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Krassimir Yankov Yankulov

**REGULATION OF TRANSCRIPTIONAL ELONGATION BY
RNA POLYMERASE II**

A thesis submitted for the degree of Doctor of Philosophy

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ABSTRACT

Transcriptional attenuation by RNA polymerase II (pol II) has been shown to regulate the expression of many genes (Spencer and Groudine, 1990) but the mechanism of this control has been poorly understood. In this thesis I present data which indicate that in *X.laevis* oocytes transactivator proteins stimulate transcriptional elongation by pol II. Transcription complexes activated by recombinant factors bound to promoter elements in synthetic genes have high competence to elongate meaning they are able to read through pausing and termination sites efficiently. Furthermore, activation domains differ in the processivity of the transcription they stimulate from a given promoter. In contrast, non-activated transcription and transcription "squelched" by a non-binding factor mostly terminates prematurely. A general transcription factor, TBP, is found to stimulate initiation, but not elongation of pol II transcription. These results suggest that programming the competence of RNAPolymerase II to elongate is an integral part of the initiation step which is controlled by activators co-operating with the basal transcriptional machinery.

The positive effect of transcriptional activators on pol II processivity is counteracted by the suppressor of transcriptional elongation DRB (Dichlororibofuranosyl-benzimidazole) and by protein kinase inhibitors such as H-8 and H-7. Here I characterise a transactivator binding CTD-protein kinase which is highly sensitive to DRB, H-7 and H-8. This protein kinase co-purifies with the general transcription factor TFIIF on affinity chromatography resins and has properties indistinguishable from the TFIIF associated kinase. I suggest that the effect of DRB on transcriptional elongation is mediated by inhibition of the TFIIF associated kinase activity.

The human protein BM28, which is analogous to the yeast MCM2 and MCM3 proteins, has been proposed to participate in DNA replication. In this thesis I include experiments which indicate that BM28 is also essential for pol II transcription in *X.laevis* oocytes.

**THIS WORK IS DEDICATED TO MY PARENTS,
ANASTASIA AND YANKO**

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ABBREVIATIONS

ADI	ATP dependent inhibitor
ATP	adenosine triphosphate
BHI	brain heart infusion
BSA	bovine serum albumin
CAT	chloramphenicol-acetyl-transferase
cpm	counts per minute
CTD	carboxyterminal domain of RNAPol II
CTP	cytosine triphosphate
dATP	deoxy adenosine triphosphate
dCTP	deoxy cytosine triphosphate
dGTP	deoxy guanosine triphosphate
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNA-PK	DNA dependent kinase
dNTP	deoxy nucleoside triphosphate
DRB	5,6-dichloro-1-b-D-ribofuranosylbenzimidazole
DTT	dithiothreitol
dTTP	deoxy thymidine triphosphate
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
GST	Glutathione Transferase
GTF	general transcription factor
GTP	guanosine triphosphate

HIV1 or HIV2	human immunodeficiency virus 1 or 2
hsp70 or hsp26	heat shock protein 70 or 26
kb	kilobases/kilobasepairs
kDa	kilodaltons
LTR	long terminal repeat
MLP	major late promoter
OD	optical density
P1 or P2	promoter 1 or promoter 2 of c- myc
PBS	phosphate buffered saline
PIC	preinitiation complex
PMSF	phenylmethylsulfonylflourid
PNA	polynucleotide kinase
RNA	ribonucleic acid
RNApol II or pol II	RNA polymerase II
RP-A	replication protein A
rpm	revolutions per minute
RT	read through
T2	terminator element 2 of c-myc
TAF	TBP associated factor
TAR	transactivation responsive element of HIV1 and HIV2
TBP	TATA binding protein
TdT	terminal deoxy transferase
TK	thimidine kinase
TM or tm	terminated
UTP	uridine triphosphate

INTRODUCTION

1.1. Regulation of Eukaryotic Class II Genes Expression

Regulation of eukaryotic class II gene expression is a complex multistep process that involves the concerted action of numerous transcriptional activators and at least eight general transcription factors (GTFs, reviewed in (Buratowski, 1994; Zawel and Reinberg, 1993) in addition to RNA polymerase II (pol II). Several stages in the RNAPol II transcription cycle, namely transcription initiation, promoter clearance, elongation and termination, are all potential targets for different control mechanisms.

Initiation includes the correct positioning of RNA polymerase II at the promoter and unwinding of the DNA strands. This process is accompanied by multiple interactions between factors, generally required for transcription from most class II promoters (the GTFs) and the polymerase itself, and is greatly influenced by transcriptional activators. Once the first phosphodiester bond is synthesised, the polymerase proceeds through a stage, referred as to promoter clearance, in which it leaves the preinitiation complex and transforms into the elongating complex. Elongation is the phase during which the enzyme moves along DNA and extends the growing RNA chain. Termination is the stage, in which RNA synthesis is suspended and the polymerase and the nascent RNA are released from the template. Elongation ceases at the 3' region of the gene or, alternatively, termination can occur within the transcription unit, as will be discussed further. Initiation is thought to be the principal stage, where gene expression is regulated and, undoubtedly, is most extensively investigated. Significant progress has been made in identifying transcription initiation factors and understanding their role in the formation of preinitiation complex and promoter clearance (for review see (Buratowski, 1994). Less is known

about factors that are generally involved in elongation and termination by RNAPolymerase II.

It is becoming evident that transcriptional elongation is also a critical stage where gene expression can be controlled. Premature termination or attenuation in response to physiological signals has been documented in a number of eukaryotic genes. Thus it is possible to modulate the mRNA levels of these genes by regulating the efficiency with which RNAPolymerase II reads through intragenic termination and pausing sites. The mechanisms, governing transcriptional processivity and attenuation, though, are much less well understood than transcriptional initiation.

A definition of premature termination, attenuation and processivity of RNAPolymerase II should facilitate this discussion. In this thesis, attenuation defines pausing of the polymerase or termination within the gene rather than at its 3' end, or both. In many cases, it is yet unclear whether pausing (that is temporary cessation of elongation, where the polymerase and the transcript remain associated with the template) or true termination takes place. "Processivity" refers to the competence of RNAPolymerase II to read through potential pausing and termination sites.

In this review I shall describe the GTFs, the common features of sequence specific transcriptional activators and the mechanisms, by which they are thought to control RNA pol II transcription. More details will be given about the GTFs, which are investigated in the experimental section of the thesis. Special attention will be paid to elongation by RNA polymerase II in several genes, where premature termination of transcription has been observed. The current knowledge about transcriptional attenuation will also be discussed.

1.1.1.RNAPol II General Transcription Factors

Eight factors (TFIIA, TFIIB, TFIID, TFIIF, TFIIIE , TFIIH, TFIIS and TFIII), see (Zawel and Reinberg, 1993) which are necessary for basal transcription from most class II genes, have been described. These factors are highly conserved from yeast to mammals and share significant functional similarities. Throughout this review I shall use predominantly the human nomenclature, since it is generally accepted and since human GTFs are most thoroughly investigated. Human analogues in *Drosophila* have identical names. *S.cerevisiae* and rat analogues of the human GTFs are given below.

human	rat	yeast
TFIIA	-	TFIIA
TFIIB	α	e
TFIID	τ	d
TFIIIE	ϵ	a
TFIIH	δ	b
TFIIF(RAP30/74)	$\beta\gamma$	g

1.1.1.1. TFIID is the factor, which binds to the TATA element of class II promoters and provides the foundation for formation of pre-initiation complex. It consists of TBP (TATA Binding Protein) and several tightly associated proteins, called TAFs (TBP Associated Factors).

TBP is one of the most highly conserved proteins in eukaryotes. The C-terminus region of TBP, which harbours the DNA binding domain, shares more than 80% homology from yeast to mammals, while the N-terminus is divergent across different species. Resolving of the crystal structure of TBP demonstrated that its shape resembles a saddle. Its inner surface contacts with

the minor groove of the TATA element, causing significant distortions in DNA, while the outer surface is accessible for other transcription factors (Kim et al., 1993a; Kim et al., 1993b). TBP alone is sufficient to direct basal in vitro transcription, when supplemented with other GTFs. Binding of TBP (TFIID) to the promoter is the first step in the formation of the preinitiation complex which nucleates further association of the rest of the factors and RNA polymerase II. Direct interaction between TBP and TFIIB on DNA (Hisatake et al., 1993), TFIIA (Maldonado et al., 1990) and the non-phosphorylated carboxyterminal domain of the catalytic subunit of RNAPol II (Usheva et al., 1992), respectively, have been documented.

TFIID is a target for numerous positive and negative regulators of transcription. TBP can interact with the transactivation domains of the Herpes Simplex Virus protein VP16 (Stringer et al., 1990), the Human Immunodeficiency Virus TAT protein (Kashanchi et al., 1994), the Adenovirus type 2 E1a protein (Horikoshi et al., 1991), the Epstein-Barr Virus Zta protein (Lieberman and Berk, 1991), the Cytomegalovirus IE2 protein (Hagemeyer et al., 1992) and the cellular p53 protein (Seto et al., 1992). Although these interactions are likely to contribute to the process of activation, they are clearly not sufficient since TBP alone does not mediate regulation by upstream binding transcription factors.

Stimulation of in vitro transcription by transactivators can be detected only if purified TFIID, but not TBP, is used (Pugh and Tjian, 1990), which suggests that at least some subunits of TFIID can function to link transactivation domains with the basal transcription machinery. TFIID was found to exist as a stable complex, composed of TBP and at least seven TAFs in *Drosophila* (Dynlacht et al., 1991; Hoey et al., 1990) and eight TAFs in man (Tanese et al., 1991; Takada et al., 1992; Zhou et al., 1993). TBP exists mainly as a free subunit in *S.cerevisiae*, which led to the conclusion that there are no TAFs

in this species. Recently, though, TAFs were also described in yeast (Poon and Weil, 1993).

Cloning and analysis of seven *Drosophila* TAFs revealed multivalent protein-protein TAF-TAF and TAF-TBP interactions, which could account for the remarkable stability of the TFIID complex (for review see (Goodrich and Tjian, 1994b). Three of the TAFs have been demonstrated to associate with transcriptional activators. TAF_{II}250 interacts directly with TBP and is identical to the described human protein CCG1, which is necessary to overcome the G1 arrest of a temperature sensitive cell line presumably by complementing a defect in transcription (Sekiguchi et al., 1991; Wang and Tjian, 1994). *In vitro*, transcription activation in extracts from these cells (hamster ts13) can be restored by addition of exogenous holo-TFIID (Wang and Tjian, 1994). TAF_{II}110 interacts with Sp1 (Hoey et al., 1990), while TAF_{II}40 interacts with the transactivation domain of VP16 (Goodrich et al., 1993). Mutations or antibodies that disrupt the TAF-activator interactions in these two cases revealed tight correlation between TAF binding and transcriptional activity. Thus, TAFs appear to be mediators of activation both *in vivo* and *in vitro* and possibly provide a wide range of contacts, which can be used selectively by different classes of transcriptional activators. Furthermore, TAFs can mediate interactions between activation domains and GTFs, other than TBP. For example, TAF_{II}40 interacts with both VP16 and TFIIB (Goodrich et al., 1993).

In addition to activators and mediators of activation, TBP was shown to interact with several proteins, which function as transcriptional repressors. NC1 and NC2 (Meisterernst and Roeder, 1991; Meisterernst et al., 1991) are human factors, which both form complexes with TBP and inhibit association of TFIIA and TFIIB with the TBP-DNA complex. In a different study two other inhibitors of basal transcription in human cells - Dr1 and Dr2, were characterised (Inostroza et al., 1992). Dr1 is a 19 kDa protein, which upon phosphorylation can stably interact with the TBP-DNA complex and displace

TFIIA. Upon dephosphorylation Dr1 cannot bind stably to TBP but precludes association with TFIIB and interferes with the assembly of the preinitiation complex (Inostroza et al., 1992). Dr2 and NC1 display similar chromatographic behaviour and might be equivalent (Merino et al., 1993). Dr2 was initially isolated as an activity that suppresses basal transcription, but potentiates the function of acidic activators. Subsequently, it was found that Dr2 is actually Topoisomerase I (Merino et al., 1993). Interestingly, mutations that abolished the topoisomerase activity of Dr2 had no effect on its function as a suppressor/activator of transcription. This fact implied that the role of Topoisomerase I in transcription initiation should not be directly connected with its enzyme properties. Dr2/Topoisomerase I was shown to interact specifically with TBP too (Merino et al., 1993).

(Auble and Hahn, 1993) reported the purification of an ATP-dependent Inhibitor (ADI) of transcription in yeast. This factor was not similar to the previously described inhibitor activities in human cells. It interacts directly with TBP and dissociates it from DNA in a ATP dependent manner. In addition, ADI suppression of transcription can be overcome by TFIIA. It was predicted that ADI prevents TBP from non-specific or weak specific interactions with DNA.

Several studies have demonstrated that TFIID is necessary for transcription from TATA-less class II promoters (for review see (Weis and Reinberg, 1992)). TBP is also a putative subunit of SL1, SNAPc and TFIIB - factors, required for RNAPol I transcription, and transcription from TATA-containing and TATA-less pol III promoters, respectively (for review see (Goodrich and Tjian, 1994b; Hernandez, 1993)). In these cases TBP is complexed with different sets of TAFs, which program the specific function of these factors at different promoters.

1.1.1.2. TFIIB is a single polypeptide of 33 kDa, which binds to and stabilises the TBP-DNA complex (Buratowski et al., 1989; Maldonado et al., 1990; Ha et al., 1991). It seems to play crucial role together with pol II in selecting the transcription start site in *S.pombe* and *S.cerevisiae* (Li et al., 1994). Binding of TFIIB to the TBP-DNA complex and subsequent association with the TFIIF-pol II complex is thought to be a rate limiting step in the in vitro transcription reactions (Lin and Green, 1991; Lin et al., 1991). Recently TFIIB was reported to interact with TAF_{II}40 (Goodrich et al., 1993) and acidic transactivation domains via positively charged amphipathic helix, positioned in its carboxyterminal domain. Mutations in this region did not affect basal transcription, but completely abolished activation of transcription by GAL4-VP16 and GAL4-AH (Roberts et al., 1993). Hence, TFIIB has distinct functions in basal and activated transcription and the effect of acidic activators is at least partially mediated by contacts with TFIIB.

1.1.1.3. TFIIA. There has been a lot of controversy considering the role and the requirement for TFIIA in transcriptional initiation. TFIIA was originally characterised as an activity, that stabilises the DB-DNA complex and stimulates basal transcription when TFIID rather than TBP was used (Zawel and Reinberg, 1993). Nevertheless, highly purified TFIID and TBP do not require TFIIA for transcription (Zawel and Reinberg, 1993). Recently it emerged that TFIIA counteracts repressors of transcription such as Dr1, Dr2 and ADI, presumably through interactions with TBP.

TFIIA consists of 35, 19 and 12 kDa subunits in man and of 30 and 20 kDa subunits in *Drosophila* (Dejong and Roeder, 1993; Yokomori et al., 1993). It was shown by affinity chromatography that *Drosophila* TFIIA, in addition to TBP, could interact with TAF_{II}110 and TBP (Yokomori et al., 1993). Tight association between the endogenous TFIID and TFIIA was further demonstrated by co-immunoprecipitation of these two factors (Yokomori et al.,

1993). This implied that TFIIA might affect formation of the preinitiation complex, which was not due solely to its anti-inhibitory properties. In support to such idea (Wang et al., 1992; Ma et al., 1993), indicated that TFIIA is essential for activated transcription, while there was no requirement for that factor in basal transcription. The novel function of TFIIA in transcriptional activation was associated with the carboxyterminal domain of the 19 kDa subunit (Ma et al., 1993), but the authors did not assay whether the same aminoacid residues were necessary for counteracting the effect of transcriptional inhibitors.

1.1.1.4. TFIIF consists of two subunits (RAP30 and RAP74, (Flores et al., 1990; Flores et al., 1988). In solution it exists as a heterotetramer. RAP30 suppresses non specific binding of RNAPol II to DNA and is responsible to recruit pol II to the DAB-DNA complex through interactions with TFIIB (Killeen and Greenblatt, 1992). Although RAP74 does not appear to be obligatory for the recruitment of RNAPol II, it stabilises the DAB-DNA-pol II complex. Both RAP30 and RAP74 are necessary for transcription initiation in vitro, since RAP30 alone can not substitute for TFIIF (Flores et al., 1991).

RAP74 is extensively phosphorylated in vivo, possibly by the TFIIF associated kinase (Ohkuma and Roeder, 1994). In addition to its function in transcriptional initiation, TFIIF was shown to stimulate elongation by suppressing RNAPol II pausing in vitro (Bengal et al., 1991 ; Chang et al., 1993). Recently, direct interactions between RAP74 and SRF or the transactivation domain of VP16, but not Sp1, respectively, were demonstrated (Zhu et al., 1994). This indicates a possible role of RAP74 in transcription activation. It is not known, though, whether phosphorylation of RAP74 affects its interaction with transactivators or its function as a stimulator of elongation in vitro.

1.1.1.5. TFIIS (initially described as RAP38, (Sopta et al., 1985)) is a 38 kDa single polypeptide, which directly interacts with the catalytic subunit of RNAPol II. Recently TFIIS was shown to be identical with RTF (Szentirmay and Sawadogo, 1993), a factor required for reinitiation *in vitro*. TFIIS does not directly participate in the second and further rounds of initiation from one and the same promoter, but rather stimulates elongation of the reinitiated complexes on templates, which already contain a paused RNAPol II at the end of the G-less cassette (Szentirmay and Sawadogo, 1993). TFIIS functions by triggering a 3'-5' RNAase activity from the catalytic subunit of RNAPol II, when the RNAPol II complex stalls on the template. After digestion of several bases backwards, TFIIS facilitates the resumption of elongation presumably by re-establishing a proper elongation conformation of RNAPol II, which might be lost upon pausing (Izban and Luse, 1992; Johnson and Chamberlin, 1994). TFIIS has been used as a tool to distinguish paused polymerases from true termination events *in vitro*, since TFIIS can promote extension of the RNAs associated with the template (Christie et al., 1994; Kerppola and Kane, 1988).

1.1.1.6. TFIIF is a not well characterised factor, that was found to contaminate different TFIIA, TFIID or TFIIF preparations (Zawel and Reinberg, 1993). It is required for transcription with highly purified TFIID or TBP and is believed to enter the preinitiation complex after the formation of DABpolIIF-DNA. Recently it has been reported that the TFIIF activity copurifies as a 55 kDa polypeptide and stimulates elongation (D. Reinberg, in preparation).

1.1.1.7. TFIIE is a heterotetramer, composed of two subunits - p34 and p56 (Inostroza et al., 1991). It is assumed that TFIIE enters the PIC and functions after the formation of DAB-DNA-polIIF complex (Zawel and Reinberg, 1993). Although the p56 subunit contains a region bearing homology

with a consensus sequence present in the catalytic loop of several kinases, no enzyme activity has been detected in that factor (Zawel and Reinberg, 1993). Recently, Ohkuma and Roeder (1994) demonstrated that TFIIE stimulates both the TFIIH dependent ATPase and kinase activities at a late stage in the assembly of the preinitiation complex. p56(TFIIE) binds the non-phosphorylated, but not the phosphorylated form of RNAPol II, RAP74 (TFIIF) and the carboxyterminal domain of TBP, while p34(TFIIE) associates with RAP30 (TFIIF) (Maxon et al., 1994). TFIIE was also shown to interact with the holo-TFIID complex or with TFIIH via the ERCC3 subunit (Maxon et al., 1994). The functional relevance of these interactions still remains obscure, although they indicate that TFIIE could enter the preinitiation complex at an early stage or even could exist in a large heterogeneous GTF conglomerate (see "the multistep model for transcription initiation").

1.1.1.8. TFIIH is a multisubunit (at least five subunits in yeast and man and at least seven in rat) versatile factor, which copurifies with DNA dependent helicase, ATPase and RNAPol II carboxyterminal domain (CTD) kinase activities (Conaway and Conaway, 1989; Fischer et al., 1992; Gerard et al., 1991; Lu et al., 1992; Schaeffer et al., 1993; Serizawa et al., 1993b), all of which have been proposed to play some role in the transition from initiation to elongation. TFIIH, as well as TFIIE, is required for transcription from linearised templates, but not from supercoiled templates (Parvin and Sharp, 1993). Surprisingly, the TFIIH kinase activity was found not obligatory for initiation, formation of open complex (melting of DNA at the transcription start site and synthesis of the first phosphodiester bond) or promoter clearance (release of the polymerase from the promoter) on both supercoiled or linearised templates in in vitro transcription assays with highly purified components (Goodrich and Tjian, 1994a; Serizawa et al., 1993a). The TFIIH kinase activity was required, though, when TBP was replaced with holo-TFIID

in vitro (Serizawa et al., 1993a). By introducing a non-hydrolysable analogue of ATP (AMP-PNP), Goodrich and Tjian demonstrate that ATP, but not GTP hydrolysis supports promoter clearance on linearised templates. Since GTP is a substrate for the TFIIH kinase (Serizawa et al., 1993b), that hydrolysis was associated with the ATPase and the helicase rather than the kinase activities of TFIIH. Thus, the functional significance of the TFIIH kinase remains obscure.

Initially it was thought that the ATPase and helicase activities of TFIIH are required to unwind the transcription start site and to form an open complex (Buratowski, 1993; Schaeffer et al., 1993). The experiments, presented in (Goodrich and Tjian, 1994a) clearly indicate that ATP hydrolysis is not necessary prior to the formation of the first phosphodiester bond both on linearised and supercoiled templates, but is required at a late stage (after the synthesis of a dinucleotide) on linearised templates. It is believed that negative supercoiling provides the energy, needed for promoter clearance and thus circumvents the requirement for TFIIE and TFIIH. TFIIH and TFIIE were found not necessary for elongation either, since addition of these two factors after promoter clearance has no effect on transcription (Goodrich and Tjian, 1994a). The former conclusion, though, could be challenged by the fact that TFIIH is dissociated upon transition from initiation to elongation, so that the p62 subunit of TFIIH remains associated with the polymerase, while ERCC2 and ERCC3 do not. TFIIE was also absent from the elongation complex (D. Reinberg, P.Kumar; personal communication). Nevertheless, the data from both groups imply that the ATPase and kinase activity of TFIIH and TFIIE are not directly engaged in elongation. Since the helicase activity was not required for DNA unwinding either, it is not clear what the precise role of the TFIIH helicase activity in transcription is.

Some of the subunits of the yeast and human TFIIH were recently cloned and characterised. Human p62 (Fischer et al., 1992) and yeast p74 (TFB1, (Gileadi et al., 1992a) are analogues, representing a putative subunit of

TFIIH in these two species. Of potential importance is the fact that human p62 can specifically interact with the transactivation domains of VP16 and p53 (J.Greenblatt, in press). No enzyme activity is associated with p62 or p74. The helicase and most of the ATPase activity of the human TFIIH were shown to be carried by p89 (also called ERCC3)(Roy et al., 1994). Two other subunits of the human TFIIH - p44 and p36, share a conserved Zn finger motif with the yeast SSL1 protein (Humbert et al., 1994; Yoon et al., 1992), and see below). In addition, p44 is 58 % homologous to the yeast SSL1 (Yoon et al., 1992). Unlike SSL1, p34 and p44 do not display any helicase or ATPase activity.

Interestingly, p89 is identical to the protein, encoded by the previously characterised excision repair gene ERCC3 (Schaeffer et al., 1993). Another excision repair protein - ERCC2, is also associated with TFIIH, although not that tightly as ERCC3 (Schaeffer et al., 1994). None of the human TFIIH subunits, cloned so far, carries a kinase activity. The functional importance of p62, p34 and p44 in RNAPol II transcription was demonstrated by inhibiting basal transcription in vitro by antibodies against these peptides (Humbert et al., 1994; Schaeffer et al., 1993). Mutations in ERCC2 and ERCC3 were previously characterised as the causes for the DNA-repair deficiency in Xeroderma pigmentosum (for review see (Tanaka and Wood, 1994).

In *S.cerevisiae*, TFIIH was also found to contain proteins, that were shown to participate in the repair of DNA damage. The yeast homologues of ERCC2 (named rad3), ERCC3 (rad25 or SSL2) and p44 (SSL1) were detected as putative subunits of transcription factor b (Feaver et al., 1993). SSL1 and SSL2 were cloned as genes, which when mutated were capable of overcoming the inhibition of translation by an artificial stem-loop structure in the 5' untranslated sequence of a mRNA (Yoon et al., 1992). Rad3 and rad25 were independently cloned, based on their ability to complement DNA repair defective *S.cerevisiae* mutants. SSL1, SSL2 (rad25) and rad3 are all shown to possess helicase activity. Temperature sensitive mutants of rad3 and rad25

demonstrated the significance of these genes for transcription (Guzder et al., 1994a; Guzder et al., 1994b; Qiu et al., 1993). Upon transition to non-permissive temperature, both pol II and pol I transcription were severely inhibited in the *rad25_{ts}* and *rad3_{ts}* mutants, while pol III activity was unaffected. Interestingly, the mutation in *rad3_{ts}* did not affect the helicase activity of this protein (Qiu et al., 1993). In contrast, mutation in *rad25*, which abolished its helicase activity, completely inhibited transcription in vitro and is lethal in vivo (Guzder et al., 1994b).

Highly purified TFIIH complemented DNA excision repair in cell-free extracts from ERCC3 mutant hamster cells (Vanvuuren et al., 1994) and from human XPB(ERCC3) and XPD(ERCC2) cells (Drapkin et al., 1994). Thus, TFIIH can directly function both in transcription and DNA repair.

1.1.2.1. RNA polymerase II

RNA polymerase II activity copurifies with several polypeptides, ranging from 220 to 10 kDa (Zawel and Reinberg, 1993). Interactions between the large subunit (200 or 220 kDa according to different authors) and some of the GTFs were described in the previous chapter.

The carboxyterminal domain of the largest subunit of RNAPol II is composed of 26 copies in yeast, 42 copies in *Drosophila* and 52 copies in mammals of a consensus heptapeptide repeat (YSPTSPA) (Zawel and Reinberg, 1993), whose function in gene regulation has been subject of considerable research and speculation. Deletion mutants that result in the loss of more than half of the heptapeptide repeats in mouse, *Drosophila* and *S.cerevisiae* are lethal, indicating that this domain is essential in vivo (Allison and Ingles, 1989; Bartolomei et al., 1988). In *S.cerevisiae*, reducing the number of the heptapeptide repeats from 26 to 13 does not alter significantly activation by

the acidic activator GAL4. Mutations, affecting the transactivation domain of GAL4, though, resulted in suppression of activation in mutants with partially deleted CTD. Extending of the CTD to more than 26 copies of the heptapeptide enhanced activation by the crippled GAL4 activator relative to the wild type CTD (Allison et al., 1989 ; Edwards et al., 1991 ; Nonet et al., 1987 ; Peterson et al., 1991). Thus it appeared that a longer CTD can complement mutations in an activation domain, suggesting that CTD plays an important role in the process of transcriptional activation. In agreement with that, mutations in several proteins that interact with CTD or TBP and suppress CTD truncation mutations (SRBs, (Thompson et al., 1993) were also required for activation in vitro and in vivo (see also chapter 1.1.5).

In vivo, the large subunit of RNAPol II exists in two forms - IIA, which is not phosphorylated, and IIO, which is extensively phosphorylated at the CTD domain. In vitro, the nonphosphorylated form of RNA polymerase II preferentially enters the preinitiation complex. Subsequently it undergoes phosphorylation of the CTD upon transition from initiation to elongation (Cadena and Dahmus, 1987; Payne et al., 1989). Since TBP interacts with non-phosphorylated, but not with phosphorylated YSPTSPA-oligopeptide, it was proposed that phosphorylation of CTD facilitates the disruption of the PIC by decreasing the affinity of CTD-TBP and other CTD interactions (Usheva et al., 1992). Phosphorylation is also believed to cause conformational changes in the CTD domain (Zhang and Corden, 1991) which could possibly lead to promoter clearance. One model suggests that phosphorylation of the CTD is necessary to trigger elongation and to prevent the transcribing polymerase from interactions with initiation factors (Peterson and Tjian, 1992). However, phosphorylation of CTD by the TFIIH kinase was not required for transcription in vitro (Serizawa et al., 1993a). In addition, the kinase activity of TFIIH had properties, distinct from the ATPase activity, necessary for promoter clearance from supercoiled templates (Goodrich and Tjian, 1994a). Clearly,

phosphorylation of CTD is not obligatory for releasing RNAPol II from the preinitiation complex on a minimal promoter. It is possible, though, that phosphorylation of CTD is required for activated transcription.

1.1.2.2. CTD-Kinase Activities from different organisms have been purified and characterised. The TFIIF associated CTD-kinase activity phosphorylates CTD during in vitro transcription reactions with highly purified components. Importantly, TFIIE and AdML promoter DNA dramatically enhance the TFIIF CTD-kinase activity (Feaver et al., 1991; Lu et al., 1992; Ohkuma and Roeder, 1994; Serizawa et al., 1993b). Phosphorylation of recombinant or synthetic CTD peptide substrates, though, is not stimulated to a similar extent by promoter DNA (Roy et al., 1994). TFIIF is a good candidate for the kinase, responsible for the transcription associated hyperphosphorylation of CTD in vivo, although the data from the in vitro experiments do not explain the necessity for that modification.

The DNA-dependent protein kinase (DNA-PK) phosphorylates in vitro several DNA binding proteins and the RNA polymerase II CTD domain ((Gottlieb and Jackson, 1993) and the references therein; (Peterson et al., 1992)). (Arias et al., 1991) reported that on immobilised linearised templates the DNA-PK is present in association with the transcription complex and phosphorylates RNAPol II in a promoter dependent manner. DNA-PK consists of a 350 kDa catalytic component (A) and a regulatory component(B), which contains two subunits of 70 and 80 kDa. Component B was recently shown to be identical with the human autoantigen Ku. It is essential for the protein kinase activity and recruits the catalytic subunit to DNA by its intrinsic ability to recognise DNA ends (Dvir et al., 1993; Gottlieb and Jackson, 1993). In this respect, it is difficult to rule out whether the CTD phosphorylation in the experiments of (Arias et al., 1991) results from co-localisation of DNA-PK and RNAPol II on DNA or whether this is a genuine promoter dependent phosphorylation.

Several other protein kinases were also shown to phosphorylate CTD. Two of these contained the p34^{cdc2}-protein (Cisek and Corden, 1989; Stevens and Maupin, 1989) and their function was directly associated with RNAPol II transcription. Two others (CTDK1 and CTDK2) were partially purified and more cautiously implicated in the control of class II gene expression (Payne and Dahmus, 1993).

1.1.3. Sequence Specific Transcriptional Activators

Transcriptional activators act through promoter elements in a sequence specific manner. A transactivator usually contains a specific DNA-binding domain (or a domain which anchors the factor to the promoter by protein-protein interactions), a dimerisation domain that allows formation of homo- or hetero- multimers, and an activation domain (Ptashne, 1988). Transactivation domains are loosely classified as acidic, glutamine rich, proline rich and serine-threonine-rich (Gileadi et al., 1992b). Interestingly, mutagenesis studies indicate that the aminoacid residues, which are most important for activation, are not necessarily the predominant residues in the domain (Cress and Triezenberg, 1991; Gill et al., 1994; Leuther et al., 1993; Vanhoy et al., 1993; Walker et al., 1993). Instead, interspersed hydrophobic residues within the acidic or glutamine aminoacids appear to be important elements of activation (Tjian and Maniatis, 1994).

The structural relationship and functional specificity of the different classes of transactivation domains remain unclear. Since no evidence for some defined secondary structure of activation domains have been obtained (O'Hare and Williams, 1992; Vanhoy et al., 1993), it is speculated that they can assume particular three-dimensional conformation upon adhering to a partner, thus undergoing induced fit (Tjian and Maniatis, 1994). Clues about the specificity of transactivation domains are suggested by the preferential association of

different transactivators to distinct TAFs (see chapter 1.1.1.1., (Goodrich and Tjian, 1994b).

There are many reported interactions between transactivation domains and general transcription factors, TAFs, SRBs and other "mediator" molecules. It is conceivable that most of the effects of transcriptional activators are mediated by these molecules. Some of the interactions between activators and GTFs were described in chapter 1.1.1. Transactivators also act to relieve the repression of histones and other chromatin factors on transcription. However, investigation of the interplay between transcriptional activators and chromatin in vitro are severely limited by the difficulty of correctly assembling chromatin (for review see (Wolffe, 1994; Wolffe and Schild, 1991) and the deficiency of highly purified transcription factors to work in such systems.

1.1.4. Initiation of Transcription by RNAPolymerase II - the Multistep Model

The current view, based predominantly on biochemical data, is that the preinitiation complex (PIC) in vitro is assembled on the promoter from free factors in a highly ordered stepwise fashion. Significant amount of data indicate that the formation of the PIC is nucleated by binding of TBP (or TFIID) to the TATA box. For basal transcription, TBP is sufficient for the subsequent incorporation of the other GTFs. Transcriptional regulation by activators, however, requires the entire TFIID complex and other factors such as the SRBs, for example. The next step, which is believed to be a rate limiting stage in the formation of PIC, is the association of TFIIB. TFIIB has at least two functions. It stabilises the TBP-DNA complex and is responsible for the recruitment of pol II-TFIIF into PIC via contacts with RAP30. At this stage it is likely that dissociation of unstable TBP-DNA complexes takes place, possibly promoted by negative regulators of transcription (if not highly purified TFIID is used). In

basal transcription, TFIIA and TFIIB antagonise the effect of these transcriptional inhibitors. Upon the entry of RNAPol II into the PIC, DNA is melted at the initiation site and the first phosphodiester bond is synthesised. TFIIIE, TFIIF and TFIIJ join the PIC after the incorporation of RNAPol II. Following the formation of a complete PIC, RNAPol II-CTD domain is phosphorylated by the TFIIF associated kinase. ATPase hydrolysis, most likely by one of the TFIIF helicase activities, is also required for the transition from initiation to elongation. Both the kinase and ATPase activity of TFIIF are believed to be essential for promoter clearance, although some uncertainty comes from recent reports (Goodrich and Tjian, 1994a; Serizawa et al., 1993a; Timmers, 1994). After promoter clearance the polymerase is released from its contacts with the initiation factors and synthesis of RNA proceeds. TFIIIS clearly is not necessary for transcription initiation. Upon pausing of the polymerase (at the end of a G-less cassette or at a natural pausing site), though, this factor is required for resumption of elongation. Other two GTFs - TFIIF and TFIIJ - have been also indicated to stimulate elongation in reconstituted *in vitro* transcription systems, although their function is not well understood.

After the disruption of the preinitiation complex TBP (TFIID) and probably TFIIA remain at the promoter, poised for reinitiation events. TFIIB and TFIIIE leave the preinitiation complex and can recycle between different templates in a template commitment assay, while both subunits of TFIIF travel along with the phosphorylated form of RNAPol II (as determined by western blot analysis of elongation complexes on immobilised templates). Most interestingly, TFIIF is decomposed upon transition from initiation to elongation, so that a phosphorylated variant of p62(TFIIF) remains with the elongating polymerase, while ERCC2 and ERCC3 leave the complex (D. Reinberg, personal communication). The disruption of TFIIF might explain why this factor can not be recycled in template commitment assay.

Of the GTFs, TFIIB, TFIID, TFIIF and pol II are required for most in vitro basal transcription systems, while TFIIE and TFIIH were found dispensable for transcription from supercoiled templates as described in chapter 1.1.1.8. (Flores et al., 1992; Parvin and Sharp, 1993; Tyree et al., 1993).

According to the stepwise model for the formation of the preinitiation complex, transactivators exert their function mainly by recruiting GTFs to the promoter via protein-protein contacts with different components of the basal transcription machinery or "adaptor" molecules. They are also believed to enhance the rate limiting step in the formation of DB-DNA complex. In addition, (Choy and Green, 1993) demonstrated that acidic transactivation domains can also enhance the stability of the preinitiation complex, possibly by increasing the affinity of interactions between the GTFs. Based on significant amount of in vitro obtained data, the major consequence of transcriptional activation appears to be increased rate in the formation of productive initiation complexes.

So far, almost no influence of transactivators on other stages in the RNAPol II transcription cycle has been suggested. It is assumed that once a preinitiation complex is formed, promoter clearance and elongation automatically take place. Not in complete agreement with that assumption, however, is the fact that in vitro longer templates are less efficiently transcribed (D.Reinberg, personal communication). One explanation for these observations is that the in vitro systems with highly purified initiation components are deficient in elongation factors. Another possibility is that in the absence of activators and auxiliary factors the transcription complexes lack the ability to elongate efficiently. That ability at present seems enigmatic, but several points of evidence indicate that transactivators might promote novel functions for GTFs as compared to basal transcription. TFIIA has been presumed as an anti-suppressor factor, but only recently shown to be necessary for activated transcription in vitro (Ma et al., 1993). It is not known whether the anti-

Using a similar approach, (Kim et al., 1994) purified a RNA polymerase II activity, which consisted of about 32 polypeptides. This activity transcribed minimal promoters with higher efficiency than the in vitro transcription system, reconstituted from purified factors, and was responsive to activators upon addition of TFIIB, TBP, TFIIE and TFIIH. Furthermore, the transcription associated phosphorylation of RNAPol II-CTD by TFIIH was about 8 fold more efficient when using the "holoenzyme" as compared to the reconstituted system. SRB2, SRB4, SRB5, SRB6, SUG1, GAL11 and the three subunits of yeast TFIIF were found in the complex, while no TBP or TAFs were detected. GAL11 was recently shown to enhance activated transcription through its effect on the basal transcription machinery rather than being a gene specific factor (Sakurai et al., 1993). The SUG1 gene was characterised as a suppressor of an activation defective GAL4 mutant (Swaffield et al., 1992). The SRBs (Suppressors of RNA polymerase B) were isolated as suppressors of a deficiency in transcription activation, caused by partial truncation of the RNAPol II CTD (Thompson et al., 1993). Initially, SRBs were copurified with RNAPol II and TBP and considered as yeast functional analogues of TAFs (Thompson et al., 1993). (Kim et al., 1994) demonstrate that highly purified "holoenzyme" is devoid of TBP or TAFs. Thus, the SRBs, GAL11 and SUG1, which were previously reported to mediate activation, were all found complexed in an activator responsive pol II complex. It is possible that these proteins comprise a novel class of "adaptors" distinct from the TAFs.

