Effect of Activators on Promoter Clearance by RNA Polymerase II

by

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Eukaryotic transcription is a complex and highly regulated process. The adenovirus major late (AdML) promoter, a model promoter for transcription by RNA polymerase II (pol II), contains a binding site for the transcriptional activator upstream stimulatory factor (USF). Previous experiments showed that deletions in the USF site in AdML promoter decrease the stability of ATP-activated transcription complexes. As a result we wanted to look at the effect USF had on the rate of promoter clearance by pol II in vitro.

To develop an assay for promoter clearance, special DNA templates were constructed such that a G at position +11 is the only G incorporated into the growing RNA in the first 36 nucleotides of RNA synthesis. Our assay for promoter clearance is designed to measure whether pol II has passed position +11 at a given time, t, and depends on the ability of the chain terminating nucleotide 3'-O-methyl GTP (added at t) to quantitatively stop transcription if it is incorporated into the RNA at +11.

Controls for the promoter clearance assay are in progress. So far controls have shown the presence of promoter specific transcripts from our templates that are distinguishable from the background. In addition, 3'-O-methyl GTP at high concentrations has been shown to terminate transcription almost completely. However, it is still not quite clear if it is efficient enough for the promoter clearance assay.
Acknowledgements

I would like to thank Dr. Peterson foremost for his kindness and guidance during the long year. I appreciate the opportunity given to me to gain valuable experience that will help me tackle my future goals. In addition, I would like to thank Lisa Dandridge for putting up with me for those many hours in lab making the experience more bearable during the, sometimes, tedious hours. Also, you were most willing to lend a helping hand when I needed it and to brighten up a day with your humor.
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LIST OF ABBREVIATIONS

adenovirus major late promoter (AdML)
carboxy terminal domain (CTD)
Cdk-activating kinase (CAK)
cyclin-dependent kinase (Cdk)
general transcription factor (GTF)
initiator element (Inr)
mouse mammary tumor virus promoter (MMTV)
RNA polymerase II (pol II)
TATA-binding protein (TBP)
TBP associated factor (TAF)
upstream stimulatory factor (USF)
INTRODUCTION

Mammals contain tens of thousands of genes encoded within their DNA. Converting the information stored within these genes to a final product, protein or RNA, involves a series of processes. Transcription, the process responsible for converting a gene's DNA sequence into an intermediary, RNA, consists of many highly regulated steps. Understanding the regulation of these steps has the potential to impact the study of many biological pathways, i.e. apoptosis, which when defective in these regulatory steps may result in a disease or disorder such as cancer. In addition, this understanding may lead to developing cures or better treatments for these diseases.

RNA polymerases are responsible for the enzymatic activity of synthesizing RNA chains. Prokaryotes possess one major RNA polymerase while eukaryotes possess three separate RNA polymerases characterized by their sensitivity to fungal toxin α-amanitin. RNA polymerase I (pol I), insensitive to α-amanitin, transcribes class I genes encoding ribosomal RNA. RNA polymerase II (pol II), sensitive to 1 μg/ml of α-amanitin, transcribes class II genes encoding mRNA and small nuclear RNA (U1-U5). RNA polymerase III (pol III), sensitive to an α-amanitin concentration of 50 μg/ml, transcribes class III genes encoding transfer RNAs, 5S ribosomal RNA and U6 small nuclear RNA. The eukaryotic RNA polymerases lack the ability to transcribe genes accurately and efficiently; thus they require additional factors to recognize their respective promoters. In the following work the studies involve pol II, thus the subsequent descriptions relate to transcription involving pol II.

 Characteristics of class II promoters

A class II promoter contains a set of DNA sequences involved in basal levels of transcription while regulators modify these levels in a positive (activator) or negative (repressor) fashion. The promoter consists of a combination of sequences including core promoter elements, proximal promoter elements, and distal enhancer elements.
Core promoter elements are necessary for accurate and site-specific transcription initiation by pol II and associated transcription factors. Two of the best-known core promoter elements are the TATA box and initiator (Inr) (Kollmar and Farnham, 1993). All known pol II promoters contain at least one of the two elements, which function independently or in conjunction. The TATA box is an AT-rich region located about 30 base pairs (bp) upstream of the transcription start site (+1) while the Inr is located at the start site. Promoters lacking TATA boxes contain GC-rich regions 5' to the +1 site and usually initiator elements (Carcamo et al., 1991; Conaway and Conaway, 1993; Smale and Baltimore, 1989).

In addition to core promoter elements, DNA binding domains for regulators exist. These domains may be adjacent to the promoter, upstream or downstream of the promoter, or thousands of bp away. In addition, many act independent of orientation. The regulatory factors bind in a trans fashion and, in addition to DNA binding domains, contain activation or repression domains. Activators have been shown to aid in transcription complex assembly. For example, GAL4-AH promotes closed transcription complex formation at the adenovirus E4 promoter (Wang et al., 1992b). Additional functions for activators are: help in recruitment of transcription factors, stabilize protein-protein or protein-DNA interactions, and stabilization of transcription complexes. However, little is known about how activators affect events beyond complex assembly. Activator GAL4-VP16 was shown to stimulate elongation processivity (Yankulov et al., 1994).

