This is to certify that the

dissertation entitled

INTERACTIONS OF TFIIF IN THE PREINITIATION COMPLEX

presented by

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has been accepted towards fulfillment
of the requirements for

M.S. degree in BIOCHEMISTRY

Major professor

Date 12/17/99

# INTERACTIONS OF TFIIF IN THE PREINITIATION COMPLEX

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Biochemistry

1999

## ABSTRACT

INTERACTIONS OF TFIIF IN THE PREINITIATION COMPLEX

By

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Transcription factor TFIIB is an essential component of the RNA polymerase II initiation complex. TFIIB acts as a bridging factor by stabilizing the TBP-TATA box interaction on the promoter and by recruiting RNA polymerase II and TFIIF. Several lines of genetic and in vitro experiments implicate that transcriptional start site selection is mediated by a complex that involves TFIIF, TFIIB, and polymerase. The N-terminus of TFIIB has long been postulated to be a scaffold for the assembly of RNA polymerase II-TFIIF. The N-terminal domain of TFIIB binds the RAP30 subunit of TFIIF and RNA polymerase II. RAP74, the large subunit of TFIIF, has been shown to interact genetically with the N-terminus of TFIIB. In addition to the functional interaction between TFIIB and TFIIF during transcription initiation, both factors may cooperate to regulate dephosphorylation of the Carboxy Terminal Domain (CTD) of RNA polymerase II after transcription termination. Here, we characterize the interaction between TFIIF and TFIIB using surface plasmon resonance (SPR) / BIACORE. We demonstrate that TFIIF binds the N-terminal domain of TFIIB. Our studies also indicate that the interaction of the TFIIF complex with TFIIB occurs largely through the C-terminus of RAP74. A truncation of the last 25 amino acids of RAP74 abrogates the binding of TFIIF with TFIIB. Using TFIIF amino acid substitution mutants, we further characterize the region

of RAP74 between 475 and 505 as being important for TFIIB interaction. Through gel mobility shift assays, we also show that the N-terminus of TFIIB is not required for the recruitment of RNA polymerase II and TFIIF *in vitro*. TFIIB sequence from amino acid 11 to 103, encompassing the zinc finger domain, is dispensable for the assembly of DBPolF complexes. However, amino acid sequence between 11 to 45 is required for basal transcription.

This is dedicated to my wife and family,
whose love and support have kept me grounded
to what is important in life.

## ACKNOWLEDGEMENTS

I would like to thank my mentor, Dr. Zach Burton, whose knowledge, support, efforts, patience, and accomplishments have served as an inspiration toward my development as a scientist. I extend a warm hand of gratitude to Drs. Ann Finkelstein, Lei Lei, Delin Ren, and David McConnell for their friendship and scientific expertise throughout the years.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| a.a. | amino acid(s) |
| AdMLP | adenovirus major late promoter |
| ATP | adenosine triphosphate |
| BRE | TFIIB recognition element |
| CTD | carboxyl terminal domain of the largest subunit of RNA polymerase II |
| CTP | cytidine triphosphate |
| DNA | deoxyribonucleic acid |
| GTP | guanosine triphosphate |
| HAT | histone acetyltransferase |
| HCA | hydrophobic cluster analysis |
| Inr | Initiator element |
| kDa | kilodaltons |
| mRNA | messenger RNA |
| NTP | nucleoside triphosphate |
| PAGE | polyacrylamide gel electrophoresis |
| PCR | polymerase chain reaction |
| RAP | RNA polymerase II associating protein |
| RNAPII | RNA polymerase II |
| sarkosyl | N-lauroylsarcosine sodium salt |
| SDS | sodium dodecyl sulfate |
| TFII | transcription factor of RNA polymerase II |
| UTP | Uridine triphosphate |

# CHAPTER 1

# LITERATURE REVIEW

**Overview**

The regulation of gene expression is at the core of cellular function and metabolism. RNA polymerase II requires the complex interaction of transcription factors with the basal RNA polymerase II transcription machinery to form the functional holoenzyme responsible for the transcription of protein-coding genes. RNA polymerase II underlies development and differentiation, it is the endpoint of signal transduction pathways and it reshapes the cell in response to metabolic need and specific environmental information. Understanding the mechanistic details by which these processes occur has a direct impact on medical problems, such as some forms of cancer in which there is a strong correlation between loss of growth inhibition and aberrant transcriptional regulation.

Control of transcription in eukaryotes requires a greater repertoire of accessory proteins than it does in prokaryotes. In prokaryotes, a single core RNA polymerase enzyme composed of three subunits is responsible for all cellular transcription. Bacterial RNA polymerase consists of an $\alpha_2\beta\beta'$ core enzyme and a $\sigma$ factor to confer promoter recognition and specificity (Platt, 1986). Eukaryotic cells contain three nuclear DNA-dependent RNA polymerases that transcribe different sets of genes. RNA polymerase I (A) synthesizes ribosomal RNA precursors. RNA polymerase II (B) transcribes pre-messenger RNA (mRNA) and some small nuclear RNA (snRNA). RNA polymerase III (C) transcribes 5S rRNA and transfer RNA (tRNA) and some snRNA. Each of these enzymes requires the aid of an assortment of additional factors for selective promoter recognition and regulated transcription initiation.

Eukaryotic mRNA synthesis proceeds through multiple stages referred to as preinitiation, initiation, elongation, and termination. As mentioned earlier, RNAP II alone is not sufficient for specific initiation (Roeder, 1976; Weil et al., 1979). Polymerase requires additional sets of protein factors for accurate initiation from promoter DNA *in vitro* (Matsui et al., 1980). These accessory factors are termed GTFs (general transcription factors) and include TFIID, TFIIA, TFIIB, TFIIF, TFIIE and TFIIH. These GTFs function in intimate association with RNA polymerase II and are required for selective binding of polymerase to its promoters, formation of the open complex, and synthesis of the first few phosphodiester bonds of nascent transcripts.

The conventional model for ordered transcription initiation by RNAPII is characterized by a distinct series of events: 1) recognition of the promoter DNA by TFIID, 2) recognition of the TFIID-promoter complex by TFIIB, 3) recruitment of a TFIIF/RNAP II complex, 4) binding of TFIIE and TFIIH to complete the preinitiation complex, 5) promoter melting and formation of an "open" initiation complex, 6) synthesis of the first phosphodiester bond of the nascent mRNA transcript, 7) release of RNAP II contacts with the promoter (escape), and 8) elongation of the RNA transcript. RNAP II and the GTFs can assemble in a defined order on promoter DNA *in vitro*, suggesting stepwise assembly of the preinitiation complex (Buratowski et al., 1989; Van Dyke et al., 1988). However, similar to the initiation of transcription by bacterial polymerase holoenzyme (Busby and Ebright, 1994), *in vivo* RNAP II may assemble into a multi-component holoenzyme complex before binding to promoter DNA. RNA polymerase II holoenzymes have been purified from many eukaryotic organisms (Chao et al., 1996; Cho et al., 1997; Kim et al., 1994; Koleske and Young, 1994; Maldonado et al.,

1996; Ossipow et al., 1995; Pan et al., 1997; Thompson et al., 1993). The subunit composition of these different preparations differs somewhat, and these differences involve the presence or absence of GTFs and regulatory factors. The existence of this RNAPII holoenzyme suggests an alternative to the paradigm of sequential assembly of GTFs and suggests that most of the initiation machinery can bind to a promoter in a single step.

Sequence-specific DNA binding proteins regulate mRNA transcription in gene-specific ways (Mitchell and Tjian, 1989). Activated transcription involves the basal transcription apparatus integrating with additional factors (activators/repressors) that bind elements upstream of the promoter DNA. Most transcriptional regulation in eukaryotes is believed to be mediated directly by transactivators or through the intercession of adapters to modulate the rate of initiation and/or elongation by RNAP II.

Transcription factors cover a wide range of functions in the regulation of accurate transcription. General transcription factors have roles in pre-initiation, initiation, and elongation. This chapter will review the basics of mRNA transcription by RNAPII on the canonical Adenovirus Major Late Promoter (AdMLP) and will include a treatise of the components of the general transcriptional machinery and the process of basal transcription. A summary of transcriptional regulation including activation and repression will also be given.

## TRANSCRIPTIONAL MACHINERY

### Promoter Structure

Three distinct families of DNA sequence elements direct transcription by RNAP II. The first family includes the core/basal promoter elements found near the site where RNAP II initiates transcription. Two classes of core elements have been identified: the TATA element, located 25-30 base pairs upstream of the transcription start site with a consensus sequence TATA(A/T)A(A/T) and the Initiator (Inr) motif, a pyrimidine-rich sequence $PyPyA_{+1}N(T/A)PyPy$ encompassing the transcription start site (Weis and Reinberg, 1992). The TATA and Inr core elements serve to nucleate the initiation complex and are recognized by components of the transcription machinery. They can function independently or synergistically. Various promoters contain one element or both elements. Two other families of *cis*-regulatory elements are the promoter-proximal elements like CCAAT and GC elements, situated 50 to a few hundred base pairs upstream of the start site, and the promoter-distal elements (enhancers) found up to tens of thousands of base pairs away from the transcription start site (Mitchell and Tjian, 1989; Ptashne, 1989). These elements contain binding sites for protein that modulate transcription through activation or repression. Transcriptional activators bind to these elements and facilitate assembly of the preinitiation complex through direct or indirect contact with the general transcription factors. The binding of transcriptional repressors to these elements impair transcription by interfering with activators or by inhibiting general transcription factors. It is the combination of all these elements that give gene-specific promoters their characteristic strength.

A new class of core promoter element located downstream of the Inr has been identified in *Drosophila.* The downstream promoter element (DPE) with a conserved sequence motif (A/G)G(A/T)CGTG, is located about 30 bp downstream of the start site (Burke and Kadonaga, 1996). It is a basal promoter element that is present in a subset of *Drosophila* TATA-box-deficient (TATA-less). DNase I footprinting and *in vitro* transcription experiments revealed that a DPE in its normal downstream location is necessary for transcription of DPE-containing TATA-less promoters and can compensate for the disruption of an upstream TATA box of a TATA-containing promoter (Burke and Kadonaga, 1997). The DPE works in conjunction with the Inr element to provide a binding site for TFIID in the absence of a TATA box to mediate transcription of TATA-less promoters. Two components of TFIID, *Drosophila* $TAF_{II}60$ and $TAF_{II}$ 40 have been found to interact specifically with the DPE (Burke and Kadonaga, 1997).

In addition to the TATA, the initiator, and the downstream promoter element, a fourth core promoter element has recently been identified (Lagrange et al., 1998). The TFIIB recognition element (BRE) is located immediately upstream of the TATA element and has a consensus sequence of (G/C)(G/C)(G/A)CGCC. As the name indicates, the BRE is specifically recognized by the general transcription factor TFIIB. It is possible that TFIIB-BRE interaction may play a role in determining the overall strength of a promoter, the order of preinitiation complex assembly at a promoter, the rate-limiting step in transcription initiation at a promoter, and the responsiveness of a promoter to specific transcriptional activators.

**RNA Polymerase II (RNAPII)**

RNA Polymerase II is a highly conserved multisubunit protein complex among eukaryotes (Young, 1991).The three largest subunits, Rpb1, Rpb2 and Rpb3, are related to the prokaryotic core RNA polymerase subunits and are largely responsible for RNA catalysis (Markovtsov et al., 1996; Mustaev et al., 1997; Severinov et al., 1996; Zaychikov et al., 1996). RNAPII is generally composed of 10 to 12 subunits. Hela cell RNAPII contains 10 subunits ranging in size from 240 kDa to 10 kDa. Yeast RNAPII consists of 12 polypeptides with molecular sizes of 220 kDa to 10 kDa. The two largest subunits RPB1 and RPB2 are structurally and functionally related to the two largest subunits of *E. coli* RNA polymerase β' and β, respectively (Sweetser et al., 1987). Both subunits are involved in DNA and nucleotide substrate binding. The two large subunits of yeast RNA polymerase I and III are also homologues of *E. coli* RNA polymerase β' and β subunits. Rpb3, the third largest subunit, and Rpb11 are related to the α subunit of *E. coli* RNA polymerase (Kolodziej and Young, 1991) and may play a role in nucleating assembly of the enzyme. Five smaller subunits (Rpb5, Rpb6, and Rpb8, Rpb10, and Rpb12) of 14-28 kDa are essential components shared by all three nuclear RNA polymerases in eukaryotes (Allison et al., 1985; Memet et al., 1988; Treich et al., 1992; Woychik et al., 1990).

**Carboxy Terminal Domain (CTD)**

The largest subunit of eukaryotic RNAPII (Rpb1) contains an unusual C-terminal domain (CTD) consisting of multiple repeats of the consensus sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser (YSPTSPS) (Corden et al., 1985). This domain is not present in eukaryotic

RNAPI, RNAPIII, and prokaryotic RNA polymerases. The number of repeats of the heptapeptide consensus sequence appears to correlate with the genetic complexity of the organism. The sequence is repeated with some degeneracy 26-27 times in yeast, 42-44 times in *Drosophila*, and 52 times in mammals (Sawadogo and Sentenac, 1990; Young, 1991). Due to its high serine, the CTD can be highly phosphorylated at the Ser-Pro repeats. As a result of phosphorylation, RPB1 is frequently resolved into two major forms, the largely unphosphorylated IIa form or the highly phosphorylated IIo form (Cadena and Dahmus, 1987; Kim and Dahmus, 1988). A third form that is sometimes isolated lacks the CTD (designated as RNAPIIb) as a result of truncation due to proteolysis. Deletion mutations that remove the CTD are lethal in yeast (Nonet et al., 1987) , *Drosophila* (Zehring et al., 1988) and mouse (Bartolomei et al., 1988). Partial truncation of the CTD in yeast has been shown to confer cold-sensitivity and inositol auxotrophy (Nonet and Young, 1989). One possible function of the CTD may be to mediate transcription activation by upstream regulators. Except with Sp1 (Zehring and Greenleaf, 1990) , the effects of activator proteins are abolished when CTD truncation mutants are introduced in RNAPII of mammalian cells (Gerber et al., 1995).

The CTD becomes extensively phosphorylated during the initiation of transcription and must be dephosphorylated for re-entry of the polymerase in an initiation complex (Lu et al., 1991; O. Brien et al., 1994). The phosphorylation is thought to play a role in the transition from initiation to elongation (Cadena and Dahmus, 1987; O. Brien et al., 1994). Multiple kinases appear to mediate CTD phosphorylation. Most notably, the kinase activity of TFIIH (Feaver et al., 1991; Lu et al., 1992; Serizawa et al., 1992) and P-TEFb (positive transcription elongation factor b) (Marshall et al., 1996) can

phosphorylate the CTD. The Srb10/Srb11 kinase cyclin pair (Liao et al., 1995), Cdc2 , a homologous protein to P-TEFb's PITARLE catalytic subunit (Cisek and Corden, 1989), and Ctk1 (Lee and Greenleaf, 1991) also have kinase activities that can phosphorylate the CTD. Whether these CTD kinases are gene specific or affect different steps during the transcription cycle remains to be determined. Recently, extensive ubiquitination of Rpb1 during transcription reactions *in vitro* was observed (Mitsui and Sharp, 1999). Phosphorylation of the CTD was a signal for ubiquitination. Specific inhibitors of the CTD cyclin-dependent kinase (cdk) complex suppressed the ubiquitination reaction. Another study demonstrated that UV-induced ubiquitination of phosphorylated CTD Rpb1 was followed by its degradation in the proteasome (Ratner et al., 1998). Interestingly, a proteasomal subunit (SUG1) copurifies with a CTD kinase / DNA repair complex, TFIIH (Friedberg, 1996; Weeda et al., 1997). This presents a model that in both regulation of transcription and DNA repair, phosphorylation of the CTD by kinases might signal degradation of the polymerase. Transcriptional arrest of RNAPII at DNA lesions could be aborted through proteasome degradation rather than through resumption of elongation after DNA repair.