(Kim et al., 1994) estimate that at least half of the RNA polymerase II molecules in the yeast cell are associated with the "holoenzyme". The holoenzyme itself can be separated into "mediator" of transactivation and "core" enzyme. The "mediator" contained about 20 polypeptides, including the SRBs, GAL11 and SUG1. It was indicated that the "core" enzyme can respond to activators upon addition of GTFs and either yeast TAFs or "mediator", but

the authors did not assay for the joint effect of the "mediator" and TAFs on activated transcription.

The integrity of the "holoenzyme" may depend on the method of fractionation, which can in part explain the discrepancy in the content of the activator responsive pol II complexes, prepared by these two groups. Notably absent from both complexes are TFIIA, TBP and TFIIE. Moreover, TFIIA was not required either for basal nor activated transcription by the holoenzyme. The current view of TFIIA is that it competes with negative factors that adhere to TFIID and block the assembly of the preinitiation complex. Hence, negative factors such as ADI (Auble and Hahn, 1993) are possibly absent in these in vitro transcription reactions. Alternatively, the holoenzyme is already in a pre-activated state, which is not sensitive to inhibitors of transcription.

The in vitro transcription assays, employed by (Koleske and Young, 1994) and (Kim et al., 1994) do not distinguish whether there are differences in the properties of the elongating polymerases, initiated by purified factors or by the "holoenzyme".

Purification of an activator responsive RNAPol II "holoenzyme" provides biochemical confirmation of the genetic evidence that the SRBs, SUG1 and GAL11 are all required for activation of transcription in yeast. It remains to be elucidated whether these proteins and the TAFs could further co-operate in activation of pol II transcription. Finally, the studies by (Koleske and Young, 1994) and (Kim et al., 1994) raise the question whether activator dependent enhancement of PIC assembly actually operates in vivo.

1.2. Control of Transcriptional Elongation by RNAPol II

As discussed in chapter 1.1.4., most transcriptional regulation in eukaryotes is believed to be mediated by transactivators modulating the rate of

initiation by pol II. There are several examples however, of regulation at the level of transcriptional processivity; that is the ability to elongate through sites where the polymerase is liable to pause or terminate prematurely (reviewed by (Greenblatt et al., 1993; Lis and Wu, 1993). It is not known whether different factors control initiation and elongation respectively. Nor is it known whether regulation of processivity is widespread or confined to a few special cases.

1.2.1. Transcriptional Attenuation in Viral Genes

1.2.1.1. HIV-1

One of the most intensely studied examples of regulation of transcriptional elongation is that of HIV-1. Efficient synthesis of HIV-1 mRNA requires the virally encoded protein TAT, which binds to a stem-loop structure in the 5' region of the viral RNA (TAR). Premature termination in that gene occurs at multiple sites downstream of the stem-loop. Nuclear run-on experiments indicated 5'-3' decline in the density of polymerases in the absence of TAT. In the presence of TAT, though, a high density of polymerases throughout the transcription unit and accumulation of long RNAs was observed both in vitro and in vivo (Kao et al., 1987; Feinberg et al., 1991; Marciniak and Sharp, 1991; Kato et al., 1992). This led to the prediction that TAT is a sequence-specific anti-termination factor (Feinberg et al., 1991; Feinberg and Green, 1992), although some evidence did not fully support such an idea. Deletion of the TAR (initially proposed to be the termination directing element) did not reduce the degree of transcriptional attenuation throughout the gene (Laspia et al., 1989), and TAR mediated stimulation of transcriptional processivity by TAT decreased as TAR was moved away from the promoter (Selby et al., 1989). TAT or the adenovirus protein E1a each stimulated

initiation from the HIV-1 LTR promoter in HeLa cells by more than 15 times (Laschia et al., 1989). E1a, however, had far smaller effect on processivity, which was low and resembled that of basal transcription from the gene. In contrast, TAT enhanced both initiation and efficient elongation through the TAR element. These results implied that TAT, in addition to suppressing of premature termination, could function like transcriptional activators in stimulating initiation.

A further indication that TAT resembles conventional DNA binding transcriptional activators came from the observation that the VP16 transactivation domain increased initiation, when targeted to the promoter via TAR by fusion with the TAT RNA binding domain (Tiley et al., 1992). This implied that TAT might function as an activator. It has also been shown that TAT stimulates transcription from a synthetic LTR promoter as a GAL4-TAT fusion protein, targeted to DNA (Southgate and Green, 1991). However, TAT function required co-operation with other transactivators, since TAT alone was not able to increase CAT expression of an HIV1-LTR-CAT reporter either via its cognate RNA binding site or when targeted to DNA by a GAL4 binding domain. When a synthetic LTR promoter was activated to high levels by GAL4-E1a or GAL4-VP16, TAT had little effect on HIV-1 transcription (Southgate and Green, 1991), implying that TAT and conventional activators stimulate HIV-1 expression in a similar way. Hence, TAT seems to function via interactions with promoter-bound factors by enhancing the formation of elongation-competent transcription complexes (Cullen, 1993). Recent findings demonstrated that TAT could directly associate with TBP and TFIID (Kashanchi et al., 1994), thus confirming its predicted capacity to intimately influence the transcription initiation machinery.

The complexity in the control of the HIV1 LTR promoter requires low levels of basal transcription in order to provide the means for TAT transactivation. Clear evidence for a independent promoter element (IST -

inducer of short transcripts), which is essential for the production of short HIV-1 transcripts, was provided by (Sheldon et al., 1993). IST was mapped between -5 and +26 relative to the start site of HIV1 LTR promoter and mutations in it substantially reduced the formation of non-processive transcription complexes, while having no effect on transactivation by TAT. In another study, the TATA element was also shown to be essential for directing non-processive transcription from the LTR promoter (Lu et al., 1993).

1.2.1.2. Control of Transcriptional Elongation in Other Viruses

In eukaryotes, attenuation was first documented in the Adenovirus type 2 by investigation of RNA synthesis in isolated nuclei (Evans et al., 1979). At a late stage in infection, transcripts from the AdMLP terminate prematurely approximately 120 and 180 bases downstream of the initiation site. These short RNAs were stable enough to be isolated from infected cells or from in vitro transcription reactions (Hawley and Roeder, 1985; Maderious and Chen, 1984). It was concluded that the short RNAs observed resulted from true termination rather than being products of pausing or processing of longer transcripts, since these RNAs can not be chased into longer transcripts. The RNA sequence preceding the termination site at +180 has the potential to form a stable stem-loop structure and is followed by a stretch of 5 U's, where termination takes place (Seiberg et al., 1987).

Attenuation of transcription was also observed during late infection of SV40. In vitro, nuclei isolated from the infected cells produced a 95 base RNA species from the viral major late promoter (Hay et al., 1982). The prematurely terminated RNA contained two mutually exclusive stem-loop structures, followed by five U's. Truncated SV40-MLP RNAs have not yet been detected in vivo, presumably because of their instability (Resnekov et al., 1989).

Cessation of transcriptional elongation at discrete sites has been also documented in the minute virus of mice (MVM) and the polyomavirus (Grass et al., 1987; Resnekov and Aloni, 1989). In these cases attenuation of transcription within the first 200 bases of the transcribed units appeared early or late during the infection, respectively. Stem-loop structures and stretches of U's in the transcribed RNAs were implicated for the possible mechanism of termination.

The premature termination of transcription from AdMLP, SV40-MLP, the P4 promoter of MVM and the early promoter of polyomavirus was temporally regulated during the course of infection (Evans et al., 1979; Grass et al., 1987; Hay et al., 1982; Resnekov and Aloni, 1989), which led to the suggestion that attenuation could serve as a control mechanism. In these cases, a stem-loop secondary structure followed by a stretch of U's in the elongating RNA seems to play an important role in the process of termination. Indeed, mutations that destabilised the stem-loop or reduced the number of U residues suppressed the efficiency of the elongation block in MVM, SV40 and Adenovirus (Bengal et al., 1991; Kessler et al., 1989; Seiberg et al., 1987). Such structure is reminiscent of the intrinsic prokaryotic rho-independent and some factor dependent terminator elements (for review see (Greenblatt et al., 1993; Spencer and Groudine, 1990b). This raises a possibility of further analogy in the patterns of termination and antitermination between eukaryotic viruses and prokaryotes. Although eukaryotic mechanisms of antitermination, resembling these mediated by phage lambda-N and Q proteins in E.coli can not be ruled out, no convincing experimental support for such hypothesis has so far been produced. The only parallel that can be drawn is between the lambda-N and the HIV-TAT proteins. Both of them carry a arginine-rich motif and exert their function via binding to RNA. Nevertheless, it seems that TAT stimulates transcription of HIV-1 in an essentially different way (at the level of

initiation, see chapter 1.2.1.1.), compared to the lambda-N protein, which modifies established elongation complexes.

1.2.2. Transcriptional Attenuation in Cellular Genes

1.2.2.1. Premature Termination of c-myc Transcription

In normal cells c-myc is subject to a complexity of control mechanisms in a diversity of cell types and under a variety of physiological conditions. C-myc transcription is upregulated by different proliferative agents such as mitogens or growth factors and conversely, it is downregulated by differentiation signals (Bentley and Groudine, 1986b; Eick and Bornkamm, 1986; Lindsten et al., 1988). In addition, c-myc expression directs the processes of programmed cell death - apoptosis (Evan et al., 1992). Not surprisingly for an important factor in cell growth and differentiation, aberrant regulation of c-myc is associated with a wide variety of neoplasms.

The steady-state level of c-myc RNA is controlled by modulating transcription initiation, elongation and mRNA stability (reviewed by (Spencer and Groudine, 1991). The mouse and human genes are transcribed by two promoters, P1 and P2, which are separated by about 160 bp. Transcripts, originating from the P1 promoter, read the full length of the gene or terminate prematurely at position T1, which overlaps the P2 TATA box (Wright et al., 1991; Roberts et al., 1992). Transcripts, originating from the P2 promoter, terminate at position T2 near the end of the first exon (Bentley and Groudine, 1988). Notably, P1 transcripts do not terminate at T2. Enhanced usage of P1, thus surpassing the block of elongation at T2, has been suggested to have an important impact for the deregulation of c-myc in Burkitt's lymphomas (Spencer and Groudine, 1990a).

The first indication that c-myc is regulated at the level of transcriptional elongation came from experiments with differentiating human HL-60 cells (Bentley and Groudine, 1986a; Eick and Bornkamm, 1986). Upon treatment with retinoic acid these cells differentiate into granulocytes and reduce the steady-state level of c-myc RNA more than 10 fold. Nuclear run-on experiments, which detect the density of RNA polymerases over discrete segments of the gene, indicated that the rapid initial downregulation of c-myc results from a 10 fold increase in a block of transcriptional elongation at the 3' end of exon 1. In normal human T-lymphocytes or tonsillar cells stimulated by mitogens, increased levels of expression of c-myc are due partially to release of that elongation block (Eick et al., 1987; Lindsten et al., 1988). Short truncated RNAs, corresponding to attenuated products at the end of exon 1 have not been detected in vivo presumably because they are highly unstable. When the murine or human c-myc are injected in *X.laevis* oocytes or analysed in vitro, though, premature termination occurs at T tracts positioned at the end of exon 1 or at the beginning of intron 1, respectively (Bentley and Groudine, 1988; London et al., 1991). These RNA species were unlikely to be products of splicing of full length transcripts, since injection of synthetic full length RNA did not result in truncated RNAs. The sequences preceding the sites of termination have a potential of forming a stem-loop structure in the transcribed RNA both in the human and the mouse c-myc. A 95 bp fragment from the human and 180 bp fragment from the murine gene were sufficient to program premature termination in *X.laevis* oocytes, when positioned downstream from some, but not all heterologous promoters (Bentley and Groudine, 1988; Roberts and Bentley, 1992). Interestingly, deletions of the T stretches (Bentley and Groudine, 1988) did not reduce the efficiency of the elongation blockage in *X.laevis* oocytes. Furthermore, the sequences around T2 were found dispensable for attenuation in human cells as determined by nuclear run-on assay. Instead, sequences upstream of position +47 of P2

conferred the attenuation of c-myc transcription (Krumm et al., 1992) and see below). The idea that promoter elements rather than terminators are essential in the control of elongation in c-myc was supported by mutational analysis of the c-myc P2 promoter (D. Bentley, published in (Yankulov et al., 1994). In these experiments, mutations of putative transactivator binding sites upstream of the murine P2 promoter (Me1a1, E2F and Me1a2) reduced the level of readthrough transcription, while mutation of the TATA element preferentially reduced the level of terminating transcription.

The efficiency of premature termination at the c-myc terminator (T2) was dependent on the distance from the start site (Bentley and Groudine, 1988; Roberts et al., 1992; Spencer et al., 1990). Higher levels of attenuated transcripts were observed when the T2 element was closer to the start site, while almost no termination was detected if this element was more than 500 bp downstream of the initiation site regardless of which promoter was used.

The results, obtained *in vitro* and in *X.laevis* oocytes by analysis of steady-state RNA and in mammalian cells by nuclear run-on assays suggested that a genuine pausing or termination of RNAPol II takes place at the exon 1/intron 1 boundary. Surprisingly, *in vivo* detection of ssDNA regions by treatment with KMnO₄ (presumably caused by open pol II complexes, *in vivo* footprinting of RNAPol II) demonstrated no paused RNAPol II at that position in HL60 cells (Krumm et al., 1992). KMnO₄ sensitive sites, however, were detected about 30 bases downstream of the P2 initiation site both in non-differentiated and differentiated HL-60 cells. No such KMnO₄ sensitive sites were detected *in vitro* or in *X.laevis* oocytes (Meulia et al., 1993). Of potential interest is the fact that just upstream of +30 in the human c-myc there is a sequence of dyad symmetry, capable of forming a stem-loop structure. High-resolution nuclear run-on analysis of c-myc elongation in HL-60 cells supported the notion of polymerase paused at +30, which was released during the assay (Krumm et al., 1992; Strobl and Eick, 1992). Interestingly, in the run-

on analysis the polymerases from differentiated cells were significantly less processive and terminated transcription in the promoter-proximal region, while those from non-differentiated cells elongated more efficiently in the run-on reaction. Sequences downstream of position +47 were found completely dispensable for the attenuation of transcription. The authors speculate that promoter-proximal pausing in c-myc provides a potential signal for modification of RNAPolymerase II, which transforms it into an elongation competent form. In *X.laevis* oocytes such a transformation was not associated with pausing at +30. It was possible that different chromatin structure of c-myc in these two systems might influence promoter-proximal pausing.

The observations of (Krumm et al., 1992; Meulia et al., 1993) raise the obvious question whether the control of c-myc transcriptional processivity in mammalian cells and in oocytes is underlayered by a common mechanism or not. Recent investigation in our laboratory suggests a positive answer to that question. Full discussion of this problem will be given in section 3.1.3.

1.2.2.2. Transcriptional Attenuation in Other Cellular Genes

Pausing or polymerase 'hold-back' close to the start site, similar to that in the human c-myc, has also been found in β 1-tubulin, glyceraldehyde-3-phosphate dehydrogenase and polyubiquitin genes in *Drosophila* (Giardina et al., 1992; Rougvie and Lis, 1990) and the human transthyretin gene (Mirkovitch and Darnell, 1992). In hsp70, a high density of RNAPol II complexes in the promoter- proximal region of the gene can be detected even in cells, which have not been heat shocked. Unlike c-myc, in vitro these complexes can be released only by high salt or sarcosyl treatment (Rougvie and Lis, 1988). Activation of HSF by high temperature in vivo facilitates release of the polymerases, which are paused at position +25 relative to the start site, and

stimulates high levels of transcription over promoter-distal regions of this gene (Rougvie and Lis, 1988; Rougvie and Lis, 1990). Mutations in the hsp70 promoter known to bind another factor (GAGA factor) reduced markedly the amount of "hold-back" polymerases (Lee et al., 1992). GAGA is believed to prevent association of nucleosomes or histone 1 with DNA, allowing access of transcription factors to the promoter (Kerrigan et al., 1991). Thus, promoter-proximal arrest in this gene is dependent on sequence specific transcriptional factors. Interestingly, polymerases stalled at the 5' end in the quiescent gene are hypophosphorylated on the CTD, whereas the elongating polymerases are a mix of hypo- and hyperphosphorylated forms (Weeks et al., 1993).

Elongation arrest mechanisms also contribute to developmental timing and tissue specificity in the expression of N-myc and L-myc genes (Xu et al., 1991). In L-myc the block of transcriptional elongation is mapped within the first intron of the gene. Loss of this block accounts for the high steady-state levels of L-myc mRNA in some small cell lung carcinomas (Krystal et al., 1988). In human pre-B cells N-myc transcription is attenuated between exon 1 and 2. Transcriptional processivity over that region is stimulated by interleukin-7 (Morrow et al., 1992). It is not known whether the mechanisms of attenuation between c-myc, L-myc and N-myc are related.

Attenuation of transcription at the 5' region of other mammalian genes has also been reported. Steady-state levels of N-ras (Jeffers and Pellicer, 1992), c-myb (Bender et al., 1988; Watson, 1988), c-fos (Mechti et al., 1991) and ADA (adenosine deaminase, (Maa et al., 1990; Ramamurthy et al., 1990) messenger RNAs are at least partially controlled at the level of premature termination of transcription. In all these cases modulations of transcriptional elongation were in response to extracellular signals or corresponded to the tissue specific distribution of mRNA.

When transcriptional elongation of the murine ADA gene was investigated in injected *X.laevis* oocytes, termination occurred mainly at

position +96 relative to the initiation start site (Ramamurthy et al., 1990). Surprisingly, deletion of 65 bp fragment beginning 8 bp 3' to the termination site decreased transcriptional blockage at +96, but increased attenuation at a secondary site 189 bp further downstream.

Interesting data, which are in tune with the notion that the actual site of termination might not be the most important determinant in attenuation, were provided by analysis of the *X.laevis* α -tubulin gene in injected oocytes (Hair and Morgan, 1993). The 3' ends of the truncated RNAs were mapped immediately downstream of a stem-loop structure in the 5' leader. Deletion of that structure did not increase the level of extended transcripts, but premature termination continued at non-specific sites farther downstream. Sequences from -200 to +19 were sufficient to program attenuated transcription. Furthermore, competition with the same fragment specifically stimulated the blockage of elongation in the wild type gene. As in the case of the *c-myc* P2 promoter, these findings indicate that promoter-dependent disruption of elongation rather than abrogation of a specific antitermination mechanism is the cause of premature termination.

1.3. Mechanisms of Control of Transcriptional Elongation

1.3.1. The Inhibitor of RNAPol II elongation DRB

The adenosine analogue 5,6-dichloro-1-b-D-ribofuranosylbenzimidazole (DRB) is a well documented inhibitor of RNAPol II transcription which acts at the level of elongation. Initial experiments revealed that DRB reduced the production of long mRNAs, while short RNAs were not affected by the drug (Tamm, 1977; Tamm and Sehgal, 1977; Zandomeni et al., 1983; Zandomeni et al., 1982). Subsequently it was demonstrated that in several genes where

natural pre-mature termination occurred, DRB inhibited only the fraction of polymerases which could read through the attenuation sites (Chodosh et al., 1989; Marciniak and Sharp, 1991; Marshall and Price, 1992; Roberts and Bentley, 1992). Based on these studies, a hypothesis of two classes of RNAPol II complexes with different processivity was introduced. Processive and non-processive transcription complexes can be distinguished by two major criteria. Non-processive polymerases are released from the template at termination sites within the first few hundred bases of the transcription unit (Marshall and Price, 1992). For this reason, non-processive complexes are mostly found in the 5' part of the transcription unit while processive polymerases are mostly found at promoter-distal positions (Marciniak and Sharp, 1991; Roberts and Bentley, 1992). Processive and non-processive transcription complexes also differ in their susceptibility to DRB which specifically inhibits the processive form. According to the hypothesis, non-processive complexes have to be converted into the processive form to allow read through of potential termination sites. DRB was predicted to inhibit that conversion (Marciniak and Sharp, 1991; Roberts and Bentley, 1992; Marshall and Price, 1992).

Several points of evidence support the idea that the conversion into processive form occurs at promoter-proximal positions or at the level of initiation. For example, addition of DRB after initiation *in vitro* (Zandomeni et al., 1983) or during the elongation stage of run-on analysis (Roberts and Bentley, 1992) had no effect on transcription. In addition, complexes which had travelled more than 500 bases were resistant to DRB (Roberts and Bentley, 1992; Kephart et al., 1992; Marshall and Price, 1992). These data clearly indicate that although DRB inhibits transcription at the level of elongation, it can not promote intragenic termination throughout the transcription unit. RNAPol II complexes, which have already been converted to high processivity mode, are not sensitive to the drug. Two studies, though, directly contradicted that model

(Chodosh et al., 1989; Marshall and Price, 1992). In these assays, addition of DRB subsequently to initiation inhibited the synthesis of longer transcripts, although continuous reinitiation events or modification of pol II complexes at early stage of elongation were not ruled out.

The molecular basis for the difference in processivity and susceptibility to DRB between different pol II complexes is unclear. Insight into the mechanism of action of DRB is provided by the fact that in vitro it can inhibit several protein kinases, including some CTD kinases (Zandomeni et al., 1986; Cisek and Corden, 1989; Stevens and Maupin, 1989). A recent study by (Dubois et al., 1994) indeed showed inhibition of the phosphorylation of RNAPol II CTD by DRB, which correlated with the level of RNA synthesis in vivo. The effect of DRB was very similar to that of two well characterised protein-kinase inhibitors: H-7 and H-8. The work of (Dubois et al., 1994), though, does not distinguish whether dephosphorylation of CTD results from inhibited transcription (hyperphosphorylation of CTD is associated with actively transcribed polymerases in vivo) or alternatively, inhibition of a CTD kinase directly suppresses transcription.

Better understanding of how exactly DRB operates in the control of transcriptional elongation is hampered by the lack of reasonable target for this drug. Marshall and Price (1992) have suggested the existence of P-TEF (Positive-Transcription Elongation Factor), which converts elongation complexes from abortive into processive mode before the polymerases have synthesised several hundred bases. Besides speculating that P-TEF could be a kinase, nothing else is known about this factor.

1.3.2. Role the Promoter in Control of RNAPol II Elongation

Throughout this review I described several cases in viral and cellular genes, where attenuation of transcription was implicated in the overall control of their expression. In some of these genes discrete sites of premature termination were characterised and shown to operate in the context of different promoters and different expression systems. Although no sequence homology between different intragenic terminators was reported, RNA stem-loop structures followed by stretches of T's in DNA were proposed to constitute the attenuation responsive elements in some, but not all terminators. Paradoxically, deletion of the T2 element from c-myc (Krumm et al., 1992), TAR from HIV-1 (Sheldon et al., 1993) and the X.laevis α -tubulin terminator (Hair and Morgan, 1993) respectively, does not abolish attenuation, but shifts the positions where the polymerases pause or cease elongation. A plausible explanation for this observation is that though they are not essential for termination per se, these elements facilitate efficient termination at discrete sites making it more easily detectable than if it occurred inefficiently at many positions. Another indication that transcriptional elongation might be controlled at positions, distal from terminator elements, is the fact that no factors, binding to the sequences that direct premature termination, have so far been discovered.

As described in the previous chapter, efforts to explain the mechanism, by which DRB inhibits elongation, led to the hypothesis of promoter-proximal modification of RNA polymerase II complexes. Thus, the question whether the promoter regulates transcriptional processivity becomes quite important.

The promoter has been implicated in the control of transcriptional elongation in the U1 and U2 snRNA, c-myc, HIV1 and Hsp70 genes. The U1 and U2 snRNA promoters are essential for the generation of transcription

complexes which recognise the 3' box terminator and therefore produce correct 3' ends (Neuman de Vegvar et al., 1986; Hernandez and Weiner, 1986). Replacement of U1 and U2 promoters with HSV TK or HIV-1 promoters, respectively, resulted in decreased usage of the 3' box and increased termination at heterologous downstream poly A sites. In Hsp 70 and Hsp 26 genes, activation of the promoter bound HSF releases paused polymerases and dramatically induces transcription over the 3' region of the gene (Lis and Wu, 1993). Similar mechanism of modulation of transcriptional processivity exists in the c-myc gene. Mutations or deletion of putative transactivator binding sites (Me1a1, E2F, Me1a2) markedly decreased transcription through the T2 termination element in oocytes (D.Bentley, published in (Yankulov et al., 1994).

Interesting possibility that promoter based factors could positively regulate elongation of pol II transcription also comes from analysis of synthetic HIV-1 genes (Southgate and Green, 1991). GAL4-VP16 and GAL-E1a stimulated transcription to high levels when targeted to the synthetic promoter. In these experiments processivity was not addressed, but in the presence of GAL4-VP16 and GAL-E1a TAT had no additional effect on the expression of the reporter CAT gene. Thus upregulation via promoter elements circumvented the requirement for a stimulator of elongation. The fact that TAT enhances both initiation and elongation (see chapter 1.2.1.1) further supported a hypothesis that strong transactivation domains could increase processivity of transcription from the HIV-1 LTR promoter (Cullen, 1993; Greenblatt et al., 1993).

Since attenuation has been observed at the 5' regions of many genes, it is reasonable to expect the existence of promoter based sequences which generate non-processive transcription. In the Hsp70 gene, such element is the GAGA factor binding site which is required to establish paused polymerase (Lee et al., 1992), whereas in the human c-myc sequences upstream +47 relative to the start site are sufficient to confer promoter pausing (Krumm et al., 1992).

Detailed analysis of the mouse c-myc P2 promoter in *X.laevis* oocytes indicated that mutations in the TATA box significantly reduced transcription, but surprisingly, completely abolished termination at the T2 site. Similar requirement for the TATA element in maintaining low levels of non-processive transcription was observed in HIV-1 too (Lu et al., 1993). In addition, production of attenuated transcription in HIV-1 also requires IST (see 1.2.1.1, (Sheldon et al., 1993) which overlaps the initiation site.

IST can support non-processive transcription when incorporated into other promoters and mutations in it do not affect TAT transactivation (Sheldon et al., 1993). Based on its autonomy and sequence, IST is reminiscent of Initiator elements, found in many cellular genes (for review see (Weis and Reinberg, 1992). Initiators are necessary and sufficient for accurate transcription initiation in vitro at TATA-less promoters. They were also found in many TATA containing promoters, but their in vivo significance in such a context has not been determined. It is possible that the IST element represents a initiator motif in the HIV-1 LTR. Most interestingly, there is a significant similarity in the sequence of the transcription start site of the human c-myc, HIV-1 LTR and the Ad2ML promoters, all of which produce relative high levels of non-processive transcription (Krumm et al., 1993). It is then possible that certain initiator-like elements specify initiation events which give rise to prematurely terminated transcripts.

In summary, promoter elements of certain genes can positively or negatively regulate processivity of pol II transcription. Negative elements in HIV-1 and c-myc coincide with sequences that are required for basal or non-activated transcription in vitro. The GAGA factor, which binds the Hsp70 promoter, functions by antagonising chromatin repression of transcription in different promoters. In contrast, in all cases described so far, upregulation of pol II processivity via the promoter is by transcriptional activators. It is not known whether the same activation domains can contribute to increased

processivity of transcription in different promoter contexts. Neither is it known whether all transactivators possess intrinsic properties to increase the elongation competence of RNAPolymerase II.

RESULTS

2.1. Transcriptional Activators Stimulate RNAPol II Processivity

2.1.1. Strategy of Investigation

As described in chapter 1.3.2., promoter elements which bind sequence specific activators, contribute to regulation of the efficiency with which polymerases read through the 5' regions of the transcription units. It was possible that this type of control is confined to c-myc, HIV-1 and some genes which are normally regulated at the level of transcriptional elongation. Alternatively, control of RNAPol II processivity by transactivators could be a widespread phenomenon.

In order to study in detail the role of transactivators on processivity of RNA polymerase II transcription a strategy of investigation, based on the injected *X.laevis* oocyte system, was designed. *X.laevis* oocytes have the advantage that they do not degrade attenuated transcripts so that the amount of terminated and readthrough RNA can be directly quantified by RNase protection assay (Bentley and Groudine, 1988). Whether or not RNA polymerases terminate at T2 or HIV-2 TAR was used as a criterion to indicate whether they are of the processive or the non-processive type (Roberts and Bentley, 1992). Previously it has been shown that processive polymerases which read through the c-myc T2 element do not terminate at a second T2 site in tandem (Roberts and Bentley, 1992). The fraction of transcripts which read through (RT/RT+TM) was a measure of the processivity of transcription. The interpretation of the experiments in my thesis is unaffected by whether or not the 3' ends detected are generated by termination at these sites or termination followed by processing.

Several synthetic genes with promoters, containing five binding sites for the yeast transcriptional activator GAL4 upstream of different TATA elements were constructed. TATA boxes were from the mouse c-myc P2 promoter (Gal5-P2CAT); Adenovirus E4 and E1b promoters (Gal5-E4mycCAT and Gal5-E1bmycCAT); from HIV2 LTR (Emerman et al., 1987) (Gal5-HIV2CAT) and from the human TK promoter with or without an initiator element - II and IV (Smale et al., 1990) - (Gal5-II-TKmycCAT and Gal5-IV-TKmycCAT). All constructs except Gal5-HIV2CAT contained the c-myc T2 terminator element (Bentley and Groudine, 1988) 100-300 bases downstream of the transcription start site. Gal5-HIV2CAT contained the HIV2 TAR terminator element. HIV-1 and HIV-2 LTR promoters are highly conserved (Emerman et al., 1987; Guyader et al., 1987). Transcription from both of them is activated via Sp1 and NF- κ B sites and is attenuated at TAR (Guyader et al., 1987). Schematic representation of the constructs used in this investigation is given in Fig. 1.

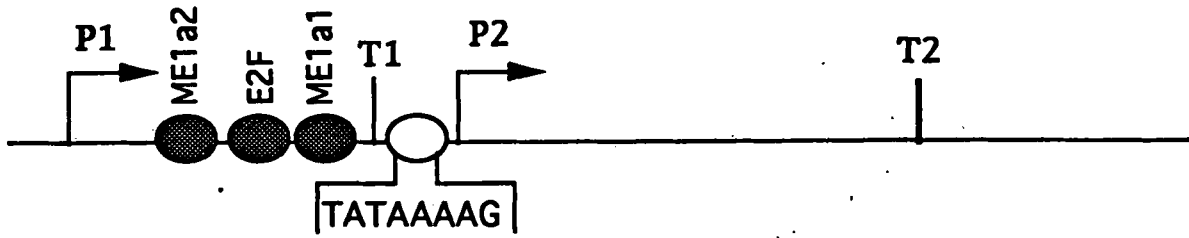
These constructs were co-injected in *X.laevis* oocytes with GAL4-fusion proteins or with BSA as a control. The GAL4-proteins contained the DNA binding domain of GAL4 (GAL4(1-147)) fused to the transactivation domain of the Herpes simplex virus protein VP16 (GAL4-VP16, (Sadowski et al., 1988)); the conserved region 2&3 transactivation domain of the Adenovirus E1a protein (GAL4-E1a, (Lillie and Green, 1989) or the synthetic acidic amphipathic domain AH (GAL4-AH, (Giniger and Ptashne, 1987). VP16 and AH transactivation domains are highly acidic, while E1a(CR2&3) is not. Human recombinant TBP was used to investigate the control of processivity directed by the TATA element. As a control for injection efficiency and RNA recovery, the Adenovirus VA1 gene which is transcribed by pol III, was co-injected with the test plasmids.

RNA from the oocytes, injected with each construct and each of the recombinant proteins was analysed by RNAase protection assay and the products were quantified by a Phosphorimager (Molecular Dynamics) or by densitometry. Processivity (RT/RT+TM) of non-activated transcription (co-

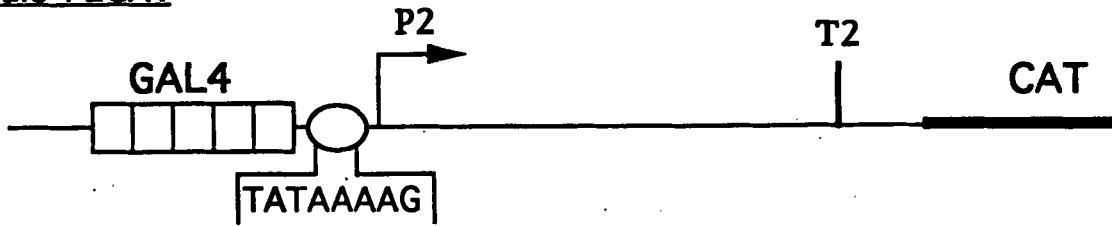
Figure 1. Schematic representation of the genes, used in the analysis of RNAPol II processivity.

The transcription initiation sites are indicated by long arrows. P1 and P2 - Promoter 1 and Promoter 2 initiation sites of the mouse c-myc gene. T1 and T2- termination site 1 and termination site 2 of the c-myc gene. TAR-the transcription activation responsive element of HIV. Short arrows indicate multiple sites of premature termination in the HIV2 TAR. TATA boxes and the TdT initiator element are represented by open circles. The sequences of the TATA boxes and the Initiator are given below each of these elements. Filled in circles mark the positions of transactivator binding sites in the mouse c-myc and the LTR HIV2 promoters. GAL4-binding sites are shown by squares. CAT-Chloramphenicol-Acetyl-Transferase encoding sequence. More details about the constructs used are given in the text and in Materials and Methods.

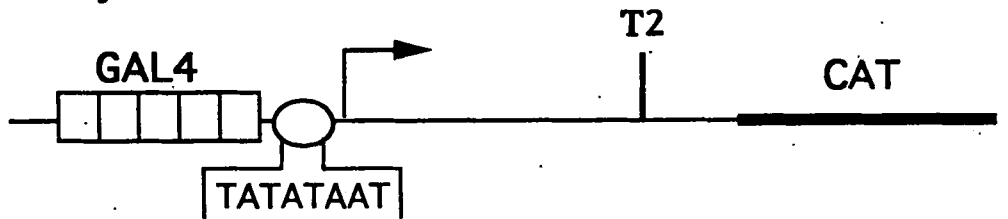
mouse c-myc (pSX943)



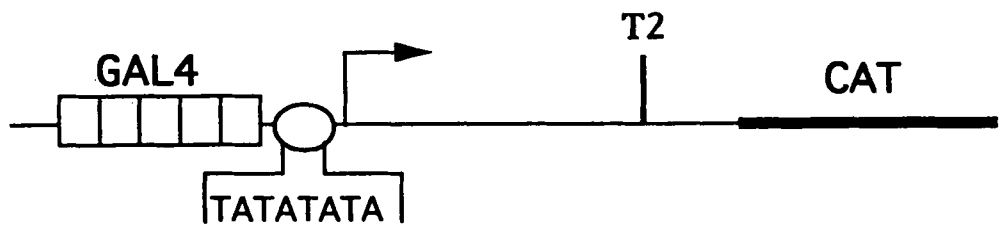
Gal5-P2CAT



Gal5-E1bmycCAT



Gal-5-E4mycCAT



injection of BSA) and transcription, driven by different activation domains or the wild type promoters was estimated.

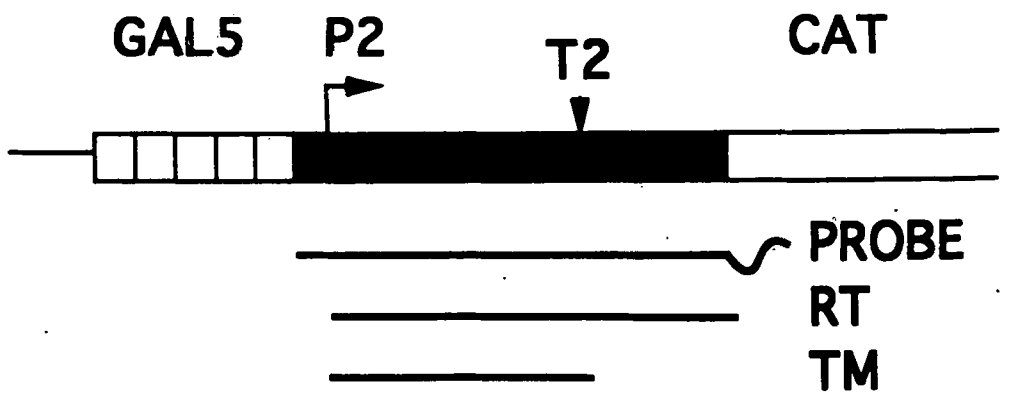
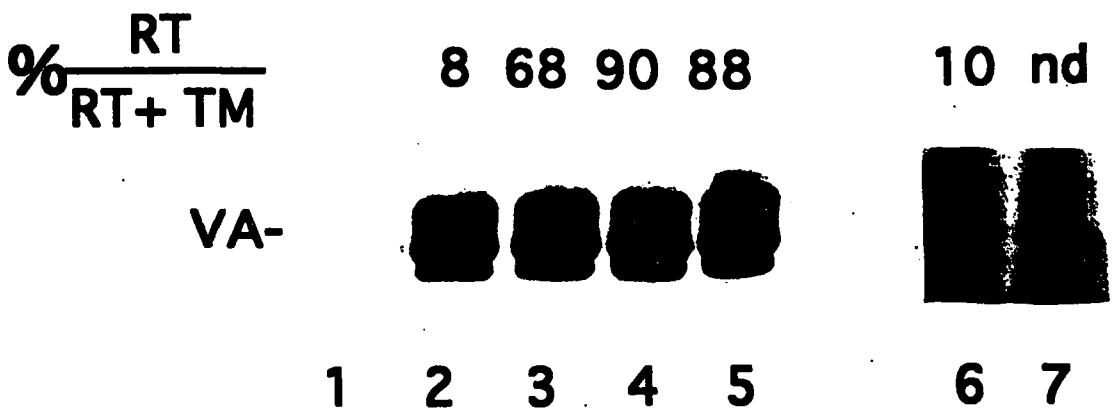
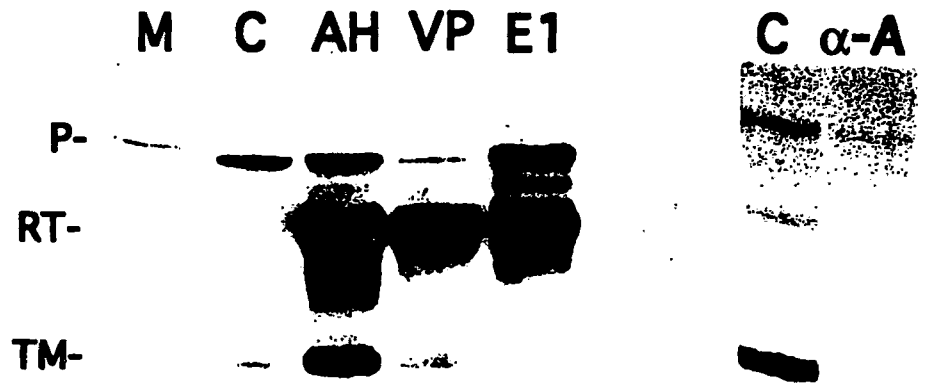
2.1.2. Synthetic Activators Enhance Processivity of c-myc Transcription

C-myc transcription is regulated by attenuation at premature termination sites. Transcripts, originating from the P1 promoter read through or terminate at the T1 site, which overlaps the P2 TATA element (Roberts et al., 1992), while transcripts from the P2 promoter read through or terminate at the T2 site (Bentley and Groudine, 1988). It is possible that this promoter interacts with a special class of activators that can regulate processivity. Alternatively, it is possible that the ability to stimulate elongation could be a general property of transcriptional activators. To address that question, I assayed whether synthetic activators could affect transcriptional processivity from a chimaeric c-myc gene, in which sequences upstream of the P2 TATA box were replaced by five binding sites for the yeast transcription factor GAL4 (pGal5-P2CAT, see Fig. 1). The plasmid was co-injected with BSA as a control or saturating amounts of the recombinant transcription factors GAL4-AH, GAL4-VP16 or GAL4-E1a. The total protein concentration injected was equalised with BSA. Processivity (RT/RT+TM) was determined after quantifying the RT and TM RNAase protection products by a Phosphorimager (Molecular Dynamics).

