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FUNCTIONAL DISSECTION OF HUMAN RAP74 IN TRANSCRIPTIONAL INITIATION, ELONGATION, AND RECYCLING

presented by

Lei Lei

has been accepted towards fulfillment
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FUNCTIONAL DISSECTION OF HUMAN RAP74 IN TRANSCRIPTIONAL INITIATION, ELONGATION, AND RECYCLING

By

Lei Lei

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ABSTRACT

FUNCTIONAL DISSECTION OF HUMAN RAP74 IN TRANSCRIPTIONAL INITIATION, ELONGATION, AND RECYCLING

By

Lei Lei

Transcription factor IIF (TFIIF) cooperates with RNA polymerase II (pol II) during multiple stages of the transcription cycle. Human TFIIF is an $\alpha_2\beta_2$ heterotetramer of RNA polymerase II-associating protein 74- and 30-kDa subunits (RAP74 and RAP30). The primary sequence indicates that RAP74 may comprise separate N- and C-terminal domains connected by a flexible loop. Functional data obtained with RAP74 deletion mutants strongly support this model for RAP74 architecture and further show that the N- and C-terminal domains and the central loop of RAP74 have distinct roles during separate phases of the transcription cycle. The N-terminal domain of RAP74 (minimally amino acid 1-172) is sufficient to deliver pol II into a complex formed on the adenovirus major late promoter with the TATA binding protein, TFIIB, and RAP30. A more complete N-terminal domain fragment (amino acid 1-217) strongly stimulates both initiation and elongation by pol II. The region of RAP74 between aa 136 and 217 is critical for both initiation and elongation, and mutations in this region have similar effects on initiation and elongation. Based on these observations, RAP74 appears to have a similar function in initiation and elongation. The central region and the C-terminal domain of RAP74 do not contribute strongly to single-round initiation or elongation stimulation but do stimulate multiple-round transcription in an extract system.

The region of RAP74 between aa 136 and 217 is highly conserved among divergent organisms including yeast, *Drosophila*, *Xenopus*, and human. Because this region is critical for both initiation and elongation, a comprehensive set of point mutations was introduced and mutant proteins were analyzed in transcription assays. Consistent with the analysis of deletion mutants, point mutations in RAP74 also affect both initiation and elongation very similarly, strongly supporting the notion that RAP74 plays an identical role in both initiation and elongation. Several amino acid residues such as L155, W164, N172, I176, and M177 appear to be most critical for the function of RAP74 in initiation and elongation. The lower activities of RAP74 mutants are not due to the inability to bind the transcription complex; in fact, even the most defective mutants have a similar affinity as wild type RAP74 for both the preinitiation complex and the elongation complex. Recent photocrosslinking experiments demonstrate that an adenovirus virus major late promoter template wraps tightly around pol II in a complex containing TBP, TFIIB, TFIIE, TFIIF, and TFIIH. RAP74 is critical for formation of the wrapped structure and the activities of RAP74 mutants in initiation, elongation, and DNA wrapping are very similar. This suggests that RAP74 may induce DNA wrapping in both the preinitiation complex and the elongation complex to facilitate DNA helix untwisting, which is necessary for both initiation and rapid elongation.

TFIIF is the only general transcription factor previously shown to stimulate both initiation and elongation by pol II. Using a sensitive elongation assay, it is demonstrated that TBP, TFIIB, TFIIA, and TFIIE stimulate the elongation activity of TFIIF, possibly by recruiting TFIIF into the elongation complex.

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Finally, I thank my parents and my wife for their unconditional love, understanding, and support.

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aa

AdMLP

AMS

ATP

bp

BSA

CKII

CTD

CTP

Da

DNA

DRB

DPE

DTT

EDTA

EGTA

FPLC

GTP

HAT

HCA

HDA

HEPES

LIST OF ABBREVIATIONS

aa	amino acid(s)
AdMLP	adenovirus major late promoter
AMS	ammonium sulfate
ATP	adenosine triphosphate
bp	base pair(s)
BSA	bovine serum albumin
CKII	casein kinase II
CTD	carboxyl terminal domain of the largest subunit of RNA polymerase II
CTP	cytidine triphosphate
Da	Dalton
DNA	deoxyribonucleic acid
DRB	5,6-dichlorobenzimidazole riboside
DPE	downstream promoter element
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
EGTA	ethyleneglycol-bis-(β-aminoethyl ether) N, N, N', N'-tetraacetic acid
FPLC	fast protein liquid chromatography
GTP	guanosine triphosphate
HAT	histone acetyltransferase
HCA	hydrophobic cluster analysis
HDA	histone deacetylase
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)

lar

kb

kDa

mRN

NMP

nt

NTP

PAG

PCR

PBS

pol I

pol II

pol III

RAP

RNA

rRNA

sarko

SDS

TAF_{II}

TATA

TBP

TFII

tRNA

Inr	Initiator element
kb	kilobase pairs
kDa	kilodaltons
mRNA	messenger RNA
NMR	nuclear magnetic resonance
nt	nucleotide(s)
NTP	nucleoside triphosphate
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PBS	phosphate buffered saline
pol I	RNA polymerase I
pol II	RNA polymerase II
pol III	RNA polymerase III
RAP	RNA polymerase II associating protein
RNA	ribonucleic acid
rRNA	ribosomal RNA
sarkosyl	N-lauroylsarcosine sodium salt
SDS	sodium dodecyl sulfate
TAF_{II}	TBP associated factor of RNA polymerase II transcription
TATA	TATA element or TATA box
TBP	TATA box-binding protein
TFII	transcription factor of RNA polymerase II
tRNA	transfer RNA

UTP

Amino Acid C

Alanine

Arginine

Asparagine

Aspartic Acid

Cysteine

Glutamic Acid

Glutamine

Glycine

Histidine

Isoleucine

Leucine

Lysine

Methionine

Phenylalanin

Proline

Serine

Threonine

Tryptophan

Tyrosine

Valine

UTP	Uridine triphosphate

Amino Acid Codes

Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Cysteine	Cys	C
Glutamic Acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

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CHAPTER 1

TRANSCRIPTION BY EUKARYOTIC RNA POLYMERASE II

Transcription of nuclear genes in eukaryotes is carried out by three different DNA-dependent RNA polymerases (reviewed in Young, 1991). RNA polymerase I (pol I) synthesizes the ribosomal RNA (rRNA); RNA polymerase II (pol II) synthesizes the messenger RNA (mRNA) and some small nuclear RNA (snRNA); and RNA polymerase III (pol III) synthesizes the 5S rRNA, transfer RNA (tRNA), and some snRNA. Each of these three eukaryotic RNA polymerases is composed of 8-14 polypeptides. All three RNA polymerases require the assistance of accessory transcription factors for specific transcription initiation. The complexity of the eukaryotic RNA polymerases and their transcription factors likely reflects the need for elaborate controls on transcription in eukaryotes.

When transcribing mRNA-encoding genes, pol II alone is not sufficient for specific initiation (Roeder, 1976; Weil et al., 1979). An additional set of protein factors is required for accurate initiation from a promoter DNA sequence in vitro (Matsui et al., 1980). These accessory factors are termed GTFs (general transcription factors) and include TBP (TATA-binding protein) or TFIID, TFIIA, TFIIB, TFIIF, TFIIE and TFIIH ("TF" for transcription factor, and "II" for RNA polymerase II) (reviewed in Conaway and Conaway, 1993; Zawel and Reinberg, 1993; Roeder, 1996; Orphanides et al., 1996; Hampsey, 1998). The entire set of GTFs is composed of about 30 polypeptides and most of the corresponding cDNAs have been isolated. Similar factors have been identified in human, yeast, rat and *Drosophila* systems. These GTFs along with pol II are often

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referred to as the general transcriptional machinery to emphasize their importance in transcription.

Although pol II and the GTFs are sufficient for accurate initiation of transcription from naked DNA templates in vitro, this transcription is neither dependent on nor responsive to the presence of transcriptional activators and is therefore known as "basal" transcription. Transcription in an eukaryotic cell, however, is a tightly regulated process. The packaging of DNA into chromatin results in global gene repression (reviewed in Paranjape et al., 1994). Moreover, sequence-specific DNA binding proteins regulate the transcription in a gene-specific manner (Mitchell and Tjian, 1989). Transcriptional repressors bind to specific DNA sequences (repressor-binding sites) and inhibit the expression of target genes, while transcriptional activators bind to specific DNA sequences (activator-binding sites) and induce the expression of target genes. Cofactors, which bridge the interaction between a DNA-bound regulator and the general transcriptional machinery, sometimes are also required for gene activation or repression. The overall outcome of gene induction is therefore a combined result of "anti-repression" and "true activation" (Roeder, 1991; Orphanides et al., 1996). Although the "basal" transcription may not occur in vivo, the components of the general transcriptional machinery and the process of basal transcription are nevertheless the ultimate targets of transcriptional regulators. Studies of the basic mechanisms of transcription and the general machinery not only improve our understanding of the fundamental concepts of gene expression, but also provide us with the framework necessary to understand how regulators work.

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In this chapter I will review the basics of mRNA transcription by pol II, including the components of general transcriptional machinery and the process of basal transcription. A brief summary of transcriptional activation and repression will also be given. At the end, an overview will be given to reemphasize the basic function of TFIIF as a guide to the following chapters.

GENERAL TRANSCRIPTIONAL MACHINERY

Promoter Architectures

The promoter of an mRNA-encoding gene can be divided into core elements and regulatory elements (reviewed in Hampsey, 1998). The core elements define the site for assembly of the preinitiation complex (PIC) and contribute to both the strength and selectivity of a promoter. The core elements include a TATA sequence (TATA), an initiator sequence (Inr), and a downstream promoter element (DPE). A promoter can have a TATA box, an Inr sequence, or both a TATA and an Inr. DPE appears to function in conjunction with the Inr element as a TFIID binding site at some TATA-less promoters. Regulatory elements, generally located outside the core promoter, are gene-specific sequences that serve as binding sites for transcriptional activators or repressors which regulate the level of transcription.

TATA

TATA elements are located about 30 base pairs (bp) upstream of the transcription start site in most eukaryotes except in yeast *Saccharomyces cerevisiae* where TATA elements are typically 40 to 120 bp upstream of the start site (Hampsey, 1998). The

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consensus sequence for TATA elements is TATAAA (Singer et al., 1990; Wobbe and Struhl, 1990). Many derivatives of this sequence also confer TATA function, although with reduced activity. The TATA sequence serves as the binding site for the TATA binding protein (TBP). The TBP-TATA association nucleates the assembly of the PIC. The binding of TBP is a step that can be rate limiting for initiation both in vitro and in vivo (Hoopes et al., 1992; Klein and Struhl, 1994).

Promoters lacking canonical TATA elements (TATA-less promoters) have also been identified, especially in many housekeeping genes such as the terminal deoxynucleotidyl transferase (TdT) gene and the dihydrofolate reductase (DHFR) gene (Weis and Reinberg, 1992). Transcription from TATA-less promoters remains TBP-dependent, although the rate-limiting step in PIC assembly at TATA-less promoters is unlikely to be TBP recruitment. Other components of the general machinery, such as the TAF_{II}s (TBP-associated factors in TFIID), may recognize promoter elements other than TATA to nucleate the PIC assembly on the TATA-less promoters.

Inr

Initiator elements are DNA sequences encompassing the transcription start site. The Inr was first identified at the TATA-less promoter of the mammalian TdT gene and subsequently found at many promoters, either TATA-containing or TATA-less (Weis and Reinberg, 1992). It now appears that most promoters contain an Inr, although the nucleotide sequence of this element is not highly conserved. Proteins that bind Inr elements include TFII-I, YY1, CIF150, E2F, USF, as well as pol II itself (reviewed in Smale, 1997; Weis and Reinberg, 1992). Genetic evidence in yeast indicated that TFIIB

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may also interact with the Inr in determining the start site (Pinto et al., 1992; Pinto et al., 1994). TAF_{II}s are also implicated in recognizing the Inr to function as promoter selective factors. Among these Inr-binding proteins, CIF150 is a homolog of *Drosophila* $TAF_{II}150$ ($dTAF_{II}150$), which binds promoter DNA overlapping the Inr region (Kaufmann et al., 1996; Kaufmann et al., 1998; Verrijzer and Tjian, 1996). A recombinant TBP-$TAF_{II}150$-$TAF_{II}250$ subcomplex is minimally required for efficient utilization of Inr and the downstream promoter element (Verrijzer et al., 1994; Verrijzer et al., 1995). A human homolog of $dTAF_{II}150$, named human $TAF_{II}150$ ($hTAF_{II}150$), was recently identified as a bona fide TFIID subunit and found to be homologous to CIF150 (Martinez et al., 1998). Therefore, the function of recognizing the Inr by TAF_{II} subunits is conserved. It appears that additional factors are essential for transcription from TATA-less promoters (Martinez et al., 1998). The functions and mechanisms of other Inr-binding proteins remain to be further determined. It is possible that different Inr-binding proteins are utilized at different promoters.

DPE

The downstream promoter element (DPE) was initially identified in *Drosophila* and is located about 30 bp downstream of the start site (Burke and Kadonaga, 1996; Burke and Kadonaga, 1997). The DPE works in conjunction with the Inr element as a TFIID binding site at some TATA-less promoters. The histone-like TAF_{II}s including $dTAF_{II}60$ and $dTAF_{II}$ 40 interact specifically with the DPE. It is interesting to note that some TAF_{II}s also contact the DNA about 30 bp downstream of the start site on the TATA-containing adenovirus major late promoter (Verrijzer et al., 1995; Oelgeschlager

et al., 1996).

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et al., 1996). One explanation is that the PIC generally contacts DNA from upstream of the TATA sequence all the way to about 30 bp downstream of the start site. In doing so, the promoter DNA is fully wrapped around the polymerase and general transcription factors (Robert et al., 1998).

RNA POLYMERASE II

Bacterial RNA polymerase consists of an $\alpha_2\beta\beta'$ core enzyme and a σ promoter specificity factor. All three eukaryotic nuclear RNA polymerases are also multi-subunit enzymes. Pol II is generally composed of 10-12 subunits; for example, yeast pol II consists of 12 polypeptides encoded by the *RPB1* to *RPB12* genes (Young, 1991). There is extensive structural conservation among the pol II subunits from diverse organisms. For example, six subunits of human pol II can functionally replace their homologs in yeast (McKune et al., 1995). Eukaryotic RNA polymerases consist of common as well as class-specific subunits. Five subunits, Rpb5, Rpb6, and Rpb8, Rpb10, and Rpb12, are essential components of all three eukaryotic RNA polymerases. The Rpb1, Rpb2, Rpb3, and Rpb11 subunits of pol II are homologous to subunits of pol I and pol III. Only Rpb4, Rpb7, and Rpb9 are unique to pol II. Among the genes encoding yeast pol II subunits, only *RPB4* and *RPB9* are not esential for cell viability.

The three largest pol II subunits are related to the bacterial core RNA polymerase subunits and are largely responsible for RNA catalysis (Young, 1991). The largest pol II subunits, Rpb1, is homologous to the β' subunit of bacterial RNA polymerase. The second largest pol II subunit, Rpb2, is homologous to the β subunit of bacterial RNA polymerase. Rpb3 is somewhat related to the α subunit of bacterial RNA polymerase.

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No subunit of pol II appears to be closely related to the bacterial σ subunit. The bacterial polymerase active site is made up of a modular arrangement of β' and β subunits, suggesting that Rpb1 and Rpb2 may constitute the active site of pol II (Mustaev et al., 1997; Severinov et al., 1996; Zaychikov et al., 1996; Markovtsov et al., 1996). Rpb1 and β' are involved in template DNA binding, while Rpb2 and β bind nucleotide substrates.

Yeast genetics provide some clues regarding the in vivo functions of individual pol II subunits. Different mutations in RPB1 and RPB2 have been found to affect either the start site selection or transcriptional elongation, demonstrating that both Rpb1 and Rpb2 are important in both initiation and elongation (Berroteran et al., 1994 Hekmatpanah and Young, 1991; Archambault et al., 1992; Powell and Reines, 1996). Rpb3 is involved in pol II assembly, a similar function as the bacterial α subunit (Young, 1991; Kolodziej and Young, 1991; Kolodziej and Young, 1989; Kolodziej et al., 1990). Rpb9 is implicated in defining the start site, perhaps through interaction with TFIIB (Hull et al., 1995; Sun et al., 1996). Both Rpb9 and TFIIB have a zinc ribbon motif which may be involved in start site selection. Biochemical analyses indicate that Rpb4 and Rpb7 are loosely associated with pol II and a form of yeast pol II without Rpb4 and Rpb7 is active in elongation in vitro (Edwards et al., 1991). However, both subunits are required for accurate initiation in vitro. The functions of other pol II subunits remain unclear.

The protein envelope structure of various RNA polymerases has been determined by two-dimensional crystallography of microcrystalline arrays (Polyakov et al., 1995; Darst et al., 1989; Asturias et al., 1997; Darst et al., 1991). Prominent features of these structures are finger-like projections in the RNA polymerase that close to form a channel large enough to accommodate the DNA template. For *E. coli* RNA polymerase, this

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channel is open in the initiating holoenzyme and closed in the elongating enzyme. This suggests that the DNA penetrates through the channel, which closes around the template during elongation to prevent termination (Polyakov et al., 1995; Darst et al., 1989). The structure of an RNA polymerase can be described as a "hand" with a "palm", "thumb", and "finger". The DNA template is suggested to run across the palm and through the channel, grasped by the thumb and finger. The active site for RNA synthesis is postulated to be close to the channel between the thumb and the finger. The yeast RNA polymerase II can display the thumb and finger either apart or together, depending on the presence of pol II subunits Rpb4 and Rpb7 and TFIIE in the complex (Asturias et al., 1997; Darst et al., 1991). Photocrosslinking experiments suggest that the DNA template is wrapped around the polymerase and general transcription factors in the pol II preinitiation complex (Robert et al., 1998; Forget et al., 1997; Kim et al., 1997). The DNA wrapping is proposed to play an important role in the formation of open complex during initiation and the advance of the transcription "bubble" during elongation (Lei et al., 1998; Robert et al., 1998).

CTD

A unique feature of pol II is the presence of tandem repeats of a heptapeptide sequence at the carboxy-terminus of its largest subunit (reviewed in Young, 1991; Dahmus, 1996). The carboxy-terminal repeat domain (CTD) has a consensus sequence YSPTSPS and is highly conserved among eukaryotic organisms. The repeat length appears to increase with increasing genome complexity, for example, there are 26 or 27

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repeats in yeast pol II, depending on the strain, and 52 repeats in human pol II (Young, 1991).

The CTD is highly phosphorylated on the two SP serines in vivo. It can also be phosphorylated on the tyrosine residues (Dahmus, 1994; Dahmus, 1995; Dahmus, 1996). Therefore, because of the repetitive sequences, about 100 to 150 potential phosphorylation sites are present within the CTD of human pol II. There are at least two forms of RNA pol II in vivo, designated pol IIO, which is hyperphosphorylated at the CTD, and pol IIA, which is hypophosphorylated. The pol IIA form preferentially enters the PIC, whereas pol IIO is found in the elongating complex (Dahmus, 1996). Conversion of pol IIA to pol IIO occurs concomitant or shortly after the transition from initiation to elongation (Lu et al., 1991; O. Brien et al., 1994). The CTD can be phosphorylated by many kinases in vitro, including TFIIH (Feaver et al., 1991; Lu et al., 1992; Serizawa et al., 1992), P-TEFb (Marshall et al., 1996), Srb10/Srb11 (Liao et al., 1995), Cdc2 (Cisek and Corden, 1989), and Ctk1 (Lee and Greenleaf, 1991). It remains to be determined whether these CTD kinases are gene specific or if they affect different steps during the transcription cycle. A phosphatase activity specific for dephosphorylation of the CTD has also been identified (Chambers and Dahmus, 1994). This CTD phosphatase activity is regulated by TFIIF and TFIIB (Chambers et al., 1995). The RAP74 subunit of TFIIF stimulates CTD phosphatase activity while TFIIB inhibits this stimulatory effect of TFIIF. Because pol IIA preferentially enters the PIC, the CTD phosphatase, TFIIF, and TFIIB appear to interact to regulate pol II recycling (Lei et al., 1998).

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Although the CTD is essential for cell viability, its function in transcription is not entirely clear. RNA pol II lacking the CTD is able to initiate transcription from TATA-containing promoters in vitro, although not from TATA-less promoters such as the DHFR promoter (Akoulitchev et al., 1995; Buermeyer et al., 1995). Moreover, the activation of many yeast genes is independent of the TFIIH kinase in vivo (Lee and Lis, 1998). This may suggest a functional redundancy among various CTD kinases.

Besides its functions in transcription, the CTD has also been implicated in pre-mRNA processing such as splicing, 5' capping and 3'-end formation (Greenleaf, 1993; McCracken et al., 1997a; McCracken et al., 1997b; Yue et al., 1997). This suggests that in eukaryotes the mRNA transcription and processing steps may be coupled through the CTD (Steinmetz, 1997).

Holoenzymes

RNA pol II and the GTFs can assemble in a defined order on promoter DNA in vitro, suggesting stepwise assembly of the PIC (Van Dyke et al., 1988; Buratowski et al., 1989; reviewed in Zawel and Reinberg, 1993). However, this model has been challenged by the identification of RNA polymerase II holoenzymes from yeast and mammalian cells. The yeast pol II holoenzyme contains all the subunits of pol II (core pol II), an SRB/mediator complex, and a subset of GTFs (Koleske and Young, 1994; Kim et al., 1994). Unlike the core pol II, the holoenzyme supports activated transcription when supplemented with only TBP (instead of TFIID) and the remaining GTFs in reconstituted transcription systems. This property is attributed to the SRB/mediator complex that contains coactivators that physically link activators to the basal transcription machinery.

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This form of pol II holoenzyme is often referred to as the SRB/mediator-containing holoenzyme. Another distinct form of yeast pol II holoenzyme has also been discovered and it appears to be less abundant and regulates only a subset of genes (Shi et al., 1997; Wade et al., 1996; Shi et al., 1996). Several forms of mammalian pol II holoenzymes have also been isolated through either immunoprecipitation or affinity chromatography (Chao et al., 1996; Maldonado et al., 1996b; Ossipow et al., 1995; Scully et al., 1997; Pan et al., 1997). These holoenzymes differ in their composition but all respond to transcriptional activators. The identification of different pol II holoenzymes from both yeast and human suggests that the assembly of a pol II holoenzyme may be a dynamic and complex process and different forms of pol II holoenzymes may coexist inside cells.

GENERAL TRANSCRIPTION FACTORS

The GTFs include TFIID, IIA, IIB, IIF, IIE and IIH and were identified initially from human HeLa cell extracts as protein factors necessary for accurate transcription initiation by the purified RNA pol II from double-stranded DNA templates in vitro (Orphanides et al., 1996; Roeder, 1996; Hampsey, 1998; Conaway and Conaway, 1993; Conaway and Conaway, 1997). The compositions and major properties of human GTFs are summarized in Table 1. Similar factors were also identified in yeast, *Drosophila* and rat cells, and the GTFs isolated from divergent organisms are highly conserved.

Table 1. Human general transcription factors

Factors	Subunits	Characteristics
TFIID	TBP	Binds TATA element, contacts TFIIB and TFIIA, nucleates PIC assembly; required for pol I, pol II, and pol III transcription
	$TAF_{II}250$	Scaffold for TFIID assembly, binds TBP and inhibits TBP-TATA interaction; cell cycle progression (G1/S), serine kinase (RAP74), HAT
	$TAF_{II}150$, CIF150	Contacts Inr
	$TAF_{II}135$	Binds Sp1
	$TAF_{II}100/95$	WD-40 repeats
	$TAF_{II}80/70$	Histone H4 similarity, Drosophila homolog ($dTAF_{II}60$) contacts DPE
	$TAF_{II}55$	
	$TAF_{II}32/31$	Histone H3 similarity, Drosophila homolog ($dTAF_{II}40$) contacts DPE
	$TAF_{II}30$	Binds estrogen receptor
	$TAF_{II}28$	Histone fold
	$TAF_{II}20/15$	Histone H2B similarity
	$TAF_{II}18$	Histone fold
TFIIA	TFIIAα, TFIIAβ, TFIIAγ	Both α and β are derived from one gene, likely posttranslationally processed; binds TBP and stabilizes TBP-TATA interaction
TFIIB	TFIIB	Binds TBP and stabilizes TBP-TATA interaction, recruits pol II/TFIIF, start site selection; zinc ribbon, cyclin repeats, conformational change
TFIIF	RAP30	σ homology, cryptic DNA-binding domain (helix-turn-helix); pol II delivery, DNA wrapping, initiation and elongation
	RAP74	Pol II delivery, DNA wrapping, initiation, elongation, and reinitiation; interacts with TFIIB, stimulates CTD phosphorylation; phosphorylated in vivo, can be phosphorylated in vitro by CKII, $TAF_{II}250$, and TFIIH kinases
TFIIE	TFIIEα, TFIIEβ	Recruits TFIIH and stimulates TFIIH activities (DNA helicases and CTD kinase); promoter melting, DNA wrapping
TFIIH	ERCC3, XPB	3'-5' helicase, required for both transcription and NER
	ERCC2, XPD	5'-3' helicase, required for NER but not transcription
	p62	NER
	p52	NER
	hSSL1	NER, zinc-binding
	cdk7, MO15	CTD kinase, component of CAK
	cyclin H	cdk7 partner, component of CAK
	MAT-1	Zinc-binding, CAK assembly factor
	p34	Zinc-binding

PIC - preinitiation complex; HAT - histone acetyltransferase; Inr - initiator element; DPE - downstream promoter element; CTD – the C-terminal domain of RNA polymerase II; CKII - casein kinase II; NER - nucleotide excision repair; CAK - cdk7-activating kinase (cdk7, cyclin H, and MAT-1)

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TFIID

TFIID is a multisubunit complex consisting of the TATA binding protein (TBP) and TBP-associated-factors (TAF_{II}s) (reviewed in Hernandez, 1993; Burley and Roeder, 1996). The binding of TBP or TFIID to a promoter is the first step in assembling an initiation complex (Nakajima et al., 1988; Zawel and Reinberg, 1993). This step is rate-limiting on many promoters both in vitro and in vivo and many regulators appear to affect the TBP-TATA interaction.

TBP

TBP is a universal transcription factor, required for initiation by all three eukaryotic RNA polymerases (Cormack and Struhl, 1992; Hernandez, 1993; White and Jackson, 1992). In each case, TBP is associated with a set of other polypeptides. Besides being a subunit of TFIID, TBP was also identified as an essential component of pol I transcription factor SL1 and pol III transcription factor TFIIIB (Comai et al., 1992; Kassavetis et al., 1992; Lobo et al., 1992; Taggart et al., 1992). TBP also associates with SNAPc, a protein complex required for transcription of certain snRNA genes by pol II or pol III (Hernandez, 1993).

TBP has been isolated from various eukaryotic organisms and it ranges in molecular weight from 22 kDa (*Arabidopsis*) to 38 kDa (human and *Drosophila*) (reviewed in Burley and Roeder, 1996). TBP can be divided into two structural domains, a highly conserved C-terminal domain and a more divergent N-terminal domain. The conserved C-terminal domain of TBP (TBPc) consists of two imperfect direct repeats. TBPc is sufficient to bind the TATA sequence and direct specific transcription initiation

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in vitro when supplemented with the remaining GTFs and pol II (Hoey et al., 1990; Horikoshi et al., 1990; Peterson et al., 1990; Zhou et al., 1993). TBPc is also sufficient for formation of the complete TFIID complex (Zhou et al., 1993). Although the N-terminal domain is divergent, it is conserved among vertebrate forms of TBP and regulates pol III transcription at the U6 promoter (Mittal and Hernandez, 1997).

The crystallographic structures of TBP and TBP-TATA complexes revealed a novel DNA binding fold, resembling a molecular "saddle" that sits astride the DNA (Nikolov et al., 1992; Kim et al., 1993; Kim et al., 1993; Nikolov et al., 1996). The DNA binding surface is a concave antiparallel β-sheet and the convex seat of the saddle consisting of four α-helices is available for interaction with other factors. Unlike many other DNA-binding proteins, TBP recognizes the minor groove of the 6-bp TATA element. Upon binding, TBP induces kinks at both ends of the TATA element and bends the DNA about 80^0 toward the major groove. The bending causes a severe change in the path of the DNA and increases the proximity of proteins bound on either side of TBP, such as TFIIA and TFIIB. This could initiate wrapping of promoter DNA around pol II and GTFs. The wrapping of DNA is suggested to be important for both transcriptional initiation and elongation (Robert et al., 1998; Lei et al., 1998).

TAF_{II}s

In addition to TBP, TFIID contains 8 to 11 additional TBP-associated factors (TAF_{II}s) ranging in size from 15 to 250 kDa (Goodrich and Tjian, 1994a; Burley and Roeder, 1996; Hampsey, 1998). TAF_{II}s have been identified from human, *Drosophila*, and yeast, and there is significant conservation among most TAF_{II} subunits isolated from

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different organisms. Human $TAF_{II}250$ ($hTAF_{II}250$) and its counterparts, *Drosophila* $TAF_{II}230$ ($dTAF_{II}230$) and yeast $TAF_{II}145$ ($yTAF_{II}145$, also known as $yTAF_{II}130$), bind TBP and each is believed to be a scaffold for the assembly of other $TAF_{II}s$ into TFIID complexes. $hTAF_{II}250$ is a bipartite protein kinase with specificity for the RAP74 subunit of TFIIF (Dikstein et al., 1996). $hTAF_{II}250$ is also a histone acetyltransferase (HAT) (Mizzen et al., 1996). Both the kinase domains and the HAT domain of $hTAF_{II}250$ are conserved in $dTAF_{II}230$, but only the HAT domain is conserved in $yTAF_{II}145$.

Earlier studies suggested that TFIID has a weaker affinity than TBP for binding the TATA element (Nakatani et al., 1990; Aso et al., 1994). Studies with a cell-free system lacking $TAF_{II}s$ revealed that $TAF_{II}s$ impair functional PIC formation (Oelgeschlager et al., 1998). Investigations using partially reconstituted TFIID complexes showed that $hTAF_{II}250$ represses the basal transcription activity of TBP (Verrijzer et al., 1995; Guermah et al., 1998). It was shown that $hTAF_{II}250$, $dTAF_{II}230$, and $yTAF_{II}145$ each inhibit TBP binding to the TATA box in vitro (Kokubo et al., 1993; Kokubo et al., 1998; Nishikawa et al., 1997). Mutagenic analyses indicated that $dTAF_{II}230$ binds both the convex side and the concave DNA binding surface of TBP (Kokubo et al., 1994; Nishikawa et al., 1997). Transcriptional activator VP16 binds competitively with $dTAF_{II}230$ to the DNA-binding domain of TBP (Nishikawa et al., 1997). The negative regulation of transcription within the TFIID multisubunit complex was further supported by the three-dimensional structure of a TBP-$dTAF_{II}230$ complex. This NMR structure of a protein complex consisting of the core domain of yeast TBP (amino acids 49-240) and the N-terminal region of $dTAF_{II}230$ (amino acids 11-77)

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confirmed that the N-terminal fragment of dTAF$_{II}$230 interacts directly and tightly with the concave DNA binding surface of TBP (Liu et al., 1998). This region of dTAF$_{II}$230, upon interacting with TBP, undergoes a conformational change and mimics the partially unwound structure of the TATA DNA sequence. It is unlikely that both the N-terminal region of dTAF$_{II}$230 and the TATA element can occupy the same DNA-binding surface of TBP. These evidences suggest that, prior to the TBP-TATA interaction, a conformational change within the TFIID complex must occur to release the DNA binding surface of TBP from interacting with other TAF$_{II}$ subunits including dTAF$_{II}$230. Transcriptional activators, such as VP16, may facilitate this process.

Biochemical and crystallographic analyses indicate that a histone octamer-like structure may exist within TFIID (Hoffmann et al., 1997; Hoffmann et al., 1996). Histone H3-, H4-, and H2B-like TAF$_{II}$s have been identified in human, *Drosophila*, and yeast TFIID complexes (Burley and Roeder, 1996; Xie et al., 1996; Nakatani et al., 1996). In the case of human TFIID, a putative hTAF$_{II}$ histone octamer-like structure would contain a heterodimer of hTAF$_{II}$31 (histone H3-like) and hTAF$_{II}$80 (H4-like) sandwiched between two hTAF$_{II}$20 (H2B-like) homodimers. No H2A-like TAF$_{II}$ has been identified. This octamer-like structure may wrap DNA on the surface of TFIID. This would explain the extended DNase I footprint and photocrosslinking of TFIID compared with those of TBP on many promoters (Verrijzer et al., 1995; Oelgeschlager et al., 1996; Burke and Kadonaga, 1996). However, it was recently discovered that the promoter DNA in the preinitiation complex (PIC) containing pol II, TBP, IIA, IIB, IIE, IIF and IIH is wrapped in a similar fashion as that in the DNA-TFIID complex (Robert et al., 1998). Because these structures occupy the same core promoter region, they can not

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both be present simultaneously. TAF_{II}s must move within the TFIID-DNA complex to accommodate assembly of the PIC. It is intriguing to note that no formation of a protein-DNA complex containing TFIID, the remaining GTFs, and pol II has been reported.

TAF_{II}s appear to play multiple roles in transcription. First, TAF_{II}s can function as promoter selectivity factors (Smale et al., 1990; Pugh and Tjian, 1991; Zhou et al., 1992; Martinez et al., 1994; Verrijzer and Tjian, 1996). TAF_{II}s are important in recognizing core promoter elements including both the Inr and DPE (Kaufmann and Smale, 1994; Purnell et al., 1994; Verrijzer et al., 1994; Verrijzer et al., 1995; Hansen and Tjian, 1995; Burke and Kadonaga, 1996; Burke and Kadonaga, 1997). Promoter hybrid experiments with conditional TAF_{II} mutants in yeast demonstrate that the requirement for $TAF_{II}145$ maps to core promoter elements (Shen and Green, 1997). Second, TAF_{II}s can function as coactivators (Verrijzer and Tjian, 1996). TAF_{II}s were previously shown to be generally required for activator-dependent transcriptional stimulation in human and *Drosophila* systems in vitro (Pugh and Tjian, 1990; Dynlacht et al., 1991; Tanese et al., 1991; Chen et al., 1994; Jacq et al., 1994; Chiang and Roeder, 1995). This paradigm was challenged by the observations that yeast TAF_{II}s are only required for transcriptional activation of a subset of genes in vivo (Apone et al., 1996; Moqtaderi et al., 1996; Walker et al., 1996). The TAF_{II} function at these promoters is associated with core promoter elements instead of elements bound by activators (Apone et al., 1996; Moqtaderi et al., 1996; Walker et al., 1997). Furthermore, the yeast SRB/mediator-containing pol II holoenzyme is capable of responding to activators in the absence of TAF_{II}s in vitro (Koleske and Young, 1994; Kim et al., 1994). It was recently reported that transcriptional activation can occur independent of TAF_{II}s in a highly purified in vitro system, although this TBP-mediated

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activation can be stimulated by the addition of TAF_{II}s (Wu et al., 1998). The earlier observations that TAF_{II}s were necessary for activation are attributed to the presence of negative factors in those systems. The TAF_{II}-independent activation was also observed using a HeLa nuclear extract depleted of the major TAF_{II}s by immunoprecipitation (Oelgeschlager et al., 1998). It was shown that the TAF_{II}-independent activation occurs predominantly at the level of productive preinitiation complex assembly. The addition of TAF_{II}s actually inhibits the formation of PIC. Human homologs of SRB proteins are implicated in mediating the transcriptional activation in the absence of TAF_{II}s (Oelgeschlager et al., 1998). Third, TAF_{II}s can function as repressors in a reconstituted system in the absence of activators (Verrijzer et al., 1995; Guermah et al., 1998; Malik et al., 1998). The addition of an activator and a coactivator can relieve this inhibition (Malik et al., 1998). The inhibitory effect of TAF_{II}s could be caused by the TAF_{II}250-TBP interaction which inhibits the binding of TBP to the TATA element (Liu et al., 1998). It could also be caused by the formation of a wrapped TFIID-promoter structure which precludes the formation a functional PIC (Oelgeschlager et al., 1996; Robert et al., 1998). Transcriptional activators and coactivators may relieve the inhibitory effects of TAF_{II}s by removing $hTAF_{II}$250 from TBP to facilitate the TBP-TATA interaction and displacing the extensive contacts between TAF_{II}s and the core promoter.

TFIIA

TFIIA associates with the PIC through interaction with TBP and stabilizes TBP-TATA binding (Buratowski et al., 1989; Imbalzano et al., 1994). Although initially defined as a GTF, TFIIA is dispensable for accurate initiation in a purified system

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directed by TBP (Matsui et al., 1980; Reinberg et al., 1987; Sayre et al., 1992; Sun et al., 1994; Yokomori et al., 1994). However, TFIIA plays important roles in transcriptional activation in vitro. First, TFIIA mediates displacement of transcriptional repressors such as Dr1-DRAP1/NC2, PC3/Dr2, HMG1, and Mot1 from the TFIID complex, a process known as "antirepression" (Auble et al., 1994; Ge and Roeder, 1994a; Inostroza et al., 1992; Meisterernst et al., 1991; Merino et al., 1993). Second, TFIIA functions in "true activation" by interacting with specific transcriptional activators and coactivators (Ozer et al., 1994; Yokomori et al., 1994; Yokomori et al., 1993; Ge and Roeder, 1994a; Shykind et al., 1995). TFIIA is required to overcome a rate-limiting step during formation of the open transcription complex (Wang et al., 1992).

cDNAs encoding TFIIA subunits have been isolated from human (DeJong and Roeder, 1993; DeJong et al., 1995; Ma et al., 1993; Ozer et al., 1994; Sun et al., 1994), yeast (Ranish et al., 1992), and Drosophila (Yokomori et al., 1993; Yokomori et al., 1994). Human and *Drosophila* TFIIA consist of three subunits with molecular masses of 37 (α subunit), 19 (β subunit) and 13 kDa (γ subunit). α and β subunits are encoded by a single gene and appear to be produced by a protein processing event. Yeast TFIIA contains two subunits with molecular masses of 32 and 13 kDa, encoded by *TOA1* and *TOA2* genes respectively. The 32 kDa TOA1 gene product is homologous to the human α subunit at its amino terminus and the human β subunit at its carboxyl terminus. The 13 kDa TOA2 product is homologous to the human γ subunit.