**Transcription complex assembly**

Transcription consists of major phases, which themselves consists of many steps, all of which may be regulated. The first major phase, initiation, begins with the first step formation of a closed transcription complex on the promoter. Two models exist for the formation of a transcription complex: ordered stepwise assembly and pol II holoenzyme assembly.
Pol II requires additional factors (Fig. 1), termed general transcription factors (GTFs), to ensure accurate initiation of transcription (Roeder, 1996). In a stepwise assembly model the GTFs and pol II assemble on the promoter DNA through a series of steps. The sequence of GTF assembly on the adenovirus major late (AdML) promoter in vitro was determined via gel mobility shift assays (Buratowski et al., 1989). The series of steps begins with TFIID and TFIIA forming a complex at the TATA box, followed by TFIIIB. The DNA-binding subunit of TFIID, the TATA-binding protein (TBP), forms a stable complex (Hoopes et al., 1992) distorting the DNA sequences upstream and downstream of the TATA box into closer proximity (Burley and Roeder, 1996; Nikolov and Burley, 1997; Roeder, 1996). TFIIIB, through interaction with TBP, contacts the DNA sequences upstream and downstream of the TATA box (Nikolov et al. 1995). Next, RNA pol II, in association with TFIIF, can become part of the complex through direct interactions with TFIIIB. Then recruitment of TFIIE, which interacts with pol II, occurs. Finally, TFIIE recruits and makes direct contacts with TFIIH completing the closed complex.

The alternative model, pol II holoenzyme assembly, challenges the stepwise assembly. A pol II holoenzyme in solution has pol II, TFIIE, TFIIF, and TFIIH preassembled together. A number of complexes purified by conventional chromatography or by antibody affinity chromatography using an anti-TFIIF antibody lacked stochiometric amounts of TFIIIB, TFIID, TFIIE, and/or TFIIH (Koleske and Young, 1994; Cho et al., 1997; Maldonado et al., 1996; Kim et al., 1994). Pol II complexes lacking only TFIIA were isolated using protein affinity chromatography or an antibody to the TFIIH subunit cdk7(Pan et al., 1997; Ossipow et al., 1995). All of these complexes are known as pol II holoenzyme complexes and are capable of basal transcription with the addition of their respective missing GTFs. In some cases the holoenzyme complexes are capable in vitro of activated transcription (Koleske and Young, 1994; Maldonado et al., 1996; Pan et al., 1997).
RNA polymerase II general transcription factors

<table>
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<tr>
<th>TFIIA</th>
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<td></td>
<td>β</td>
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<td>cyclin H</td>
<td>37kD</td>
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<td></td>
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<td></td>
<td>MAT-1</td>
<td>32kD</td>
<td>kinase assembly factor</td>
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Fig. 1. The table above lists the RNA polymerase II general transcription factors, their size, and known functions.
RNA polymerase II

Transcription in bacteria involves a single RNA polymerase composed of three types of subunits $\beta'$, $\beta$, and $\alpha$. One or more additional proteins ($\sigma$ subunits) confer selective promoter recognition and regulatory functions upon the core prokaryotic polymerase (Helmann and Chamberlin, 1988; McClure, 1985). Three types of RNA polymerases exist within eukaryotes. These eukaryotic polymerases consist of 8 to 14 polypeptides. In addition, the polymerases require many additional factors to confer selective promoter recognition and regulatory functions (Geiduschek and Tocchini-Valentini, 1988; Mitchell and Tjian, 1989; Ptashne, 1988; Struhl, 1989). This suggests that eukaryotes need more intricate transcriptional control.

Transcriptionally active pol II has been purified from many different eukaryotes, such as yeast (Scntenac, 1985) and humans (Freund and McGuire, 1986), through column chromatography or immunological methods (Kim and Dahmus, 1988; Ossipow et al., 1995). Throughout the eukaryotes three features of pol II are conserved. First, pol II consists of 8 to 12 subunits. Second, two large subunits of about 220 kDa and 140 kDa are present consistently. In yeast these two subunits are functional homologues to the large prokaryotic subunits, $\beta'$ and $\beta$ (Allison et al., 1985; Sweetser et al., 1987). Third, three pol II subunits ranging in size from 14 kDa to 28 kDa are also associated with pol I and III.

A difference between the largest prokaryotic subunit and largest pol II subunit is the presence of a carboxy terminal domain (CTD) in the eukaryotic subunit. The highly conserved CTD consists of many heptapeptide repeats of the consensus sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser (Allison et al., 1985; Bird and Riddle, 1989; Corden et al, 1985). Pol II purified from different eukaryotic species vary in the length of the repeats. For example, yeast 26 to 27 repeats (Allison et al., 1985) while humans have 52 repeats (Corden et al., 1985; Young, 1991). Many different electrophoretic forms have been isolated based on the degree of phosphorylation of the CTD (Allison et al., 1985; Corden et al., 1985).
During the formation of a closed transcription complex at the promoter, only the nonphosphorylated form of pol II can bind (Laybourn and Dahmus, 1990). In the shift from transcriptional initiation to elongation the CTD becomes hyperphosphorylated indicating a potential function of phosphorylation in promoter clearance and elongation (Parada and Roeder, 1996). Deletion mutations of all or most of the CTD in yeast and *Drosophila* cells are lethal (Allison et al., 1988; Pinto et al., 1994). In vitro transcription experiments using human HeLa nuclear extracts indicate in some promoters CTD is needed for efficient promoter-dependent transcription (Buratowski et al., 1989; Thompson et al., 1989).