CTD kinases and CTD phosphatases may contribute to mediating differential CTD phosphorylation. A phosphatase activity specific for dephosphorylation of the CTD has been identified (Archambault et al., 1997; Chambers and Dahmus, 1994; Chambers and Kane, 1996; Cho et al., 1999; Dubois et al., 1999). This CTD phosphatase activity is regulated by both TFIIF and TFIIB (Chambers et al., 1995). The RAP74 subunit of TFIIF stimulates CTD phosphatase activity while TFIIB inhibits this stimulatory effect of TFIIF. The CTD phosphatase allows recycling of RNAPII because the

hypophosphorylated IIA form of polymerase preferentially enters the preinitiation complex. The phosphatase dephosphorylates the CTD allowing efficient incorporation of RNAPII into transcription initiation complexes, which results in increased transcription.

**Holoenzymes**

RNA polymerase II requires multiple general transcription factors to initiate site-specific transcription. These proteins have been shown to assemble *in vitro* in an ordered fashion onto promoter DNA (Buratowski et al., 1989; Van Dyke et al., 1988) .Recent evidence indicates that many of the polymerase's accessory factors assemble into a large complex, called the RNA polymerase holoenzyme. Holoenzyme is a form of RNA polymerase II that potentially is readily recruited to promoters *in vivo*. Characterization of the yeast holoenzyme has identified several initiation factors including TFIIB, TFIIF, TFIIH, SRB/mediator complex and other regulatory proteins (Kim et al., 1994; Koleske and Young, 1994). Several forms of mammalian RNA polymerase II holoenzymes have also been isolated (Chao et al., 1996; Maldonado et al., 1996; Ossipow et al., 1995; Pan et al., 1997; Scully et al., 1997). Unlike core RNA polymerase II, holoenzyme responds to transcriptional activators *in vitro*. Transcription by RNAPII holoenzyme is stimulated by the activator protein GAL4-VP16 (Koleske and Young, 1994). Other distinct forms of yeast RNAP II holoenzyme have been discovered containing various subsets of general initiation factors (Kim et al., 1994; Koleske and Young, 1994), the SWI/SNF chromatin remodeling complex (Wilson et al., 1996), and Paf1/Cdc73 complexes that regulate only a subset of genes (Shi et al., 1997; Shi et al., 1996; Wade et al., 1996). These holoenzymes differ in their composition but all respond to transcriptional activators.

**Basal Transcription Factors**

The initiation of mRNA transcription is an essential stage in the regulation of gene expression. For RNA polymerase II to transcribe a gene, an array of more than 20 polypeptides must assemble at the promoter. A universal set of proteins, termed the basal apparatus, recognizes a core promoter and initiates transcription. This basal apparatus comprises polymerase II and the general transcription factors (GTFs) TFIID (TBP + TAFs), TFIIB, TFIIA, TFIIE, TFIIH, and TFIIF. Almost all core promoters employ the same set of basal transcription factors and the subunits are conserved between yeast and man.

**TFIID**

Transcription factor TFIID is a multisubunit protein complex (≈ 750 kDa) essential for RNAPII transcription from class II promoters. It was initially isolated from a *Drosophila* nuclear extract as a complex capable of binding the TATA DNA sequence (Parker and Topol, 1984). Mammalian TFIID was identified as a chromatographic fraction of Hela cell nuclear extract, capable of protecting the TATA box and regions therein on the Adenovirus Major Late Promoter (AdMLP) (Horikoshi et al., 1988; Reinberg et al., 1987). The TFIID complex consists of the TATA binding protein (TBP) and several accessory proteins called TBP- associated factors ($TAF_{II}s$) (Burley and Roeder, 1996; Gill and Tjian, 1992; Goodrich and Tjian, 1994; Hernandez, 1993). TFIID's promoter binding activity makes it one of the most important molecules in RNAPII transcription. It is responsible for nucleating the assembly of a transcriptionally competent initiation complex (Nakajima et al., 1988; Zawel and Reinberg, 1993).

Recognition of the core promoter by TFIID is likely to be one of the first rate-limiting steps in the transcription initiation process (Buratowski et al., 1989). Two other TBP-TAF complexes exist in the nucleus of eukaryotic cells for transcription by RNAPI and RNAPIII. Distinct sets of $TAF_{II}$s in these TBP-TAF complexes provide promoter selectivity for transcription by these polymerases. SL1 is the TBP-TAF required for RNAPI transcription of ribosomal RNA (Comai et al., 1992). RNAPIII transcription of transfer RNA, 5S RNA and small nuclear RNA is carried out through TBP-TAF complexes, designated TFIIIB (Huet and Sentenac, 1992; Kassavetis et al., 1992; Lobo et al., 1992; Simmen et al., 1992; Taggart et al., 1992) and SNAPc (Hernandez, 1993). SNAPc is a bifunctional complex that, depending on snRNA structure, directs either RNAP III or RNAP II transcription of snRNA (Hernandez, 1993).

**TBP**

TATA Binding Protein's (TBP) primary function is promoter recognition through binding of the TATA box. TBP is an essential transcription factor for accurate transcription and plays a requisite role in transcription initiation by all three RNA polymerases (Cormack and Struhl, 1992; Sharp, 1992). TBP is also important for nucleation of functional preinitiation complexes on TATA-less promoters (Martinez et al., 1994; Weis and Reinberg, 1992). Amino acid sequence analysis of TBP from yeast, *Drosophila*, *Arabidopsis* and human reveals that the C-terminal domain of the protein comprising 180 amino acids is highly conserved (Hernandez, 1993; Pugh and Tjian, 1990). Basal *in vitro* transcription experiments have shown yeast TBP to be functionally interchangeable with mammalian TBP (Cavallini et al., 1988; Hahn et al., 1989).

However, human TBP fails to functionally substitute yeast TBP *in vivo* (Cormack et al., 1991; Gill and Tjian, 1991). Although highly conserved and functionally analogous, TBP's molecular weight ranges from 22 kDa (*Arabidopsis*), 27kDa (yeast), to 38 kDa (human and *Drosophila*) (Burley and Roeder, 1996).

The conserved C-terminal domain of TBP or as it is called TBP "core domain" (TBPc) forms the basis of two imperfect direct repeats that are sufficient for both binding to the TATA box and basal transcription (Hoey et al., 1990; Horikoshi et al., 1990; Peterson et al., 1990) . This domain is also important for interaction with general transcription factors TFIIA and TFIIB (Buratowski et al., 1989; Maldonado et al., 1990). TBPc has also been shown to be sufficient for support of normal cellular growth in yeast *in vivo* (Lieberman et al., 1991).

Crystal structures of *Arabidopsis*, yeast and human TBPc and TBPc bound to DNA have been solved (Chasman et al., 1993; Kim et al., 1993; Kim et al., 1993; Nikolov et al., 1996; Nikolov et al., 1992). The structure of the molecule resembles a "saddle" made up of two equal pseudo-symmetrical halves. Each α/β domain corresponds to each of the C-terminal direct repeats, composing two α helices and five antiparallel β–sheets that are connected in the order S1-H1-S2-S3-S4-S5-H2 (S for β-sheet; H for α-helix). The α–helical loop between the first and second β–sheets (S1-H1-S2) form the basis of the "stirrups" represented on both sides of the molecule that are responsible for interactions with TFIIA and TFIIB (Buratowski et al., 1989; Maldonado et al., 1990). TBP sits astride the DNA and recognizes its binding site through minor groove contacts (Lee et al., 1991; Starr and Hawley, 1991). Binding of TBP to the TATA box induces extensive distortion in the DNA helix and introduces a severe 80° kink or

bend in the DNA (Horikoshi et al., 1992). This bending serves to increase the proximity of other factors forming the preinitiation complex and may also serve to align upstream regulatory factors closer to the basal machinery.

The N-terminal domain of TBP is less well-conserved in eukaryotes (Cormack et al., 1991; Gill and Tjian, 1991) and may mediate interactions with coactivators (Pugh and Tjian, 1990; Zhou et al., 1991). However, the N-terminus among vertebrate forms of TBP is well conserved and regulates RNA polymerase III transcription at the U6 promoter (Mittal and Hernandez, 1997).

**$TAF_{II}s$**

Although TBP is sufficient for promoter recognition and the assembly of subsequent general transcription factors into a functional preinitiation complex, transcriptional activation is observed only when TFIID is used (Hoffman et al., 1990; Pugh and Tjian, 1990). TBP is the universal transcription factor that is required by all three RNA polymerases (Hernandez, 1993). The presence of distinct TBP Associating Factors ($TAF_{II}s$) bound to TBP programs these transcription complexes to discriminate between promoters designated for different classes of RNA polymerases. In each case, it is decorated with distinct sets of associated factors. *Drosophila* TFIID has been entirely reconstituted from recombinant proteins of TBP and eight $TAF_{II}s$ with molecular weight of 30 to 250 kDa (Chen et al., 1994). Human TFIID, contains 8 to 11 additional $TAF_{II}s$ ranging from 15 to 250 kDa to form the complex (Burley and Roeder, 1996; Goodrich and Tjian, 1994; Hampsey and Reinberg, 1997). Mammalian homologues have been described for all the *Drosophila* $TAF_{II}s$. In yeast, TFIID lacks an evident homologue of

$dTAF_{II}110$ /$hTAF_{II}130$ and $hTAF_{II}105$ (Dikstein et al., 1996). The largest *Drosophila* TAF, $dTAF_{II}250$ and its human and yeast counterpart are critical in the assembly of TFIID and help anchor TBP in the TFIID complex (Chen et al., 1994). $TAF_{II}$s appear to play multiple roles in transcription that include promoter recognition, catalysis, and can serve as activation domain targets. $TAF_{II}$s are important in recognizing core promoter structures including both the Inr and DPE (Burke and Kadonaga, 1997; Hansen and Tjian, 1995; Kaufmann and Smale, 1994; Purnell et al., 1994; Verrijzer et al., 1995). The different sequence recognition properties of TBP and $TAF_{II}$s may allow TFIID to recognize different promoter elements, either individually or in combination, thereby permitting activation from a diverse array of promoters (Roeder, 1996). In addition to participating in functionally relevant interactions with promoter DNA, $TAF_{II}$s contact other GTFs in the preinitiation complex (Goodrich et al., 1993; Hisatake et al., 1995; Ruppert and Tjian, 1995; Yokomori et al., 1993).

It was observed that $dTAF_{II}30$, $dTAF_{II}40$, $dTAF_{II}60$ and their human homologues are related in sequence and structure to the core histone proteins H2B, H3, and H4 (Burley and Roeder, 1996). Biochemical and crystallographic analyses indicate that a histone octamer-like structure may exist within TFIID (Hoffmann et al., 1996; Hoffmann et al., 1997). This raises the possibility that $TAF_{II}$s bind DNA in a similar fashion to the their core histone counterparts. Mimicry of a nucleosome like structure changes the topology of the promoter and could allow wrapping of DNA to permit the multiple contacts by TFIID with core promoter elements or with transcriptional activators.

TFIID possesses two known catalytic activities, including histone acetylation and basal factor phosphorylation. TFIID's histone acetyl-transferase (HAT) activity resides in

hTAF$_{II}$250 (Mizzen et al., 1996). The HAT activity of TAF$_{II}$250 is conserved in yeast, *Drosophila*, and man. Histone acetylation is an important step in the conversion of inactive chromatin into a transcriptionally competent form. Acetylation of lysine residues in histones weakens histone-DNA interactions, thereby permitting access to the DNA by activators and general transcription factors. TAF$_{II}$250 is a bipartite protein kinase which can phosphorylate itself and the RAP74 subunit of TFIIF (Dikstein et al., 1996). Sequence alignment between human TAF$_{II}$250 and its yeast counterpart TAF$_{II}$145/ TAF$_{II}$130 reveal that neither the kinase domain nor the TFIIF interaction domain is conserved in the yeast protein. Phosphorylation of TFIIF in higher eukaryotes may influence activator-dependent recruitment of RNA polymerase II into promoter complexes or may affect the initiation and elongation properties of RNA polymerase II.

TAF$_{II}$s can serve as coactivators to receive gene-specific transcriptional activation signals. TFIID is the target of many activator domains as the binding of activators to TAF$_{II}$s can enhance recruitment of TFIID to the promoter. TBP can replace TFIID fractions in basal transcription, but fails to support activated transcription in human and *Drosophila* systems *in vitro* (Chen et al., 1994; Chiang and Roeder, 1995; Dynlacht et al., 1991; Jacq et al., 1994; Pugh and Tjian, 1990; Tanese et al., 1991). TFIID fractions containing TAF$_{II}$s are essential for regulated transcription. As such, numerous activator-TAF interactions have been described (Pugh, 1996; Zawel and Reinberg, 1995). Gln-rich Sp1 and Bicoid bind dTAF$_{II}$110 (Gill et al., 1994; Sauer et al., 1995; Sauer et al., 1995). Acidic activation domain proteins VP16 and p53 bind dTAF$_{II}$40 and dTAF$_{II}$60 (Goodrich et al., 1993; Klemm et al., 1995).

**TFIIA**

Yeast TFIIA is a heterodimer of 32 kDa and 13.5 kDa (Ranish and Hahn, 1991). Deletion of either of the yeast genes encoding the two polypeptides (TOA1 and TOA2) is lethal (Ranish et al., 1992). Human TFIIA (DeJong et al., 1995; DeJong and Roeder, 1993; Ma et al., 1993; Ozer et al., 1994; Sun et al., 1994) and *Drosophila* TFIIA (Yokomori et al., 1993; Yokomori et al., 1994) are composed of three subunits with homology to the yeast proteins. The 37 kDa α and 19 kDa β subunits share sequence similarity with the large subunit in yeast, while the 13 kDa γ subunit is homologous to the smallest yeast subunit. The α and β subunits of human and *Drosophila* TFIIA are generated by protein processing from a single polypeptide precursor.

TFIIA facilitates and stabilizes the binding of TFIID to DNA through direct interaction with TBP (Buratowski and Zhou, 1992; Imbalzano et al., 1994; Lee et al., 1992). X-ray crystallographic structures of TBP-DNA-TFIIA complexes have been solved (Geiger et al., 1996; Tan et al., 1996). TFIIA binds to the N-terminal stirrup structure of TBP and makes contact with the DNA backbone upstream of the TATA box. TFIIA binds with high affinity to both TBP and certain $TAF_{II}$s (Yokomori et al., 1993) in the TFIID complex. It has been shown that cloned TFIIA plays no apparent role in basal transcription and is in fact dispensable for accurate initiation in *in vitro* transcription systems when TBP and not TFIID is used (Sayre et al., 1992; Sun et al., 1994; Yokomori et al., 1994). However, TFIIA plays an essential role in *in vivo* transcription, because of the lethal phenotype it displays when its yeast genes are deleted (Ranish et al., 1992).