Crystal structures of yeast TFIIA-TBP-TATA complexes have been solved (Geiger et al., 1996; Tan et al., 1996). The TOA1 and TOA2 polypeptides interact extensively in the complexes. The carboxyl termini of TOA1 and TOA2 form a compact

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β-sheet structure termed β sandwich or β barrel. The amino termini of TOA1 and TOA2 form a four-helix bundle. TFIIA interacts with the amino-terminal region of core TBP and with DNA upstream of the TATA element. The β-sandwich domain of TFIIA interacts with both TBP and DNA and stabilizes TBP binding to the TATA element. The four-helix bundle domain of TFIIA is positioned to interact with other proteins such as transcriptional activators. The crystal structure provides insight on the function and mechanism of TFIIA. It was found that the β and γ subunit of human TFIIA can function in antirepression, whereas the α subunit is additionally required for activation (Ma et al., 1996). From the yeast TFIIA structure and the sequence similarity between yeast and human TFIIA subunits, it is possible that the β and γ subunits of human TFIIA fold into the β-sandwich structure that binds TBP and DNA, while the α subunit is required to form the four-helix bundle domain that binds transcriptional activators.

TFIIB

Human TFIIB exists as a single polypeptide of 35 kDa (Ha et al., 1991). TFIIB has also been identified in *Drosophila* (Wampler and Kadonaga, 1992; Yamashita et al., 1992), yeast (Pinto et al., 1992), and archaebacteria (Ouzounis and Sander, 1992; Qureshi et al., 1995). TFIIB enters the PIC after TBP binding to the TATA element and recruits RNA pol II and TFIIF (Buratowski et al., 1989). Consistent with this role, TFIIB interacts directly with the TBP-DNA complex, pol II, and TFIIF (Buratowski et al., 1989; Maldonado et al., 1990; Ha et al., 1993; Fang and Burton, 1996). TFIIB is involved in start site selection in yeast (Pinto et al., 1992; Li et al., 1994).

TFIIB contains a zinc-ribbon motif at its amino-terminus and two imperfect direct repeats which form the carboxy-terminal core (Zhu et al., 1996; Barberis et al., 1993; Malik et al., 1993). The carboxy-terminal core of TFIIB (TFIIBc) is capable of binding the TBP-TATA complex, but is deficient in transcription, likely due to its inability to support efficient recruitment of the pol II/TFIIF complex (Barberis et al., 1993; Buratowski and Zhou, 1993; Ha et al., 1993; Yamashita et al., 1993). The three-dimensional structures of TFIIBc and the TFIIBc-TBP-DNA complex have been solved (Bagby et al., 1995; Nikolov et al., 1995). Each repeat of TFIIBc consists of five α-helices, which form a compact globular domain with structural similarity to cyclins involved in cell cycle control (Gibson et al., 1994; Jeffrey et al., 1995). TFIIBc binds underneath and on one side of the TBP-DNA complex and it interacts with both TBP and DNA, as suggested by footprinting and photocrosslinking experiments (Lee and Hahn, 1995; Lagrange et al., 1996). The basic amino-terminal repeat of TFIIB contacts the acidic carboxy-terminal "stirrup" of TBP. The bending of the TATA element by TBP allows TFIIB to interact with the phosphodiester backbone of DNA both upstream and downstream of the TATA sequence. Therefore, similar to TFIIA, TFIIB stabilizes the TBP-TATA interaction through both protein-protein contacts and protein-DNA contacts. Recent experiments demonstrate that TFIIB is a sequence-specific DNA-binding protein that recognizes a novel sequence element found in certain promoters (Lagrange et al., 1998). TFIIB and TFIIA bind to different sides of the TBP-DNA complex. Although missing from the crystal structure, the N-terminal zinc-ribbon domain is speculated to bind DNA in the vicinity of the transcription start site and may stabilize the melting of the promoter.

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TFIIF

Human TFIIF is a heterotetrameric factor consisting of RAP30 (26 kDa) and RAP74 (58 kDa) subunits. RAP is an acronym for an RNA polymerase II-associating protein. The subunits of TFIIF were originally identified on the basis of their affinity for immobilized pol II and shown to be essential for pol II transcription (Sopta et al., 1985; Burton et al., 1988). TFIIF was later purified independently as an indispensable pol II initiation factor (Flores et al., 1988; Flores et al., 1989; Flores et al., 1990). TFIIF was also known in human cells as factor FC (Kitajima et al., 1990), in rat cells as factor βγ (Conaway and Conaway, 1991), in Drosophila cells as factor 5 (Price et al., 1989), and in yeast cells as factor g (Henry et al., 1992). cDNAs encoding TFIIF subunits have been isolated from human (Sopta et al., 1989; Finkelstein et al., 1992; Aso et al., 1992), *Drosophila* (Kephart et al., 1993; Frank et al., 1995; Gong et al., 1995), *Xenopus* (Gong et al., 1992), and yeast (Henry et al., 1994; Sun and Hampsey, 1995). TFIIF in higher eukaryotes consists of two subunits, homologous to human RAP74 and RAP30 respectively. Yeast TFIIF has three subunits, the two larger ones (Tfg1 and Tfg2) are homologous to RAP74 and RAP30 respectively, while the third subunit (Tfg3) is also a component of yeast TFIID and SWI/SNF complexes. Unlike *TFG1* and *TFG2*, the *TFG3* gene is not essential for yeast cell viability and Tfg3 protein is not required for transcription in vitro (Henry et al., 1992; Henry et al., 1994). Two human leukemogenic proteins, AF-9 and ENL, share considerable sequence similarity with Tfg3 (Cairns et al., 1996). Tfg1 (also known as Ssu71) is implicated in the start site selection in yeast (Sun and Hampsey, 1995).

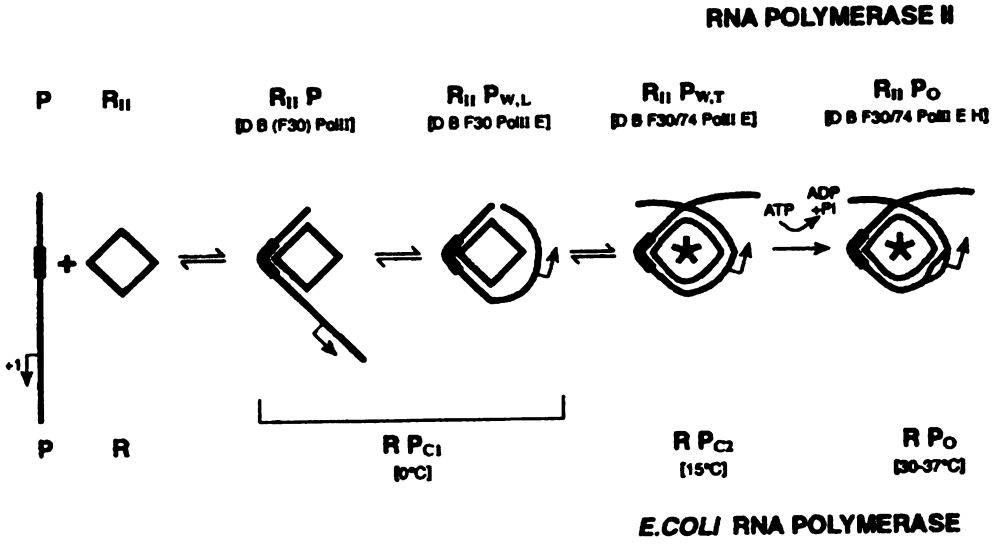


Figure 1. A model for the wrapping of the promoter DNA around RNA polymerase during formation of the preinitiation complex. This model can account for the results obtained for both *E. coli* RNA polymerase and RNA polymerase II. The asterisks indicate isomerization of the complex. Abbreviations: P, promoter; R, *E. coli* RNA polymerase; R_{II}, RNA polymerase II; D, TBP; B, TFIIB; F30, RAP30; F30/74, RAP30/74; E, TFIIE; H, TFIIH; RP_{C1}, closed complex I; RP_{C2}, closed complex II; RP_O, open complex; $R_{II}P_{W,L}$, loosely wrapped complex; $R_{II}P_{W,T}$, tightly wrapped complex.

Primary sequence analysis indicates that RAP30 contains two distinct regions with sequence similarity to bacterial σ factors (Sopta et al., 1989; Garrett et al., 1992). One of the σ-homology regions in RAP30 is thought to interact with pol II, and TFIIF binds to the same surface on *E. coli* RNA polymerase as σ^{70} does (McCracken and Greenblatt, 1991). Moreover, RAP30 has a crytic DNA-binding domain at its carboxyl terminus, a feature also shared by bacterial σ^{70} (Tan et al., 1994). The NMR structure of the C-terminal region of human RAP30 displays a helix-turn-helix fold, similar to the linker histone H5 and the hepatocyte nuclear transcription factor HNF-3/*fork head* (Groft et al., 1998). The N-terminal region of RAP30 binds RAP74 and TFIIB (Fang and Burton, 1996).

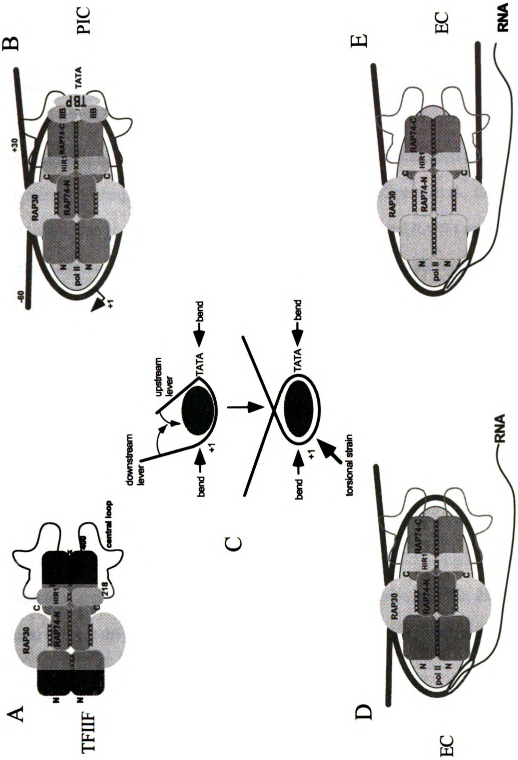
The RAP74 protein can be divided into three regions based on sequence analysis: the N-terminal globular domain, the C-terminal globular domain, and a flexible central region (Wang and Burton, 1995; Lei et al., 1998). No three-dimensional structure of RAP74 has been reported to date. The N-terminal domain of RAP74 binds RAP30 while the C-terminal domain binds TFIIB and pol II (Wang and Burton, 1995; Yonaha et al., 1993). The C-terminal domain of RAP74 also stimulates a CTD phosphatase activity that removes phosphate groups from the CTD of the largest subunit of pol II (Chambers et al., 1995). This stimulation by RAP74 can be blocked by TFIIB, indicating a dynamic interaction between TFIIF, TFIIB, and CTD phosphatase.

One important role of TFIIF is to deliver pol II to the preinitiation complex (Conaway et al., 1991; Flores et al., 1991; Killeen et al., 1992). Pol II alone cannot stably

associate with the TBP-TFIIB-DNA complex (DB complex), and must be escorted to the promoter by TFIIF to form the TBP-TFIIB-pol II-TFIIF-DNA (DBPolF) complex. RAP30 alone in some cases is sufficient for delivering pol II to the DB complex, however, RAP74 is stimulatory and enhances the stability of the DBPolF complex (Killeen et al., 1992; Flores et al., 1991; Tyree et al., 1993; Tan et al., 1994). In another study, both RAP30 and RAP74 were found to be necessary for the formation of a DBPolF complex (Lei et al., 1998; Chapter 2). The regions of RAP30 and RAP74 that are critical for the interaction between these two polypeptides are both required for forming the DBPolF complex.

TFIIF plays a key role in transcriptional initiation after PIC assembly. Using photocrosslinking approaches, the locations of pol II and GTFs on the adenovirus major late promoter (AdMLP) were mapped (Coulombe et al., 1994; Forget et al., 1997; Robert et al., 1996; Robert et al., 1998). Surprisingly, TFIIF induces a conformational change of pol II and wraps the promoter DNA around pol II and TFIIF (Forget et al., 1997; Robert et al., 1998; see Fig. 1). Both RAP30 and RAP74 are required for forming the wrapped complex. Analyses of RAP74 deletion mutants demonstrated that the activities of RAP74 fragments in wrapping the DNA correlate very well with their activities in transcriptional initiation and elongation (Lei et al., 1998; Robert et al., 1998). The DNA wrapping is also an important feature in prokaryotic transcription and is critical for the formation of a transcription-competent "open" complex (Schickor et al., 1990; Travers, 1990; Craig et al., 1995; Polyakov et al., 1995). In the pol II preinitiation complex, the DNA is first bent at the TATA box by TBP and likely bent again near the transcription start site due to the wrapping of promoter DNA around pol II. It is postulated that the

Figure 2. Proposed functions of TFIIF in transcriptional initiation and elongation. A) Domain structure of RAP74 in the TFIIF complex. The RAP74 N-terminal domain is drawn as three connected dark gray segments. Rows of Xs represent contacts between subunits. HIR1 and HIR2 are proposed to include RAP74 dimerization domains. B) Preinitiation complex (PIC). TBP and the C-terminal repeats of TFIIB (IIB) are shown interacting with the TATA box. C) Isomerization of the PIC. D and E) Elongation complexes (EC).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bending and wrapping cause a superhelical torsion on the promoter and destabilize the two DNA strands near the start site. This strand unwinding then allows the access of TFIIH helicase to separate the template strands in the presence of ATP to form an open complex.

In addition to its role in initiation, TFIIF also stimulates the elongation rate of RNA polymerase II (Bengal et al., 1991; Izban and Luse, 1992a; Kephart et al., 1994; Price et al., 1989; Tan et al., 1994; Lei et al., 1998). Both RAP30 and RAP74 are required for this stimulation (Lei et al., 1998; Tan et al., 1994). It was previously proposed that TFIIF stimulates the elongation rate by suppressing transient pausing of the RNA polymerase (Bengal et al., 1991; Price et al., 1989; Izban and Luse, 1992a). Recent data demonstrate that TFIIF does not affect the position of pausing sites but decreases the dwell time of pol II at many sites (Chapter 3). Because the same region of RAP74 is required for the activities of TFIIF in DNA wrapping, initiation, and elongation, it is suggested that the DNA template is also wrapped around pol II and TFIIF during elongation to maintain the open complex and therefore enhance the rate of RNA synthesis (Lei et al., 1998; Robert et al., 1998; Fig. 2). In the elongation complex, the DNA template may either be fully wrapped (Fig. 2D) or partially wrapped (Fig. 2E) but the torsional strain at the position of phosphodiester bond formation is maintained in both cases.

TFIIF is also involved in the recycling of pol II (Lei et al., 1998). TFIIF stimulates a CTD phosphatase activity which converts pol IIO, the elongation form of pol II, to pol IIA, the initiation form (Chambers et al., 1995). The C-terminal domain of

RAP74 is sufficient for this stimulation. It was recently demonstrated that the C-terminal domain and the central region of RAP74 are necessary for continuous RNA initiation in an extract system, possibly by stimulating the CTD phosphatase activity to regenerate pol IIA (Lei et al., 1998; Chapter 4).

RAP74 is highly phosphorylated in vivo, consistent with the presence of many putative phosphorylation sites in its central region (Sopta et al., 1985; Burton et al., 1988; Flores et al., 1989; Finkelstein et al., 1992). Many kinases including $TAF_{II}250$ (Dikstein et al., 1996) and TFIIH (Malik et al., 1998) can phosphorylate RAP74 in vitro. Both the initiation and elongation activities of TFIIF are moderately stimulated by the phosphorylation of RAP74 (Kitajima et al., 1994). However, the phosphorylation of RAP74 is not required for either initiation or elongation because recombinant RAP30 and RAP74 produced in *E. coli* are active in a highly purified system which do not contain either TFIIH or TAF_{II}s (Malik et al., 1998; Chapter 5).

TFIIE

TFIIE enters the PIC after pol II and IIF and recruits TFIIH in vitro (Buratowski et al., 1989; Flores et al., 1989; Flores et al., 1992; Maxon et al., 1994). Consistent with this role, TFIIE interacts directly with pol II, TFIIF, and TFIIH (Flores et al., 1989; Maxon and Tjian, 1994). The cDNAs encoding TFIIE proteins have been cloned from human (Ohkuma et al., 1991; Peterson et al., 1991; Sumimoto et al., 1991) and yeast (Feaver et al., 1994). Similar to TFIIF, Human TFIIE is a heterotetramer of 56 kDa (IIEα) and 34kDa (IIEβ) subunits (Ohkuma et al., 1990; Inostroza et al., 1991). Yeast TFIIE may exist as an heterodimer (Leuther et al., 1996). TFIIEα has a zinc-ribbon motif

and yeast TFIIE binds single-stranded DNA, which suggests that TFIIE may participate to form or stabilize the melted DNA in the initiator region (Kuldell and Buratowski, 1997). The functions of TFIIE include recruitment of TFIIH to the PIC, stimulation of the CTD kinase activity of TFIIH, and stimulation of the ATP-dependent DNA helicase activity of TFIIH (Lu et al., 1992; Ohkuma and Roeder, 1994; Ohkuma et al., 1995). Additionally, TFIIE contributes to DNA wrapping within the PIC (Robert et al., 1996; Robert et al., 1998). Two-dimensional crystallography of a TFIIE-pol II complex suggests that TFIIE promotes a conformational switch at the active center upon pol II-DNA interaction (Leuther et al., 1996). TFIIE has also been implicated as the direct target of some transcriptional regulators (Sauer et al., 1995; Zhu and Kuziora, 1996).

TFIIH

The entry of TFIIH completes the assembly of a preinitiation complex (Flores et al., 1992). TFIIH was identified from human, rat, and yeast cells (Conaway and Conaway, 1989; Flores et al., 1992; Gerard et al., 1991). TFIIH is a multisubunit complex and contains several enzymatic activities including a CTD kinase and two ATP-dependent DNA helicases (reviewed in Drapkin and Reinberg, 1994; Orphanides et al., 1996).

TFIIH is required for transcriptional initiation from linear DNA in vitro. TFIIH has a critical role in forming the open transcription complex (Dvir et al., 1996; Holstege et al., 1996). One of the two DNA helicases in TFIIH, namely ERCC3 in human and Rad25 in yeast, is essential for transcriptional initiation and believed to be necessary for promoter melting to form the "open" complex (Park et al., 1992; Feaver et al., 1993;

Guzder et al., 1994a; Guzder et al., 1994b; van Vuuren et al., 1994). The requirement for TFIIE, TFIIH, and ATP can be bypassed when transcribing from the DNA template either with a high negative superhelical density, which favors DNA unwinding, or with an artificially premelted region at the start site (Parvin and Sharp, 1993; Tyree et al., 1993; Holstege et al., 1995; Pan and Greenblatt, 1994). These experiments directly link TFIIE, TFIIH, and ATP to promoter melting.

TFIIH is also important for the transition form initiation to elongation, likely mediated by the CTD kinase activity of TFIIH. Presumably, phosphorylation of the CTD causes a conformational change in the PIC, disrupting the interaction between the CTD and TBP and leading to promoter clearance (Usheva et al., 1992; Goodrich and Tjian, 1994). TFIIH also promotes the transition from very early elongation complexes to stable elongation complexes (Dvir et al., 1997). The downstream DNA sequence to position +40 is required for promoter escape, perhaps because DNA is wrapped around TFIIH during this transition.

In addition to its roles in transcription, TFIIH is also implicated in nucleotide excision repair (NER) and cell cycle progression (Drapkin et al., 1994a; Drapkin et al., 1994b; Orphanides et al., 1996). First, five of the nine subunits of TFIIH for which cDNAs have been isolated have dual roles in transcription and NER. It is proposed that TFIIH plays a central role in coupling transcription and DNA repair (Drapkin et al., 1994c; Friedberg, 1996; Sancar, 1996). Second, human THIIH also contain a CAK (cdk-activating kinase) complex consisting of the protein kinase cdk7 (also known as MO15), its cyclin partner cyclin H, and an activating protein MAT-1 (reviewed in Orphanides et al., 1996). The CAK complex is thought to play a pivotal role in cell-cycle regulation by

activating the cyclin-dependent kinases (cdks). The cdk7 kinase is required for transcription from the TATA-less DHFR promoter, perhaps at the stage of promoter clearance (Akoulitchev et al., 1995). However, a direct role for TFIIH in cell-cycle progression remains controversial. TFIIH kinase activity does not vary during the cell cycle, perhaps due to the association of MAT-1 with cdk7 and cyclin H which makes cdk7 constitutively active (Adamczewski et al., 1996). Moreover, the CAK complex is not tightly associated with other TFIIH subunits and in fact only 20% of the total cellular CAK activity is associated with TFIIH (Drapkin et al., 1996). Furthermore, although the yeast TFIIH kinase Kin28 phosphorylates the CTD and is required for transcription, it does not have CAK activity in vitro (Feaver et al., 1994; Cismowski et al., 1995). Rather, the yeast CAK activity resides in a single polypeptide which does not phosphorylate the CTD and is not a component of TFIIH (Kaldis et al., 1996; Kaldis et al., 1998). The yeast CAK and human CAK appear to have different substrate specificities (Kaldis et al., 1998).

TRANSCRIPTIONAL INITIATION

Transcriptional initiation by pol II proceeds through four stages: PIC assembly, open complex formation, abortive initiation, and promoter escape or promoter clearance (Zawel and Reinberg, 1995). Unlike *E. coli* RNA polymerase, eukaryotic pol I or pol III, the initiation of transcription by pol II is dependent on energy, requiring hydrolysis of the β-γ phosphoanhydride bond of ATP (Bunick et al., 1982; Lofquist et al., 1993). The ATP hydrolysis presumably supports the helicase activity of TFIIH to form the open complex (Holstege et al., 1996).

PIC Assembly

The assembly of PIC is a prerequisite for transcriptional initiation to occur. Two models, the step-wise assembly model and the holoenzyme model, have been proposed.

Previous in vitro experiments have led to a step-wise model for the assembly of a PIC: 1) recognition of the TATA element by TBP; 2) binding of the TBP-promoter complex by TFIIB; 3) recruitment of pol II and TFIIF; 4) recruitment of TFIIE; and 5) recruitment of TFIIH by TFIIE to complete the PIC formation (Zawel and Reinberg, 1993). Although TFIIA is not required for forming a PIC, TFIIA can enter the complex after the binding of TBP and incorporation of TFIIA stabilizes the complex. This sequential assembly model implies that transcriptional regulators can affect the assembly process by affecting any single step or multiple steps through interacting with one or more general factors (Choy and Green, 1993). Indeed, in vitro interactions between activators and TBP, TFIIA, TFIIB, TFIIF, TFIIE, and TFIIH have all been reported (reviewed in Triezenberg, 1995).

On TATA-containing promoters, TBP is sufficient to nucleate the formation of a transcription-competent PIC and direct initiation. However, TBP is not sufficient for initiation from TATA-less promoters. TFIID instead of TBP is required for initiation from TATA-less promoters (Weis and Reinberg, 1992; Martinez et al., 1998). Other polypeptides within the TFIID complex (TAF_{II}s) may recognize the Inr and DPE of TATA-less promoters to initiate the preinitiation complex assembly. However, as mentioned earlier, it is unclear whether TAF_{II}s can coexist with pol II and other GTFs in

the transcription complex because TAF_{II}s appear to occupy the same DNA sequence as pol II and the GTFs do.

The step-wise model for assembly of a PIC has been challenged recently by the discovery that a subset of the GTFs exist in a preassembled form in an RNA pol II "holoenzyme", which suggests that most of the initiation machinery can bind to a promoter in a single step (Koleske and Young, 1995). The pol II holoenzymes contain transcriptional coactivators and respond to transcriptional activators when supplemented with TBP and the remaining GTFs. One important implication is that a substantial portion of transcriptional control is mediated directly through the basal transcriptional mechanism and does not require TAF_{II}s. Although the components of the SRB/mediator-containing pol II holoenzyme are well defined both genetically and biochemically, the mammalian holoenzymes are not yet well characterized. Different preparations of mammalian pol II holoenzymes appear to contain very different components, such as DNA repair proteins, splicing and polyadenylation factors, histone acetyltransferases, or even the breast cancer tumor supressor BRCA1 (Maldonado et al., 1996b; McCracken et al., 1997; Scully et al., 1997; Cho et al., 1998). Therefore, two questions need to be addressed: 1) does each mammalian holoenzyme complex reported so far represent a homogenous complex or many heterogenous subcomplexes? and 2) what is the relative abundance of the holoenzyme compared with the free pol II and GTFs inside the cells? Since numerous GTF-GTF interactions and GTF-pol II interactions have been reported, it should not come as a surprise that GTFs and pol II can associate when not bound to promoter DNA (Conaway and Conaway, 1993). One concern of the various affinity chromatography approaches used for isolating pol II holoenzymes is that the immobilized

ligand may serve as a surface to assemble holoenzyme complexes from relatively free components during the process of isolation. More extensive and careful biochemical analyses have to be carried out to firmly establish the presence of distinct mammalian pol II holoenzymes in vivo. My view is that the assembly of pol II holoenzyme complexes inside cells is dynamic and transient, and the assembly process itself may be subject to regulation by transcription factors and signal transduction pathways.

Interestingly, under some circumstances, RNA pol II can accurately initiate transcription in the absence of a full complement of general transcription factors. For instance, initiation can occur in the absence of ATP, TFIIE and TFIIH on supercoiled templates (Parvin and Sharp, 1993; Tyree et al., 1993). Using a superhelical IgH promoter, TFIIF is also dispensable (Parvin and Sharp, 1993). Because negative supercoils facilitate the unwinding of DNA strands, these observations are interpreted as evidences for the involvement of TFIIH, TFIIE, and ATP in promoter melting to form an open complex on linear DNA template. The roles of TFIIE, TFIIH, and ATP hydrolysis in promoter melting were further supported by elegant experiments using an artificially "premelted" promoter as the template. When using a premelted promoter with a short region of mismatched heteroduplex DNA at the start site, the transcription becomes independent of TFIIE, TFIIH, and hydrolyzable ATP (Holstege et al., 1995; Pan and Greenblatt, 1994). These results suggest that the ATP-dependent DNA helicase activity of TFIIH is essential for promoter melting when using linear DNA templates. Besides recruiting TFIIH, TFIIE may play a direct role in promoter melting in the absence of TFIIH (Holstege et al., 1995).

Another minimal transcription system has also been reported. Although it is generally believed that TBP is required for transcription from all eukaryotic promoters in vivo, transcription from the adeno-associated virus (AAV) P5 promoter in vitro can occur independent of TBP (Usheva and Shenk, 1994). In this case, YY1 (an Inr-binding protein), TFIIB, and pol II are sufficient to direct basal transcription from a supercoiled template. YY1 binds within the AAV-P5 Inr and cooperates with TFIIB to recruit pol II to the promoter. TFIIB was recently identified as a sequence-specific DNA binding protein (Lagrange et al., 1998). It will be interesting to test whether the DNA-binding activity of TFIIB is required for transcription in this minimal system.

Although TBP is usually associated with additional polypeptides, such as TAF_{II}s in the TFIID complex, a TBP-free-TAF-containing complex (TFTC) was recently isolated from human cells (Wieczorek et al., 1998). This complex contains many known TAF_{II}s and some unidentified polypeptides but does not have TBP. The most intriguing feature of the TFTC complex is that, when supplemented with pol II, TFIIB, TFIIE, TFIIF, and TFIIH, it can direct specific initiation from several promoters, both TATA-containing and TATA-less. The addition of recombinant TBP only stimulated the level of transcription. Because a TBP-related factor (TRF) has been reported (Hansen et al., 1997), it is important to identify the unknown polypeptides in the TFTC complex and determine if a TBP-like function resides on one of these unidentified polypeptides. The isolation of TFTC and the presence of TAF_{II}s in other protein complexes, such as the mammalian P/CAF complex (Ogryzko et al., 1998) and the yeast SAGA complex (Grant et al., 1998), reemphasize the concept of dynamic interaction among transcription factors. The specific functions of each TAF_{II} in these complexes need to be further determined.

Open Complex Formation

In order to gain access to the nucleotides of the template strand, DNA-dependent RNA polymerases require separation of the two DNA strands prior to initiation of transcription. The DNA strand separation can be detected by modifications of single-stranded DNA with chemical reagents such as $KMnO_4$ that reacts with single-stranded thymidines (Holstege et al., 1996; Holstege et al., 1997; Jiang et al., 1995; Jiang et al., 1996). A prerequisite for pol II promoter opening is the presence of ATP hydrolyzable at the β-γ bond (Wang et al., 1992; Jiang et al., 1993). This is a unique feature of pol II transcription because other DNA-dependent RNA polymerases, including eukaryotic pol I and pol III, and bacterial RNA polymerases, do not require ATP hydrolysis for initiation (Bunick et al., 1982; Lofquist et al., 1993; Eick et al., 1994). Promoter opening at the adenovirus major late promoter requires formation of the complete PIC containing TBP, TFIIB, TFIIF, TFIIE, TFIIH, and pol II (Holstege et al., 1996). The promoter opening occurs in two steps: first, dependent on ATP but prior to initiation, the –9 to +1 region becomes single-stranded; and second, formation of the first phosphodiester bond results in expansion of the open region to the +8 position. These observations lead to the model that the TFIIH-associated DNA helicase activity is crucial for creating a single-stranded region during formation of the open complex. This then gives pol II access to the nucleotides of the template strand and allows expansion of the open region upon formation of the first phosphodiester bond (Holstege et al., 1996). By analyzing the open regions and RNA products of various stalled elongation complexes, it was further shown

that the TFIIH helicase activity is required for maintaining the open region before formation of a four-nucleotide RNA product (Holstege et al., 1997).

TFIIH is the only general transcription factor known to possess ATP-dependent enzymatic activities: a CTD kinase and two DNA helicases (ERCC-2 and ERCC-3) (Feaver et al., 1991; Feaver et al., 1993; Lu et al., 1992; Serizawa et al., 1992; Schaeffer et al., 1993; Schaeffer et al., 1994; reviewed in Drapkin and Reinberg, 1994b). However, the CTD kinase activity of TFIIH is not generally required for initiation from all promoters tested. For example, the CTD kinase activity of TFIIH plays no role in transcription from the TATA-containing AdMLP although it is required for initiation from the TATA-less DHFR promoter (Serizawa et al., 1993; Makela et al., 1995; Akoulitchev et al., 1995). Therefore, the general requirement for β-γ bond hydrolysis of ATP may result from the TFIIH-associated DNA helicase activities for promoter opening. It was recently shown that one of the TFIIH-associated DNA helicases, ERCC-3, is essential for transcription while the ERCC-2 helicase is required for DNA repair (Drapkin and Reinberg, 1994b; Orphanides et al., 1996). Using negative supercoiled templates and premelted templates allowed the requirement for TFIIE, TFIIH and ATP to be bypassed, which further supported the conclusion that TFIIH, TFIIE, and ATP function in promoter opening.

TFIIE is implicated in promoter opening because it recruits TFIIH into the PIC (Flores et al., 1992). In addition to its function as a tethering factor, TFIIE stimulates transcription from negatively supercoiled templates independent of TFIIH (Tyree et al., 1993). This stimulatory effect of TFIIE is increased by conditions that enhance the stability of the DNA duplex and therefore make the DNA more resistant to opening, thus

implicating TFIIE in promoter melting in a direct manner (Holstege et al., 1995). TFIIE binds single-stranded DNA, possibly through the zinc-ribbon motif of TFIIE-α, the large subunit of TFIIE (Kuldell and Buratowski, 1997). TFIIE may participate to form or stabilize the single-stranded DNA in the melted region. Recent photocrosslinking studies indicate that TFIIE stabilizes the wrapping of promoter DNA around pol II and TFIIF in the PIC (Robert et al., 1998; Robert et al., 1996). It is proposed that the bending and wrapping of DNA have a direct consequence for topological untwisting of the helix around the transcription start site (Robert et al., 1998). Because TFIIF is essential for forming the wrapped structure in the PIC, these studies implicate TFIIF in formation of the open complex.

Abortive Initiation

Similar to bacterial RNA polymerases, RNA polymerase II goes through an abortive phase of transcription in which short RNA transcripts are synthesized and released before productive elongation occurs (Luse and Jacob, 1987). The abortive initiation may reflect a conformational change that has to occur to convert the initiation complex to a productive elongation complex. It may serve as a checkpoint for pol II and transcriptional regulators before pol II embarks on elongating a full length RNA transcript.

Promoter Escape or Promoter Clearance

Promoter escape, also called promoter clearance, is the transition from abortive initiation to productive elongation. Many promoters, such as *Drosophila* hsp70 and

mammalian *c-myc*, appear to be regulated at the step of promoter escape in vivo (Lee et al., 1992; O. Brien et al., 1994; Krumm et al., 1995). It is believed that pol II must break some protein-protein contacts and protein-DNA contacts that hold it on the promoter to commence elongation. Presumably, this transition requires a conformational change in pol II and release of some initiation factors. For instance, promoter escape in bacterial systems involves the release of σ factor and significant changes in polymerase comformation and template contacts (Metzger et al., 1989). In the pol II system, it is suggested that the phosphorylation of the pol II CTD contributes to promoter escape. As mentioned earlier, pol II enters the preinitiation complex with an unphosphorylated CTD and the pol II CTD is highly phosphorylated in the elongation complex. Moreover, the phosphorylation of the CTD occurs concomitant or shortly after initiation. The phosphorylation of the CTD may break some protein contacts because the unphosphorylated CTD interacts with several general transcription factors including TBP and TFIIE (Flores et al., 1989; Usheva et al., 1992).

It is not clear which general transcription factors are present in the elongation complex after initiation and promoter escape. Using a template competition approach in a reconstituted system containing pol II, TBP, TFIIB, TFIIF, TFIIE, and TFIIH, it was shown that after initiation, all general transcription factors dissociate from pol II (Zawel et al., 1995). Only TFIIF can reassociate with the elongation complex when pol II encounters pausing sites and suppresses pausing. This implies that TFIIF is the only general factor that functions in both initiation and elongation, and other general factors function only as initiation factors. However, it is unclear which general transcription factors accompany pol II in the elongation complex in vivo. For example, other factors

that normally hold some of the general factors in the elongation complex may be missing in this reconstituted system. It was recently found that recombinant TBP, TFIIA, TFIIB, and TFIIE can stimulate the elongation rate of pol II in cooperation with TFIIF (Chapter 5). These factors may recruit and stabilize TFIIF in the elongation complex through protein-protein contacts and possibly protein-DNA contacts. It is possible that, like in the PIC, these factors function in concert with TFIIF to wrap the DNA around pol II to maintain the transcription bubble during productive elongation (Robert et al., 1998). It remains important to test whether the elongation stimulation by TBP is TATA element-dependent or it can happen when the elongation complex is away from the promoter. It is also important to find out whether any of these general factors does exist in the elongation complex in vivo.

TRANSCRIPTIONAL ELONGATION

For many genes transcribed by pol II, elongation is rate limiting for the production of full-length transcripts (reviewed in Bentley, 1995). Transcriptional control at the level of elongation has been observed in many viral transcriptional units such as the HIV-1 and cellular genes such as *c-myc*, *c-myb*, *c-fos*, and hsp70.

Mechanics of Elongation

Two models have been proposed for explaining the mechanism of transcript elongation: monotonic versus "inchworming" (reviewed in von Hippel, 1998). It was proposed that the elongation complex moves mostly in a monotonic fashion and enters

the "inchworming" cycle only when it encounters specific signals in the DNA template and the nascent RNA (Nudler et al., 1994).

A central feature of the elongation complex is a transiently open transcription bubble (~ 18 bp in length) which moves with the RNA polymerase through the otherwise double-stranded DNA while the polymerase catalyzes template-directed transcript elongation (von Hippel, 1998). Elongation of transcription has been perceived as a monotonous process in which each nucleotide addition is accompanied by a one-base pair translocation of RNA polymerase, which was considered as an inflexible and rigid structure. However, recent observations suggest a substantial conformational flexibility of the ternary elongation complex. The DNA:RNA hybrid, protein-DNA contacts, and protein-RNA contacts all contribute to the stability and movement of the elongation complex (Nudler et al., 1996; Nudler et al., 1997; Nudler et al., 1995). *E. coli* RNA polymerase is proposed to have two RNA-binding sites: a 3'-proximal "loose" RNA-binding site, and an upstream "tight" RNA-binding site. A third flexible element within the polymerase, termed the "front-end domain", contacts the double-stranded DNA just downstream of the transcription bubble (Nudler et al., 1994). The elongation complex tends to stay in a preferred, relaxed conformation as it advances along the template. As the RNA chain grows, the filling of the loose binding site alternates with the threading of the recently synthesized RNA through the tight binding site so that an optimal distance between the catalytic center and the front end is maintained. However, certain sequence signals encountered on the way cause the ternary complex to rearrange. The rearrangement is induced by the "anchoring" of the front-end domain to the DNA and accompanied by the cessation of threading of the newly synthesized RNA through the

tight RNA-binding site. At the same time, the growth of the RNA chain continues, resulting in progressive filling of the loose RNA-binding site. This causes a buildup of internal strain within the elongation complex. The strained conformation is reverted to the relaxed form only when the anchoring contacts are broken and the front end leaps forward with simultaneous threading of the transcript through both RNA-binding sites, a process termed "inchworming". By comparing stalled complexes at many positions, it is shown that the inchworming cycle is an incidental event rather than intrinsic to elongation. In the absence of the signal, the RNA polymerase translocates concomitantly with the growth of the transcript and the elongation proceeds in a monotonous fashion. Only when encountering the signal, the ternary complex becomes strained and the inchworming cycle occurs. Entering an inchworming cycle may be a mechanism to slow elongation for entry into pause, arrest, or termination mode.