**TFIID**

TFIID consists of a TBP subunit and nine TBP associated factors (TAFs). TFIID activity was first discovered in human nuclear extracts (Matsui et al., 1980), but difficulties existed in purifying and studying the factor. On the other hand, a polypeptide of 27 kDa isolated from transcriptionally active yeast extracts possessed the same activity as TFIID (Cavallini et al., 1989; Hahn et al., 1989b; Hernandez, 1993; Schmidt et al., 1989). As a result, a cDNA encoding the yeast TBP (Cavallini et al., 1989; Hahn et al., 1989b; Horikoshi et al., 1989; Schmidt et al., 1989) led to the cloning of TBP in other eukaryotes such as humans (Hoffman et al., 1990; Kao et al, 1990; Peterson et al., 1990). These TBPs facilitated transcription like mammalian TFIID but did not respond to transcriptional activators (Hoey et al., 1990; Peterson et al., 1990; Pugh and Tjian, 1990; Smale et al., 1990). It was deduced from TFIID's molecular weight of ~750 kDa (Conaway et al., 1990; Nakajima et al., 1988) that other factors existed that functioned in transcriptional activation. TAFs (Dynlacht et al., 1991; Tanese et al. 1991), also known as coactivators (Peterson et al., 1990; Pugh and Tjian, 1990), have a possible role in transcriptional activation.

The TBP subunit varies in size in different species, 22 kDa to 38 kDa. It consists of two domains, a carboxy-terminal TATA-binding domain and an amino terminal domain.
While the TATA-binding domain is highly conserved, the amino terminal domain shows little conservation between species. The function of the amino terminal domain is not clear, but the TATA-binding domain has been shown to function in specific transcription initiation and TFIID complex formation (Hoey et al., 1990; Zhou, 1993).

The TATA-binding domain has the shape of a saddle (Chasman et al., 1993; Nikolov et al., 1995) where the concave underside binds DNA and the convex outer surface serves as a binding site of other GTFs (Nikolov et al., 1997). The carboxy terminal domain binds TATA elements with high affinity (Hahn et al., 1989a). It interacts with the minor groove (Starr and Hawley, 1991) leading to partial unwinding and bending of the DNA (Kim et al., 1993). The bending of the DNA may help in bringing proximal DNA elements to the start site or assist in the interactions of GTFs with activators and pol II.

TFIID has at least twelve TAFs ranging in size from 15 to 250 kDa. Most of the human pol II TAFs have been cloned and are highly conserved. In vitro TAFs are required for promoter recognition and activator-dependent transcriptional stimulation in both humans and Drosophila systems, however the role in vivo is not clear (Nakatani et al., 1990; Orphanides et al., 1996).

**TFIIA**

TFIIA has been isolated from yeast (Ranish et al., 1992), humans (De Jong et al., 1993; Ma et al., 1993; Ozer et al., 1994), and *Drosophila* (Yokomori et al., 1994). Human and *Drosophila* TFIIA consists of three subunits (Cortes, 1992): α (37kDa), β (19kDa), and γ (13kDa) while yeast consists of two (Ranish et al., 1992). The subunits of yeast and humans share some homology.

The need for TFIIA varies in different reconstituted transcription systems. TFIIA stimulates transcription reactions involving TFIID (Ozer et al., 1994; Sun et al., 1994; Yokomori et al., 1994), possibly by neutralizing the activity of some repressors, but does not stimulate reactions involving recombinant TBP (Cortes, 1992). However, transcription using TFIID immunopurified to homogeneity and homogenous preparations of the other
GTFs are stimulated by recombinant TFIIA (Ozer et al., 1994; Sun et al., 1994). This indicates TFIIA has a role in the initiation of basal transcription. TFIIA binds both TBP (Cortes, 1992; Ranish and Hahn, 1991) and TBP-DNA complexes (Buratowski et al., 1989; Cortes, 1992) increasing TBP affinity for the TATA element (Imbalzano et al., 1994) and enhancing its ability to compete with other proteins bound at the promoter. Besides neutralizing repressive activity TFIIA also enhances activation by some activators (Yokomori et al., 1994; Ozer et al., 1994). The β and γ subunits are necessary for antirepression, while all three subunits are responsible for activation (Ma et al., 1996).

TFIIB

TFIIB probably aids in linking promoter-bound TFIID to pol II/TFIIF complexes, in addition to specifying the start site of transcription. TFIIB isolated from humans consists of a single polypeptide of ~35 kDa (Ha et al., 1991) and possesses homology to TFIIB found in yeast (Pinto et al., 1992) and Drosophila (Wampler and Kadonaga, 1992). The polypeptide consists of amino- and carboxy-terminal domains (Barberis et al., 1993; Malik et al., 1993). The amino-terminal domain contains a zinc-binding domain (Bagby et al., 1995) that may stabilize melting of the promoter by binding near the transcription start site (Orphanides et al., 1996). Support for this idea comes from experiments involving mutations next to the zinc-binding domain that alter start site selection (Pinto et al., 1994).