Despite TFIIA's ambiguous role in *in vitro* basal transcription, TFIIA is required for activator stimulated transcription *in vitro* (Ma et al., 1993; Ozer et al., 1994; Sun et

al., 1994; Yokomori et al., 1994; Zhou et al., 1993). It does so through direct interactions with specific transcriptional activators such as Zta (Ozer et al., 1994) , Sp1, NTF-1, and VP16 (Yokomori et al., 1994) and coactivators such as PC4 (Ge and Roeder, 1994). It has also been proposed that TFIIA can function as an anti-repressor, through interactions with TBP, to remove negative regulatory components present in crude TFIID chromatographic fractions (Cortes et al., 1992; Ge and Roeder, 1994; Ma et al., 1996; Zawel and Reinberg, 1995). Pursuant to those ideas, the β and γ subunit of human TFIIA have been found to function in antirepression, while the α subunit is required for activation (Ma et al., 1996).

**TFIIB**

Transcription factor TFIIB is a single polypeptide of 35 kDa (Ha et al., 1991) that is an essential component of the RNA polymerase II initiation complex. It enters the preinitiation complex after TBP binding to the TATA box. TFIIB acts as a bridging factor by stabilizing the TBP-TATA interaction on the promoter complex and recruiting RNA polymerase II and TFIIF (Buratowski et al., 1989). Because of its bridging role, TFIIB bears two unique structural features that reinforce its importance in preinitiation assembly and function. TFIIB contains at its N-terminus a $Zn^{2+}$ finger domain that is dispensable for interaction with TBP, but is important for efficient recruitment of polymerase-TFIIF and to support basal transcription initiation (Barberis et al., 1993; Buratowski and Zhou, 1993; Hisatake et al., 1993). Mutations at the N-terminus inhibit assembly of transcription intermediates. At the C-terminus lies two imperfect direct repeats similar to the core domain of cell cycle regulatory proteins (Bagby et al., 1995;

Gibson et al., 1994). The two cyclin-like C-terminal repeats of TFIIB (TFIIBc) are necessary and sufficient for interaction with the TBP-TATA complex (Hisatake et al., 1993). TFIIBc is deficient in transcription due to its inability to support efficient recruitment of the RNAPII/TFIIF complex (Barberis et al., 1993; Buratowski and Zhou, 1993; Ha et al., 1993; Yamashita et al., 1993).

Based on the solved three-dimensional structure of TFIIBc (Bagby et al., 1995) and TFIIBc-TBP-DNA ternary complex (Nikolov et al., 1995), the C-terminal region of TFIIB binds TBP, activators (Roberts and Green, 1994) and DNA . TBP-TFIIB contacts are mainly between the basic amino-terminal repeat of TFIIB and the acidic carboxy-terminal "stirrup" of TBP. 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). This interaction may stabilize the melting of the promoter near the transcription start-site. The N-terminus is postulated to be a scaffold for the assembly of RNA polymerase II-TFIIF . In addition to the functional interaction between TFIIB and TFIIF during transcription initiation, both factors cooperate with each other to regulate dephosphorylation of the CTD of RNA polymerase II after transcription termination (Chambers et al., 1995).

**TFIIF (RAP30/74)**

By itself, RNA polymerase II cannot stably associate with the preinitiation complex subassembly, and must be escorted to the promoter by TFIIF. The subunits of TFIIF, RAP30 and RAP74, were purified from HeLa cell extract by affinity chromatography on a column containing immobilized calf thymus RNA polymerase II as

a ligand (Sopta et al., 1985). Based on their affinity to bind RNA polymerase II this group of proteins was termed RNA polymerase II-Associated Proteins (RAPs), the number indicating the apparent molecular weight in kDa. RAP30 and RAP74 were shown to be required for accurate transcription initiation from several promoters, defining the TFIIF complex as a general initiation factor that binds RNAPII (Flores et al., 1988). Antibodies directed against RAP30 co-immunoprecipitate RAP74, indicating that RAP30 and RAP74 are normally associated in a complex (Burton et al., 1988). Both subunits of TFIIF are required for accurate initiation in RAP30-immunodepleted HeLa cell nuclear extract and *in vitro* assay systems reconstituted with purified components (Burton et al., 1988; Burton et al., 1986; Flores et al., 1989). TFIIF was not initially identified as one of the four original HeLa cell chromatographic fractions (TFIIA, TFIIB, TFIID, and TFIIE) required for accurate initiation by RNAPII (Sawadogo and Roeder, 1985). Further purification resolved the TFIIE fraction into two factors, TFIIE and TFIIF, both of which are essential for initiation (Flores et al., 1989). Purification of the TFIIF fraction defined the two subunits, identical to RAP30 and RAP74 (Flores et al., 1990). In solution and in the preinitiation complex, TFIIF is a heterotetramer of two RAP30 and two RAP74 subunits (Conaway and Conaway, 1989; Flores et al., 1990; Robert et al., 1998). Gel filtration studies have shown that the apparent molecular weight of the TFIIF complex is 220-280 kDa suggesting a heterotetrameric $\alpha_2\beta_2$ structure (Conaway and Conaway, 1989; Flores et al., 1990; Kitajima et al., 1990).

TFIIF homologues have been identified and purified from rat liver as initiation factor $\beta\gamma$ , from *Drosophila* as Factor 5, and from yeast as factor g (Conaway and Conaway, 1989; Henry et al., 1992; Kitajima et al., 1990; Price et al., 1989). Yeast TFIIF

(factor g) is composed of three subunits with apparent molecular masses of 105, 54, and 30 kDa encoded by genes TFG1, TFG2, and TFG3, respectively (Henry et al., 1992). Tfgl and Tfg2 are the functional and structural homologues of mammalian RAP74 and RAP30 (Henry et al., 1994). Tfg3 represents a dispensable subunit which is less tightly associated and slightly stimulatory for yeast TFIIF activity . The gene for TFG3 was previously isolated as ANC1, a gene involved in cytoskeletal function (Welch et al., 1993). Tfg3 has no known human counterpart but bears close sequence similarity to human leukemia proteins ENL and AF-9 (Henry et al., 1994; Welch and Drubin, 1994). Tfg3 has been identified as a TFIID subunit , $yTAF_{II}30$ (Poon et al., 1995) , and is a component of the SWI/SNF chromatin-remodeling complex (Cairns et al., 1996).

Similar to bacterial σ factors, TFIIF prevents spurious initiation by inhibiting and reversing the binding of polymerase to non-promoter sites on DNA (Conaway and Conaway, 1990; Killeen and Greenblatt, 1992). In fact both subunits exhibit limited sequence similarity to bacterial σ factors (Garrett et al., 1992; McCracken and Greenblatt, 1991; Yonaha et al., 1993). It is not clear whether these structural similarities reflect functional similarities. However, RAP30 appears to be partially analogous to bacterial E.coli $\sigma^{70}$ . Human RAP30-RAP74 can bind *E. coli* RNA polymerase and can be displaced by $\sigma^{70}$ (McCracken and Greenblatt, 1991). Two conserved regions have been identified (Garrett et al., 1992; Sopta et al., 1989). Primary structure analysis of human RAP30 revealed that the greatest sequence homology between RAP30 and *E. coli* $\sigma^{70}$ is located in the 2.1 σ conserved region of $\sigma^{70}$ (McCracken and Greenblatt, 1991). This region of $\sigma^{70}$ is required for binding to *E. coli* core polymerase (Lesley and Burgess, 1989). The carboxy terminus of the RAP30 has weak sequence similarity with

region 4 of bacterial σ factors; in particular, it exhibits significant similarity to the carboxyl-terminal regions 4.1 and 4.2 of SpoIIIC (*Bacillus subtilis* $\sigma^K$) (Garrett et al., 1992). The C-terminus of mammalian transcription factor RAP30 has been found to be a cryptic DNA-binding domain (Tan et al., 1994). Strikingly, the DNA-binding domain of RAP30 is homologous to σ factor region 4, believed to direct recognition and binding of RNA polymerase at the -35 element of bacterial promoters (Dombroski et al., 1992). The NMR structure of the C-terminus of human RAP30 possesses 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 human cDNAs encoding RAP30 and RAP74 (Finkelstein et al., 1992; Aso et al., 1992) have been cloned (Aso et al., 1992; Finkelstein et al., 1992; Sopta et al., 1989). RAP30 and RAP74 produced in *E. coli* can replace natural human RAP30/74 to direct accurate transcription *in vitro* (Finkelstein et al., 1992). Some of the functions of TFIIF can be carried out by RAP30 alone. The RAP30 subunit has the property of suppressing nonspecific DNA binding of RNAPII. RAP30 can deliver the polymerase to the promoter to support transcription initiation in the absence of RAP74 (Killeen et al., 1992). In addition, the TFIIF complex can remove RNAPII from nonspecific DNA sites, whereas RAP30 alone can only prevent it from binding to these sites (Conaway and Conaway, 1990; Killeen et al., 1992).

*In vitro* evidence suggests that TFIIF binds to RNAP II primarily through RAP30. RNAPII protects and prevents the phosphorylation of RAP30 by protein kinase A in the polymerase core homology region to $\sigma^{70}$ (McCracken and Greenblatt, 1991). Recombinant RAP30 also binds RNAPII, indicating that the RAP30 subunit directs

binding of TFIIF to polymerase (Killeen et al., 1992). It is also likely that a RAP30-DNA interaction can enhance the stability of the preinitiation complex. This is consistent with the photocrosslinking of RAP30 to sequences between the TATA box and the transcription start site (Coulombe et al., 1994; Kim et al., 1997; Robert et al., 1996).

Although RAP30 can carry out many of the functions of TFIIF alone, the full complement of TFIIF activities requires the presence of RAP74. RAP74 appears to play roles in at least three stages of transcription, i.e. initiation, promoter escape, and elongation. For initiation, RAP74 participates in TFIIF's binding to polymerase by stabilizing either the interaction between RAP30 and polymerase or polymerase and the DNA (Garrett et al., 1992; Tyree et al., 1993). Indeed, RAP74 can interact directly with RNAPII through its C-terminus, which is masked by the N-terminus and central region (Wang and Burton, 1995). Curiously human RAP74 can enhance the binding of bacterial RNA polymerase to a promoter and stimulate its transcription *in vitro* (Chibazakura et al., 1994). RAP74 protein can be divided into three regions including an N-terminal and C-terminal globular domain and a flexible highly charged central region (Lei et al., 1998; Wang and Burton, 1995). Deletion mutagenesis studies have indicated that the N-terminal regions of both RAP30 and RAP74 are responsible for their interaction and are essential for transcriptional activity (Wang and Burton, 1995; Yonaha et al., 1993). Genetic studies have implicated RAP74 in start site selection. A mutation in the yeast TFIIB gene (SUA7) that causes aberrant start site selection is suppressed by secondary mutations in the yeast RAP74 homologue (Sun and Hampsey, 1995). This thereby restores the use of the normal start site. In support of this idea, RAP74 is able to interact

with TFIIB (Fang and Burton, 1996) and can be crosslinked to promoter sequences between the TATA element and the start site (Robert et al., 1996).

RAP30 and RAP74 were initially thought to function exclusively in initiation and in elongation, respectively, however both subunits are now known to function in both processes (Tan et al., 1994). RAP74 has also been implicated in RNAPII promoter escape (Chang et al., 1993). RAP74 is dispensable for initiation but is required for very early elongation. The requirement for RAP74 in promoter escape may be due to its function in late complex assembly. TFIIF can increase the rate of transcription elongation by suppressing transient pausing of the polymerase (Bengal et al., 1991; Bradsher et al., 1993; Flores et al., 1989; Izban and Luse, 1992; Price et al., 1989). The C-terminal domain of RAP74 also stimulates a CTD phosphatase activity that removes phosphate groups from the CTD of polymerase (Archambault et al., 1997; Archambault et al., 1998; Chambers et al., 1995). This stimulation by RAP74 can be blocked by TFIIB, indicating a dynamic interaction between TFIIF, TFIIB, and CTD phosphatase thought to be involved in the recycling of RNAPII (Dahmus, 1996; Roeder, 1996). It has been shown that the C-terminal domain and the central region of RAP74 are necessary for continuous RNA initiation in an extract system, possibly by the stimulation of CTD phosphatase activity (Lei et al., 1998).

Photocrosslinking studies have defined the topology of a TBP-TFIIB-TFIIF-RNA Polymerase II-TFIIE promoter complex. RAP74 and RAP30 bind promoter DNA between the TATA box and start site, a region where TFIIE and RNAPII also crosslink (Coulombe et al., 1994; Kim et al., 1997; Robert et al., 1996). RAP74 also binds DNA upstream of TATA, inducing a conformational change that affects the position of

RNAPII relative to the DNA template and wraps the promoter DNA around polymerase and TFIIF (Forget et al., 1997). From these studies and analysis of RAP74 deletion mutants, a clear correlation was drawn from the activities of RAP74 fragments in wrapping the DNA with their behavior in transcription (Lei et al., 1998; Robert et al., 1998). Due to the wrapping of promoter DNA around polymerase in the preinitiation complex, the DNA is first bent at the TATA box by TBP and bent again near the transcription start site. It is postulated that the bending and wrapping cause a superhelical distortion on the promoter that destabilizes the 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. The DNA-wrapping model has important implications for transcription initiation and elongation and the role of TFIIF in converting the polymerase into an isomerized or active state.

TFIIF can undergo several covalent modifications. Both TFIIF subunits are highly specific substrates for covalent poly ADP- ribosylation (Rawling and Alvarez-Gonzalez, 1997). The role of this modification remains unknown. RAP74 is heavily phosphorylated *in vivo*, in accordance with the presence of several potential phosphorylation sites in its sequence (Aso et al., 1992; Finkelstein et al., 1992; Kitajima et al., 1994). RAP74 has been shown to be phosphorylated *in vitro* by casein kinase II, protein kinase A, the TFIID subunit $TAF_{II}250$ (Dikstein et al., 1996; Yonaha et al., 1997) and TFIIH (Ohkuma and Roeder, 1994). RAP74 was recently shown to possess a serine/threonine kinase activity, allowing an autophosphorylation of serine 385 and threonine 389. These sites are phosphorylated by casein kinase II-like kinases and $TAF_{II}250$, a component of TFIID (Rossignol et al., 1999). The precise role of RAP74 phosphorylation has not been

established, although *in vitro* data suggest that both the initiation and elongation activities of TFIIF are stimulated by phosphorylation (Kitajima et al., 1994). Recently it was suggested that the CK2α and its phosphorylation of RAP74 can affect the *in vivo* recruitment of TFIIF to promoter DNA (Egyhazi et al., 1999).