Although the discontinuous mechanism of transcriptional elongation is mainly derived from the study of bacterial RNA polymerases, a similar mechanism also appears to be utilized in eukaryotic systems. Conformational changes in pol II accompanying "inchworm" movement have been demonstrated by probing isolated ternary complexes with nucleases and RNA cleavage factors (Izban and Luse, 1993a; Linn and Luse, 1991). *E. coli* RNA polymerase apparently switches from monotonic progress to the inchworm mode in response to DNA sequences that lock the loose RNA-binding site and the front-end domain of polymerase onto the DNA (Nudler et al., 1994). It is likely that pol II behaves in a similar way during elongation.

Pausing, Arrest, and Termination

Under physiological conditions, the optimal elongation rate of RNA polymerases is about 1,200-1,500 nucleotides (nt)/min in higher eukaryotes (Ucker and Yamamoto, 1984; Izban and Luse, 1992a). Furthermore, many of the genes transcribed are up to 10^4 or 10^5 bp in length, demanding a high processivity of the elongation complex. During elongation, however, intrinsic signals in template DNA and nascent RNA can divert a fraction of RNA polymerases from the path of rapid and processive elongation (reviewed in Landick, 1997). Three types of such signals are: 1) pause signals, which can stop the RNA polymerase temporarily to await interaction of a regulatory molecule, but from which the elongation complex can escape spontaneously; 2) terminators, which can irreversibly release RNA and DNA from the RNA polymerase (RNA polymerases never extend released transcripts; to the contrary, DNA polymerases can rebind and extend the 3' ends of released DNA molecules); and 3) arrest signals, which can cause RNA polymerase to backtrack its catalytic center to an internal phosphodiester in the nascent RNA chain. The escape from an arrest site then requires hydrolysis of the transcript, catalyzed by the RNA polymerase, to realign the 3' end of the nascent RNA and the catalytic center correctly. This RNA cleavage reaction is stimulated by GreA or GreB in bacteria and by SII in eukaryotes (Borukhov et al., 1993; Borukhov et al., 1992; Izban and Luse, 1993a; Izban and Luse, 1992b). During elongation, therefore, RNA polymerase must hold the DNA template and nascent RNA tightly enough to avoid dissociation yet loosely enough to translocate rapidly along the template, and it must be

able to reverse these properties efficiently at the template positions where pausing or termination is programmed.

Although multiple classes of pause, termination, and arrest signals are identified in bacterial systems, the signals in eukaryotes are poorly understood (Landick, 1997; von Hippel, 1998). For all RNA polymerases at each position in a gene, the ternary complex may have a slightly different structure induced by the template sequence that affects the energetic barriers to the polymerization reaction. As a result, enormous variation is seen in the polymerase "dwell time" at different positions (von Hippel and Yager, 1992). The difference in dwell time may reflect the difference in K_m for NTP at the next position. Pauses can be affected by many factors including the DNA sequence, DNA bending, nucleotide availability and misincorporation, RNA secondary structure, and DNA-binding proteins that may act as roadblocks. Pausing appears to be a universal prerequisite for termination, but certainly not all pause sites are termination sites. Although the mechanism is still unclear, the discontinuous movements of RNA polymerases may mediate steps in pausing, termination, and arrest (Nudler et al., 1994; Landick, 1997). Both the DNA:RNA hybrid and the protein-nucleic acid contacts within the ternary complex are key determinants in these processes.

Elongation Factors

Several general pol II elongation factors have been identified from human, yeast, rat, and *Drosophila* (Reines et al., 1996). These include TFIIF, SII, SIII, ELL, P-TEFb, and N-TEF. Among these factors, TFIIF, SIII, and ELL stimulate the elongation rate and suppress transient pausing of pol II. SII stimulates the intrinsic RNA cleavage activity of

pol II to overcome pause sites and arrest sites. P-TEFb (positive elongation factor b) increases the processivity of pol II, while N-TEF (negative elongation factor) causes premature termination. One component of N-TEF, namely *Drosophila* factor 2, is an ATP-dependent transcript release factor and a member of the SWI2/SNF2 ATPase family (Xie and Price, 1997; Liu et al., 1998). The human homolog of factor 2 also displays the double-stranded DNA-dependent ATPase activity and induces pol II termination (Liu et al., 1998).

TFIIF has dual roles in both initiation and elongation (Bengal et al., 1991; Izban and Luse, 1992a; Kephart et al., 1994; Tan et al., 1994; Lei et al., 1998). Both RAP30 and RAP74 subunits are required for supporting initiation and stimulating the elongation rate (Tan et al., 1994; Lei et al., 1998). It was suggested that TFIIF stimulates the elongation rate by suppressing the transient pausing of pol II (Bengal et al., 1991; Izban and Luse, 1992a; Price et al., 1989). TFIIF does not appear to affect the positions of pausing but generally decreases the dwell time of pol II at each position (Chapter 3). By analyzing a series of TFIIF mutant proteins in elongation, it is clear that the activity of TFIIF in stimulating elongation rate correlates very well with its ability in decreasing the dwell time of pol II at each pause site. However, the cause-effect relationship between the rate stimulation and the pause suppression of TFIIF activities is still unclear.

Biochemical and genetic evidences suggest that SII (also known as TFIIS or RAP38) interacts with pol II (Sopta et al., 1985; Agarwal et al., 1991; Archambault et al., 1992). SII does not enhance the elongation rate but promotes the passaging of pol II through intrinsic DNA arrest sites and pause sites (Bengal et al., 1991; Izban and Luse, 1992a; Reines et al., 1989; Reinberg and Roeder, 1987). SII stimulates an endonuclease

activity of pol II that hydrolyzes the nascent RNA, resetting the pol II catalytic center at the 3' end of the transcript and allowing elongation to resume (Izban and Luse, 1992b; Johnson and Chamberlin, 1994; Powell et al., 1996). The increment of SII-facilitated transcript cleavage varies dramatically between paused and arrested ternary complexes, indicating that paused complexes and arrested complexes have distinct conformations (Izban and Luse, 1993a; Izban and Luse, 1993b). The pol II subunit Rpb9 appears to interact with SII to regulate the transcript cleavage and the read-through activities of pol II (Awrey et al., 1997). By stimulating the intrinsic 3'-5' RNA cleavage activity of pol II, SII also enhances the fidelity of transcription (Jeon and Agarwal, 1996; Yoon et al., 1998).

P-TEFb was originally identified from *Drosophila* as a factor important for the production of DRB-sensitive transcripts in vitro (Marshall and Price, 1995). DRB, an ATP analog and kinase inhibitor, is known to inhibit elongation both in vitro and in vivo (Zandomeni et al., 1983; Zandomeni and Weinmann, 1984; Chodosh et al., 1989; Yankulov et al., 1995). The human homolog of P-TEFb was also identified (Peng et al., 1998a). Both *Drosophila* and human P-TEFb are cyclin-dependent kinases which can specifically phosphorylate the CTD of pol II (Marshall et al., 1996; Peng et al., 1998a; Peng et al., 1998b). In addition to its role as a general elongation factor, P-TEFb also has a specific function in the HIV-1 Tat-mediated transcriptional activation (Zhu et al., 1997; Fujinaga et al., 1998). Tat, a virally encoded transcriptional activator, is required for the synthesis of full-length RNA molecules from the HIV-1 virus genome (reviewed in Jones and Peterlin, 1994). In the absence of Tat, short transcripts are formed and released, a process called premature termination. HIV-1 Tat is a unique transcriptional activator

because, instead of bindng a DNA element, it binds an RNA sequence (termed TAR) which is located near the 5' end of the nascent transcript. The large subunit of P-TEFb, cyclin T, interacts specifically with HIV-1 Tat and enhances the affinity and specificity of the Tat:TAR interaction (Wei et al., 1998). The interaction between Tat and P-TEFb in turn also recruits P-TEFb to the paused early elongation complex. Presumably, the CTD kinase activity of P-TEFb can then phosphorylate the pol II CTD and convert the paused elongation complex to a processive elongation complex. Alternatively, the kinase activity of P-TEFb can phosphorylate another transcription factor that is specifically required for productive transcription of the HIV-1 genome.

SIII and ELL have similar functions to TFIIF in stimulating the overall elongation rate and suppressing the transient pausing of pol II (Bradsher et al., 1993; Bradsher et al., 1993; Shilatifard et al., 1996; Shilatifard et al., 1997a; Shilatifard et al., 1997b). Interestingly, both ELL and SIII are implicated in the development of certain human cancers. The gene encoding ELL is a frequent target for t(11; 19) chromosomal translocations in acute myeloid leukemias (Shilatifard et al., 1996). SIII, also called elongin, is a target for regulation by the protein product of the VHL (von Hippel-Lindau) tumor suppressor gene, which is mutated in several types of tumors (Aso et al., 1995; Duan et al., 1995). The normal tumor suppressor function of the VHL protein likely involves the down-regulation of SIII (elongin) transcriptional activity. SIII (elongin) consists of a transcriptionally active A subunit and two regulatory B and C subunits. The elongin B and elongin C subunits form a stable binary complex that interacts with elongin A and strongly induces its elongation activity (Takagi et al., 1996; Aso et al., 1996). The VHL tumor suppressor protein is capable of binding stably to the elongin BC complex

and preventing it from activating elongin A (Pause et al., 1996; Takagi et al., 1997; Conaway et al., 1998). The interaction of the VHL protein with the BC complex is mediated in part by a short VHL region and many naturally occuring VHL mutants found in tumors have mutations that fall within this critical region. A number of these VHL mutants exhibit reduced binding to elongin BC complex in vitro.

TRANSCRIPTIONAL TERMINATION AND REINITIATION

The signal and mechanism of termination in pol II transcription are poorly understood. It is possible that the dephosphorylation of the CTD may serve as a trigger for pol II to terminate, although this remains to be further tested. *Drosophila* and human ATP-dependent transcript release factors have recently identified. Both factors exhibit dsDNA-dependent ATPase activities and induce pol II termination from stalled elongation complexes (Xie and Price, 1996; Xie and Price, 1997; Liu et al., 1998).

The CTD of pol II in the elongation complex is highly phosphorylated (Dahmus, 1996), however, pol IIA is preferentially associated with the preinitiation complex (Lu et al., 1991). Therefore, the CTD has to be dephosphorylated so that pol II can re-enter the preinitiation complex for a successive round of transcription to occur, a process called transcriptional recycling. It should be noted that the transcriptional recycling is only one form of transcriptional reinitiation. Transcriptional reinitiation in vitro can have different forms: 1) the same DNA template is "fired" multiple times. As soon as the first polymerase molecule exits the promoter, a second preinitiation complex then assembles on the same template and new initiation occurs; 2) the same pol II molecule "fires" multiple times, i.e., transcriptional recycling. After finishing the synthesis of one RNA

chain, the same polymerase molecule is re-delivered to a promoter and initiates another round of transcription; and 3) different pol II molecules "fire" from different DNA templates at different times. Although they are mechanistically distinct, one shared requirement by these different forms of reinitiation is believed to be the conversion of pol IIO to pol IIA. In the case of transcriptional recycling, the dephosphorylation occurs on a transcriptionally active polymerase right before or shortly after termination. In the two other cases, the CTD dephosphorylation may occur on free polymerases to maintain the pool of pol IIA. Indeed, the CTD phosphatase is capable of dephosphorylating the pol II CTD in the absence of DNA (Chambers and Dahmus, 1994). The yeast Srb10 CTD kinase inhibits transcription by phosphorylating the pol II CTD prior to formation of the initiation complex on promoter DNA and therefore preventing PIC assembly (Hengartner et al., 1998). Cdk8, the mammalian homolog of Srb10 kinase, is shown to be associated with an mammalian pol II holoenzyme and may also phosphorylate the CTD independent of DNA (Cho et al., 1998). Mammalian TFIIH can also phosphorylate the pol II CTD independent of DNA although the yeast TFIIH kinase Kin28 preferentially phosphorylate the CTD only when the transcription apparatus is associated with DNA (Hengartner et al., 1998). Therefore, it is likely that the phosphorylation status of pol II is tightly controlled by both CTD kinases and CTD phosphatase to regulate the abundance of pol IIA. Interestingly, the CTD phosphatase is stimulated by the RAP74 subunit of TFIIF and TFIIB blocks this stimulation (Chambers et al., 1995). The C-terminal domain of RAP74 is sufficient for stimulating CTD phosphatase activity. Consistent with its ability to stimulate the CTD dephosphorylation, full length RAP74 supports multiple rounds of transcription in an extract system. RAP74 mutants containing only the N-terminal

domain do not stimulate the CTD dephosphorylation and are incapable of transcriptional reinitiation (Lei et al., 1998; Chapter 4). Because the CTD phosphatase, TFIIF, and TFIIB all bind pol II directly and function in the dephosphorylation reaction, it is possible that these factors cooperate to regulate transcriptional reinitiation by controlling the conversion of pol IIO to pol IIA. Additional factors may also be involved. The inhibitory effect of TFIIB on CTD phosphatase activity may serve as a brake during elongation to prevent premature dephosphorylation, which would be expected to cause termination.

TRANSCRIPTIONAL ACTIVATION

In eukaryotic cells, packaging of DNA into chromatin causes overall gene repression (Paranjape et al., 1994). DNA sequences within the chromatin structure are generally inaccessible to transcription factors and pol II. Covalent modifications of histones affect the packaging of nucleosomal DNA. For example, acetylation of the lysine residues at the N-terminal tails of core histones is believed to loosen the nucleosomal structure and expose the DNA sequence, while deacetylation of these residues is believed to tighten the nucleosome and make the DNA sequence less accessible (reviewed in Pazin and Kadonaga, 1997; Hampsey, 1997). In addition to the compacted chromatin structures, there are many general or gene-specific transcriptional repressor proteins in eukaryotic cells (Hanna Rose and Hansen, 1996). An important function of transcriptional activators is to relieve the repression caused by the chromatin structure and/or repressor proteins, an effect often referred to as "anti-repression" or "derepression". In addition, activators possess the ability to facilitate the intrinsic

transcription reaction by acting on the general machinery to increase the efficiency of the transcription process. This is sometimes referred to as "true activation" (Paranjape et al., 1994). Therefore, an activator may function both to counteract the repression and to increase the level of transcription.

Many components of the general transcription machinery, including TBP, TFIIA, TFIIB, TFIIF, TFIIE, and TFIIH, have been implicated as direct targets of transcriptional activators (Triezenberg, 1995). Another group of molecules, termed coactivators, mediators or adaptors, may also be involved in gene activation (Pugh and Tjian, 1990; Kelleher et al., 1990; Berger et al., 1990). Transcriptional coactivators are distinct from activators in that most coactivators do not bind DNA directly and none identified so far appears to bind DNA in a sequence-specific manner. Coactivators are distinct from the GTFs in that coactivators are dispensable for basal transcription in vitro (Hampsey, 1998). In some cases, coactivators seem to bridge the interaction between gene-specific activator proteins and the general transcription apparatus, while in other cases, coactivators seem to facilitate remodeling of chromatin structure. The interaction between a DNA-bound activator and a component of the yeast pol II holoenzyme is capable of inducing gene expression in vivo (Barberis et al., 1995; Farrell et al., 1996).

Transcriptional Activators

Transcriptional activators usually have separable domains for recognizing target genes (DNA-binding domains) and for stimulating the transcriptional machinery (activation domains) (Mitchell and Tjian, 1989; Triezenberg, 1995). The three-dimensional structures of many DNA-binding domains reveal various protein motifs for

recognizing specific DNA sequences, such as the helix-turn-helix motif, Zn-fingers, and the leucine-zipper motif (Harrison, 1991). To the contrary, little is known about the structures of transcriptional activation domains. Activators have traditionally been classified according to the most prevalent amino acid of their activation domains, such as acidic, glutamine-rich, proline-rich, or serine/threonine-rich (Triezenberg, 1995). However, mutational analyses indicate that a pattern of bulky hydrophobic amino acid residues may be more important than the more obvious features initially used to distinguish activation domains.

Many activators appear to be able to make multiple contacts with various GTFs and coactivators. For example, VP16 has been shown to interact with TBP, TFIIB, TFIIA, TFIIH, Ada2, hTAF$_{II}$32, and dTAF$_{II}$40 (Barlev et al., 1995; Goodrich et al., 1993; Ingles et al., 1991; Klemm et al., 1995; Kobayashi et al., 1995; Lin et al., 1991; Xiao et al., 1994). VP16 also interacts with the yeast pol II holoenzyme containing the SRB/mediator complex (Hengartner et al., 1995). This multitude of interaction may reflect the intrinsic complex nature of PIC formation, which involves many protein-protein and protein-DNA interactions. The ability to interact with multiple targets by one activator may synergistically facilitate the assembly of PIC. Alternatively, an activator may interact with different targets at different transitions during transcription. It is also possible that an activator may interact with different targets at different promoters.

Transcriptional Coactivators

Transcriptional coactivators function either to bridge the interaction between an enhancer-bound activator and the general transcriptional machinery, or to change the

structure of chromatin (Hampsey, 1998; Kaiser and Meisterernst, 1996). These include mammalian USA (upstream stimulatory activity), the SRB/mediator complex, the SWI/SNF and related chromatin-remodeling complexes, and the yeast SAGA and related complexes that catalyze nucleosomal histone acetylation. Although TFIID and TFIIA were originally identified as general factors, it is clear that both TFIIA and the TAF_{II} components of TFIID are not required for basal transcription in vitro and therefore sometimes also classified as coactivators. In addition to these general coactivators that presumably are involved in the activation of many genes, some gene-specific or cell-specific coactivators have also been identified such as OCA-B and TRAPs (thyroid hormone receptor-associated proteins) (Kim et al., 1996; Fondell et al., 1996; Yuan et al., 1998). The coactivator functions of USA, SRB/mediator, chromatin-remodeling complexes, and histone acetyltransferase (HAT) complexes are summarized in the following paragraphs.

USA was initially identified in HeLa cells as a fraction required for transcriptional activation in a purified in vitro system (Meisterernst et al., 1991). USA includes both positive cofactors (PCs) and negative cofactors (NCs). Several USA components have been defined, including PC1 (poly[ADP-ribose] polymerase), PC2, PC3 (topoisomerase I), and PC4 (Meisterernst et al., 1997; Kretzschmar et al., 1993; Kretzschmar et al., 1994; Merino et al., 1993; Ge and Roeder, 1994b). Among these factors, PC4 binds TBP and several activators and it mediates functional interactions between DNA-bound activators and the PIC (Ge and Roeder, 1994b).

Yeast **SRB/mediator** is a multisubunit complex consisting of Srb2 to Srb11, Med1 to Med4, Med6, Med7, Med8, Gal11, Sin4, Rgr1, and Rox3 (reviewed in

Hampsey, 1998). SRB/mediator, in contrast to TAF_{II}s, plays a more general role in transcriptional activation. The SRB/mediator complex is a component of yeast pol II holoenzyme and has several defined activities: 1) stimulation of basal transcription in a highly purified system; 2) response to transcriptional activators in vitro; and 3) stimulation of phosphorylation of the pol II CTD by TFIIH (Kim et al., 1994; Koleske and Young, 1994). The SRB (suppressor of RNA polymerase B) genes were originally identified in a genetic screen based on suppression of the cold-sensitive growth phenotype associated with trunctions of the pol II CTD, suggesting interactions between the SRB proteins and the CTD (Nonet and Young, 1989). Several SRB proteins indeed bind the pol II CTD in vitro (Koleske et al., 1992; Thompson et al., 1993). The mediator complex was independently isolated from yeast using a biochemical approach based on its requirement for transcriptional activation by RNA polymerase II in a reconstituted system (Kim et al., 1994). The mediator complex interacts directly with the pol II CTD. Although there are some differences, genetic and biochemical evidence indicates that the SRB complex and the mediator complex likely exist as a single complex present in the pol II holoenzyme (Wilson et al., 1996; Li et al., 1996). This form of pol II holoenzyme is therefore referred to as the SRB/mediator-containing holoenzyme. Not all of the cellular RNA pol II is found in the holoenzyme form as determined by quantitative Western blots (Kim et al., 1994; Koleske and Young, 1994). It was suggested that the holoenzyme is the form of pol II recruited to most promoters in vivo (Thompson and Young, 1995), although this remains to be further tested. Not all genes encoding the SRB/mediator subunits are essential for cell viability and little is known about the mechanisms of their functions. Among these different subunits, Srb2 physically

associates with the PIC and binds TBP directly, revealing a functional link between the CTD and TBP (Koleske et al., 1992). Srb5 is a component of the PIC and is required for efficient transcription initiation (Thompson et al., 1993). Srb10 and Srb11 constitute a kinase/cyclin pair and the Srb10 kinase can phosphorylate the pol II CTD (Liao et al., 1995). The phosphorylation of CTD by Srb10 prevents formation of the PIC and inhibits transcription (Hengartner et al., 1998). Gal11, originally identified in a genetic selection for protein factors required for full expression of galactose-inducible genes, copurifies as a SRB/mediator component of the pol II holoenzyme (Kim et al., 1994). Gal11 enhances basal transcription and facilitates activation by many gene-specific activators. Gal11 is shown to interact with TFIIE and stimulate the CTD kinase activity of TFIIH (Sakurai and Fukasawa, 1997; Sakurai and Fukasawa, 1998). Several mammalian homologs of yeast SRB/mediator components have been identified and shown to be present in mammalian pol II holoenzymes (Chao et al., 1996; Cho et al., 1998).

SWI/SNF and related complexes facilitate transcriptional activation by affecting nucleosome structure in an ATP-dependent manner (reviewed in Burns and Peterson, 1997; Kingston et al., 1996). The yeast SWI/SNF complex is a multisubunit complex and binds DNA with high-affinity (Cairns et al., 1994; Quinn et al., 1996). Among the SWI/SNF subunits, Swi2/Snf2 is a DNA-dependent ATPase. Swp29 is identical to the Tfg3 subunit of TFIIF and to the $TAF_{II}30$ subunit of TFIID, implying a link between the SWI/SNF, TFIIF, and TFIID complexes (Cairns et al., 1996). The SWI/SNF complex was also reported to be a component of the SRB/mediator-containing pol II holoenzyme, however, an independent preparation of pol II holoenzyme does not contain SWI/SNF (Wilson et al., 1996; Li et al., 1996). Although with interesting properties, the SWI/SNF

complex is only required for activation of several yeast genes, including *HO*, *SUC2*, *Ty*, *ADH1*, *ADH2*, *INO1*, and *STA1* (Hampsey, 1998). Many promoters are not dependent on SWI/SNF for activation, indicating that there may be alternative mechanisms for activation from the SWI/SNF-independent genes. Human SWI/SNF complex is also capable of altering the nucleosome structure and enhancing the binding of transcriptional activators (Kwon et al., 1994; Imbalzano et al., 1994). Human SWI/SNF can also stimulate transcriptional elongation by overcoming the nucleosome-enhanced pausing on the hsp70 gene (Brown et al., 1996). Other chromatin-remodeling factors include *Drosophila* NURF, CHRAC, and yeast RSC complexes. NURF facilitates the GAGA-dependent formation of nuclease hypersensitive sites within a nucleosome array in vitro (Tsukiyama and Wu, 1995). CHRAC facilitates the accessibility of DNA in chromatin and chromatin assembly (Varga-Weisz et al., 1997). RSC (remodels the structures of chromatin) was isolated from yeast on the basis of homology to components of the SWI/SNF complex (Cairns et al., 1996). Similar to SWI/SNF, RSC is a multisubunit complex and has a DNA-dependent ATPase. However, there is yet no evidence that CHRAC or RSC plays a direct role in transcription.

Histone acetyltransferases (HATs) catalyze the acetylation of lysine residues at the N-terminal tails of histones. The acetylation of lysine residues neutralizes their positive charge and would presumably reduce the interaction of the core histone tails with DNA, which is postulated to cause gene activation (Paranjape et al., 1994). The direct link between histone acetylation and gene activation was established when the yeast transcriptional coactivator Gcn5 was found to possess HAT activity (Brownell et al., 1996). Gcn5 is a component of the yeast adapter complex, consisting of Ada1, Ada2,

Ada3, Gcn5, and Ada5 (Berger et al., 1990; Berger et al., 1992; Candau and Berger, 1996; Horiuchi et al., 1995; Horiuchi et al., 1997; Marcus et al., 1994; Marcus et al., 1996; Pina et al., 1993; Roberts and Winston, 1996). The adapter complex is required for full activation by a subset of transcriptional activators. Both the HAT activity and the interaction with Ada2 were essential for Gcn5 function in vivo (Candau et al., 1997). Recently, several nucleosomal HAT complexes were isolated from yeast (Grant et al., 1997). One of such complexes is named SAGA (Spt-Ada-Gcn5-Acetyltransferase) and contains Gcn5, Ada2, Spt3, Spt7, Ada5/Spt20 and several TAF_{II}s sununits (Grant et al., 1997; Grant et al., 1998). This SAGA complex links nucleosomal histone acetylation with transcriptional activation associated with Ada, TAF_{II} and Spt proteins. The SPT genes were originally identified in a genetic screen for suppressors of a Ty element insertion in the *HIS4* promoter (Eisenmann et al., 1989). *SPT15* is identical to TBP and many Spt proteins have functions in chromatin dynamics. Similar to SWI/SNF, Gcn5 and Ada proteins are only required for activation from a subset of genes in vivo. Genetic evidence suggests some functional overlap between SWI/SNF, SAGA, and SRB/mediator complexes in their roles as coactivators of gene expression (Pollard and Peterson, 1997; Roberts and Winston, 1996; Roberts and Winston, 1997). SWI/SNF and SAGA complexes may cooperate to alter the chromatin structure. Alternatively, there may be yet unidentified factors involved in changing the structure of chromatin to facilitate transcriptional activation. It is also possible that the promoter regions of many yeast genes are not tightly packaged into nucleosomes and the alteration of chromatin structure is not essential for formation of functional transcription complexes at these genes. Several HATs newly identified in mammalian cells are also implicated in

transcriptional control. These mammalian HATs include human Gcn5, p300/CBP, $hTAF_{II}250$, P/CAF, and ACTR (Mizzen et al., 1996; Candau et al., 1996; Yang et al., 1996; Chen et al., 1997; Martinez-Balbas et al., 1998). P/CAF exists in a multisubunit complex that also contains some TAF_{II} subunits (Ogryzko et al., 1998). The p300/CBP coactivator, in cooperation with the ligand-activated estrogen receptor, stimulates transcription initiation from chromatin templates containing an estrogen response element in vitro (Kraus and Kadonaga, 1998). In addition to using histones as substrates, p300/CBP also acetylates the tumor suppressor protein p53 and enhances its DNA-binding and transcriptional activity (Gu and Roeder, 1997). Another factor, named FACT (facilitate chromatin transcription), was isolated from HeLa cells and moderately stimulates transcript elongation from nucleosomal DNA templates (Orphanides et al., 1998).

Although some transcriptional coactivators, such as chromatin-remodeling complexes and HAT complexes, are capable of changing the chromatin structures, the functional relationship between the sequence-specific transcriptional activators and these coactivators is still unclear. If the chromatin structure has to be disturbed before an activator can bind its target DNA sequence, how is the chromatin-remodeling complex or HAT initially recruited to a target gene? On the other hand, if an activator recruits the chromatin-remodeling complex or HAT to a target gene, how does the activator bind its target sequence within the compacted nucleosome? Perhaps the chromatin is not a static structure and not all DNA-binding sites are tightly wrapped within nucleosomes. The promoter-recognition by activators and the chromatin-modification by SWI/SNF and HAT coactivators may be a cooperative process. An activator may have a limited access

to its cognate DNA binding site in the absence of nucleosome alternation or histone acetylation. The binding of the activator to the promoter can then be enhanced by the action of SWI/SNF and HAT after they are recruited to the promoter through the activator. The stable association of an activator with the promoter and the alteration of nucleosome structure then facilitate assembly of the transcription initiation complex.

Mechanisms of Activation

Transcriptional activators can function by relieving the inhibitory effect of chromatin and by stimulating the general transcription machinery. In principle, activators can stimulate various steps along the pathway to the synthesis of a mRNA molecule. These include 1) alteration of the chromatin structure; 2) recruitment of GTFs and pol II to a promoter; 3) stimulation of open complex formation; 4) stimulation of promoter escape; 5) stimulation of elongation; and 6) stimulation of reinitiation.

Alteration of the Chromatin Structure

Chromatin-mediated repression of transcription can be alleviated by the addition of transcriptional activators (Kamakaka et al., 1993; Lorch et al., 1992; Workman et al., 1991). Transcriptional activators may recruit chromatin-remodeling complexes and HATs to specific promoters to alter the local nucleosomal structure and allow assembly of the PIC. It is possible that transcriptional activators work synergistically with chromatin-remodeling complexes and/or HAT complexes to achieve this effect. SWI/SNF has been shown to facilitate both activator binding and TBP binding to promoter DNA (Imbalzano et al., 1994; Cote et al., 1994). Many interactions between

activators and HATs have also been reported. Among the histone acetyltransferases, $TAF_{II}250$ is a subunit of TFIID and has been shown to interact with both activators and basal factors (Ruppert and Tjian, 1995; Wang et al., 1997). CBP and p300 interact with activators including CREB, E1A, and nuclear receptors (Arany et al., 1994; Kwok et al., 1994; Yang et al., 1996; Kamei et al., 1996). Both ACTR and P/CAF also interact with nuclear receptors (Chen et al., 1997).

Recruitment of GTFs and pol II

The recruitment of GTFs by activators is extensively documented and a large number of activator-GTF interactions have been reported. TFIIA, TBP, IIB, IIE, IIF, and IIH have all been implicated as direct targets for various activators in vitro. The pol II holoenzyme can also be recruited to a promoter by an activator. The recruitment of GTFs and pol II holoenzymes by activators presumably facilitates formation of a functional PIC but may also affect late steps in transcription.

Activators such as VP16, Zta, and T antigen bind directly to TFIIA, and the binding correlates with their ability to enhance TFIIA-TFIID-TATA complex formation (Damania et al., 1998; Kobayashi et al., 1995; Kobayashi et al., 1998; Ozer et al., 1994). Activators may assist TFIIA in overcoming the slow step of TBP binding in PIC formation (Chi and Carey, 1993; Chi and Carey, 1996).

The tethering of TBP to a promoter overcomes the requirement for an activator, suggesting that the binding of TBP to a promoter is a slow step that can be accelerated by activators in vivo (Chatterjee and Struhl, 1995; Klages and Strubin, 1995; Majello et al., 1998; Xiao et al., 1995). TBP has been shown to bind in vitro to many activators

including VP16, Sp1, Oct-1, Oct-2, Gal4, C-Jun, c-Myc, E2F1, and P53 (Ingles et al., 1991; Emili et al., 1994; Zwilling et al., 1994; McEwan et al., 1996; Melcher and Johnston, 1995; Franklin et al., 1995; Pearson and Greenblatt, 1997; Liu et al., 1993; Truant et al., 1993; Chang et al., 1995). Activators can also recruit TFIID through interacting with TAF_{II} subunits. Many activators including VP16, Sp1, NTF-1, E1A, progesterone receptor, Bicoid, ERM, ICP4, and estrogen receptor have been shown to bind TAF_{II} subunits in vitro (Goodrich et al., 1993; Klemm et al., 1995; Chen et al., 1994; Gill et al., 1994; Sauer et al., 1995; Schwerk et al., 1995; Defossez et al., 1997; Mazzarelli et al., 1997; Jacq et al., 1994; Carrozza and DeLuca, 1996).

TFIIB interacts with several activators such as VP16, Gal4-AH, CTF1, Gal4, RXRβ, CREB, HIV-1 Vpr, and Vitamin D receptor (Agostini et al., 1996; Blanco et al., 1995; Leong et al., 1998; Xing et al., 1995; Choy and Green, 1993; Kim and Roeder, 1994; Lin et al., 1991; Roberts et al., 1993; Wu et al., 1996; Leong et al., 1998). Activators may induce a comformational change in TFIIB which facilitates the binding of TFIIB to the TBP-DNA complex (Roberts and Green, 1994; Hayashi et al., 1998).

TFIIF interacts with androgen receptor, SRF, and VP16 (McEwan and Gustafsson, 1997; Zhu et al., 1994; Joliot et al., 1995). The interaction between the RAP74 subunit of TFIIF and SRF is important for activation by SRF from the *c-fos* promoter (Zhu et al., 1994; Joliot et al., 1995). The activation domain of androgen receptor interacts with both TBP and TFIIF, but only the interaction with TFIIF appears to be essential for its activity (McEwan and Gustafsson, 1997).

TFIIE interacts with Gal11, a component of the yeast pol II holoenzyme (Sakurai et al., 1996). Genetic evidences suggest that TFIIE and Gal11 work in a common

pathway to regulate transcription in vivo (Sakurai et al., 1997; Sakurai and Fukasawa, 1997). Gal11 cooperates with TFIIE to stimulate the CTD phosphorylation by TFIIH in vitro (Sakurai and Fukasawa, 1998). TFIIE also interacts with the Epstein-Barr virus activator EBNA 2 through a coactivator p100 (Tong et al., 1995).

TFIIH binds VP16, p53, HIV-1 Tat, and RARα (Xiao et al., 1994; Rochette-Egly et al., 1997; Garcia-Martinez et al., 1997). Both the activation domains of RARα and p53 can be phosphorylated by the kinase activity of TFIIH and the phosphorylation correlates with their activities in transcription (Lu et al., 1997; Rochette-Egly et al., 1997). The interaction of Tat with TFIIH stimulates the phosphorylation of pol II CTD (Garcia-Martinez et al., 1997).

The pol II holoenzyme can also be recruited to a promoter through the interaction with activators such as VP16 and Gal4 (Hengartner et al., 1995; Koh et al., 1998). Furthermore, tethering of the holoenzyme to a promoter by fusing a holoenzyme component with a DNA binding domain is sufficient for activation (Farrell et al., 1996).

Stimulation of Open Complex Formation

The formation of an open complex requires the ATP-dependent DNA helicase activity of TFIIH. It is possible that binding of activators to TFIIH stimulates its helicase activity and therefore facilitates formation of the open complex. Both VP16 and HIV-1 Tat have been shown to enhance transcription beyond the step of TBP-TATA interaction (White et al., 1992; Xiao et al., 1997). Furthermore, mutations in the VP16 activation domain have been shown to affect formation of the open complex (Jiang et al., 1994). Activators may stimulate formation of the open complex indirectly through binding

TFIIE, as TFIIE recruits TFIIH and stimulates its DNA helicase activity. TFIIF was implicated in formation of the open complex by inducing the promoter DNA to wrap around PIC, which may lead to untwisting of the helix near the start site. Transcriptional activators that target TFIIF may stimulate the open complex formation by facilitating DNA wrapping.

Stimulation of Promoter Escape

Promoter escape is an important transition from initiation to productive elongation. This step in vivo is tightly regulated at many promoters. For example, at the *Drosophila* hsp70 promoter, pol II actively synthesizes short RNA transcript but the transcription complex is stalled near the start site before heat shock. Upon heat shock, the heat shock transcription factor (HSF) stimulates the release of pol II from the promoter and productive elongation begins (O. Brien and Lis, 1991; Rasmussen and Lis, 1993; Lis and Wu, 1993). The CTD of pol II is hypophosphorylated in the stalled complex but highly phosphorylated in the productive elongation complex, suggesting that HSF may stimulate the CTD phosphorylation to stimulate promoter escape (O. Brien et al., 1994). Therefore, by setting up an initiated complex and using the promoter escape as a switch, the cell can respond quickly to stress signals such as heat.

Stimulation of Elongation

Although many activators stimulate initiation, some appear to primarily stimulate elongation (Blau et al., 1996). Transcriptional activators VP16, E1A, HIV-1 Tat, p53, and E2F1 have all been shown to stimulate elongation (Blau et al., 1996; Yankulov et al.,

1994). Activators may recruit elongation factors to the transcription complex and affect both the rate and processivity of the elongation complex. For example, HIV-1 Tat stimulates transcription of the HIV-1 viral genome primarily by increasing the processivity of pol II, presumably by stimulating the phosphorylation of pol II CTD (Garcia-Martinez et al., 1997; Parada and Roeder, 1996). Several activators including SRF and androgen receptor interact directly with TFIIF and may enhance transcript elongation. Activators may also target other known elongation factors such as P-TEFb, SII, SIII, and ELL.

Stimulation of Reinitiation

DNA-bound activators may hold certain initiation factors at the promoter when pol II begins elongation. This would facilitate the subsequent assembly of an initiation complex and stimulate multiple rounds of transcription. Both Gal4-VP16 and Oct-2 remain bound to the DNA binding sites after initiation and stimulate reinitiation from the templates (White et al., 1992; Arnosti et al., 1993). The ligand-bound estrogen receptor tightly associates with its cognate DNA site and stimulates reinitiation by promoting the reassembly of the preinitiation complex (Kraus and Kadonaga, 1998). The CTD phosphatase may be targeted by some transcriptional activators to regulate the pool of pol IIA, which is important for transcriptional reinitiation.