TFIIB possesses three binding sites for TFIIF (Ha et al., 1993), pol II (Fang and Burton et al., 1996; Ha et al., 1993), and the TFIID-DNA complex (Buratowski et al., 1989; Maldonado et al., 1990). Once TFIID has bound the promoter, TFIIB binds the TFIID-promoter complex forming a ternary complex increasing stability (Orphanides et al., 1996). This ternary complex is then capable of binding the pol II/TFIIF complex.

The core of TFIIB consists of a two domain α-helical polypeptide believed to interact with the carboxy-terminal domain of TBP and the phosphoribose backbone of DNA upstream and downstream of the TBP-distorted TATA element (Nikolov and Burley,
1997). As a result TFII B may be responsible for determining the correct polarity of the TATA element thus aiding in forming an energetically favorable complex.

**TFIIE**

TFIIE is a heterotetramer composed of two types of subunits (Inostroza et al., 1991; Ohkuma et al., 1990), α (56 kDa) and β (34 kDa). The α subunit has an acidic carboxy-terminus while the β subunit has a basic carboxy-terminus distinguishing the subunits as acidic and basic respectively (Peterson et al., 1991). Also, the α subunit has a possible zinc-binding domain responsible for stabilizing the melted promoter (Peterson et al., 1991). The β subunit contacts the DNA at positions −2 and −14 of the promoter to aid in stabilizing the melted region (Robert et al., 1996).

TFIIE may enter the complex assembly after TFIID, TFIIA, TFII B, TFII F, and pol II or at the same time as the pol II/TFII F complex since TFIIE has an high affinity for pol II (Orphanides et al., 1996). TFIIE recruits TFIIF to complete a transcription complex ready to initiate transcription (Flores et al., 1990). In addition, TFIIE stimulates transcription from supercoiled templates, which may be required during promoter melting (Goodrich and Tjian, 1994; Timmers, 1994).

**TFII F**

TFII F is a heterodimer with two subunits (Flores et al., 1988; Flores et al., 1990), RAP30 (30 kDa) and RAP74 (74 kDa). It forms a stable complex with pol II allowing the pol II/TFII F complex to enter the transcription complex. Crosslinking studies show that RAP30 and RAP74 bind at positions −5, −15, and −19 of the promoter indicating a location between the TATA element and transcription start site (Robert et al., 1996).

Besides forming a complex with pol II, TFII F prevents and reverses binding at nonpromoter sites in a manner similar to σ factors in bacteria (Conaway and Conaway, 1990; Killeen and Greenblatt, 1992). Not surprisingly, TFII F has two regions with sequence similarity to *Escherichia coli* σ factors (Garrett et al., 1992; Sopta et al, 1989). In addition, RAP30 has been shown to contain a cryptic DNA-binding domain at its
carboxy-terminus similar to *E. coli* σ^70* (McCracken and Greenblatt, 1991). RAP30 binds pol II (McCracken and Greenblatt, 1991), TFIIB (Ha et al., 1993), and DNA (Tan et al., 1994). Via the RAP30-DNA interaction the closed transcription complex may be stabilized (Orphanides et al., 1996).

RAP30 needs the assistance of RAP74 to assist in some TFIIF functions. RAP74 is needed to remove pol II already bound to DNA nonspecifically (Conaway and Conaway, 1990; Killeen and Greenblatt, 1992). An additional TFIIF function, particularly of RAP74, includes increasing pol II transcription by stably associating with pol II and increasing the rate of elongation (Bengal et al., 1991; Chang et al., 1993; Kephart et al., 1994). RAP74 may also help in start site selection through its interaction with TFIIB (Fang and Burton, 1996).

The structure of TFIIF may undergo conformational changes during the phases of initiation and elongation. In vivo RAP74 is highly phosphorylated, but in the absence of phosphorylation, TFIIF's activity in initiation and elongation are greatly reduced (Kitajima et al., 1994), suggesting that phosphorylation may play a role in the regulation of TFIIF activity (Dikstein et al., 1996).

**TFIIH**

TFIIH consists of nine subunits ranging in size from 39 kDa to 89 kDa. It was first shown to be required in transcription in a system derived from rat liver (Conaway and Conaway, 1989), but subsequently it has been shown to be required in yeast and human systems. TFIIF possesses many catalytic activities such as DNA-dependent (d)ATPase (Conaway and Conaway, 1989; Roy et al., 1994b), (d)ATP-dependent DNA helicase (Schaeffer et al., 1993), and a serine/threonine kinase that can phosphorylate the CTD of pol II (Feaver et al., 1991; Lu et al., 1992). TFIIE affects the ATPase, DNA helicase, and kinase activities (Drapkin et al., 1994; Lu et al., 1992). TFIIE and TFIIH have a high binding affinity for each other resulting in a close association throughout the transcription process.
The ATPase/DNA helicase and kinase activities are probably involved in the ATP
dependent events required during the transition from the closed complex through
productive elongation. The ATPase/DNA helicase activity requires the hydrolysis of the β-
γ phosphoanhydride bond of ATP. A cyclin-dependent kinase (Cdk) MO15/Cdk7 of
TFIIH (Roy et al., 1994a; Serizawa et al., 1995; Shiekhatter et al., 1995) has been shown
to have dual functions: CTD-kinase and catalytic subunit of Cdk-activating kinase (CAK).
CAK is responsible for the phosphorylation and activation of many proteins important to
the cell-cycle progression (Shiekhatter et al., 1995). In addition, the entire CAK complex
is part of TFIIH (Serizawa et al., 1995; Shiekhatter et al., 1995). TFIIH’s kinase activity
may be responsible for release of pol II from the transcription complex by phosphorylation
of CTD or indirectly regulating pol II by phosphorylating another kinase that
phosphorylates CTD. CTD phosphorylation, which could serve as an indicator for
promoter clearance, may allow disassociation of pol II and association of factors for
elongation (Svejstrup et al., 1996).