In the absence of transactivators most of the transcripts from Gal5-P2CAT terminated prematurely at the T2 site (Fig. 2, lanes 2 and 6). The average processivity (RT/RT+TM), measured in 5 experiments, was 10% (Table 1 and Fig. 6). In contrast, the intact c-myc P2 promoter is typically transcribed with about 75% processivity (see for example Figure 12, lane 1). Most of the non-activated transcription was inhibited by 2 µg/ml α -amanitin (Fig. 2, lane 7) as

Figure 2. Activation of processive transcription from a chimaeric c-myc promoter by GAL4 fusion proteins.

RNase protection of transcripts from oocytes injected with Gal5-P2 CAT plasmid plus BSA (C), GAL4-AH (AH), GAL4-VP16 (VP) or GAL4-E1a (E1). The antisense XhoI-BamHI probe was transcribed from pSX943 by T3 RNA polymerase. A map of Gal5-P2 CAT with a diagram of the RNase protection strategy is shown in the lower panel of the figure. M: MspI cut pBR322 markers 404, 309, 242, 238 bp. Probe, P, and protection products corresponding to correctly initiated readthrough (RT) and T2 terminated (TM) RNA are marked. Full length protection of the probe corresponds to RNAs which read all the way around the plasmid. Processivity values (RT/RT+TM) based on Phosphorimager analysis are shown below each lane (The TM band has 1.25 times fewer labelled residues than the RT band. nd: not determined). Lanes 6 and 7 are from a different experiment in which oocytes were injected with BSA with or without 2 µg/ml α-amanitin (final intracellular concentration). RNase protection products from the co-injected Adenovirus VA1 gene (VA) are shown below.



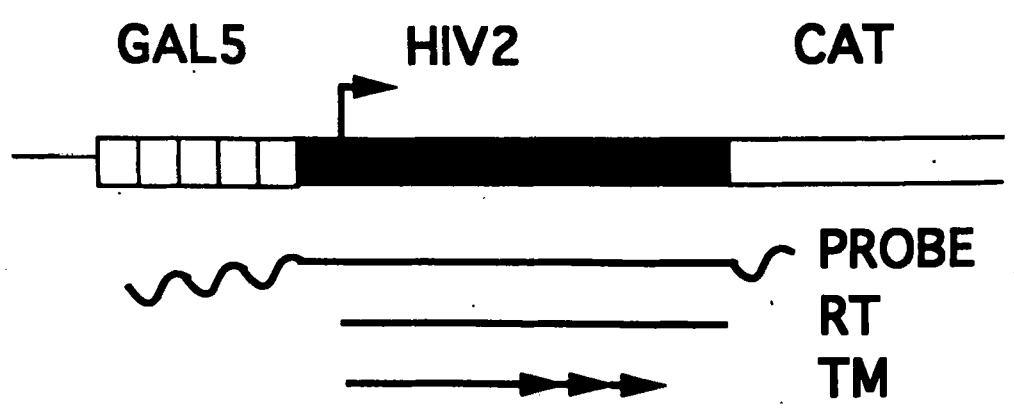
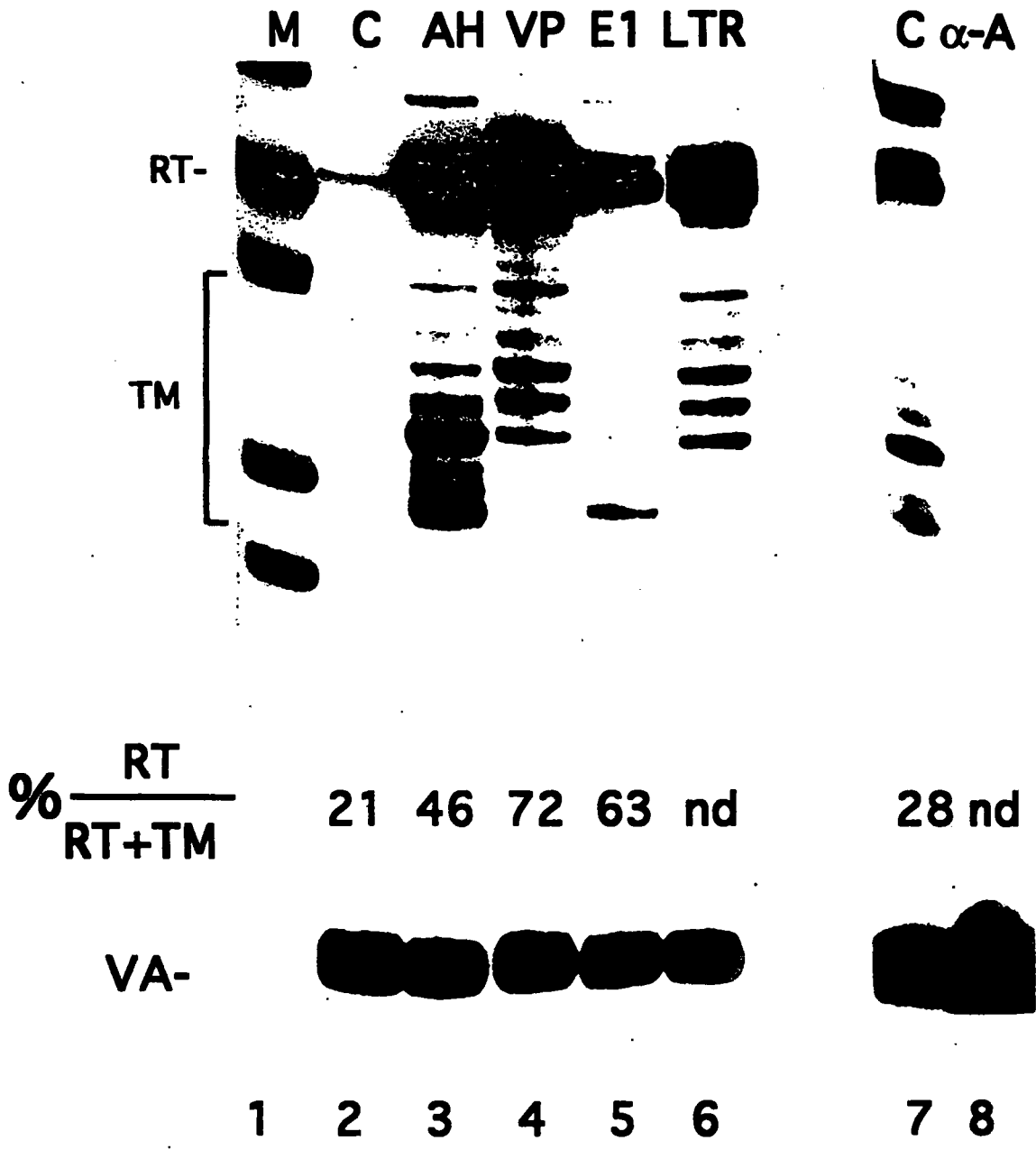
expected for pol II transcription, while transcription of the co-injected VA1 gene was unaffected. (Lanes 1-5 and lanes 6, 7 are from different batches of oocytes).

Next, transcription activated by recombinant transactivators was examined. GAL4-AH, -VP16 and -E1a stimulated transcription from the Gal5-P2 CAT gene approximately 15 fold relative to BSA injected controls (see Table 1 and Fig. 6). Interestingly, the GAL4 activators increased not only the total amount of transcription but also its processivity. In the presence of GAL4-VP16 or GAL4-E1a, the processivity of Gal5-P2 CAT transcription increased from 8% to about 90% (Fig. 2, compare lane 2 with lanes 4,5). GAL4-AH activated transcription had a processivity value of 68% (Fig. 2, lane 3) which is much higher than that of non-activated transcription but significantly lower than GAL4-VP16 or GAL4-E1a activated transcription. Average values of processivity from several independent experiments are given in Table 1 and Figure 7.

In conclusion, the truncated c-myc P2 promoter in which sequences upstream of the TATA box were replaced by GAL4 binding sites was transcribed with far lower processivity in the non-activated state, as compared to the wild type gene. Chimaeric GAL4 transactivators dramatically stimulated processivity of transcription from this promoter however GAL4-VP16 and GAL4-E1a had a consistently larger effect than GAL4-AH.

2.1.3. Synthetic Activators Enhance Processivity of HIV2 Transcription

I wanted to ask whether the effect of synthetic transactivators on transcriptional elongation applied to genes other than c-myc. Initially, a chimaeric HIV2 construct, which has terminator and basal promoter elements, unrelated to c-myc, was tested. Like c-myc, HIV2 produces prematurely



terminated transcripts in oocytes. The 3' ends of these truncated RNAs are in the TAR region 120-145 bases from the start site (Fig. 3, lane 6) in agreement with the 3' ends previously mapped in HeLa transcription extracts (Toohey and Jones, 1989). A HIV2 LTR CAT fusion gene was constructed, in which sequences upstream of the TATA box were replaced by five binding sites for GAL4 (Gal5-HIV2CAT). Processivity of transcription (RT/RT+TM) from this gene was determined in injected *Xenopus* oocytes by quantifying RNAase protection products as for the Gal5-P2CAT gene.

The Gal5-HIV2 gene was transcribed with 25 % processivity on average in the absence of transactivators (Fig. 3, lanes 2 and 7 and Table 1) in contrast to the intact HIV2 LTR which is transcribed with about 70 % processivity (Fig. 3, lane 6). Most of the non-activated transcription was by RNA polymerase II as shown by its sensitivity to 2 μ g/ml α -amanitin (Fig. 3, lane 8. Lanes 2-6 and lanes 7, 8 are from different batches of oocytes.). When saturating amounts of GAL4-VP16 or GAL4-E1a protein were co-injected with the template, processivity increased from 21% with BSA alone to 72% and 63%, respectively (Fig. 3, compare lane 2 with lanes 4,5). Transcription activated by these two proteins closely resembled transcription from the intact HIV2 LTR which is activated by endogenous oocyte factors (Fig. 3, lane 6). GAL4-AH also stimulated processivity relative to the BSA control (46% versus 21%, Fig. 3, compare lanes 2 and 3) but consistently less well than GAL4-VP16 or GAL4-E1a. The results of several experiments in different batches of oocytes are summarised in Table 1 and Fig. 6.

Interestingly, truncated HIV2 transcripts from the intact LTR and from Gal5-HIV2 CAT in the presence of GAL4-VP16 were about 10 bases longer on average than those made in the presence of GAL4-AH, GAL4-E1a or in the absence of activator. 5' end-mapping showed that the HIV2 start site was unaffected by any of the activators (see Fig. 10). This variation in the preferred site of termination may reflect different elongation properties of transcription

complexes activated in different ways. I conclude that synthetic activators stimulate transcriptional elongation in both the HIV2 and the c-myc constructs in a similar manner.

2.1.4. Activation Domains Differ in Their Ability to Stimulate Processivity

GAL4-VP16 and GAL4-E1a consistently stimulated transcription of higher processivity from the Gal5-P2 and Gal5-HIV2 promoters when compared to GAL4-AH (Figs. 2 and 3). This difference could reflect some special property of the HIV2 and c-myc genes which are normally regulated at the level of elongation or a genuine dissimilarity in the functional properties of the three activation domains. I asked whether these activators would have similar effect on processivity of transcription from synthetic promoters, unrelated to c-myc or HIV2. Two constructs composed of five GAL4 binding sites, TATA elements from the Adenovirus E4 or E1b genes and the c-myc T2 element positioned about 110 bases downstream the initiation site were chosen for these assays (pGal5-E4mycCAT and pGal5-E1bmycCAT, see Figure 1). In the experiment presented in Figure 4 the plasmids were injected in *X.laevis* oocytes along with BSA or saturating amounts of recombinant GAL4-AH, GAL4-VP16 or GAL4(1-94) proteins. Correctly initiated readthrough and terminated transcripts were detected by RNase protection assay and quantified by a Phosphorimager (see also Table 1).

Gal5-E4mycCAT and Gal5-E1bmycCAT genes produced low levels of non-activated transcription with less than 5% processivity (Fig. 4, lane 1 and 5), whereas GAL4-AH and GAL4-VP16 activated transcription was far more processive. (The signal in the control lanes, injected with BSA only, is too weak to be seen in Figure 4. Representative signals can be observed under longer exposure of the gels, for example see Fig. 8 and Fig. 13). Non-activated transcription was by pol II, as shown by its sensitivity to 2 $\mu\text{g/ml}$ α -amanitin in

Figure 4. Activation of processive transcription from synthetic promoters by GAL4 fusion proteins.

RNase protection of transcripts from oocytes injected with pGal5-E1bmycCAT or pGal5-E4mycCAT and BSA (C), GAL4(1-94), GAL4-AH (AH), or GAL4-VP16 (VP), respectively. The mapping strategy is diagrammed below using probes derived from pVZ-Gal5E4myc and pVZ-Gal5E1bmyc. RNAase protection of the co-injected VA1 control (VA) is shown below. (The lanes in this gel do not align with the corresponding lanes in the upper gel). Lanes 9,10: Gal5-E4myc transcripts activated by GAL4-AH from oocytes incubated in the presence (DRB) or absence (C) of 75 mM DRB. The slight difference in mobility of the TM bands with and without DRB were not confirmed when the two samples were mixed. Experiments in lanes 1-4, 5-8 and 9,10 are from three different batches of oocytes. RT: readthrough, TM: terminated, nd: not determined. The positions of 201 and 110 bases marker bands (MspI digested pBR322) are also indicated. TM products in lanes 1 and 5 can be observed under longer exposure of the gel. Processivity values (RT/RT+TM), based on Phosphorimager analysis, are given below each lane. The TM product contains 1.4 less labelled U residues than the TM product. "<5" indicates detection of TM signal only.

GAL5-E4
GAL5-E1b
GAL5-E4xAH

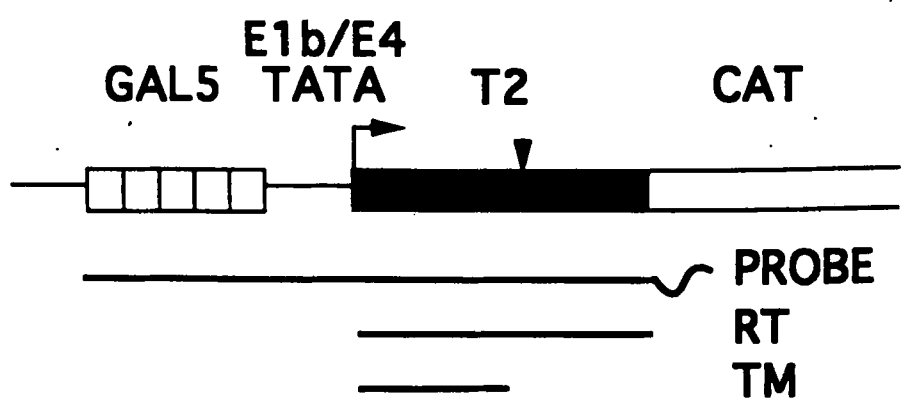
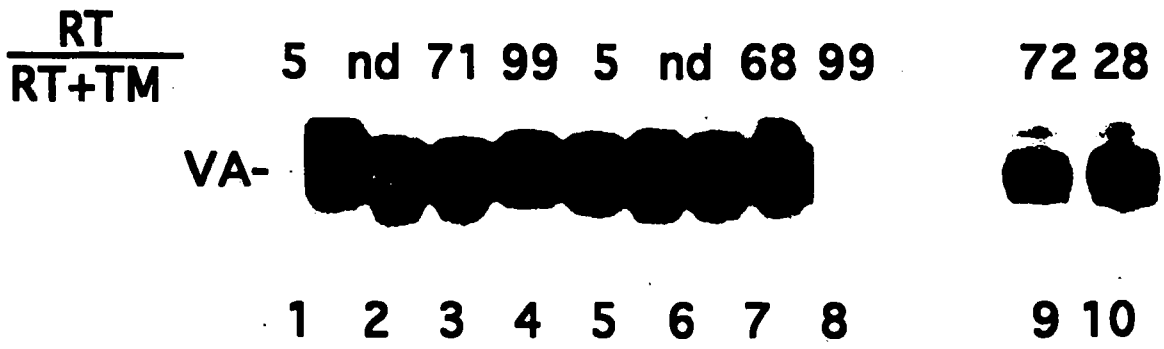
C 1-94 AH VP
C 1-94 AH VP
C DRB



←201



←110



(Fig. 8, lane 2). GAL4-VP16-stimulated transcription was more than 99% processive with no detectable premature termination at T2 for both of the constructs used (Fig. 4, lanes 4 and 8). A significant fraction of GAL4-AH-stimulated transcripts terminated prematurely, giving 71% and 68% processivity for Gal5-E4mycCAT and Gal5-E1bmycCAT, respectively (Fig. 4, lane 3 and 7). However, GAL4-AH activated transcription was still far more processive than non-activated transcription from these two constructs (Fig. 4, lane 1 and 4). For reasons I do not understand, GAL4 (1-94) suppressed the low level transcription observed in the presence of BSA for all the constructs I tested in oocytes (Fig. 4, lane 2 and 5 and data not shown).

The adenosine analogue DRB specifically inhibits the bulk of processive but not non-processive RNAPol II transcription in many cellular genes (Tamm et al., 1976). This general effect of DRB was confirmed in the specific cases of the *c-myc* gene in vivo and HIV1 in vitro (Roberts and Bentley, 1992; Marciniak and Sharp, 1991). It was important to test whether DRB also affected transcription from synthetic promoters, activated by recombinant transactivators. pGal5-E4mycCAT was co-injected with GAL4-AH and the oocytes were incubated in medium containing 75 μ M DRB. DRB reduced the processivity of transcription (RT/RT+TM) of this gene from 72% to 28% (Fig. 4, lanes 9 and 10). Similar effects of DRB were observed in experiments with the Gal5-P2CAT, Gal5-E1bmycCAT and Gal5-HIV2CAT constructs (see Fig. 13 and data not shown). Hence processive transcription activated by GAL4 fusion proteins resembles transcription of many genes in vivo in its sensitivity to DRB.

In conclusion, the processivity of transcription from synthetic promoters can be stimulated by activators and inhibited by DRB in a similar way to the wild type *c-myc* and HIV2 genes implying that these properties are quite general and not restricted to a special class of genes. The results indicated that GAL4-AH driven transcription had consistently lower processivity than GAL4-VP16 or GAL-E1a driven transcription. A summary of the effects of several

chimaeric GAL4 activators on the transcription of four different reporter genes in *Xenopus* oocytes is shown in Table 1 and Fig. 6.

2.1.5. The Initiator Element Does not Affect Processivity in *X.laevis* Oocytes

The initiation sites of HIV1-LTR (CTGGGTCTCT), the human c-myc P2 (CTAACTCGCT)(Krumm et al., 1993), HIV2-LTR (TTCGGTCGCT) and the mouse c-myc P2 (CTCGACTCGCT) share certain level of homology and constitute a Initiator-like element (Krumm et al., 1993). No such sequence is present in Gal5-E4mycCAT or Gal5-E1bmycCAT. Interestingly, at non-activated state Gal5-P2CAT and GAL5-HIV2CAT always produced higher levels of transcription than Gal5-E4mycCAT and Gal5-E1bmycCAT. Activated transcription from Gal5-P2CAT was slightly less processive as compared to Gal5-E4mycCAT and Gal5-E1bmycCAT. Note that these three synthetic genes had identical transactivator and terminator elements. These differences could reflect some quality of the initiator-like element in the c-myc P2 promoter (and that in the LTR-HIV2) to support high levels of non-processive transcription. I assayed the possible role of the initiator element in the control of transcriptional elongation by using pGal5-II-TKmycCAT and pGal5-IV-TKmycCAT. The only difference between these two promoters is that pGal5-IV-TKmycCAT contains the human terminal transferase initiator element downstream of the TK TATA box, while pGal5-II-TKmycCAT does not (see Figure 1).

pGal5-II-TKmycCAT and pGal5-IV-TKmycCAT were injected in oocytes and transcription was activated by GAL4-AH. Both constructs produced similar low levels of non-processive transcription, when co-injected with BSA (Fig. 5, lanes 1 and 5, these products can be observed under longer exposure of the gel). GAL4-AH stimulated transcription with almost equal processivity (69 % for pGal5-II-TKmycCAT and 75 % for pGal5-IV-TKmycCAT) independently of the

Figure 5. The Initiator element does not affect processivity in *X.laevis* oocytes.

RNAse protection of transcripts from oocytes injected with pGal5-IITKmycCAT or pGal5-IVTKmycCAT and BSA or GAL4-AH (AH), respectively. Antisense probes were synthesised by Sp6 RNA polymerase from BglII linearised pVZ-Gal5IITKmyc and pVZ-Gal5IVTKmyc templates. The mapping strategy is diagrammed in the lower panel of the figure. RNAse protection of the co-injected VA1 control (VA) is shown below (The lanes in this gel do not align with the corresponding lanes in the upper gel). RT: readthrough, TM : terminated. 201 and 123 bases marker bands are as marked by arrows. TM products in lanes 1 and 5 can be observed under longer exposure of the gel. "+" and "-" correspond to oocytes, injected or not with α -amanitin at 2 μ g/ml final intracellular concentration.

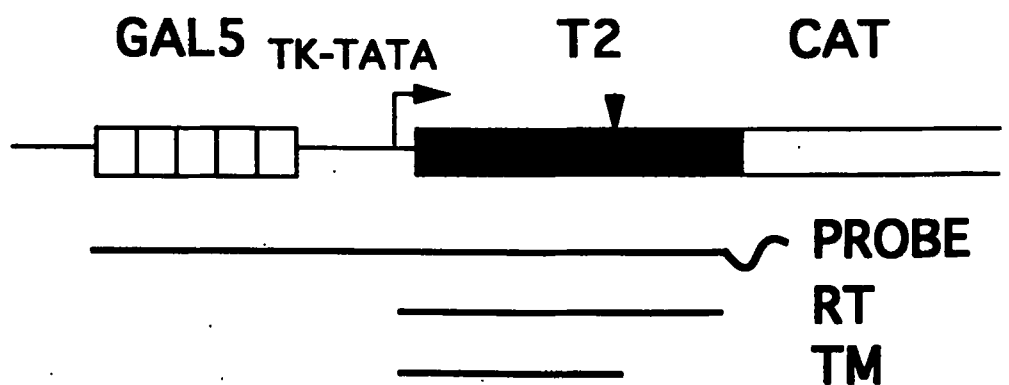
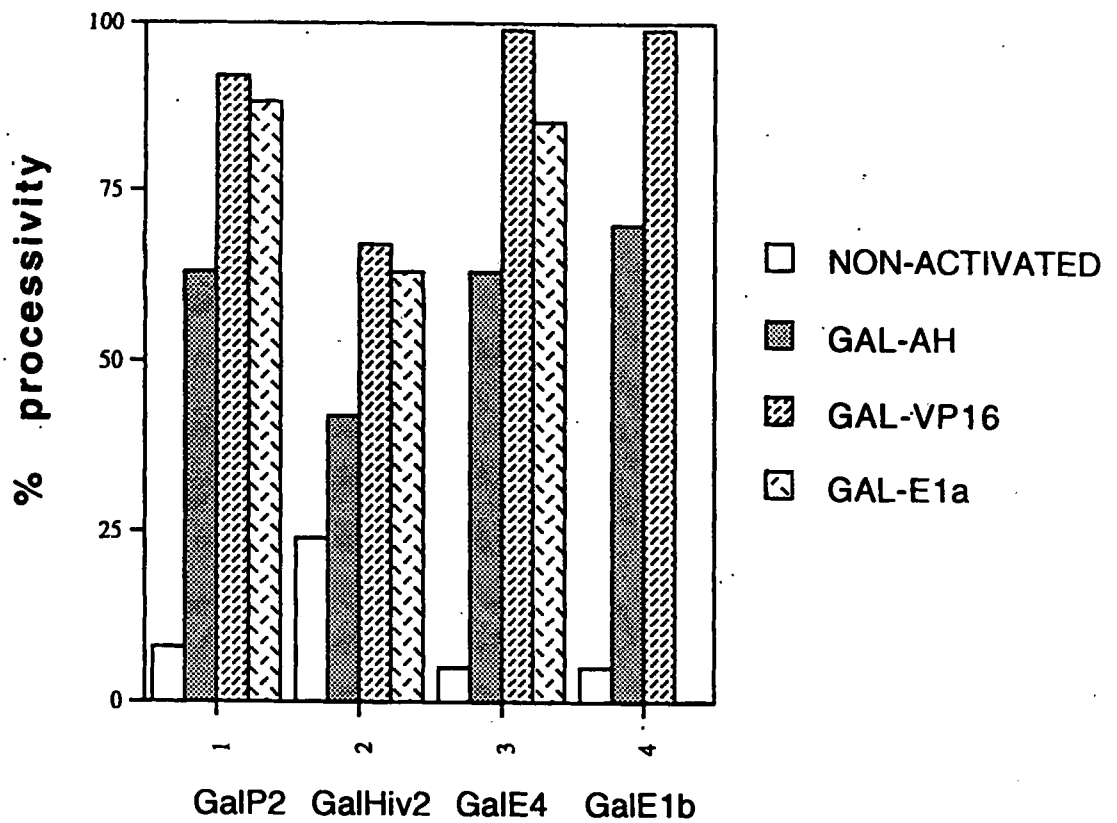


Figure 6. Summary of the effect of GAL4 activators on the processivity (RT/RT+TM) of transcription from four reporter plasmids in injected *X.laevis* oocytes: Gal5-P2CAT, Gal5-HIV2CAT, Gal5-E4mycCAT and Gal5-E1bmycCAT. Average values are given for n independent experiments in different batches of oocytes. The processivity of Gal5-E1bmycCAT was not determined with the GAL-E1a activator. The graph is based on the data in Table 1.

Table 1. Summary of transcriptional processivity from four Gal5-promoters in non-activated state and stimulated by recombinant GAL4-AH, GAL4-VP16 or GAL4-E1a in injected *Xenopus* oocytes. Processivity is expressed as the percentage of total transcription which reads through the termination sites in each gene (RT/RT+TM). The data represent analysis of RNase protection assays as described in the text and in the figures. Average values are given for the number of experiments (n) which were quantified by Phosphorimager. The average fold stimulation (X) of total transcription relative to non-activated state is also given.



Promoter	NON-ACTIVATED	GAL4-AH	GAL4-VP16	GAL4-E1a
Gal5-P2	10% (n = 5)	63% (n = 3) 15X	92% (n = 2) 15X	88% (n = 1) 15X
Gal5-HIV2	25% (n = 6)	42% (n = 4) 8X	67% (n = 4) 45X	63% (n = 1) 7X
Gal5-E1b	< 5% (n = 3)	63% (n = 8) >50X	99% (n = 3) >50X	85% (n = 1) >50X
Gal5-E4	< 5% (n = 3)	70% (n = 6) >50X	99% (n = 2) >50X	N. D.

presence of the initiator element in the promoter (Fig. 5, lanes 3 and 7). In all cases transcription was by RNAPol II as demonstrated by its sensitivity to 2 $\mu\text{g/ml}$ α -amanitin (Fig. 5, lanes 2,4,6 and 8). In summary, in injected *X.laevis* oocytes the TdT initiator element does not influence the the levels of non-activated transcription or the processivity of RNA polymerase II in the presence of an activator. This experiment does not directly address the role of the c-myc P2 or HIV-2 initiation sequences in regulating the processivity of transcription. It does not establish whether specific TATA and initiator elements can cooperate to support high levels of non-processive transcription.

2.1.6. Controls

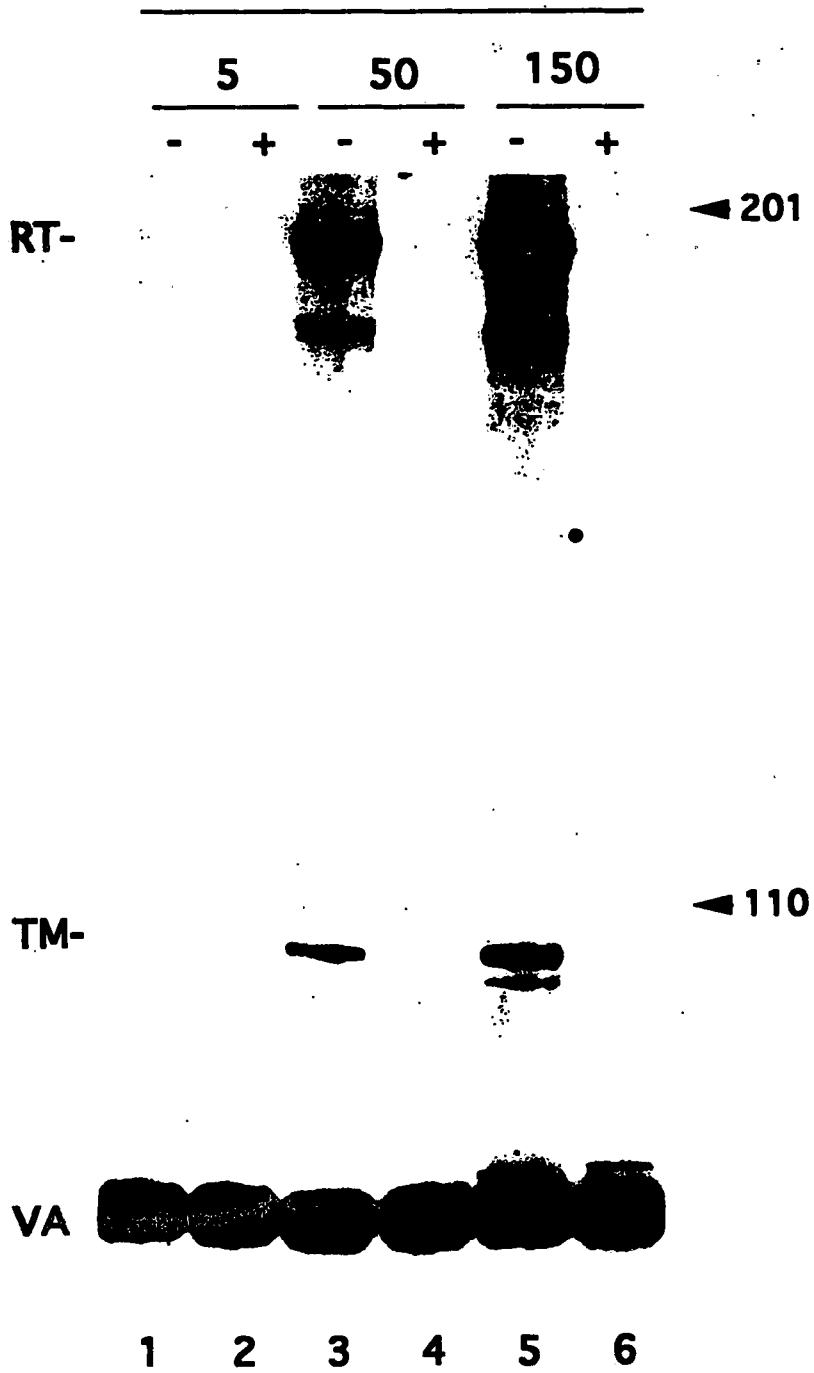
Xenopus oocytes do not contain GAL4-binding activity as determined by gel mobility shift assay (data not shown).

All GAL4 fusion proteins were injected at about 5X molar excess to the GAL4 binding sites in the plasmids (about 100 $\mu\text{g/ml}$). The amount of GAL4 protein injected was equalised using a gel mobility shift assay with CTGCAGTCGGAGGACAGTACTCCGACCCGGG as a probe (data not shown). There was a possibility that some of the effects I observed could be due to minor differences in the concentration of the transactivators. To control for such potential mis-interpretation of the results I titrated GAL4-AH and GAL4-VP16 at fixed concentration of the templates (10 $\mu\text{g/ml}$). The proteins were injected between 5 $\mu\text{g/ml}$ and 150 $\mu\text{g/ml}$. The results demonstrated reduced level of expression of the reporter plasmids at lower concentration of the transactivators, but no significant change in processivity of transcription was observed. In Figure 7, titration of GAL4-AH with Gal5-E4mycCAT is shown.

Figure 7. Titration of GAL4-AH with Gal5-E4mycCAT as a template.

RNAse protection of transcripts made in oocytes injected with pGal5-E4mycCAT together with 5, 50 and 150 $\mu\text{g/ml}$ GAL4-AH, respectively. RNAase protection was as in Fig.2. "+" and "-" correspond to oocytes, injected or not with α -amanitin at 2 $\mu\text{g/ml}$ final intracellular concentration. RT: readthrough, TM: terminated. VA1 controls are shown below. The positions of 201 and 110 bases the MspI-pBR322 markers is denoted by arrows.

GalE4 x GAL4-AH



E. coli proteins, purified in the same way as the GAL4-fusion proteins, were used in mock-injection experiments to check whether some impurities in the protein preparations could alter the pattern of transcription from our templates. No effect of these proteins was observed (data not shown).

I also assayed whether GAL4-AH and GAL4-VP16 acted specifically through the GAL4 binding sites on the plasmids and not in any other way. Δ GalHIV2 plasmid, in which the five GAL4 binding sites of pGal5-HIV2 were deleted, was co-injected separately with each of the proteins. The resulting RNA was analysed as for pGal5-HIV2CAT. No effect of these proteins on the minimal promoter activity or processivity of transcription was observed (data not shown).

Transcription in *X. laevis* oocytes, stimulated by the three GAL4-fusion proteins from all templates used, was by RNA polymerase II, as determined by its sensitivity to injecting of 20 μ g/ml α -amanitin in the cytoplasm (the final intracellular concentration is about 2 μ g/ml)(data not shown).

All probes used were tested for artifactual cleavage that can result from the RNAase protection assay. Standard amounts of labelled RNA probes (70 000 cpm) were mixed with sense transcripts from the corresponding plasmids and *X. laevis* RNA, extracted from one oocyte, and proceeded in parallel with the test samples. No bands at the positions of RT or TM products of any of the genes assayed were resulting from the RNAase protection (data not shown).

2.2. TBP Stimulates Non-processive Transcription

2.2.1. Activators and TBP Differ in the Processivity of Transcription They Stimulate

Point mutations of the c-myc P2 TATA box significantly decrease the strength of the promoter, but surprisingly, almost completely eliminate termination at the T2 site of the gene (D.Bentley, published in (Yankulov et al., 1994). These results pointed out the important role of the TATA box in directing non-processive transcription and suggested that a high rate of initiation is not necessary in order to support highly efficient elongation. Nevertheless, I wanted to rule out the possibility that the effect of activators on processivity could be a secondary consequence of an increased initiation rate, which might result in titration of a limiting termination factor, for example. In order to stimulate initiation without using an activator, I coinjected into oocytes recombinant human TATA-binding protein (TBP) together with some of the synthetic genes, used in my experiments. Previously TBP has been shown to stimulate TATA-containing promoters in *Drosophila* Schneider cells (Colgan and Manley, 1992).

The Gal5-E4mycCAT and Gal5-P2CAT genes were initially investigated. These constructs were injected in *X.laevis* oocytes with 100 µg/ml TBP or 1 mg/ml BSA as a control (Final concentration in the TBP containing injections was brought to 1 mg/ml with BSA). α -amanitin was injected separately into the cytoplasm (Fig. 8, lanes 2,4,5 and 7) at 20 µg/ml. Both Gal5-E4mycCAT (Fig. 8, lane 1) and Gal5-P2CAT (Fig. 8, lane 6) produced low levels of transcripts, most of which terminated prematurely at the T2 site of the genes as observed before (longer exposures were necessary to see the bands from the Gal5-E4mycCAT analysis). TBP stimulated transcription from both promoters 3-4 fold (Fig. 8, lanes 3 and 8), but unlike transactivators it did not increase the processivity as

Figure 8. TBP stimulates non-processive transcription from the Gal5-E4mycCAT and Gal5-P2CAT constructs.

RNAse protection of transcripts made in oocytes injected with pGal5-E4mycCAT or pGal5-P2CAT and BSA as a control (C) or recombinant human TBP (TBP), respectively. RNAase protection was as in Fig.2 for Gal5-P2CAT and as in Fig. 4 for pGal5-E4mycCAT. "+" and "-" correspond to oocytes, injected or not with α -amanitin at 2 μ g/ml final intracellular concentration. P: probe, RT: readthrough, TM: terminated. VA1 controls are shown below. The positions of some of the MspI-pBR322 markers are denoted by arrows. Lanes 1-4 and 5-8 are from different batches of oocytes:

GAL5-E4			
C		TBP	
-	+	-	+

GAL5-P2			
C		TBP	
+	-	+	-

RT ▶

◀ 201 404 ▶

◀ P

◀ R

309 ▶

◀ T

TM [

◀ 110

1 2 3 4

5 6 7 8

VA-



Figure 9. TBP stimulates non-processive transcription from the Gal5-HIV2 construct.