TRANSCRIPTIONAL REPRESSION

In principle, transcriptional repression is equally important as activation for appropriate gene expression in vivo (reviewed in Clark and Docherty, 1993; Hanna Rose

and Hansen, 1996). Both gene-specific repressors, which inhibit the transcription from specific promoters, and general repressors, which inhibit the transcription from multiple and unrelated promoters, have been identified. The repression of transcription can result from competitive binding between a repressor and an activator, squelching of a GTF or a cofactor necessary for activation, direct targeting of the basal machinery by a repressor, or alteration of the chromatin structure. Several repressors appear to directly affect the TBP-TATA interaction.

Mot1, originally identified in yeast, inhibits the binding of TBP to the TATA sequence in an ATP-dependent manner (Auble and Hahn, 1993; Auble et al., 1994). The inhibitory effect can be overcome by the addition of TFIIA or TFIIB. Mot1 is a member of the swi2/snf2 family of ATPase, but the Mot1 ATPase activity is not stimulated by DNA (Auble et al., 1997). Although functioning as a repressor on some promoters, Mot1 can also function as a coactivator on other promoters presumably by removing TBP from nonfunctional TATA sequences (Collart, 1996; Madison and Winston, 1997).

The **Not** (negative on TATA) complex from yeast are global repressors that target the general transcriptional machinery and preferentially affects basal, rather than activated, transcription (Collart and Struhl, 1994). While *mot1* mutations decrease transcription from TATA-less promoters, *not* mutations increase transcription from TATA-less promoters (Collart, 1996). Genetic evidence suggests that NOT proteins negatively regulate the activity of factors such as Spt3 and TFIIA that promote TBP-TATA interaction. NOT proteins, Mot1, Spt3, and TFIIA may functionally interact to regulate the distribution of TBP on strong and weak promoters.

The human **Dr1-DRAP1/NC2** complex represses transcription by blocking the association of TBP with TFIIA and TFIIB (Inostroza et al., 1992; Meisterernst and Roeder, 1991). Both Dr1 and DRAP1 proteins contain histone-fold motifs that appear to mediate the interaction between these two subunits (Mermelstein et al., 1996). Dr1 also interacts directly with TBP (Meisterernst and Roeder, 1991; Inostroza et al., 1992).

Histone deacetylases (HDAs) are also implicated in repressing transcription. A direct link between histone deacetylation and transcriptional repression was established when the human histone deacetylase HDA was found to be homologous to yeast Rpd3, a known transcriptional repressor identified in a genetic screen (Taunton et al., 1996; Vidal and Gaber, 1991). Besides Rpd3, several other histone deacetylases have also been identified in yeast, suggesting a possible functional redundancy (Carmen et al., 1996; Rundlett et al., 1996). The yeast histone deacetylase Rpd3 exists in a multisubunit complex containing corepressor Sin3 and other polypeptides (Kasten et al., 1997). Large HDA complexes containing Rpd3 and Sin3 homologs have also been identified in mammalian cells and they are implicated in mediating repression by unliganded nuclear hormone receptors and by Mad-Max, in each case dependent upon mRpd3 and mSin3 (Alland et al., 1997; Hassig et al., 1997; Hassig et al., 1998; Heinzel et al., 1997; Laherty et al., 1997; Nagy et al., 1997; Zhang et al., 1997). It was proposed that the replacement of the HDA complex with a HAT complex, upon either receptor-hormone binding or removal of Mad-Max by Myc-Max, switches the transcriptional repression to activation. Certainly, more thorough and insightful experiments have to be carried out to generate a clear view on how transcriptional regulators function through altering the chromatin structure.

OVERVIEW

Human TFIIF is an $\alpha_2\beta_2$ tetramer of RAP30 and RAP74 subunits. It was previously known that TFIIF plays important roles in both transcriptional initiation and elongation. It was suggested that TFIIF may also be involved in transcriptional recycling based on its interaction with pol II. However, the mechanisms of TFIIF in these distinct steps were unclear.

One primary function of TFIIF is to escort pol II to the PIC. Both RAP30 and RAP74 are essential for forming a stable DBpolF complex. RAP30 binds RAP74, TFIIB, and pol II in vitro. RAP30 shares sequence similarities with bacterial σ factors. The central region of RAP30 exhibits significant sequence similarity with the polymerase-binding domains of σ^{70} and σ^{43}. The C-terminal region of RAP30 has a cryptic DNA-binding domain similar to the conserved region 4 of bacterial σ factors. The NMR structure of the DNA-binding domain of RAP30 reveals its similarity with histone H5 and other members of the "winged" helix-turn-helix family. Mutational analyses demonstrated that transcriptional activities of RAP30 are mediated by separable domains. It was shown that the pol II-binding region of RAP30 is only necessary for elongation, while the cryptic DNA-binding domain of RAP30 is only necessary for initiation. The region of RAP30 necessary for association with RAP74 is essential for both initiation and elongation.

After the cDNA encoding human RAP74 was cloned, a number of RAP74 deletion mutants were generated in our laboratory. It was shown that the N-terminal domain of RAP74 directly binds RAP30, while the C-terminal domain of RAP74 binds

both TFIIB and pol II. The binding of RAP74 to TFIIB prevented the interaction between TFIIB and RAP30, indicating dynamic interactions between TFIIF and TFIIB. The C-terminal domain of RAP74 also stimulated the dephosphorylation of the pol II CTD by a CTD phosphatase. The N-terminal domain of RAP74 appeared to be critical for transcription, although the deletion of C-terminal regions moderately affects initiation and elongation. RAP74 can be phosphorylated by protein kinases such as casein kinase II, $TAF_{II}250$, and TFIIH. RAP74 also interacts with TFIIEα and transcriptional activators including SRF, androgen receptor, and VP16. The interaction with RAP74 is important for the SRF-mediated activation of the *c-fos* promoter.

My research project focuses on the functions and mechanisms of RAP74 in transcription by pol II. I developed several assays to dissect the functional domains of RAP74. In Chapter 2, I demonstrate that the N-terminal domain of RAP74 is necessary and sufficient to support both preinitiation complex formation, initiation and elongation. The same region is also critical for inducing a tight DNA wrap around pol II in the preinitiation complex as detected by our collaborators using photocrosslinking techniques. These novel findings, along with evidence in the literature, lead to the model that the DNA bending and wrapping are important in both transcriptional initiation and elongation. The analysis of RAP74 deletion mutants indicates that a small region, amino acids 136 to 217, of RAP74 is critical for DNA wrapping, initiation, and elongation. This region of RAP74 is highly conserved and may be important for formation of the TFIIF tetramer. In Chapter 3, I describe a series of RAP74 proteins with site-directed mutations introduced in this critical region. Using a highly sensitive assay, I analyzed the activities of these mutant proteins in elongation and calculated the average elongation rate for each

TFIIF mutant. Several amino acid residues in RAP74 were found to be critical for stimulating the elongation rate of pol II. It was previously suggested that TFIIF stimulates the elongation rate by suppressing the transient pausing of pol II. Comparing TFIIF containing wild type RAP74 or RAP74 mutants, it is clear that TFIIF does not change the positions at which pol II pauses, but TFIIF affects the dwell time of pol II at each nucleotide position. The activities of RAP74 mutants in elongation match very well with their activities in initiation, further strengthening the notion that, in contrast to RAP30, RAP74 has an identical role in both initiation and elongation. The capacity of these RAP74 point mutants in wrapping DNA around pol II in the PIC remain to be tested.

I was puzzled by the finding that both the central region and the C-terminal domain of RAP74 are not required for either initiation or elongation, particularly because these regions are involved in many protein-protein interactions and the C-terminal domain of RAP74 is highly conserved. In Chapter 4, I analyze RAP74 deletion mutants in the multiple-round assay and the single-round assay. It is clear that the C-terminal domain and the central region of RAP74 are required for continuing initiation at later reaction times, although both regions are dispensable for the first-round of transcription. This is the first direct evidence that TFIIF is involved in transcriptional reinitiation. Using a G-less cassette, I further demonstrate that transcription reinitiation in the extract system is not limited by the physical amount of DNA template or pol II molecules; rather, a kinetic event, possibly the conversion of pol IIO to pol IIA, limits the capacity of the system to reinitiate. This model is consistent with the finding that the C-terminal domain of RAP74 stimulates the conversion of pol IIO to pol IIA by a CTD phosphatase.

In Chapter 5, I test the activities of other general transcription factors in elongation assays. As predicted by the DNA wrapping model, TBP, TFIIA, TFIIB, and TFIIE cooperate to stimulate the elongation rate in a TFIIF-dependent manner. This observation suggests that these factors may indeed work in concert with TFIIF to wrap DNA template around pol II during elongation.

The results of my research project provide many insights into the functions of RAP74, and the general mechanisms of initiation and elongation. Several assays are well developed and should be very useful in the future study of TFIIF. Directions for future study are discussed.

CHAPTER 2

FUNCTIONS OF THE N-TERMINAL DOMAIN OF RAP74 IN INITIATION AND ELONGATION

INTRODUCTION

RNA polymerase II (pol II) interacts with a number of general and regulatory factors to initiate transcription accurately from a promoter (reviewed in Orphanides et al., 1996; Zawel and Reinberg, 1993). In the pathway toward initiation, promoter DNA is bent, and DNA may be wrapped around pol II (Kim et al., 1997; Robert et al., 1998). General factors TBP (or TFIID), TFIIB, TFIIF, and TFIIE cooperate with pol II to strain the DNA helix around the transcriptional start site before ATP-driven helix opening by TFIIH (Orphanides et al., 1996; Zawel and Reinberg, 1993). After initiation, pol II releases from the promoter (promoter clearance or promoter escape), elongates the RNA chain, terminates transcription, and recycles. TFIIF, made up of RAP30 (RNA polymerase II-associating protein 30 kDa) and RAP74 (58 kDa) subunits, may participate in each of these stages of the transcription cycle.

Inspection of its 517 amino acid (aa) sequence indicates that human RAP74 can be divided into three regions: 1) a highly basic N-terminal domain with significant globular structure (aa 1-217); 2) an overall acidic, highly charged central region lacking in hydrophobic amino acids but rich in E, D, K, R, S, T, G, and P (aa 218-398); and 3) a very basic C-terminal domain with globular structure (aa 399-517) (Aso et al., 1992; Finkelstein et al., 1992). The N-terminal domain is important for RAP30 binding (Wang and Burton, 1995; Yonaha et al., 1993), preinitiation complex assembly (this chapter), and elongation stimulation (Kephart et al., 1994; this chapter). The C-terminal domain of

RAP74 makes contact with TFIIB (Fang and Burton, 1996) and pol II (Wang and Burton, 1995) and stimulates the activity of a pol II carboxy-terminal domain (CTD) phosphatase that may have roles in initiation, elongation, termination, and recycling (Chambers et al., 1995).

A pathway has been defined for assembly of preinitiation complexes on TATA box-containing promoters (Orphanides et al., 1996; Zawel and Reinberg, 1993). The TATA-binding protein (TBP) subunit of TFIID binds to the TATA sequence. Insertion of TBP into the DNA minor groove at TATA induces a 95^0 bend (Kim et al., 1993; Kim et al., 1993). TFIIB can then enter to form the DB complex, made up of TBP (or TFIID), TFIIB, and promoter DNA. The C-terminal repeats of TFIIB bind DNA upstream and downstream of TATA, stabilizing the DNA bend (Lagrange et al., 1996; Nikolov et al., 1995). The N-terminal domain of TFIIB may extend toward the transcriptional start site as a scaffold on which to assemble pol II and TFIIF (Orphanides et al., 1996).

To bind efficiently to the promoter, pol II must first bind TFIIF (Conaway et al., 1991; Flores et al., 1991; Killeen et al., 1992). In some cases, the RAP30 subunit has been sufficient to deliver pol II to the promoter (Flores et al., 1991; Killeen et al., 1992; Tyree et al., 1993), but the RAP74 subunit contributes to proper assembly, complex stability, and initiation. For promoters with weak TATA boxes, both RAP30 and RAP74 contribute to template commitment of TFIID, TFIIB, and pol II (Tan et al., 1994). Furthermore, RAP74 strongly stimulates initiation from supercoiled and pre-melted templates that are dependent only on TBP, TFIIB, pol II, and TFIIF for accurate transcription (Pan and Greenblatt, 1994; Parvin et al., 1994). In most contexts, therefore,

both the RAP30 and RAP74 subunits are important for TFIIF function in complex assembly and initiation.

After fulfilling its role in initiation, TFIIF stimulates the elongation rate of pol II (Bengal et al., 1991; Izban and Luse, 1992; Kephart et al., 1994; Price et al., 1989; Tan et al., 1994). On non-chromatin DNA templates and in the absence of other general factors, TFIIF can accelerate polymerization to up to 1,500 nucleotides per min, close to the estimated physiological rate (Izban and Luse, 1992a). TFIIF suppresses pausing by pol II (Bengal et al., 1991; Izban and Luse, 1992a; Price et al., 1989), but whether this is a cause or effect of rate stimulation is not known. Both the RAP30 and RAP74 subunits of TFIIF are required for elongation stimulation (Kephart et al., 1994; Tan et al., 1994), and preliminary mapping studies indicate that the N-terminal domain of RAP74 is most important for elongation (Kephart et al., 1994). Tan et al. (Tan et al., 1995) identified a class of RAP30 mutants that are impaired for both elongation stimulation and accurate initiation, and these mutants are also defective for binding RAP74, consistent with the requirement of both subunits for elongation. They have also identified classes of RAP30 mutants that are defective only for elongation stimulation or for initiation, but not for both. In contrast to their results with RAP30, however, we find that a region within the N-terminal domain of RAP74, that is not essential for RAP30 binding, is nonetheless strongly stimulatory for both initiation and elongation.

Materials and Methods

Transcription factors and extracts

Recombinant *Saccharomyces cerevisiae* TBP and human recombinant TFIIB were the kind gifts of Steven Triezenberg and Fan Shen. The clone for production of TFIIB was the kind gift of Danny Reinberg. Recombinant human RAP30, RAP74, and RAP74 mutants were prepared and quantitated as described (Fang and Burton, 1996; Wang et al., 1993; Wang et al., 1994; Wang and Burton, 1995). Construction of new mutants is described below. Calf thymus pol II used in electrophoretic mobility shift experiments was prepared by the method of Hodo and Blatti (Hodo and Blatti, 1977) and was primarily in the IIb form, lacking the carboxy terminal domain (CTD). Gel mobility shift experiments with calf thymus pol IIa (the kind gift of Richard Burgess) gave similar results (data not shown).

Human HeLa cells were purchased from the National Cell Culture Center (Minneapolis, MN). Extracts of HeLa cell nuclei were prepared as described (Shapiro et al., 1988). A TFIIF-depleted extract was prepared by immunoprecipitation of TFIIF with anti-RAP30 and anti-RAP74 antibodies (Burton et al., 1988; Finkelstein et al., 1992). The TFIIF-depleted extract was completely dependent on the re-addition of RAP30 for activity and was strongly stimulated by addition of RAP74.

Construction of RAP74 mutants

RAP74(1-217) was constructed by polymerase chain reaction amplification of a plasmid clone encoding RAP74 with primers 5'-CATATGGCGGCCCTAGGCCCT-3' and 5'-CTCGAGAGACATTTCCAGGT-3' and subcloning between the *Nde*I and *Xho*I sites (cloning sites are underlined) of pET21a (Novagen). Three triple-alanine mutations

were constructed in RAP74(1-217), using the Quick Change Site-Directed Mutagenesis Kit (Stratagene) and appropriate primers. These mutant proteins are named RAP74(1-217)170A3, 173A3, and 176A3. Each of these mutant proteins has the sequence AAA beginning at the indicated amino acid, so, for example, 170A3 carries V170A, L171A, and N172A (Fig. 5). Mutated RAP74 genes were confirmed by DNA sequencing.

Electrophoretic mobility shift assay

The DNA probe for the gel shift assay was the adenovirus major late promoter from position -53 to +14 relative to the transcriptional start site. The probe was synthesized by the polymerase chain reaction using the primers: 5'-^{32}P-CAGGTGTTCCTGAAGG-3' and 5'-ATGCGGAAGAGAGTGA-3'. The template for PCR is pML containing the wild type adenovirus major late promoter (-258 to +196). The upstream primer was radiolabeled using γ-^{32}P-ATP and T4 polynucleotide kinase. After amplification, the DNA probe was gel-purified using the Qiaex kit (Qiagen). Mobility shifts were performed according to Wang and Burton (Wang and Burton, 1995) with some modifications. The reaction mixtures (15 ul) contained 20 mM HEPES pH 7.9, 20 mM Tris pH 7.9, 50 mM KCl, 2 mM DTT, 0.5 mg/ml BSA (bovine serum albumin), 10% v/v glycerol, radiolabeled DNA probe, and proteins, incubated at 30 ^{o}C. *Saccharomyces cerevisiae* TBP (0.3 pmol) was combined with the DNA probe (approximately 40 fmol) for 15 min. Recombinant human TFIIB (0.3 pmol) was then added and incubated for 15 min. Calf thymus pol II (0.15 pmol) was incubated with human recombinant TFIIF (0.1 pmol) for at least 5 min prior to addition to the DB complex. For reactions involving separate TFIIF subunits, pol II was incubated with RAP30 for 5 min and then mixed with RAP74 or a RAP74 mutant and pre-incubated for

an additional 5 min before addition to DB and further incubation for 15 min. It appeared that prior addition of RAP30 to pol II aided assembly of RAP74 into DBPolF. Reaction mixtures were loaded onto a 4% polyacrylamide gel containing 0.09 % bisacrylamide, 2.5 % glycerol and 0.5X TBE (tris-borate-EDTA). Dried gels were analyzed by autoradiography.

Transcription assays

Immobilized templates

Preparation of immobilized templates was adapted from published methods (Arias and Dynan, 1989; Marshall and Price, 1992). DNA containing the adenovirus major late promoter was synthesized by the polymerase chain reaction using an upstream 5'-biotinylated primer. The template for amplification was a pBluescript II SK(-) vector (Stratagene) containing the adenovirus major late promoter (-258 to +196) subcloned between the XhoI and HindIII sites of the plasmid. The sequence of the upstream primer was 5'-biotin-CCCTCGAGCGGTGTTCCGCGGTCCTCCTCG-3', and the sequence of the downstream primer was 5'-CGGTGGCGGCCGCTCTAGAACTAGTGGATC-3'. The template extended from positions -263 to +251. Biotinylated DNA was incubated with streptavidin paramagnetic beads (CPG) in 2 M NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.5 for 15 min at room temperature. Immobilized templates were collected with a magnetic particle separator (CPG), washed 4 times and stored at 4°C in phosphate buffered saline pH 7.2 containing 1 mg/ml BSA and 0.03% NaN_3.

Pulse-spin and pulse-chase single-round assays

A 20 ul reaction mixture consisted of 2 ul paramagnetic beads (about 0.6 ug DNA) carrying the adenovirus major late promoter and TFIIF-depleted transcription

extract (72 ug total protein) supplemented with recombinant RAP30 and RAP74 or a RAP74 mutant (10 pmol each), in transcription buffer (12 mM HEPES, pH 7.4, 12% glycerol, 0.12 mM EDTA, 0.12 mM EGTA, 1.2 mM DTT) containing 60 mM KCl and 12 mM $MgCl_2$. Preinitiation complexes were formed for 60 min at 30 °C. 100 uM ATP, CTP, GTP and 1 uM α^{32}P-UTP (10 μCi per reaction) were added to initiate transcription for 1 min. For the pulse-spin protocol, template-associated complexes were diluted with 200 ul transcription buffer containing 60 mM KCl and 1 mg/ml BSA, isolated by centrifugation, and extracted from beads by boiling in 20 ul 90 % v/v formamide, 1 % SDS, 10 mM Tris pH 7.9, 1 mM EDTA, 0.01 % bromophenol blue and 0.01 % xylene cyanol. For the pulse-chase protocol, instead of diluting and centrifuging samples, 1 mM ATP, UTP, GTP, and CTP were added 1 min after addition of NTPs, and elongation continued for 10 min. Reactions were stopped by addition of 200 ul 0.1 M sodium acetate pH 5.4, 0.5 % SDS, 2 mM EDTA, and 100 ug/ml tRNA, followed by phenol-chloroform extraction and ethanol precipitation. Samples were electrophoresed in a 10 % polyacrylamide gel containing 1X TBE and 50 % w/v urea. For quantitation of the gel, the signal in the presence of RAP30 and the absence of added RAP74 was used to estimate background (Figure 2, lanes 21 and 22). The weak signal obtained in the absence of added RAP74 was attributed to residual RAP74 remaining in the TFIIF-depleted extract.

Elongation stimulation assay

Stimulation of pol II elongation was determined by adding TFIIF subunits to transcriptionally engaged pol II molecules that were washed free of associated elongation factors. A 20 ul reaction mixture consisted of 2 ul paramagnetic beads carrying the

adenovirus major late promoter, transcription extract derived from HeLa cell nuclei (108 ug total protein), and transcription buffer containing 60 mM KCl and 12 mM $MgCl_2$. Samples were incubated 60 min at 30 ^oC to form preinitiation complexes. Transcription was initiated by addition of 100 uM ATP, GTP, CTP, and 1 uM (10 uCi) α-^{32}P-UTP (2 ul volume). After 1 min, elongation complexes were diluted in 200 ul transcription buffer containing 500 mM KCl and 1 mg/ml BSA and isolated by centrifugation. The purpose of this treatment was to remove accessory factors from the elongation complex. The 500 mM KCl wash appeared to be effective because elongation rate stimulation was highly dependent on addition of both RAP30 and RAP74 (Fig. 3). Complexes were diluted with transcription buffer containing 60 mM KCl and 1 mg/ml BSA and isolated by centrifugation two times. Complexes were then resuspended in 20 ul transcription buffer containing 60 mM KCl and 12 mM $MgCl_2$, recombinant RAP30 and RAP74 or a RAP74 mutant were added (10 pmol each) and incubated for 5 min. 100 uM ATP, GTP, CTP, and UTP were added in 2 ul, and transcripts were elongated for 2 min. Transcripts were isolated by phenol extraction and ethanol precipitation and electrophoresed in a 6% polyacrylamide gel, as described above. Accurate transcription was quantitated for all of the transcripts from +122 to +251. Elongation stimulation was determined by subtracting the average value for samples containing RAP30 but no RAP74 as background (Fig. 3A, lanes 14 and 15) and expressed as percent of the highest signal obtained for RAP74 (lane 3).

RESULTS

The N-terminal domain of RAP74 supports preinitiation complex assembly

A primary function of TFIIF in accurate initiation is to deliver pol II to the promoter, so we used an electrophoresis mobility shift assay to determine which regions of RAP74 were required to bring pol II into a stable complex with adenovirus major late promoter DNA, TBP (D), TFIIB, and RAP30 (DBPolF complex) (Fig. 1). By itself, TBP did not efficiently induce a shift of the promoter fragment (lane 2). Upon addition of TFIIB, however, a DB complex consisting of TBP, TFIIB, and promoter DNA was observed (lane 3). When pol II was added to DB, a weak DBPol shift was seen (lane 4). RAP30 alone did not stimulate pol II binding to DB (lane 6), nor did RAP74 (data not shown). The TFIIF complex and separately added RAP30 and RAP74 subunits, however, supported assembly of DBPolF (lanes 5 and 7). RAP74(1-172) was minimally required to support assembly (lane 10). A number of RAP74 mutants that have been shown to be defective for RAP30 binding (Wang and Burton, 1995; Yonaha et al., 1993) failed to support formation of DBPolF (lanes 11-15). The different mobilities of complexes containing RAP74 deletion mutants may be attributable to differences in the charge or the degree of DNA bending or flexibility in the complex. Because RAP74(1-517), (1-296), (1-205), and (1-172) are predicted to carry charges of 0, -4, +6, and +7, however, it is difficult to account for all observed mobility differences solely on the basis of charge. For instance, RAP74(1-172) is predicted to be more basic than RAP74(1-205) and yet supported a DBPolF complex with a faster mobility. Furthermore, RAP74(1-205) and (1-172) have different transcriptional activities (see below).

Figure 1. An electrophoretic mobility shift assay was used to analyze the requirement for RAP74 to form DBPolF. The probe was the adenovirus major late promoter between positions -53 and +14. Key: D) recombinant yeast TBP (0.3 pmol); B) recombinant human TFIIB (0.3 pmol); Pol) calf thymus pol II (0.15 pmol); F) recombinant human TFIIF complex or RAP30 and RAP74 or a RAP74 mutant, added separately (0.1 pmol). DBPolF* indicates the different mobilities of complexes containing different RAP74 mutants.

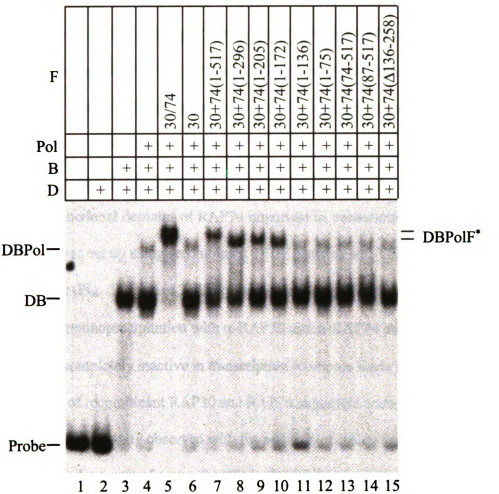

F
Pol
B
D
30
30/74
30+74(1-517)
30+74(1-296)
30+74(1-205)
30+74(1-172)
30+74(1-136)
30+74(1-75)
30+74(74-517)
30+74(87-517)
30+74(Δ136-258)
DBPol
DB
Probe
DBPolF*
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Figure 1

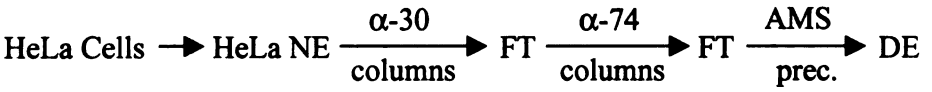
Tyree et al. (Tyree et al., 1993) reported DBPolF30 and DBPolF74 complexes, which formed with only the RAP30 or the RAP74 subunit of TFIIF, but these investigators used a different promoter and *Drosophila* TBP, TFIIB, pol II and human TFIIF, rather than yeast TBP, human TFIIB, human TFIIF, and bovine pol II, as used in this study. Killeen et al. (Killeen et al., 1992) and Flores et al. (Flores et al., 1991) also reported DBPol30 and DABPol30 complexes using very different buffer conditions from those used here. Although RAP74 was not essential for assembly in those studies, it was strongly stimulatory. This is the first report that shows the minimal region of RAP74 that stimulates incorporation of pol II into DBPolF.

The N-terminal domain of RAP74 supports accurate initiation

To map the functional domains of RAP74 important in transcription, a reconstitution assay was set up using recombinant TFIIF proteins and TFIIF-depleted HeLa nuclear extracts (Fig. 2). Native TFIIF proteins were depleted from the HeLa nuclear extracts by immunoprecipitation with α-RAP30 and α-RAP74 antibodies. The depleted extract was completely inactive in transcription (compare lanes 3 and 2, Fig. 2A), and the addition of recombinant RAP30 and RAP74 supported accurate transcription (lanes 6-9, Fig. 2A). The activity observed with the addition of recombinant RAP30 was attributed to the residual amount of native RAP74 in the depleted extract (lane 4). When a saturating amount of RAP30 was used, the level of transcription saturated in the presence of 2 pmol of RAP74 (lanes 3-6, Fig. 2B).

To determine which regions of RAP74 are most important for initiation, RAP74 mutants were tested for accurate initiation and runoff transcription from the adenovirus major late promoter (Fig. 3). RNA within the early elongation complex was visualized

Figure 2. Accurate initiation from an adenovirus major late promoter in the HeLa extract is dependent on both RAP30 and RAP74 subunits of TFIIF. A TFIIF-depleted extract was prepared by immunochromatography with α-RAP30 and α-RAP74 antibody columns, and concentrated by amonium sulfate (AMS) precipitation. A) Reactions (20 ul each) contained 800 ng pML digested with SmaI, 4 ul HeLa nuclear extracts (NE, lanes 1 and 2), or various amounts of TFIIF-depleted extracts as indicated (DE, lanes 3-9) supplemented with 10 pmol recombinant human RAP30 (lane 4), or RAP74 (lane 5), or RAP30/RAP74 complex (lanes 6-9), in transcription buffer containing 60 mM KCl and 12 mM $MgCl_2$. In lane 1, 200 ng α-amanitin was also included. After 60 min incubation, 600 uM ATP, GTP, CTP, and 25 uM α-^{32}P-UTP (10 uCi per reaction) were added and reactions continued for 30 min at 30 ^{0}C. The reactions were stopped and RNA transcripts were isolated and analyzed on a 6% polyacrylamide gel containing 50% urea. B) Similar to A, except increasing amounts of recombinant RAP74 (0, 1, 2, 5 pmol) were added in lanes 3-6. 10 pmol recombinant RAP30 was added in lanes 3-6. Reactions also contained either 4 ul NE (lane 1) or 6 ul DE (lanes 2-6).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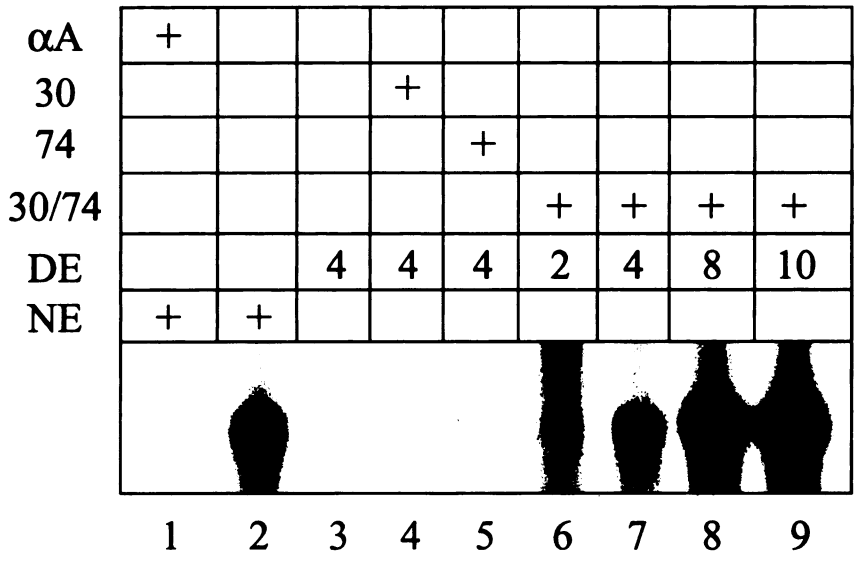
HeLa NE
α-30
columns
FT
α-74
columns
FT
AMS
prec.
DE

A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30
74
30/74
DE
NE
+
4 4 4 2 4 8 10
1 2 3 4 5 6 7 8 9

B

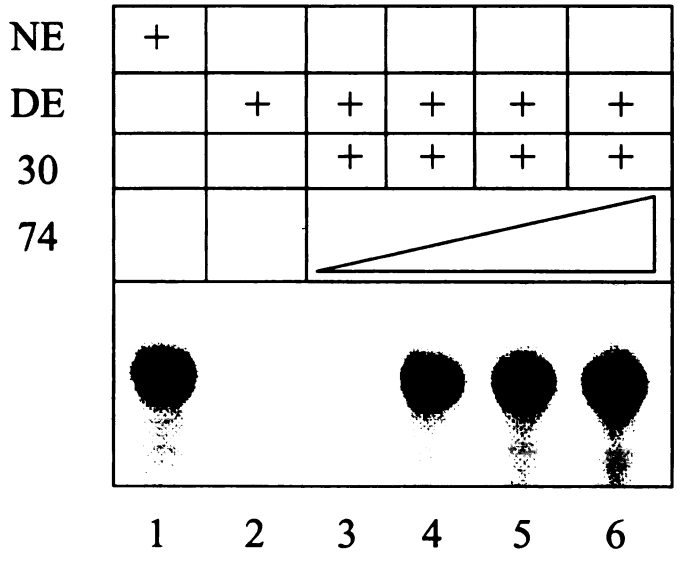
NE
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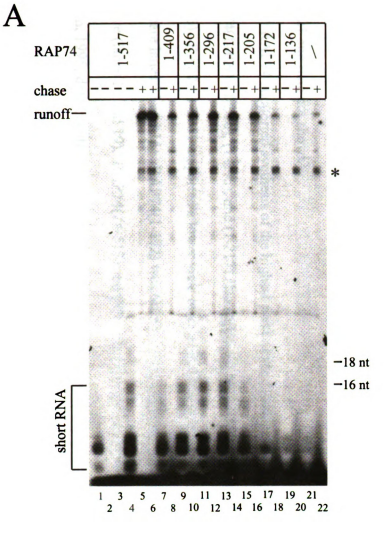
Figure 2

Figure 3. Regions of RAP74 required for accurate initiation. A) Short and runoff RNAs accurately initiated from the adenovirus major late promoter. All lanes contained TFIIF-depleted extract (DE; 72 μg total protein), recombinant human RAP30 (10 pmol), and RAP74 or a RAP74 mutant (10 pmol), preincubated with immobilized template for 60 min. Transcripts were initiated with all four NTPs and radiolabeled with α^{32}P-UTP (ACGU*) for 1 min. For the pulse-chase protocol, samples were chased by addition of 1 mM each NTP for 10 min (+). For the pulse-spin protocol, initiated complexes were diluted with transcription buffer and centrifuged briefly to isolate short, template-associated RNAs (-). The approximate sizes of short RNAs can be estimated by comparison to 5'-phosphorylated 16 and 18 nucleotide (nt) DNA markers. In lane 1, AMPPCP (a β-γ non-hydrolyzable ATP analogue) and 2'-3' dideoxy ATP (ddA) were substituted for ATP. In lane 2, AMPPCP was substituted for ATP. In lane 3, 1 μg/ml α-amanitin was included in the reaction. The gel band indicated with an asterisk is not a pol II transcript because it is synthesized in the presence of 1 μg/ml α-amanitin (data not shown). B) Phosphorimager quantitation of the data shown in "A" combined with data from two other experiments, reported as average +/- standard deviation. Short transcripts generated in the presence of RAP74 are expressed as 100%.


A
RAP74
1-517
1-409
1-356
1-296
1-217
1-205
1-172
1-136
—
chase
runoff
*
−18 nt
−16 nt
short RNA
1 3 5 7 9 11 13 15 17 19 21
2 4 6 8 10 12 14 16 18 20 22

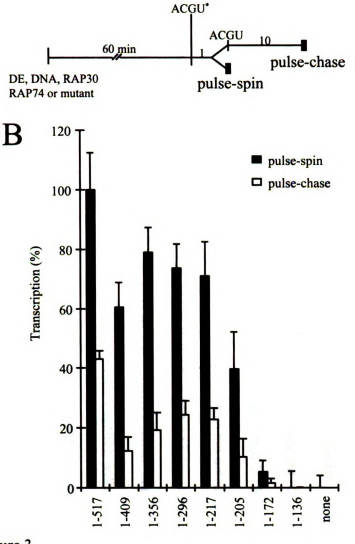

ACGU*
ACGU
10
60 min
1
pulse-chase
pulse-spin
DE, DNA, RAP30
RAP74 or mutant
B
pulse-spin
pulse-chase
Transcription (%)
120
100
80
60
40
20
0
1-517
1-409
1-356
1-296
1-217
1-205
1-172
1-136
none

Figure 3

directly on gels in the "pulse-spin" protocol or extended to the runoff position in the "pulse-chase" protocol. Both protocols limited transcription to a single round. In the pulse-spin protocol, pol II remained close to the promoter, blocking a second round of transcription. In the pulse-chase protocol, a 1000-fold excess of unlabeled UTP was added during the "chase"; so, although reinitiation could occur, it was not detected.

By several criteria, short transcripts produced in the pulse-spin protocol were inferred to be accurately initiated from the adenovirus major late promoter. Short RNAs were template-associated because they could be isolated by centrifugation with beads (lanes designated "-" for no chase). Short RNAs were chased to the predicted runoff position of +251 with addition of NTPs (lanes designated "+"). As expected for pol II transcripts, synthesis of short RNAs was completely dependent on ATP with a hydrolyzable β-γ bond (compare lanes 1 and 2). Synthesis of short RNAs was sensitive to 1 ug/ml α-amanitin (compare lanes 3 and 4) and was RAP30- and RAP74-dependent (lane 21 and data not shown).

RAP74 supported synthesis of the highest yield of short RNAs (lane 4), but RAP74(1-409), (1-356), (1-296), and (1-217) also supported transcription (lanes 7, 9, 11, and 13). RAP74(1-205) supported a lower yield of short RNA than RAP74(1-217) (compare lanes 13 and 15). RAP74(1-172) was barely active, and RAP74(1-136) was inactive (lanes 17 and 19). RAP74(74-517) and (87-517) were also inactive (Wang and Burton, 1995; data not shown). For accurate initiation, therefore, the region of RAP74 between aa 1-205 was necessary, and the region between aa 205-217 was stimulatory.

Comparison of the activities of deletion mutants showed that the regions between aa 136-217 and 1-74 were very important for initiation.