TFIIH has been shown to be necessary during transcriptional elongation (Yankulow et
al., 1996). During elongation TFIIH’s association with pol II is less stable, but the ability
of activators to stimulate elongation may be associated with their TFIIH affinity (Yankulow
et al., 1994). During elongation CTD phosphatase acts exclusively on elongating pol II
(Chambers et al., 1995), so TFIIH kinase activity may be necessary to counteract this.

It has been established that transcription and DNA repair are coupled mechanistically.
So, not surprisingly, TFIIH has an additional function in DNA excision repair. Five of
TFIIH’s nine subunits have both transcription and DNA repair functions coupling the two
processes (Dombroski et al., 1992; Drapkin et al., 1994; Humbert et al., 1994; Schaeffer et
al., 1994; Schaeffer et al., 1993).

Initiation and Elongation

After a closed transcription complex has assembled at the TATA element, the next step
of initiation involves forming an open transcription complex. The formation of an open
complex occurs during promoter melting, the process of partially unwinding the DNA around the start site to provide access to pol II. The initial melting ranges from $-9$ to $+1$ (Jiang and Gralla, 1995; Kuhn et al., 1993). One of the ATP-dependent helicases of TFIIH may be responsible for the initial melting, which requires hydrolysis of the $\beta$-$\gamma$ phosphoanhydride bond of ATP (or dATP). The open complex, less stable than an closed complex, is stabilized by the formation of the first phosphodiester bonds. The formation of these first phosphodiester bonds, which requires ATP, allows the expansion of the melted region of DNA to $+8$ (Holstege et al., 1996; Holstege et al., 1997), also ATP dependent (Wang et al., 1992a). After formation of three phosphodiester bonds in adenovirus major late promoter (AdML) promoter, the open complex is considered stable and no longer ATP dependent (Holstege et al., 1997). The ATP-activated complex can undergo abortive RNA synthesis until pol II has cleared the promoter and becomes an elongation complex.

Once pol II has moved past the first 11 to 16 base pairs, promoter clearance has occurred (Goodrich and Tjian, 1994; Dvir et al., 1997; Holstege et al., 1997). In the case of the AdML promoter, clearance occurs upon addition of the eleventh nucleotide in the transcript. TFIIE and TFIIH have been shown to be important for promoter clearance in initiation and full length transcription assays (Goodrich and Tjian, 1994). Preinitiation polymerases lack phosphorylation (Lu et al., 1991; Laybourn and Dahmus, 1989), but once transcription has begun, the CTD repeats are phosphorylated. Phosphorylation of the CTD requires ATP possibly utilized by the kinase activity of the cdk7 subunit or helicase activity of the ERCC3 subunit of TFIIH (Jiang et al., 1996; Dvir et al., 1997). The phosphorylation of the CTD, although not required for transcription, may help stalled transcription complexes continue (Jiang et al., 1996; O'Brien et al., 1994). In addition, the hyperphosphorylated CTD may play a role in promoter clearance, the shift from an initiation complex to an elongation complex. The phosphorylation of the CTD may allow the release of GTFs involved in transcription and make room for the association of factors
involved in elongation. Experiments involving RNA chain terminator 3'-O'-methyl-GTP or kinase inhibitor N-(2-[methylamino]ethyl)-5-isoquinolinesulfonamide (H8) have shown complexes with a phosphorylated CTD elongate more efficiently (Lee and Greenleaf, 1997; Dvir et al., 1997).

Once pol II clears the promoter, the GTFs are released except TFIIF. TFIIF stimulates the rate of elongation. Additional elongation factors, such as TFIIIS, associate with pol II. TFIIIS helps alleviate stalled complexes by stimulating a pol II nuclease activity that cleaves dinucleotides from the transcript allowing realignment of pol II's active site with the 3' end of the transcript (Izban and Luse, 1992b; Izban and Luse, 1993; Bengal et al., 1991; Cipres-Palacin and Kane, 1994).

**Adenovirus major late promoter**

The adenovirus major late (AdML) promoter serves as one of the two originally proposed model promoters in this research along with mouse mammary tumor virus (MMTV), but now serves as the only model due to circumstances discussed later. Adenoviruses infect the respiratory and urinary tracts, eye, lymphocytes, and entcrocytes of mammals, birds, and amphibians. The virus is transmitted through direct contact, aerosols, or oral ingestion. The adenovirus contains a linear, double stranded DNA molecule of 40kbp. A human adenovirus known to cause sarcoma provided a model for viral oncogenesis. In the later stages of viral infection, the AdML promoter drives late transcription of the viral DNA encoding virion capsid proteins (Horowitz, 1990).