**TFIIE**

Human TFIIE is a heterotetramer of 56 kDa (IIE-α) and 34kDa (IIE-β) subunits (Conaway et al., 1991; Inostroza et al., 1991; Ohkuma et al., 1990). However, two dimensional crystallography of a TFIIE- RNAP II complex suggests that yeast TFIIE may exist as an heterodimer (Leuther et al., 1996). Structurally, both subunits include defined structural motifs. TFIIE-α has a zinc ribbon motif, a helix-turn-helix motif, a leucine repeat and protein kinase consensus sequences (Ohkuma et al., 1991). TFIIE-β contains a consensus nucleotide binding site. TFIIE is essential for basal transcription. It enters the preinitiation complex after RNAP II and TFIIF. Its incorporation during preinitiation complex assembly is required for the recruitment of TFIIH (Buratowski et al., 1989; Flores et al., 1992; Flores et al., 1989). TFIIE has been demonstrated to interact physically with RNAP II, TFIIH and TFIIF (Maxon et al., 1994). TFIIE is functionally associated with TFIIH. Other functions of TFIIE include the stimulation of the CTD kinase activity of TFIIH, stimulation of TFIIH dependent ATP hydrolysis (Lu et al., 1992; Ohkuma et al., 1995; Ohkuma and Roeder, 1994) and promotion of DNA wrapping within the preinitiation complex (Robert et al., 1998; Robert et al., 1996). Specifically, TFIIE-α mediates the helicase activity of TFIIH either negatively (Drapkin and Reinberg, 1994) or positively (Serizawa et al., 1994). It has been shown that TFIIE in association

with TFIIH and ATP hydrolysis are required for promoter clearance (Goodrich and Tjian, 1994).

**TFIIH**

TFIIH is a multiprotein complex consisting of nine proteins whose molecular weights range from 34 kDa to 89 kDa (Drapkin and Reinberg, 1994; Maldonado and Reinberg, 1995). Its yeast counterpart contains 5 subunits (Feaver et al., 1993). Recently, a functionally active human TFIIH was reconstituted from baculovirus recombinant polypeptides (Moreland et al., 1999). TFIIH is by far the most complex basal transcription factor that contains at least three identifiable enzymatic activities (Orphanides et al., 1996), including two ATP-dependent helicase (Schaeffer et al., 1993; Serizawa et al., 1993), a DNA-dependent ATPase (Conaway and Conaway, 1989; Roy et al., 1994) and a kinase specific for CTD phosphorylation of the largest subunit of RNAP II (Feaver et al., 1991; Lu et al., 1992; Serizawa et al., 1992).

TFIIH plays a fundamental role in transcription because it is implicated in the transition from transcription initiation to elongation. Part of this step is mediated by the CTD kinase activity of TFIIH. The concept has been advanced that phosphorylation of the CTD causes a conformational change in the preinitiation complex that facilitates promoter escape (Goodrich and Tjian, 1994; Usheva et al., 1992). The CTD kinase activity resides with the MO15/Cdk7 subunit, a cyclin-dependent kinase (Feaver et al., 1994; Roy et al., 1994). Cyclin H, a regulatory partner of Cdk7, is also present in TFIIH (Serizawa et al., 1995). In combination with an activating protein MAT-1, the ternary complex cdk7/Cyclin H/MAT-1 forms what is called a CAK (cdk-activating kinase)

complex (Drapkin et al., 1996; Orphanides et al., 1996). The presence of Cak in TFIIH suggests the possibility of a link between cell-cycle regulation and transcription that has not clearly been established. In addition to its CTD kinase role, TFIIH also specifically phosphorylates TBP, TFIIE-α and TFIIF's RAP74 subunit (Ohkuma and Roeder, 1994).

Another crucial step that affects the transition from transcription initiation to elongation is TFIIH's role in forming the open transcription complex (Dvir et al., 1996; Holstege et al., 1996). TFIIH is required for transcriptional initiation from linear DNA *in vitro*. The requirement for TFIIE, TFIIH, and ATP can be obviated when transcription takes place on a DNA template that is partially unwound, such as negatively supercoiled DNA or artificially premelted DNA (Holstege et al., 1995; Pan and Greenblatt, 1994; Parvin and Sharp, 1993; Tyree et al., 1993). Because TFIIE contains no know enzymatic activity, open complex formation appeared to be mediated by TFIIH DNA helicase activity. 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 (Feaver et al., 1993; Guzder et al., 1994; Guzder et al., 1994; Park et al., 1992; van Vuuren et al., 1994). In yeast, Rad25p was also demonstrated to be required *in vivo* (Moreland et al., 1999).

TFIIH's ERCC3 subunit is identical to the XPB excision repair protein (Schaeffer et al., 1993). XPB/ERCC3 (Excision repair cross-complement) is a helicase implicated in the human DNA-repair disorders xeroderma pigmentosum (XP) (Weeda et al., 1990) and Trichothiodystrophy (TTD) (Weeda et al., 1997) . TFIIH has been proposed to play a central role in coupling transcription and DNA repair (Friedberg, 1996; Sancar, 1996). This role in nucleotide excision repair (NER)/ DNA repair was established by the ability

of purified TFIIH to rescue the repair deficiency of mammalian and yeast TFIIH mutants (Drapkin et al., 1994; van Vuuren et al., 1994; Wang et al., 1994).

## TRANSCRIPTION BY RNA POLYMERASE II

### Initiation

The general process of initiation is similar to that catalyzed by bacterial RNA polymerases. Binding of RNA polymerase generates a closed complex, which is converted at a later stage to an open complex in which the DNA strands have been separated. A separate stage of the initiation reaction requires ATP hydrolysis, release of transcription factors and polymerization of RNA. Transcriptional initiation by RNA polymerase II proceeds through four distinct stages: PIC assembly, open complex formation, abortive initiation, and promoter clearance (Zawel and Reinberg, 1995).

### Preinitiation Complex Assembly (PIC)

*In vitro* experiments have established a stepwise pathway for the assembly of the preinitiation complex (PIC) on the AdMLP promoter (Conaway and Conaway, 1990; Flores et al., 1991; Van Dyke et al., 1988) and identified various intermediate steps that remain stable after challenges such as nuclease-protection, gel mobility shift and template competition assays (Buratowski et al., 1989; Van Dyke et al., 1989; Zawel and Reinberg, 1993). In addition, protein crosslinking and X-ray crystallography has established the relative locations of DNA, RNAPII, TBP, TFIIA, TFIIB, TFIIE and TFIIF (Coulombe et al., 1994; Forget et al., 1997; Geiger et al., 1996; Kim et al., 1993; Kim et al., 1997; Kim

et al., 1993; Nikolov et al., 1995; Robert et al., 1996; Tan et al., 1996; Tang et al., 1996). The first step in the assembly of the initiation complex is binding of TFIID to the TATA box. For basal transcription, a single TBP is sufficient for TATA element recognition and subsequent incorporation of the other basal factors. TFIID instead of TBP is required for initiation from TATA-less promoters possibly through DNA-TAF interactions (Martinez et al., 1998; Weis and Reinberg, 1992). In addition, transcription regulation by upstream regulatory factors requires the TFIID complex. Binding of TBP to the TATA element through minor-groove contacts forms a very stable complex and involves dramatic DNA distortion (Burley and Roeder, 1996; Nikolov et al., 1996). The resulting bend brings sequences upstream and downstream of the TATA closer together (Geiger et al., 1996; Tan et al., 1996). TFIIA then joins the complex after TFIID binding, forming the "DA" complex. While TFIIA is not essential for basal transcription with purified factors, it can function to establish and maintain the committed complex under more physiological conditions (Lee et al., 1992). TFIIA stabilizes the TFIID-DNA interaction through direct contacts with both TBP and upstream DNA sequences (Geiger et al., 1996; Tan et al., 1996) . Although the precise mechanism is unknown, TFIIA can relieve the repressive effects of certain factors including Dr1 (Inostroza et al., 1992), topoisomerase I (Ma et al., 1996; Merino et al., 1993) and MOT1 (Auble et al., 1994) . TFIIA can displace repressors that are associated with TBP (Cortes et al., 1992; Coulombe et al., 1992; Ma et al., 1996; Merino et al., 1993; Ranish and Hahn, 1991) and enhance TBP's affinity for promoter DNA (Auble et al., 1994) thereby allowing TBP to nucleate the PIC. Binding of TFIIB, through direct interactions with TBP and with DNA sequences both upstream and downstream of the TATA element, results in a "DAB" or "DB" complex in which the

direct-repeat domain of core TFIIB is oriented towards the initiation site. TFIIB serves as a bridge between TBP and RNAPII, and this interaction is important for transcription start site selection (Leuther et al., 1996; Li et al., 1994; Pinto et al., 1992). Similar to TFIIA, TFIIB can stabilize artificially weakened TBP -TATA interactions (Imbalzano et al., 1994). Binding of a pre-formed TFIIF- RNAPII complex, through direct interactions of TFIIB with both TFIIF and polymerase, follows the binding of TFIIB (Leuther et al., 1996; Zawel and Reinberg, 1995) , forming a "DBPolF" or "DABPolF" complex. Polymerase by itself is poorly recruited to the "DB" or "DAB" complex without TFIIF. Although TFIIF plays a direct role in promoter targeting of RNA Polymerase II, it also plays an indirect role by reducing RNAP II binding to non-specific sites in DNA (Zawel and Reinberg, 1995). The small subunit of TFIIF, RAP30, appears sufficient for RNAP II recruitment (Conaway and Conaway, 1993; Flores et al., 1991; Killeen and Greenblatt, 1992; Tyree et al., 1993), however genetic and biochemical studies suggest that both RAP30 and RAP74 are necessary for stabilization of the PIC intermediate, through TFIIB interactions (Fang and Burton, 1996; Sun and Hampsey, 1995; Tan et al., 1994). Forming the "DABPolFE" or "DBPolFE" complex, the next step in the pathway is the binding of TFIIE, through its direct interactions with RNAP II, TBP, and potentially TFIIF (Maxon et al., 1994). TFIIE incorporation is necessary for subsequent recruitment of TFIIH (Flores et al., 1992). Binding of TFIIH completes assembly of the "DABPolFEH" preinitiation complex. Within the complete PIC, protein-DNA contacts now extend to position +30 (Buratowski et al., 1989; Van Dyke et al., 1988).

Initiation can also occur in the absence of some of the general transcription factors such as TFIIE, TFIIH and TFIIF on supercoiled templates (Parvin and Sharp,

1993; Tyree et al., 1993). Negative supercoiling of DNA can facilitate the unwinding of DNA strands, and obviate the need for the TFIIH, TFIIE, and ATP hydrolysis in promoter melting to form an open complex on linear DNA templates (Timmers, 1994). Experiments with pre-opened heteroduplex templates indicate the dispensability of TFIIH, TFIIE, and ATP hydrolysis (Holstege et al., 1996).

Limited studies have been performed with TATA-less (Inr) promoters and have suggested that PIC formation may be distinct for TATA and Inr promoters (Smale, 1997). Preinitiation complex assembly on TATA-less promoters requires specific $TAF_{II}s$ (Kaufmann and Smale, 1994; Purnell et al., 1994), Inr-binding factors such as TFII-I (Carcamo et al., 1991) and RNAPII (Roy et al., 1993). Inr-binding factors that have been identified include YYI, TFII-I, USF and E2F. Using a particular supercoiled DNA promoter, YYI, TFIIB and RNAPII were sufficient to direct accurate transcription (Usheva and Shenk, 1994). In this case, YYI appears to substitute for TBP, as a factor that binds to the core promoter through its Zinc finger and recruits the polymerase to the initiation complex.

The PIC can also be preassembled in the form of an RNA polymerase II "holoenzyme" containing a subset of the GTFs, requiring only TBP, the remaining GTFs and DNA for transcription initiation in response to activators (Koleske and Young, 1995). This form of RNAPII holoenzyme is often referred to as the SRB/mediator-containing holoenzyme. The SRB/mediator complex contains coactivators that physically link activators to the basal transcription machinery. RNA polymerase II holoenzymes have been purified from many eukaryotic organisms (Chao et al., 1996; Cho et al., 1997; Kim et al., 1994; Koleske and Young, 1994; Maldonado et al., 1996; Ossipow et al., 1995; Pan

et al., 1997; Thompson et al., 1993). The subunit composition of these different preparations differs somewhat involving the presence or absence of GTFs and regulatory factors. Some protocols lead to the purification of RNA polymerase II holoenzymes containing all of the GTFs (Ossipow et al., 1995; Pan et al., 1997; Thompson et al., 1993), whereas other protocols generate holoenzymes in which TFIIF is the only GTF that remains associated (Kim et al., 1994). Some yeast holoenzyme preparations contain stoichiometric levels of Swi-Snf (Kim et al., 1994), whereas others lack any detectable Swi-Snf protein (Cairns et al., 1996). Preparations of mammalian RNAPII holoenzymes appear to contain very different components, such as polyadenylation factors (McCracken et al., 1997), histone acetyltransferases (Cho et al., 1998), and the breast cancer tumor suppressor BRCA1 (Scully et al., 1997). The current model to explain the presence of holoenzymes suggests that transcription activation at many promoters involves recruitment of the transcription initiation apparatus in two steps (Myer and Young, 1998). Recruitment of a TBP-containing complex (Lee and Young, 1998) and a holoenzyme containing the remaining GTFs would be required for activation. Recruitment of either complex to the promoter could direct assembly of the other. Activators are thought to target components of both complexes and/or multiple components within a single complex. Given the large number of promoters present in living cells and the diverse mechanisms known to regulate gene expression, transcription initiation at some promoters could involve recruitment of components in many steps, and initiation at other promoters could involve recruitment of the entire apparatus in a single step.

**Open Complex Formation**

In addition to nucleotide triphosphates required for RNA polymerization, transcription by RNAPII necessitates the use of hydrolyzable β–γ bond of ATP or dATP (Bunick et al., 1982; Jiang et al., 1993; Sawadogo and Roeder, 1984; Wang et al., 1992). While eukaryotic RNAP I, III, and bacterial RNA polymerases do not require β–γ bond ATP hydrolysis, RNA Polymerase II requires it for initiation (Bunick et al., 1982; Eick et al., 1994; Lofquist et al., 1993). RNAP II requires separation of the two DNA strands prior to initiation of transcription. Specifically opening of the DNA helix requires ATP hydrolysis at the β-γ phosphoanhydride bond (Jiang et al., 1993; Wang et al., 1992). This process is known as "promoter melting" and is followed by formation of the first phosphodiester bond of the nascent RNA. Open complex formation allows access of nucleotide triphosphates to the template strand. TFIIH's ATPase activity mediates the activation of the basal initiation complex. TFIIH's associated DNA helicase activity is crucial for creating a single-stranded region during formation of the open complex. TFIIH helicase activity is required for maintaining the open region before formation of a four-nucleotide RNA product (Holstege et al., 1997). TFIIE may also play a direct role in promoter melting in the absence of TFIIH by stabilizing the single-stranded DNA in the melted region (Holstege et al., 1995) by binding single-stranded DNA through its α subunit zinc-ribbon motif (Kuldell and Buratowski, 1997). Photocrosslinking studies indicate that TFIIE stabilizes the wrapping of promoter DNA around RNAPII and TFIIF in the PIC (Robert et al., 1998; Robert et al., 1996). It is proposed that DNA wrapping has a direct role for topological untwisting of the helix around the transcription start site (Robert et al., 1998).