A very similar conclusion was reached from inspection of runoff transcripts (Fig. 3A, lanes designated "+"; Fig. 3B, white bars). Because RNA was labeled by comparable procedures in the pulse-chase and pulse-spin reactions, yields of transcript in the pulse-chase reactions are reported as percent of the highest signal observed using the pulse-spin protocol. RAP74 had the highest activity in runoff transcription (lanes 5 and 6). RAP74(1-296) and (1-217) were about 75 % as active as RAP74 (lanes 12 and 14). RAP74(1-205) had reduced activity (lane 16). RAP74(1-172) was almost inactive (lane 18), and RAP74(1-136) was inactive (lane 20). These data confirmed that the most important region of RAP74 for supporting accurate initiation was located within aa 1-217 and the region between aa 172-217 was critical for activity.

RAP74(1-409) and (1-356) had surprisingly low activities in runoff transcription (lanes 8 and 10), a result that has been reproduced in several experiments. RAP74(1-409) also has a partial defect in elongation stimulation (data not shown). Deletion of sequence between aa 296-356 relieves these defects, because RAP74(1-296) and (1-217) have higher activities than (1-409) and (1-356) in runoff transcription (compare lanes 8, 10, 12, and 14). Because RAP74(1-409) and (1-356) appeared to initiate more efficiently than to form runoff transcripts, these mutants may form complexes with a tendency to release abortive transcripts.

Background transcription in this and other experiments appeared to be due to residual RAP74 in the TFIIF-depleted extract (lanes 19-22). Accurate transcription was not detected when RAP30 was omitted from the reaction (data not shown) indicating that

RAP30 was more efficiently depleted than RAP74. Another observation was that a significant proportion of short RNAs were released as abortive transcripts. Comparing the ratio of accurately initiated transcripts in the pulse-spin protocol (Fig. 3B; black bars) to runoff products observed in the pulse-chase protocol (white bars), less than half of the short transcripts were recovered at the runoff position.

The N-terminal domain of RAP74 stimulates elongation by pol II

Because preinitiation complex assembly (Fig. 1) and single-round initiation (Fig. 3) were supported by the N-terminal domain of RAP74, we wondered whether the same or distinct regions might stimulate elongation by pol II (Fig. 4). Consistent with previous reports (Kephart et al., 1994; Tan et al., 1994), both RAP30 and RAP74 were required to stimulate elongation (Fig. 4A, lanes 1 and 14-17). RAP74(1-217) stimulated elongation to about the same extent as RAP74(1-296) and RAP74. RAP74(1-205), however, stimulated elongation at a reduced level, and RAP74(1-172) was inactive or nearly so. The region between aa 1-205, therefore, appeared to be essential for elongation stimulation, and the region between aa 205 and 217 was strongly stimulatory. RAP74(74-517) and (87-517), from which N-terminal sequences were deleted, did not stimulate pol II elongation (Kephart et al., 1994; data not shown).

The activities of the RAP74 mutants for elongation stimulation (Fig. 4B) appeared to be very similar to the activities obtained for accurate initiation and runoff transcription (Fig. 3B), as if very similar RAP74 N-terminal domain sequences were important for both processes. To challenge this idea, pulse-spin, pulse-chase, and elongation stimulation data were plotted on the same graph, using the sample containing RAP30 but no RAP74 as background and the sample containing RAP74(1-296) as 100% signal. The

Figure 4. The region of RAP74 between aa 172-217 is critical for both accurate initiation and elongation stimulation. A) Elongation stimulation assay. ^{32}P-labeled short RNAs were accurately initiated from the adenovirus major late promoter immobilized on beads. Complexes were washed with 0.5 M KCl to remove associated elongation factors from pol II. RAP30 (10 pmol) and RAP74 or a RAP74 mutant (10 pmol) were added and incubated for 5 min. All four NTPs (100 μM each) were added and RNA chains elongated for 2 min. B) Phosphorimager quantitation of the data shown in "A" for transcripts between +137 and +251 in length. C) Comparison of elongation stimulation (Figure 4B), pulse-spin, and pulse-chase data (from Fig. 3). Values for RAP74(1-296) are reported as 100 % of signal.

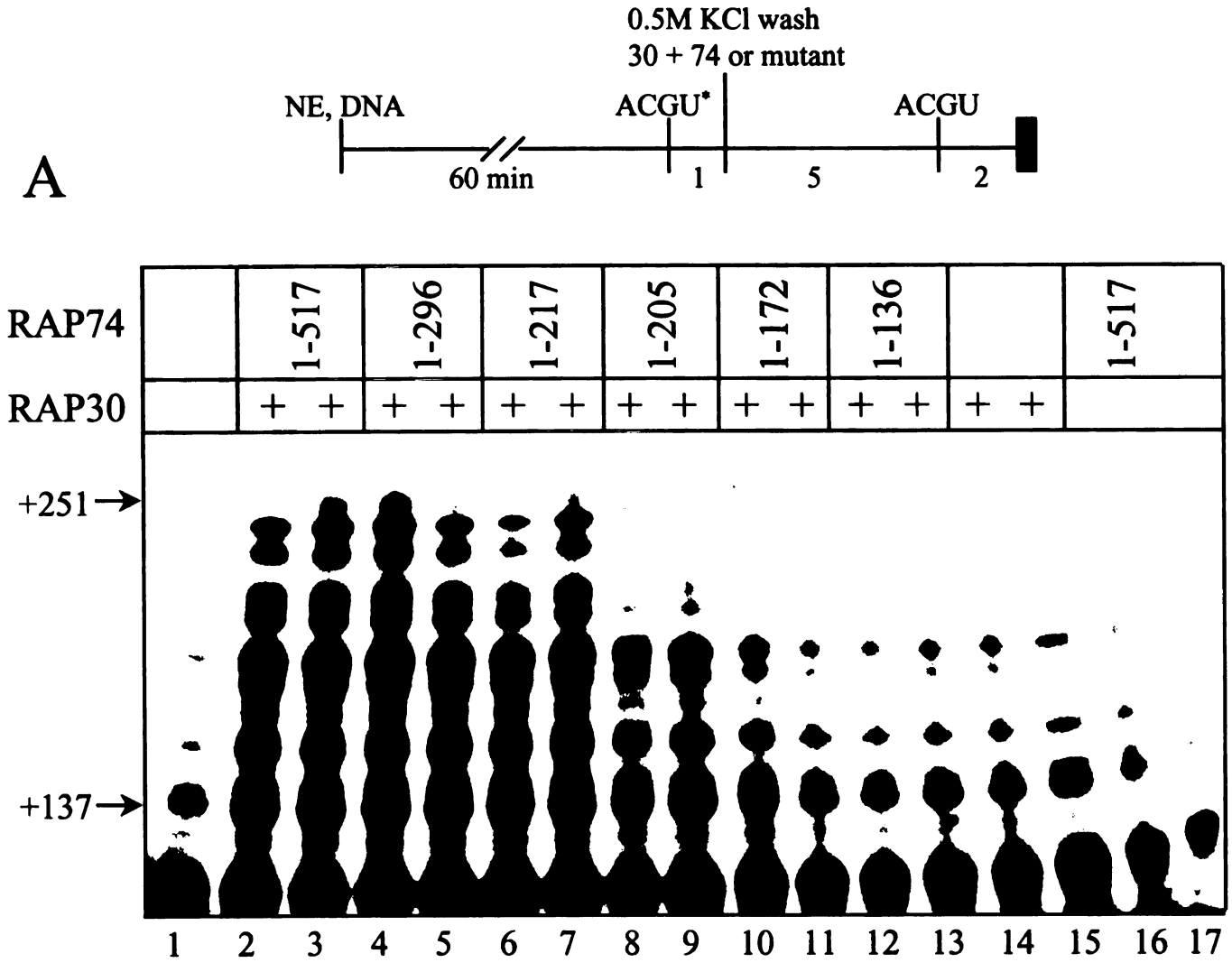

A
0.5M KCl wash
30 + 74 or mutant
NE, DNA
ACGU*
ACGU
60 min
1
5
2
RAP74
RAP30
1-517
1-296
1-217
1-205
1-172
1-136
1-517
+251
+137
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

Figure 4

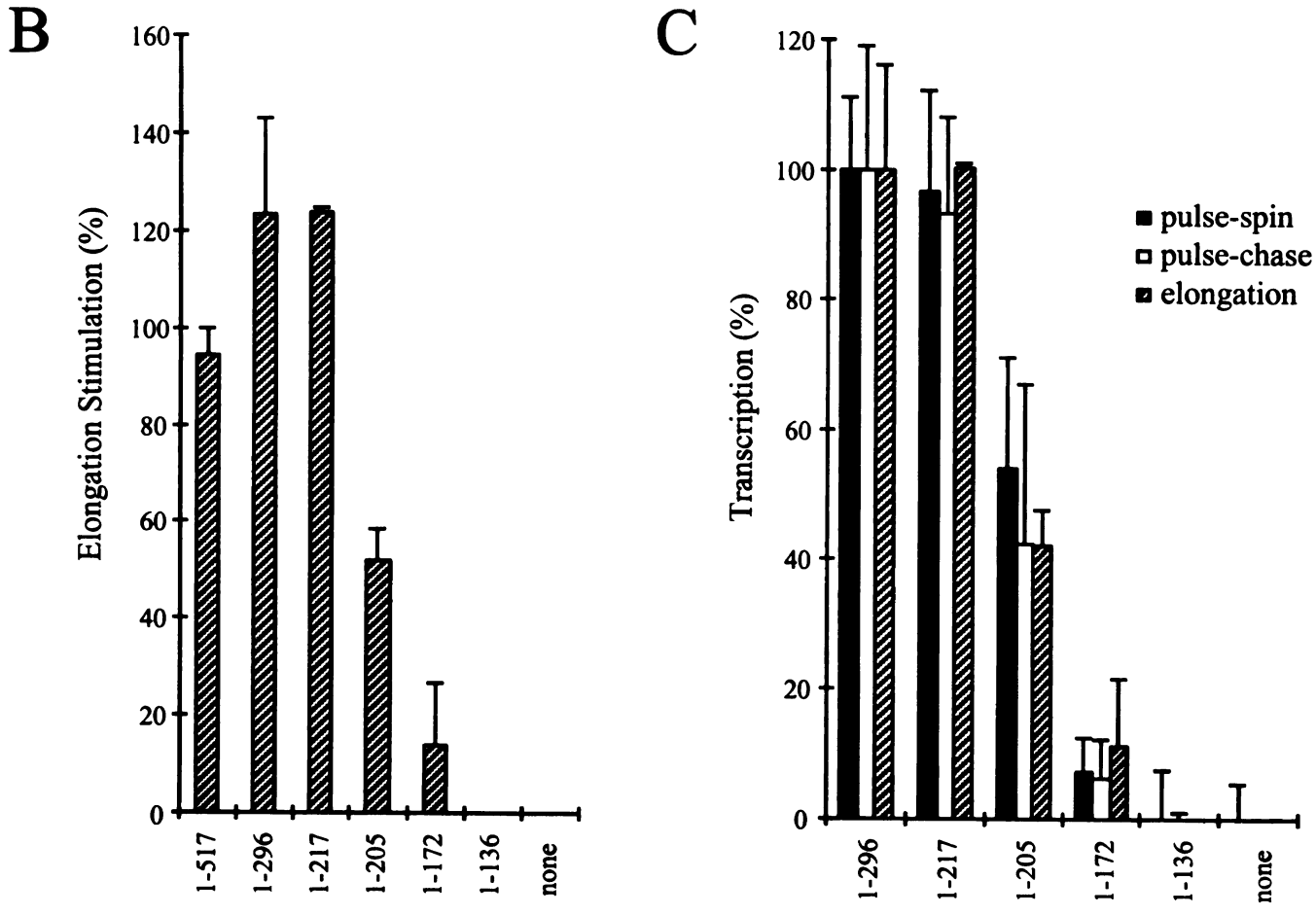

B
Elongation Stimulation (%)
160
140
120
100
80
60
40
20
0
1-517
1-296
1-217
1-205
1-172
1-136
none
C
Transcription (%)
120
100
80
60
40
20
0
pulse-spin
pulse-chase
elongation
1-296
1-217
1-205
1-172
1-136
none

Figure 4

RAP74(1-296) sample was selected as the highest value in order to eliminate the influence of the C-terminal domain on initiation from the comparison. As can be seen in Fig. 4C, the region of RAP74 required to support accurate initiation, runoff transcription, and elongation stimulation was the same.

To investigate this issue in more detail, three triple-alanine substitution mutants were constructed within this critical region (Fig. 5). The target for mutation, between aa 170-178, was selected because this region is evolutionarily conserved (Wang and Burton, 1995), and interestingly, this sequence is strongly predicted to be α-helical for diverse eukaryotic species including yeast, *Drosophila*, *Xenopus*, and human. RAP74(1-217)170A3, 173A3, and 176A3 were constructed in the RAP74(1-217) deletion mutant. The triple-alanine mutants showed significant defects in both accurate initiation and elongation stimulation (Fig. 5), as expected from the results with deletion mutants (Fig. 4C). The region of RAP74 between aa 170-178, therefore, was very important for both accurate initiation and elongation.

Figure 5. A conserved region of RAP74 between amino acids 170-178 is important for both initiation and elongation. A) Triple-alanine substitutions, 170A3, 173A3, and 176A3, constructed in RAP74(1-217), are indicated. Related sequences from human (hRAP74), *Xenopus* (xRAP74), *Drosophila* (dF5a), and yeast (ySsu71/Tfg1) are shown. B) RAP74(1-217)170A3, 173A3, and 176A3 were compared with RAP74(1-517) and (1-217) in pulse-chase initiation (top panel) and in elongation stimulation (lower panel) assays. RAP74 samples were reconstituted with RAP30 in vitro prior to assay. Pulse-chase initiation reactions contained TFIIF-depleted extract with the indicated TFIIF or TFIIF mutant (10 pmol TFIIF complex) combined with an adenovirus major late promoter template digested with SmaI at position +217. The protocol for the initiation assay was as in Fig. 2, except that the DNA template was not immobilized and the elongation time was 30 min. Elongation stimulation reactions contained salt-washed elongation complexes supplemented with the indicated TFIIF or TFIIF mutant protein (20 pmol) (as in Fig. 3). Lane 1 contains reconstituted TFIIF and 1 μg/ml α-amanitin. Lane 14 contains no added TFIIF. Lanes 2 and 3 contain 10 pmol and lanes 15 and 16 contain 20 pmol RAP30 but no added RAP74. All other lanes are as indicated above both panels. C) Phosphorimager quantitation of the data shown in "B". Elongation stimulation was calculated for the +192 to +251 transcripts.