The AdML promoter serves as the model promoter in in vitro studies of eukaryotic transcription with pol II (Horowitz, 1990). AdML is one of the strongest basal promoters known. In contrast, the MMTV promoter is very weak. The AdML promoter has three sequence elements (Fig. 2) that serve as binding sites during transcription. The first element, an upstream stimulatory factor (USF) binding site, is located at -64 to -52. Downstream of the USF binding site at -33 to -22 exists a TATA box, and finally at -1 to +5, an Inr element (Berk, 1986; Smale and Baltimore, 1989).
AdML promoter

Fig. 2. The AdML promoter has a USF binding site, a TATA box, and an initiator element (Inr).
USF, a heterodimer of 43 kDa and 44 kDa subunits, serves as an activator during transcription. Under reducing conditions affecting key cysteine residues, each subunit bound as a homodimer can activate transcription (Pognonec et al., 1992; Pognonec et al., 1991). In vitro activation occurs via two different domains located in residues 15 to 59 and 93 to 156 (Kirschbaum et al., 1992). However, USF activation in highly purified systems is significantly reduced, but can be restored with the presence of TAFs (Kokubo, 1993). It has been shown that mutations of the USF site in the AdML promoter decrease the stability of ATP-activated transcription complexes (Kopytck, 1997).

The TATA element serves as a binding site for the TBP subunit of TFIID. The Inr element assists the TATA box in specific transcriptional initiation and may regulate the strength of the promoter (O'Shea-Greenfield and Smale, 1992). Many different proteins can bind the Inr in AdML, such as USF, TFII-I (120 kDa), and cap binding factor (Du et al., 1993; Garfinkel et al., 1990; Roy et al., 1991). TFII-I may also bind in association with other proteins at the USF site (Roy et al., 1991).

**Objectives**

Determine the effect of the activator USF on AdML promoter clearance. This involves: 1) designing wild type and USF deleted AdML promoter-containing templates with G-free cassettes at +12 to +36 of the template (promoter clearance occurs after +11), 2) perform controls to determine if promoter clearance assay will work, and 3) perform time courses for promoter clearance assay.
MATERIALS AND METHODS

All DNA restriction and modification enzymes came from Promega or Gibco BRL. The ADPROCLR promoter came from Life Technologies.

Plasmids

Two G-free templates, pwtPrCl and p∆USFPrCl, containing AdML sequences were constructed as follows. Plasmid pBMLA-12 (AdML wild type), a G-free template, contains AdML sequences from -65 to +10 and specifies a transcript of 144 nt. Plasmid pUSFMLA (lacks USF site), a G-free template derived from pBMLA-12, contains AdML promoter sequences from -41 to +10, and produces a 144 nt transcript. Plasmids pwtPrCl and p∆USFPrCl were derived from pBMLA-12 and pUSFMLA respectively through PCR amplification of the promoter and G-free cassette. The first primer, a reverse sequencing primer (5’-AACAGCTATGACCATG-3’) hybridized upstream of the promoter in a region of the vector plasmid possessing a Hind III site. The second primer (ADPROCLR) contains a 3’ overhang with a Kpn I restriction site (5’-CGGGGTACCGTATAGATGGAGGAAGGGTATTGGGCGGAAGAGAGT-3’) that hybridizes within the G-free cassette, with the exception of intended mismatches, at sequences +1 to +36, resulting in the generation of a G-free cassette from +12 to +36. The PCR products were isolated from a 4% acrylamide gel, ethanol precipitated, resuspended in 50 μl of distilled water and Multicore buffer, and digested with both Hind III and Kpn I. After running part of the digested product on a 4% acrylamide gel to check for the presence of a band of correct molecular weight; the product was phenol extracted, ethanol precipitated, and resuspended in 10 μl of distilled water. Also, pUC 19 was digested with Hind III and Kpn I and checked for linearization on a 1% low-melt agarose gel. All of the insert was added to ~100 ng of large vector fragment and ligated at 37°C using T4 ligase for 60 minutes. Then the plasmids were transformed into HB101 competent cells. The templates were sequenced to assure they were as intended using the dideoxy method. After
determining the sequence was correct, large scale plasmid purification (using alkali lysis) was used to obtain templates for transcription reactions.

**In vitro transcription reactions**

Nuclear extract (45 µg of protein) was incubated on ice for 15 minutes in TM0.1 buffer (50 mM Tris-HCl (pH 7.9 at room temperature), 1 mM EDTA, 12.5 mM MgCl₂, 20% glycerol, and 100 mM KCl) and 5 mM DTT. The DTT prevents loss of the nuclear extract's activity during storage at -80°C. Transcription complexes were assembled in batch with about 1 µg of template per reaction. Assembly occurred for 60 minutes at 30°C. Nucleotides and DEPC-treated water were added to make the final volume of the reactions 35 µL. The final concentrations for the reagents were 300 µM ATP, 300 µM UTP, 1 µM CTP, 1 µM GTP (when used), 0 µM – 1 mM 3'-O-methyl GTP (Pharmacia), and 50 µM (10 µCi) [α-³²P]CTP (New England Nuclear). RNA synthesis occurred for 30 minutes at 30°C. The reactions were stopped with 90 mM EDTA (removes Mg²⁺) and treated with 0.2 µl of RNase T1 (BRL) at 37°C for 15 minutes. Then, the reaction was treated with 2 µL of proteinase K and 350 µl of stop buffer solution. Stop buffer consists of 50 mM Tris-HCl (pH 7.5), 1% SDS, 5mM EDTA, and 25 µg/ml tRNA (Sigma). The reactions were extracted with 385 µL of chloropane. Chloropane is a 1:1 mixture of phenol and chloroform with a salt solution consisting of 100 mM Tris-HCl (pH7.5), 10mM sodium acetate, 100 mM NaCl, and 1mM EDTA. Finally the reactions were ethanol precipitated and resuspended in transcription dye (0.1% xylene cyanol, 0.1% bromophenol blue). The RNA products were examined on 20% polyacrylamide gels in the presence of 8M urea.