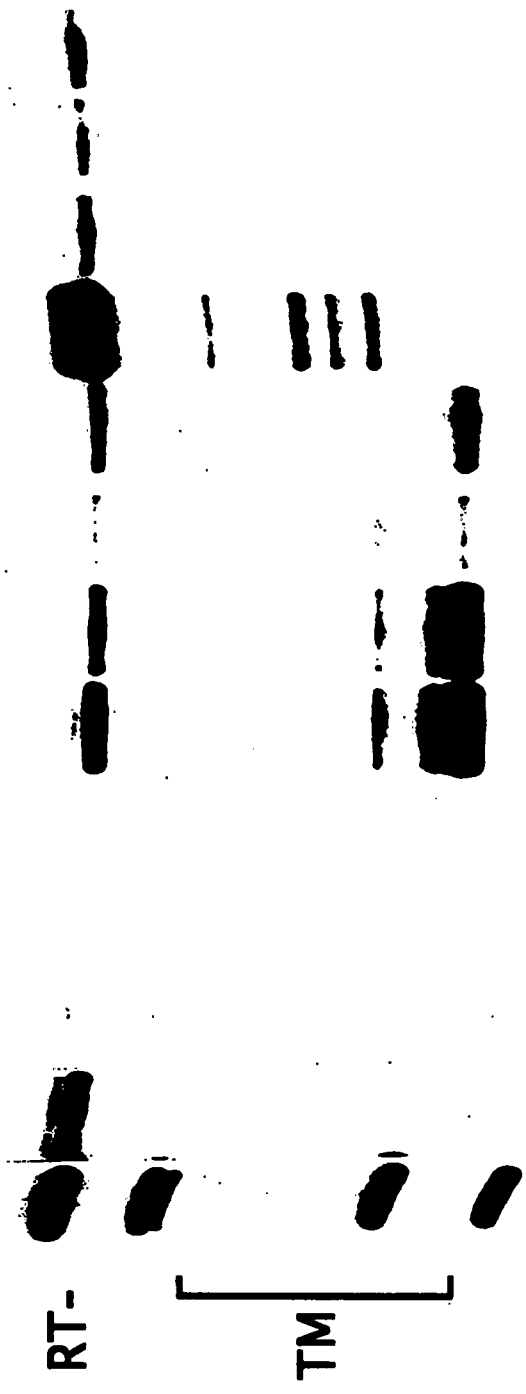
RNAse protection of transcripts from oocytes injected with BSA, human recombinant TBP, or GAL4-VP16 was as described in Fig. 3. Controls (C: Lanes 2, 3, 6, 10) were compared with oocytes co-injected with α -amanitin (2 μ g/ml intracellular concentration) (Lanes 4, 7, 11), anti-CTD antibodies (8WG16, 10 μ g/ml final intracellular concentration) (Lanes 5, 8, 12) or both (Lanes 9,13). Lane 2 is a 5 fold longer exposure of lane 3. M: markers 160, 147, 123, 110 bp. RT: readthrough, TM: terminated. VA1 controls (VA) for lanes 3-13 are shown below.

BSA

TBP

GAL4-VP16

M - - α -A α -II - α -A α -II α -A α -II - α -A α -II α -A α -II



1 2 3 4 5 6 7 8 9 10 11 12 13

VA-



no increase in the RT signal appeared. Significant amount of the TBP stimulated transcription in both cases was not sensitive to α -amanitin (Fig. 8, lane 4). Both non-activated and TBP stimulated transcription from Gal5-P2CAT was not completely sensitive to α -amanitin too.

2.2.2. TBP Stimulates Transcription. Sensitive to anti-RNAPol II Antibodies

The c-myc T2 termination site (also present in Gal5-E4mycCAT and Gal5-P2CAT) coincides with a run of T residues which can function as a RNAPol III terminator (Bentley et al., 1989). Since TBP is also a pol III factor, TBP stimulated α -amanitin resistant transcripts terminating at this position could actually be products of pol III. On the other hand, injecting of the wild type c-myc gene in *X.laevis* oocytes at high concentration can produce certain amount of α -amanitin resistant prematurely terminating transcription (Bentley et al., 1989). This data were interpreted as evidence that RNAPol III can compete successfully for the c-myc promoter when RNAPol II factors are titrated out. Gal5-E4 promoter containing genes, though, had never been reported to be transcribed by pol III. I decided to clarify whether TBP stimulated pol II or pol III transcription by using the Gal5-HIV2 construct and to introduce a monoclonal antibody against the CTD domain of the large subunit of RNAPol II (8WG16, (Thompson et al., 1989) as a specific inhibitor of RNAPolymerase II. This approach circumvented three of the major problems in the previous experiments. First, like the Gal5-E4 synthetic promoter, the HIV2 LTR promoter has never been reported to be transcribed by RNAPol III. Second, the HIV2 TAR terminator element does not contain any runs of T's which could act as a pol III terminator. Third, the 8WG16 antibody is unlikely to inhibit pol III transcription and has been previously reported to inhibit pol II transcription (Thompson et al., 1989). The results of an experiment in which I compared the sensitivity of Gal5-HIV2 transcription

In conclusion, TBP enhanced initiation of all the TATA-containing promoters I tested, but unlike the GAL4 activators it did not increase the processivity of pol II transcription from the same promoters. That data is consistent with the idea that the greater processivity of activated transcription was mediated by a specific effect of activation domains on the transcriptional machinery and is not a secondary effect of the increased initiation rate.

2.2.3. Transactivators and TBP Do not Change the HIV2 Transcription Start Site

Truncated HIV2 transcripts from the intact LTR and from Gal5-HIV2 CAT in the presence of GAL4-VP16 were about 10 bases longer on average than those made in the presence of TBP, GAL4-AH, GAL4-E1a or in the absence of an activator (see Figures 3 and 9). 5' end-mapping showed that the HIV2 start site was unaffected by different transactivators or TBP (Fig. 10), which proved that the difference in the length of the TM RNAase protection products in Figures 3 and 8 resulted from premature termination at different positions. This variation in the preferred site of termination may reflect some difference in the processivity of transcriptional complexes that read the 5' portion of HIV-2 in *X.laevis* oocytes.

2.2.4. TBP Does Not Affect Activated Transcription

TBP and the GAL4 recombinant activators displayed opposite effects on processivity of RNAPol II transcription. Since all my experiments were performed under non-physiological concentrations of TBP on non-activated

C AH VP E1 T VP

P —————▶ 405

hiv2 —————▶ 110

1 2 3 4 5 6

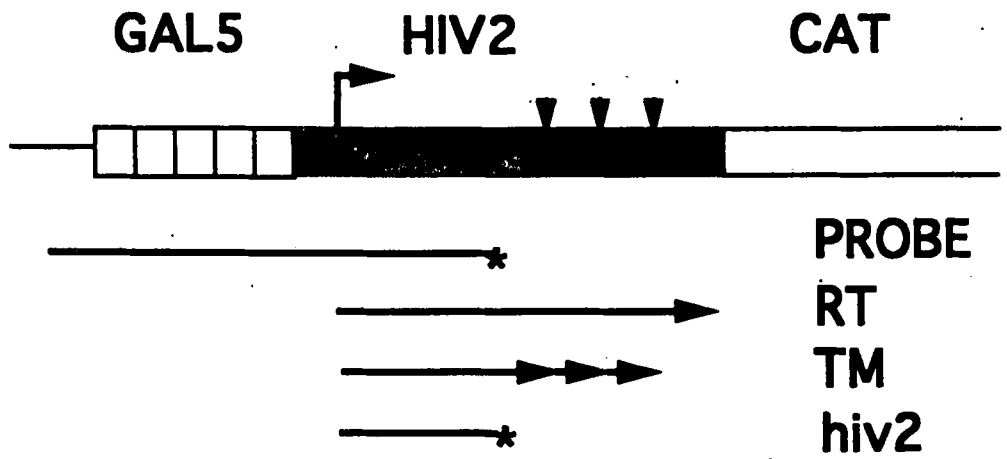
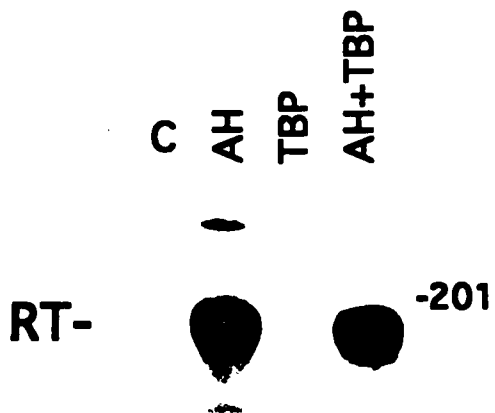


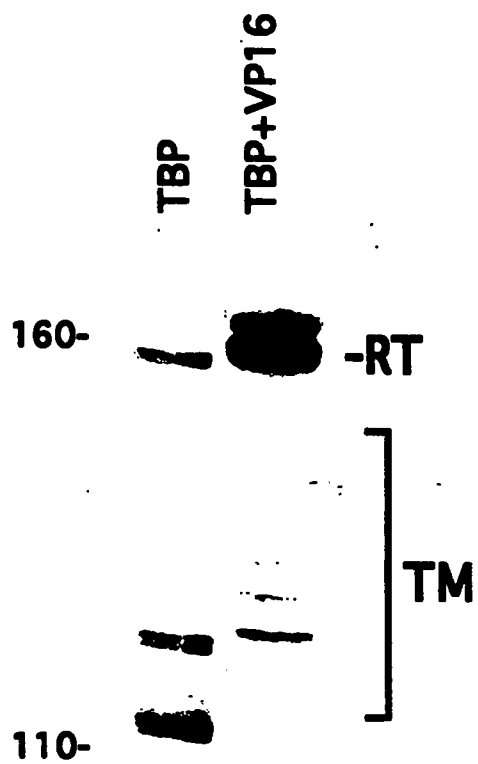
Figure 11. Transactivators overcome the effect of TBP.

RNAse protection of transcripts made in oocytes injected with pGal5-E1bmycCAT or pGal5-HIV2CAT. Oocytes were co-injected with BSA (C), TBP, GAL4-AH (AH), GAL4-VP16 (VP16) or combination of TBP and transactivator. RNAase protection was as shown in Fig. 3 for Gal5-HIV2CAT and in Fig. 4 for pGal5-E1bmycCAT. P: probe, RT: readthrough, TM: terminated. VA1 controls are shown below each lane. The positions of some of the MspI-pBR322 markers are indicated. Lanes 1-4 and 5, 6 are from different batches of oocytes.

GAL5-E1b



GAL5-HIV2



5 6



templates, I asked whether TBP could also affect the level of initiation and processivity of activated transcription. To check that, I injected pGal5-E1bmycCAT together with BSA, TBP, GAL4-AH or both GAL4-AH and TBP, respectively (Fig. 11, lanes 1-4). In a separate experiment I compared the processivity of transcription from the Gal5-HIV2 gene, which was co-injected with TBP or TBP plus GAL4-VP16 (Fig. 11, lanes 5 and 6).

In the presence of BSA, TBP and GAL4-AH transcription from pGal5-E1bmycCAT was identical to that, demonstrated in my previous experiments (Fig. 11, lanes 1-3. Compare to Fig. 4 and Fig. 8). GAL4-AH activated transcription with 65 % processivity, while TBP stimulated the synthesis of low levels of prematurely terminating RNAs. When TBP and GAL4-AH were injected together, the resulting transcription closely resembled that, observed in the presence of GAL4-AH alone (Fig. 11, lane 4). Processivity of transcription (RT/RT+TM) in the presence of GAL4-AH and TBP was 67 %.

A similar dominant effect of a transactivator over that of TBP was observed when pGal5-HIV2CAT was used as a template. In the presence of GAL4-VP16 and TBP Gal5-HIV2CAT was transcribed with processivity, characteristic for activated transcription from that template (see for example Fig. 9, lane 10) rather than TBP-stimulated transcription (Fig. 11, lanes 5 and 6).

This data indicate that in *X.laevis* oocytes TBP can stimulate low processivity RNA polymerase II transcription from non-activated templates, but the effect of TBP can be almost completely suppressed by co-injecting of transcriptional activators.

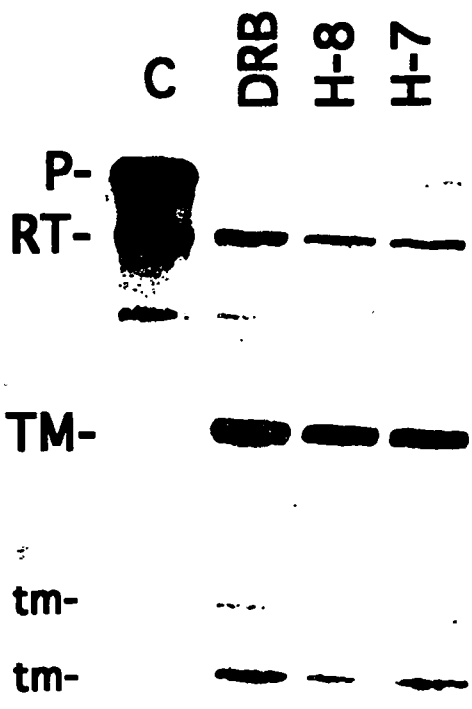
2.3. Protein Kinase Inhibitors Reduce RNAPol II Processivity

2.3.1. Kinase Inhibitors Reduce Transcriptional Processivity in the c-myc Gene

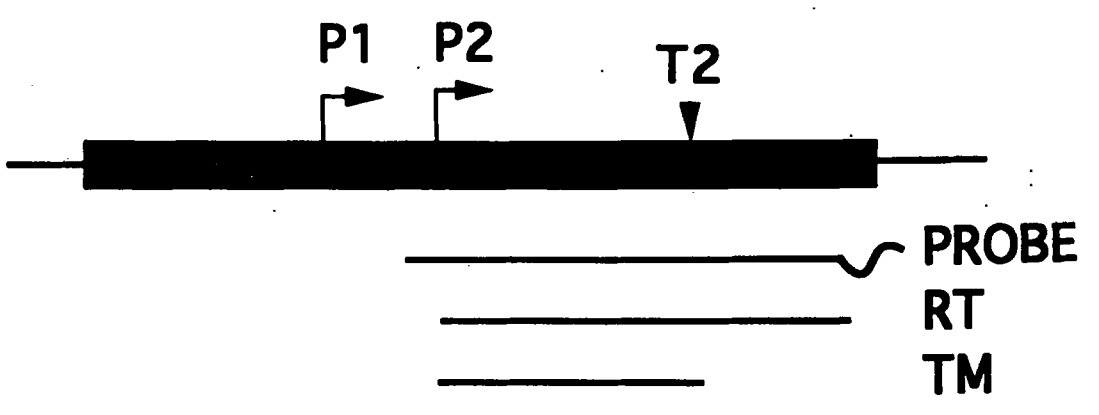
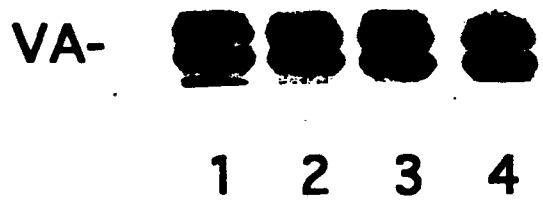
It has been previously demonstrated that the adenosine analogue DRB is an inhibitor of RNAPol II elongation (Sehgal et al., 1976; Tamm et al., 1976) (Roberts and Bentley, 1992; Marciniak and Sharp, 1991; Marshall and Price, 1992). DRB also inhibits several CTD kinase activities in vitro (Cisek and Corden, 1991; Stevens and Maupin, 1989). To address the question whether a protein kinase is involved in DRB suppression of pol II elongation I tested whether two well characterised kinase inhibitors (H-7 and H-8) (Serizawa et al., 1993b) can also inhibit pol II processivity (RT/RT+TM). H-7 and H-8 do not affect basal transcription in vitro (Serizawa et al., 1993a; Serizawa et al., 1993b), however their effects on activated transcription have not been reported.

Processivity of pol II transcription was studied in injected *X.laevis* oocytes by RNAase protection using templates that were shown to produce detectable levels of prematurely terminated RNAs (pSX943, pLTR-HIV2, pGal-E4-mycCAT and pGal-E1b-mycCAT, see Figure 1). The strategy of investigation was the same as described in Chapter 2.1.1 of this thesis.

Initially I compared the effect of DRB, H-7 and H-8 on transcription of the wild type mouse c-myc gene (pSX943). In oocytes this gene produces full length transcripts (RT) as well as discrete prematurely terminated RNA species (TM) of 300 bases (Fig. 12, lane 1). In Fig. 12 I present an experiment, where *X.laevis* oocytes were injected with pSX943 together with pSPVA1 as an internal control for pol III transcription and RNA recovery. The inhibitors (DRB at 50 μ M; H-7 and H-8 at 200 μ M) were added to the oocyte incubation media. The resulting RNA was analysed by RNAase protection assay as shown in Fig. 12 (lower panel) and quantified by a Phosphorimager (Molecular Dynamics).



$\% \frac{RT}{RT+TM}$ 73 24 25 23



DRB, H-7 and H-8 significantly reduced RT-transcription and lowered processivity (RT/RT+TM) over the c-myc gene from 73 % to 23-25% (Fig. 12. Only RT and TM signals were used to calculate the RT/RT+TM ratio). In addition to the increased termination at the previously defined T2 site (TM) (Bentley and Groudine, 1988) shorter RNAs, not detected in the control sample (tm) were observed when transcription was performed in the presence of DRB, H-7 or H-8. The results also indicated that DRB, H-7 and H-8 did not reduce significantly the rate of initiation at the c-myc P2 promoter, but prevented the polymerases from efficiently elongating through the 5' region of the gene. The control VA1 gene was unaffected by any of these inhibitors.

DRB, H-7 and H-8 also decreased severely processivity of transcription (RT/RT+TM) when the wild type LTR HIV-2 gene was used as a template to inject *X.laevis* oocytes (data not shown). In both cases (LTR HIV-2 and c-myc) the three inhibitors had similar effect in dramatically suppressing the efficiency of elongation, but did not affect pol III transcription.

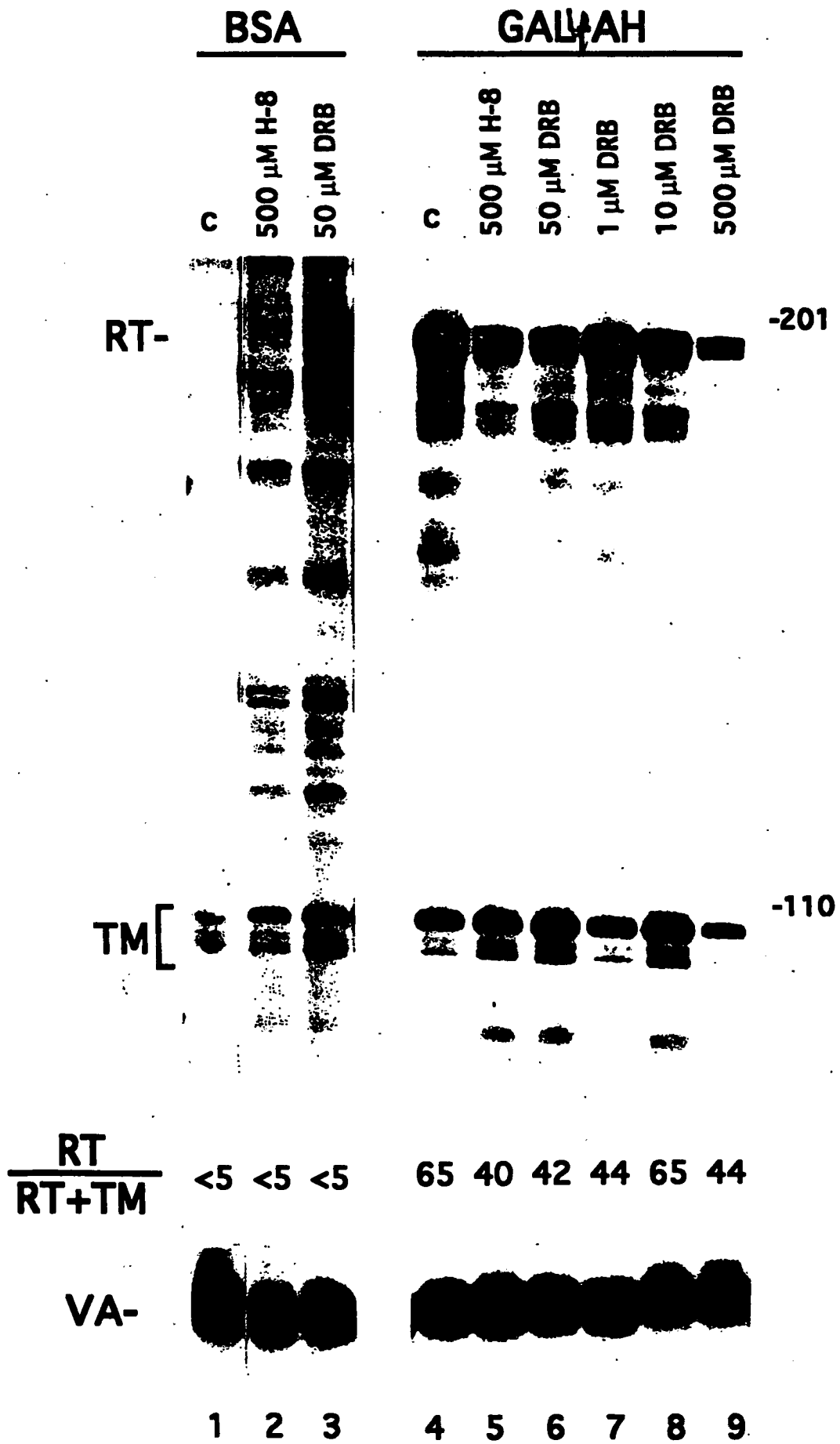
2.3.2. DRB, H-7 and H-8 Inhibit Processivity of Transcription, Driven by Synthetic Promoters

In the chapter 2.2. of this thesis it was demonstrated that the efficiency of pol II elongation in *X.laevis* oocytes is controlled predominantly at the promoter level by transactivators. It was possible that DRB, H-7 and H-8 inhibit a kinase which specifically upregulated a factor, responsible for high the processivity of transcription from the wild type c-myc and HIV2 LTR promoters. Alternatively, DRB, H-7 and H-8 could inhibit a kinase activity, generally required for high RNAPol II processivity. To distinguish between these possibilities, I assayed

Figure 13. DRB, H-7 and H-8 inhibit processivity of transcription from a synthetic promoter.

RNAase protection of transcripts from oocytes injected with pGal5-E1bmycCAT and BSA or GAL4-AH. DRB and H-8 were added to the oocyte incubation media at the concentrations, given above each lane. RNAase protection was as in Fig. 4. Abbreviations: C: control, RT: readthrough, TM: terminated, VA: RNAase protection products from the coinjected Adenovirus VA1 gene. Processivity values (RT/RT+TM), based on Phosphorimager analysis are shown below each lane. The TM band has 1.4 fewer labelled residues than the RT band. The positions of the 201 and 110 bases markers are as indicated.

GAL5-E1bmycCAT



whether these three kinase inhibitors could function independently of the nature of the promoter by using one of our synthetic GAL4 activated genes.

pGal5-E1bmycCAT (see Figure 1) was injected together with GAL-AH (Fig. 13, lanes 4-9) or 1 mg/ml BSA (Fig. 12, lanes 1-3). The final protein concentration in the GAL-AH injections was brought to 1 mg/ml with BSA. The injected oocytes were incubated with 500 μ M H-8 (Fig. 13, lanes 2 and 5) or with different concentrations of DRB (50 μ M - lanes 3 and 6; 1 μ M - lane 7; 10 μ M - lane 8; 500 μ M - lane 9). Processivity of transcription was determined by RNAase protection assay as described before (see Fig. 4).

H-8 (500 μ M) and DRB (50 μ M) had no effect on the typically non-processive transcription from GAL5-E1bmycCAT in the presence of BSA only (Fig. 13, compare lanes 1-3. Lanes 1-3 are from a longer exposure of the gel). In contrast, the same concentrations of H-8 and DRB caused increase of the TM signal and decrease of the RT signal, when transcription was activated by GAL-AH. Processivity (RT/RT+TM) was reduced from 65% in the control to 44% and 40% for H-8 and DRB, respectively (Fig. 13, lanes 4-6). Titration of DRB revealed that it did not affect the efficiency of elongation when applied at 1 μ M (Fig. 13, lane 7). 10 μ M DRB lowered the processivity values down to 42%, while much higher concentration (500 μ M) inhibited not only processivity, but the overall transcription too (Fig. 13, lanes 8 and 9). The VA1 gene, which was co-injected as a control for pol III transcription, was unaffected by either H-8 or DRB. Similar sensitivity of transcription to H-8 and DRB was observed in experiments, where template was pGal5-E4mycCAT (data not shown).

The results in Fig. 12 and Fig. 13 indicated that two kinase inhibitors (H-7 and H-8) inhibited RNAPol II transcription in a way, similar to that of DRB. DRB, though, was more efficient than H-8 and H-7. The effect of DRB, H-7 and H-8 does not confine to some special class of genes, as demonstrated by the uniform response of divergent promoters to these inhibitors. Since non-

activated transcription was not sensitive to DRB, H-7 and H-8, it seemed that activation was a prerequisite for the action of the drugs.

2.3.3. Inhibition of Processivity under "Squelching" Conditions

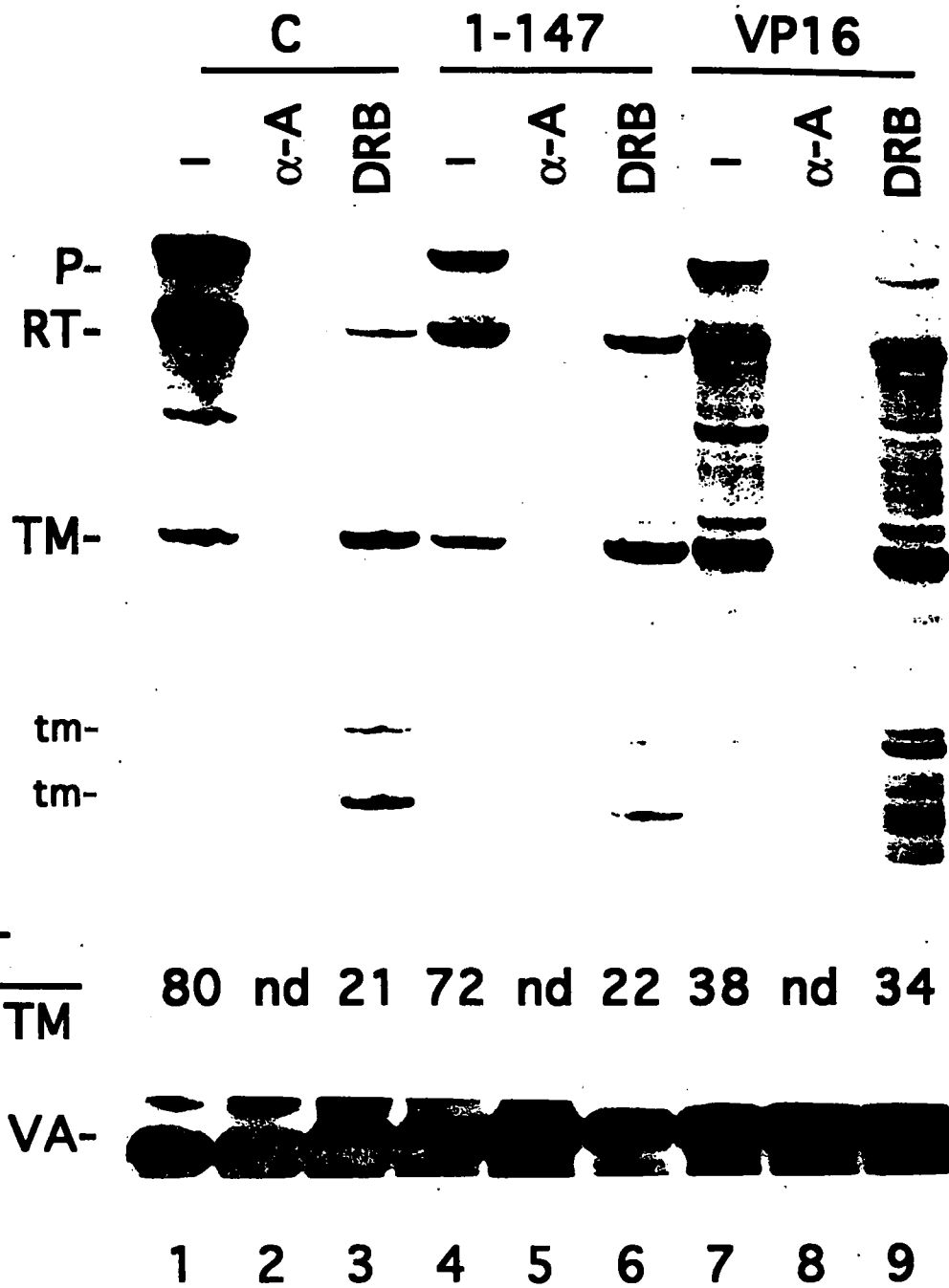
In many systems, a high concentration of transactivation domains inhibits gene expression presumably because they sequester general transcription factors. This phenomenon was termed "squelching" (Gill and Ptashne, 1988). The results, presented in section 2.1. of this thesis demonstrated that transactivators can stimulate processivity of pol II transcription. Hence, it was likely that some factor(s), which were required for high efficiency of elongation, could interact with transactivation domains and can be sequestered under "squelching" conditions. It was possible that DRB was suppressing the activity of some of these factor(s) or alternatively acted in transactivator-independent manner. These questions were addressed by asking whether high concentration of non-binding VP16 transactivation domain would inhibit RNAPol II processivity and whether DRB and VP16 would have additive effect on reducing the efficiency of elongation.

X.laevis oocytes were co-injected with the mouse c-myc gene either with 0.8 mg/ml GAL4(1-147)VP16 or 0.8 mg/ml GAL4 (1-147), which is a much weaker activator in oocytes (data not shown). α -amanitin was injected separately in the cytoplasm. DRB was added at 50 μ M to the oocyte incubation medium. Processivity of transcription was determined by quantifying RNase protection products as in Figure 12.

At 0.8 mg/ml GAL4(1-147)VP16 and GAL4(1-147) reduced the total amount of c-myc transcription to 44% and 68% (relative to the VA1 gene), as compared to the BSA control, respectively (Fig. 14, lanes 1, 4 and 7). Contrary to its effect on promoters with GAL4-binding sites, GAL4-VP16 decreased the

processivity of c-myc transcription (RT/RT+TM) from 80% in the BSA control to 38% (Fig. 14, compare lanes 1 and 7). GAL4 (1-147) had little effect, reducing processivity from 80% to 72%, (Fig. 14, lanes 1 and 4). Neither of the GAL4 proteins affected the α -amanitin sensitivity of transcription (Fig. 14, lanes 2, 5 and 8). Interestingly, when GAL4(1-147)VP16 injected oocytes were treated with 50 μ M DRB, RT/RT+TM values remained almost unchanged (38% and 34%; Fig. 14, lanes 7 and 9). In contrast, 50 μ M DRB inhibited processivity of transcription in the BSA and GAL(1-147) injected oocytes to the typical for this gene values of 20-25% (Fig. 14, lanes 3 and 6). The control VA1 gene which is transcribed by RNAPol III was unaffected by GAL(1-147)VP16 or DRB.

Figure 14 shows that the elongation of c-myc transcripts is markedly inhibited under "squenching" conditions. This observation implies that the factors which are titrated out by a non-binding activation domain include activities required for processive transcription. Inhibition of elongation by "squenching" further supports the idea that one general property of transactivation domains is to interact with factors that stimulate transcriptional processivity. In addition, the reduced sensitivity of "squenching" transcription to DRB indicates that DRB and high concentration of the VP16 transactivation domains could use the same pathway to decrease the elongational capacity of RNAPol II complexes. One plausible explanation is that VP16 sequesters a transcription factor (most likely a kinase) which is a DRB target.



$\% \frac{RT}{RT+TM}$
80
nd
21
72
nd
22
38
nd
34

2.4. The TFIIF Associated Kinase Activity Is Essential for High Processivity of RNAPol II Transcription

2.4.1. CTD-kinase Activity Binds VP16

The data presented so far suggested that the activity of a kinase, required for high efficiency of elongation in *X.laevis* oocytes (and in in vitro transcription experiments with HeLa Nuclear extracts: D.Bentley, unpublished results) was sensitive to DRB, H-7 and H-8. Several studies had shown that DRB, H-7 and H-8 can inhibit the phosphorylation of CTD (RNAPol II carboxiterminal domain) in vitro (Cisek and Corden, 1991; Serizawa et al., 1993b; Stevens and Maupin, 1989) and in vivo (Dubois et al., 1994a; Dubois et al., 1994b). On the other hand, DRB did not inhibit transcription "squashed" by GAL4(1-147)VP16, which raised a possibility of either direct or indirect interaction between this hypothetical kinase and the VP16 transactivation domain. To further explore that possibility I fractionated HeLa nuclear extract on different affinity resins and looked for CTD-kinase activities that bound specifically to the VP16 transactivation domain.

GST, GST-SW6 and GST-VP16 proteins were expressed in vitro and immobilised at saturation levels on Glutathione-Sepharose 4B beads (Pharmacia). GST-VP16 contained the same fragment (aminoacid residues 410-490) of the VP16 protein, which was present in GAL4-VP16. GST-SW6 is a fusion with the SW6 mutant of the VP16(410-490) activation domain in which Phe residues at positions 442, 473, 475 and 479 are substituted with Pro, Ala, Ala and Ala respectively. Although it still remains highly negatively charged, this mutant has almost no transcriptional activity (Walker et al., 1993).

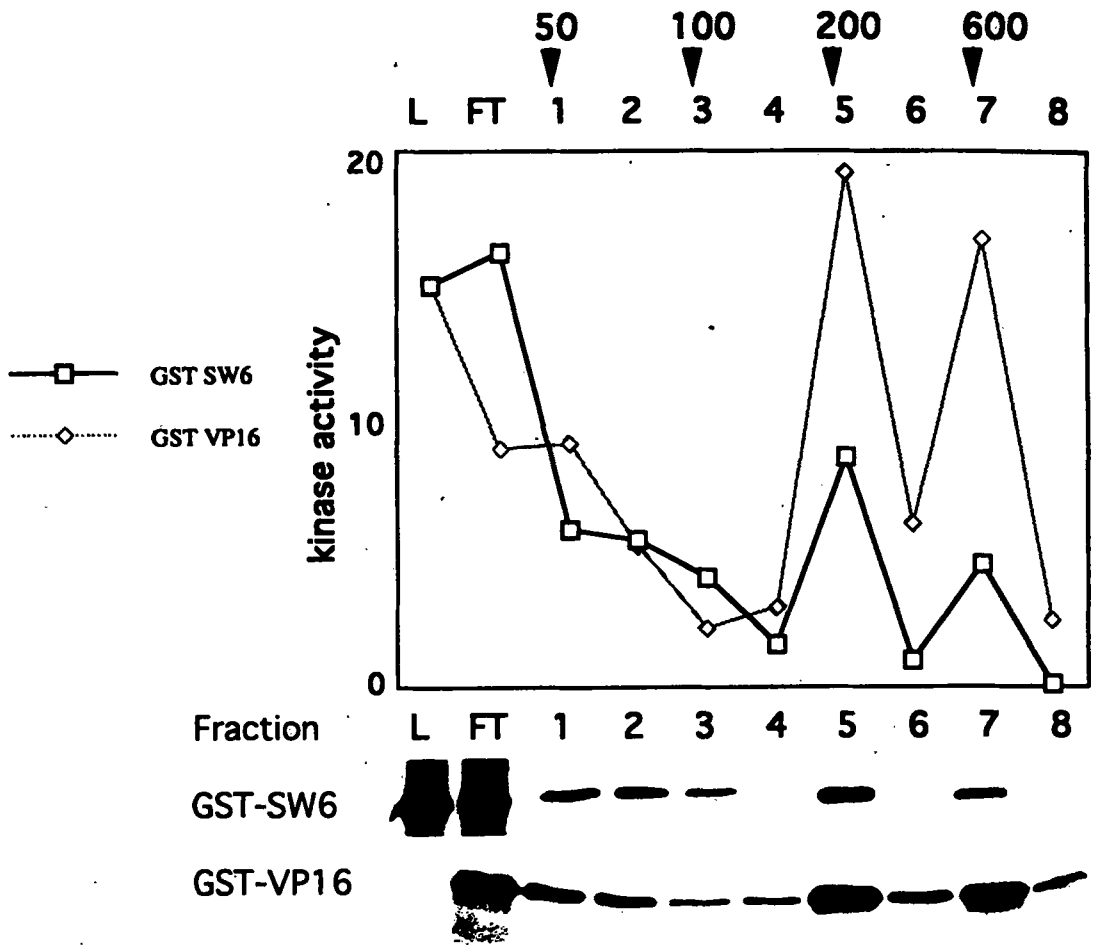
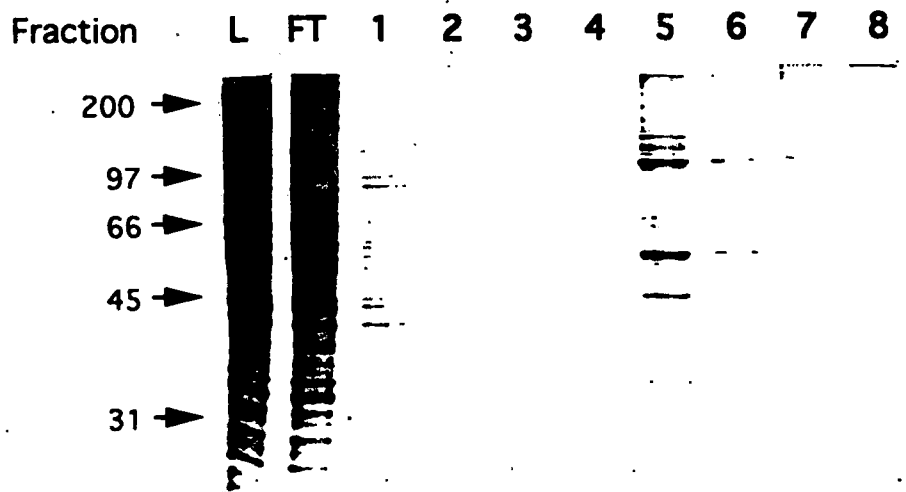
10 mg aliquots of HeLa nuclear extract proteins in buffer D (50 mM KCl) were loaded on 0.6 ml of each resin and the columns were subsequently eluted

Figure 15. A CTD kinase activity binds specifically to GST-VP16 affinity resin.