A

```
                          (173A3)
                           AAA
                    AAA          AAA
                  (170A3)      (176A3)
hRAP74  168  NK VLNHFSIMQ QRRLK  183
xRAP74  168  NK VLNHFTIMQ QRRLK  183
dF5a    195  KK VMNYFSLML RKRLR  210
ySsu71  422  RW LMKHLDNIG TTTTR  437
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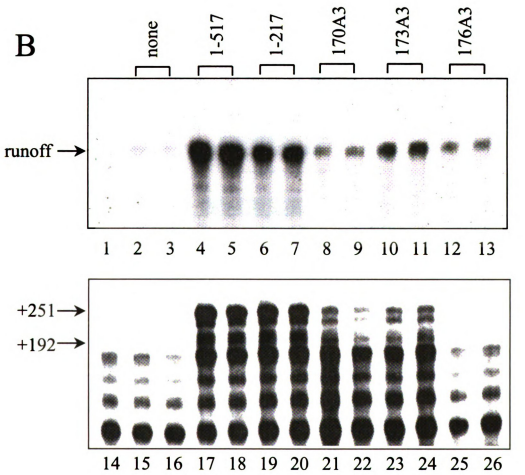

Figure 5

C

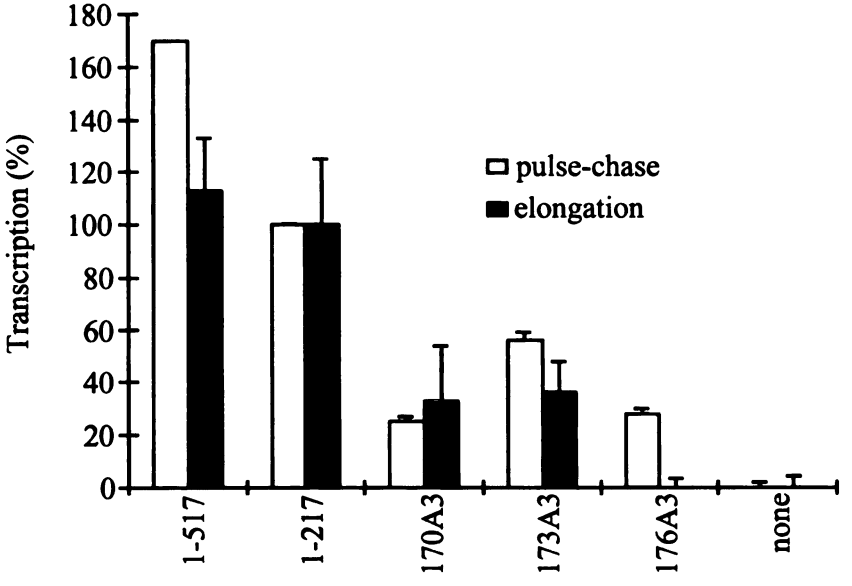
180
160
140
120
100
80
60
40
20
0
Transcription (%)
pulse-chase
elongation
1-517
1-217
170A3
173A3
176A3
none

Figure 5

DISCUSSION

From primary sequence (Aso et al., 1992; Finkelstein et al., 1992), RAP74 is proposed to have highly basic N- and C-terminal domains separated by a highly charged, overall acidic, and flexible central region that is rich in charged amino acids, E, D, K, and R, and also S, T, P, and G. In this chapter, we show that these primary sequence features correspond to distinct N- and C-terminal functional domains. The N-terminal domain is sufficient to support preinitiation complex assembly, single-round initiation, and elongation by pol II.

The RAP74 N-terminal domain extending from aa 1-217 supports most RAP74 functions for preinitiation complex assembly (Fig. 1), accurate initiation (Fig. 3), and elongation stimulation (Figs. 4 and 5). In this work, we have mapped a critical region for these functions between aa 136-217. RAP74(1-172) binds tightly to the RAP30 subunit, but RAP74(1-136) does not (Wang and Burton, 1995; Yonaha et al., 1993). Consistent with its capacity to bind RAP30, RAP74(1-172) is minimally sufficient to support pol II assembly into DBPolF (Fig. 1). Fulfilling this role in assembly, however, is not sufficient to support TFIIF function in initiation or elongation, which minimally requires RAP74(1-205) (Fig. 3). RAP74(1-217) is significantly more active than RAP74(1-205) in both accurate initiation and elongation stimulation (Figs. 3 and 4).

Recent site-specific DNA photocrosslinking studies show that RAP74 induces a significant conformational change within the DBPolFE preinitiation complex (Forget et al., 1997; Robert et al., 1996; Robert et al., 1998). This conformational change is referred to as "isomerization" to compare it with isomerization of the *E. coli* preinitiation complex, which involves similar changes in protein conformation (Roe et al., 1985) and

wrapping of promoter DNA around RNA polymerase (Craig et al., 1995; Polyakov et al., 1995). Interestingly, RAP74(1-205), which is minimally required for accurate initiation, also minimally supports isomerization of DBPolFE as indicated by development of a number of specific DNA photocrosslinks with the RPB1 and RPB2 subunits of pol II, with RAP30, and with TFIIE34 (Forget et al., 1997; Robert et al., 1996; Robert et al., 1998). Because RAP74(1-172) is sufficient for tight RAP30 binding and DBPolF assembly but insufficient for isomerization of DBPolFE, accurate initiation or elongation stimulation, RAP74(1-217) and (1-205) appear to have functions that are not communicated to pol II directly through the RAP30 subunit. Photocrosslinking studies have indicated that RAP74 approaches the adenovirus major late promoter at numerous positions extending all the way from –56/-61 upstream of TATA to +26 downstream of +1 (about 280 angstroms of B-form DNA) (Robert et al., 1998; Forget et al., 1997). This extensive crosslinking "footprint" is the basis for one argument in favor of DNA wrapping around pol II in DBPolFE and also for an $\alpha_2\beta_2$ heterotetrameric form of TFIIF in the complex (Robert et al., 1998). Because RAP74 interacts with promoter DNA and induces isomerization of DBPolFE, RAP74 appears to support DNA wrapping both by contacting DNA directly and by modifying the contacts of RAP30, pol II, and TFIIE34 with DNA (Robert et al., 1998; Robert et al., 1996; Forget et al., 1997). The region of RAP74 between aa 172-205, therefore, appears to stimulate transcription by helping DNA to wrap around pol II. Wrapping may result from direct interactions between DNA and aa 172-205 or this region may be involved in protein-protein interactions that facilitate wrapping. Recent work indicates that aa 172-205 is involved in dimerization of RAP74 (Robert et al., 1998). It is not clear whether the adjacent region of RAP74 from

aa 205-217, which also stimulated initiation and elongation, contributed to DNA wrapping or another function. It is interesting to note that DBPolF complexes containing RAP74(1-517), (1-296), (1-205), and (1-172) had different electrophoretic mobilities that could have been caused by differences in the degree of DNA bending or flexibility (Fig. 1). Differences in mobility might relate to the degree of DNA bending caused by partial or complete isomerization of DBPolFE in the presence of TFIIF mutants.

RAP74(1-217), (1-205), and (1-172) showed a spectrum of decreasing activities in both accurate initiation and elongation stimulation (Fig. 4C). Triple alanine mutants in this region, RAP74(1-217)170A3, 173A3, and 176A3, were also significantly affected for both initiation and elongation (Fig. 5). The 170A3 mutant was more defective in initiation than 173A3, but they had very similar defects in elongation. Perhaps more interestingly, the 176A3 mutant was partially active in initiation but inactive for elongation stimulation. The initiation assay is more complex than the elongation assay, because initiation is influenced by all of the general transcription factors and some regulatory factors in the extract system, and the elongation assay may only involve elongating pol II and TFIIF. In the initiation assay, therefore, general or regulatory factors could partially complement TFIIF activity. 176A3, therefore, might be partially complemented for its function in initiation by interaction with a general or regulatory transcription factor, which is present in the cell extract but absent in salt-washed elongation complexes. Conceivably, re-addition of such a factor to the elongation complex might relieve the 176A3 defect in elongation.

Because the same region of RAP74 contributed strongly to both initiation and elongation, RAP74 may perform a similar role in both processes. In preinitiation

complex assembly, the role of RAP74 appears to be to wrap DNA around pol II and to isomerize the complex (Robert et al., 1998). If RAP74 has a similar role in elongation, it is also likely to involve DNA wrapping around pol II. In initiation, DNA wrapping is induced around eukaryotic RNA polymerase I (Schultz et al., 1993), RNA polymerase II (Kim et al., 1997; Robert et al., 1998) and prokaryotic RNA polymerase (Craig et al., 1995; Polyakov et al., 1995).

It was somewhat surprising that the sequence requirements for RAP74 were so similar for initiation and elongation, because a very different conclusion was reached for the RAP30 subunit of TFIIF (Tan et al., 1995). Although RAP30 mutations that fail to bind RAP74 were found to be severely defective for both initiation and elongation, mutations in other regions of RAP30 affected initiation and elongation in different ways. RAP30 mutations within a presumed pol II binding region were defective in elongation stimulation but not in initiation. RAP30 mutations within a DNA-interacting region were defective for accurate initiation but not elongation stimulation (Tan et al., 1995). These results may indicate that RAP30 has distinct roles in initiation and elongation, although RAP74 appears to fulfill a common role in both processes. Because DNA-binding regions of RAP30 appear to be critical for initiation but dispensable for elongation, RAP30 may interact specifically with DNA in the preinitiation complex. During elongation the DNA sequence encountered by pol II is in flux, and RAP30 may make less extensive template contacts. RAP30 is also likely to make reduced protein-protein contacts as initiation factors dissociate from the elongation complex.

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CHAPTER 3

SITE-DIRECTED MUTAGENESIS OF THE N-TERMINAL DOMAIN OF RAP74: CRITICAL AMINO ACID RESIDUES INVOLVED IN ELONGATION

Introduction

Accurate initiation from human pre-messenger RNA promoters requires the cooperation of RNA polymerase II (pol II) and general transcription factors (Orphanides et al., 1996; Conaway and Conaway, 1993; Roeder, 1996). An ordered pathway for assembly of an active transcription complex on a promoter containing a TATA box has been defined in vitro (Orphanides et al., 1996; Conaway and Conaway, 1993; Zawel and Reinberg, 1993). TBP binds to the TATA element. TFIIB then associates with TBP and promoter DNA to form a TBP-TFIIB-promoter complex. TFIIF escorts pol II into the TBP-TFIIB-promoter complex, and TFIIF is required for stable binding and retention of pol II. TFIIE further stabilizes the complex and is necessary to recruit TFIIH. TFIIH has ATP-dependent DNA helicase and CTD kinase activities. The TFIIH helicase activities are believed to open the DNA helix for formation of the open complex prior to initiation (Orphanides et al., 1996).

Human TFIIF is an $\alpha_2\beta_2$ heterotetramer of RAP30 and RAP74 subunits. Both subunits participate in recruitment of pol II to the promoter and both are necessary for accurate initiation from linear DNA templates in vitro (Lei et al., 1998; Tan et al., 1994). Deletion mutagenesis of the gene encoding the 517 amino acid (aa) RAP74 subunit revealed an important region extending from aa 136 to 217 (Lei et al., 1998; Chapter 2). RAP74(1-136) does not bind to RAP30 and is inactive in forming the transcription

complexes and in transcription assays. RAP74(1-172) forms transcription complexes readily but has weak activity in transcription. RAP74(1-205) is more active than RAP74(1-172), and RAP74(1-217) has similar activity to RAP74(1-517, wt).

TFIIF was recently shown to have a fundamental role in isomerization of the pol II preinitiation complex (Forget et al., 1997; Robert et al., 1998). Site-specific photocrosslinking analyses of complexes containing an adenovirus major late promoter (AdMLP), TBP, TFIIB, TFIIF, TFIIE, and RNA polymerase II demonstrate that TFIIF induces promoter DNA to wrap tightly around RNA polymerase II and the general factors. TFIIF containing RAP74(1-172), although fully assembled, did not support the tightly wrapped DNA structure. TFIIF containing RAP74(1-205) and more complete versions of RAP74, however, did support this isomerized and more active structure. The region of RAP74 between aa 172 to 205, therefore, appears to have a critical function in complex isomerization, and the isomerization appears to involve tight DNA wrapping around pol II and the general factors.

In addition to its roles in preinitiation complex assembly, isomerization and initiation, TFIIF also stimulates the rate of elongation by RNA polymerase II (Price et al., 1989; Izban and Luse, 1992a; Bengal et al., 1991). Both the RAP30 and RAP74 subunits are required for elongation rate stimulation (Lei et al., 1998; Tan et al., 1994). Interestingly, RAP74(1-136), (1-172), (1-205), (1-217) and (1-517, wt) were found to have very similar activities in both initiation and elongation stimulation, indicating that the region of RAP74 between aa 136 to 217 makes a similar contribution to multiple stages of the transcription cycle (Lei et al., 1998). Because DNA wrapping induced by TFIIF has been shown to be important for initiation, perhaps DNA wrapping around

RNA polymerase II is a feature of rapid elongation complexes as well. The region of RAP74 between aa 136 and 217 is highly conserved from yeast to human. In this chapter we report a detailed mutational analysis of the RAP74 subunit of TFIIF in this region.

Materials and Methods

Construction of RAP74 mutants

Site-directed mutants of human RAP74 were constructed using the Quick Change Site-Directed Mutagenesis Kit (Stratagene) and appropriate primers. Mutations were confirmed by DNA sequencing. RAP74 mutant proteins were produced in *E. coli*, purified to near homogeneity, and assembled with recombinant human RAP30 to generate TFIIF complexes (Wang et al., 1993; Wang et al., 1994).

Transcription assays

Immobilized templates

Immobilized templates were prepared as described (Lei et al., 1998; Chapter 2). The template for amplification and the upstream biotinylated primer were the same as described (Chapter 2). The sequence of the downstream primer was 5'-GTGCTCATCATTGGAAAACGTTCTT-3'. The template extended from positions -263 to +1,250 relative to the transcription start (+1). Biotinylated DNA was incubated with streptavidin paramagnetic beads (Vector Laboratories) in 2 M NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.5 for 15 min at room temperature. Immobilized templates were collected with a magnetic particle separator (CPG), washed 2 times and stored at 4°C in phosphate buffered saline pH 7.2 containing 1 mg/ml BSA and 0.03% NaN_3.

Initiation assay: pulse-sarkosyl chase

This was modified from the pulse-chase protocol previously described (Chapter 2). 0.25% sarkosyl was added during the "chase" to prevent premature termination. Each 20 ul reaction mixture contained 800 ng DNA template (pML digested with *Sma* I at +217), TFIIF-depleted extract (72 ug total protein), recombinant TFIIF or TFIIF

mutant (10 pmol each), in transcription buffer (12 mM HEPES, pH 7.4, 12% glycerol, 0.12 mM EDTA, 0.12 mM EGTA, 1.2 mM DTT) containing 60 mM KCl and 12 mM $MgCl_2$. Preinitiation complexes were formed for 60 min at 30 °C. 100 uM ATP, CTP, GTP and 1 uM α^{32}P-UTP (10 μCi per reaction) were added to initiate transcription for 1 min. 1 mM ATP, UTP, GTP, CTP, and 0.25% sarkosyl were added, and elongation continued for 30 min. Reactions were stopped by addition of 200 ul 0.1 M sodium acetate (pH 5.4), 0.5 % sodium dodecyl sulfate, 2 mM EDTA, and 100 ug/ml tRNA, followed by phenol-chloroform extraction and ethanol precipitation. Samples were electrophoresed in a 6% polyacrylamide gel containing 50 % urea (w/v). For quantitation of the gel, the signal in the presence of RAP30 and the absence of added RAP74 was used to estimate background (Figure 1, lane 11). The weak signal obtained in the absence of added RAP74 was attributed to residual RAP74 remaining in the TFIIF-depleted extract.

Elongation stimulation assay: sarkosyl and high salt wash

This was modified from the method previously described (Chapter 2). A 20 ul reaction mixture consisted of 2 ul paramagnetic beads carrying the adenovirus major late promoter, transcription extract derived from HeLa cell nuclei (108 ug total protein), and transcription buffer containing 60 mM KCl and 12 mM $MgCl_2$. Samples were incubated 60 min at 30 °C to form preinitiation complexes. Transcription was initiated by addition of 100 uM ATP, GTP, CTP, and 1 uM (10 uCi) α-^{32}P-UTP (2 ul volume). After 1 min, elongation complexes were washed extensively with transcription buffer containing 1% sarkosyl, 500 mM KCl and 1 mg/ml BSA. This treatment appeared to be effective in removing the accessory factors because the elongation rate of pol II was strongly

stimulated by TFIIF (Figs. 1, 3, and 4). Complexes were then washed with transcription buffer containing 60 mM KCl and 1 mg/ml BSA. Complexes were resuspended in 20 ul transcription buffer containing 60 mM KCl and 12 mM $MgCl_2$, recombinant TFIIF complexes were added (20 pmol each or as indicated) and incubated for 5 min. 1 mM ATP, GTP, CTP, and UTP were added in 2 ul, and transcripts were elongated for various amounts of time as indicated. Transcripts were isolated by phenol extraction and ethanol precipitation and electrophoresed in a 10% polyacrylamide gel. The gel was quantitated using a Phosphorimager (Molecular Dynamics).

Results

Detailed mutational analysis of RAP74 in the critical region between aa 136 and 217

Previous work had indicated that the N-terminal domain of RAP74 was very important for both initiation and elongation of transcription (Lei et al., 1998; Chapter 2). This hypothesis was tested in the experiment shown in Fig. 1, using human recombinant TFIIF containing RAP30 and RAP74 or a RAP74 deletion mutant. For these studies, improvements were made in accurate initiation and elongation assays. For the initiation assay, an extract derived from human HeLa cells was depleted of TFIIF by immunoprecipitation with anti-RAP30 and anti-RAP74 antibodies. Recombinant TFIIF or a TFIIF mutant was added back to the depleted extract to restore accurate initiation. The template for transcription was the plasmid pML containing the adenovirus major late promoter. The DNA was treated with restriction enzyme *Sma* I that has a cleavage site at position +217 relative to the transcription start site. Transcription was initiated with a 1 min pulse of 100 μM ATP, CTP, GTP, and 1 μM α^{32}P-UTP. Sarkosyl was then added to 0.25 % and elongation continued for 30 min with 1 mM each NTP. In this protocol, sarkosyl removes elongation and termination factors from RNA polymerase II so that initiated chains are recovered efficiently at the +217 position (data not shown). As a result, this runoff assay very accurately reflects single-round initiation from the adenovirus major late promoter.

Elongation complexes were prepared on bead templates containing the adenovirus major late promoter. In an extract derived from HeLa cell nuclei, transcription was accurately initiated from the promoter with a 1 min pulse of 100 μM ATP, CTP, GTP, and 1 μM α^{32}P-UTP. Nascent elongation complexes were washed free of elongation and

Figure 1. Identification of a region of RAP74 that is critical for transcription initiation and elongation stimulation. A) Accurate initiation from the adenovirus major late promoter. An extract derived from HeLa cell nuclei was depleted of TFIIF by immunoprecipitation with anti-RAP30 and anti-RAP74 antibodies. Activity was restored by addition of 10 pmol TFIIF formed with RAP74 (1-517, wt) (designated F517) or deletion mutants F217, F205, F172, or F136. The reaction in lane 12 was identical to those in lanes 1 and 2 except that 1 μg/ml α-amanitin was included in the reaction. B) Phosphorimager quantitation of the data shown in "A". C) Elongation stimulation by TFIIF deletion mutants. Elongation complexes were formed on immobilized templates close to the adenovirus major late promoter and washed with 1% sarkosyl and 0.5 M KCl to remove nascent elongation factors. 20 pmol TFIIF was added to washed elongation complexes and 1 mM each NTP was added for 30 s (samples designated "chase"). M is a single-stranded, 5' end-labeled DNA marker.

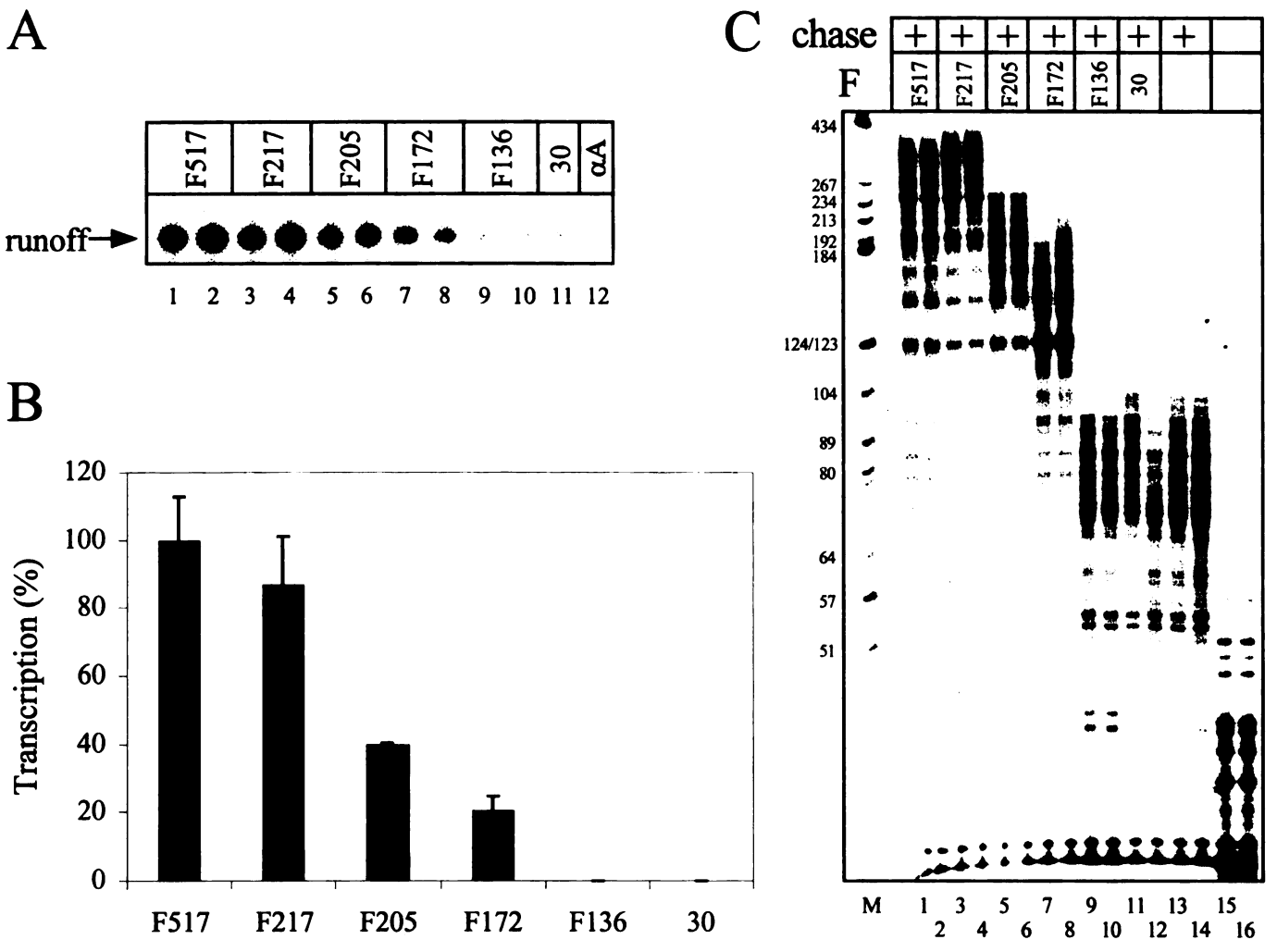

A
F517
F217
F205
F172
F136
30
αA
runoff
1 2 3 4 5 6 7 8 9 10 11 12
B
Transcription (%)
120
100
80
60
40
20
0
F517
F217
F205
F172
F136
30
C
chase
F
F517
F217
F205
F172
F136
30
434
267
234
213
192
184
124/123
104
89
80
64
57
51
M 1 3 5 7 9 11 13 15
2 4 6 8 10 12 14 16

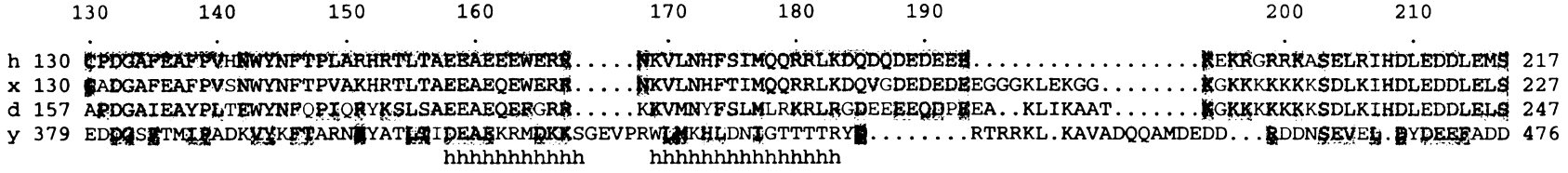
Figure 1

termination factors with buffer containing 1% sarkosyl and 0.5 M KCl. Complexes were re-equilibrated with transcription buffer, and TFIIF was added to the reaction. For the assays shown in Fig. 1, elongation was for 30 seconds in the presence of 1 mM each NTP (Fig. 1C). TFIIF containing RAP74(1-217) had almost as much activity as wild type TFIIF in initiation and slightly higher activity than wild type TFIIF in elongation. TFIIF containing RAP74(1-205), which is minimally required to support isomerization of the preinitiation complex, had reduced activity in both assays. RAP74(1-172) was further reduced in activity, and RAP74(1-136) was inactive. RAP74(1-136) is the only one of these mutants that does not stably assemble with the RAP30 subunit of TFIIF. This experiment shows that amino acids 136 to 217 of human RAP74 are very important for both accurate initiation and elongation of transcription.

To determine which amino acids in the region from aa 136 to 217 were most important for RAP74 function, a large set of site-directed mutants was constructed (Fig. 2). In Fig. 2A an alignment of human RAP74 with homologues from *Xenopus*, *Drosophila*, and yeast is shown. PHD analysis indicated that aa 157 to 182 of human RAP74 may constitute an extended α-helix and this structure is likely to be preserved in RAP74 homologues. Single, double, and triple amino acid substitutions are indicated beneath the sequence and categorized according to their activities in initiation and elongation assays (Figs. 3-6). Single substitutions are named according to the RAP74 amino acid that is substituted, L155A, W163A, etc. Multiple mutants are named for the

Figure 2. TFIIF mutagenesis. A) RAP74 homologues from eukaryotic species including human (h), *Xenopus* (x), *Drosophila* (d for *Drosophila* Factor 5a), and *S. cerevisiae* (y for yeast Ssu71, suppressor of *sua7*; also designated Tfg1 for transcription factor "g"). PHD analysis indicates that a large segment of this sequence is α-helical (hhhhhhh). B) RAP74 mutants analyzed in this study. Mutants are designated as "Critical", "Moderate", or "Less important" depending on their observed defects in initiation and elongation (Figs. 3-6). The most important residues are boxed in the sequence. Underlined mutants have slightly different properties in initiation and elongation assays (Fig. 6).

A


130 140 150 160 170 180 190 200 210
h 130 CPDGAFEAFPVHNWYNFTPLARHRTLTAEEAEEEWERRNKVLNHFSIMQQRRLKDQDQDEDEEEKEKRGRRKASELRIHDLEDDLEMS 217
x 130 CADGAFEAFPVSNWYNFTPVAKHRTLTAEEAEQEWERRNKVLNHFTIMQQRRLKDQVGDEDEDEEGGGKLEKGGKGKKKKKKKSDLKIHDLEDDLELS 227
d 157 APDGAIEAYPLTEWYNFQPIQRYKSLSAEEAEQERGRRKKVMNYFSLMLRKRLRGDEEEEQDPEEAKLIKAATKGKKKKKKKSDLKIHDLEDDLELS 247
y 379 EDDQSETMIPADKVYKFTARNYATLQIDEAEKRMDKKSGEVPRWLMKHLDNIGTTTTRYRRTRRKL.KAVADQQAMDEDDRDDNSEVELDYDEEEADD 476
hhhhhhhhhhh hhhhhhhhhhhhhhh

B

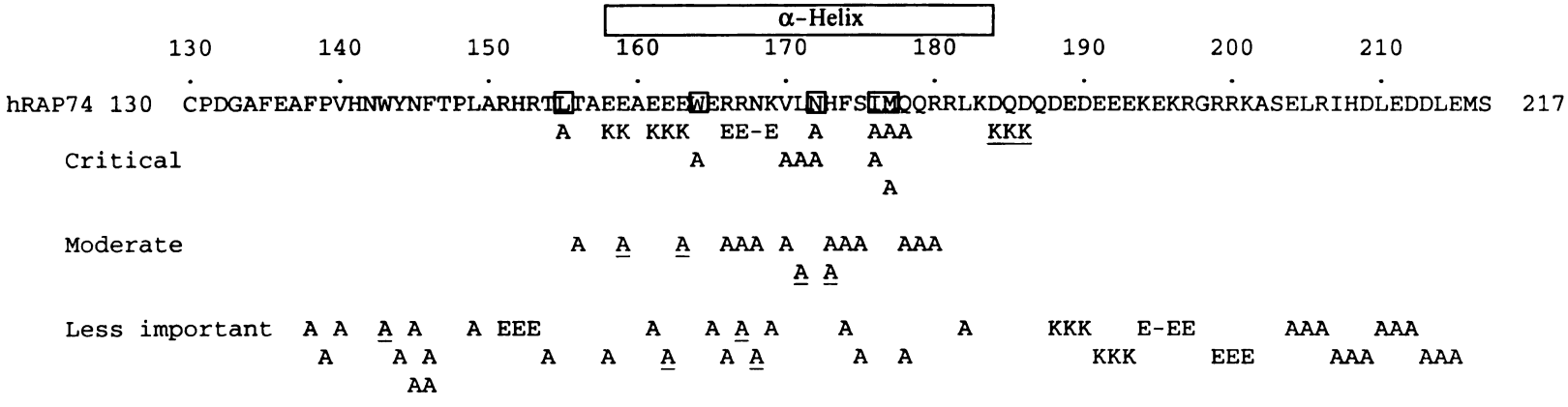

α-Helix
130 140 150 160 170 180 190 200 210
hRAP74 130 CPDGAFEAFPVHNWYNFTPLARHRTLTAEEAEEEWERRNKVLNHFSIMQQRRLKDQDQDEDEEEKEKRGRRKASELRIHDLEDDLEMS 217
A KK KKK EE-E A AAA KKK
Critical
A AAA A
A
Moderate
A A A AAA A AAA AAA
A A
Less important A A A A A EEE A A A A A A KKK E-EE AAA AAA
A A A A A A A A KKK EEE AAA AAA
AA

Figure 2

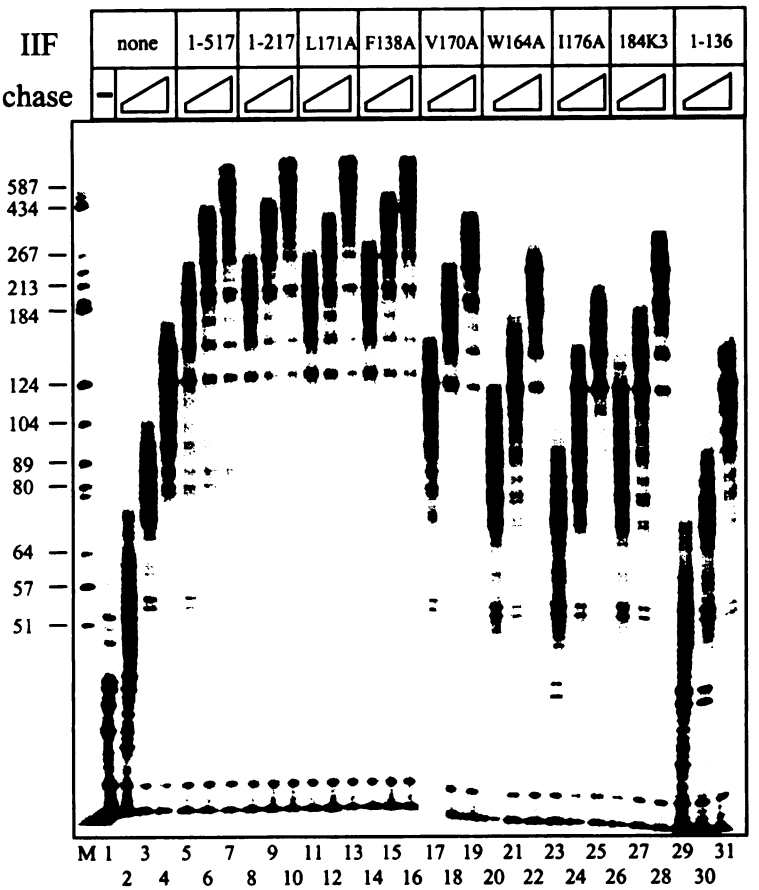
position of the first substituted amino acid, 161K3, 176A3, etc. A combination of alanine substitutions and charge-reversal mutations was constructed based on the notion that these mutations would cause changes in activity without inducing long-range changes in protein conformation. Human recombinant RAP74 mutants were produced in *E. coli*, purified to near homogeneity, combined in vitro with human recombinant RAP30 in buffer containing 4 M urea. The TFIIF complex was reconstituted by dialysis into buffer without urea (Wang et al., 1994). Mutants with the most pronounced transcriptional defects have been analyzed by gel filtration chromatography. This analysis indicates that RAP74 mutants form $\alpha_2\beta_2$ heterotetramers in complex with RAP30 as does wild type TFIIF (Wang et al., 1994; data not shown).

TFIIF mutants affect both initiation and elongation very similarly

Mutants were analyzed for their ability to stimulate the elongation rate of RNA polymerase II (Fig. 3). As described in Fig. 1, sarkosyl- and salt-washed elongation complexes were prepared, a TFIIF sample was added, and elongation was continued with 1 mM ATP, GTP, CTP, and UTP for 15, 30, or 60 s. A phosphorimager was used to determine the average length of transcripts in each gel lane and this value was plotted versus the elongation time. The slope of the line is reported as the average elongation rate. Quantitation of elongation rates was very precise in single experiments with an r^2 of 0.99 to 1.0. Variation between experiments for a TFIIF sample was less than 10 % (data not shown). The value for TFIIF wt was used to scale data between experiments. RNA polymerase II pauses at many sites along the template. The selection of pause sites and the efficiency of pausing appear to be identical whether TFIIF is present or absent from

Figure 3. Analysis of representative TFIIF mutants in elongation. A) The elongation rate stimulation assay. Washed elongation complexes were prepared as described in Fig. 1. After addition of 20 pmol TFIIF, 1 mM of each NTP was added for 15, 30, or 60 s as indicated (chase). The size marker (M) is a 5' end-labeled, single-stranded DNA marker for estimation of transcript sizes. TFIIF mutants are named for the RAP74 mutations they contain (i.e. L171A). B) Quantitation of the data shown in "A". A phosphorimager was used to estimate the midpoint of the distribution of RNA bands. Average transcript length is plotted against elongation time. The slope is reported as the average elongation rate. Slopes vary between r^2 of 0.99 and 1.0.

A


IIF
chase
none
1-517
1-217
L171A
F138A
V170A
W164A
I176A
184K3
1-136
587
434
267
213
184
124
104
89
80
64
57
51
M 1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30

B

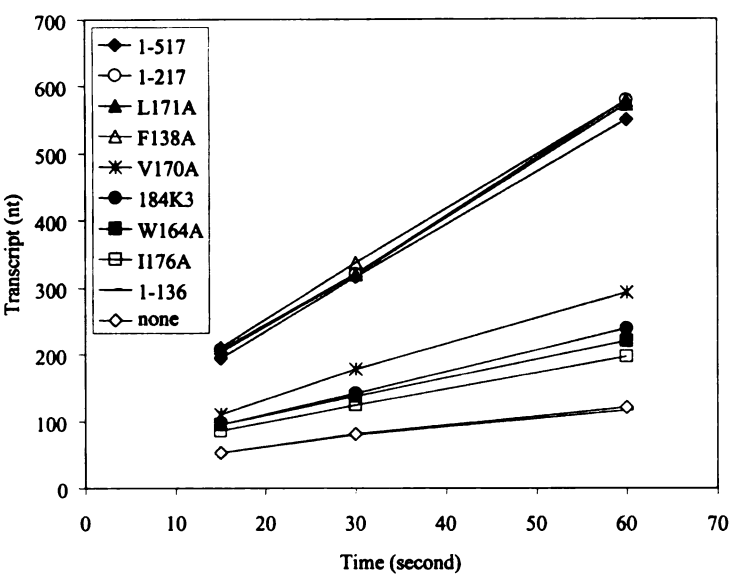
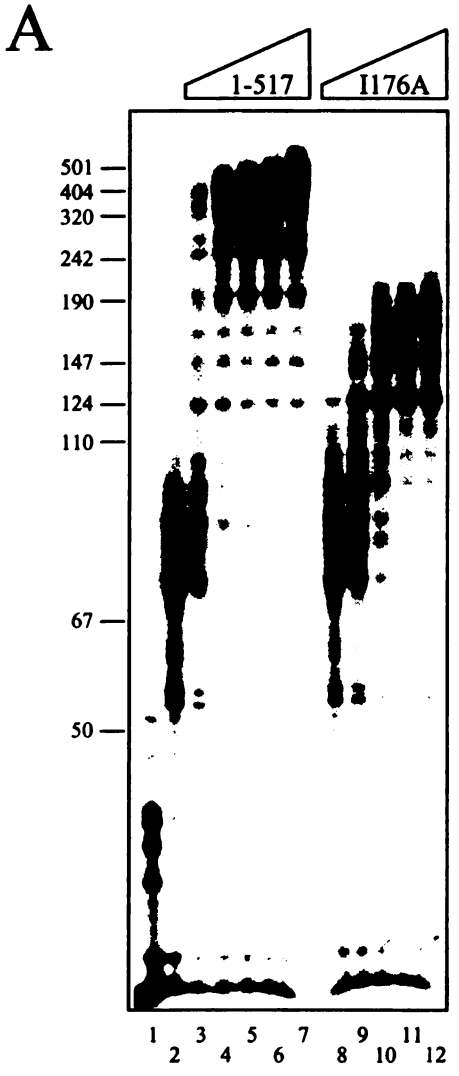

Transcript (nt)
Time (second)
1-517
1-217
L171A
F138A
V170A
184K3
W164A
I176A
1-136
none
0
100
200
300
400
500
600
700
0
10
20
30
40
50
60
70

Figure 3

Figure 4. TFIIF containing RAP74(I176A) has a similar affinity as TFIIF wt for elongation complexes but a lower activity. A) Elongation stimulation by TFIIF wt and TFIIF containing RAP74(I176A). The chase was for 30 s with 1 mM each NTP. Lanes 2-7 and 8-12 contained 5, 10, 20, 40, or 60 pmol of the indicated TFIIF complexes. B) Quantitation of the data shown in "A". A phosphorimager was used to estimate the average size of the RNA transcripts in each lane.


A
1-517
I176A
501
404
320
242
190
147
124
110
67
50
1 2 3 4 5 6 7 8 9 10 11 12

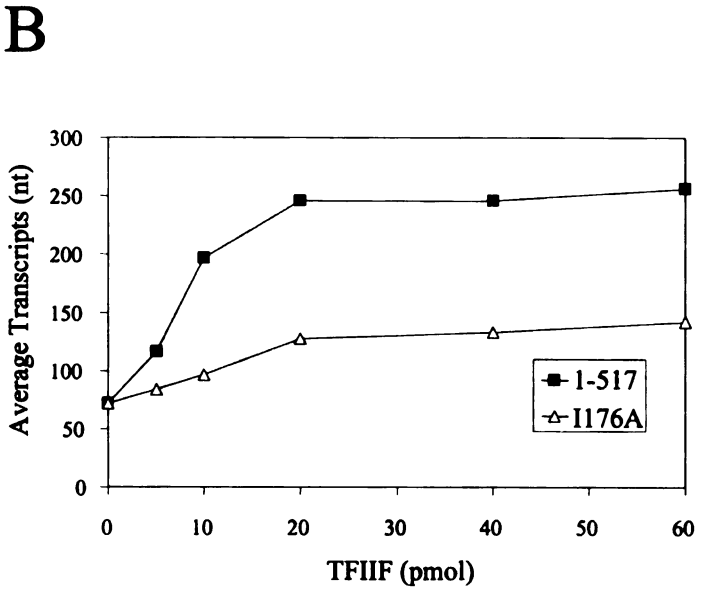

B
Average Transcripts (nt)
300
250
200
150
100
50
0
0 10 20 30 40 50 60
TFIIF (pmol)
1-517
I176A

Figure 4

the reaction (Fig. 3 and data not shown). TFIIF appears to decrease the dwell time at all sites.

TFIIF containing RAP74 mutants within this N-terminal domain appeared to have a very similar affinity for transcription complexes compared to TFIIF containing RAP74 wt (Fig. 4, and data not shown). Several lines of evidence supported this claim. First, the ability of TFIIF mutants to form the DNA-TBP-TFIIB-pol II-TFIIF complex was tested by a gel mobility shift assay (Lei et al., 1998; Chapter 2). Even the most defective TFIIF mutants, except 1-136 which is completely inactive, recruited pol II as efficiently as TFIIF wt (unpublished results, Delin Ren and Zachary Burton). Second, TFIIF mutants competed with TFIIF wt in initiation assays (Lei et al., 1998; unpublished results, Delin Ren and Zachary Burton). Addition of a 10-fold molar excess of a TFIIF mutant (such as I176A) over TFIIF wt reduces transcription to the level supported by the mutant alone. Furthermore, the concentration of TFIIF necessary to support accurate initiation and elongation stimulation was determined. There was no clear difference between the concentration requirement for TFIIF wt and TFIIF mutants either for accurate initiation (unpublished results, Delin Ren and Zachary Burton) or for elongation stimulation (Fig. 4). Additionally, these experiments showed that the concentrations used for analysis of mutants were saturating for both initiation and elongation (Figs. 1, 3, and 6), so low activities of mutants were due to specific defects other than an altered affinity for transcription complexes.

The elongation rates of TFIIF mutants are compared in Fig. 5. TFIIF containing RAP74(1-136) is inactive for elongation stimulation. RAP74(1-158), however, and all substitution mutants within the aa 136 to 217 region have some ability to stimulate

Figure 5. The average elongation rates of TFIIF and TFIIF mutants. Elongation stimulation assays were done as in Fig. 3. An asterisk (*) indicates that a TFIIF mutant was constructed in RAP74(1-217) rather than RAP74(1-517, wt).

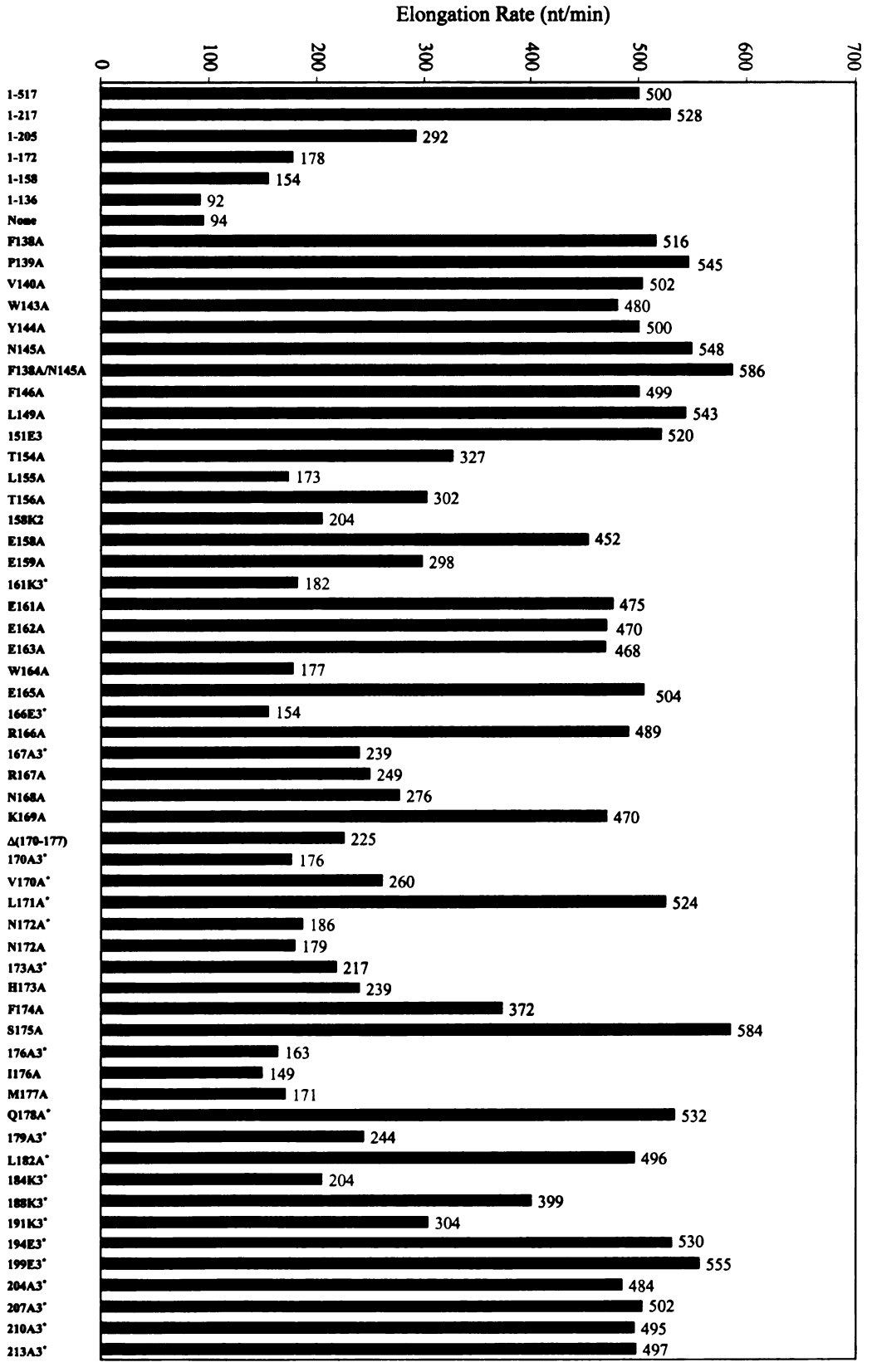

Elongation Rate (nt/min)
0
100
200
300
400
500
600
700
1-517 500
1-217 528
1-205 292
1-172 178
1-158 154
1-136 92
None 94
F138A 516
P139A 545
V140A 502
W143A 480
Y144A 500
N145A 548
F138A/N145A 586
F146A 499
L149A 543
151E3 520
T154A 327
L155A 173
T156A 302
158K2 204
E158A 452
E159A 298
161K3* 182
E161A 475
E162A 470
E163A 468
W164A 177
E165A 504
166E3* 154
R166A 489
167A3* 239
R167A 249
N168A 276
K169A 470
Δ(170-177) 225
170A3* 176
V170A* 260
L171A* 524
N172A* 186
N172A 179
173A3* 217
H173A 239
F174A 372
S175A 584
176A3* 163
I176A 149
M177A 171
Q178A* 532
179A3* 244
L182A* 496
184K3* 204
188K3* 399
191K3* 304
194E3* 530
199E3* 555
204A3* 484
207A3* 502
210A3* 495
213A3* 497

Figure 5

elongation. Substitution mutants having the lowest elongation rates (less than 200 nt/min) include L155A, 161K3*, W164A, 166E3*, 170A3*, N172A*, N172A, 176A3*, I176A, and M177A. L155, W164, N172, I176, and M177 all have hydrophobic side chains that appear to be critical for RAP74 function. It is likely that these amino acid residues form a hydrophobic patch important for protein-protein interactions. Single alanine substitutions also indicate that the individual side chain beyond β-carbon at the positions of aa 161 (E), 162 (E), 163 (E), 166 (R), 167 (R), 169 (K), 170 (V), 171 (L), or 178 (Q) is not critical for RAP74 function. The defects associated with 161K3* and 166E3* are caused by the combinations of multiple substitutions, because none of the single substitutions at these positions is critical. The C-terminal domain and the central region do not affect elongation because the same substitutions constructed in either RAP74(1-517, wt) or RAP74(1-217) have similar effects (compare N172A and N172A*, and data not shown). Interestingly, several substitutions, such as P139A, N145A, F138A/N145A, L149A, S175A, and 199E3* appear to increase the elongation activity of RAP74.

Initiation and elongation activities for the entire collection of TFIIF mutants are shown in Fig. 6. The striking result from this analysis is that mutants that are adversely affected for elongation rate stimulation are similarly affected for accurate initiation. As previously shown, TFIIF containing RAP74(1-136) is inactive for accurate transcription and elongation stimulation (Fig. 1). RAP74(1-158), however, and all substitution mutants within the aa 136 to 217 region have some ability to support accurate initiation and elongation stimulation. As the data is displayed, the elongation rate data appear to mirror the results from the initiation assays, demonstrating that amino acids in this region

of RAP74 make very similar contributions to both the initiation and elongation phases of the transcription cycle.

Figure 6. TFIIF mutants have very similar activities in accurate initiation and elongation rate stimulation. The activities of TFIIF mutants in elongation were compared with their activities in initiation. Accurate initiation assays were done as in Fig. 1.

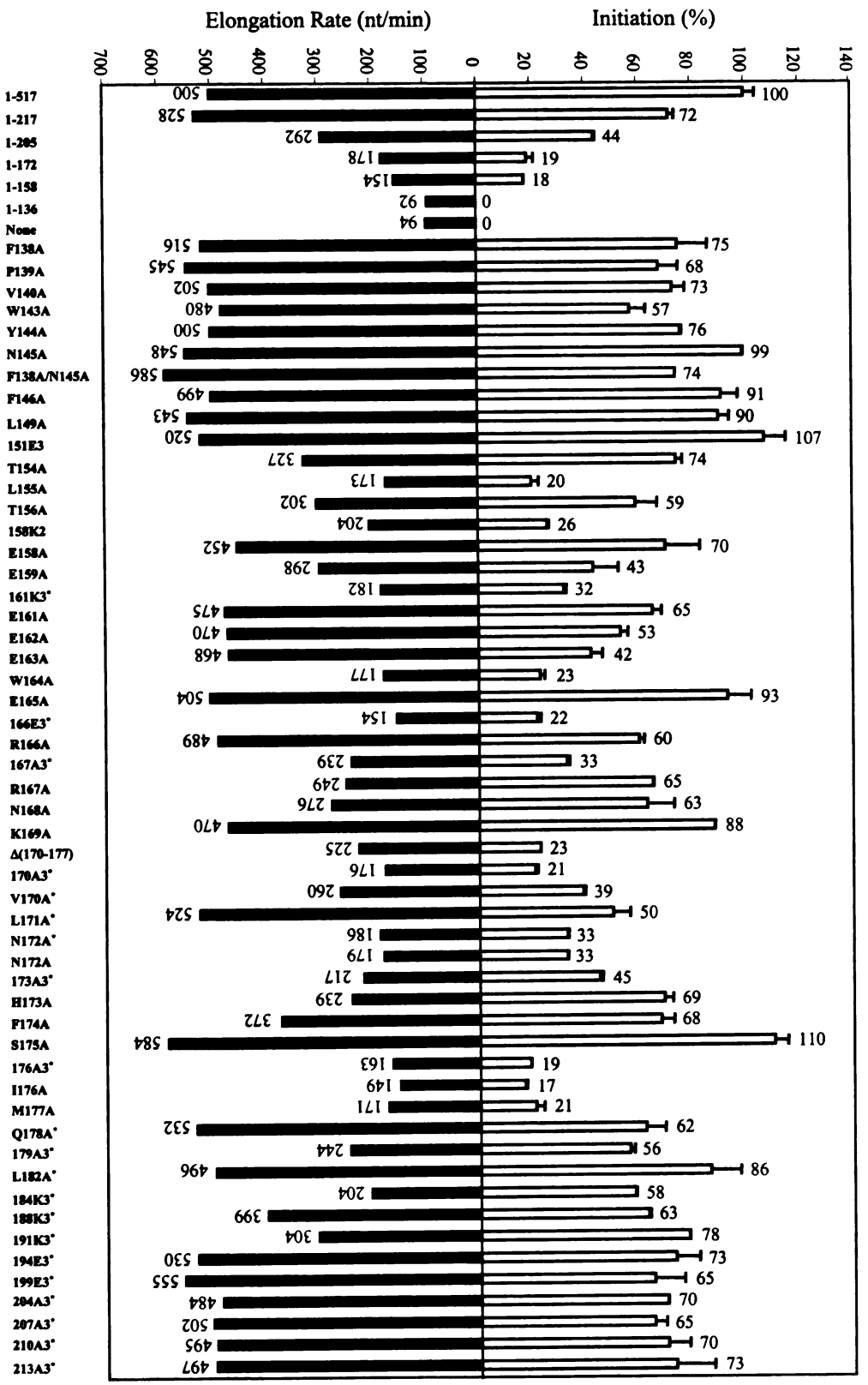

Elongation Rate (nt/min)
Initiation (%)
700
600
500
400
300
200
100
0
20
40
60
80
100
120
140
1-517
1-217
1-205
1-172
1-158
1-136
None
F138A
P139A
V140A
W143A
Y144A
N145A
F138A/N145A
F146A
L149A
151E3
T154A
L155A
T156A
158K2
E158A
E159A
161K3*
E161A
E162A
E163A
W164A
E165A
166E3*
R166A
167A3*
R167A
N168A
K169A
Δ(170-177)
170A3*
V170A*
L171A*
N172A*
N172A
173A3*
H173A
F174A
S175A
176A3*
I176A
M177A
Q178A*
179A3*
L182A*
184K3*
188K3*
191K3*
194E3*
199E3*
204A3*
207A3*
210A3*
213A3*
500 100
528 72
292 44
178 19
154 18
92 0
94 0
516 75
545 68
502 73
480 57
500 76
548 99
586 74
499 91
543 90
520 107
327 74
173 20
302 59
204 26
452 70
298 43
182 32
475 65
470 53
468 42
177 23
504 93
154 22
489 60
239 33
249 65
276 63
470 88
225 23
176 21
260 39
524 50
186 33
179 33
217 45
239 69
372 68
584 110
163 19
149 17
171 21
532 62
244 56
496 86
204 58
399 63
304 78
530 73
555 65
484 70
502 65
495 70
497 73

Figure 6

Discussion

The region of RAP74 between aa 136 and 217 is very important for accurate initiation from the adenovirus major late promoter and elongation rate stimulation. It is striking that RAP74 mutants in this region have almost equivalent effects in such different assays. The accurate initiation assay was done in a reconstituted HeLa cell extract system. Transcription from the adenovirus major late promoter was dependent on: 1) ATP hydrolysis; 2) presumably, all of the general transcription factors; and 3) the presence of the DNA binding site for the transcriptional activator USF/MLTF (upstream sequence factor/major late transcription factor). Many general and regulator proteins, therefore, could modulate TFIIF activity in this system. Because of extensive washing of elongation complexes, the elongation stimulation assay is likely dependent only on RNA polymerase II that is transcriptionally engaged and TFIIF. It is therefore a surprise that results in the initiation and elongation assays mirror one another so closely. It is possible that the TFIIF activity in these assays depends solely on interactions between TFIIF and RNA polymerase II and/or the DNA template.

The L155 to M177 region of RAP74 is conserved in evolution and is likely to form an α-helical structure (Fig. 2). Amino acid substitutions in L155, W164, N172, I176 and M177 have the greatest defects in accurate initiation and elongation. A common feature of these amino acid residues is that they all have hydrophobic side chains that are likely involved in protein-protein interactions. These mutants, however, appear by various criteria to enter transcription complexes with wild type affinity (Fig. 4 and data not shown). Unfortunately, the molecular structure of this region of RAP74 has not yet been reported. Mutagenic analysis indicates that, if this region is α-helical, there