**Synthesis of RNA markers and recovery control**

RNA markers and recovery control were produced using the SP6 promoter of pGEM (Promega). The markers were synthesized in separate reactions using pGEM linearized with Hind III, Hinc II, Sal I, Xba I, and Bam HI, resulting in marker sizes of 11, 24, 26, 32, and 38-mer respectively. In the reaction 2 µg of linear template was added to
transcription buffer containing 40 mM Tris-HCl (pH 7.5), 6 mM MgCl$_2$, 2 mM spermidine, 10 mM NaCl, and 10 mM DTT supplemented with 2.5 mM ATP, GTP, UTP, 0.25 mM CTP, 1 U/ml RNasin (Promega), and 10 μCi [$\alpha$-$^{32}$P]CTP. To synthesize the markers 20 U of SP6 RNA polymerase (BRL/Gibco) was used at 37°C for 60 minutes. Reactions were terminated with the addition of 350 μl of stop solution, chloropane extracted, ethanol precipitated, and resuspended in 100 μl of DEPC-treated water. The 11-mer, 24-mer, and 32-mer were selected as the best markers upon analysis. The 11-mer contained an additional three bands, which probably differ by one base pair.

The recovery control was performed in much the same way with the following exceptions. The pGEM was linearized with Pvu II, which cleaves 98 base pairs downstream from the transcription start site. SP6 RNA polymerase reactions were performed using 1 μg of template and 10 U of the SP6 polymerase. Dilutions of the final product were made to determine what amount is best to serve as a recovery control.
RESULTS

Controls for promoter clearance assay

In theory the promoter clearance assay is very simple (Fig. 3). At the time, t, when 3'-O-methyl GTP is added if pol II has not cleared the promoter (transcribed beyond +11) the chain terminator will be incorporated at +11 terminating transcription resulting in the present of an 11-mer. If at time t pol II has cleared the promoter (transcribed beyond +11), pol II will be able to complete transcription through the entire G-free cassette. Upon treatment with T1 a 26-mer will be generated by all transcription complexes that had cleared the promoter at the time of 3'-O-methyl GTP addition. Based on this premise the concentration of the 26-mer at the various time points, t, can be used to determine the rate of promoter clearance. After determining the rates for the plasmids, pwtPrC1 and pAUUSFPrC1, it can be determined if USF affects the rate of promoter clearance.

Currently the controls for the promoter clearance assay are in progress. The controls answer many questions necessary to prove this assay will work since it has never been attempted before. First, does the template work. Second, what concentration of 3'-O-methyl GTP terminates transcription in a 100% effective way. Third, what background interference will be observed from the vector since it does contain many G-free regions. To answer these questions a series of in vitro transcription reactions were conducted with the method discussed in the MATERIALS AND METHODS.

The reactions were performed on pUC 18 (which is the same as pUC 19 but in an opposite orientation) and pwtPrC1. The stopped reactions were all treated with RNase T1. One of the reactions was lacking GTP thus it should result in an 11-mer while another reaction lacked 3'-O-methyl GTP that should result in a 26-mer. The results were as follows. In pwtPrC1 reactions a promoter dependent band was observed (not observed in pUC 18) that decreased with increased concentrations of 3'-O-methyl GTP (Fig. 4, lanes 10-14). However at the time there was a lack of markers to distinguish the size of the band, although the band is probably the 26-mer. Any band the size of an 11-mer was
Fig. 3. Outcomes of promoter clearance assay.
Fig. 4. Control for promoter clearance assay using both pwtPrCl and pUC 18, treated with RNase T1. Lanes 1-7 and 8-14 used pwtPrCl and PUC 18. Lanes 1, 2, 8, and 9 lacked GTP, but had 500 μM (lanes 1 and 8) and 1000 μM (lanes 2 and 9). Reactions in lanes 3-7 and 10-14 each had GTP (1 μM) and increasing [3'-O-Me-GTP], 0 μM-1000 μM.
impossible to distinguish. The amount of bands observed in the pUC decreased substantially with increased concentrations of 3'-O-methyl GTP.

The second attempt at the controls was performed in the same manner with the following modifications. The CTP concentration in the reactions was reduced to 0.4 μM. In addition, a series of reactions were performed with pwtPrClI that lacked T1 treatment. In pwtPrClI reactions lacking T1 if the 3'-O-methyl GTP concentration is high enough only an 11-mer should be observed. In the reaction that lacks 3'-O-methyl GTP there should be no distinguishable bands due to many different bands of various sizes. In the reactions lacking GTP only 11-mers should be distinguishable. The results of the reactions involving T1 treatment were the same as in the first attempt. However it was observed in the reactions lacking T1 treatment at the higher concentrations of 3'-O-methyl GTP a 37-mer was observed (Fig. 5, lanes 1, 2, 6, and 7) meaning the reactions did not terminate at +11 as expected, but terminated at +37. So termination was more efficient at +37 then +11. A possible explanation for the read through at +11 was misincorporation of an NTP other than GTP. At +38 of the template strand another location for GTP or 3'-O-methyl GTP to be incorporated exist which possibly lead to more efficient termination at the end of the G-free cassette.