**Abortive Initiation**

Abortive initiation, leading to synthesis and release of oligomers up to ten nucleotides, competes with productive initiation which leads to the formation of stable elongating complexes. Bacterial RNA polymerases (Gralla et al., 1980; Levin et al., 1987; Tintut et al., 1995) go through an abortive phase of transcription in which approximately 75% of the time the short RNA is aborted and synthesis must be restarted. Similarly, accurate initiation of productive RNA synthesis *in vitro* at the adenovirus major late promoter is accompanied by abortive initiation of very short transcripts (Bunick et al., 1982; Jacob et al., 1994; Linn and Luse, 1991; Luse and Jacob, 1987). Abortive initiation may represent an energy barrier that RNAP II has to overcome in order to begin productive elongation. Alternatively, abortive initiation may reflect a conformational change that has to occur to convert the initiation complex to a productive elongation complex (Buratowski et al., 1989).

**Promoter Escape**

Promoter escape is the event which results in the transition from abortive initiation to productive elongation. This transition must break protein-protein and protein-DNA contacts that establish the initiation complex. This transition requires a conformational change in RNAPII (Samkurashvili and Luse, 1998) and release of some initiation factors. RNA polymerase promoter escape in bacterial systems involves the release of a $\sigma$ factor and changes in polymerase conformation and template contacts (Metzger et al., 1989). After promoter escape, TBP remains bound to the promoter, whereas TFIIB, TFIIE, TFIIF, and TFIIH are released from the complex (Zawel and

Reinberg, 1995). Phosphorylation of the RNAP II CTD has long been associated with the transition from initiation to elongation (Laybourn and Dahmus, 1990; Payne et al., 1989). This observation has suggested a role for CTD phosphorylation in promoter clearance (Conaway and Conaway, 1993), and the TFIIH CTD kinase activity is known to mediate this modification (Svejstrup et al., 1996). It was found in a defined transcription assay, that TFIIE, TFIIH, and ATP hydrolysis are not required for abortive initiation, but instead required by promoter clearance from a linear template (Goodrich and Tjian, 1994). As described above, this requirement could be related to DNA strand separation rather than CTD phosphorylation. The role for TFIIH in promoter clearance may explain why helicase and CTD kinase activities are found in one complex. DNA unwinding may stimulate promoter clearance, while CTD phosphorylation may mark polymerases that have successfully progressed into elongation. TFIIH-mediated phosphorylation of the CTD within the PIC has been proposed to promote conformational changes that release RNAPII from general initiation factor contacts thought to be important for either PIC assembly, activation or initiation steps. Interactions that are reversed by phosphorylation include CTD-TBP and RNAPII TFIIE interactions (Flores et al., 1989; Maxon et al., 1994; Usheva et al., 1992; Zawel and Reinberg, 1993). SRB proteins and other factors in the RNAPII holoenzyme complex that interact with the CTD might also be reversed by CTD phosphorylation. The requirement for CTD phosphorylation by TFIIH remains unclear. In the purified basal factor system, a role for CTD phosphorylation in promoter clearance has not been shown for the AdML promoter (Makela et al., 1995; Serizawa et al., 1993). However, CTD phosphorylation is required for promoter clearance at the Adenovirus E4 (AdE4) promoter (Jiang et al., 1996). CTD kinase activity of TFIIH at a

post initiation phase is also required at the dihydrofolate reductase (DHFR) promoter (Akoulitchev et al., 1995). ATP hydrolysis, therefore, may result from the TFIIH-associated DNA helicase activities required for promoter opening. Additionally, in cell extract systems, the RAP74 subunit of TFIIF (Chang et al., 1993) and P-TEFb (Marshall and Price, 1995) are required for productive promoter escape.

**Elongation**

After polymerase has escaped from the promoter, translocation of RNAPII occurs processively on the DNA as it enters the elongation phase during which the transcription complex synthesizes nascent RNA. The synthesis reaction is powered by free energy liberated by nucleotide polymerization. The elongation stage of RNAPII is a complex process that involves a number of regulatory factors and is increasingly being recognized as a major regulatory process of gene expression (Bentley, 1995; Greenblatt et al., 1993; Spencer and Groudine, 1990; Uptain et al., 1997; von Hippel, 1998; Wright, 1993). Well documented examples of transcription regulated at the elongation level include oncogenes c-myc, c-fos, and c-myb (Kerppola and Kane, 1991), HIV (Greenblatt et al., 1993) and *Drosophila* hsp70 (Lis and Wu, 1993).

Messenger RNA synthesis in eukaryotic cells can take place at rates of 1200-2000 nucleotides per minute (Tennyson et al., 1995; Thummel et al., 1990; Ucker and Yamamoto, 1984), while elongation by purified mammalian RNAPII *in vitro* proceeds at rates of only 100-300 nucleotides per minute (Izban and Luse, 1992). Detailed mechanistic studies have shown that purified RNAPII is highly susceptible to sustained pausing and arrest during elongation on naked DNA and chromatin templates (Aso et al.,

1995; Izban and Luse, 1992; Uptain et al., 1997). Promoter-proximal pausing is released by DNA- or RNA-binding activators that recruit or stimulate positive-acting transcription elongation factors. Release of RNAP II from stalled complexes is a rate-limiting step in transcription of some inducible eukaryotic genes (Izban and Luse, 1992; Kerppola and Kane, 1991; Tennyson et al., 1995). Taken together, these findings suggest the existence of a class of factors that stimulate the overall rate of elongation of eukaryotic mRNAs by suppressing pausing and arrest by RNA polymerase II (Bentley, 1995).

**Pausing, Arrest, and Termination**

Throughout the elongation phase, RNA polymerase can encounter blocks that result in transcriptional pauses, transcriptional arrest, or transcript termination (Landick, 1997). 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). Transcriptional pause and arrest signals can be intrinsic, a result of the RNA polymerase's interaction with sequences in the nascent transcript and the DNA template (Reines et al., 1987). During a transcriptional pause, RNA polymerase temporarily stops RNA synthesis for a finite period of time before spontaneously resuming transcript elongation. The molecular basis for arrest is not well understood, but a connection has been suggested between arrest and a transient failure of the polymerase to translocate along the template. An arrested elongation complex is unable to efficiently resume transcript synthesis without the aid of accessory factors. These factors can cause RNA polymerase to backtrack its catalytic center to an internal phosphodiester in the nascent RNA chain. Throughout both pauses and arrests, RNA polymerase remains

catalytically active, stably bound to the DNA template, and retains the nascent transcript. Paused complexes and arrested complexes have distinct conformations (Izban and Luse, 1993). Pausing appears to be a universal prerequisite for termination (Bentley, 1995). This effect is true for ρ-dependent termination in bacteria; however, not all pauses result in termination. Whether pausing is a necessary prerequisite for all termination events is unknown. Terminator signals cause the polymerase to irreversibly release RNA and DNA from the ternary complex. RNA polymerases can also undergo blocks to elongation in response to DNA binding proteins that physically hinder the progression of ternary complexes or under artificial conditions in which one or more of the four nucleotide substrates is not available.

**Elongation Factors**

Arrested elongation complexes have not terminated, but they can only resume chain elongation very slowly. Protein factors that influence transcript elongation have been isolated from human, yeast, rat, and *Drosophila* and have been separated into two classes (Reines et al., 1996). The first, TFIIS (SII/RAP38) helps RNAPII to read-through the template when it becomes arrested in ternary complexes (RNA-DNA-Protein) because of transcriptional blocks (Reinberg and Roeder, 1987). TFIIS has been shown to interact both genetically and biochemically with RNAPII (Agarwal et al., 1991; Archambault et al., 1992; Sopta et al., 1985).The RNAPII subunit, Rpb9, appears to specifically interact with SII (Awrey et al., 1997). The interaction of TFIIS with the arrested RNAPII stimulates the cleavage of nascent RNA from the 3' end, causing the polymerase to move backwards (Izban and Luse, 1992; Izban and Luse, 1993; Johnson

and Chamberlin, 1994; Powell et al., 1996). This RNA cleavage reaction is stimulated by GreA or GreB in bacteria and by SII in eukaryotes (Borukhov et al., 1992; Borukhov et al., 1993) . The cleavage reaction is carried out by the RNAPII itself, releasing a short RNA fragment of 7-17 nucleotides long (Rudd et al., 1994). Because of this endonuclease activity of RNAPII, SII can increase the fidelity of transcription (Jeon and Agarwal, 1996; Yoon et al., 1998). Transcription restarts from the newly exposed 3' end and RNAPII moves through the block.

A second class of structurally unrelated elongation factors include TFIIF, Elongin (SIII), ELL& ELL2 (Shilatifard et al., 1997; Shilatifard et al., 1996), DSIF (Selby and Sancar, 1997) and CSB (Selby and Sancar, 1997).These factors increase the overall elongation rate of RNAP by suppressing its the transient pausing (Bengal et al., 1991; Bradsher et al., 1993; Bradsher et al., 1993; Izban and Luse, 1992; Price et al., 1989; Shilatifard et al., 1997; Shilatifard et al., 1997; Shilatifard et al., 1996). As with SII, these proteins can bind to RNAPII but function in a distinct manner because they cannot release polymerase from arrest or promote polymerase backtracking.

TFIIF is unique among the basal transcription factors in that it has roles in both initiation and elongation (Bengal et al., 1991; Izban and Luse, 1992; Kephart et al., 1994; Lei et al., 1998; Tan et al., 1994). TFIIF can decrease the duration of pausing by RNAPII and protects the elongation complex from arrest (Gu and Reines, 1995). It has been proposed that stimulation of elongation is potentiated through the phosphorylation of the RAP74 subunit (Kitajima et al., 1994). The kinase responsible for RAP74 phosphorylation *in vivo* has not been identified. A number of protein kinases are capable of phosphorylating RAP74 *in vitro*. They include casein kinase II, protein kinase C & A,

(Kitajima et al., 1994), $TAF_{II}250$ (Dikstein et al., 1996) and RAP74 , which can weakly autophosphorylate itself (Rossignol et al., 1999). Analysis with RAP74 mutants have suggested that only the N-terminal of the protein is required for elongation (Kephart et al., 1994) whereas a RAP30 central region is required for elongation (Tan et al., 1995).

The Elongin complex (SIII) functions through a direct interaction with elongating polymerase and suppresses pausing by increasing the time polymerase spends in an active conformation (Bradsher et al., 1993; Moreland et al., 1998). Elongin is a heterotrimer composed of a transcriptionally active A subunit of 110 kDa and two smaller positive regulatory B and C subunits of 18 and 15 kDa respectively (Aso et al., 1996; Takagi et al., 1996). A role for the elongin BC complex in oncogenesis was brought to light by the discovery that elongin B and C are components of a multiprotein complex containing the product of the von Hippel-Lindau (VHL) tumor suppressor gene (Duan et al., 1995; Kibel et al., 1995). VHL disease is an autosomal dominant familial cancer syndrome that predisposes affected individuals to a variety of tumors. Like elongin A, the VHL protein contains a consensus elongin BC box (Kibel et al., 1995). Binding of elongin A and VHL to the elongin BC complex is mutually exclusive and VHL is capable of inhibiting the activity of the elongin complex *in vitro* by binding tightly to the elongin BC complex and preventing it from interacting with elongin A (Duan et al., 1995).

P-TEFb is a unique elongation factor because it promotes elongation by RNAPII possibly through phosphorylation of the CTD (Marshall et al., 1996). P-TEFb is a heterodimer composed of 43 and 120 kDa subunits. It was originally identified from *Drosophila* as a factor important for the production of DRB-sensitive transcripts *in vitro* (Marshall and Price, 1995). DRB is a nucleotide analog which has been shown to be a

potent inhibitor of eukaryotic mRNA synthesis *in vivo* and in crude transcription systems *in vitro* (Chodosh et al., 1989; Marshall and Price, 1992; Zandomeni et al., 1983). *Drosophila* and human P-TEFb are capable of phosphorylating the CTD of RNA polymerase II (Marshall et al., 1996; Peng et al., 1998; Peng et al., 1998). The CTDs of actively elongating polymerases are highly phosphorylated (Bartholomew et al., 1986; Cadena and Dahmus, 1987; O. Brien et al., 1994; Payne et al., 1989). Polymerases containing hypophosphorylated CTDs preferentially enter the preinitiation complex (Chesnut et al., 1992; Laybourn and Dahmus, 1990; Lu et al., 1991; Serizawa et al., 1993), where they are subsequently phosphorylated during or shortly after initiation (Laybourn and Dahmus, 1990; O. Brien et al., 1994). DRB inhibits phosphorylation of the CTD by P-TEFb which correlates with DRB-induced inhibition of elongation by RNA polymerase II. P-TEFb has also been implicated in the HIV-1 Tat-mediated transcriptional activation (Fujinaga et al., 1998; Zhu et al., 1997).

**Transcriptional Termination And Recycling**

The mechanism controlling RNAPII termination is poorly understood. Termination results when RNAPII stops its processive elongation and releases the nascent RNA (Kerppola and Kane, 1991). *E. coli* RNA polymerase recognizes specific sites in the DNA template which serve as termination signals. This recognition for the most part occurs independently of accessory factors and can occur intrinsically by prokaryotic RNA polymerases. However, protein factors, such as bacterial rho protein, are also required at some sites in prokaryotes. RNAPII requires release factors downstream of the polyadenylation addition signal. The most notable of eukaryotic

release/termination factors is the *Drosophila* N-TEF protein. The *Drosophila* N-TEF protein and its human counterpart function in an ATP-dependent manner to induce release of transcripts synthesized by RNA polymerase II (Liu et al., 1998; Xie and Price, 1997; Xie and Price, 1996). One of the major eukaryotic termination factors is the 3' processing machinery for RNA polymerase II transcripts (Proudfoot, 1989). Termination of the nascent transcripts is coupled to processing in ways that are not understood. Accurate and efficient termination of transcription requires the sequences and machinery required for proper 3'end formation (Pandey et al., 1994; Proudfoot, 1989).

After polymerase has terminated and been released, it is free to reassemble the preinitiation complex at the same or a distinct TFIID bound promoter. The same polymerase molecule is re-delivered to a promoter and initiates another round of transcription, a process called recycling. The polymerase must be made competent to efficiently re-enter into the PIC. RNAPII binds to the preinitiation complex preferentially with its CTD in the underphosphorylated RNAPIIa form (Lu et al., 1991). Transition from a preinitiation complex to an elongation complex is accompanied by phosphorylation of the CTD, yielding to the RNAPIIo form. To recycle the polymerase, there must be a conversion from IIo to IIa, facilitated by a phosphatase. A CTD phosphatase that catalyzes the dephosphorylation of RNA Polymerase II has been isolated (Chambers and Dahmus, 1994; Chambers and Kane, 1996). Polymerase recycling may be regulated by the coordinated activities of at least TFIIF, TFIIB, and the CTD phosphatase in a multimeric complex. The C-terminal domain of the RAP74 subunit of TFIIF has been shown to strongly stimulate the CTD phosphatase (Chambers et al., 1995). RAP74 mutants containing only the N-terminal domain do not stimulate the

CTD dephosphorylation and are incapable of transcriptional reinitiation (Lei et al., 1998). However, TFIIB binding to RAP74 blocks the RAP74-dependent stimulation of the CTD phosphatase activity (Chambers et al., 1995). The inhibitory effect of TFIIB on CTD phosphatase activity may serve to prevent premature dephosphorylation during productive elongation, which could cause premature termination.