Preparation of the affinity resins, fractionation of HeLa Nuclear Extract and kinase activity assay were as described in Materials and Methods. The concentration of KCl (50, 100, 200 and 600 mM respectively) in the affinity column fractions is indicated by arrows. L: load; FT flowthrough.

A. 0.3 μ l of each fraction were assayed in standard kinase reactions (see Materials and Methods) with GST-CTD as a substrate. Fixed dried gels were quantified by a Phosphorimager (Molecular Dynamics) and the data plotted on a graph. The GST-SW6 and the GST-VP16 column CTD-kinase profiles are shown below the graph.

B. SDS-polyacrylamide gel analysis of the GST-VP16 column fractions. 5 μ l of fractions L and FT, 15 μ l of fractions 1 and 2, and 30 μ l of fractions 3-8 respectively, were separated on 10 % SDS-acrylamide gel according to Laemmli (1970) and stained with Coomassie. The arrows indicate the mobility of Rainbow (Amersham) protein molecular weight markers.

A**B**

with buffer D supplemented with 50, 100, 200 and 600 mM KCl (two fractions of two bed volumes for each KCl concentration, respectively). Schematic representation of the experiment is given in Fig. 15. Very little or no protein was recovered in fractions 3 to 8 from the GST column as judged by Bradford assay (BioRad), while GST-SW6 and GST-VP16 fractions contained detectable amounts (0.05-0.3 mg/ml) of total protein. Each of the GST-SW6 and GST-VP16 fractions were further analysed by SDS-gel electrophoresis and by a kinase assay using GST-CTD as a substrate (Fig. 15, A and B).

SDS-electrophoresis showed multiple polypeptide bands throughout the chromatographic profile from both GST-SW6 and GST-VP16 columns. No significant difference in the polypeptide content of the corresponding fractions from the two columns was observed (Fig. 15 B, only the GST-VP16 profile is shown). This similarity was likely to result from the ion-exchange properties of the resins, since both SW6 and VP16 were highly acidic. Differences in the chromatography of concrete proteins will be given in the next chapter.

GST-CTD kinase activity was found in all fractions from both GST-SW6 and GST-VP16 columns (Fig. 15 A). The protein kinase reaction was specific for CTD, since no phosphorylation of GST only was detected (data not shown). Fixed and dried gels were quantified by a Phosphorimager and the data plotted on a graph to compare the levels of CTD phosphorylation. Most of the CTD-kinase activity was recovered in the 0.2 and 0.6 M KCl fractions from both of the columns. In fractions 5 to 7 the CTD-kinase activity from the GST-VP16 column was several fold higher than the corresponding fractions from the GST-SW6 column and ten fold higher than the preceding fractions from the GST-VP16 column (Fig. 15 A). Thus, it occurred that a CTD-kinase activity bound to the VP16 transactivation domain with greater affinity than to the SW6 mutant.

2.4.2. The VP16 Binding CTD-Kinase Activity Co-elutes with TFIIF

Several CTD-kinase activities have been implicated in the control of RNA polymerase II transcription. Some of them contain the p34^{cdc2} kinase (Cisek and Corden, 1989). Other two are the DNA-dependent kinase (Dvir et al., 1993; Gottlieb and Jackson, 1993) and the TFIIF associated kinase (Fischer et al., 1992; Lu et al., 1992). I assayed whether the peak of CTD-kinase activity from the VP16 affinity column would coincide with the presence of any of these CTD-kinases. The pattern of fractionation of p34^{cdc2}, the Ku subunit of the DNA-dependent kinase and the p62 subunit of TFIIF was investigated by western blot analysis. In addition, antibodies against proteins that were reported to interact with VP16 ((RP-A (Li and Botchan, 1993; He et al., 1993) and TBP (Stringer et al., 1990)) were used to estimate the relative affinity of interaction between VP16 and other proteins. The results are shown in Fig. 16.

None of the proteins bound to the GST-Sepharose resin. As expected, both TBP and RP-A were eluted in the higher salt fractions (5 to 8) from the GST-VP16 column, confirming their previously described affinity to the VP16 transactivation domain. Interestingly, the mutant GST-SW6 did not retain either of these two proteins. p34^{cdc2} and the Ku antigen did not bind specifically to GST-SW6 or GST-VP16. Although Ku was detected in fractions 1 to 5 from the GST-VP16 column, the signal was weak (the filters with the anti-Ku antibody were overexposed in order to see these bands) and did not follow the pattern of fractionation of TBP or RP-A, indicating relatively low, if any, affinity of interaction. Unlike p34^{cdc2} and the Ku antigen, p62 was almost entirely depleted from the nuclear extract and found predominantly in fractions 5, 6 and 7 from the GST-VP16 column. Far less, although significant amount of p62 was detected in fraction 5 from the GST-SW6 column, but the protein was not depleted from the FT fraction. The elution pattern of p62 on the GST-VP16 column resembled that of RP-A and TBP and demonstrated comparable affinity

Figure 16. Western blot analysis of the GST, GST-SW6 and GST-VP16 affinity column fractions.

5 μ l of fractions L and FT, 15 μ l of fractions 1 and 2, and 30 μ l of fractions 3-8 from each column, respectively, were separated on 10 % SDS-acrylamide gels and transferred to Immobilon membranes. Western blot analysis (0.5-3 μ g/ml for the monoclonal antibodies and 1:200 dilution for the rabbit antisera. For detailed description of the antibodies see Table 3) was performed in parallel with the three filters for each antibody as described in Materials and Methods. The filters treated with the anti-Ku antibody were overexposed to bring up the bands in lanes 1-5 from the GST-VP16 column. After each analysis the filters were washed in 2% SDS, 100 mM 2mercaptoethanol at 50°C and reused several times. Anti-RPA antibody in these experiments was 70C.

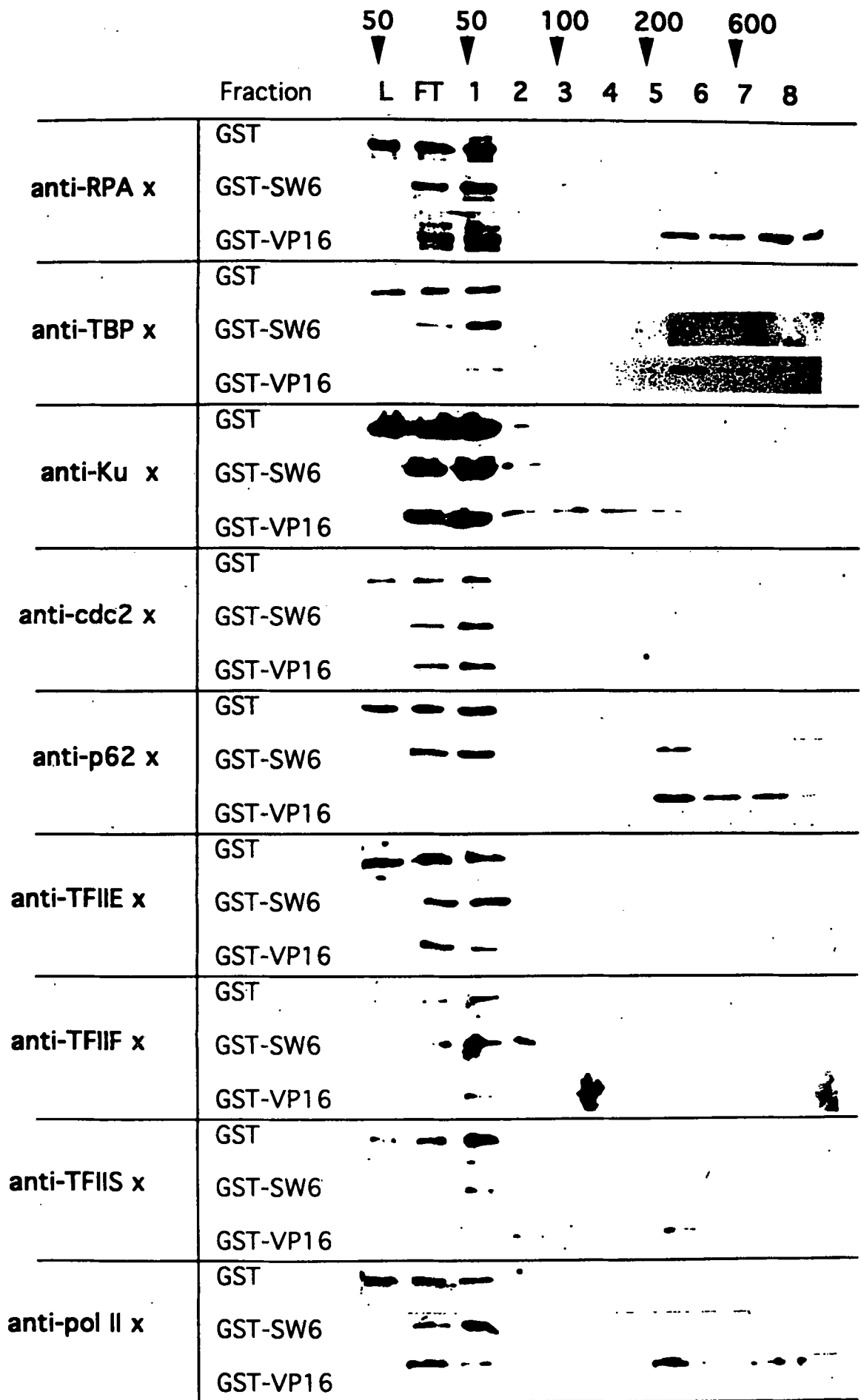
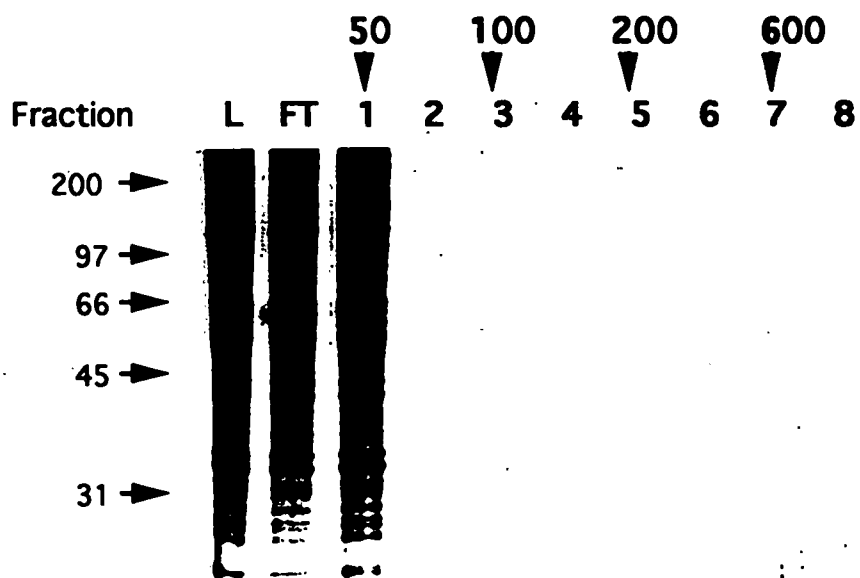
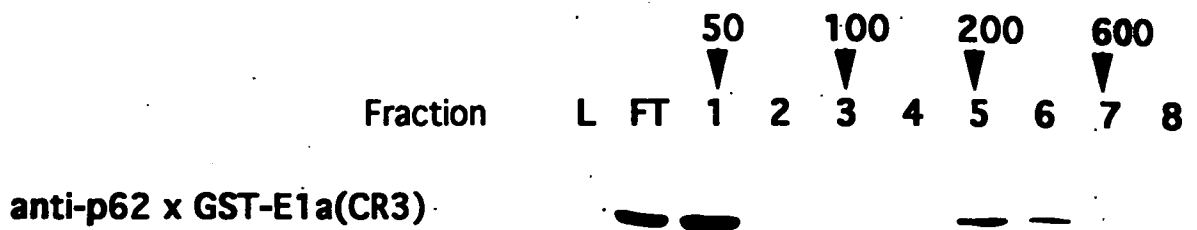


Figure 17. TFIID associates with E1a (CR2&3)

Preparation of the GST-E1a(CR2&3) affinity beads, affinity chromatography and western blot analysis were identical to the experiments with GST, GST-SW6 and GST-VP16 resins. 10% Coomassie stained SDS-acrylamide gel, showing the polypeptide profile of the GST-E1a(CR2&3) column, is presented below the results from the western. The arrows indicate the mobility of Rainbow (Amersham) protein molecular weight markers.



for the VP16 transactivation domain. The mutations in the SW6 protein did not completely abolish association with TFIIH, nevertheless the efficiency of interaction was substantially reduced. Most interestingly, TFIIH (p62) followed closely the fractionation of the CTD-kinase activity both on the GST-SW6 and GST-VP16 columns.

Similar affinity chromatography experiment was performed with GST resin, in which the conserved regions 2 and 3 of the Adenoviral protein E1a was used as a ligand. The results showed that human p62 was retained by the GST-E1a(CR2&3) column and eluted at 0.2 M KCl (Fig. 17). In addition, p62 from *X.laevis* oocyte extract interacted specifically with the GST-VP16 resin (data not shown). This data imply that the association of TFIIH with transactivation domains (direct or indirect) is not species specific and is not restricted to VP16 only.

Other pol II GTFs (TFIIF, TFIIE , TFIIS) and the large subunit of RNA polymerase II were also detected in fraction 5 from the GST-VP16 column, although they were not effectively depleted as in the case of TFIIH and TBP (Fig. 16). TFIIE and the large subunit of RNAPol II were found mostly in the 0.2 M KCl fraction, while TFIIF and TFIIS were present in the lower salt fractions too. TFIIF, TFIIE, TFIIS and large subunit of RNA polymerase II were not retained by neither GST or GST-SW6 resins.

The experiments presented in this chapter do not distinguish whether any of the interactions observed were directly between VP16 and the assayed proteins or were mediated by other proteins (see Discussion).

2.4.3. The VP16 Kinase is Highly Sensitive to DRB and Displays TFIIH Substrate Specificity

Several points of evidence presented so far suggested that a kinase might be involved in the control of pol II processivity. Indication about the nature of that kinase was provided by the fact that a CTD-kinase specifically interacted with the transactivation domain of VP16 and was coeluted with the p62 subunit of the general transcription factor TFIIF. Based on the results in Fig. 14, it was predicted that such a kinase may be sensitive to DRB. To test that prediction, it was essential to assay whether DRB (and H-8 and H-7) would inhibit the VP16-binding kinase. Another important issue, raising from the results in Fig. 16, was whether it would phosphorylate other substrates, characteristic for the TFIIF kinase (RAP74 (TFIIF), p56(TFIIE) and TBP (Ohkuma and Roeder, 1994). Initially, the peak 200 mM KCl CTD-kinase activity fraction from the GST-VP16 column (VP16-fraction 5) was assayed for its ability to phosphorylate TFIIF substrates. In Fig. 18 A it is shown that VP16-fraction 5 phosphorylates with almost equal efficiency (15-20 pmoles Pi/mg protein) RAP74 (TFIIF), p56 (TFIIE) and CTD (GST-CTD) and less efficiently TBP (about 7 pmoles Pi/mg protein) in a kinase reaction that was linear for more than 1 h. Endogenous activity, which can phosphorylate these proteins in the absence of TFIIF was not detected in the preparations of the recombinant substrates used (data not shown). In agreement with the previously reported substrate specificity of TFIIF (Ohkuma and Roeder, 1994), the VP16-binding kinase did not phosphorylate RAP30 (TFIIF) and p34(TFIIE) (data not shown). As demonstrated in Fig. 18 A, the kinase reaction with all four substrates was highly sensitive to DRB.

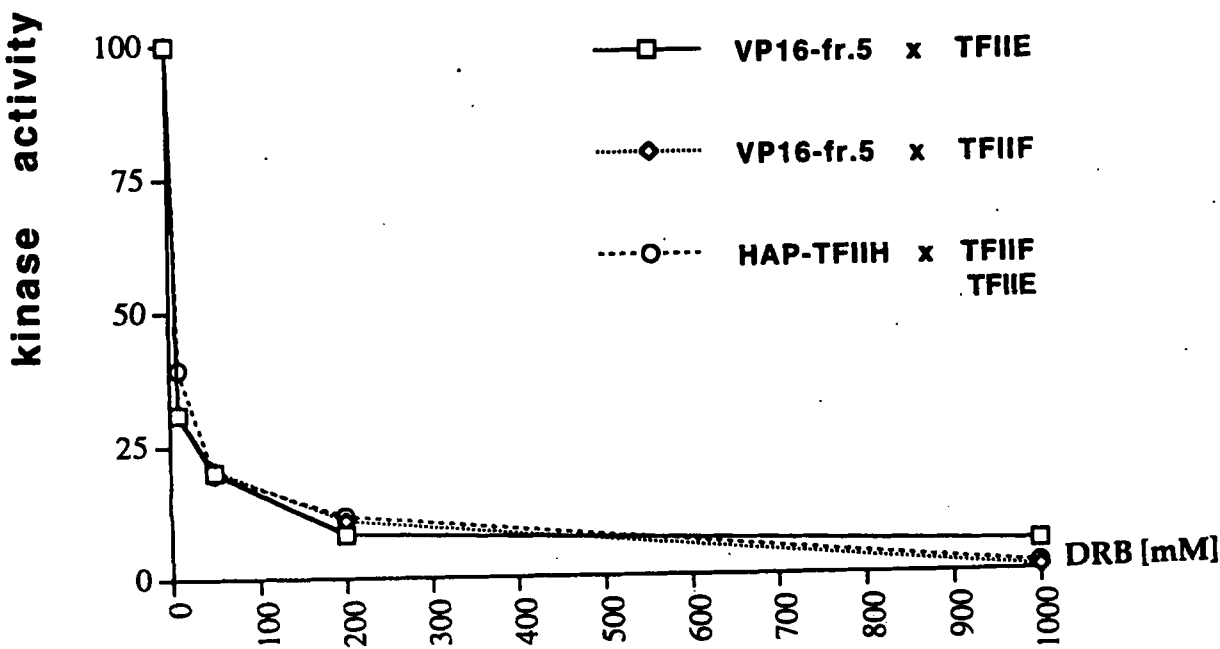
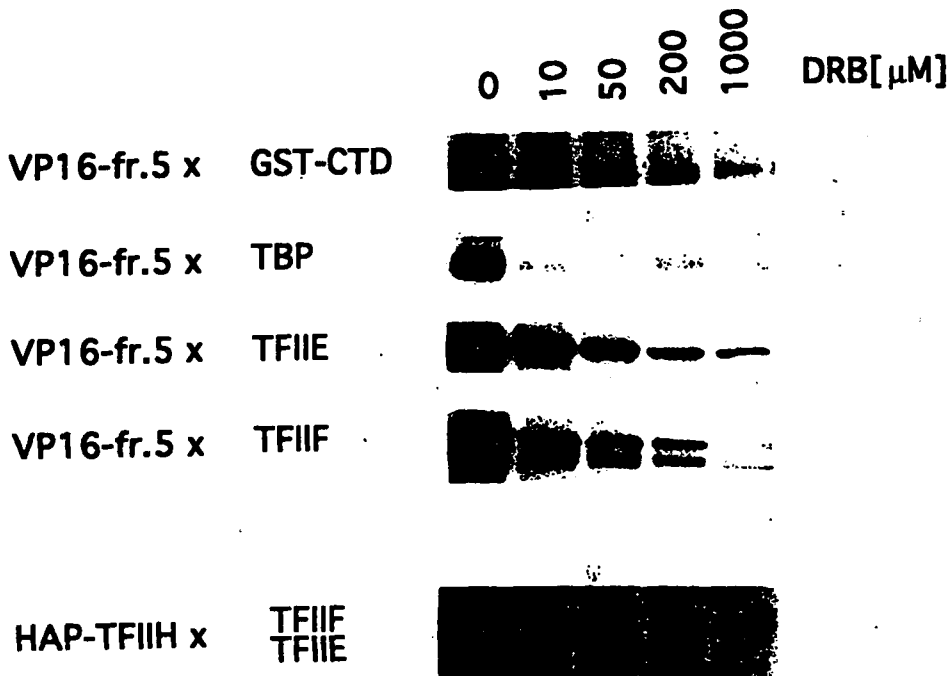
In a separate experiment I tested the DRB sensitivity of TFIIF, which had been passed through five columns before the final Hydroxyapatite purification step (HAP-TFIIF). This preparation was a gift from J.M. Egly. Both HAP-TFIIF and VP16 fraction 5 kinases were equally sensitive to DRB with RAP74(TFIIF) and p56 (TFIIE) as substrates (Fig. 18 A). (The signal from the gel with the HAP-purified TFIIF was about 20 times weaker as compared to the VP16-fraction 5 kinase). The Phosphorimager data obtained with the TFIIF and TFIIE

Figure 18. Characteristics of the GST-VP16 fraction 5 kinase.

0.3 μ l from fraction 5 from the GST-VP16 column were used in standard kinase reactions which were linear for more than 1 h. Substrates were GST-CTD (40 μ g/ml), TBP (40 μ g/ml), TFIIE (60 μ g/ml) and TFIIF (45 μ g/ml). 3.3 μ g/ml pAdH, linearised with EcoRI, and the kinase inhibitors (DRB, H-7 and H-8) were added to the reactions 30 min before the substrates and γ -³²P ATP.

A. The GST-VP16 fraction 5 kinase and highly purified TFIIF kinase are uniformly sensitive to DRB.

The concentration of DRB (μ M) in the samples is shown above the lanes. 0.1 μ l of Hydroxyapatite purified human TFIIF, 45 μ g/ml TFIIF and 40 μ g/ml TFIIE were used as kinase activity and substrates respectively, in HAP-TFIIFxTFIIF samples. The gels from GST-VP16 fraction 5 kinase experiments were exposed 30-120 min for the different substrates, while the gel from the HAP-TFIIFxTFIIF experiment was exposed for 18 h. Fixed dried gels were phosphorimaged and the data were plotted on the graph, shown in the lower part of the figure. The activity of the non-inhibited reactions was normalised to 100%, although there were differences in the specific phosphate incorporation.



substrates were plotted on the graph, shown in the lower panel of the figure. The activity in the non-inhibited reactions with these two substrates was normalised to 100%, although there were differences in the specific phosphate incorporation. Signals, relative to the non-inhibited samples were then plotted to demonstrate the uniform sensitivity of the two kinase activities to DRB.

The VP16-fraction 5 kinase activity was further characterised by comparing its sensitivity to DRB, H-7 and H-8. Each of the four substrates (GST-CTD, TFIIF, TFIIE and TBP) were used. Fixed dried gels were quantified by a Phosphorimager (Molecular Dynamics) and the data plotted on a separate graph for each substrate. A graph, characteristic for GST-CTD, TBP and p56(TFIIE), is shown in Figure 18 B. In that particular case the substrate was GST-CTD. DRB typically inhibited the kinase activity to 50% of the control when applied between 10 and 50 μM , while the same effect with H-7 and H-8 was reached at 200 μM or more. Note that similar concentrations are necessary to inhibit processivity of pol II transcription both in vitro (D.Bentley, unpublished results) and in injected *X.laevis* oocytes (Figs 12-14). The phosphorylation of RAP74(TFIIF) was as sensitive to DRB and H-8 as the rest of the substrates. For reasons I do not understand, phosphorylation of TFIIF was not inhibited but slightly stimulated by relatively low concentrations of H-7 (data not shown).

2.4.4. Other Characteristics of the VP16 Binding Kinase

Phosphorylation of the large subunit of RNA polymerase II and some synthetic CTD substrates by the TFIIF associated kinase is markedly stimulated by promoter DNA (Lu et al., 1992; Roy et al., 1994; Serizawa et al., 1993b) and TFIIE (Ohkuma and Roeder, 1994). Another feature of this kinase activity is that GTP and dATP, but not UTP and CTP, can compete with ATP for the catalytic

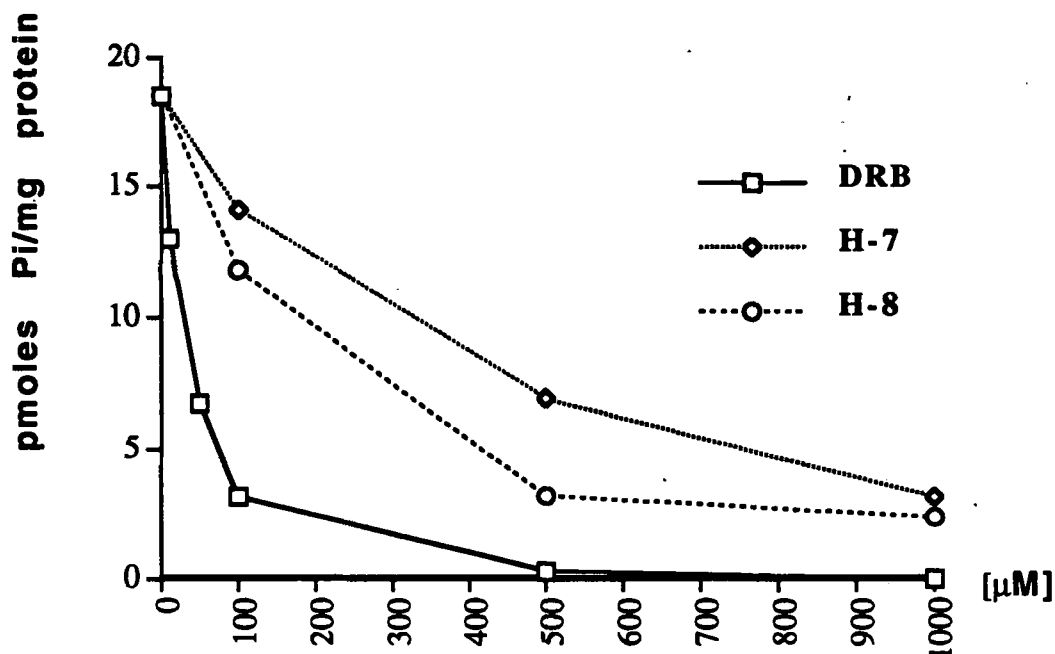


Figure 18 B. Inhibition of GST-CTD phosphorylation by the VP16-fraction 5 kinase in the presence of DRB, H-7 and H-8.

Kinase reactions were performed in the presence of several concentrations (0-1 mM) of the kinase inhibitors. The radioactive band in the lane, corresponding to the non treated with inhibitors sample, was excised and counted to estimate the phosphate incorporation in GST-CTD. The gel was quantified by a Phosphorimager (Molecular Dynamics), the figures were normalised for phosphate incorporation based on the counts from the excised band and the data were plotted on a graph.

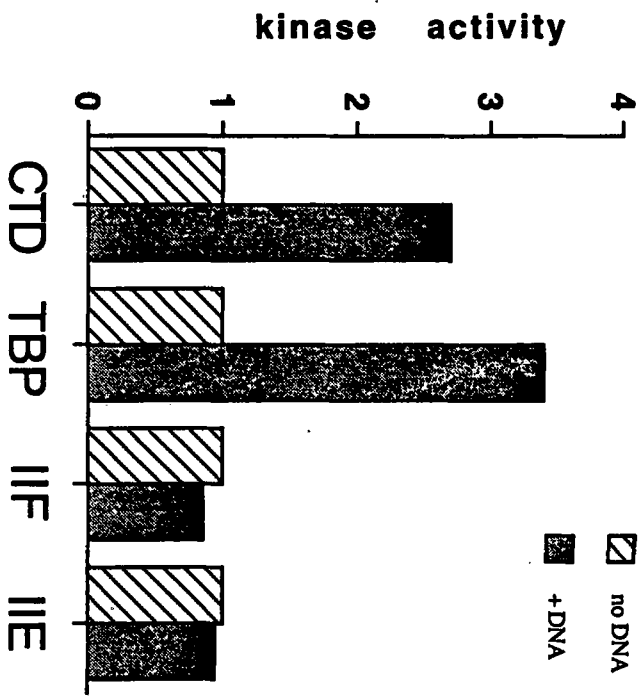
Figure 18 C. Stimulation of the GST-VP16 fraction 5 kinase activity by DNA.

Relative kinase activities in reactions, performed in the presence or absence of 3.3 $\mu\text{g/ml}$ pAdH linearised with EcoRI, is presented. The activity in all samples without DNA is normalised to 1, although the specific phosphate incorporation for different substrates was not equal.

Figure 18 D. The VP16 fraction 5 kinase activity is selectively competed by GTP and dATP.

Standard kinase reactions with GST-CTD as a substrate were performed in the presence of 0.5 mM ATP, GTP, dATP, CTP and UTP. The fixed dried gel was quantified by a Phosphorimager (Molecular Dynamics) and the data plotted on the graph. The bars represent percentage of the GST-CTD kinase activity relative to the control (C).

DNA STIMULATION



NTP COMPETITION

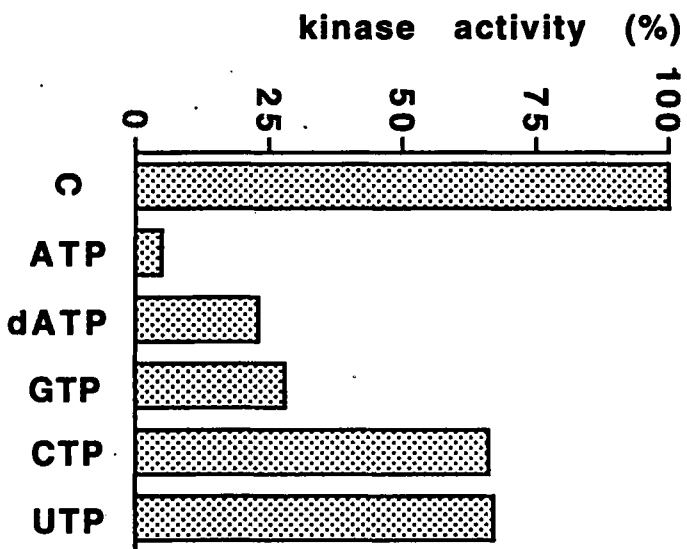
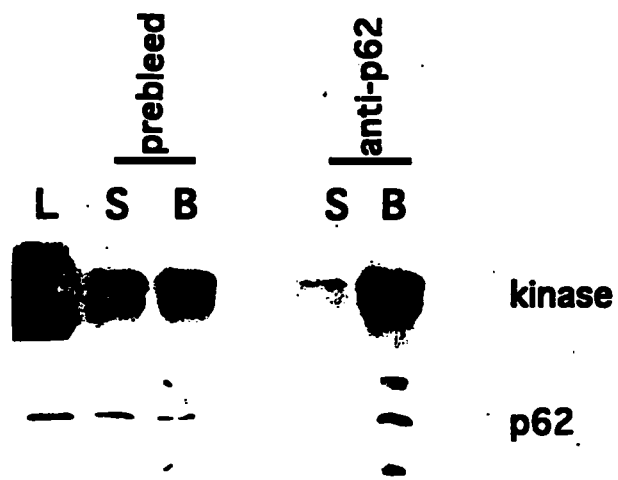


Figure 19. Immunodepletion of the kinase activity in VP-16-fraction 5 with anti-p62 antibodies.

Rabbit prebleed, polyclonal and monoclonal antibodies against p62(TFIIF) were immobilised on Protein A-Sepharose as described in Materials and Methods. VP-16-fraction 5 was incubated with the prebleed and the affinity resins for three hours. Kinase: 2 μ l of the input (L), 2 μ l of the resulting supernatant fractions (S) and 2 μ l settled volume of the washed beads (B) were analysed by standard kinase reactions with TFIIF as substrate. p62: 20 μ l of each fraction and 20 μ l settled volume of the beads were analysed by western blot analysis as in Figure 16.



site of the enzyme. I assayed whether the same characteristics apply for the VP16 binding kinase.

Linearised plasmid, containing AdML promoter DNA stimulated the phosphorylation of GST-CTD and TBP about three times, while having no effect on the phosphorylation of RAP74 (TFIIF) and p56 (TFIIE) (Fig. 18 C). Addition of recombinant TFIIE to VP16-fraction 5 did not enhance the kinase activity (data not shown), but it should be emphasised that endogenous TFIIE had already been detected in this fraction (see Fig. 16).

Previously I hypothesised that a kinase is involved in transcriptional activation. Since the affinity purified kinase activity had all the characteristics of the hypothetical kinase, it was interesting to check whether the VP16 transactivation domain would stimulate it. GAL4-VP16 had no effect on the phosphorylation of GST-CTD, but subsequent western analysis indicated the presence of GST-antibodies reactive polypeptide with the mobility of GST-VP16. Thus, it was possible that GST-VP16 leakage from the affinity resin could have interfered with that assay (data not shown).

Addition of excess unlabelled NTPs to the kinase reaction with GST-CTD as a substrate demonstrated that GTP and dATP, but not UTP and CTP, competed effectively with $\gamma^{32}\text{P}$ -ATP (Fig. 18 D). The same substrate specificity was reported for highly purified TFIIF (Roy et al., 1994; Serizawa et al., 1993b).

2.4.5. Immunodepletion of VP16-associated kinase with anti-TFIIF antibody

The results presented so far showed that the VP16-associated kinase had many of the properties of TFIIF and contained the bulk of p62(TFIIF) from HeLa nuclear extract. Although the kinase subunit(s) of TFIIF have not been identified, the activity is known to associate tightly with p62 (Schaeffer et al.,

1993). In order to further establish whether the VP16-associated kinase was indeed TFIIF, I reacted it with a mixture of monoclonal and polyclonal anti-p62 antibodies immobilised on Protein-A Sepharose (Pharmacia) and monitored the kinase activity of the washed beads (B) and the supernatant (S) using TFIIF as a substrate. The presence of TFIIF was assayed by Western blot analysis with monoclonal 3C9 antibodies against p62 (TFIIF). As a control, pre-immune serum from the same rabbit was used. Despite the high background binding to the pre-immune beads, the result in Figure 19 demonstrates that the kinase activity eluted from the VP16 column is quantitatively depleted by anti-p62 antibodies (Fig. 19, compare the activity of the input sample with that of the immune supernatant). Western blotting of the proteins bound to the beads and in the supernatant confirmed that p62 was depleted along with the kinase activity by the immune antibody (Fig. 19).

In summary, a VP16 binding kinase activity from HeLa nuclear extract co-fractionated with the GTF TFIIF and had substrate specificity and properties, indistinguishable from the kinase activity of this factor.

2.4.6. Processivity of Pol II Transcription is Inhibited by Antibodies Against TFIIF (p62)

Inhibition of processivity of the mouse c-myc gene by "squenching" implied that factor(s), required for pol II elongation, can be sequestered by the VP16 transactivation domain. To clarify whether one of these factors could be TFIIF, the effect of monoclonal antibodies against p62(TFIIF)(Schaeffer et al., 1993) on the transcription of c-myc was investigated in *X.laevis* oocytes.

X.laevis oocytes were injected with the mouse c-myc gene together with mouse monoclonal antibodies against p62 (3C9); against human RP-A (70C or 34A, (Kenny et al., 1990) or human C-MYC (9E10, (Evan et al., 1985) as controls.

Figure 20. Antibodies against TFIID (p62) inhibit processivity of c-myc transcription.

RNAase protection of transcripts from oocytes injected with pSX943. Injections and RNAase protection was as in Fig.12. Abbreviations: C: control, P: probe, RT: readthrough, TM : terminated, VA: RNAase protection products from the coinjected Adenovirus VA1 gene. Full length protection of the probe corresponds to transcripts from the P1 promoter or RNAs that read all the way around the plasmid. The injection samples contained 1 mg/ml BSA (lane 1 and 7) or the following mouse monoclonal antibodies: α -p62 (3C9)-0.3 mg/ml (lanes 2, 8, 9 and 10) or 0.03 mg/ml (lane 3); α -RPA (70C) - 0.15 mg/ml (lane 4); α -RPA (34A) - 0.15 mg/ml (lane 5); α -C-MYC (9E10)- 0.15 mg/ml (lane 6). The total protein injected was made up to 1 mg/ml with BSA. The samples (10 μ l) presented in lanes 7 and 9 were premixed with 10 μ l settled volume of p62-NTA-Agarose. DRB (50 μ M) was added to the oocyte incubation media. Lanes 1-6 and lanes 7-10 are from different experiments.