is no particular relationship between the face of the helix on which side chains are displayed and the transcriptional defects of substitutions at those positions. In the future, a molecular structure of human TFIIF will assist the interpretation of these mutants.

The region between aa 172 and 205 is important for self-association of RAP74 in protein affinity chromatography experiments (Robert et al., 1998). The critical mutations identified in this work, therefore, may lie within a dimerization region that is important in maintaining the TFIIF heterotetramer. The gel filtration chromatography analysis of the native molecular weight of TFIIF samples, however, indicated that even the most severely affected mutants in this region, including RAP74(I176A), RAP74Δ(170-177) and RAP74(1-158), formed heterotetramers with RAP30 (data not shown).

Site-specific DNA-protein photocrosslinking studies have recently demonstrated that the region of RAP74 between aa 172 and 205 is critical for forming a tight wrap of adenovirus major late promoter DNA around a preinitiation complex containing TBP, TFIIB, RNA polymerase II, TFIIF and TFIIE (Robert et al., 1998). RAP74(1-172) assembles into this complex but fails to support a tightly wrapped structure and fails to support formation of many specific photocrosslinks to general transcription factors and RNA polymerase II within the core promoter. RAP74(1-205), on the other hand, appears to support DNA wrapping, apparently with the same efficiency as RAP74 wt. RAP74(1-205) is not as active in transcription as RAP74 wt (Fig. 1), but its transcriptional defect has not yet been revealed in photocrosslinking experiments. The interpretation of the photocrosslinking data was that the region of RAP74 between aa 172 and 205 is necessary for forming the tight DNA wrap around RNA polymerase II and for isomerization of the preinitiation complex. Isomerization was hypothesized to include

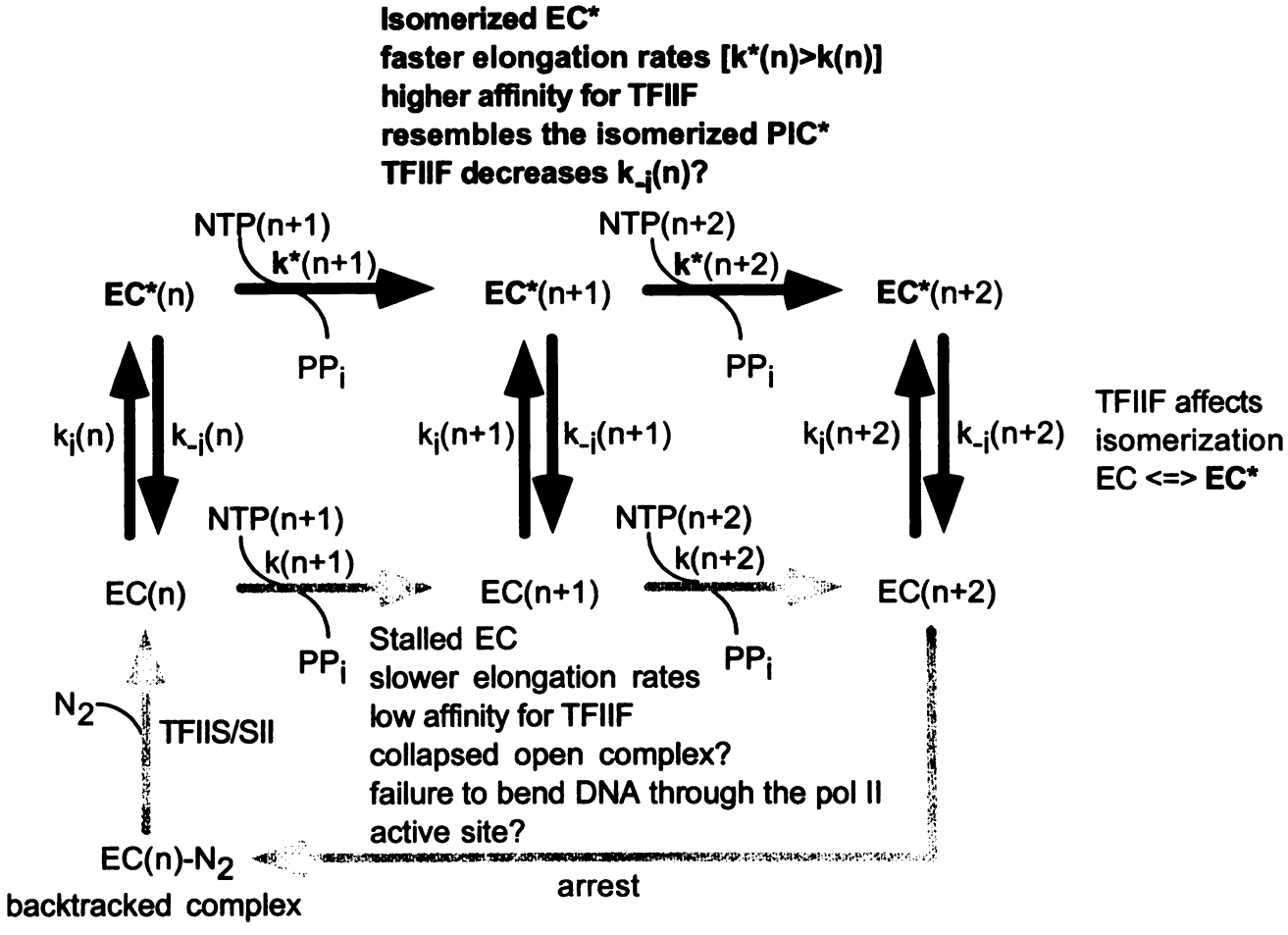
tight wrapping of promoter DNA around pol II and the general factors and sharp bending of DNA through the RNA polymerase II active site. The critical region between L155 and M177 that we identified by mutational analysis appears to correspond to this region of RAP74 that is critical for isomerization. Dr. Benoit Coulombe's laboratory is currently testing these amino acid substitution mutants in photocrosslinking experiments to determine whether they can support a tight promoter DNA wrap around pol II.

Assuming that the defect of these RAP74 mutants in initiation is due to a failure to isomerize the preinitiation complex, what is their defect in elongation? Because these mutants have such similar effects on initiation and elongation, they may also fail to properly isomerize the elongation complex. Because DNA wrapping and bending appear to be the primary characteristics of the isomerized preinitiation complex, we hypothesize that sharp DNA bending through the RNA polymerase active site may similarly stimulate elongation by RNA polymerase II and that TFIIF supports this bent structure during elongation. A model to describe these ideas is shown in Fig. 7.

This model takes into consideration: 1) asynchrony of RNA polymerase II elongation; 2) DNA bending and possibly wrapping in the elongation complex; 3) studies of stalled elongation complexes; 4) isomerization of the elongation complex induced by TFIIF; and 5) the observation that pause site selection is not affected by TFIIF. RNA polymerase II elongates transcription asynchronously, as if polymerase is partitioning between active (EC*) and inactive (EC) forms during elongation. Asynchronous elongation has been described most clearly for RNA polymerase III, although this also is

Figure 7. A model for the role of TFIIF in isomerization of the elongation complex. TFIIF is proposed to affect partitioning between unisomerized and isomerized complexes during elongation. TFIIS is suggested to work on the unisomerized elongation complex to overcome irreversible transcriptional arrest. Isomerization of complexes is suggested to involve wrapping of template DNA around RNA polymerase II and sharp bending of the template through the RNA polymerase active site.

Figure 7


Isomerized EC*
faster elongation rates [k*(n)>k(n)]
higher affinity for TFIIF
resembles the isomerized PIC*
TFIIF decreases k-i(n)?
NTP(n+1)
k*(n+1)
EC*(n)
PPi
EC*(n+1)
NTP(n+2)
k*(n+2)
PPi
EC*(n+2)
ki(n)
k-i(n)
ki(n+1)
k-i(n+1)
ki(n+2)
k-i(n+2)
TFIIF affects
isomerization
EC <=> EC*
NTP(n+1)
k(n+1)
EC(n)
PPi
EC(n+1)
NTP(n+2)
k(n+2)
PPi
EC(n+2)
Stalled EC
slower elongation rates
low affinity for TFIIF
collapsed open complex?
failure to bend DNA through the pol II
active site?
N2
TFIIS/SII
EC(n)-N2
backtracked complex
arrest

a feature of RNA polymerase II and *E. coli* RNA polymerase (Matsuzaki et al., 1994). In this model, **EC*** is depicted with a DNA template that is sharply bent through the RNA polymerase II active site, and the template is less sharply bent in EC. Our interpretation of studies of stalled elongation complexes is that the stalled complex must resemble the unisomerized EC, which is more likely to be an entry point into an editing mode than an active elongation mode. Stalled elongation complexes do not appear to interact strongly with TFIIF (data not shown), although TFIIF stimulates elongation about 6-fold (Figs. 3 and 6). Stalled elongation complexes have short exonuclease III, DNase I, and hydroxyl radical footprints, indicating that the DNA template is not wrapped around RNA polymerase in EC (Samkurashvili and Luse, 1998; Selby et al., 1997). TFIIS/SII may interact with the EC form to resolve backtracked complexes (Izban and Luse, 1992b; Izban and Luse, 1993a; Awrey et al., 1998). The proposed role of TFIIF in this model is to help support the **EC*** form of the elongation complex. TFIIF might do this by accelerating k_i and inhibiting k_{-i}. TFIIF is proposed to help maintain the DNA template in a sharply bent conformation through the RNA polymerase II active site during elongation. TFIIF is not expected to interact directly with the RNA polymerase II active site. In this regard, TFIIF may not affect $\mathbf{k}^*(n)$, the rate of phosphodiester bond formation. If TFIIF affected this step, TFIIF would alter the selection of pause sites, which has not been observed.

Acknowledgements

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CHAPTER 4

FUNCTIONS OF THE C-TERMINAL DOMAIN AND CENTRAL REGION OF RAP74 IN RECYCLING OF RNA POLYMERASE II

INTRODUCTION

An intriguing feature of the RPB1 subunit of pol II is the carboxy terminal domain (CTD) which has the consensus sequence YSPTSPS tandemly repeated 52 times in human pol II (Dahmus, 1996). Phosphorylation and dephosphorylation of the CTD by the regulated activities of CTD kinases (Feaver et al., 1991; Lu et al., 1992; Marshall et al., 1996; Serizawa et al., 1992; Serizawa et al., 1993) and phosphatases (Chambers and Dahmus, 1994) appear to control progression through the transcription cycle. Pol II enters the preinitiation complex with its CTD in a largely unmodified form designated pol IIA (Laybourn and Dahmus, 1989; Lu et al., 1991). During elongation, pol II is converted to the pol IIO form (Bartholomew et al., 1986; Laybourn and Dahmus, 1989) which is heavily phosphorylated on the SP serines of the YSPTSPS consensus sequence, so hyperphosphorylation of the CTD is thought to be important to establish and maintain the elongation complex. Removal of phosphates from the CTD may be a signal to terminate transcription and recycle pol II to a promoter.

A recently identified CTD phosphatase that catalyzes the dephosphorylation of pol IIO to pol IIA is stimulated by the C-terminal domain of the RAP74 subunit of TFIIF (Chambers et al., 1995). Interestingly, RAP74-dependent stimulation of CTD phosphatase activity is blocked by addition of TFIIB. The C-terminal domain of RAP74 binds to TFIIB (Fang and Burton, 1996) and pol II (Wang and Burton, 1995), so TFIIF, TFIIB, and the CTD phosphatase may be components of a multiprotein complex that

binds pol II and regulates pol II recycling. In this chapter we demonstrate that the central region and the C-terminal domain of RAP74 stimulate multiple-round transcription in an extract system consistent with a role for RAP74 in transcriptional recycling.

Materials and Methods

Transcription factors and extracts

Recombinant human RAP30, RAP74, and RAP74 mutants were prepared and quantitated as described (Chapter 2). Construction of new RAP74 mutants is described below. The TFIIF-depleted extract was completely dependent on the re-addition of RAP30 for activity and was strongly stimulated by addition of RAP74.

Construction of RAP74 mutants

Internal deletion mutants RAP74(Δ306-351), (Δ276-351), and (Δ219-351) were constructed using the Quick Change Site-Directed Mutagenesis Kit (Stratagene). All mutants were made using the same primer for the amino acid 351 position, 5'-GACATTGACAGCGAGGCCTCCTCAGCCCTCTTCATGGCG-3'. For the second oligonucleotide primers, 5'-GGAGGCCTCGCTGTCAATGTCGCTCTGCTCATCGACACCATTGGG-3', 5'-GGAGGCCTCGCTGTCAATGTCTGACATGTAGTCCACCTCTTGGCC-3', and 5'-GGAGGCCTCGCTGTCAATGTCGGAGGACATTTCCAGGTCGTCTTCAAGGTC-3' were used, respectively. The underlined sequences represent the complimentary overlap between the two mutant primers. Mutated RAP74 genes were confirmed by DNA sequencing.

Multiple-round and sarkosyl block assays

Transcription was initiated from an adenovirus major late promoter template digested with *Sma*I to produce a +217 base runoff transcript. The source of transcription factors was TFIIF-depleted extract (72 ug total protein) supplemented with recombinant RAP30 and RAP74 (10 pmol each, except as noted). For all reactions, preinitiation

complexes were formed for 60 min at 30 °C. For the multiple-round assay, 600 uM ATP, CTP, GTP and 25 uM α^{32}P-UTP (10 μCi per reaction) were added and transcription continued for the indicated times (Figs. 1-3). For the 10 min cold--multiple round assay, 600 uM ATP, CTP, GTP and 25 uM UTP were added and incubated for 10 min, and then α^{32}P-UTP (10 μCi per reaction) was added and transcription continued for 60 min (Fig. 1). For the single-round sarkosyl block assay (Hawley and Roeder, 1985), 600 uM ATP, CTP, and 25 uM α^{32}P-UTP (10 μCi per reaction) were added and incubated for 1 min. 600 uM GTP and 0.25 % w/v sarkosyl were added and transcription continued for 59 min (Fig. 1), 79 min (Fig. 2B and 2C), or 99 min (Fig. 3A). 0.05 % sarkosyl was previously shown to be sufficient to block new initiation by pol II (Hawley and Roeder, 1985), so sarkosyl was added in five-fold excess over the amount necessary to constrain transcription to a single round. As a control, sarkosyl was added to reactions before NTPs, causing initiation to be completely blocked, indicating that the level of detergent added in experiments was sufficient to block reinitiation (data not shown).

For the experiment shown in Fig. 2A, using the multiple-round transcription protocol, 0.25 % sarkosyl was added at the indicated times to block new initiation, and transcription was continued for an additional 30 min to complete all previously initiated chains (Hawley and Roeder, 1985). This was a control experiment to demonstrate that new initiation occurs throughout the course of the reaction. Transcription was quantitated and background selected as described above.

G-less cassette pol II trap

To characterize multiple-round transcription in the extract system, a "G-less cassette" trap for pol II was used (Szentirmay and Sawadogo, 1994). The template was

plasmid pML(C_2AT)19, the kind gift of Michele Sawadogo, containing the adenovirus major late promoter fused to a G-less cassette at position +11 (Sawadogo and Roeder, 1985a; Sawadogo and Roeder, 1985b). Preinitiation complexes were formed for 1 hr. Reactions contained TFIIF-depleted extract supplemented with 10 pmol recombinant RAP30 and RAP74. 600 uM ATP, GTP, and CTP, 1 mM 3'-O-methyl GTP, and 25 uM α^{32}P-UTP (10 uCi per reaction) were added to reactions as indicated in Fig. 4. At t = +1 min, 0.05 % sarkosyl was added to some reactions to estimate single-round transcription. In the presence of ATP, CTP, UTP, and 3'-O-methyl-GTP a transcript of 390 bases was synthesized. Under this condition, pol II stalled after insertion of 3'-O-methyl-GMP into the RNA chain at position +390. The template was digested with *Pvu*II to allow simultaneous detection of stalled transcription at +390 and runoff transcription at position +602. In control experiments 0.05 % sarkosyl was found to be sufficient to constrain transcription to a single round (data not shown). 0.05 % sarkosyl was used in this experiment because, unexpectedly, the early elongation complex formed from the G-less cassette template was much more sensitive to disruption with sarkosyl than that initiated from the wild type promoter (data not shown). The promoters in these two plasmids are identical from positions -256 to +10, and therefore it appears that the DNA sequence downstream from +10 may contribute to the observed difference in sarkosyl sensitivity. For chase reactions, 1 mM GTP and UTP were added and elongation continued for 10 or 60 min, as indicated in Fig. 4.

RESULTS

The central region and C-terminal domain of RAP74 stimulate multiple-round transcription

RAP74 mutants were tested for the ability to support multiple-round transcription *in vitro* (Fig. 1). In one protocol, NTPs were added with α^{32}P-UTP radiolabel and transcription was allowed to continue for 60 min. In a modified protocol, unlabeled NTPs were added for 10 min before addition of radiolabel (10 min cold--multiple round), and transcription continued for 60 min. For comparison, a single-round protocol was done in which sarkosyl (0.25 %) was added 1 min after addition of NTPs to block new initiation (Hawley and Roeder, 1985). The final specific activity of radiolabel was the same in all three procedures, so the intensities of transcription signals can be compared directly. For the sarkosyl block assay (Fig. 1A), the observed transcriptional activities of RAP74 mutants were very similar to those determined in the pulse-spin initiation assay (Chapter 2).

Cycles of transcription were estimated by dividing the yield of transcripts in the absence of sarkosyl (multiple-round) by the yield of transcripts in the presence of sarkosyl (single-round). By this estimation, full length RAP74 supported approximately four cycles of transcription in 60 min (Fig. 1B). However, RAP74(1-409), (1-356), (1-296), and (1-217) supported only about two rounds of accurate transcription. The region between aa 409-517, therefore, was important for multiple round transcription. For RAP74(1-517), (1-409), and (1-356), cycles of transcription were not diminished using the 10 min cold--multiple round protocol. The transcription system, therefore, did not become limiting for factors or substrates within 70 min. In contrast, RAP74(1-217) and

Figure 1. The C-terminal domain of RAP74 stimulates multiple-round transcription. A) Single-round and multiple-round transcription assays. All samples contained TFIIF-depleted extract (DE; 72 μg protein), recombinant human RAP30 (10 pmol) and RAP74 or a RAP74 mutant (10 pmol). In the single-round protocol, reinitiation was blocked by addition of the anionic detergent sarkosyl. In the multiple-round protocol, transcription was allowed to proceed for 60 min. In the 10 min cold--multiple round protocol, transcription with all four NTPs was allowed to proceed for 10 min before addition of radiolabel and incubation for 60 min. Reactions labeled αA contained RAP74 and 1 μg/ml α-amanitin. B) Phosphorimager quantitation of the data in "A".

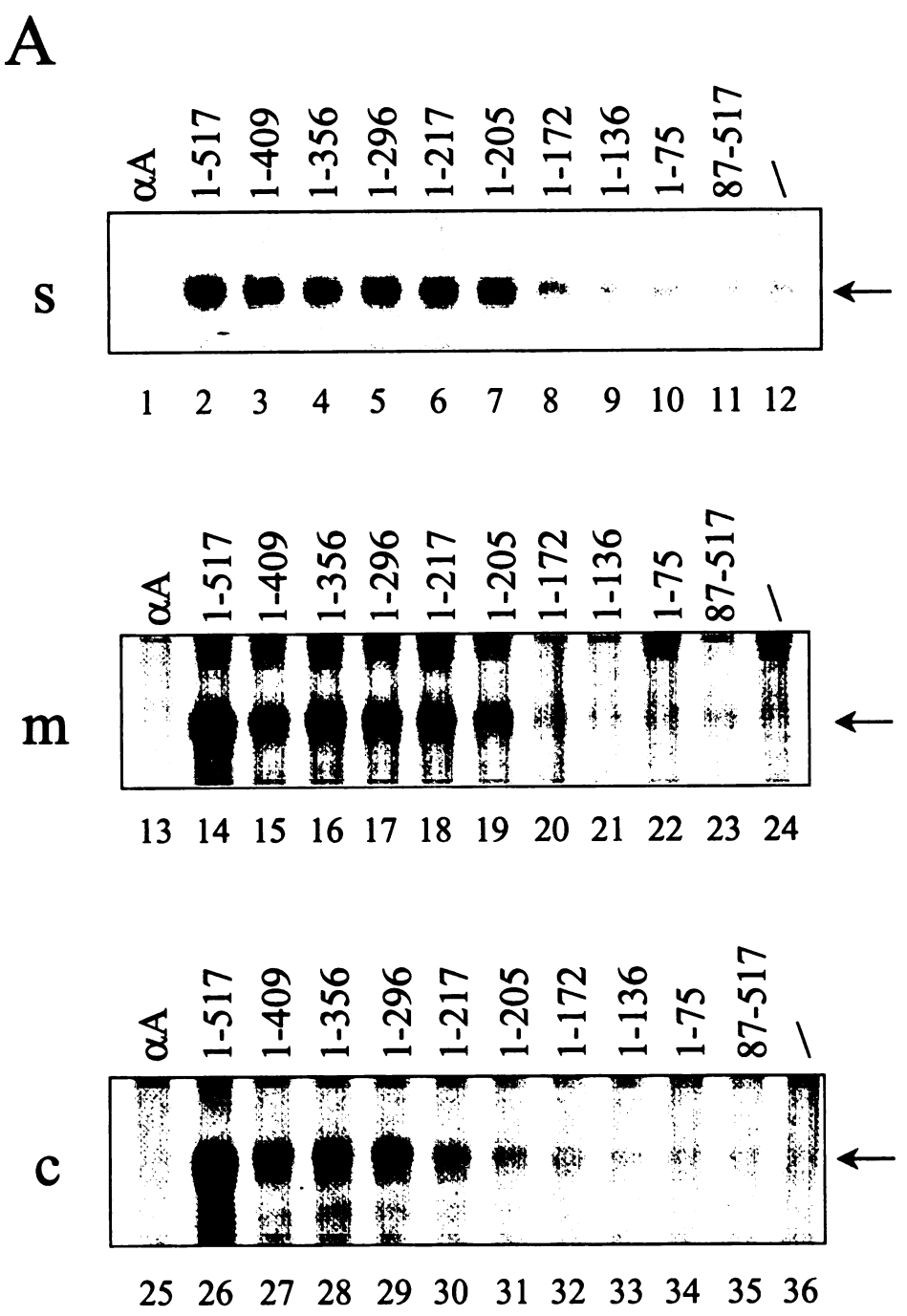
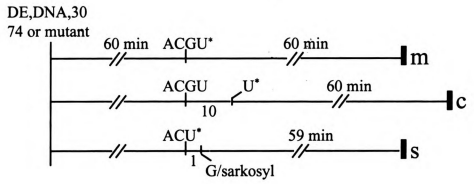

A
αA
1-517
1-409
1-356
1-296
1-217
1-205
1-172
1-136
1-75
87-517
—
s
1 2 3 4 5 6 7 8 9 10 11 12
m
13 14 15 16 17 18 19 20 21 22 23 24
c
25 26 27 28 29 30 31 32 33 34 35 36

Figure 1


DE,DNA,30
74 or mutant
60 min
ACGU*
60 min
m
ACGU
U*
60 min
10
c
ACU*
59 min
1
G/sarkosyl
s

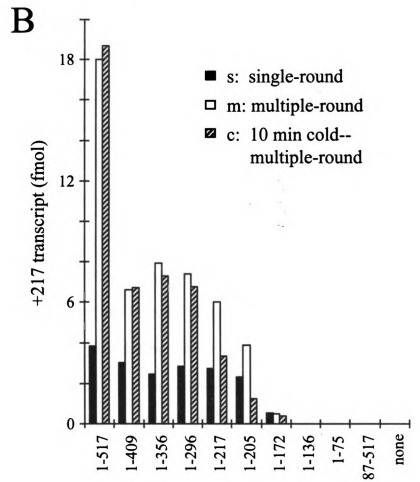

B
18
12
6
0
+217 transcript (fmol)
s: single-round
m: multiple-round
c: 10 min cold--
multiple-round
1-517
1-409
1-356
1-296
1-217
1-205
1-172
1-136
1-75
87-517
none

Figure 1

(1-205) had a reduced ability to support multiple rounds of transcription after a 10 min incubation with unlabeled NTPs. In the presence of RAP74(1-217) and (1-205), therefore, some transcription factor(s) appeared to become limiting for initiation at later reaction times.

In order to characterize new initiation in the extract, the yield of transcripts was determined at different times after addition of NTPs, and transcripts continued to accumulate for more than 90 min (Fig. 2A). The increase in transcription was due to new initiation rather than slow elongation of chains initiated at earlier times, because when sarkosyl was added to rescue all previously-initiated chains, transcripts nonetheless continued to accumulate for the entire 90 min (open squares). If chains were initiated at early times and slowly elongated, the sarkosyl rescue curve (open squares) would achieve its maximal value at an early time, instead of tracking the curve without sarkosyl (filled squares), as observed.

To better understand the activities of RAP74 mutants in multiple round transcription, cycles of transcription were determined as a function of time for RAP74 mutants (Figs. 2B and 2C). Mutants for which the graph has a positive slope at the +40 and +80 min time points were judged to be capable of supporting multiple-round transcription. By this criterion, RAP74 was most active followed by RAP74(1-409) and (1-356). RAP74(1-296) had a very weak capacity to support multiple-round transcription, and RAP74(1-217) and (1-205) were inactive. Although defective for multiple-round transcription, RAP74(1-296) and (1-217) were highly active in single-round transcription (Chapter 2). The region of RAP74 between aa 409-517 was

Figure 2. The central region and the C-terminal domain of RAP74 cooperate to stimulate multiple-round transcription. A) New initiation occurred throughout the 90 min course of the reaction. Assays were done either by the multiple-round protocol (Fig. 5) and stopped at the indicated times (filled squares), or instead of stopping the reactions, sarkosyl was added to block new initiation and transcription continued for an additional 30 min to complete any previously initiated chains (open squares). B and C) Both the central region and the C-terminal domain of RAP74 contributed to multiple-round transcription. Multiple-round transcription was determined using the protocol in Fig. 5, except that reactions were stopped at the indicated times. Single round transcription was estimated using a sarkosyl block procedure with a 79 min elongation. Cycles of transcription were estimated as transcription in the absence (time indicated)/presence of sarkosyl (79 min elongation).

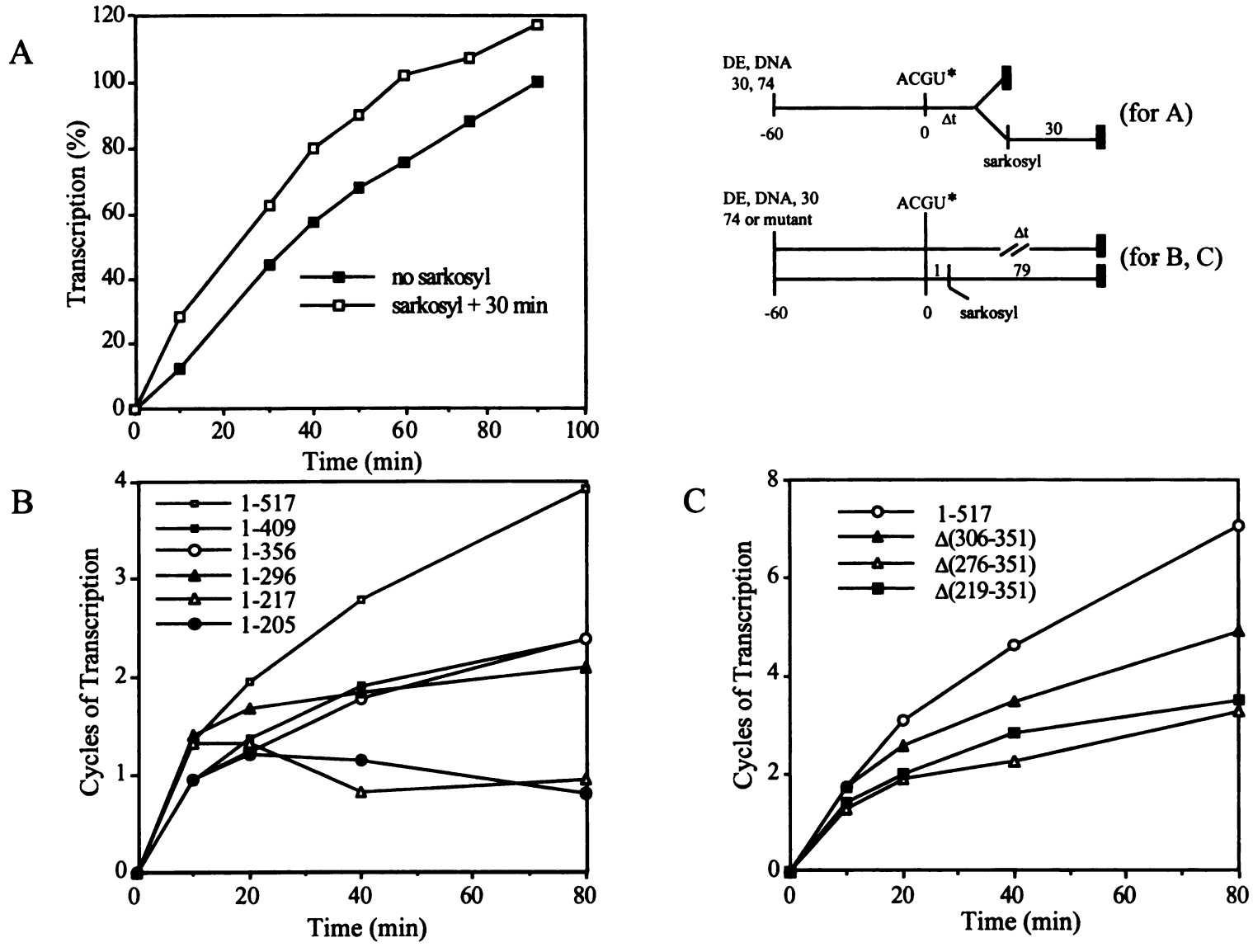

A
Transcription (%)
Time (min)
no sarkosyl
sarkosyl + 30 min
DE, DNA
30, 74
ACGU*
Δt
-60
0
30
sarkosyl
(for A)
DE, DNA, 30
74 or mutant
ACGU*
Δt
1
79
-60
0
sarkosyl
(for B, C)
B
Cycles of Transcription
Time (min)
1-517
1-409
1-356
1-296
1-217
1-205
C
Cycles of Transcription
Time (min)
1-517
Δ(306-351)
Δ(276-351)
Δ(219-351)

Figure 2

important for multiple-round transcription, but the region between aa 217-356 stimulated multiple-round transcription, even in the absence of the C-terminal domain. To further test the importance of the central region, three internal deletion mutants were constructed and tested (Fig. 2C). RAP74(Δ306-351), (Δ276-351), and (Δ219-351) all were shown to support multiple-round transcription at a reduced level compared to RAP74. Each of these mutants, however, supported single-round transcription almost as well as RAP74 (about 75% wild type activity (data not shown), similarly to RAP74(1-217)). RAP74(Δ306-351) was most active for multiple-round transcription, and RAP74(Δ276-351) and (Δ219-351) had lower activity. Although the central region stimulated multiple-round transcription, the C-terminal domain supported this activity in the absence of aa 219-351, as indicated by the positive slope of the graph for RAP74(Δ276-351) and (Δ219-351) at late time points (Fig. 2C; open triangles and filled squares). Therefore, both the C-terminal domain and the central region of RAP74 contributed to multiple-round transcription.

An alternative explanation for the observed defects of RAP74 deletion mutants in multiple-round transcription might be that mutants were less stable than RAP74 during the reaction time course. We did not believe that this would be the case because the central loop of RAP74 appears to be the region that is most sensitive to proteolysis (data not shown), and RAP74(1-296) and (1-217), which show the most dramatic defects in multiple round transcription, have most or all of the central region removed. Also, a spectrum of defects in multiple-round transcription was noted (Figs. 2B and 2C), so different mutants would have to have different stabilities in approximate proportion to their length, which seemed unlikely. Furthermore, a 5-10 fold functional excess of each

mutant protein was added to reactions during a one hour preincubation, so RAP74 mutants would have to be stable in the extract under reaction conditions during the preincubation but not after addition of NTPs, which seemed unlikely. In a Western blot analysis, RAP74(1-517), (1-217), (1-172), and RAP30 remained intact throughout the reaction time course (data not shown). Most convincingly, experiments shown in Fig. 3 demonstrated the stability of RAP74 mutants during the reaction and the selective defect of RAP74(1-217) in multiple round transcription. The experiment shown in Fig. 3A demonstrated that F217 (TFIIF complexes containing RAP74(1-217)) and F172 appeared to compete with TFIIF for formation of transcription complexes. Addition of F217 to a reaction containing TFIIF inhibited multiple-round transcription (compare columns 1 and 2) but not single-round transcription (compare columns 4 and 5). This was expected because RAP74(1-217) is active for single-round but not multiple round transcription (Fig. 2B). Since RAP74(1-172) was nearly inactive for transcription (Chapter 2), this mutant inhibited both multiple-round (compare columns 1 and 3) and single-round transcription (compare columns 4 and 6). The inhibitory effect of RAP74(1-172) was consistent with our observation that this mutant assembled into the DBPolF complex (Chapter 2). The competitive effects of RAP74(1-217) and (1-172) persisted during the 100 min time course indicating that these proteins remained active for complex assembly. The experiment shown in Fig. 3B shows that late addition of TFIIF rescued a reaction containing F217 for multiple-round transcription (compare column 1 to columns 3-5) but that re-addition of F217 did not (compare columns 2 and 8). Rescue of the reaction with TFIIF was not complete because of inhibition by F217 (Fig. 3A). If the defect of F217 at late reaction times were due to degradation or inactivation, re-addition of F217 would be

Figure 3. RAP74(1-217) is specifically defective for multiple-round transcription. A) TFIIF containing RAP74(1-217) is dominant-negative for multiple-round transcription. Reactions in columns 1-6 contained 2 pmol TFIIF; reactions in columns 2 and 5 also contained 2, 4, 10, or 20 pmol F217 (data points were combined within the error bar because the effect was essentially maximal with 2 pmol F217); reactions in columns 3 and 6 contained 2 or 20 pmol F172. B) The defect of TFIIF containing RAP74(1-217) can be rescued by TFIIF wt. The reaction in column 1 contained 2 pmol TFIIF; reactions in columns 2-8 contained 2 pmol F217; reactions in columns 3, 4, and 5 contained 2, 4, or 10 pmol TFIIF added at +10 min; reactions in columns 6, 7, and 8 contained 10 pmol RAP30, RAP74, or F217 added at +10 min.

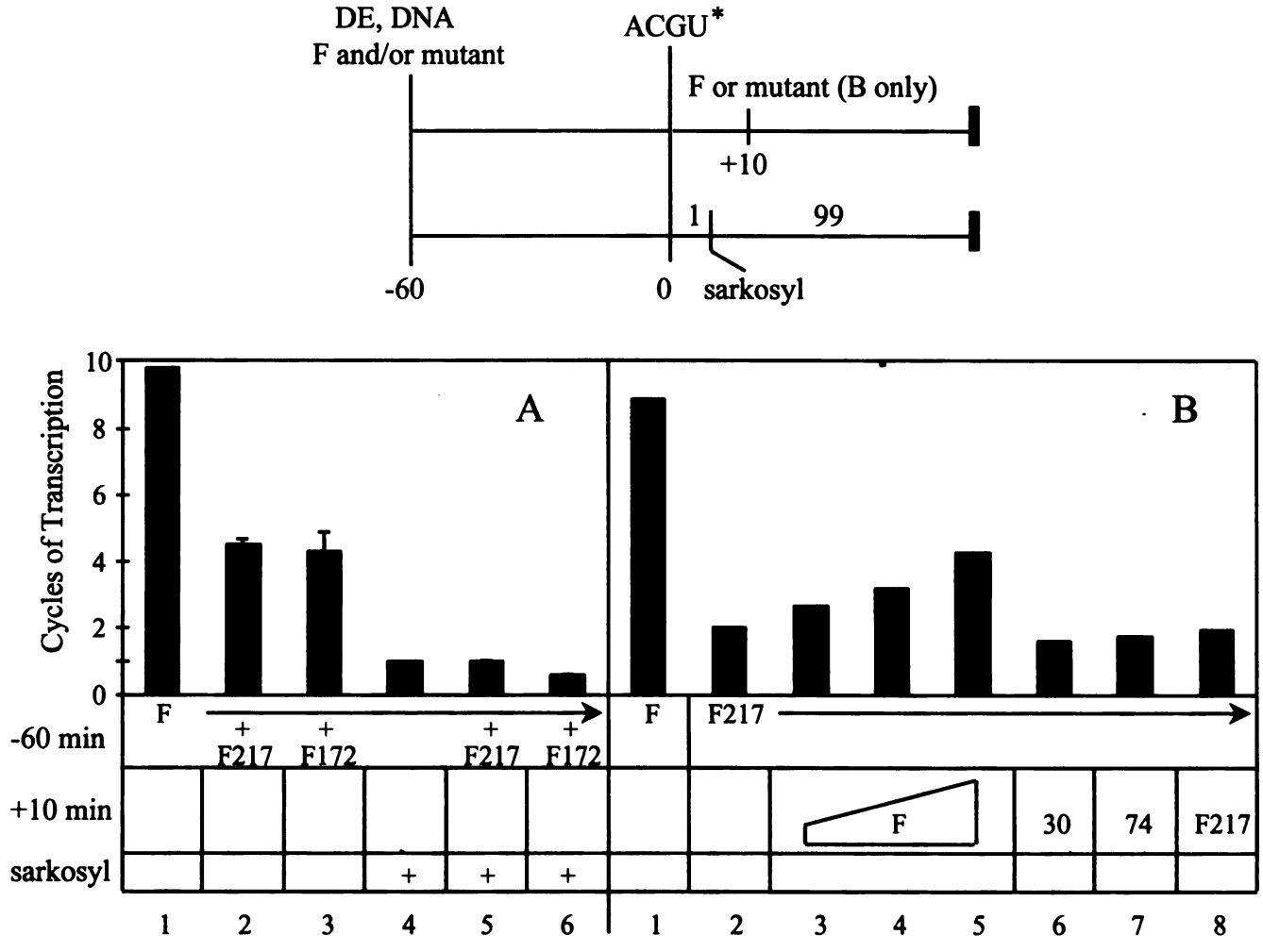

DE, DNA
F and/or mutant
ACGU*
F or mutant (B only)
+10
1
99
-60
0
sarkosyl
Cycles of Transcription
10
8
6
4
2
0
A
B
F
+
F217
+
F172
+
F217
+
F172
F
F217
-60 min
+10 min
sarkosyl
F
30
74
F217
+
+
+
1
2
3
4
5
6
1
2
3
4
5
6
7
8

Figure 3

expected to rescue transcription, but this was not observed (column 8). Furthermore, because re-addition of TFIIF did rescue multiple-round transcription, the presence of F217 did not cause the irreversible inactivation of another general factor, such as TFIIB or pol II. Therefore, these data demonstrated that RAP74(1-217) had a specific defect in multiple- but not single-round transcription and that this defect was not attributable to degradation or inactivation of the mutant protein. These experiments strengthen our argument that the C-terminal domain and central region of RAP74 have a specific role in transcriptional recycling.

New initiation resulted from previously unused pol II molecules initiating from previously unused promoters

In the extract system, new initiation events could involve re-use of promoters, re-use of pol II, or initiation from many promoters from which pol II is enabled to initiate at various times. We used a G-less cassette template as a pol II "trap" to discriminate between these possibilities (Szentirmay and Sawadogo, 1994). With the G-less cassette, an adenovirus major late promoter transcript can be synthesized with only ATP, CTP, and UTP, omitting GTP (Sawadogo and Roeder, 1985a). Pol II stalls at the first position at which GMP must be incorporated. If promoters are re-used, multiple pol II molecules pile-up at the end of the G-less cassette resulting in a ladder of transcripts each one shorter by about 30 nucleotides (Szentirmay and Sawadogo, 1994). The number of rungs on the ladder corresponds to the number of pol II molecules that have stalled on the template. If pol II must be re-used for new initiation at later reaction times, trapping pol II at the end of the G-less cassette is expected to inhibit new initiation.

We have therefore considered three models to characterize multiple-round transcription in the extract system: 1) promoter limitation (promoter re-use); 2) pol II limitation (pol II re-use); and 3) kinetic limitation (new initiation from different promoters using different pol II molecules at various times). Promoter limitation results if only a small number of promoters are bound by the necessary set of DNA-binding transcription factors (i.e. TFIID, major late transcription factor, etc.), and these factors remain committed to the same promoter through multiple transcription rounds. Dr. Michele Sawadogo's laboratory has set up a transcription system with purified and recombinant components in which functional promoter limitation was demonstrated (Szentirmay and Sawadogo, 1994). Alternatively, in the pol II limitation model, only a small fraction of pol II molecules have the capacity to accurately initiate and reinitiate. If pol II is limiting in concentration and if pol II molecules are trapped at the end of the G-less cassette, multiple round transcription will be inhibited. Pol II limitation can arise by functional limitation of any transcription factor that remains tightly associated with pol II through the transcription cycle. A third possibility is the kinetic limitation model. If a transcription factor has to be in a particular form (i.e. phosphorylation state) to be active, then the availability of active transcription complexes at any one time might be controlled.

To discriminate between these models, we did the experiment shown in Fig. 4. The template contained the adenovirus major late promoter fused to a G-less cassette (Sawadogo and Roeder, 1985a). We anticipated that a low level of GTP in the extract might allow elongation past the end of the cassette, and in fact, this is what we have observed (data not shown). This problem was overcome by addition of the chain

Figure 4. Multiple-round transcription in an extract system can be described by a kinetic limitation model. The template contains the adenovirus major late promoter fused to a G-less cassette that extends to position +389. The template was digested with *Pvu* II at position +602. Transcripts formed in the presence of the chain terminator 3'-O-methyl-GTP (mG) and in the absence of GTP stalled at the end of the G-less cassette. When GTP was included in the reaction, pol II continued transcription to the +602 runoff position. The template and protocols are summarized at the top of the figure. Expected results for the promoter limitation, pol II limitation, and kinetic limitation models are shown in the left panel and discussed in the text. Experimental data are shown in the right panel.

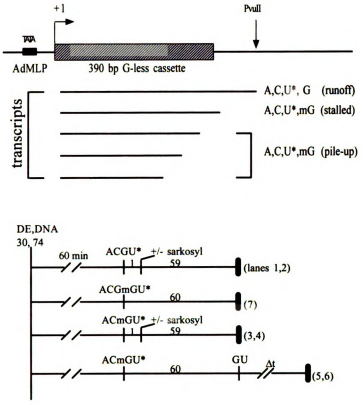

+1
PvuII
TATA
AdMLP
390 bp G-less cassette
transcripts
A,C,U*, G (runoff)
A,C,U*,mG (stalled)
A,C,U*,mG (pile-up)
DE,DNA
30, 74
60 min
ACGU* +/- sarkosyl
59
(lanes 1,2)
ACGmGU*
60
(7)
ACmGU* +/- sarkosyl
59
(3,4)
ACmGU*
60
GU
At
(5,6)
simulated results
experimental data
10
60
GU chase
ACGmGU*
ACGU*
ACmGU*
sarkosyl
runoff
stalled or terminated
"pile-up"
promoter limitation
pol II limitation
kinetic limitation
1 2 3 4 5 6 7

Figure 4

terminator 3'-O-methyl-GTP. Because the plasmid template was digested with *Pvu*II at position +602, stalling at the end of the cassette could be compared to runoff transcription.

Simulated results based on the three models are indicated with experimental data that is most consistent with a kinetic limitation model (Fig. 4). RAP74 supported approximately 4 to 10 rounds of initiation in 80 min (Figs. 1-3), and this result was confirmed for the G-less cassette template by comparing multiple-round (- sarkosyl) and single-round (+ sarkosyl) runoff transcription (Fig. 4, compare lanes 1 and 2). When 3'-O-methyl-GTP (mG) was included in the reaction, multiple-round transcription was not noticeably inhibited (compare lanes 3 and 4 with lanes 1 and 2). These data most closely resemble the results expected for the kinetic limitation model. Stalling of pol II at the end of the G-less cassette was efficient, because the runoff transcript was barely detectable in the presence of the chain terminator (lane 3). A single "pile-up" product was observed above background corresponding to approximately 10% of stalled transcripts. This gel band appears to be a pile-up product because it was chased to the runoff position with addition of GTP (compare lane 3 with lanes 5 and 6), and it was not observed in lanes 1, 5, or 6, as expected for a terminated transcript. We have not detected a second pile-up product (which would be expected at a level of only 1% of the stalled transcript), so few promoters appear to be used as many as three times (lane 3). Because about four cycles of transcription were observed and only 10% of promoters were used even twice, the extract system was generally in active promoter excess. Because new initiation was not inhibited by trapping pol II, the data were not consistent with a pol II limitation model (compare lanes 3 and 4 with lanes 1 and 2). A very slow rate of true pol

II recycling may occur in this system, but most new initiation required the recruitment of unused pol II molecules.

About 20% of the transcripts stalled at the end of the G-less cassette were terminated or arrested, because they were not chased with addition of GTP (compare lane 3 with lanes 5 and 6). This low frequency of escape from the pol II trap was not sufficient to complicate interpretation of the experimental results because escape of 20% of pol II molecules cannot account for 4 rounds of new initiation. Also, the sample in lane 7 showed that inclusion of 3'-O-methyl-GTP in the reaction did not affect multiple-round transcription.

DISCUSSION

From primary sequence (Aso et al., 1992; Finkelstein et al., 1992), RAP74 is proposed to have highly basic N- and C-terminal domains separated by a highly charged, overall acidic, and flexible central region that is rich in charged amino acids, E, D, K, and R, and also S, T, P, and G. These primary sequence features correspond to distinct N- and C-terminal functional domains (Fig. 5). The N-terminal domain is sufficient to support preinitiation complex assembly, single-round initiation, and elongation by pol II (Chapter 2). The central region and C-terminal domain of RAP74 have a small stimulatory effect on initiation but their most important function is in multiple-round transcription. Deletion of just the central region or just the C-terminal domain of RAP74 creates a protein with partial function in multiple-round transcription.

When cycles of transcription were plotted against time for multiple-round transcription reactions, the resulting curve showed a fast initial rate of RNA synthesis followed by a slower rate (Figs. 2B and 2C). The initial burst of synthesis persisted for 10-15 min, and then the slower rate dominated the reaction. This slower rate represents "multiple-round" transcription and RAP74 mutants from which C-terminal and/or central regions were removed were found to be defective for this slow rate of transcription at later reaction times.

In Figure 4, we showed that multiple-round transcription in the extract conformed to what we call a kinetic limitation model. If this system had fit the pol II limitation model, this would have demonstrated true pol II recycling, because release of pol II from the template would then have been required to support new initiation. To establish a pol

Figure 5. N- and C-terminal domains of RAP74, which were originally proposed from sequence analysis, correspond to distinct functional domains. The N-terminal domain has most of the required functions for single-round initiation and elongation. The central region and C-terminal domain of RAP74 function in multiple-round transcription. Regions shaded dark are very important for activity. Key: IIB) TFIIB; CTDP) CTD phosphatase; PIC) preinitiation complex.