## TRANSCRIPTIONAL REGULATION

### Transcriptional Activation

Under normal conditions, the minimal protein apparatus required for accurate transcription initiation consists of GTFs and RNAPII. This transcription is not dependent on the presence of regulatory factors and is known as "basal" transcription. Transcriptional activation of eukaryotic genes during development or in response to extracellular signals involves the regulated assembly of multiprotein complexes on enhancers and promoters. DNA packaged into chromatin results in general repression of gene transcription (Hanna Rose and Hansen, 1996). Activated transcription is a process by which an activator protein induces an increase in RNAPII transcription of a certain gene. Activators are specific regulatory proteins that bind DNA sequences located upstream of the core promoter to ultimately increase the rate of transcription. Activators can stimulate various steps along the pathway for mRNA synthesis and may serve to counteract repression and increase transcription. These can include : 1) removal of repressor molecules from promoters by recruiting, for example, chromatin remodeling enzymes (Kingston et al., 1996), 2) recruitment of GTFs and RNAPII to the promoter to

facilitate PIC assembly (Paranjape et al., 1994; Ptashne and Gann, 1997), 3) stimulation of promoter clearance and elongation (Bentley, 1995; Triezenberg, 1995), 4) induction of covalent modifications such as phosphorylation of proteins in the PIC.

**Activators**

Protein fusion experiments have defined two modular functional regions of cellular activators (Brent and Ptashne, 1985; Ptashne, 1988). One "promoter-targeting" region or DNA-binding domain directs the protein specifically to the DNA, enabling the other "activating domain" to stimulate transcription (Mitchell and Tjian, 1989). Activators have loosely been classified according to the most prevalent amino acid of their activation domains. Activators have been termed acidic (VP16, GAL4, GCN4), glutamine-rich (Sp1, Oct-1, Oct-2, Jun), proline-rich (CTF/NF1), or serine/threonine-rich (Triezenberg, 1995). However, a pattern of bulky hydrophobic amino acid residues in these activation domains may be more important than the prevalence of characteristic amino acids (Cress and Triezenberg, 1991; Gill et al., 1994; Triezenberg, 1995). It is likely that interaction and cohesion of activation domains and their targets is driven by hydrophobic forces. Structural information of activation domains has been difficult to obtain because these domains largely appear to be unstructured and have no secondary structure (Shen et al., 1996). However, activation domain have been known to undergo an induced fit, assuming more constrained structures in the presence of certain GTFs (Shen et al., 1996; Uesugi et al., 1997).

The DNA-binding domains of activators reveal canonical protein motifs for recognizing specific DNA sequences, including the helix-turn-helix motif, Zn-fingers,

and the leucine-zipper motif (Harrison, 1991). The overall potency of a transcriptional activator depends on a number of factors: the affinity for its site on the DNA and the subunit interactions necessary to assemble a functional activator, as well as the strength of the interaction between an activation domain and its target.

**Transcriptional Coactivators**

The ability to reconstitute activator dependent transcription *in vitro* with biochemically defined components has enabled a search for partners that interact with specific activation domains. Transcriptional coactivators, also known as mediators or adapters are required for transcriptional activation to transduce the signal to the basal machinery by bridging the interaction between gene-specific activator proteins and GTFs (Berger et al., 1990; Kelleher et al., 1990; Pugh and Tjian, 1990). These factors are distinct from activators in that most do not directly bind DNA and none appears to bind DNA in a sequence-specific manner (Hampsey, 1998). Coactivators can also facilitate chromatin remodeling (Kaiser and Meisterernst, 1996). Several functionally distinct classes of coactivators have been described. These include the TAF components of TFIID (Goodrich and Tjian, 1994), the SRB/mediator complex that associates with RNAPII's CTD (Hengartner et al., 1995), TFIIA, SAGA and related complexes that catalyze nucleosomal histone acetylation (Grant et al., 1998), and the SWI/SNF and related chromatin-remodeling complexes (Chiba et al., 1997).

**Template Activation**

Chromatin presents a sizable barrier to the process of RNA transcription, inhibiting both the accessibility of the general transcription machinery to promoter sequences and the binding of upstream regulatory proteins. *In vitro* experiments have established that RNAPII and the GTFs cannot efficiently bind promoters packaged into nucleosomes (Paranjape et al., 1994). Promoter binding would require the assistance of factors that alter or remodel nucleosomes to allow access to the promoter. This derepression would cause the chromatin structure to be decondensed and unfolded, or modify the nucleosome components so as to affect their availability or structure. One candidate for this activity is the multiprotein SWI/SNF complex, which has been implicated in chromatin remodeling by genetic and biochemical studies (Peterson, 1996). SWI/SNF can facilitate TBP binding to promoter DNA and activator binding to upstream regulatory sequences (Cote et al., 1994; Imbalzano et al., 1994; Peterson and Tamkun, 1995). Various subunits of SWI/SNF have been associated with both yeast and human RNAP holoenzymes (Liao et al., 1995; Neish et al., 1998; Wilson et al., 1996).Yeast RNAPII holoenzyme containing SWI/SNF has been shown to remodel nucleosomes (Gaudreau et al., 1997). The SWI/SNF complex requires ATP hydrolysis for its role in transcription stimulation and possesses a DNA-dependent ATPase activity. Interestingly, the SWI/SNF complex possesses a weak sequence specific DNA-binding activity, that may suggest a role for targeting RNAPII holoenzyme to specific promoters (Quinn et al., 1996). It is unlikely that SWI/SNF is the universal nucleosome remodeler, in light of other identified remodeling activity, such as NURF and RSC (Cairns et al., 1996; Tsukiyama et al., 1995; Tsukiyama and Wu, 1995).

Chromatin mediated repression of transcription can be alleviated by the addition of transcriptional activators such as GAL4-VP16, Sp1 and PHO4 (Croston et al., 1991; Kamakaka et al., 1993; Lorch et al., 1992; Svaren et al., 1994; Workman et al., 1991). Transcriptional activators are directly involved in the modification of chromatin to derepress its effect on gene transcription. Activators may recruit chromatin-remodeling complexes to specific promoters to alter the local nucleosomal structure and allow assembly of the PIC.

Histone acetylation is also involved in nucleosomal disruption (Struhl, 1998; Vettese-Dadey et al., 1996). Acetylation of histone makes chromatin less condensed and, therefore, more accessible to the transcription machinery (Hebbes et al., 1988). Two yeast transcription coactivators, ADA and SAGA complexes, contain the specific histone acetyl transferase GCN5 (Grant et al., 1997; Horiuchi et al., 1995). $TAF_{II}250$ is a subunit of the basal transcription factor TFIID that possesses histone acetyltransferase activity. $TAF_{II}250$ can interact with both activators and basal factors (Imhof et al., 1997; Ruppert and Tjian, 1995; Wang et al., 1997). Transcriptional activators appear to work synergistically with chromatin remodeling complexes such as SWI/SNF and HAT complexes to facilitate derepression.

**Recruitment of GTFs**

Activator proteins work to enhance the rate at which GTFs assemble to form the preinitiation complex and/or induce its isomerization to an activated form. Direct interactions between activators and several components of the basal transcription machinery (TFIIA, TBP, IIB, IIE, IIF, and IIH) have been detected (Hori and Carey,

1994; Zawel and Reinberg, 1995). In this model, activators bind to upstream DNA sequences and assist the assembly of the PIC through interactions with GTFs and by looping the DNA to permit the interaction (Kingston and Green, 1994). Alternatively, recruitment may function to retain some GTFs at the promoter after initiation (Zawel et al., 1995). By interacting directly with promoter bound GTFs, activators may prevent their dissociation to facilitate reinitiation.

The association of TBP with the promoter has been characterized as being slow *in vitro* (Schmidt et al., 1989). *In vivo* studies with the yeast acidic activator GCN4 have suggested that TBP-TATA binding is rate limiting and activation domains can increase the recruitment of TBP to promoters (Klein and Struhl, 1994). Similar, experiments using GAL4-TBP fusion protein show that TBP recruited by the GAL4 DNA-binding domain to promoters bearing a GAL4-binding site can interact with the TATA element and direct high levels of transcription *in vivo* (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, Zta, Tat, and p53 (Chang et al., 1995; Emili et al., 1994; Franklin et al., 1995; Ingles et al., 1991; Liu et al., 1993; McEwan et al., 1996; Melcher and Johnston, 1995; Pearson and Greenblatt, 1997; Truant et al., 1993; Zwilling et al., 1994). Activators may also enhance the stability of the TBP-TATA binary complex (Chen et al., 1993). Activators can also recruit TFIID by interacting with TAF subunits (Carrozza and DeLuca, 1996; Chen et al., 1994; Defossez et al., 1997; Gill et al., 1994; Goodrich et al., 1993; Jacq et al., 1994; Klemm et al., 1995; Mazzarelli et al., 1997; Sauer et al., 1995; Schwerk et al., 1995).

The association of the TBP to DNA promoters is a rate-limiting step in gene expression. It was recently suggested that slow promoter binding might be related to TBP's ability to occlude its DNA binding domain through TBP-TBP dimerization (Coleman and Pugh, 1997). In fact, TBP and TFIID can exist as dimers in solution (Coleman and Pugh, 1997; Jackson-Fisher et al., 1999; Taggart and Pugh, 1996). TBP dimerization may be a mechanism to prevent unregulated gene expression and its own degradation (Jackson-Fisher et al., 1999). This suggests that the purpose of certain activators may be to disrupt TBP dimerization in order to enhance the rate of TBP-DNA binding. Pursuant to this idea, VP16 has been found to induce a conformational change in TBP that affects TBP binding to DNA (Liljelund et al., 1993). Recently, TFIIA has also been shown to regulate TBP/TFIID monomerization and accelerate the kinetics of DNA binding by TBP (Coleman et al., 1999).

TFIIA is dispensable for basal transcription but is required for activator mediated transcription *in vitro* and *in vivo* (Ma et al., 1993; Ozer et al., 1998; Ozer et al., 1994). Recruitment of TFIIA by certain activators can promote DA complex formation and help overcome the slow step of TBP binding in PIC formation (Chi and Carey, 1996; Chi and Carey, 1993). 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).

Transcription activation is thought to entail conformational changes in the preinitiation complex prior to initiation of RNA synthesis. The presence of an activator can dramatically alter the Dnase I footprint of TFIID on a promoter (Burley and Roeder,

1996). It has been suggested that VP16 may induce a conformational change in TFIIB that facilitates the binding of TFIIB to the TBP-DNA complex by disrupting intramolecular interactions within TFIIB and stimulates RNAPII/TFIIF recruitment (Hayashi et al., 1998; Roberts and Green, 1994). TFIIB mutants defective in activated, but not basal transcription have been identified (Roberts et al., 1993). Indeed, TFIIB can interact 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; Choy and Green, 1993; Kim and Roeder, 1994; Leong et al., 1998; Lin et al., 1991; Roberts et al., 1993; Wu et al., 1996; Xing et al., 1995).

The roles of TFIIF and TFIIE in transcriptional activation are not clear. TFIIE interacts with the Epstein-Barr virus activator EBNA 2 through a coactivator p100 (Tong et al., 1995). The RAP74 subunit of TFIIF has been known to interact with transactivation proteins serum response factor (SRF), androgen receptor, and VP16 (McEwan and Gustafsson, 1997; Zhu et al., 1994). A correlation of SRF-RAP74 binding and transcriptional activation suggests that RAP74 is a critical target for SRF-activated transcription (Joliot et al., 1995). Such a correlation has not been defined for androgen receptor and VP16.

**Stimulation of Promoter Escape and Elongation**

Initiation is not the only stage of RNAPII transcription that is stimulated by activators. As described above, post initiation steps such as promoter clearance and elongation are also subject to regulation. Stimulation of promoter clearance not only enhances the rate of RNA synthesis but also facilitates the entry of a second polymerase.

The formation of an open complex requires the ATP-dependent DNA helicase activity of TFIIH. Binding of activators to TFIIH or TFIIE may stimulate the helicase activity and therefore facilitate formation of the open complex.

The complete preinitiation complex contains at least two CTD specific protein kinases, including TFIIH and the mediator/SRB complex of the holoenzyme (Liao et al., 1995). Phosphorylation of the CTD is required for the polymerase to enter an elongation mode. It is conceivable that activators could increase the rate of initiation by promoting CTD phosphorylation. For example, upon the activity of a heat shock activator HSF near the promoter of *Drosophila* hsp70, enhanced phosphorylation of the CTD can induce promoter proximal escape of the polymerase (Lis and Wu, 1993; O. Brien et al., 1994; O. Brien and Lis, 1991; Rasmussen and Lis, 1993). TFIIH binds activator proteins VP16, p53, RARα, and Tat (Garcia-Martinez et al., 1997; Rochette-Egly et al., 1997; Xiao et al., 1994). Mutations in the VP16 activation domain have been shown to affect formation of the open complex (Jiang et al., 1994). RARα and p53 activation domains can be phosphorylated by TFIIH and the phosphorylation correlates with their activities in transcription (Lu et al., 1997; Rochette-Egly et al., 1997). The Tat protein is a transcriptional activator that is required for efficient human immunodeficiency virus 1 (HIV-1) gene expression . The interaction of Tat with TFIIH stimulates CTD phosphorylation and activation of HIV-1 transcription (Garcia-Martinez et al., 1997; Parada and Roeder, 1996; White et al., 1992). Interestingly, a coactivator responsible for Tat transactivation, Tat-SF, binds and co-immunoprecipitates with TFIIF's small subunit, suggesting a role for RAP30 as a target in controlling elongation by Tat (Kim et al., 1999).

Activators may also target known elongation factors such as P-TEFb, TFIIF, SII, SIII, and ELL. These factors act by stimulating the catalytic rate of RNA synthesis (TFIIF, SIII, and ELL) or by facilitating the passage of RNAPII through various arrest sites (SII). Activators could stimulate elongation by recruiting these factors to the polymerase and affecting the processivity of the elongation complex. The recent implication that TFIIF serves to form the open complex by inducing promoter DNA to wrap around the PIC presents another potential target for activators (Coulombe and Burton, 1999; Robert et al., 1998).

# CHAPTER 2

# INTERACTION BETWEEN TRANSCRIPTION FACTORS TFIIF AND TFIIB

## INTRODUCTION

The general transcription factor TFIIB plays an essential role in RNAPII transcription, and together with RNAPII and TBP, defines the minimal set of factors necessary for *in vitro* promoter-dependent transcription of a supercoiled DNA template (Orphanides et al., 1996). TFIIB is involved in the selection of transcription start sites, as mutations in the *Saccharomyces cerevisiae* TFIIB homologue *SUA7* gene (Pinto et al., 1992) alters transcription start site selection *in vivo* and *in vitro* (Bangur et al., 1997; Pinto et al., 1994). TFIIB acts as a bridge between promoter-bound TFIID/TBP and RNAPII/TFIIF. In both ordered-assembly and holoenzyme-recruitment models of preinitiation complex formation, TFIIB recognizes promoter-bound TFIID and facilitates the assembly of the remaining GTFs and RNAPII. Consistent with this role, TFIIB interacts with DNA adjacent to the TATA box (Lagrange et al., 1998) and binds to TBP (Buratowski et al., 1989; Ha et al., 1993; Maldonado et al., 1990), the TBP-associated factor $TAF_{II}40$ (Goodrich et al., 1993), RNAPII (Bangur et al., 1997; Bushnell et al., 1996; Ha et al., 1993), and both subunits of TFIIF (Fang and Burton, 1996; Ha et al., 1993). TFIIB may also play a role in the regulation of transcription by gene-specific regulatory proteins, as many of these regulatory factors bind TFIIB directly (Chiang et al., 1996; Colgan et al., 1995; Franklin et al., 1995; Hadzic et al., 1995; Lin et al., 1991; MacDonald et al., 1995; Sauer et al., 1995; Xing et al., 1995).