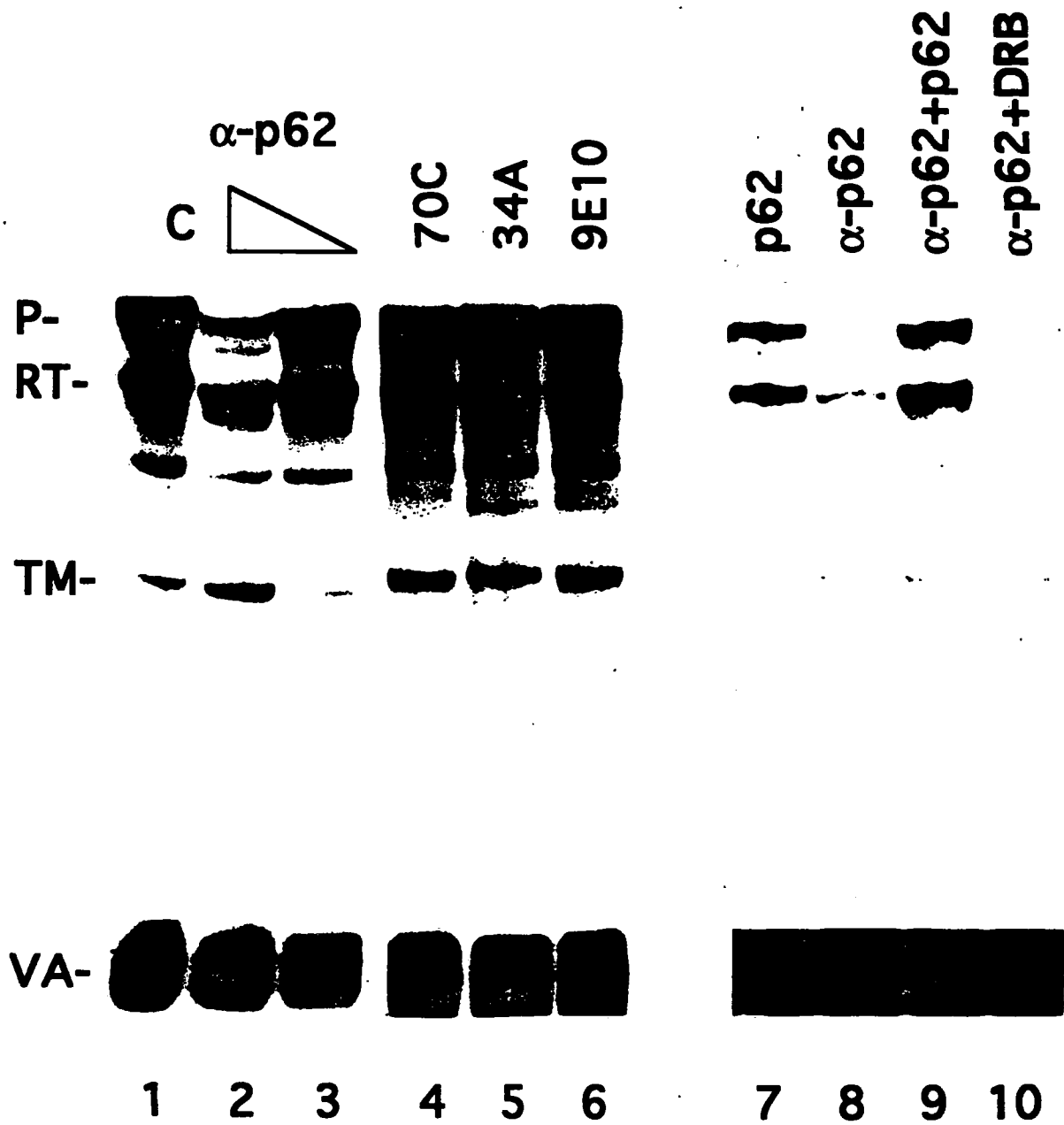
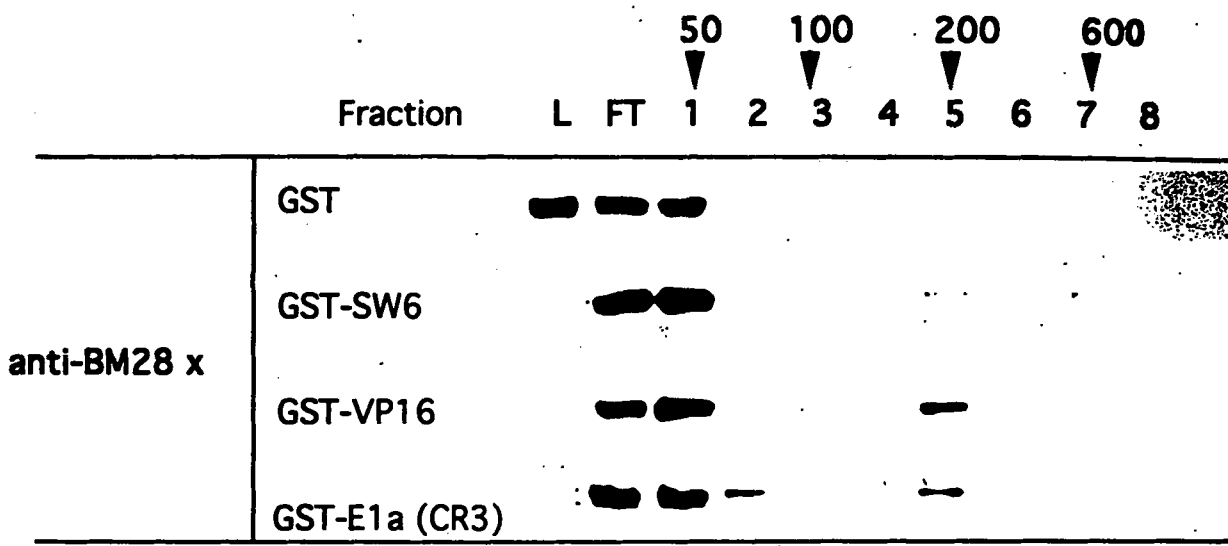


Figure 21. BM28 binds specifically to GST-VP16 and GST-E1a(CR2&3).

All fractions from the GST, GST-SW6, GST-VP16 and GST-E1a(CR2&3) chromatography experiments were analysed by Western blot analysis with antibodies against BM28 as described in Fig. 16.



The final protein concentration in the injections was equalised to 1 mg/ml with BSA. The 3C9 and 70C monoclonal antibodies reacted with 62 and 70 kDa *X.laevis* oocyte polypeptides, respectively, while 34A and 9E10 did not recognise any antigen as determined by western blot analysis (data not shown).

The anti-p62 antibody, injected at 0.3 mg/ml, decreased significantly processivity (RT/RT+TM) of transcription of the *c-myc* gene as compared to the BSA control (Fig. 20, lanes 1 and 2). Ten times less 3C9 antibody (0.03 mg/ml) and the three control monoclonal antibodies (70C, 34A and 9E10, each injected at 0.15 mg/ml, respectively) had no effect on *c-myc* transcription (Fig. 20, lanes 3-6). The effect of the anti-p62 antibodies was not due simply to interfering with pol II transcription, since antibodies against RNAPol II CTD (8WG16) completely inhibited the expression of *c-myc* (see Figure 22 A). The control VA1 gene was unaffected by any of these antibodies.

The specificity of the effect of 3C9 antibodies was further assayed by preincubating the injection mixtures with p62, immobilised on NTA-agarose beads (Novagen). DNA was mixed with p62-beads, 3C9 antibodies at 0.3 mg/ml, or both as described in Fig. 20. As shown in Fig. 20 (lane 7), preincubation of the injection sample with p62-beads did not alter *c-myc* transcription (compare lanes 1 and 7). The effect of 3C9 antibodies on pol II processivity (lane 8) was highly specific to p62, since premixing of the antibody and p62-beads completely reconstituted the pattern of transcription observed in the control lane (Fig. 20, compare lanes 7,8 and 9). Some of the oocytes, injected with 3C9 antibodies were treated with 50 μ M DRB to check whether the drug could inhibit RNAPol II elongation independently of TFIIF. In the presence of anti-p62 antibodies DRB was not able to significantly decrease the processivity of *c-myc* transcription (Fig. 20, compare lanes 8 and 10). This result was consistent with the idea that most, if not all, of the effect of low concentrations of DRB on processivity is mediated by inhibition of the TFIIF kinase activity.

2.5. BM28 Regulates RNAPol II Transcription

The human protein BM28 was recently cloned and shown to share significant level of homology with the *S.cerevisiae* proteins MCM2 and MCM3, *S.pombe* CDC21 and the mouse protein P1 (Todorov et al., 1994). All these proteins have been considered to be involved in the onset of DNA replication in yeast (Chen et al., 1992; Yan et al., 1991) and mouse (Thommes et al., 1992). Injection of antibodies against BM28 protein in G1 cells delayed entry in S-phase and inhibited RNA and DNA synthesis (Todorov et al., 1994); I.Todorov, unpublished observations). So far, no direct participation of BM28 or its yeast analogues in RNA synthesis has been reported. In collaboration with Dr. I.Todorov we investigated whether BM28 could play some role in RNAPol II transcription.

2.5.1. BM28 Binds to Transcriptional Activation Domains

Initially we asked whether BM28 could associate with transactivation domains of sequence specific RNAPol II factors. All fractions from the affinity chromatography experiments described in Chapter 2.4.2. were assayed for the presence of BM28 by western blot analysis (Fig. 21). BM28 was found with the bulk of the GTFs and the large subunit of RNAPolymerase II in the 0.2 M KCl fractions of the GST-VP16 and the GST-E1a(CR2&3) columns. Non-specific association with the control GST-Sepharose resin was not detected. Interestingly, BM28 interacted with the mutant SW6 domain, although much less efficiently as compared to the VP16 domain (Fig. 21). That pattern of fractionation of BM28 was reminiscent of p62(TFIID), p56(TFIIE), RAP74(TFIIF) and the large subunit of RNAPolymerase II (see Fig. 16). A major difference between these proteins, though, was that while the amount of many of the GTFs

in the FT fractions of the affinity columns was substantially decreased, a relatively small proportion of BM28 associated with the transactivation domains.

2.5.2. Antibodies Against BM28 Inhibit c-myc Transcription

The results from the affinity chromatography experiments indicated that BM28 interacted specifically with RNAPol II transcription factors. Further support of that observation was provided by immunoprecipitation assays with the anti-BM28 antibodies (I.Todorov, unpublished). In order to clarify if these interactions had any functional significance, we asked whether the same antibodies would affect pol II transcription in injected *X.laevis* oocytes.

First I studied the effect of anti-BM28 antibodies on transcription of the mouse c-myc gene. *X.laevis* oocytes were co-injected with pSX943 together with BSA (Fig. 22 A, lane 1) or antibodies against p62(TFIIF) (3C9, Fig. 22 A, lane 2); against CTD (8WG16, Fig. 22 A, lane 3) or against BM28 (Fig. 22 A, lane 4). As demonstrated in chapter 2.4.6., the anti-p62 antibodies suppressed RNAPol II processivity without significantly decreasing the level of initiation. The anti-CTD antibodies almost completely inhibited c-myc transcription, although some resistant non-processive RNA species was also detected. Previously I had observed similar effect of the anti-CTD antibodies on Gal5-HIV2 transcription (see Fig. 9). The anti-BM28 antibodies were very efficient in inhibiting RNAPol II transcription and abolished c-myc expression. Neither of the antibodies used had any effect on the VA1 control gene.

2.5.3. Antibodies Against BM28 Inhibit Transcription of a Synthetic Gene

It was possible that BM28 function could be specific for certain genes,

Figure 22. Anti-BM28 antibodies inhibit RNAPol II transcription in *X.laevis* oocytes.

RNAase protection of transcripts from *X.laevis* oocytes, injected with pSX943 (A and C) and pGal5-P2CAT (B). Injections and RNAase protection were as in Fig.12 and Fig. 2, respectively. Abbreviations: C: control, P: probe, RT: readthrough, TM : terminated, VA: RNAase protection products from the coinjected Adenovirus VA1 gene.

A. Oocytes were injected with pSX943 together with BSA (C) or antibodies against p62(TFIIH) (3C9, 0.3 mg/ml), the carboxyterminal domain of RNAPol II (8WG16, 0.15 mg/ml) or against BM28 (0.15 mg/ml). All samples contained 1 mg/ml BSA. Full length protection of the probe corresponds to transcripts from the P1 promoter or RNAs that read all the way around the plasmid.

B. Oocytes were injected with pGal5-P2CAT together with BSA (C) or antibodies against RPA (70C, 0.15 mg/ml) or against BM28 (0.15 mg/ml). Transcription from this construct was activated by co-injecting of 0.1 mg/ml GAL4-AH. The protein concentration of the samples was equalised with 1 mg/ml BSA.

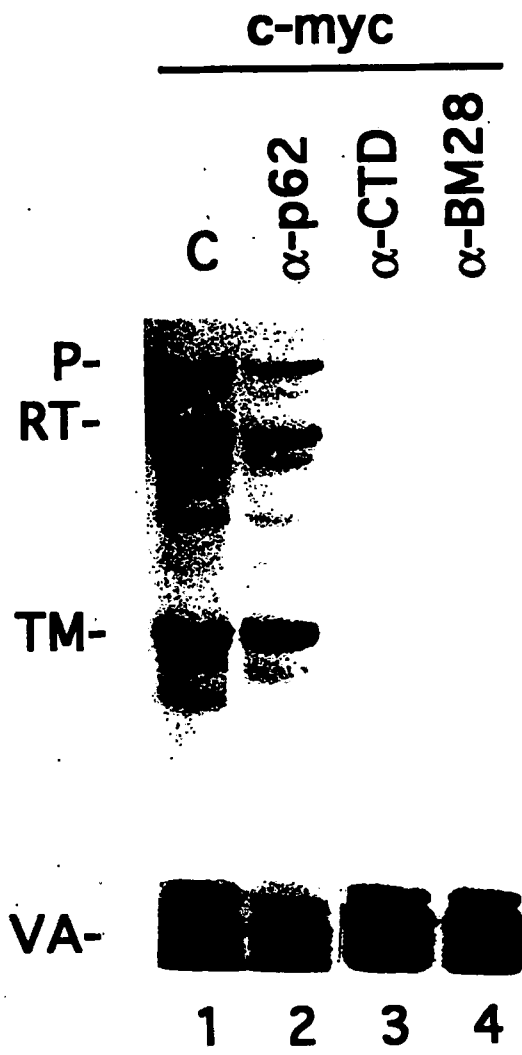
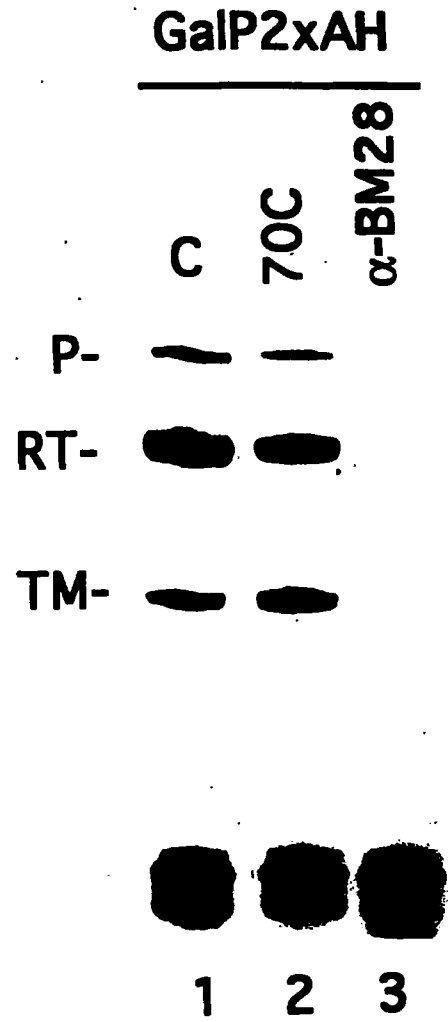
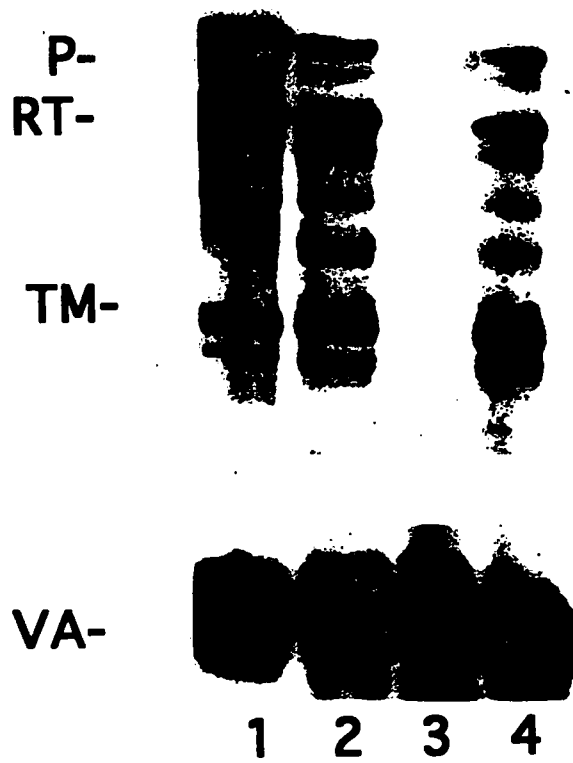
A**B**

Figure 22 C. Oocytes were injected with pSX943 and anti-BM28 antibodies (α -BM28) as in Fig. 21A. Recombinant BM28 was injected at 0.8 mg/ml (lanes 2 and 4). BSA (0.8 mg/ml) was added to the samples, presented in lanes 1 and 3.

c-myc

-	-	+	+	α -BM28
-	+	-	+	BM28



including c-myc. Alternatively, it could be a factor that is generally required for RNAPol II transcription. To distinguish between these possibilities, the effect of BM28 antibodies on transcription from a synthetic GAL5-P2mycCAT construct was investigated. The GAL5-P2mycCAT plasmid was co-injected with GAL4-AH and anti-BM28 antibodies (Fig. 22 B, lane 3) or antibodies against RP-A (70C, Fig. 22 B, lane 2). These samples were compared to a control, injected with the plasmid and GAL4-AH only (Fig. 22 B, lane 1). While the 70C antibodies had no effect on both pol II and pol III transcription, the anti-BM28 antibodies abolished pol II transcription as in the case of the wild type c-myc gene. The anti-BM28 antibodies also inhibited completely transcription from the wild type HIV-2 LTR promoter (data not shown). These results indicated that the BM28 was required for the expression of a wide range of class II genes.

2.5.4. The Effect of the Anti-BM28 Antibodies is Specific for BM28

I further investigated the specificity of the in vivo effect of the anti-BM28 antibodies by blocking them with excess of BM28. In the experiment presented in Fig. 22 C pSX943 was injected with BSA (lane 1), recombinant BM28 at 0.8 mg/ml (lane 2), anti-BM28 antibodies at 0.15 mg/ml (lane 3) or both BM28 and anti-BM28 antibodies (lane 4). BM 28 caused some decrease in processivity of c-myc transcription in a way, similar to that of "squelched" c-myc expression (see Fig. 14). When BM28 and the antibodies against it were co-injected, pol II transcription was partially recovered from the effect of the antibody (Fig. 22 C, compare lanes 3 and 4), although the level of premature termination was still high. The VA1 control was unaffected by either the antibodies or BM28. Thus, the antibodies against BM28 act specifically to inhibit RNAPol II transcription.

DISCUSSION

Transcriptional activators stimulate the expression of eukaryotic class II genes by specifically interacting with their promoters. At present, it is assumed that the major, if not the only function of transactivators is to increase the low basal level of initiation, observed in vitro at minimal promoters (see Chapter 1.1.3.). Most of the published models postulate that transactivators stimulate transcription by direct and indirect interaction between activation domains and general transcription factors thus facilitating assembly of the preinitiation complex (reviewed in (Drapkin et al., 1993)). It is likely that transactivators can further affect the incidence with which initiation takes place by inducing higher affinity interactions within the preinitiation complex (Choy and Green, 1993). (Hahn, 1993) hypothesised that there is a qualitative difference between initiation at activated and non-activated states of the promoter. According to that hypothesis, activators promote "effective" initiation, while in the non activated state "abortive" initiation complexes, which can not trigger synthesis of RNA, are formed. Transactivators in general have never been implicated to control subsequent stages of RNApolymerase II transcription such as promoter clearance and elongation. Some of the experiments presented here and studies from other laboratories suggest that this view should be revised.

3.1.1. Activators Enhance Transcriptional Processivity

In chapter 2.1. of this thesis I show that transactivators not only stimulate the rate of initiation, but substantially increase the efficiency with which RNApolymerase II reads through sites of pausing or premature termination. The experiments in figures 2-6, 8, 9 and 11 demonstrate fundamental qualitative difference between the processivity of activated and non-activated RNAPol II

transcription in injected *X.laevis* oocytes. Non-activated transcription from truncated c-myc P2 and HIV-2 LTR promoters lacking their natural sequences upstream of the TATA box has low processivity (see Figs. 2 and 3). In contrast, transcription activated from the wild type promoters by endogenous oocyte factors is highly processive and elongates efficiently through potential pausing and intragenic termination sites (compare Figs. 2, 3 and 12, for example). Similarly, recombinant GAL4 activators injected into oocytes stimulated transcriptional processivity in reporter genes containing GAL4 binding sites, which were fused to basal promoter elements from HIV-2, the mouse c-myc, the human TK and the Adenovirus E4 and E1b genes (Figs. 2-6 and Table 1). On the other hand, "squelching" by GAL4-VP16 severely inhibited the processivity of c-myc transcription (see Fig. 14), possibly by sequestering factors, necessary for efficient elongation. This observation suggests that among the factors sequestered by an excess of the VP16 activation domain in trans are proteins which stimulate pol II elongation. The nature of the "elongation" factors will be discussed in detail later on.

Upregulation of transcriptional processivity by transactivators is not an exceptional feature of the injected *X.laevis* oocyte system. Some of the synthetic constructs described in Fig. 1 were assayed for processivity of transcription in vitro (D.Bentley, unpublished results) or in transfected mammalian cells (D.Bentley, published in (Yankulov et al., 1994); J. Blau, unpublished). In agreement with the results obtained in *X.laevis* oocytes, these systems produced activated transcription which was far more processive than non-activated transcription. In transfected mammalian cells RNAPol II processivity has been estimated by nuclear run-on assays, which employs different criteria for the efficiency of elongation. Thus, my observation that RNAPol II processivity is modulated by transcriptional activators was confirmed in a different system and with a different assay.

The similarity in the results, obtained in *X.laevis* oocytes and other systems strongly suggest that activator mediated stimulation of transcriptional elongation by RNAPol II is a wide spread phenomenon. Together, these observations imply that regulation of processivity is not promoter-specific nor is it peculiar to one type of transcriptional activator. For example whereas the AH- and VP16- activation domains are highly acidic, E1a- is not.

An important outcome from the recent work in our laboratory, part of which is presented in section 2.1 of the thesis, is that transactivators stimulate RNAPolymerase II to efficiently elongate at promoter-distal sequences of the genes, while non-activated complexes fall off the template soon after leaving the promoter. A logic consequence of this observation is that a qualitatively different initiation events might occur in the presence or in the absence of transactivators. Experimental support of such a hypothesis is provided by Fig. 9 and section 2.3. of the thesis and by previous studies by (Roberts and Bentley, 1992) and (Marciniak and Sharp, 1991). This issue will be discussed in detail later on.

In conclusion, the effect of transactivators on pol II transcription is not confined to increasing the frequency of initiation but also affects the elongation properties of the RNAPol II complex once it is released from the promoter.

3.1.2. Implications for the Control of Transcriptional Processivity in HIV-1 and HIV-2

A connection between stimulated initiation and the competence for extensive elongation has been established previously only for HIV transcription activated by TAT (Laspia et al., 1989; Marciniak and Sharp, 1991). The VP16 C-terminal domain activated transcription from the HIV1 LTR promoter as effectively as TAT when measured by CAT reporter activity but it was not assayed whether this effect was due to stimulated initiation or enhanced elongation

(Southgate and Green, 1991; Tiley et al., 1992). Southgate and Green (1991) observed that when the HIV1 promoter was activated by GAL4-VP16, TAT had a significantly smaller effect than in the absence of GAL4-VP16. This fact suggested that these two activators operate through some common pathway. My results show that a strong activator such as VP16 produces the same effect as TAT on the processivity of HIV2 transcription (Figs. 4 and 9). Therefore I hypothesise that in this respect the effect of TAT is not unique but quite general and is due to its property to activate transcription from the HIV LTR promoter.

The first direct evidence that a factor other than TAT may affect processivity was reported by (Laspia et al., 1990) who found that E1a weakly stimulated processivity of HIV1 transcription. Furthermore E1a and TAT synergised to increase processivity. Based on these observations it was predicted that VP16 and other strong activators may mimic the effect of TAT on processivity (Cullen, 1993; Greenblatt et al., 1993). This prediction is confirmed by the data presented here (Fig. 4).

TAT itself stimulates both initiation and elongation (Laspia et al., 1989) and was recently shown to interact with TBP and TFIID (Kashanchi et al., 1994). These characteristics are reminiscent of VP16 and E1a, as well as other transactivators (see chapter 1.1.1.1). In my experiments, promoter binding transactivators displayed properties similar to these reported for TAT in different systems, indicating that most of the effect of TAT on processivity of HIV-1 and HIV-2 transcription might be achieved through the promoter by interactions with promoter-bound factors. Additional implication from my research and others studies (see chapter 1.2.1.) is that production of long mRNAs from the HIV-1 LTR promoter is not critically dependent on TAT. This point predicts that some inducible sequence specific factors that bind the LTR promoter can provide temporary stimulation for processive transcription. In this way the initial synthesis of TAT will be primed and a positive feedback loop can be established (Cullen, 1993). It is not clear whether the relatively high non-activated transcription from

the LTR promoter, observed in my experiments, can contribute to the initial synthesis of long mRNAs, for example by facilitating the response to weak activation signals. A certain level of basal transcription, though, is essential for TAT transactivation, since this protein is tethered to the promoter via its RNA-binding domain.

3.1.3. Implications for the Control of Transcriptional Processivity in c-myc and other genes

Transcription from the c-myc P2 promoter is attenuated within the first 500 bases of the transcription unit in both the murine and human genes (Bentley and Groudine, 1988). The 3' ends of the truncated prematurely terminated c-myc RNAs have been mapped at T-rich sequences close to the exon1/intron1 boundary, called T2, and detected both in *X.laevis* oocytes and in vitro (Bentley and Groudine, 1988; Nepveu and Marcu, 1986). In mammalian cells, though, such short c-myc RNAs have not been observed presumably because of their instability. Surprisingly, deletion of the sequences, contributing to premature termination at the T2 site in vitro and *X.laevis* oocytes do not abolish the gradient in the density of RNAPol II complexes over the gene (Krumm et al., 1992). High resolution run-on assay confirmed the elevated RNAPolymerase II density in the promoter proximal region of the gene as compared to the promoter distal regions, but no sharp decline downstream of T2 was observed (Krumm et al., 1992; Strobl and Eick, 1992). This data clearly indicated that modulation of a specific conditional block of elongation at T2 was not the cause of attenuation in c-myc. Instead, pausing of the elongating complex immediately downstream of the initiation site in a way, similar to that previously described in the *Drosophila* Hsp70 and other genes, was proposed to be the principal mechanism by which c-myc transcriptional processivity is controlled in mammalian cells (Krumm et al., 1992).

In the *Drosophila* Hsp70 gene, the promoter bound GAGA factor facilitates the establishing of a paused polymerase, while release of the paused complex requires activation of the HSF factor (Lee et al., 1992; Rougvie and Lis, 1990).

Promoter-proximal pausing downstream of P2 was confirmed when the Gal5-P2mycCAT gene was transfected in 293 cells, although considerable decrease in polymerase density 3' of the T2 site was also detected. When transcription from this construct was activated by GAL4-VP16, both promoter pausing and attenuation at the exon1/intron1 boundary was suppressed (D.Bentley, published in (Yankulov et al., 1994)). Gal5-P2mycCAT contains the mouse c-myc sequences downstream of position -44 relative to the P2 start site. In the human c-myc, sequences upstream of position +47 relative to the P2 start site were sufficient to program polymerase pausing (Krumm et al., 1992). Taken together, these observations show that sequences, consisting of the TATA element and the P2 initiation site comprise an element that directs pausing of RNAPolymerase II, while upstream sequences are responsible for activation and stimulation of transcriptional elongation. Further mutational analysis of the sequences upstream of the P2 TATA box demonstrated that transactivator responsive elements upregulate the efficiency with which RNAPol II reads through the T2 site in *X.laevis* oocytes (D.Bentley, published in (Yankulov et al., 1994)).

In *X.laevis* oocytes, a paused RNAPol II complex downstream of P2 was not detected (Meulia et al., 1993). Nevertheless, sequences downstream of -44 relative to the P2 start site were sufficient to support transcription which terminated prematurely at T2; and to support GAL4-transactivators stimulated transcription with high processivity, respectively (Fig. 2 and Fig. 8). These similarities strongly suggest that the same mechanisms of transcriptional elongation control operate in *X.laevis* oocytes and in mammalian cells. The difference in the manifestation of low processivity transcription (promoter proximal pausing or preferential termination at T2, respectively) is likely to result from the chromatin structure of the c-myc gene in these two experimental systems (Meulia et al., 1993).

Over 20 cellular and viral genes have been identified which transcription is regulated by modulating the efficiency of elongation through pausing and termination sites (Spencer and Groudine, 1990). Based on the results, described in Figs. 2-6, it is likely that regulated transcriptional elongation in these genes is controlled by the activation domains of "conventional" transcription factors.

3.1.4. Role of Terminator Elements in the Control of Transcriptional Elongation

Although discrete sites of pausing and premature termination by RNAPol II have been identified in a number of genes including c-myc (Bentley and Groudine, 1988), adenosine deaminase (Ramamurthy et al., 1990), c-fos (Mechti et al., 1991), α -tubulin (Hair and Morgan, 1993), and HIV1 and HIV2 (Toohey and Jones, 1989), these sites may not be essential in order for RNAPol II transcription to be regulated at the level of elongation. As discussed in chapter 3.1.3., the T2 terminator element of the human c-myc gene is dispensable for transcriptional attenuation (Krumm et al., 1992). It has also been demonstrated that the efficiency of elongation in mammalian cells can be controlled by transactivators even over sequences of prokaryotic origin (CAT), which are unlikely to possess specific eukaryotic terminators (D.Bentley, published in (Yankulov et al., 1994; Laspi et al., 1990).

In *X.laevis* oocytes, deletion of the ADA +96 terminator element (Ramamurthy et al., 1990) or the α -globin terminator element (Hair and Morgan, 1993), respectively, did not abolish attenuation in these genes, but shifted the position of premature termination further downstream. In a different study, the levels of premature termination at the c-myc T2 site in *X.laevis* oocytes were found inversely dependent on the distance between the start site and the terminator element (Roberts and Bentley, 1992). These results are consistent with the idea that the competence of RNAPol II to read through the termination sites rather than

sequence specific termination factors control the amount of attenuation at a particular position in the gene. Indeed, my investigation on transcriptional processivity in *X.laevis* oocytes indicates that the block of transcriptional elongation at T2 or TAR in synthetic genes can be almost entirely overcome by the strong transactivation domain of VP16 (Figs. 2, 3, 4).

All these observations suggest that at non-activated state, RNAPol II elongation is inefficient regardless of the template sequence, although some sequence elements clearly block the progress of RNAPolymerase II far more effectively than others.

3.1.5. Processivity and Promoter Strength

The levels of premature termination at the c-myc T2 site in injected *X.laevis* oocytes is significantly increased at high levels of injected template DNA (Meulia et al., 1993; Middleton and Morgan, 1990; Spencer and Kilvert, 1993). These results were interpreted as evidence for a limiting elongation factor in *X.laevis* oocytes, which might be titrated out under conditions of high levels of pol II transcription (Spencer and Kilvert, 1993).

The experiments in this thesis were conducted at constant concentration of the injected DNA, which was significantly lower than those, used in the above mentioned studies. Furthermore, I always injected an excess of recombinant transactivators together with the test plasmids. The results, obtained in my investigation are not consistent with the interpretation of Spencer and Kilvert (1993), since transcription can be activated to high levels by injecting GAL4-VP16 or GAL4-E1a proteins and any of the constructs without incurring increased premature termination (Figs. 2-6). Therefore, my results do not support the idea of a limiting elongation factor in oocytes. Here I suggest an alternative explanation for the template titration results of (Spencer and Kilvert, 1993), namely that high

levels of DNA sequester transcriptional activators thus promoting non-activated transcription with low processivity.

In certain cases (Figs. 2 and 4), the processivity of GAL4-VP16 activated transcription approximated 100%. Is it possible that high levels of transcription titrate out some termination factor(s)? In Fig. 2, GAL4-AH, GAL4-VP16 and GAL4-E1a each stimulated the overall c-myc expression about 15 fold, nevertheless the intensity of premature termination differed significantly. Another argument against the existence of a limiting termination factor in oocytes is the fact that c-myc TATA box mutants were expressed poorly, but with higher than 90% processivity (D.Bentley, published in (Yankulov et al., 1994). Thus, no correlation between promoter strength, intensity of transcription and pol II processivity can be made.

3.1.6. Transactivation Domains Differ in Their Capacity to Stimulate Transcriptional Processivity

Three different transactivation domains, fused to the GAL4 DNA binding domain, were used to investigate processivity of activated RNAPol II transcription in injected *X.laevis* oocytes. Two of them - AH and VP16 - were acidic, while the E1a domain was not. GAL4-AH consistently produced less processive transcription with all the constructs used as compared to the VP16 and E1a transactivation domains (see Figure 6 and Table 1). On the other hand, GAL4-E1a and GAL4-VP16 activated transcription with similar processivity when Gal5-P2mycCAT was used as a template (Fig. 2), while in the case of Gal5-HIV2CAT GAL4-E1a was not as efficient as GAL4-VP16 (Fig. 3). This data indicate that different transactivation domains can stimulate transcription with different degrees of processivity in an identical promoter context; and that at different promoters transactivators could display distinct properties. It is also necessary to

mention that all constructs, used in my investigation, contained five GAL4 binding sites. It is possible that reducing the number of these sites (and in this way the number of transactivation domains at the promoter) could reveal even greater dissimilarities between the activation domains.

What is the principle that governs the differences in the "elongation" capacity of different domains? It seems that the type of transactivation domain is not of major importance, since AH and VP16, although being acidic, did not equally stimulate transcriptional elongation (see Fig. 6 and Table 1). Furthermore, E1a which is non-acidic, resembled VP16 (Fig. 6 and Table 1). Hence, other qualities are essential for the elongation capacity of the transactivation domains.

Extensive research has documented specific associations between some activation domains and TBP (TFIID), TFIIB and/or other general transcription factors. Many, but not all of these were described in Chapter 1.1.1.1. It is clear that different transactivators can establish different interactions with the basal transcription machinery and other factors. It is possible that the affinity and the range of interactions, which a transactivation domain can form may reflect its competence to stimulate initiation and/or processivity of transcription. Such an assumption suggests that the exceptionally potent transactivation domain of VP16 should interact with a wide range of GTFs. Support for this hypothesis is provided by the affinity chromatography experiment in Fig. 16. Moderate transactivators, which need co-operation with additional transactivation domains (for example Sp1 and TAT in the case of HIV-1 (Southgate and Green, 1991), might have a narrower range of interactions with the GTFs.

3.2.1. Role of Basal Transcription Elements in Transcriptional Processivity

All synthetic constructs used in this study contained an identical block of five GAL4-binding sites positioned upstream of different basal transcription

elements (see Fig. 1). However, basal transcription elements such as the TATA box and the initiator-like elements could also influence the regulation of transcriptional elongation.

The TATA element seems to affect directly that regulation. For example, GAL4-AH consistently activated slightly more processive transcription from the Gal5-E4 promoter than from Gal5-E1b which differs only in the sequence of its TATA box (see Fig. 1 and Table 1). The TATA sequence is also important in determining the processivity of transcription from the c-myc P2 promoter. Point mutation in this TATA box in the context of the complete mouse P2 promoter almost abolished non-processive transcription (D. Bentley, published in (Yankulov et al., 1994). Similar observations were made for TATA box mutations of the human c-myc gene and HIV-1 (Meulia et al., 1993; Lu et al., 1993). All these data imply that the TATA element might be involved in the maintenance of some basal levels of non-processive transcription and in modulating the response to activators. How the TATA sequence affects processivity is unknown. One possible explanation is that different forms of TFIID (Timmers and Sharp, 1991) which bind preferentially to different TATA elements, are responsible for promoting transcription by complexes with different degrees of processivity.

As discussed in 2.1.5., the Gal5-HIV2 and the Gal5-P2myc promoters are transcribed with higher efficiency in the non-activated state than the Gal5-E4, Gal5-E1b and Gal5-TK promoters. LTR-HIV1, LTR-HIV2, the mouse and the human c-myc P2 and the AdMLP promoters contain a similar Initiator-like element (Krumm et al., 1993), see also chapter 2.1.5), which might contribute to the significant levels of basal transcription observed from the Gal5-P2mycCAT and Gal5-HIV2CAT. Another important similarity between HIV-1, HIV-2, c-myc and the AdML genes is that they all are controlled at the level of transcriptional elongation (Krumm et al., 1993). It has been reported that mutations in the IST (Initiator of Short Transcripts) sequence, which overlaps with the Initiator-like element in HIV-1, completely inhibited the production of abortive transcripts

(Sheldon et al., 1993). Mutations in the TATA box of HIV-1 LTR promoter have the same effect (Lu et al., 1993). Thus, it was interesting to assay whether basal transcription elements could co-operate in supporting non-processive transcription.

In Fig. 5, insertion of the TdT Initiator element (Smale et al., 1990) downstream of the TK TATA box had no effect on both non-activated and activated transcription. The result suggests that not any TATA-Initiator combination can serve as an effective non-processive transcription element. Alternative explanation is that Initiators and TATA boxes do not co-operate at all or at least not in *X.laevis* oocytes. It is noteworthy, though, that a mutant HIV1-LTR, in which the TATA box was replaced by the TdT Initiator, was unresponsive to TAT (Berkhout and Jeang, 1992).

The amount of experiments in the direction of cooperation between basal transcription elements allows restricted field for speculation. Nevertheless, it is essential to check whether mutations in the Initiator-like elements in c-myc P2 and AdML promoters would have a similar effect as in HIV1-LTR before making general conclusions.

3.2.2. TBP Enhances Non-Processive Transcription

Analysis of the HIV-1 LTR and the c-myc P2 TATA boxes suggested that non-processive transcription can be directed through this element (see chapter 3.2.1.). Since many of the TATA box functions are believed to be mediated by TBP (TFIID), a possible way to check that hypothesis was to assay for the effect of TBP on non-activated transcription. In Figs. 8, 9 and 11 I demonstrate that in *X.laevis* oocytes, coinjecting of human TBP stimulates non-processive transcription from three different minimal promoters - Gal5-P2, Gal5-E4 and Gal5-HIV2 (see Fig. 1 for

details about these promoters). Elevated transcription from TATA-containing promoters by cotransfection of TBP was previously also reported by (Colgan and Manley, 1992). While initiation at Gal5-P2 and Gal5-E4 was enhanced about 3-4 fold by TBP (Fig. 8), transcription from the Gal5-HIV2 promoter was increased by more than 20 fold (Fig. 9). The reason for that difference is unknown.

In contrast to transcriptional activators, TBP enhanced initiation, but not elongation, since most of the transcripts made in the presence of TBP terminated prematurely at the T2 or TAR sequences, respectively (Figs. 8 and 9). Processivity values for non-activated and TBP-stimulated transcription in all experiments were almost equal (compare Figs. 2, 3, 4 with Figs. 8 and 9), which suggests that stimulation by TBP is not changing qualitatively the properties of the elongating polymerase.