multiple-round transcription stimulation
276 296 356 409 517
IIB-binding, CTDP stimulation
358 517
Pol II binding
363 444 486
1 217 399 517
N
Globular domain
E, D, K, R-rich
S, T, P, G-rich
Globular domain
C
1 74 136 172
RAP30-binding, delivery of Pol II to PIC
1 74 172 205
DBPolFE rearrangement, DNA wrapping
1 74 172 205 217
accurate initiation, elongation stimulation

Figure 5

II limited system that can support multiple-round transcription will require that recycling be much more efficient than in the extract system. According to the kinetic limitation model, new initiation events at later reaction times were largely dependent on unused pol II molecules that were recruited to (or activated at) previously unused promoters. The extract system was found to have an excess of active promoters, and pol II and general factors were estimated to be in significant excess over the level of adenovirus major late promoter transcripts that were produced (Burton et al., 1986; Cochet-Meilhac et al., 1974; Lu et al., 1992). In a system that contains an excess of active promoters and a presumed excess of transcription factors, it is somewhat difficult to understand why all the active promoters are not occupied and utilized at once. One way to view this kind of regulation is that a particular factor may require modification (i.e. phosphorylation or dephosphorylation) to be activated for initiation, so that although the factor is not limiting in concentration, it is limiting in activity.

In the extract system, the rate at which CTD phosphatases convert pol IIO to pol IIA may become limiting for initiation. When ATP and GTP are added, CTD kinases such as TFIIH (Feaver et al., 1991; Lu et al., 1992; Serizawa et al., 1992; Serizawa et al., 1993) and P-TEFb (Marshall et al., 1996) can phosphorylate pol IIA to pol IIO. If this conversion is efficient, most pol II will be in the pol IIO form, but only the small fraction that remains in the initiating pol IIA form is expected to assemble into preinitiation complexes (Chambers and Dahmus, 1994; Laybourn and Dahmus, 1989). Pol IIO requires a CTD phosphatase to convert it to the pol IIA form for new initiation. The initial burst of RNA synthesis observed in Figs. 2B and 2C may reflect utilization of the

pool of pol IIA in the extract. The slow rate of RNA accumulation at later reaction times may reflect the rate of conversion from pol IIO to pol IIA. In our system, true recycling of pol II after template runoff was not efficient, because trapping pol II at the end of a G-less cassette did not inhibit multiple-round transcription (Fig. 4). On the other hand, multiple-round transcription in the extract appeared to reflect a physiological recycling system, because this process involved activation of transcription complexes as a function of time. Additionally, multiple-round transcription was completely dependent on the presence of either the central region or C-terminal domain of RAP74 (Figs. 2B and 2C). As an example, RAP74(1-217) was completely inactive for multiple-round transcription because this mutant failed to support new initiation at late reaction times, but RAP74(1-217) was highly active for the initial burst of transcription (Fig. 2B).

RAP74, TFIIB, and the CTD phosphatase may be components of a pol II recycling mechanism. The C-terminal domain of RAP74 stimulates a CTD phosphatase (Chambers et al., 1995), and this region of RAP74 binds TFIIB (Fang and Burton, 1996) and pol II (Wang and Burton, 1995). TFIIB blocks stimulation of CTD phosphatase activity by RAP74 (Chambers et al., 1995). Dephosphorylated pol IIA preferentially enters the preinitiation complex, and CTD kinases phosphorylate pol II within the preinitiation complex or shortly after initiation (Chambers and Dahmus, 1994; Laybourn and Dahmus, 1989). During elongation, pol II is hyperphosphorylated on the CTD (Chambers and Dahmus, 1994; Lu et al., 1991). Because RAP74 stimulates and TFIIB blocks stimulation of CTD phosphatase activity (Chambers et al., 1995), we suggest that TFIIB may be present in elongation complexes to block CTD dephosphorylation in order to prevent premature termination. RNA processing beyond the 3' mRNA cleavage and

polyadenylation sequence (AAUAAA) may provide a signal to relieve the TFIIB block to CTD phosphatase activity, allowing RAP74 to stimulate conversion of pol IIo to pol IIa. Interestingly, the 3' end cleavage factor complex (CPSF/CstF) interacts with the CTD and associates with elongating pol II (McCracken et al., 1997b). We suggest that conversion of transcriptionally engaged pol IIO molecules to the pol IIA form may induce termination, with pol IIA released in the appropriate form to recycle to a promoter.

RAP74 has distinct functions in bringing pol II to the promoter, in isomerization of the preinitiation complex, in elongation stimulation, perhaps in termination, and in pol II recycling (Fig. 5). These specialized functions must be regulated for progression through the transcription cycle. For instance, RAP74 is unlikely to stimulate elongation rate and the activity of the CTD phosphatase during elongation, because this would simultaneously stimulate elongation and induce premature termination. It will be of great interest to identify all of the components of the initiation, elongation, termination, and recycling complexes to begin to unravel how pol II monitors its progress through the transcription cycle.

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CHAPTER 5

FUNCTIONS OF GENERAL TRANSCRIPTION FACTORS IN ELONGATION

Introduction

General transcription factors TBP, TFIIB, TFIIE, TFIIF, and TFIIH are required for pol II to initiate transcription specifically from a linear template in vitro (Orphanides et al., 1996; Roeder, 1996; Conaway and Conaway, 1993). TBP, TFIIB, and TFIIF are involved in recognizing the promoter and recruiting pol II. TBP is divided into two regions, a divergent N-terminal region and a highly conserved C-terminal region (TBPc). TBPc is necessary and sufficient for pol II transcription (Orphanides et al., 1996; Burley and Roeder, 1996). Both the RAP30 and RAP74 subunits of TFIIF are required for initiation from a linear template (Lei et al., 1998; Tan et al., 1994), but RAP30 is minimally sufficient for initiation from a supercoiled template (Tyree et al., 1993). RAP74 is not essential but strongly stimulates the level of transcription from the supercoiled template (Tyree et al., 1993). TFIIH and TFIIE are involved in promoter melting. The requirement of TFIIH in initiation can be bypassed using a negatively supercoiled template (Parvin and Sharp, 1993; Holstege et al., 1996). TFIIE is not essential when using a supercoiled template but TFIIE stimulates the level of transcription, presumably by facilitating formation of the open complex (Holstege et al., 1995). TFIIA, originally identified as a general transcription factor, is dispensable for basal transcription but important for transcriptional activation (Orphanides et al., 1996; Hampsey, 1998). Various interactions among these general factors and pol II have been reported (Hampsey, 1998; Orphanides et al., 1996).

TFIIF is the only general transcription factor previously shown to stimulate elongation (Bengal et al., 1991; Izban and Luse, 1992a; Price et al., 1989). Using a dC-tailed template and purified pol II, it was shown that *Drosophila* TFIIF (factor 5) had a weak affinity for the elongation complex and entered the elongation complex when pol II was paused (Price et al., 1989). Using a template competition assay and a purified system, it was demonstrated that TBP, TFIIB, TFIIE, TFIIF, and TFIIH all dissociated from pol II after initiation, and only TFIIF was able to reassociate with the elongation complex when pol II encountered pausing sites (Zawel et al., 1995). However, it is not clear which general factors remain in the elongation complex under physiological conditions because the protein factors that may normally maintain some of these general factors in the elongation complex are missing from both assays.

The DNA template in the TBP-TFIIB-TFIIF-TFIIE- pol II complex appears to be wrapped around pol II and general factors (Robert et al., 1998). DNA wrapping is proposed to induce the strand separation and formation of the open complex in initiation. TFIIF plays a critical role in forming the wrapped structure. Because the activities of TFIIF mutants in elongation are very similar to the activities in initiation, TFIIF is proposed to wrap the DNA template around pol II in elongation (Chapters 2 and 3). Wrapping is expected to facilitate DNA strand separation at the position of phosphodiester bond formation and therefore stimulate the elongation rate of pol II. TBP, TFIIB, TFIIA, TFIIE, and TFIIH might be expected to stabilize TFIIF in the elongation complex and participate in wrapping DNA around pol II as they do in the preinitiation complex. This would increase the affinity of TFIIF for the elongation complex and enhance the elongation activity of TFIIF.

Materials and Methods

Transcription factors and extracts

HeLa nuclear extracts were prepared as described (Chapter 2). Recombinant RAP30, RAP74, and RAP74 mutants were prepared and quantitated as described (Chapters 2 and 3). Calf thymus pol II was prepared by the method of Hodo and Blatti (Hodo and Blatti, 1977). Human recombinant TBPc containing the conserved C-terminal core of TBP was the generous gift of Dr. Sean Juo. Human recombinant TFIIB and yeast TBP were the generous gifts of Drs. Fan Shen and Steve Triezenberg.

The clones for production of human TFIIEα and TFIIEβ were kindly provided by Dr. Danny Reinberg. Recombinant TFIIE subunits were produced in *E. coli*, purified, and TFIIE complexes were reconstituted as described (Maldonado et al., 1996a). Recombinant TFIIA subunits (p55 and p13) in 6 M guanidine hydrochloride were the generous gifts of Dr. Jim Geiger, and TFIIA complexes were reconstituted by the method of sequential dialysis (Wang et al., 1994). TBPc, TFIIB, TFIIA, TFIIE, TFIIF, and TFIIF mutants were purified to near homogeneity as detected by the commassie blue staining of SDS-PAGE gels (data not shown).

Transcription assays

Transcription with purified components

A transcription system consisting of purified calf thymus pol II, recombinant human TBPc, TFIIA, TFIIB, TFIIE, TFIIF, and a negatively supercoiled DNA template was modified from (Parvin and Sharp, 1993; Malik et al., 1998). The template was the plasmid pML(C2AT)Δ71, a kind gift of Michele Sawadogo, containing the adenovirus major late promoter fused to a G-less cassette at position +11 (Sawadogo and Roeder,

1985a; Sawadogo and Roeder, 1985b). A 20 ul reaction mix, typically composed of 120 ng pol II, 14 ng TBPc, 30 ng TFIIB, 125 ng TFIIE, 1 pmol (~ 80 ng) TFIIF, 68 ng TFIIA, 400 ng pML(C2AT)Δ71, 20 ug BSA, and 2 u RNase inhibitor (Promega) was assembled at room temperature. The buffer contains 60 mM KCl and 6 mM $MgCl_2$. Different combinations of protein factors were used as noted. 600 uM ATP, CTP, and 25 uM [α-^{32}P]UTP (10 uCi per reaction) were added to each reaction and reactions were stopped after 1 hr incubation at 30 ^{0}C. Transcripts were isolated and analyzed on a 6% polyacrylamide gel containing 50% urea as described (Chapter 2).

Elongation stimulation assays

Stimulation of pol II elongation was determined by adding transcription factors to the transcriptionally engaged elongation complexes that were washed free of accessory factors with 1% sarkosyl and 0.5 M KCl (Chapter 3). The amounts of factors added are indicated in the figure legends. In some experiments BSA was used as a negative control. 1 mM ATP, GTP, CTP, and UTP were then added to elongate the RNA chains and the reactions were stopped after 30 seconds. Transcripts were isolated and analyzed on a 10% polyacrylamide gel containing 50% urea as described (Chapter 2).

Results

RAP74 stimulates transcription from a supercoiled template

To test the activities of recombinant TBPc, TFIIB, TFIIA, TFIIE, and TFIIF, these factors were first analyzed in the transcription assay using a defined system (Fig. 1). Consistent with previously published results (Malik et al., 1998), TBPc, TFIIB, TFIIE, TFIIF, and pol II were sufficient to actively initiate transcription from the supercoiled template pML(C2AT)Δ71 that contains a G-less cassette downstream from the transcriptional start site (lanes 2). Both bands (denoted with the arrows) represent specific products because they were sensitive to α-amanitin (lane 1), indicating pol II-specificity, and resistant to RNase T1 that would cut RNA at the position of guanosine, indicating the correct transcription start (lanes 2 and 3). TBP, TFIIB, TFIIF, and pol II were all necessary for transcription (lanes 2, 4-7). Neither TFIIA nor TFIIE was necessary but the removal of TFIIE reduced the transcription to about 50% (compares lanes 2, 8, and 9). The level of transcription was saturated at about 0.5 pmol TFIIF (lanes 10-13). RAP30 alone supported a minimal level of transcription (lanes 14 and 15), while RAP74 alone was inactive (lanes 16 and 17). The presence of RAP74, however, stimulated the level of transcription by about 10-fold (compare lanes 11 and 14).

TBPc, TFIIB, TFIIA, and TFIIE stimulate elongation by pol II

Surprisingly, TBPc, TFIIA, TFIIB, and TFIIE also stimulated elongation by pol II (Fig. 2A). First, the addition of TFIIA, TBPc, TFIIB, and TFIIE (ATBE) stimulated the synthesis of longer RNA chains (compare lanes 2 and 4), while the addition of BSA did not (lanes 3). Furthermore, ATBE cooperated with TFIIF in stimulating elongation by pol II. Although capable of stimulating the elongation rate, TFIIF was shown to have a

Figure 1. RAP74 stimulates transcription from a supercoiled template pML(C2AT)Δ71. The "+" signs denote reactions to which factors were added: pol II (120 ng), TBP (TBPc, 14 ng), F (RAP30/74 complex, 1 pmol or 80 ng), B (TFIIB, 30 ng), A (TFIIA, 68 ng), E (TFIIE, 125 ng). In lanes 10-13, 0.5, 1, 2, or 4 pmol RAP30/74 complex was added. In lanes 14 and 15, 1 or 4 pmol RAP30 instead of TFIIF was added. In lanes 16 and 17, 1 or 4 pmol RAP74 instead of TFIIF was added. In lane 1, 200 ng α-amanitin was included, and in lane 3, 2 u of RNase T1 was included. Transcripts were resolved on a 6% polyacrylamide gel containing 50% urea.

αA
T1
74
30
E
A
B
F
TBP
pol II
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
0.5
1
2
4
1
4
1
4

Figure 1

Figure 2. General transcription factors stimulate elongation by pol II.

A) A combination of human recombinant TFIIA, TBPc, TFIIB, and TFIIE (ATBE) recruits TFIIF (F) to the elongation complex. Transcription was initiated from the adenovirus major late promoter in a HeLa nuclear extract with 100 uM ATP, CTP, GTP and 1 uM α^{32}P-UTP for 1 min. Elongation complexes were prepared by washing with buffer containing 0.5 M KCl and 1% sarkosyl. A (TFIIA, 10 pmol), T (TBPc, 10 pmol), B (TFIIB, 10 pmol), E (TFIIE, 6 pmol), F (RAP30/74 complex, 2 pmol or 20 pmol), and BSA (bovine serum albumin, 2 ug) were added as indicated. The chase was with 1 mM each NTP for 30 seconds. B) Recruitment of TFIIF (1 pmol) by general transcription factors (compare lane 3 with lanes 4 through 18). The method was the same as in "A".

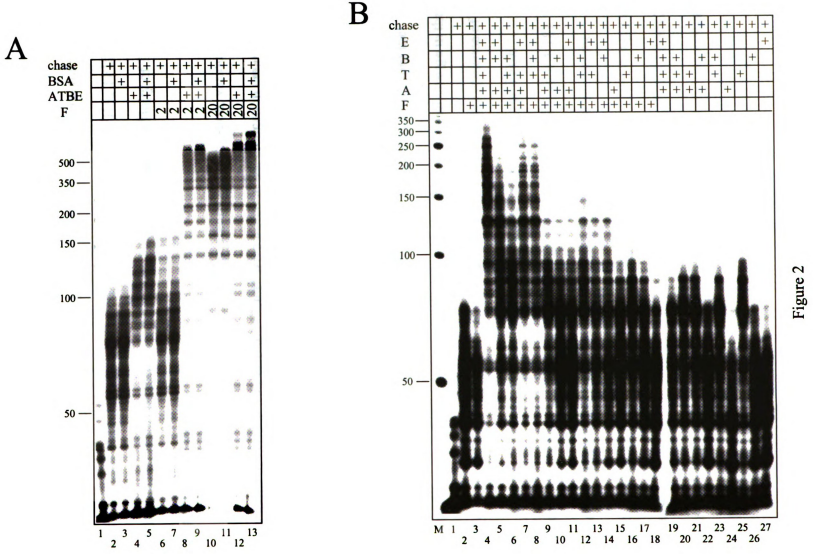
A
chase
BSA
ATBE
F
500
350
200
150
100
50
1 3 5 7 9 11 13
2 4 6 8 10 12
B
chase
E
B
T
A
F
350
300
250
200
150
100
50
M 1 3 5 7 9 11 13 15 17 19 21 23 25 27
2 4 6 8 10 12 14 16 18 20 22 24 26

Figure 2

weak affinity for the stalled elongation complex (Price et al., 1989; also see Chapter 3). The addition of ATBE stimulated elongation at both limiting (2 pmol) and saturating (20 pmol) amounts of TFIIF (lanes 6, 8, 10, and 12). The addition of BSA had no effect (lanes 7, 9, 11, and 13). Because various interactions among these general transcription factors and pol II were previously reported, it was likely that TBP, TFIIA, TFIIB, and TFIIE stimulated the elongation activity of TFIIF by increasing the affinity of TFIIF for the elongation complex.

To identify the necessary components for stimulating elongation, various combinations of general transcription factors were tested in the elongation assay (Fig. 2B). 1 pmol of TFIIF did not stimulate elongation above background (compare lanes 3 and 2), while the addition of ATBE slightly enhanced elongation (lanes 19 and 2). However, the addition of ATBE dramatically increased elongation in the presence of 1 pmol TFIIF (lanes 4, 3, and 19). The presence of ATBE may recruit TFIIF into the elongation complex. Alternatively, ATBE may induce a conformational change in the stalled elongation complex, which favors rapid elongation. Although the presence of all factors gave rise to the strongest activity, none of TBPc, TFIIB, TFIIA, or TFIIE appeared to be essential for stimulating the elongation activity of TFIIF (lanes 4-18). This result suggests that there are redundant pathways for recruiting TFIIF into the elongation complex albeit with different activities. TBPc slightly stimulated elongation in the absence of TFIIF but neither TFIIA, TFIIB, nor TFIIE had this capacity (lanes 24-27). TFIIA, TFIIB, and TFIIE did not affect the ability of TBPc in stimulating elongation (lanes 19-27).

Figure 3. TBPc, TFIIA, TFIIB, and TFIIE stimulate the elongation activities of TFIIF and TFIIF mutants. The method was the same as in Fig. 2, except that increasing amounts of TFIIF or TFIIF mutants (1, 5, 20 pmol) were added as indicated.

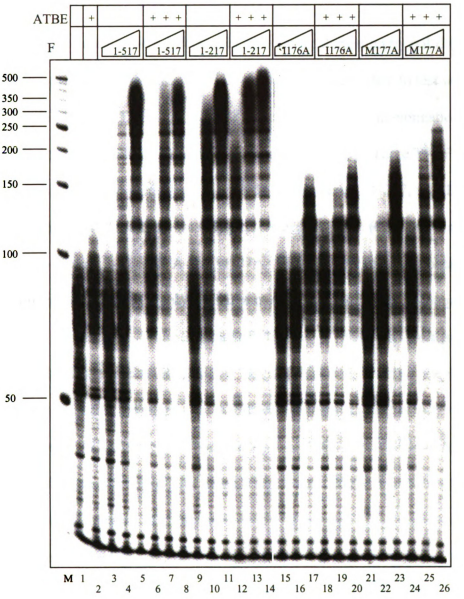
ATBE
F
1-517
1-517
1-217
1-217
1176A
1176A
M177A
M177A
500
350
300
250
200
150
100
50
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26

Figure 3

TBPc, TFIIB, TFIIA, and TFIIE stimulate the elongation activities of TFIIF and TFIIF mutants

Many TFIIF mutants were defective in stimulating elongation by pol II (Chapters 2 and 3). Because these factors aided in TFIIF recruitment, we wished to test whether TBPc, TFIIA, TFIIB, and TFIIE would rescue these TFIIF mutants in elongation or merely function to stabilize these mutants in the complex (Fig. 3). Three TFIIF mutants (1-217, I176A, M177A) and wild type TFIIF (1-517) were tested. 1-217, a TFIIF complex containing RAP74(1-217), was fully active in elongation (Chapters 2 and 3; compare lanes 9-11 with 3-5). I176A and M177A, TFIIF complexes each containing a point mutation in RAP74, were highly defective in elongation (Chapter 3; compare lanes 15-17 and 21-23 with 3-5). 1, 5, and 20 pmol of TFIIF or TFIIF mutant were tested in the presence or the absence of ATBE (TFIIA, TBPc, TFIIB, and TFIIE) in the elongation assay. As shown in Fig. 3, the addition of ATBE increased the elongation activities of TFIIF and all three TFIIF mutants. Although I176A and M177A were stimulated by the addition of ATBE, the activities of both mutants remained very low (lanes 15-26). Therefore, the intrinsic defects of I176A and M177A in elongation could not be fully rescued by the general factors.

It was previously demonstrated that the C-terminal region of RAP74 (aa 358-517) interacted with TFIIB in vitro (Fang and Burton, 1996), however, this interaction was dispensable for ATBE to stimulate the elongation activity of TFIIF because both 1-517 and 1-217 was stimulated by ATBE to a similar extent (compare lanes 12-14 with lanes 6-8). This was consistent with the result that TFIIB was not essential for stimulating the

elongation activity of TFIIF (lane 7, Fig. 2B). Other contacts among TBPc, TFIIA, TFIIB, TFIIE, and TFIIF presumably helped to recruit TFIIF into the elongation complex.

Discussion

RAP74 in initiation

Both RAP30 and RAP74 were required for transcription from a linear template in the HeLa extract (Lei et al., 1998) and in a defined system consisting of TBP, TFIIB, TFIIE, TFIIH, and pol II (Tan et al., 1994). However, RAP30 alone was sufficient to support transcription from a supercoiled template when supplemented with pol II, TBP, and TFIIB (Tyree et al., 1993). RAP74 was not essential but stimulated the level of transcription. In order to understand the different requirements for RAP74, a defined system was established with recombinant human TBPc, TFIIB, TFIIA, TFIIE, TFIIF, purified calf thymus pol II, and a negatively supercoiled template pML(C_2AT)Δ71 (Fig. 1). Clearly, pol II, TBPc, TFIIB, TFIIE, and TFIIF were sufficient for initiation in this system. Consistent with previous results (Tyree et al., 1993), the addition of RAP30 instead of TFIIF indeed supported a minimal level of transcription (lanes 14 and 15). Although RAP74 alone was inactive (lanes 16 and 17), the presence of RAP74 strongly stimulated the level of transcription (compare lanes 11 and 14). Because RAP74 was required for transcription from a linear template but not from a supercoiled template, the negative supercoils in the DNA template apparently relieved the requirement of RAP74 in initiation. This result is not unexpected because RAP74 helps to induce DNA wrapping around pol II and isomerization of the preinitiation complex. Similar to DNA wrapping, negative supercoiling may cause a torsional strain on the template to facilitate formation of the open complex, thus bypassing the requirement for RAP74 in template isomerization. The addition of RAP74 may induce DNA wrapping on the supercoiled template and stimulate transcription by further enhancing isomerization. RAP74 is

necessary for initiation from a linear template (no superhelical tension) because under this condition the DNA wrapping becomes essential for isomerization. A similar observation was made for TFIIE. TFIIE is necessary for transcription from a linear template but partially dispensable for transcription from a supercoiled template (lane 9). The requirement of TFIIE in the defined system depends on the stability of the DNA template (Holstege et al., 1995). Under conditions such as low salt in which DNA strand separation is favored, TFIIE weakly stimulates transcription. Under conditions in which DNA strand separation is more difficult, TFIIE strongly stimulates transcription. These observations led to the conclusion that TFIIE plays a direct role in open complex formation. It remains important to analyze RAP74 in the defined system under different conditions to test whether the requirement for RAP74 is directly related to the stability of the double helix.

Human TBPc stimulates elongation by pol II

TBP is a universal transcription factor required for accurate initiation by all three eukaryotic RNA polymerases (Hernandez, 1993). The primary function of TBP in pol II transcription is to bind the TATA element and nucleate the assembly of the PIC. TBPc, the C-terminal core of TBP, is sufficient for PIC assembly and initiation (Orphanides et al., 1996). It is generally believed that TBP remains bound to the TATA element when pol II exits from the promoter and begins elongation, although the evidence in support of this view is insufficient. Fig. 2 demonstrated, for the first time, that human TBPc did stimulate elongation when added to the stalled elongation complex which was washed free of accessory factors. Although the stimulation by TBPc is weak, it is highly reproducible. Neither BSA, TFIIA, TFIIB, nor TFIIE stimulates elongation by pol II.

Because the elongation complex is stalled near the start site, it is possible that TBPc binds the TATA element and induces a conformational change in the stalled elongation complex that favors rapid elongation. It remains important to test whether TBPc or full length TBP can stimulate elongation independent of a TATA element. Several approaches are proposed: 1) stall the elongation complex farther downstream from the start site and test whether TBPc or TBP can stimulate elongation; 2) stall the elongation complex, remove the upstream DNA sequence including the TATA element by restriction digestion, and then test whether TBPc or TBP can stimulate elongation; 3) test whether TBP mutants that are defective for DNA binding can stimulate elongation.

General transcription factors stimulate the elongation activity of TFIIF

TBPc, TFIIB, TFIIA, and TFIIE stimulate the elongation activity of TFIIF (Fig. 2). None of these factors (TBPc, TFIIB, TFIIA, and TFIIE; "ATBE") is essential for the stimulation but the addition of all four factors gives rise to the strongest stimulation observed. TFIIF has been shown to have a weak affinity for the stalled elongation complex. Therefore, TBPc, TFIIB, TFIIA, and TFIIE may recruit TFIIF into the elongation complex through protein-protein contacts and possibly protein-DNA contacts. These general factors may cooperate with TFIIF to induce DNA wrapping in the elongation complex as they do in the preinitiation complex (Robert et al., 1998). DNA wrapping is proposed to maintain the elongation complex in an isomerized state that is essential for phosphodiester bond formation.

TBPc, TFIIB, TFIIA, and TFIIE also stimulate the elongation activities of several TFIIF mutants tested (Fig. 3). The C-terminal domain and the central region of RAP74 are dispensable for stimulation by ATBE, because TFIIF complexes containing either

wild type RAP74 or RAP74(1-217) are stimulated to the same extent. I176A and M177A are highly defective in elongation but both can be stimulated by ATBE. However, the activities of I176A and M177A in the presence of ATBE remain low. This demonstrates that the intrinsic defects of I176A and M177A in elongation can not be rescued by ATBE, suggesting that recruitment of TFIIF mutants into elongation complex is not sufficient to stimulate rapid elongation.

This report demonstrates for the first time the novel activities of TBP, TFIIB, TFIIA, and TFIIE in transcript elongation. These general factors may recruit TFIIF and possibly help wrap the DNA template in the elongation complex. It remains important to test whether TBP, TFIIB, TFIIA, TFIIE stay in the elongation complex or they simply recruit TFIIF into the elongation complex and subsequently release. These studies strongly suggest that factors that have previously been considered to be only involved in initiation are very likely to have roles in elongation as well. It will be important to determine whether TBP, TFIIB, TFIIA, and TFIIE associate with elongation complexes in vivo.

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FUTURE RESEARCH

The functional domains of RAP74 have been mapped in this study. The N-terminal domain of RAP74 is important for both initiation and elongation stimulation. Several amino acid residues in a highly conserved region of the N-terminal domain have been found to be most critical for both initiation and elongation. These results suggest that RAP74 plays an identical role in both initiation and elongation. RAP74 is proposed to induce DNA wrapping in both the preinitiation complex and the elongation complex to facilitate isomerization. The C-terminal domain and the central region of RAP74 do not contribute strongly to single-round initiation or elongation stimulation but do stimulate multiple-round transcription. Additionally, a novel function for TBP, TFIIB, TFIIA, and TFIIE in elongation is demonstrated. These factors, previously considered to be involved only in initiation, stimulate the elongation activity of TFIIF, apparently by recruiting TFIIF into the elongation complex. TBPc alone slightly stimulates elongation by pol II.

Several projects can be developed from these results to further improve our understanding of the mechanisms of RAP74 in transcriptional initiation, elongation, and recycling.

RAP74 is proposed to facilitate formation of the open complex by inducing DNA wrapping in initiation. A series of site-directed mutants of RAP74 has been constructed and many have reduced activities in initiation. However, even the most defective mutants of RAP74 are capable of binding RAP30 and delievering pol II to the preinitiation complex. RAP74 mutants that are defective in initiation need to be tested in the photocrosslinking assay to determine whether these mutants are defective in DNA

wrapping, and these tests are in progress in collaboration with Dr. Benoit Coulombe and his laboratory.

How TFIIF stimulates the elongation rate of pol II is not yet known. Detailed studies of TFIIF in the elongation complex may face significant technical difficulties. By analogy, RAP74 is proposed to induce DNA wrapping in the elongation complex as RAP74 does in the preinitiation complex. The elongation complex at any template position may have multiple configurations. DNA wrapping is expected to maintain pol II in the isomerized state that is active in phosphodiester bond formation. RAP74 mutants that are defective in elongation stimulation are expected to be defective in maintaining the elongation complex in the isomerized state. Plasmids pML20-42 and pML20-49 containing the adenovirus major late promoter with modified sequences were kindly provided by Dr. Donal Luse. Immobilized templates containing the modified sequences were prepared and tested to be active in transcription assays (data not shown). These immobilized templates will be used to generate homogenous populations of stalled elongation complexes. Several approaches are designed to analyze the effect of RAP74 on the elongation complex. First, RAP74 can be tested for the ability to affect the DNA boundary of the elongation complex. DNA wrapping is expected to increase the extent of DNA-protein contact within the elongation complex particularly in the downstream direction. Preliminary results demonstrated that TFIIF did not affect the DNA boundary as mapped by exo III footprinting (personal communications with Dr. Donal Luse). However, TFIIF has a weak affinity for the stalled complex and may not bind tightly enough to induce changes in the footprint. Because TBPc, TFIIB, TFIIA, and TFIIE appear to recruit TFIIF into the elongation complex, the DNA boundary of the elongation

complex will be mapped in the presence or the absence of TFIIF, TBPc, TFIIB, TFIIA, and TFIIE. Second, RAP74 can be tested for the ability to affect the transcription bubble in the elongation complex by $KMnO_4$ footprinting. DNA wrapping is expected to stabilize the open complex and therefore enhance the intensity of the $KMnO_4$ footprint. Furthermore, RAP74 may affect the Km of the NTP substrates in elongation. This can be tested by analyzing the elongation rate in the presence or the absence of RAP74 and under various NTP concentrations. RAP74 mutants that are defective in elongation stimulation will be used as controls.

General factors TBPc, TFIIB, TFIIA, and TFIIE apparently stimulate the elongation activity of TFIIF by recruiting TFIIF into the elongation complex. This may be directly demonstrated by analyzing the elongation complex in an RNA gel mobility shift assay. Synchronously stalled elongation complexes will be made using the immobilized templates containing the modified DNA sequence, and the accessory factors will be removed by washing with 1% sarkosyl and 0.5 M KCl. TFIIF or TFIIF mutants will be added in the presence or the absence of general factors. After a brief incubation, the flanking DNA sequence will be removed by restriction enzyme digestion and the elongation complexes will be analyzed on a native polyacrylamide gel. The radioactive labeled RNA transcript will serve to identify the complex. This approach may also demonstrate whether general factors remain in the elongation complex because different shifts will be observed if general factors are present.

TFIIF, TFIIB, and CTD phosphatase are proposed to be components of a transcriptional recycling apparatus that regulates the conversion of pol IIO to pol IIA. TFIIF, TFIIB, and FCP-1, an essential component of CTD phosphatase, are all present in

the pol II holoenzyme prepared from HeLa extracts using GST-SII as an affinity ligand. It will be interesting to test whether this holoenzyme is capable of transcriptional recycling. If it does recycle, the pol II holoenzyme can be used to identify additional factors important for transcriptional recycling by the method of fractionation and reconstitution. It will also be easier to track the phosphorylation status of the CTD in the pol II holoenzyme than HeLa extracts because of higher background in the extracts. The phosphorylation status of the CTD will be analyzed in the presence of RAP74 or RAP74 mutants to determine whether the activities of RAP74 mutants in supporting multiple-round transcription coorelate directly with the extent of CTD dephosphorylation. A GST-SII clone has been kindly provided by Francois Robert with the permission of Dr. Jack Greenblatt.

The three-dimensional structures of TBP, TBP-TATA complex, TFIIB-TBP-DNA complex, and TFIIA-TBP-DNA complex have provided many insights into the functions and mechanisms of these transcription factors. The three-dimensional structure of the DNA binding domain of RAP30 was recently solved by NMR. However, no three-dimensional structure of RAP74 has been reported. We are currently in collaboration with Dr. Jim Geiger and his colleagues to determine the structures of RAP74 and TFIIF using X-ray crystallography techniques.

During the course of this work, several in vitro assays have been established for studying the functions of RAP74. These include the gel mobility shift assay, various initiation assays (pulse-spin, pulse-chase, and pulse-sarkosyl chase), a sensitive elongation stimulation assay, and a recycling assay. These assays will be useful in analyzing new RAP74 mutants and other transcription factors such as RAP30.

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