conjunction with a 5 % acrylamide stacking gel. Gels with a thickness of 0.75 mm were separated using a constant current of 30 mA / gel in 1 x SDS running buffer, with cooling. Following separation, gels were used for Western blotting (5.5.4).

10 x SDS-PAGE running buffer: 0.25 M Tris-base; 2.5 M glycine; 0.5 % (v / v) SDS

5.5.4 Western-blotting and immuno-detection of proteins

The western-blotting technique involves the immobilisation of proteins resolved by SDS-PAGE, and their detection using specific antibodies.

Proteins separated on SDS-PAGE gels were transferred to a PVDF membrane using a wet transfer system with all components soaked beforehand in transfer buffer. The gel was placed on top of a piece of Whatman 3MM filter paper and sponge and overlaid with PVDF membrane, pre-washed in Methanol and equilibrated in transfer buffer. The blotting sandwich was completed by the addition of a second layer of filter paper and sponge. Transfer in a cassette assembly was carried out at 150 mA for 90 ‘.

Following transfer, PVDF membranes were washed repeatedly in 1 x TBS / Tween-20 to remove any gel remnants from the transfer procedure. The remaining protein-binding capacity of the gel was neutralised by incubation in blocking buffer >1 h, with shaking. Excess blocking reagent was removed by washing 3 x using the appropriate wash buffer, before incubation of the membrane with primary antibody diluted to its correct working concentration in 5 % BSA / washing buffer, 1 h, at RT. Membranes were washed 3 x with wash buffer to remove excess primary antibody. A peroxidase-conjugated, secondary antibody, specific for the isotype of the primary antibody used, was

applied to the membrane at its correct working concentration, diluted in 5 % BSA / washing buffer, for 1 h, at RT. Excess antibody was removed by washing 3 x in wash buffer.

Protein-antibody complexes were detected on X-ray film using a chemoluminesence reaction catalysed by the antibody-conjugated peroxidase. The ECL solution was used as substrate for this reaction, following the manufacturer's protocol.

Transfer buffer:	1 x SDS-PAGE running buffer; 10 % (v / v) methanol
Blocking buffer:	5 % (w / v) fat-free milk powder; wash buffer
Wash buffer I:	1 x PBS; 0.05 % Tween-20
Wash buffer II:	1 x TBS; 0.05 % Tween-20

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7. Appendix

A *Curriculum Vitae*

Rob Daniel Chapman
Born 30th of August, 1976 in Leeds, UK

School Education

1981-1983	Featherbank Infant School, Horsforth, Leeds
1983-1987	Newlaithes Junior School, Horsforth, Leeds
1987-1992	Benton Park Comprehensive School, Rawdon, Leeds
1992-1994	Benton Park Sixth-Form College, Rawdon, Leeds

University Education

1994-1997	B.Sc. 2.1 (HONS) Medical Biochemistry, Department of Molecular Biology & Biotechnology, The University of Sheffield, UK

Research Experience

1997-1998	Research Assistant Laboratory of Dr. E. Qwarnstrom, Department of Molecular Medicine, The Royal Hallamshire Hospital, University of Sheffield, UK
1998-1999	Research Assistant Laboratory of Prof. Dr. D. Eick GSF - Institute for Clinical Molecular Biology and Tumour Genetics, Munich, Germany

Doctoral Thesis

1999-2002	"A Functional Analysis of the RNA Polymerase II Large Subunit Carboxy-Terminal Domain" GSF - Institute for Clinical Molecular Biology and Tumour Genetics, Munich, Germany Prof. Dr. D. Eick

B Publications

Meininghaus, M., Chapman, R. D., Horndasch, M., and Eick, D. (2000). Conditional expression of RNA polymerase II in mammalian cells. Deletion of the carboxy-terminal domain of the large subunit affects early steps in transcription. J Biol Chem *275*, 24375-24382.

C Oral Presentations

Mechanisms of Eukaryotic Transcription 2001
Cold Spring Harbour Laboratory, 30th of August 2001:
"Removal of the last repeat of RNA Polymerase II causes a global defect in transcription"
Cold Spring Harbour, New York, USA

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