How TBP enhanced transcription from the minimal promoters is not clear. It is possible that TBP directly interacts with the TATA element, most likely together with endogenous oocyte factors, and increases the rate of initiation. Such an explanation might be supported by the results of (Cormack and Struhl, 1993). They reported that yeast TBP mutants, which were defective in pol III transcription, produced higher levels of mRNA, which indicates that competition for limiting amounts of TBP between the RNAPolymerase activities takes place in vivo. So far, though, we have not observed any indication of limiting amounts of pol II factors in *X.laevis* oocytes. In addition, injection of TBP did not enhance activated transcription, nor did it reduce its processivity although the synthesis of RNA was much more intensive (Fig. 11).

Alternatively, the injected TBP counteracts some inhibitors of pol II transcription such as NC1, NC2, Dr1 and DR2 (see chapter 1.1.1.1.) and releases otherwise suppressed basal transcription. In this respect, injecting of TBP with a mutant DNA binding domain will provide evidence to distinguish between these possibilities.

Whatever the mechanism of the effect of TBP on initiation is, the resulting elongation complexes are obviously deficient in processivity, since most of them terminated prematurely at T2 or TAR. Hence, TBP stimulates transcription, but can not substitute for transactivators in promoting high efficiency of elongation. A simplified conclusion from that observation is that different initiation events take place in the presence of excess of TBP and transcriptional activators, respectively.

In summary, the data presented in Figs. 8-12 clearly indicate that processive and non-processive transcription can be regulated independently. Factors which interact with the TATA box and elements such as the IST in the HIV-1 LTR stimulate non-processive transcription while transactivators preferentially stimulate processive transcription. Another important conclusion from these experiments is that increased initiation is not necessarily coupled to increased efficiency of elongation. Hence, the effect of transactivators on processivity is specific.

3.2.3. Is TBP Stimulated Transcription Driven By RNAPolymerase II

The c-myc P2 promoter can support α -amanitin resistant transcription which terminates at the T2 site, when a high concentration of template DNA is injected into *X.laevis* oocytes (Bentley et al., 1989). The c-myc T2 site coincides with a run of T residues, which can serve as a RNAPol III termination signal (Bogenghagen and Brown, 1981). Therefore, this data was interpreted as evidence for a shift in the P2 promoter specificity when RNAPol II factors were insufficient. On the other hand, significant proportion of the TBP stimulated transcription in *X.laevis* oocytes at low template levels was resistant to low concentration of α -amanitin (Figs. 8 and 9) and terminated at the T2 site (Fig. 8). TBP is a putative subunit of TFIIB - a factor required for pol III transcription (Hernandez,

1993). Is it possible then that TBP actually shifted the specificity of the minimal promoters used and activated pol III transcription? Several points of evidence argue against that. When Gal5-HIV2CAT was used as a template, TBP stimulated the production of prematurely terminating RNAs (Fig. 9). The same effect of TBP was observed with the Gal5-P2mycCAT and Gal5-E4mycCAT constructs. In the case of Gal5-HIV2CAT, though, the sites of premature termination did not coincide with runs of T's. Furthermore, this non-processive transcription was resistant to α -amanitin, but was sensitive to anti-RNAPol II antibodies (Fig. 9). In contrast, transcription of the coinjected Adenovirus VA1 gene, which is transcribed by RNAPol III, was not sensitive to these antibodies.

The definition of α -amanitin resistant RNAPol II transcription should be cautiously introduced. Nevertheless, the comparison between transcription from a genuine pol III promoter (VA1) and the Gal5-HIV2CAT construct indicates that the TBP stimulated non-processive transcription is not driven by RNAPolymerase III because it is sensitive to anti-RNAPolymerase II antibodies. Therefore, the difference in the α -amanitin sensitivity of non-activated(non-processive) and activated(processive) transcription could reflect the properties of biochemically distinct RNAPolymerase II forms. A possible connection between α -amanitin sensitivity and processivity of RNAPol II has been previously pointed out by (Coulter and Greenleaf, 1985; Chen et al., 1993), who observed that a mutation in the large subunit of *Drosophila* RNAPol II which conferred α -amanitin resistance also reduced the elongation rate in vitro. Additional evidence for biochemical distinction between non-activated and activated elongation complexes is provided by the experiments in chapters 2.3. and 2.4. and will be discussed in chapter 3.3.

3.3. Protein Phosphorylation and Transcriptional Processivity

DRB (5,6-dichloro-1-b-D-ribofuranosyl-benzimidazole) inhibits RNAPolymerase II at the level of transcriptional elongation (Sehgal et al., 1976;

Tamm, 1977). It has also been reported that in HIV-1 and c-myc DRB inhibited only the fraction of polymerases which read through the sites of premature termination (Marciniak and Sharp, 1991; Marshall and Price, 1992; Roberts and Bentley, 1992). The results, presented in Figs. 4, 12 and 13 indicate that DRB is not simply preventing the formation of elongation competent complexes, but rather shifts the population of transcribing polymerases from high processivity to a predominantly low processivity form. As discussed in chapter 3.1., both transcription initiation and RNAPol II processivity are regulated by transactivators. In this respect, DRB seems to predominantly antagonise the stimulatory effect of activators on RNAPol II elongation. Therefore, identification of physiological substrate(s) for DRB and understanding the mechanism of DRB-mediated inhibition of transcription would provide information of how exactly transactivators influence processivity.

So far, the only indication about the mechanism by which DRB might suppress pol II transcription is that DRB is an adenosine analogue and inhibits several protein kinases in vitro, including some RNAPol II CTD kinase activities (Cisek and Corden, 1989; Stevens and Maupin, 1989; Zandomeni et al., 1986). In this chapter (3.3.) I discuss the possibility that the target of DRB is the TFIIF associated kinase activity.

3.3.1. Protein Kinase Inhibitors Decrease RNAPolymerase II Processivity

In Figs. 12 and 13 I demonstrate that two well characterised isoquinoline-sulfonamide kinase inhibitors (H-7 and H-8, (Hidaka and Kobayashi, 1992; Serizawa et al., 1993) can suppress RNAPol II elongation in *X.laevis* oocytes in a way, equivalent to that of DRB. This similarity between the effects of H-7, H-8 and DRB; and the previously reported properties of DRB as a kinase inhibitor (Cisek and Corden, 1989; Stevens and Maupin, 1989; Zandomeni et al., 1986) strongly

suggest that DRB works by inhibition of some protein kinase. The experiments in Figs. 12 and 13 do not distinguish whether the three inhibitors have the same target or not. Nevertheless, it seems unlikely that DRB, H-7 and H-8 inhibit a kinase(s) which positively regulates specific transcriptional activator(s). If each inhibitor separately inhibits a specific kinase, one would expect to observe differences between the suppression of processivity in different genes. HIV2-LTR, the mouse c-myc and the two synthetic promoters used (Gal5-E1b and Gal5-E4), besides the TATA box, share no common element, but all these genes responded uniformly to DRB, H-7 and H-8.

An additional indication that DRB, H-7 and H-8 exert their function through a similar or identical target comes from the comparison of the effective concentrations of these inhibitors in different systems. I found that DRB is a more potent inhibitor of transcriptional elongation than H-7 and H-8 (Figs. 12 and 13). Maximal inhibition by DRB on the transcription of four different genes in injected *X.laevis* oocytes was observed when the drug was applied at 10 μ M (Fig. 13), while equal effect with H-7 and H-8 was achieved at 100-200 μ M. Similar concentrations of DRB, H-7 and H-8 were necessary to significantly suppress transcriptional elongation of Gal5-HIV2CAT when this gene was transcribed in HeLa nuclear extracts (D.Bentley, unpublished observations). The incorporation of 3 H-uridine and the expression of a luciferase reporter or heat shock genes in HeLa cells (Dubois et al., 1994a; Dubois et al., 1994b) were also inhibited more efficiently by DRB as compared to H-7 and H-8. It is noteworthy that (Dubois et al., 1994b) detected inhibition of the RNAPol II transcription in vivo at drug concentrations, similar to those I used in oocytes for specific genes. I suggest that in the experiments of Dubois et al. (1994) the overall inhibition of RNA synthesis is mediated by reduced efficiency of elongation by RNAPol II.

The data discussed in this chapter indicate that DRB, H-7 and H-8 have a general effect on RNAPol II transcription rather than a specific effect on the expression of particular genes. As discussed in chapter 2.3.2., the TFIIF kinase is

sensitive to these inhibitors at concentrations that inhibit RNAPol II elongation. Hence, I propose that the major effect of H-7, H-8 and low concentrations of DRB on RNAPol II transcription is mediated by inhibition of the TFIIF kinase activity.

3.3.2. A DRB Sensitive Protein Kinase is Involved in Coupling Activation and Transcriptional Processivity

"Sqelching" of c-myc transcription by a non-binding VP16 transactivation domain and DRB are not additive in suppressing RNAPol II processivity (Fig. 14). This result implies that "sqelching" and DRB could work through a common mechanism. As discussed in the previous chapter, DRB is likely to inhibit a function, generally required for pol II transcription. If VP16 "sqelches" by interacting with factors necessary for transcriptional elongation, one of these factors might be the target of DRB. In support to that model, a CTD kinase activity which binds specifically to the VP16 transactivation domain, was isolated by affinity chromatography (Fig. 15). The CTD-kinase bound less well to a control mutant VP16 domain (SW6), which was a poor activator, but retained the negative charge of the wild-type domain (Walker et al., 1993).

The most intriguing property of this kinase activity was its high sensitivity to DRB (Fig. 18, A and B). As demonstrated in Fig. 18, 50% inhibition of the kinase (I_{50}) was achieved at 10 to 50 μ M DRB, while I_{50} for H-7 and H-8 were between 200 and 500 μ M. These values are in good agreement with the minimal effective drug concentrations, which inhibit transcriptional elongation in vitro (D.Bentley, unpublished observations) and in *X.laevis* oocytes (Figs. 12 and 13). An additional point of interest was that the peak of CTD-kinase activity coincided with the presence of RNAPol II and the bulk of GTFs, which also were specifically retained by the GST-VP16 column (Fig. 16). Thus, the kinase, isolated by VP16 affinity chromatography had all the characteristics of a hypothetical factor that couples

activation and transcriptional processivity : it is sensitive to DRB, interacts with a transcriptional activator (directly or indirectly) and is associated with the general transcription factors.

3.3.3. Comparison Between the VP16-associated Kinase and TFIIH

The affinity chromatography fractions from Fig. 15 were further characterised by Western blotting and kinase assays. Special attention was paid to the distribution of components of three protein kinases that phosphorylate the RNApol II CTD *in vitro*, namely p34^{cdc2}, the DNA-dependent kinase and TFIIH (see chapter 1.1.2.2.). The elution profile of the TFIIH p62 subunit, but not p34^{cdc2} or the Ku subunit of the DNA-dependent kinase, closely followed the profile of the CTD kinase activity both on the GST-VP16 and GST-SW6 columns (Fig. 16). This result suggested that TFIIH is a likely candidate for the activator coupled kinase. An additional similarity between the TFIIH kinase and the VP16-associated kinase was that both were sensitive to H-7 and H-8 (Fig. 18 B and (Serizawa et al., 1993)).

Subsequent analysis demonstrated the close similarity between the VP16 associated and the TFIIH kinases. Both enzymes phosphorylated CTD, TBP, p56 (TFIIE), RAP74(TFIIF), but not RAP30(TFIIF) and p34(TFIIE)(Fig. 18 A and (Ohkuma and Roeder, 1994)). dATP and GTP, but not UTP or CTP compete with ATP for the catalytic site of the VP16 associated kinase (Fig. 18 D). The same nucleotide specificity was previously established for highly purified TFIIH kinase activity (Roy et al., 1994). Like TFIIH (Serizawa et al., 1992; Serizawa et al., 1993), the VP16-associated kinase was stimulated by promoter containing DNA when substrates were GST-CTD and TBP, but not TFIIE(p56) and TFIIF(RAP74) (Fig. 18 C). My experiments do not rule out whether this difference results from the nature of my *in vitro* kinase assay or represents some significant promoter dependent

preference of the kinase for its substrates. The VP16 associated kinase activity was not stimulated by addition of recombinant TFIIE (data not shown) as reported for TFIIH (Ohkuma and Roeder, 1994), but significant amount of endogenous TFIIE was detected in the same fraction.

In an Immunodepletion experiment, most of the kinase activity and p62(TFIIH) in the VP16-fraction 5 were depleted by anti-TFIIH (p62) antibodies (Fig. 19). This result strongly indicates that the major kinase which associates with VP16 is TFIIH, although a minor contribution of other kinases with similar properties can not be eliminated.

Inhibition of the TFIIH kinase by DRB has never been reported. In Fig. 18 (A and B) I demonstrate that the VP16-associated kinase and highly purified TFIIH are equally sensitive to DRB when a substrate was TFIIF. The same results were obtained when a substrate was GST-CTD (data not shown). In the experiments described in chapter 2.4.3. the I_{50} for H-7 and H-8 was 200 μ M or more for the different substrates. (Serizawa et al., 1993) have reported that the rat analogue of TFIIH (factor δ) kinase activity was sensitive to lower concentrations of H-7 and H-8, when substrates were the large subunit of RNA polymerase or a CTD peptide. One reason for this difference could be the different species source of the kinase. Other reason could be the nature of the substrates (GST-CTD versus CTD peptide or the large subunit of RNAPol II). It is also worth mentioning that some of the delta factor properties - DNA stimulation, kinase activity, dependence on TFIIE, were altered at different KCl concentration or with different CTD substrates -peptide or the large subunit of RNAPol II. In yeast, the TFIIH (factor b) kinase was less sensitive to H-8 as compared to the delta factor from rat (Li and Kornberg, 1994; Serizawa et al., 1993). In the assays of Li and Kornberg (1994), the same concentration of H-8 inhibited RNAPol II transcription with higher efficiency when purified factors were used instead of crude yeast extract. However, the levels of CTD phosphorylation and transcription were not compared. ..

If TFIIH is an activator coupled kinase, it might be stimulated by transactivation domains. So far that question has not been addressed, although RNAPol II isolated as an activator responsive "holoenzyme" (see chapter 1.1.5.) is much more efficiently phosphorylated by TFIIH as compared to the pure RNAPolymerase II (Kim et al., 1994). In my assays, GAL4-VP16 had no effect on the phosphorylation of GST-CTD (data not shown), but there was an indication of contamination with GST-VP16 from the affinity resin. (Ohkuma and Roeder, 1994) reported that the TFIIH kinase activity is stimulated conditionally by TFIIE at a late stage in the formation of the preinitiation complex. So, it is also possible that transactivators can indeed stimulate the TFIIH kinase activity, but only in the context of a promoter bound activator responsive complex.

3.3.4. Importance of CTD, TBP, TFIIF and TFIIE Phosphorylation for RNAPol II Transcription

TFIIH phosphorylates TBP, the RNAPolymerase II CTD and the large subunits of TFIIF and TFIIE in vitro (Fig. 18 A and (Ohkuma and Roeder, 1994)). The relevance of the phosphorylation of these substrates for transcription in vivo is not well understood. It is not known whether TBP is phosphorylated in vivo or not. Neither is it known whether phosphorylation of TBP would alter its functions, the integrity of the TFIID complex or the affinity of interactions with GTFs and transcription activators. The most likely position for phosphorylation in TBP is a CTD-like sequence in the N-terminus of the protein (Ohkuma and Roeder, 1994), but the significance of that site is unknown. Since TBP phosphorylation is stimulated by promoter DNA in a way, similar to that of CTD (Fig. 18 C), it is tempting to speculate that this event could be coupled to initiation or promoter clearance.

Maxon et al. reported that TFIIE interacts with TBP, TFIIF, TFIIH and the nonphosphorylated form of RNAPolymerase II (Maxon et al., 1994). All these data, though, were obtained with non-phosphorylated TFIIE. The authors did not assay whether phosphorylation of p56(TFIIE) by TFIIH will change the spectrum of TFIIE interactions. Nothing is known about whether phosphorylation will alter the properties of TFIIE in basal transcription reactions either. In my kinase assays, phosphorylation of TFIIE, as well as TFIIF, was not enhanced by promoter DNA (Fig. 18 C). Clearly, further work is necessary to establish the functional significance of TFIIE phosphorylation.

TFIIF is known as an elongation factor which suppresses pausing of RNAPolymerase II in vitro (Bengal et al., 1991). Therefore, the phosphorylation of TFIIF by TFIIH suggests a possible function in controlling elongation. One possibility is that phosphorylation of RAP74 might disrupt the interaction of this protein with other GTFs such as TFIIE, and to stimulate elongation by stabilising the interaction with RNAPol II which is quite labile (Price et al., 1989). It is also intriguing that RAP74 interacts with the transactivation domains of VP16 and SRF (Zhu et al., 1994), implying that it might be directly involved in transcriptional activation. In this respect, the kinase activity of TFIIH might modulate the association between activation domains and RAP74.

The strongest candidate for an in vivo substrate of the TFIIH kinase is the RNAPol II CTD. The phosphorylation of CTD by TFIIH is promoter and TFIIE dependent (Lu et al., 1992; Ohkuma and Roeder, 1994; Serizawa et al., 1993c); and the transition from transcriptional initiation to elongation is accompanied by hyperphosphorylation of CTD in vivo (Payne et al., 1989) and in vitro (Lu et al., 1992). Importantly, polymerases stalled at the 5' end of the quiescent *Drosophila* Hsp70 gene are unphosphorylated whereas actively elongating polymerases in the heat-shocked state are a mixture of hypo- and hyperphosphorylated forms (O'Brien et al., 1994). On the other hand, DRB treatment of cells causes a shift in mobility of the pol II large subunit from the hyperphosphorylated IIO form to the

nonphosphorylated IIA form (Dubois et al., 1994b). This data along with the evidence that TFIIF is inhibited by DRB *in vitro* (Fig. 18 A) suggest that the CTD is a substrate of TFIIF *in vivo* and that this phosphorylation is essential for RNAPol II elongation.

3.3.5. Implication for the Function of TFIIF in Activated Transcription

There is a lot of controversy about the mechanism by which TFIIF regulates RNAPol II transcription. It has been proposed that a helicase functions at a late stage in the formation of preinitiation complex to unwind DNA and allow the formation of the first phosphodiester bond (Buratowski, 1993). A CTD-kinase activity which will phosphorylate the large subunit of RNAPolymerase II and will release CTD from its contact with TBP to trigger elongation, was also predicted (Usheva et al., 1992). The discovery of such enzyme activities, associated with TFIIF (Lu et al., 1992; Schaeffer et al., 1993; Serizawa et al., 1992; Serizawa et al., 1993) seemed to explain the role of this factor in RNAPol II transcription. Surprisingly, both the helicase and the kinase activity of TFIIF are dispensable for transcription under certain conditions.

Several studies have established a requirement for the ATPase and helicase activity of ERCC3 (TFIIF) in promoter clearance on linear templates (Goodrich and Tjian, 1994; Roy et al., 1994; Timmers, 1994). On supercoiled templates, though, the TFIIF dependence of basal transcription is specific, so that for certain promoters such as IgH and Adenovirus MLP TFIIF is dispensable (Parvin and Sharp, 1993). The helicase activity of TFIIF is not required for the unwinding of DNA and establishing of an open transcription complex either on supercoiled or linearised templates, since on both templates addition of highly purified TFIIF is not critical for the initial synthesis of RNA (Goodrich and Tjian, 1994). Nevertheless, the helicase activity of rad3 (the yeast homologue of ERCC2) but not

that of rad25 (the yeast homologue of ERCC3) is essential for pol II transcription both in vivo and in vitro (Guzder et al., 1994a; Guzder et al., 1994b; Qiu et al., 1993). Mutations in rad25 also abolished pol II transcription in vivo, but they did not affect the helicase activity of this protein (Qiu et al., 1993). While these findings demonstrate that the helicase activity of rad3 has a direct role in RNAPolymerase II transcription, they do not specify whether it is essential for open complex formation or some other process.

When TFIIE and TFIIH were added after promoter clearance to an in vitro transcription reaction, no effect was observed on transcriptional elongation (Goodrich and Tjian, 1994). Goodrich and Tjian (1994) interpreted their results as evidence that these two factors are not involved in regulation of transcriptional elongation. Kumar et al. (D. Reinberg, personal communication), though, demonstrated that TFIIH is disintegrated and redistributed at the stage of promoter clearance, so that the p62 subunit remains associated with the elongating polymerase, while ERCC2 and ERCC3 leave the complex. Hence, the experiments of (Goodrich and Tjian, 1994) do not rule out possible role of TFIIE and TFIIH for transcriptional elongation, but indicate that their function is not mechanistic participation in the elongating RNAPol II complex. My results predict that TFIIH actually controls elongation, but by establishing a elongation-competent RNAPolymerase II complex at the promoter level.

So far, a function for the TFIIH associated CTD kinase activity in transcription has not been defined. The initial prediction that phosphorylation of CTD is essential for promoter clearance (Payne et al., 1989) was directly contradicted by the results of Serizawa et al.(1993b). In their experiments with highly purified transcription factors (this system does not contain any CTD-kinase activity other than TFIIH) inhibition of the TFIIH kinase and CTD phosphorylation by H-8 had no effect on the transcription from a minimal AdML promoter. Another proposal, namely that phosphorylation of CTD prevents the elongating polymerase from contacts with the initiation factors (Peterson and

Tjian, 1992), is not consistent with the results of Serizawa et al (1993b) either. Transcription in vitro can also proceed in the absence of ATP or GTP (up to the points where A or G are incorporated), which are substrates for the TFIIF kinase (Goodrich and Tjian, 1994). It is important to note that the results described in this paragraph were obtained by basal transcription assays. They do not address whether activated transcription requires CTD phosphorylation and the TFIIF kinase. Furthermore, these assays are designed to detect initiation, but not the efficiency of elongation. My results also indicate that transcriptional initiation in *X.laevis* oocytes is not very sensitive to kinase inhibitors, which might explain why Serizawa et al. (1993) did not detect any effect of the inhibited TFIIF kinase activity (Figs. 12 and 13).

Although it seems clear that the TFIIF kinase activity is not required for basal transcription, the experiments in chapters 2.2. and 2.3. of this thesis strongly suggest that it is necessary for activated transcription. Inhibition of pol II transcription and the TFIIF kinase by DRB, H-7 and H-8 does not markedly reduce overall initiation or promoter clearance but it does dramatically reduce the elongational capacity of activated transcription complexes in *X.laevis* oocytes (Figs 12 and 13). Similar effect of these inhibitors was observed in vitro when transcription was performed in crude HeLa nuclear extract (D.Bentley, unpublished observations). These results support the idea that phosphorylation of neither CTD nor other substrates by TFIIF is essential for initiation. Instead, the TFIIF kinase activity fulfils a significant function in transforming the initiation complex into an elongation competent complex. A significant, but probably not the only modification of the elongation complex is the phosphorylation of CTD.

Price and colleagues have suggested that DRB inhibits a factor (P-TEF, positive transcription elongation factor) which is able to function in the short interval after initiation and before polymerases have extended 500 bases (Marshall and Price, 1992). CTD phosphorylation in vivo appears to occur in the period

immediately following initiation. The available evidence is therefore consistent with the possibility that P-TEF corresponds to TFIIF.

3.3.6. Role of RNAPolymerase II CTD in Activated Transcription

Both non-specific or promoter-specific basal transcription *in vitro* are unaffected by the absence of CTD in yeast (Li and Kornberg, 1994). In contrast, truncation of more than 50% of the CTD heptapeptides is lethal *in vivo* (Edwards et al., 1991; Nonet et al., 1987; Allison and Ingles, 1989). CTD truncation mutants, containing half of the CTD, display a phenotype characterised by retarded growth and defects in inducible gene expression (Allison and Ingles, 1989; Peterson et al., 1991). Interestingly, some of the suppressors of CTD-truncation mutants appear to be proteins which mediate transcriptional activation (Thompson et al., 1993; Kim et al., 1994). Furthermore, the level of transcriptional stimulation by activation domain fragments of GAL4 and GCN4 is modulated by the length of CTD (Allison and Ingles, 1989), see also chapter 1.1.2.1.). Therefore, an intact CTD seems to be essential for normal activator function in yeast.

It is known that elongation *in vitro* is inhibited on chromatin templates (Izban and Luse, 1991). Since two of the suppressors of CTD-truncation mutants appeared to be histone H3 and SIN1 (a HMG-like protein) (Kruger et al., 1991; Peterson et al., 1991) CTD might also be involved in stimulation of elongation through chromatin structures *in vivo*. If CTD is essential both for activation and elongation of RNAPolIII transcription, and if there is a direct link between activation and elongation, then one could explain why a partial CTD deletion mutation can be complemented by unrelated proteins such as mediators of activation (SRBs) and chromatin factors (H3 and SIN1).

Finally, my results, presented in chapters 2.3 and 2.4 suggest that not CTD itself, but rather phosphorylated CTD is responsible to mediate both transcriptional activation and processivity.

3.3.7. Role of GTFs in Control of Transcriptional Elongation

What is the difference between processive and non-processive RNAPolymerase II complexes? According to the data presented in 2.3. and 2.4. a major constituent in the control of transcription elongation is the TFIIF protein kinase activity. As discussed in chapter 3.3.4. the most likely substrate for TFIIF kinase activity *in vivo* is the CTD domain of the large subunit of RNAPol II. While I assume that highly processive elongation complexes require phosphorylation of CTD, the role of other basal transcription factors in the control of pol II processivity remains to be established.

TFIIF (RAP30/RAP74) which recruits RNAPolymerase II into the pre-initiation complex through contacts with TFIIB (Drapkin et al., 1993; Flores et al., 1989) also stimulates elongation by suppressing pausing of RNAPolymerase II. (Bengal et al., 1991; Kato et al., 1992) have reported that TFIIF and TAT work through similar pathways since the effects of TAT and TFIIF on HIV1 transcription *in vitro* were not additive. Furthermore, anti-RAP74 antibody inhibited activation by TAT *in vitro* (Kato et al., 1992). Recently it was demonstrated that RAP74(TFIIF) interacted with some transactivation domains (VP16 and SRF, but not Sp1 (Zhu et al., 1994)), however it is not clear to what extent these interactions contribute to transcriptional activation and processivity. Another indication of direct functional involvement of TFIIF in elongation is provided by the observation that both RAP30 and RAP74 travel along with the phosphorylated form of RNAPol II in experiments with immobilised templates *in vitro* (D.Reinberg, personal communication).

Two other activities, termed TFIIX (Bengal et al., 1991) and TFIIJ (Flores et al., 1992) were reported not to be required for initiation, but to stimulate transcriptional elongation. These factors are not well characterised and the mechanism of their function is not known.

TFIIS is a factor with defined function during RNAPol II elongation. It triggers 3'-5' RNAase activity from the catalytic subunit of RNAPolymerase II upon pausing thus facilitating resumption of elongation (Izban and Luse, 1992; Johnson and Chamberlin, 1994). It remains unclear whether TFIIS is an integral subunit of the elongation complex or whether this protein interacts with RNAPolymerase II upon pausing. However, it is noteworthy that TFIIS was initially characterised by affinity chromatography as a protein that specifically associates with RNAPol II (RAP38, (Sopta et al., 1985)). My results from analysis of the proteins that were retained by the GST-VP16 resin showed that TFIIS coelutes with several GTFs and the large subunit of RNAPol II (Fig. 16). This indicates that TFIIS might be incorporated into the elongation complex at the early stages of transcription due to its affinity to the initiation factors or RNAPol II itself. Such a hypothesis, though, requires further investigation.

3.3.8. Is There Differential Stringency in the Requirement for CTD Phosphorylation

The model proposed in chapter 3.3.5. assumes that the TFIIH kinase activity is required for high processivity of activated transcription, but not for basal or non-activated transcription. Indeed, basal transcription in vitro (Serizawa et al., 1993b), as well as non-activated transcription in *X.laevis* oocytes (Fig. 13, lanes 1-3) are not sensitive to the kinase inhibitors DRB, H-7 and H-8. In contrast, processivity of activated transcription was markedly suppressed by the same inhibitors (Figs. 12-14; D.Bentley, unpublished results). Hyperphosphorylation of CTD, however, takes place in basal in vitro transcription experiments (Lu et al.,

1992; Serizawa et al., 1993a). What contributes to the differential processivity of activated and non-activated transcription then? The available data can not answer that question. One possibility is that in basal transcription phosphorylation of CTD (and/or other substrates) is inefficient. Support for this idea is provided by the fact that in the activator responsive "holoenzyme" phosphorylation of CTD is about 8 fold higher as compared to the "core" enzyme ((Kim et al., 1994), see also chapter 1.1.5.). It is also equally possible that activated transcription complexes incorporate elongation factors and obtain novel characteristics (e.g. high processivity) relative to non-activated complexes. Phosphorylation of CTD itself might not exemplify these characteristics, but is essential for them. Thus, phosphorylation of CTD might be differentially required in basal and activated transcription.

Indication of differential necessity of CTD is provided by the experiments of (Li and Kornberg, 1994). Yeast CTD-less RNAPolymerase II is active and indistinguishable from the intact polymerase in basal transcription with the CYC1 promoter. In contrast, the CTD-less RNAPolymerase II is completely inactive when transcription is performed with crude extracts (presumably activated) with the same promoter.

Differential requirement for CTD phosphorylation between activated and non-activated transcription (and phosphorylation of other substrates) may explain why "squelched" transcription is less sensitive to DRB (Fig. 14). In activated state, the c-myc promoter directs transcription which is highly sensitive to DRB, so that processivity is reduced to 15-20% upon treatment with that drug (Figs. 12 and 14). "Squelching" by VP16 at the concentration used competes with the c-myc specific transactivators for TFIIF and other factors required for efficient elongation. As a result of that, RNAPol II processivity over the c-myc gene is substantially reduced and mimics non-activated transcription. Therefore, the requirement for phosphorylation of CTD is abolished and transcription is not any further sensitive to DRB.

3.4. Evidence for RNAPol II "holoenzyme" in HeLa cells

In the affinity chromatography experiments presented in Figs. 15 and 16, RNAPolymerase II, TBP, TFIIH, TFIIH, TFIIF and TFIIS were all found to co-purify in the 0.2 M KCl fractions of the VP16 affinity column. Previous experiments have shown that TBP (Stringer et al., 1990) and TFIIB (Lin and Green, 1991) can interact directly with VP16. Affinity chromatography with crude extracts, which was applied in my investigation, confirmed the interaction between VP16 and TBP, but can not distinguish whether these general transcription factors bind directly to the GST-VP16 column. Binding of TFIIH (p62) to VP16, though, appears to be stronger as compared to the rest of the GTFs (Fig. 16), which might be an indication that TFIIH and VP16 interact directly. Indeed TFIIH and TBP were almost quantitatively depleted from the extract by passage over the VP16 column, while the rest of the tested GTFs and RNAPol II were not. Recently, a direct interaction between VP16 and TFIIH was reported (J.Greenblatt, in press).

The co-elution of five general transcription factors during affinity chromatography under mild conditions raises an interesting possibility that a transactivator responsive RNAPol II holoenzyme may exist in HeLa nuclear extract. A foundation for such a complex could be provided by the multiple affinity interactions between the GTFs. Recently, RNAPol II "holoenzymes" have been isolated from *S.cerevisiae*. They contain homologues of several of the factors which co-purify from HeLa extract on the VP16 column. (Koleske and Young, 1994) isolated a complex which contains TFIIH, TFIIB, and TFIIF homologues in addition to SRB proteins and RNAPol II in its hypophosphorylated form. Kornberg and colleagues (Kim et al., 1994) isolated a different complex which lacked TFIIH and TFIIB. Both "holoenzymes" conferred responsiveness to the VP16 activation domain in transcription assays. My results suggest that a similar

holoenzyme(s) which can be substantially enriched by VP16 affinity chromatography may exist in mammalian cells.

An alternatively possibility is that the VP16 transactivation domain could nucleate the formation of a RNAPol II complex in the absence of promoter DNA. In that situation, less potent transactivation domains which have restricted capacity to interact with general transcription factors, will form different complexes. In vivo, cooperation between transactivators associating with discrete sets of GTFs could be required to activate gene expression.

3.5. A Model For the Regulation of RNAPolymerase II Elongation

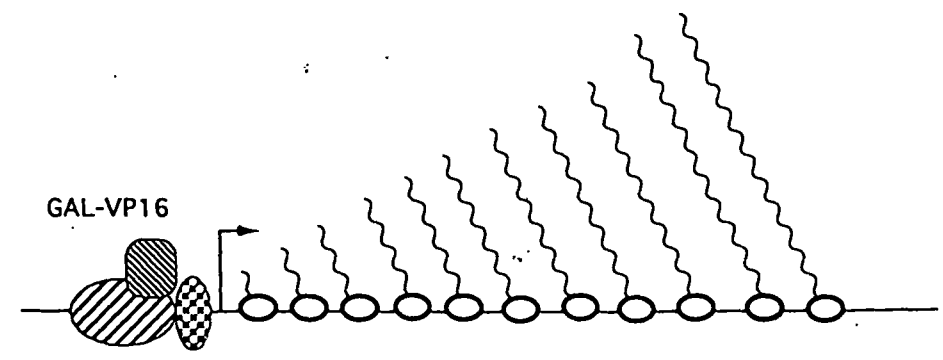
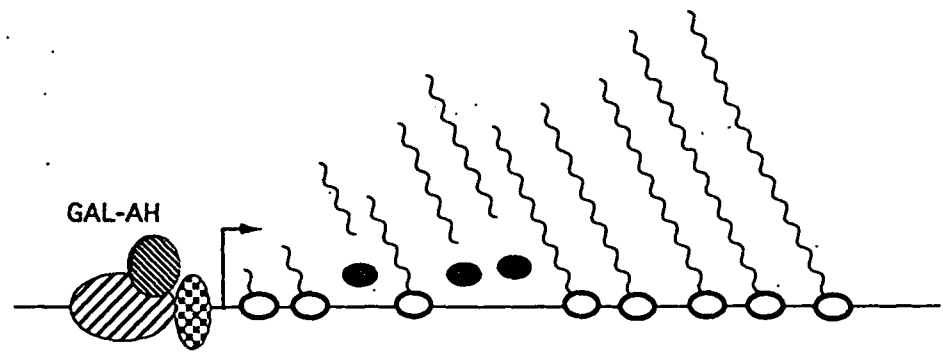
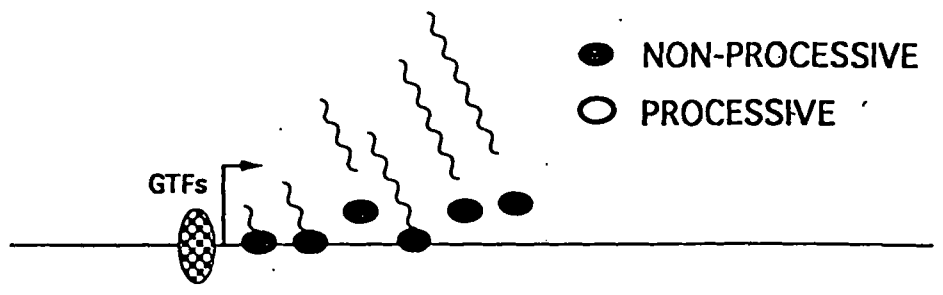
A model which describes my investigation, as well as other experimental data from our laboratory, is presented in Figure 23. We suggest that there are two types of transcription complex: non-processive and processive, although it is also possible that processivity varies continuously. The idea of different levels in the elongation competence of RNAPolymerase II is inspired by the observation that in HIV-2 VP16 activated transcripts terminate at promoter distal sites as compared to transcripts, activated by the relatively weaker activator GAL4-AH (Fig. 3). In the non-activated state and when transcription is stimulated by TBP, the non-processive form predominates whereas in the activated state the processive form predominates. As discussed in chapter 3.1.6., activators differ quite extensively in the processivity of the transcription they stimulate. These differences are represented in Fig. 23 by moderate (middle) and high processivity (bottom) modes of transcription. We propose that the balance between processive and non-processive elongation is determined by basal promoter elements including the TATA box, and by the repertoire of activation domains bound to promoter elements. Highly potent transactivation domains such as VP16 and E1a will recruit more efficiently elongation factors to the promoter, while weaker activators will

require cooperation for elevated gene expression. Significant levels of initiation does not necessarily lead to effective elongation, as demonstrated by the effect of TBP on transcription from minimal promoters in Figs. 8 and 9. In this respect, it is possible that transactivators stimulate initiation and elongation in different ways. The idea of independent regulation of initiation and elongation by transactivators is also supported by the fact that kinase inhibitors do not suppress significantly initiation, but do prevent the polymerase from efficient elongation. Our observations imply that different types of initiation event occur in the presence or absence of activators resulting in the assembly of processive or non-processive transcription complexes respectively.

Is stimulated processivity essential for the overall activation of gene expression? Although the results obtained in my investigation do not directly answer that question, it has been demonstrated by nuclear run-on assay that increased initiation rate and enhanced processivity both make an important contribution to the synthesis of functional mRNAs (D. Bentley, published in (Yankulov et al., 1994). We consider the low processivity of non-activated transcription as a mechanism, which reduces gene expression in the absence of specific activators while permitting the promoter to maintain an open "standby" configuration, which may permit rapid activation in response to environmental stimuli.

Figure 23. Model describing processivity of transcription in the basal and activated states.

Two classes of transcription complex are represented : non-processive (filled) and processive (open), however it is also possible that processivity could be modulated continuously. Only one copy of each GAL4 activator is shown although multiple dimers were bound to each promoter in our experiments. Non-activated or TBP-stimulated transcription (top) is predominantly non-processive. The GAL4-AH (middle) and GAL4-VP16 (bottom) activated states are characterised by increasing levels of transcriptional processivity.



3.6. Involvement of BM28 in RNAPol II transcription

In section 2.5 of this thesis I present data indicating a possible involvement of BM28 (Todorov et al., 1994) in RNAPolymerase II transcription (Figs. 21-22). The first piece of evidence is that BM28 associated specifically with VP16 and E1a(CR2&3) as demonstrated by affinity chromatography (Fig. 21). BM28 also interacted with the activation-deficient mutant of VP16 (SW6), although less efficiently than VP16 (Fig. 21). Similar fractionation was observed for TFIIF(p62) (Fig. 16 and 17), but any further exploration of that fact at present will be hugely speculative. The nature of the association between BM28 and transactivation domains is unclear, although it should be emphasised that only a small proportion of BM28 was retained by the affinity resins. Since the ligand proteins were in excess, it is conceivable that the activation domain-BM28 interactions were mediated by other less abundant proteins. An important point, arising from these experiments, is that BM28 was found to coelute with the bulk of GTFs and RNAPol II. The significance of that co-purification is worth considering, since antibodies against BM28 specifically and completely inhibit pol II transcription in *X.laevis* oocytes (Fig. 22). Following that line, it is not unlikely that BM28 participates in a RNAPol II "holoenzyme" complex (see chapter 3.4.).