TFIIB is comprised of a highly conserved N-terminal region and a C-terminal core domain. It is a 33 kDa polypeptide that contains two imperfect repeats encompassing the C-terminal two-thirds of the molecule and an N-terminal zinc binding motif. The C-terminal domain forms a protease resistant core (cIIB) (Barberis et al., 1993; Malik et

al., 1993) that interacts with the N-terminal domain (nIIB) (Roberts and Green, 1994). In addition to the zinc binding domain, nIIB includes a conserved sequence that links the zinc binding domain to cIIB (Na and Hampsey, 1993). Intact TFIIB has evaded structural analysis, however crystallographic structural data have been obtained for the C-terminal domain of human TFIIB (Nikolov et al., 1995). NMR structures have been obtained for the core domain of human TFIIB and for a portion of the N-terminal region of the TFIIB from archaebacteria *Pyrococcus furiosis* (Bagby et al., 1995; Zhu et al., 1996).

The solution structure for human cIIB (Bagby et al., 1995), and a co-crystal structure for a TATA-TBP-cIIB ternary complex (Nikolov et al., 1995), revealed that the core C-terminal region is mainly α-helical, arranged in pseudo-dyad symmetry. The end of the first direct repeat forms a basic amphipathic α helix while the second direct repeat contains a surface cluster of hydrophobic amino acids and a helix-turn-helix motif that interacts with specific DNA sequences located upstream of the TATA box (Lagrange et al., 1998; Qureshi and Jackson, 1998). The free structure of cIIB is similar to its TATA-TBP-cIIB co-crystal counterpart, except that free cIIB is more compact and the relative orientation of the two repeats is different, suggesting a TFIIB conformational change upon the assembly of the ternary complex (Hayashi et al., 1998).
NMR structural data have been obtained for the N-terminus of *Pyrococcus furiosus* TFIIB, revealing a zinc ribbon β-sheet similar to that present in the elongation factor TFIIS (Zhu et al., 1996). The crystal structure of the TFIIB C-terminal domain in a complex with TBP bound to DNA (Nikolov et al., 1995) has long suggested that the N terminus of TFIIB would project downstream toward the region of transcriptional initiation and may interact with TFIIF to recruit RNAPII. The high degree of amino acid

conservation of the N-terminal region of TFIIB suggests that important functions reside in this portion of the protein.

Biochemical and structural evidence have suggested that the N- and C-terminal domains of native TFIIB are engaged in an intramolecular interaction (Bangur et al., 1997; Hayashi et al., 1998; Roberts and Green, 1994). Presumably in order to form a productive PIC, TFIIB must undergo a conformational change that would disturb the intramolecular interaction, thereby exposing domains that are required for further assembly of the PIC. A conformational change in TFIIB may facilitate the interaction between TFIIB and RNAPII/TFIIF.

Interestingly TFIIB has an intimate relationship with TFIIF as part of a CTD dephosphorylation complex. As mentioned in the previous chapter, 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. A CTD phosphatase that catalyzes the dephosphorylation of RNAPII has been shown to interact (Archambault et al., 1997; Archambault et al., 1998) and is stimulated by the C-terminal domain of the RAP74 subunit of TFIIF (Chambers et al., 1995). 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 RNAPII (Wang and Burton, 1995); therefore, TFIIF, TFIIB, and the CTD phosphatase may be components of a multiprotein complex that binds RNAPII and regulates the polymerase recycling mechanism, affecting the pool of competent polymerases available for transcription of genes.

From amino acid 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 (Figure 1). The N-terminal domain is sufficient to support preinitiation complex assembly, single-round initiation, and elongation by RNAP II. 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 *in vitro*. Deletion of the central region or just the C-terminal domain of RAP74 creates a protein with partial function in multiple-round transcription (Lei et al., 1998). Multiple-round transcription is likely to represent a recycling mechanism for RNAPII or an essential polymerase transcription factor. In an effort to map the domain of RAP74 responsible for multiple-round transcription and to elucidate the role of the TFIIB-TFIIF interaction in transcription, we have generated and characterized a collection of RAP74 mutants in *in vitro* transcription, gel mobility shift and protein-protein interaction assays. Using surface plasmon resonance, we have mapped the domain of RAP74 responsible for interaction with TFIIB. In marked contrast to previously published results (Barberis et al., 1993; Buratowski and Zhou, 1993), we also show that the N-terminus of TFIIB is not required for the recruitment of RNAPII and TFIIF *in vitro*.

Figure 1. 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 1

## MATERIALS AND METHODS

Transcription Factors and Extracts.

Recombinant human RAP30, RAP74, and RAP74 mutants were prepared and quantitated as described (Fang and Burton, 1996; Wang and Burton, 1995; Wang et al., 1993; Wang et al., 1994). Calf thymus RNAPII 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). Recombinant yeast TBP and human recombinant TFIIB were the kind gifts of Steven Triezenberg and Fan Shen. The clones for production of wt TFIIB and TFIIB truncation mutants were the kind gifts of Danny Reinberg, James Geiger, and Masami Horikoshi. 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., 1996). TFIIB mutants were purified as described and stored in 1M guanidine HCl (Hisatake et al., 1993).

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.

RAP74 Mutagenesis and Reconstitution of Recombinant TFIIF

RAP74 mutants, 1-492,1-472, and 1-450, were constructed with appropriate primers by polymerase chain reaction amplification of a plasmid clone encoding RAP74 and subcloning between the *Nde*I and *Xho*I sites of pET21a (Novagen). Site-directed mutants of human RAP74 were constructed using the Quick Change Site-Directed Mutagenesis Kit (Stratagene) and appropriate primers. Double and triple alanine and charge reversal mutagenesis was done in the 470 to 517 region of RAP74 to identify regions of the C-terminal domain that are important for transcriptional function. The following are the RAP74 point mutation mutants that were constructed : LL473AA, KK475EE, KK480EE, EQT487AAA, VL492AA, IL496AA, KR498EE, LNP500AAA, RK504EE, MI506AA, MHF511AAA. Mutated RAP74 genes were confirmed by DNA sequencing. RAP74 mutants and the RAP30 constructs have a C-terminal six histidine tag. Recombinant proteins were overexpressed in *E. coli* BL21(DE3) and purified to near homogeneity from inclusion bodies by affinity chromatography on Qiagen Ni-NTA resin in buffer containing 4M urea. (Wang et al., 1993; Wang et al., 1994). The TFIIF complex was formed by adding equimolar amounts of RAP30 to each RAP74 mutant protein. Urea was removed by dialysis into high salt buffer lacking urea to renature and reconstitute the complex. Protein concentration was determined by absorbance at 280 nm using calculated extinction coefficients.

Multiple-round and Single-round Transcription Assays

Transcription was initiated from an adenovirus major late promoter template digested with *Sma*I to produce a +217 base runoff transcript. A 20 μl reaction mixture

consisted of 0.8 μg adenovirus major late promoter DNA and TFIIF-depleted transcription extract (72 μg 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 $MgCl_2$. For all reactions, preinitiation complexes were formed for 60 min at 30 °C. For multiple round transcription, 600 μM ATP, CTP, GTP and 25 μM $\alpha^{32}$P -UTP (10 μCi per reaction) were added and transcription continued for 60-90 minutes. 0.25 % sarkosyl was added afterwards to block new initiation, and transcription was continued for an additional 30 min to complete all previously initiated chains (Hawley and Roeder, 1985). For the single-round sarkosyl block assay (Hawley and Roeder, 1985), 600 μM ATP, CTP, and 25 μM [$\alpha$-$^{32}$P]UTP (10 μCi per reaction) were added and incubated for 1 min. 600 μM GTP and 0.25 % w/v sarkosyl were added and transcription continued for 59 min. 0.05 % sarkosyl was previously shown to be sufficient to block new initiation by RNAPII (Hawley and Roeder, 1985), so sarkosyl was added in five-fold excess over the amount necessary to constrain transcription to a single round. Reactions were stopped by addition of 200 μl 0.1 M sodium acetate pH 5.4, 0.5 % sodium dodecyl sulfate, 2 mM EDTA, and 100 μg/ml tRNA, followed by phenol-chloroform extraction and ethanol precipitation. Samples were electrophoresed in a 6% polyacrylamide gel containing 50 % urea (w/v). Dried gels were analyzed by autoradiography and the yield of the accurately initiated transcript was quantitated using a Molecular Dynamics phosphorimager.

Transcription with Purified Components

A transcription system consisting of purified calf thymus RNAPII, recombinant human TBPc, TFIIA, TFIIB, TFIIE, TFIIF, and a negatively supercoiled DNA template was modified from (Malik et al., 1998; Parvin and Sharp, 1993). The template was the plasmid pML(C2AT)Δ71, a gift of Michele Sawadogo, containing the adenovirus major late promoter fused to a G-less cassette at position +11 (Sawadogo and Roeder, 1985; Sawadogo and Roeder, 1985). A 20 μl reaction mix, typically composed of 0.3 pmol of calf thymus RNAPII, 0.8 pmol TBPc, 1 pmol TFIIB, 1 pmol TFIIE, 1 pmol TFIIF, 1 pmol TFIIA, 200 ng of supercoiled pML(C2AT)Δ71, 20 μg BSA, and 2 U RNase inhibitor (Promega) was assembled at room temperature. The buffer contains 60 mM KCl and 6 mM $MgCl_2$. 600 μM ATP, CTP, and 25 μM [$\alpha$-$^{32}$P]UTP (5 μCi 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. Dried gels were analyzed by autoradiography.

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 upstream primer was radiolabeled using $\gamma$-$^{32}$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 µl) 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* TBPc (0.6 pmol) was combined with the DNA template (approximately 40 fmol) for 15 min. Recombinant human wild type or mutant TFIIB (0.6 pmol) was then added and incubated for 15 min. Calf thymus RNAPII (0.3 pmol) was incubated with human recombinant wt or mutant TFIIF (1 pmol) for at least 5 min prior to addition to the DB complex. RNAPII was incubated with TFIIF and TFIIE (where indicated) for 5 min before addition to DB and further incubation for 30 min. 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.

Biosensor Kinetic Measurements.

The binding affinities of the TFIIF mutants with TFIIB were determined using a BIACORE 2000 real time kinetic interaction analysis system (Biacore, Inc., Piscataway, NJ) to measure association ($k_a$) and dissociation ($k_d$) rates (Schuck, 1997). Carboxymethylated Dextran biosensor chips (CM5) were activated with EDC [N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide hydrochloride] and NHS (N-hydroxysuccinimide) according to supplier instructions. For immobilization, recombinant TFIIB or TFIIF mutants in 10 mM sodium acetate, pH 4.5 were injected onto the biosensor chip at a concentration of 20 µg/ml to yield approximately 400-700 RU's (resonance response units) of covalently coupled protein. Unreacted groups were blocked with an injection of 1M ethanolamine. Kinetic measurements were carried out in binding

buffer (10 mM HEPES, pH 7.9, 150 mM NaCl, 0.005 % Surfactant P20) by injecting serial dilutions of recombinant proteins at 25°C using a flow rate of 15 µl/min. Association *($k_a$)* and dissociation *($k_d$)* rates were calculated using the 1:1 Langmuir association kinetic model in the BIACORE evaluation software. The equilibrium dissociation constant ($K_D$) was calculated as $k_a/k_d$.

## RESULTS

*Multiple- And Single- Round Transcription With RAP74 Truncation Mutants Reveal The Importance Of The RAP74 C-Terminus.*

The RAP74 N-terminal domain extending from a.a. 1 to 217 supports most RAP74 functions for preinitiation complex assembly, accurate initiation, and elongation stimulation. RAP74(1-217) had a specific defect in multiple- but not single-round transcription and this defect was not attributable to degradation or inactivation of the mutant protein. Deletion of just the C-terminal and/or central regions of RAP74 creates a protein with partial function in multiple-round transcription. The region of RAP74 between a.a. 409-517 has been found to be important for multiple-round transcription (Lei et al., 1998). The central region between a.a. 217-356 was weakly able to stimulate multiple-round transcription in the absence of the C-terminal domain.

An important question that remains to be answered is what C-terminal sequence between a.a. 409-517 contributes to multiple round transcription. Implicit with this question is whether the effect on multiple round transcription is due to a defect in initiation, elongation, promoter escape, recycling of polymerase through decreased CTD phosphatase stimulation, TFIIB binding, or polymerase binding. To determine what region in the C-terminal domain is important for multiple round transcription, RAP74(1-492), RAP74(1-472), RAP74(1-450) truncation mutants were constructed, reconstituted with RAP30 to form TFIIF (Figure 2), and tested for the ability to support single- and multiple- round transcription *in vitro*.

RAP74 C-terminal mutants 1-492, 1-472, and 1-450 all showed significant defects in multiple round transcription (Figure 3B). RAP74 (1-492) mutant, which

Figure 2. SDS-PAGE of TFIIF C-terminal truncation protein complexes stained with Coomassie blue : lane 1, 2 μg/band protein marker standard; lane 2, wild type TFIIF [30/74(1-517)]; lane 3, TFIIF(1-492); lane 4, TFIIF(1-472); lane 5, TFIIF(1-450). Histidine-tagged recombinant RAP30 and RAP74 were overexpressed in *E. coli* BL21(DE3) and purified to near homogeneity from inclusion bodies by affinity chromatography on $Ni^{2+}$-NTA resin in buffer containing 4M urea. The TFIIF complex was formed by adding equimolar amounts of RAP30 to each RAP74 mutant protein. Urea was removed by dialysis into high salt buffer lacking urea to renature and reconstitute the complex.

TFIIF C-terminal Truncation Mutants

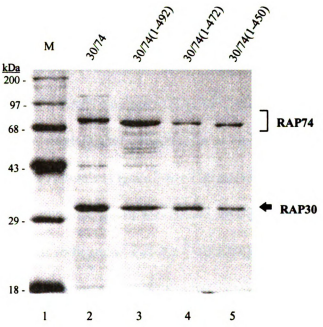



Figure 2

represented a truncation of 25 amino acids, was significantly reduced for multiple round transcription. This truncation reduced the total transcription signal to 38 % of wt TFIIF activity. Further truncation to 1-472 and 1-450 (data not shown) did not significantly aggravate the defect, indicating the importance of amino acids 492-517. Multiple round transcription for RAP74 C-terminal mutants 1-492, 1-472, and 1-450 did not show significantly higher activity than the 1-217 mutant. As previously characterized, TFIIF RAP74(1-217) mutant was shown to be defective for multiple round transcription but shows near maximal activity for single round transcription compared to wt RAP74 (Figure 3A). This mutant serves as a control for comparing multiple and single round defects that may be present in RAP74 mutants. RAP74 C-terminal mutant 1-492 did not show a significant defect in single round transcription as its activity approximated both wt RAP74 and RAP74(1-217) levels (Figure 3A). The activity of 1-472 and 1-450 (data not shown) in multiple round transcription was below 50% of wt TFIIF activity. These results corroborated previous indications that the C-terminal domain of RAP74 stimulates multiple round transcription (Lei et al., 1998) and further define sequence within the domain that is important for this activity.