The third attempt at the controls was quite different from the others. Reactions were performed on both pUC 18 and pwtPrClI with and without T1 treatment. The CTP concentration in the reactions was 0.5 μM. The concentration in the reactions of both ATP and UTP were reduced to 100 μM (to reduce the chance of misincorporation) and GTP reduced to 0.5 μM. A concentration of 2 mM 3'-O-methyl GTP was used in reactions except for those lacking it. The actual elongating period of the transcription reactions was reduced to 5 minutes to give the polymerase less time to read through the G-free cassette. Three reaction conditions existed: 1) no GTP, 3'-O-methyl GTP present; 2) GTP, no 3'-O-methyl GTP; and 3) both GTP and 3'-O-methyl GTP present. In this experiment markers were employed of the following size 11-mer, 24-mer, and 32-mer. This experiment was
Fig. 5. Control for promoter clearance assay using pwtPrClI not treated with RNase T1. Reactions in lanes 1 and 2 lack GTP, but have 3'-O'Me-GTP at 500 µM and 1000 µM respectively. Reactions in lanes 3-7 have GTP (1 µM) and increasing [3'-O'Me'GTP], 0 µM-1000 µM.
not nearly as clean as the other experiments, but some observations were possible. First, a 37-mer was present in a T1 treated reaction involving pwtPrCl that had GTP, but did not have 3\'-O-methyl GTP (Fig. 6, lane 5). This supports a misincorporation, because a 37-mer should not be present. Second, a 37-mer was present in a reaction involving pwtPrCl, not treated with T1, that lacked GTP, but had 3\'-O-methyl GTP supporting that termination at +11 is not efficient or misincorporation (Fig. 6, lane 1).
Fig. 6. Control for promoter clearance assay using both pwtPrCl and pUC 18. Lanes 1-6 and 7-12 used pwtPrCl and pUC 18 respectively. Lane 13 has no plasmid. Lanes 4-6 and 10-12 were treated with RNase T1. Lanes 1, 4, 7, and 10, reactions lacked GTP, but had 2 mM 3'-O-Me-GTP. Lanes 2, 5, 8, and 11, reactions had 0.5 μM GTP, but lacked 3'-O-Me-GTP. Lanes 3, 6, 9, 12, and 13, reactions had 0.5 μM and 2 mM 3'-O-Me-GTP. Lanes 14 and 15 are the 32 and 24-mer RNA markers respectively.
DISCUSSION

There are more control experiments to be performed in an attempt to show that the promoter clearance assay is feasible. However, the early indications have shown possible misincorporation or a low efficiency at terminating at +11 during the controls. In attempts to combat misincorporation the concentration of the NTPs could be reduced even more, but the difficulty of getting transcription increases. If read-through at +11 still occurs after stretching the reactions conditions as much as possible that probably indicates a low efficiency of termination at +11. Also, read-through at +11 may not be promoter or pol II dependent, which can be determined by using α-amanitin in some reactions. As a result the templates will have to be redesigned to be able to incorporate another GTP or 3’-O-methyl GTP at +12. Of course this could possibly result in a doublet of a 26 and 25-mer. In hindsight this probably would have been a better design to begin with for the templates. Also, another option that may be considered as a further control is to design a TATA mutant with the G-free cassette in the appropriate place to show that the presence of the 26-mer is promoter dependent.

Controls so far have shown the presence of promoter specific transcripts distinguishable from the background. In addition, 3’-O-methyl GTP at high concentrations terminates transcription almost completely. However, it is still not quite clear if it is efficient enough for the promoter clearance assay.

Assuming the controls work proving that the promoter clearance assay is feasible. The next step will be running a time course using the promoter clearance assay for plasmids, pwtPrCl and pΔUSFPrCl. This differs from the previous transcription reactions because the 3’-O-methyl GTP is added at a time, t, after the transcription reaction began. The series of times chosen for the time courses will constitute a very short period of time. It is highly likely that a stop-flow machine will be needed to conduct the reactions. The use of the stop-flow machine may change the amount of reactants needed.
If USF is found to have an effect on the rate of promoter clearance, there are plans to study it further. For example, templates with heteroduplexes at the start site will be used to help discern the transcription step the activator, USF, affects. Also, if these experiments work in determining the effect of activators on promoter clearance the promoter clearance assay can be applicable to determine the effect different activators have on specific promoters concerning promoter clearance.

As a side note many attempts over a six-month period were made to find a MMTV transcript that would transcribe so experiments, including the promoter clearance assay, could be conducted. However despite these efforts a working template was never found. Possibly the frozen stock of the plasmids was contaminated with something such as RNase. Another option is the nuclear extract was not as good as past nuclear extracts making it difficult to use MMTV promoter-containing templates since it is a very weak promoter. MMTV possesses binding sites for both repressors and activators. MMTV as a weak promoter would have provided a nice contrast to AdML in the experiments.
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