A second evidence that BM28 plays a role in pol II transcription comes from the experiments, presented in Fig. 22. Anti-BM28 antibodies abolished pol II transcription in *X.laevis* oocytes without incurring any decrease in pol III transcription. Importantly, the effect of anti-BM28 antibodies was identical to that of anti-CTD antibodies (Fig. 22 A).

By what mechanism does BM28 control pol II transcription? BM28 (Todorov et al., 1994) is a human homologue of the *S.cerevisiae* proteins MCM2, MCM3 (Yan et al., 1991) and CDC46/MCM5 (Chen et al., 1992). MCM2, MCM3 and CDC46/MCM5 are all believed to be involved in an early step in DNA

replication (for a review see (Diffley et al., 1994) and to be members of yeast "replication licensing factor" (Yan et al., 1993). The "licensing factor" itself, as predicted by (Blow and Laskey, 1988), is required for initiation of replication and can gain access to chromatin only during mitosis. It activates replication and limits the initiation of replication to once per origin per cell cycle. MCM2, MCM3 and CDC46 are present in yeast nuclei until the onset of S phase and are tightly associated with DNA during G1 (Yan et al., 1993). In addition, *mcm2*, *mcm3* and *cdc46* mutants are defective in maintaining minichromosomes. Therefore, they fulfil some of the properties required for a "licensing factor". As for BM28, it has nuclear localisation throughout the cell cycle and microinjection of antibodies against it delays entry into S phase (Todorov et al., 1994). At present, close functional relationship between MCM2, MCM3 and BM28 is only proposed, but not experimentally confirmed.

It is surprising that BM28 which has a significant homology with replication factors might be involved in pol II transcription. No indication of any participation of *mcm2*, *mcm3* and *cdc46* in transcription has been published so far. Nevertheless, the results in Fig. 22 strongly suggest that BM28 is essential for pol II transcription in *X.laevis* oocytes. The effect of anti-BM28 antibodies should not be connected to some interference between replication and transcription factors in that system, since antibodies against a genuine replication factor such as RP-A (Kenny et al., 1990) had no effect. At present it is not clear whether BM28 would be required for pol II transcription in systems, different from injected *X.laevis* oocytes. Neither is known how exactly it affects that process. Therefore, it is preliminary to speculate about the mechanism which couples BM28 and transcription.

MATERIALS AND METHODS

4.1. Materials

All chemicals used were analytical grade, purchased mainly from Sigma, BDH or Fissons. Enzymes were purchased from Boeringer, Stratagene, Promega, New England Biolabs or Sigma.

4.2. Chromatography Supports and Resins

Bio-Rad Economy Columns, tubings and other accessories were generally used for chromatography experiments and protein purification. Chromatography resins were purchased as follows:

S-Sepharose Fast Flow, Q-Sepharose Fast Flow, Glutathione-Sepharose 4MB, Heparin-Sepharose 4MB, Protein-A Sepharose 4MB and Sephadex G-70 were from Pharmacia.

dsDNA-Cellulose was from Sigma.

NTA-Agarose was from Qiagen.

Disposable Bio-Rad P-10 columns were used for fast buffer exchange of small samples.

Small volume dialysis was performed in Pierce Microdialysis Unit.

4.3. Inhibitors

PMSF (Sigma) stock solution was 50 mg/ml in DMSO.

DRB (Sigma) was kept as 50 mM suspension in EtOH at -20°C.

Microcistin (500 µM), H-8 (20 mM), H-7 (20 mM), α -amanitin (1 mg/ml), Leupeptin (1 mg/ml), aprotonin (1.8 mg/ml) and β -Glycerophosphate

(1M) were purchased from Sigma and stored as solutions in water at -20°C. Pepstatin (Sigma) was dissolved in MeOH at 1 mg/ml and stored at -20°C. Benzamidine (Sigma) was added to the buffers prior to use.

Ampicilin and IPTG were from Sigma.

4.4. Buffers and Solutions

MBS (Modified Barths Solution)

10 mM HEPESNa pH 7.6

0.088 M NaCl

1 mM KCl

2,4 mM NaHCO₃

0.8 mM MgSO₄

0.7 mM CaCl₂

50 µg/ml Gentamycin

BUFFER A

10 mM HEPES pH 7.9

100 mM NaCl

10 mM 2-mercaptoethanol

100 µM ZnCl₂

XL wce EXTRACTION BUFFER

30 mM Tris HCl pH 8

100 mM KCl

10 mM 2-Glycerophosphate

2 mM EGTA

1 mM DTT

2 mM Benzamidine

BUFFER D

20 mM K HEPES pH 7.9

50 mM KCl

0.2 mM Na EDTA

0.2 mM K EGTA

2 mM DTT

FORMAMIDE LOADING BUFFER

99% Formamide (Fluka)

10 mM EDTA

0.1 % Bromphenol Blue

0.1 % Xylene Cyanol

10 X GLYCEROL LOADING BUFFER

50% Glycerol (w/v), 2% Orange G, in TE

GEL EXTRACTION BUFFER

0.5 M Ammonium Acetate pH 6.5

1 mM EDTA

0.1 % SDS

GEL FIXING SOLUTION

10% Acetic Acid, 10% MeOH

XL EXTRACTION BUFFER

1% SDS

0.1 M Tris-HCl , pH 8.0

10 mM EDTA

XL GUANIDIUM BUFFER

4 M Guanidium HCl

5 mM Na Citrate

0.1 M 2-ME

0.5% Sarcosyl

FORMAMIDE HYBRIDISATION BUFFER

80% Formamide

0.4 M NaCl

20 mM PIPES pH 6.4

1 mM EDTA

SDS-LOADING BUFFER

2% SDS

10% Glycerol

50 mM Tris pH 6.8

100 mM DTT or 2-ME

0.02 % Bromphenol Blue

TBE running buffer

89 mM Tris Borate, 89 mM Boric Acid, 2 mM EDTA, pH 8.3

TAE running buffer

20 mM Tris Acetate, 10 mM Sodium Acetate, 5 mM EDTA, pH 8.0

PROTEIN TRANSFER BUFFER, pH 9.2

48 mM Tris

39 mM Glycine

10% Methanol

0.03 % SDS

PROTEASE INHIBITORS were added where indicated to the following final concentrations:

1 mM Benzamide

50 µg/ml PMSF

1 µg/ml leupeptin,

1 µg/ml pepstatin,

1.8 µg/ml aprotonin

4.5. Gel Electrophoresis of Nucleic Acids

ACRYLAMIDE GEL ELECTROPHORESIS

4.5 - 6.5 % denaturing acrylamide gels were used for RNA mapping (RNAase and S1 protection assays) and purification of RNA or DNA probes for analysis of RNA. The gels contained 1X TBE, 7M Urea, 20:1 mixture of Acrylamide/bis-Acrylamide and were run in 1X TBE. Samples were heated at 95°C for 2-3 min in more than 70% Formamide Loading Buffer just before loading. Short gels were run at 25W and long - at 38W.

4.5 % non-denaturing gels were used for gel mobility shift assay and for purification of probes for that assay. The gels contained 0.5X TBE and 37.5:1 Acrylamide/bis-Acrylamide mixture and were run in 0.5X TBE at 200 V.

Samples were loaded in Glycerol Loading Buffer or Gel Shift Buffer (see GEL MOBILITY SHIFT ASSAY).

All gels were run in Cambridge Electrophoresis Ltd. tanks.

AGAROSE GEL ELECTROPHORESIS

1-2% Agarose (SeaKem GTG) gels in TAE were used for analysis of plasmid preparations and restriction enzyme digestions, as well as for purification of restriction digest fragments. Gels were run at 80-100 V in Ellard Instrumentation Ltd. tanks. TAE and the gels contained 10 µg/ml Ethidium Bromide.

4.6. Protein Electrophoresis and Blotting

Protein electrophoresis was performed according to (Laemmli, 1970). The samples were heated for 5 min at 95°C in SDS-Loading Buffer. The gels were run in BioRad Mini Protean gel tanks at 130 V for 1.5-3.5 hours.

PAA gel separated proteins were transferred to Immobilon-P PVDF membranes (Millipore) in a Trans-Blot SD Semi-Dry Transfer Cell (BioRad) following the manufacturer's instructions. Immobilon membranes were soaked for 2 min in Methanol and then equilibrated for at least 15 min in Transfer Buffer. PAA gels were equilibrated in Transfer Buffer for 10-15 min. Blotting was at 20V/200 mA for 25-90 min (depending on the concentration of the gel and the molecular weight of the protein(s) of interest) between three sheets of soaked 3MM paper (Whatman) at each electrode.

4.7. Gel Mobility Shift Analysis

The DNA binding activity of GAL4-fusion proteins was measured by a gel mobility shift assay with the oligonucleotide probe

CTGCAGTCGGAGGACAGTACTCCGACCCGGG

The probe was annealed to a complementary oligonucleotide, giving CTGC sticky ends. 20 ng of the annealed oligo were labelled with Sequenase 2.0 (USB) in 10 μ l filling in reaction with 40 μ Ci α^{32} P-dATP and 100 μ M cold dCTP, dGTP and dTTP in Sequenase buffer. This labelling gives typically about 200 000 cpm/ng oligo. The labelled oligonucleotide was purified from a 4.5 % non-denaturing acrylamide gel by excising the hot band and extraction in Gel Extraction Buffer. The eluted signal was counted, the oligo precipitated in 2.5 volumes EtOH, 20 mM Mg₂Cl and resuspended at 100 000 cpm/ μ l.

Gel retardation assays were performed with less than 0.5 ng of the fusion proteins and 50 000 cpm of the labelled oligo in a 20 μ l reaction with final concentration:

50 mM NaCl

20 mM Tris HCl 7.5

50 μ g/ml poly(dI.dC) (Pharmacia)

1 mM EDTA

1 mM DTT

5% Glycerol

0.1 % NP-40

1 mg/ml BSA

50 μ M ZnSO₄

2.5 mM MgCl₂

0.1 % Bromophenol Blue

The reaction mixtures were incubated on ice for 30 min and 15 μ l were loaded on a 4.5 % Acrylamide gel (37.5:1 Acrylamide : Bis-Acrylamide) in 0.5X TBE. The gel was pre-run for 30 min and run for 90 min at 200 V, dried on DEAE-paper (Whatman) and exposed to Kodak XAR films or quantified by a Phosphorimager (Molecular Dynamics).

4.8. Injecting of *X.laevis* Oocytes

X. laevis oocytes were obtained from the ICRF Animal Unit at Clare Hall and kept in MBS at room temperature. They were separated by treatment with collagenase type II (Sigma) at 1 μ g/ml in MBS for at least 5 hours and then extensively washed. After this treatment the oocytes can be maintained in MBS for 2-3 days.

Injections were performed following centrifugation of the oocytes at 1400 rpm. for 11 min in a IEC Centra-7C centrifuge. This allows the nuclei to surface and become visible under a stereoscope. Each oocyte was injected with 46 nl mixture, containing 0.46 ng of the test plasmid, 0.46 ng of pSP65-VA1 control plasmid and protein where indicated with a Drummond "Nanoject" apparatus according to the instructions of the manufacturer. α - amanitin was injected at 20 μ g/ml into the cytoplasm to give final intracellular concentration of 1-2 μ g/ml. DRB, H-8, H-7 were added to the incubation media at concentrations, indicated in the text.

4.9. Oocyte RNA Isolation and Analysis

RNA Isolation

RNA isolation was as described (Bentley et al., 1989; Bentley and Groudine, 1988). Briefly, 10-20 healthy oocytes were harvested 16-20 h after

injection, drained and crushed by a pipette in 250 μ l of XL Extraction Buffer. 250 μ l of XL Guanidium Buffer were added and the homogenate was extracted once with 500 μ l of phenol/chloroform and once with 400 μ l of chloroform. The final extract (500 μ l) was added to 1 ml of absolute EtOH and the RNA was stored at -20°C .

4.10. RNAase and Nuclease S1 Protection Analysis

RNAase Protection Assay

RNA isolated from 1 oocyte was co-precipitated with 70 000 cpm of the VA and 70 000 cpm of the test gene RNA probes for RNAase protection. Hybridisation was for 3 h or overnight in 10 μ l Formamide Hybridisation Buffer at 48°C . The samples were then digested at 37°C for 30 min in 100 μ l RNAase reaction (0.3 M NaCl, 10 mM TrisHCl pH 7.5, 5 mM EDTA, 5 μ g/ml RNAase T1 and 0.5 μ g/ml RNAase A). The RNAase reaction was terminated by adding 2 μ l 10% SDS and 2 μ l 10 mg/ml Proteinase K and incubated for additional 15 min at 37°C to inactivate any residual RNAase activity. The RNA was then Phenol/Chloroformed, precipitated in EtOH, dissolved in 2.5 μ l water and 5.5 μ l Formamide Loading Buffer and separated on denaturing Acrylamide/Urea Gels. Fixed, dried gels were exposed on Kodak X-Omat films or quantified on Phosphorimager (Molecular Dynamics).

S1 nuclease Protection Assay

Co-precipitation and hybridisation were the same as for RNAase protection assay except that 50 K of the 5'-labelled DNA probe was used. The samples were digested in 100 μ l S1 reaction (30 mM NaCOOH pH 4.6, 2 μ M ZnSO₄; 0.3 M NaCl, 25 U S1 nuclease (Boeringer)) for 30 min at room

temperature, EtOH precipitated and analysed as in the RNAase protection assay.

4.11. Probes for RNA Analysis

Probes for S1 Protection Assay

HindIII end $\gamma^{32}\text{P}$ -ATP labelled HindIII-EcoRI dsDNA fragment, derived from pGal5-HIV2CAT, was used for mapping of the 5' ends of HIV2CAT transcripts. The fragment was labelled at the HindIII site by PNK and $\gamma^{32}\text{P}$ -ATP. This probe was made by D.Bentley.

Probes for RNAase Protection Assay

Probes for RNAase protection assays were synthesised for 45 min at 37°C from linearised plasmids in 10 μl reaction which contained :

- 1 μl template (1 mg/ml linearised plasmid)
- 2 μl 5X transcr. buffer (Stratagene)
- 1 μl 100 mM DTT
- 1.5 μl N mix (1.88 mM ATP, GTP, CTP; 0.375 mM UTP)
- 4 μl $\gamma^{32}\text{P}$ UTP (800 Ci/mmol)
- 0.5 μl RNA polymerase

The reaction was terminated by adding of 20 μl Formamide Loading Buffer and heating the samples for 2 min at 95°C. The probes were purified from 4.5% denaturing gels by excision and extraction of the hot band in 500 μl Gel Extraction Buffer for 2 hours at 65°C. Typically 75 000 cpm were used per sample in the RNAase protection analysis.

The plasmids, the restriction enzymes and the RNA polymerases used are listed in Table 2. The lengths of the probes and the important products of the assays are also shown.

Table 2. Probes for RNAase Protection Assay

probe	plasmid	Linearised with (enz)	Synthesised by (enz)	Length (nucleotides)		
				probe	RT	TM
IITK	pVZGalIITK	BglII	Sp6	340	185	130
IVTK	pVZGalIVTK	BglII	Sp6	340	220	130
E1bmyc	pVZGalE1bmyc	HindIII	T7	350	195	105
E4myc	pVZGalE4myc	HindIII	T7	350	200	105
943myc	pSX943	BamHI	T3	400	356	302
hiv2	pVZHIV2	EcoRI	T3	410	165	130*
VA	p5'VA	XbaI	T7	130	70	-

*multiple bands from 120 to 140 bases

RT - readthrough band

TM - terminated band

Sense RNA was produced from the same plasmids, using T3 RNA polymerase for pGalIITK, pGalIVTK, pGalE1bmyc and pGalE4myc. T7 RNA polymerase was used for the synthesis of sense c-myc RNA from pSX943. All these RNAs were synthesised with 100 μ M cold NTPs only.

4.12. X. Laevis Whole Cell Extract

X.laevis oocytes were collagenased and washed in MBS in the same way as for injecting. Finally the oocytes were washed twice in ice-cold XL wce Extraction Buffer and transferred to 15 or 30 ml Corex tubes so that to fill about 75 % of the tube volume. The tube was topped with XL wce Extraction Buffer and overlayers with 1 ml of liquid paraffin (Boots). This helps to keep the yolk vesicles at the phase boundary and facilitates the collecting of the clear extract. Protease inhibitors were added to the water phase. The oocytes were crushed and extracted in a single step by spinning for 20 min at 10 000 rpm in a Sorvall HB-4 rotor. The midphase was carefully collected by a syringe and immediately respun for 90 min at 40 000 rpm in a Beckman SW-40 rotor. The clear midphase was collected, avoiding carefully the upper yolk layer, brought to 20% glycerol, aliquoted and frozen in liquid nitrogen. After thawing the extract was filtered through 0.45 μ m filter.

4.13. HeLa Cell Nuclear Extract

The protocol follows that of (Shapiro et al., 1988). I used the 1985 freezing of Hela from Clare Hall. All manipulations were on ice or at 4°C. 20 litre cells were harvested at 5×10^5 per ml (10^{10} cells total). The cells were pelleted at 2000 rpm for 5 min in Beckman J-6 rotor, washed twice with cold PBS and transferred to 50 ml Falcon tubes (7.5 ml packed cells per tube). The tube was filled with hypotonic lysis buffer plus protease inhibitors and the cells were allowed to swell for 10 min on ice. After repelleting the final volume of the suspension was brought to 27 ml with hypotonic buffer plus protease inhibitors and the cells were homogenised in a pre-chilled 40 ml Wheaton

Dounce by 8 strokes with the B pestle. 3 ml (1/10 vol.) sucrose restore buffer were added to the homogenate and mixed in with 2 gentle strokes. The crude nuclear fraction was quickly pelleted in 30 ml Corex tubes by spinning in a swinging bucket Sorvall rotor HB4 at 10,000 rpm for 30 seconds. The turbid supernatant was discarded and the nuclei resuspended by gentle douncing in a total volume of 58.5 ml nuclear resuspension buffer plus protease inhibitors. 6.5 ml (1/10 vol.) of saturated neutralised Ammonium Sulphate were added dropwise while stirring to give final concentration of 0.41 M. The suspension was stirred in ice bath for 30 min and then spun in a Beckman TI45 rotor at 45 000 rpm for 90 min at 4°C. Solid ammonium sulphate (0.33g/ml) was added slowly to the supernatant and stirred for 20 min. The pellet was collected in 10 ml of nuclear dialysis buffer plus protease inhibitors, dialysed twice in 2 litres dialysis buffer for 90 min, clarified by spinning for 10 min at 10 000 rpm in a Sorvall HB-4 rotor, aliquoted and frozen in liquid nitrogen.

Solutions for HeLa Nuclear Extract

HYPOTONIC BUFFER

10mM HEPES pH 7.9

0.75mM spermidine

0.15mM spermine

0.1mM EGTA

0.1mM EDTA

10mM DTT

10mM KCl

NUCLEAR DIALYSIS BUFFER (BUFFER D)

20mM K HEPES pH 7.9

100 mM KCl

0.2mM Na EDTA

0.2mM K EGTA

2mM DTT

20% glycerol

NUCLEAR RESUSPENSION BUFFER

20mM K HEPES pH 7.9

0.75 mM spermidine

0.15 mM spermine

0.2 mM Na EDTA

2mM EGTA

2 mM DTT

25% glycerol

10 X SALT

0.5 HEPES pH 7.9

7.5 mM spermidine

1.5 mM spermine

100 mM KCl

2mM EDTA

10mM DTT

SUCROSE RESTORE

9 vols. 75% sucrose, 1 vol 10X salts

4.14. Affinity Chromatography

Preparation of affinity GST-; GSTSW6-; GSTVP16- and GSTE1a(CR2&3)-Sepharose resins is described in Production of GST-fusion Proteins. The resins (0.6 ml) were packed in Bio-Rad Economy Columns and equilibrated with buffer D (50 mM KCl). 1 ml HeLa Nuclear Extract (about 10 mg/ml initially, diluted twice with equal volume of buffer D without KCl to bring down the salt concentration to 50 mM KCl) or 4 ml of X.laevis Whole Cell Extract (2-3 mg/ml) were passed three times through the columns. The resins were subsequently washed and eluted with the following buffers (2 fractions of 1.35 ml each, equal of 4.5 resin bed volumes): buffer D+50 mM KCl (fractions 1 and 2); buffer D+100 mM KCl (fractions 3 and 4); buffer D+200 mM KCl (fractions 5 and 6); buffer D + 600 mM KCl (fractions 7 and 8). In all cases buffer D was supplemented with 20 % v/v Glycerol and protease inhibitors. Fractions were immediately transferred to -20°C. Aliquots were used for subsequent analysis.

4.15. Preparation of Anti-p62 Beads and Immunodepletion

100 µl Protein-A Sepharose beads were mixed with 1.2 ml of rabbit antiserum, raised against the p62 subunit of TFIIF (this antiserum was prepared by D.Bentley) and 5 µg of 3C9 monoclonal antibodies or prebleed serum, respectively, and rocked for two hours at 4°C. The beads were then washed three times with PBSA and twice with 200 mM Na-Borate buffer pH 8. 1 ml of 40 mM Dimethyl Pimilmaleimid (Sigma) in Borate buffer was mixed with the resin and rocked for 1 h to cross-link the antibodies and Protein A. Finally the beads were washed extensively with PBSA and equilibrated with buffer D+50 mM KCl. 50 µl of the VP16 fraction 5 were diluted with 150 µl

buffer D without KCl to lower the KCl concentration to 50 mM, mixed with the beads and rocked for three hours at 4°C. The supernatants were collected and the beads washed three times with 1 ml of buffer D+50 mM KCl.

4.16. Standard Protein Kinase Assay

Kinase activity was assayed in a 20 µl standard reactions with final concentration:

50 mM KCl
20 mM Tris HCl pH 8
7 mM MgCl₂
2 mM DTT
5 mM 2-Glycerophosphate
1 mM Microcistin
3.3 µg/ml pAdH3, linearised by EcoRI
100 µg/ml BSA
25 µM ATP
4 µCi $\gamma^{32}\text{P}$ -ATP
1X protease inhibitors

Substrates were used at the following concentrations:

GST-CTD , at 40 µg/ml
GAL-CTD, at 120 µg/ml
human recombinant TBP, at 40 µg/ml
human recombinant TFIIF, at 45 µg/ml
human recombinant TFIIE, at 60 µg/ml

TBP, the two subunits of TFIIF and the two subunits of TFIIE were expressed and purified by D.Bentley and T.Purton. The two subunits of TFIIF

and of TFIIIE, respectively, were mixed after purification of each individual component.

Test fractions were preincubated in Falcon 96 well microtiter plates for 40 min at 30°C with 5 μ M cold ATP (and inhibitors, where indicated) to prevent high autophosphorylation signals from the sample. Subsequently the final cold ATP concentration was brought to 25 μ M, and the substrates and 4 μ Ci γ ³²P-ATP were added to initiate the reaction. After 1 h at 30°C the reactions were terminated by 5 μ l 5X SDS-Loading Buffer and the samples were heated for 5 min at 90°C. The products of the reaction were separated on 10-12 % SDS-Polyacrylamide gels for 90 min at 150 V. Finally the gels were washed extensively (at least 2 h) in Gel Fixing Solution, dried and exposed on Kodak XAR film or phosphorimaged.

4.17. Western Blot Analysis

Proteins were separated and blotted as described in PROTEIN ELECTROPHORESIS AND BLOTTING. Rainbow Markers (Amersham) were always used to control for the quality of separation and transfer. After blotting the filters were marked with a pencil and protein binding sites were saturated for 4-15 hours at 4°C in milk-PBSA (5% Marvel Fat Free Dry Milk, 0.25 % Tween-20 in PBSA).

Monoclonal antibodies and affinity purified rabbit antibodies were used at 0.3-5 μ g/ml. Polyclonal sera were diluted 100-400X. All antibodies were diluted in 3% Bovine Albumin (Fraction V, Sigma), 0.25 % Tween-20, 0.02 % NaN₃ in PBSA and used several times.

Secondary anti-mouse and anti-rabbit immunoglobulin HR-peroxidase conjugated antibodies were purchased from Daco (Copenhagen) and were diluted 1:5000 in milk-PBSA.

Filters were incubated with the primary and secondary antibodies for 1 h at room temperature. After each incubation the filters were washed 4-5 times in 100 ml of milk-PBSA for 5 min. Finally the filters were rinsed in PBSA. Detection of the immune complexes was for 1 min by ECL reagent (Amersham) according to the instructions of the manufacturer.

After each antigen detection all the antibodies were stripped from the filters by incubation in STRIP (2% SDS; 100mM 2-Mercaptoethanol in PBSA) for 15 min at 50°C. The filters were then extensively washed in PBSA, incubated for 1 h in milk-PBSA and used for immunodetection of other antigens.

4.18. Expression and Purification of Recombinant Proteins

GAL-fusion proteins

GAL4-AH was produced from the plasmid pTMC2. GAL4-VP16 was expressed from pET21bGAL4VP16. This protein contains the Herpes simplex virus VP16 protein sequence 412-490 and LDRSVEHHHHHH C-terminal of VP16 residue 490. GAL4-E1a(CR2&3) was produced from pET21dGAL4-E1a and contains E1a residues 121-222 (Zhou et al., 1992). This protein has a K to A substitution at position 2 and contains the sequence RAALEHHHHHH C-terminal of the E1a sequence. GAL4 (1-147) was expressed from pET21bGAL4 (1-147) and contains the sequence PVDKLA AALEHHHHHH C-terminal to residue 147. GAL4 (1-94) was expressed from pET21bGAL4 (1-94). C-terminal of amino acid 94 the sequence is AALEHHHHHH. Host strains were XA90 for GAL4-AH and BL21 DE3 (pLysS) for the others.

Cultures were initiated from either fresh plates or 100 times diluted overnight cultures. Cells were grown in BHI media at 37°C to OD₆₀₀=0.6. The

temperature was then decreased to 30°C and protein expression was initiated by adding of 1 mM IPTG. After 3h the cells were collected and washed in buffer A. All subsequent manipulations were at 4°C. The cells were sonicated (6 x 10 s), the homogenate was supplemented with protease inhibitors and 0.2 % NP40, rocked for 30 min at 4°C and centrifuged for 10 min at 10, 000 rpm (Sorvall, HB-4 rotor). GAL4 (1-94), GAL4(1-147), GAL4-AH and GAL4-VP16 were further purified by adding of 5 ml settled volume of Heparin-Sepharose CL6B (Pharmacia) to the supernatant. After 15 min the resin was pelleted, transferred to a column and washed with 10 column volumes of buffer A. Proteins were eluted with 0.6 M NaCl in buffer A and mixed with 2 ml of S-Sepharose Fast Flow (Pharmacia). The mixture was diluted six times with buffer A without NaCl. After 15 minutes the resin was transferred to a column and washed with 0.2 M NaCl in buffer A. GAL4-fusion proteins were collected in 0.4 M NaCl in buffer A without protease inhibitors. Aliquots were stored at -70 °C.

GAL4-E1a was purified from inclusion bodies which were washed in 2% Na-Deoxycholate and then solubilised in 6M Guanidine HCl in buffer A. The proteins were loaded on a Ni-NTA-Agarose column (Qiagen) and eluted with 100 mM Imidazole in buffer A and 6M Guanidine HCl. Subsequent renaturing was by step-wise dialysis in 3M, 1.5 M and 0.75 M Guanidine HCl in buffer A, supplemented with 1 mM ZnCl₂ (Each step was 1.5 h). After the final dialysis the renatured protein was purified by mixing with 1 ml of DNA-Cellulose (Sigma) and slowly (30 min) diluting the mixture to 0.3 M Guanidine HCl with buffer A. The resin was transferred to a column, washed with buffer A without protease inhibitors and eluted with 0.4 M NaCl in the same buffer.

p62

p62 was expressed from pET21Dp62SA in Topp2 and purified in the same way as GALE1a(CR2&3). It was either eluted and used as antigen for

immunising rabbits or renatured together with the resin as for GALE1a and used as affinity beads.

GST-fusion proteins

GST, GST-VP16 and the mutant GST-SW6 were expressed using derivatives of the pGEX2T vector (Pharmacia). The VP16 fragment fused to GST was the EcoRI fragment derived from pSGVPA490 (Sadowski et al., 1988) encoding residues 410-490 of VP16. GST-SW6 was constructed by substitution of the SphI-StyI fragment of pSW6 (Walker et al., 1993) into the GST-VP16 plasmid. GST-E1a was expressed from pGST-E1a. pGST-E1a(CR2&3) and GST-CTD was expressed from pGCTD (Peterson et al., 1992) or from a derivative of this plasmid pET21a-GCTD. pGST, pGSTVP16, pGSTSW6 and pGSTE1a(CR2&3) and pGCTD, respectively, were transfected in Topp2 (Promega) cells. Cells were grown in BHI media at 37°C to OD₆₀₀=0.6. Protein expression was initiated by adding 1 mM IPTG. After 90 min the cells were collected and washed in buffer NENT. Expression for shorter periods were introduced when not-full length recombinant GST binding species were produced in the cells (GST-VP16 and GST-E1a(CR2&3) were expressed for 30 min only). All subsequent manipulations were at 4°C. Protease inhibitors were added prior and NP-40 - after sonication. The homogenate was rocked for 30 min, spun for 15 min in HB-4 Sorvall rotor and the supernatant loaded directly to 2 ml of GST-Sepharose (Pharmacia). The resin was washed with buffer NENT plus 1M NaCl and either stored for affinity chromatography experiments or eluted with 15 mM Glutathion (reduced form, Sigma). The proteins were dialysed against buffer D, aliquoted and snap-frozen.

BM28 was a gift from Dr. I.T. Todorov. This protein was expressed in insect cells by baculovirus and purified over three columns.

Human TBP was expressed and purified by D.Bentley and T. Purton.

Human TFIIH was a gift from Dr. J.M. Egly. This protein has been extensively purified over six columns (Roy et al., 1994).

4.19. Antibodies

The antibodies used are listed in Table 3.

Table 3. List of Antibodies

Antibody	Antigen	Type	Reference
anti-CTD 8WG16	RNApol II -CTD domain	MAB, IgG2a ascitie	(Thompson et al., 1990)
anti-TBP (MTB6)	TBP	MAB Prot.A-IgG	(Chatterjee et al., 1993)
anti-RPA (70C)	the large (70 kDa) subunit of RP-A	MAB Prot.A-IgG	(Kenny et al., 1990)
anti-p62 (3C9)	62 kDa subunit of TFIIH	MAB, IgG2a ascite fluid	(Fischer et al., 1992)
anti-Ku (N3H10)	Ku subunit of DNA-PK	MAB, IgG2b ascite fluid	(Wang et al., 1993)
anti-CDC2	CDC2 protein from X.laevis	MAB Prot.A-IgG	(Kobayashi et al., 1992)
anti-myc (9E10)	human c-myc	MAB Prot.A-IgG	(Evan et al., 1985)
anti-BM28	N-terminus of human BM28	R-PAb Prot.A- IgG	(Todorov et al., 1994)
anti-GAL	yeast GAL(1-147)	R-PAb serum	

anti-IIS	human TFIIIS	R-PAb serum	
anti-rap30	30 kDa subunit of TFIIIF (rap 30)	R-PAb serum	
anti-IIIE	the small (34 kDa) subunit of TFIIIE	R-PAb serum	

MAB - mouse monoclonal antibody

R-PAb - rabbit polyclonal antibody

Prot.A-IgG - Protein A-Sepharose purified antibodies

All MAb were gifts from the authors. Anti-IIS, anti-IIIF(rap30) and anti-IIIE were made by D.Bentley and T.Purton.

4.20. Plasmid Preparation

200 ml overnight cultures were pelleted and resuspended in 25 ml cold TES (25 mM Tris-HCl pH 7.5, 10 mM EDTA, 15 % sucrose). 50 ml of 0.2 M NaOH, 1 % SDS were then added and the suspension was vigorously mixed to lyse the cells. After 10 min on ice 37.5 ml 2,7 KAcetate pH 4.8 were added and the mixture left for 30 min on ice. After spinning (10 min, 4 500 rpm in Beckman rotor J-6) the supernatant was carefully collected (decanted through Miracloth, Calbiochem) and the plasmid precipitated with equal volume of isoprpanol at room temperature for 5 minutes. The pellet was dissolved in 5 ml TE and 5 ml 5M LiCl were added. After 5 min on ice the precipitate was discarded by centrifugation (10 min at 3000 rpm, J-6) and the supernatant precipitated by adding of 25 ml EtOH for 5 min at room temperature. The final pellet was dissolved in 2.5 ml TE. 4.2 g CsCl were slowly dissolved together with 0.2 ml of 10 mg/ml EtBromide. The plasmid solution was underlayered beneath 8 ml 55

% CsCl and spun overnight at 50000 rpm. The plasmid band was collected by punching the tube with a 24 g needle. EtBr was extracted 3 times with NaCl saturated butanol. 3 volumes of water were added to the extracted DNA and the plasmid precipitated with 2.5 volumes EtOH. After one more precipitation with EtOH and AmAcetate the plasmid prep was dissolved in water. The concentration was measured by OD260 and the quality of the prep - by agarose electrophoresis.

4.21. Plasmids

pSX943 contains the 943 base Sma I-Xho I fragment of the mouse c-myc exon I cloned into the Sma I - Sal I sites of the Bluescribe derivative pVZ (Henikoff and Eghtedarzadeh, 1987).

pGal5-P2CAT was made by substituting the TATA box of pGAL4/E1bTATA (Lillie and Green, 1989) with the BamHI-SacI fragment of c-myc exon 1 which starts 15 bases upstream of the TATA sequence.

pGal5-E1bmycCAT was derived from GAL4/E1bTATA by insertion of the mouse c-myc exon 1 180 b.p. NotI-SacI fragment between the BamHI and SacI sites of the polylinker.

pGal5-E4mycCAT contains the c-myc 180 b.p. NotI-SacI fragment between the BamHI and SacI sites of the polylinker of pGal5-E4 CAT (Flint and Jones, 1991) pGal5-E4 CAT is identical to GAL4/E1b TATA except for substitution of the E4 TATA box, CTATATATACTCGC, for the E1b sequence AGGGTATATAATG between the XbaI and BamHI sites of the polylinker.

pGal5-IITKmycCAT contains the mouse c-myc *Not I-Xho I (the T2 element), inserted downstream of five GAL4 binding sites and the human TK TATA element in pSP72.

pGal5-IVTKmycCAT contains the TdT initiator element (GGCCCCTCATTCTGGAGAC), inserted downstream the TK TATA box of pGal5-IITKmycCAT.

pHIV2-LTRCAT-556/+156 was described in (Emerman et al., 1987).

pGal5-HIV2CAT was made by substituting the TATA of GAL4/E1bTATA with a PCR product extending from -32 to +156 of HIV2 between the XbaI and SmaI sites. The HIV2 sequence was derived from the plasmid pHIV2-LTR-CAT-556/+156.

pΔGal5-HIV2CAT was made by excising of the EcoR1-HindIII fragment from pGal5-HIV2CAT. That fragment contained the five GAL4 binding sites.

pSP65-VA contains the XbaI-SalI 258 base fragment of Adenovirus 2 containing the VA1 gene subcloned from pMHVA (Mellits and Mathews, 1988). This plasmid was a gift of K. Mellits.

pVZGal5-P2, pVZGal5-E1bmyc, pVZGal5-E4myc, pVZGal5-IITKmyc and pVZGal5-IVTKmyc. T7 RNA probes complementary to Gal5-P2CAT, Gal5-E1bmycCAT, Gal5-E4mycCAT, Gal5-IITKmycCAT and Gal5-IVTKmycCAT transcripts were synthesised from pVZ subclones of these plasmids in which HindIII-SacI fragments containing the GAL4 sites, TATA elements and myc sequences were inserted into the Bluescribe derivative pVZ (Henikoff and Eghtedarzadeh, 1987).

pVZHIV2 contains the 415 b.p. KpnI-HindIII fragment of HIV2-LTR-CAT-556/+156 extending from -259 to +156 subcloned in pVZ. Antisense RNA probe was synthesised from this template for analysis of Gal5-HIV2 and HIV2-LTR-CAT-556/+156 transcripts.

p5'VA was used for preparation of AdVA probe. It contains the XbaI-BamHI fragment that includes the first 73 bases of the transcribed sequence (Herrmann and Mathews, 1989).

pGSTCTD was described in (Peterson et al., 1992).

pGSTVP16 is a derivative of the pGEX2T vector (Pharmacia). The VP16 fragment fused to GST was the EcoRI fragment derived from pSGVPA490 (Sadowski et al., 1988) encoding residues 410-490 of VP16.

pGSTSW6 was constructed by substitution of the SphI-StyI fragment of pSW6 (Walker et al., 1993) into the GST-VP16 plasmid.

pGSTE1a(CR2&3) was cloned by inserting the EcoRI-Xba I fragment (encoding residues 121-222) of pET21dGAL4-E1a (Zhou et al., 1992) into the BamHI site of pGEX20T (J.Armstrong).

pTMC2 (Lin et al., 1988) was used for expression of GAL1-147.

pET21bGAL4VP16 was derived from pSGVPA490 (Sadowski et al., 1988). The plasmid contains the Herpes simplex virus VP16 protein sequence 412-490 and was used for expression of GAL4VP16.

pET21dGAL4-E1a was a subclone of the NcoI-XbaI fragment of pET8cGAL4-E1a containing E1a residues 121-222 (Zhou et al., 1992).

pET21bGAL4 (1-147) was made by insertion of the GAL4 (1-147) sequence in the plasmid pGST-GAL4 (1-147)-CREB (S. Goodbourn, personal communication) into NheI-SalI cut pET21b.

pET21bGAL4 (1-94) was made by cloning the XbaI-HpaI fragment of pET21bGAL4 (1-147) into pET 21b cut with XbaI and NotI (filled in).

pET21Dp62SA was made by insertion of the the BsmI 1.7 kb fragment, encoding the full length p62(TFIIH) (Fischer et al., 1992a) into the SmaI site of pET21D.

pAdH3 contains the 2.1 kb Sma I - Hind III of Adenovirus 2, containing the MLP, in the pVZ derivative of Bluescribe.

Most of these plasmids had been cloned by D.Bentley and generously donated for my experiments.

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