*Biosensor Measurements Of TFIIF, RAP30, And RAP74 Binding To TFIIB.*

RAP74, TFIIB, and the CTD phosphatase represent essential players in polymerase recycling. Polymerase enters the preinitiation complex with its CTD in the IIa state. Progression through elongation is stimulated by CTD phosphorylation by CTD kinases. In order for the polymerase to re-enter the transcription cycle after termination, the CTD must become dephosphorylated. It has been demonstrated that the C-terminal

Figure 3. Single and Multiple Round Transcription with TFIIF C-terminal truncations 1-217, 1-492, and 1- 472. *In vitro* transcription was done with a TFIIF depleted Hela nuclear extract (DE). Transcription was initiated from the adenovirus major late promoter digested with restriction endonuclease SmaI to produce a +217 base runoff transcript. 5 pmol of TFIIF complex was used for all reactions. Preinitiation complexes were allowed to form for 60 min at 30 °C. Panel A) for the single-round sarkosyl block assay, ATP, CTP, and radiolabeled UTP were added and incubated for 1 min. GTP and 0.25 % sarkosyl were added and transcription continued for 60 min. Reinitiation was blocked by sarkosyl. Panel B) for the multiple-round assay, ATP, CTP, and radiolabeled UTP were added. ATP, CTP, GTP and radiolabeled UTP were added and transcription continued for 60 minutes. 0.25 % sarkosyl was added afterwards to block new initiation, and transcription was continued for an additional 30 min to complete all previously initiated chains. Samples were electrophoresed in a 6% polyacrylamide gel containing 50 % urea (w/v). The accurately initiated transcript was quantitated using a phosphorimager.

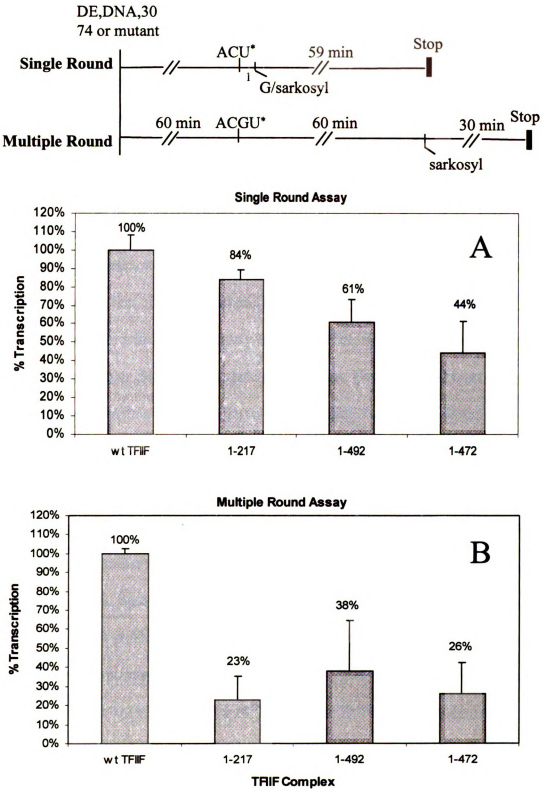
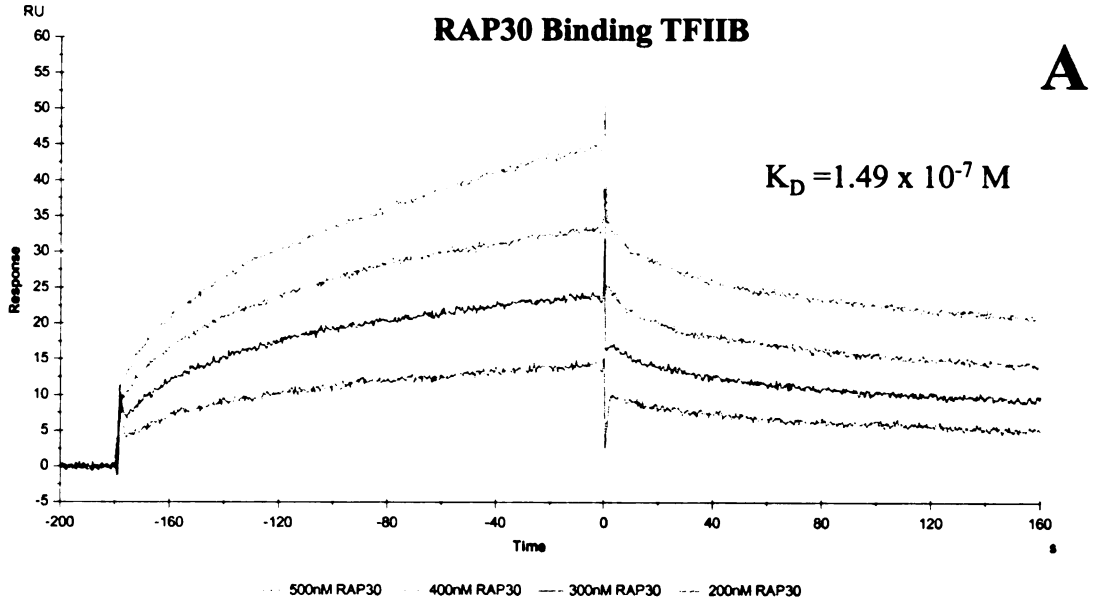

DE,DNA,30
74 or mutant
Single Round
ACU*
59 min
Stop
G/sarkosyl
Multiple Round
60 min
ACGU*
60 min
30 min
Stop
sarkosyl
Single Round Assay
% Transcription
120%
110%
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
100%
84%
61%
44%
A
wt TFIIF
1-217
1-492
1-472
Multiple Round Assay
% Transcription
100%
23%
38%
26%
B
wt TFIIF
1-217
1-492
1-472
TRIIF Complex

Figure 3

domain of RAP74 is able to bind TFIIB and polymerase and stimulate the CTD phosphatase. TFIIB antagonizes stimulation of the phosphatase by RAP74. Evidence seems to suggest that TFIIB and TFIIF cooperate to modulate the interconversion of the two forms of polymerase.

Wild-type and mutant TFIIF proteins were assayed for stable association with TFIIB, because changes in how these two proteins interact could affect polymerase recycling and provide clues to explain defects in multiple round transcription (Figure 3B). Binding kinetics was determined using a BIACORE biosensor system, a machine designed for real-time interactive biomolecular interaction analysis (Schuck, 1997). The detection principle relies on an optical phenomenon that detects changes in the refractive index of the solution close to the surface of the sensor. The on and off rates of the interaction are measured and a binding constant is calculated. Concentration-dependent binding studies were conducted to allow calculation of kinetic parameters. Non-linear analysis of the association and dissociation parts of the sensorgrams showed good curve fitting to the 1:1 ($A + B \Leftrightarrow AB$) association model. The $A + B \Leftrightarrow AB$ association model was used to determine the dissociation binding constant for TFIIF, RAP74, and RAP30 binding to TFIIB. Both TFIIF subunits are capable of binding TFIIB individually. RAP30 binding to TFIIB yielded a $K_D$ of $1.49 \times 10^{-7}$ M (Figure 4 A) while RAP74 binding to TFIIB had a dissociation constant of $K_D = 4.22 \times 10^{-7}$ M (Figure 4 B). The TFIIF:TFIIB binding constant had a $K_D$ of $1.43 \times 10^{-8}$ M (Figure 5). Both TFIIF subunits have dissociation constants within the same magnitude, however the TFIIF complex binds TFIIB with a 3.4 to 10 fold higher affinity. TFIIB-TFIIF contact is largely maintained through RAP74. It is possible that a conformational change occurs in RAP74

Figure 4. Binding kinetics analysis of TFIIF subunits RAP30 and RAP74 binding immobilized TFIIB. The physical interaction of both RAP30 and RAP74 with TFIIB was measured by surface plasmon resonance using the BIACORE biosensor. The test ligand was immobilized on a CM5 sensor chip by amine coupling. Concentration-dependent binding studies were conducted to allow calculation of kinetic parameters. Non-linear analysis of the association and dissociation parts of the sensorgrams showed good curve fitting to the 1:1 ($A + B \Leftrightarrow AB$) association model. The $A + B \Leftrightarrow AB$ association model was used to determine the dissociation binding constant ($K_D$) for RAP30 and RAP74 with TFIIB as indicated on the figures. Interaction of TFIIF with Bovine Serum Albumin protein was used as control to subtract non-specfic interactions that may occur between TFIIF and the carboxymethyl matrix on the chip.


RAP30 Binding TFIIB
A
RU
Response
Time
s
$K_D$ =1.49 x $10^{-7}$ M
500nM RAP30
400nM RAP30
300nM RAP30
200nM RAP30

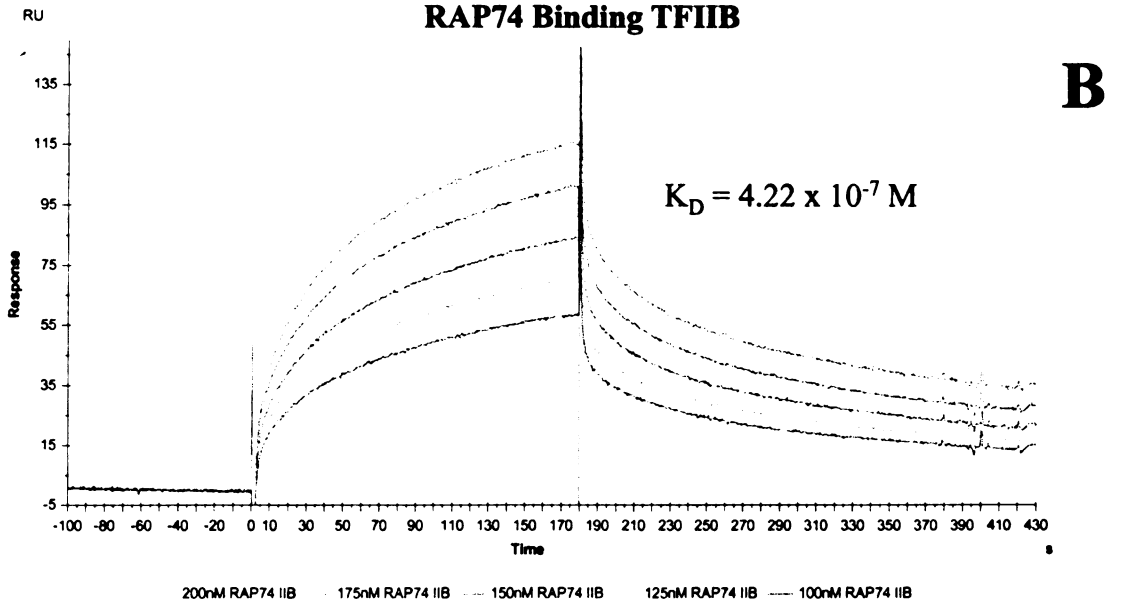

RAP74 Binding TFIIB
B
RU
Response
Time
s
$K_D$ = 4.22 x $10^{-7}$ M
200nM RAP74 IIB
175nM RAP74 IIB
150nM RAP74 IIB
125nM RAP74 IIB
100nM RAP74 IIB

Figure 4

Figure 5. Binding kinetics analysis of wild type TFIIF binding immobilized TFIIB protein. Determination of kinetic parameters was identical to Figure 4.

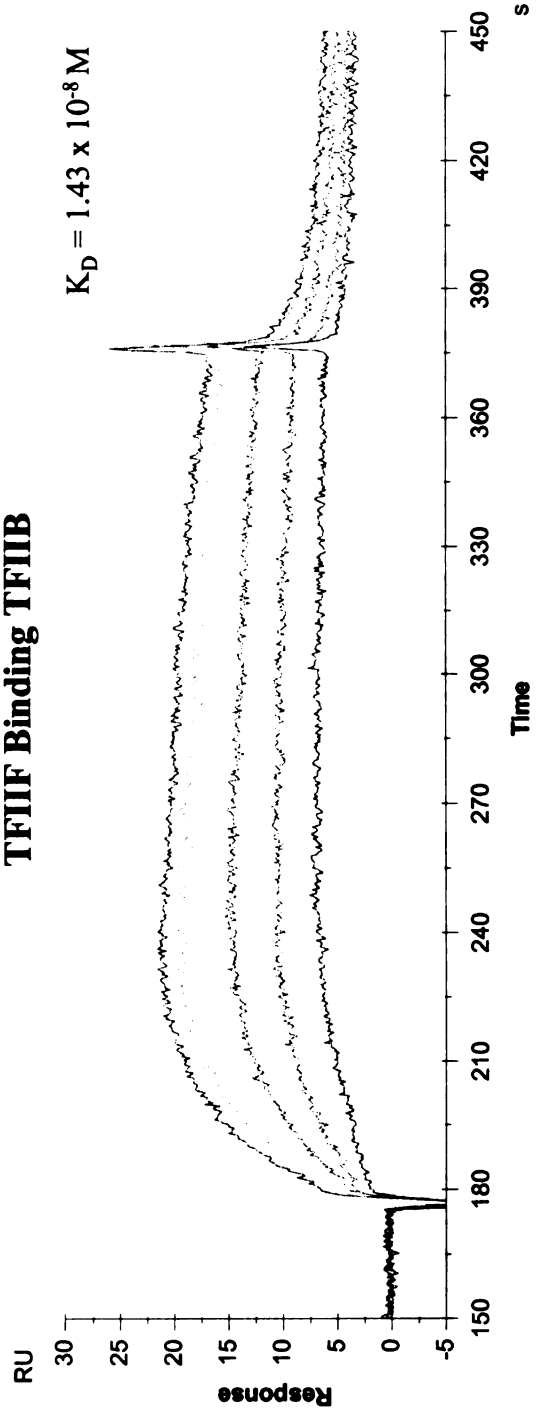

TFIIF Binding TFIIB
$K_D = 1.43 \times 10^{-8}$ M
RU
Response
30
25
20
15
10
5
0
-5
150
180
210
240
270
300
330
360
390
420
450
s
Time
100nM TFIIF
80nM TFIIF
60nM TFIIF
40nM TFIIF
20nM TFIIF

Figure 5

Figure 6. Structural and functional domains of TFIIB and SDS-PAGE of TFIIB truncation mutants stained with Coomassie blue. Two TFIIB deletion mutants, TFIIB-N (1-113) and TFIIB-C (114-316), were used to determine which domain interacts with TFIIF. TFIIB has two domains : a 192 amino acid C-terminal region containing two imperfect direct repeats, and a 124 amino acid cysteine rich N-terminal region which contains a zinc-ribbon domain. The end of the first direct repeat forms a basic amphipathic α helix. The C-terminal direct repeats are important for TBP-DNA interaction and basal transcription. By contrast, the N-terminus is dispensable for formation of the DB complex but is required for basal transcription initiation. SDS-PAGE of TFIIB truncation mutants: Lane 1) 2 μg/band protein marker standard. Lane 2) wild type TFIIB. Lane 3) TFIIB- N. Lane 4) TFIIB-C.

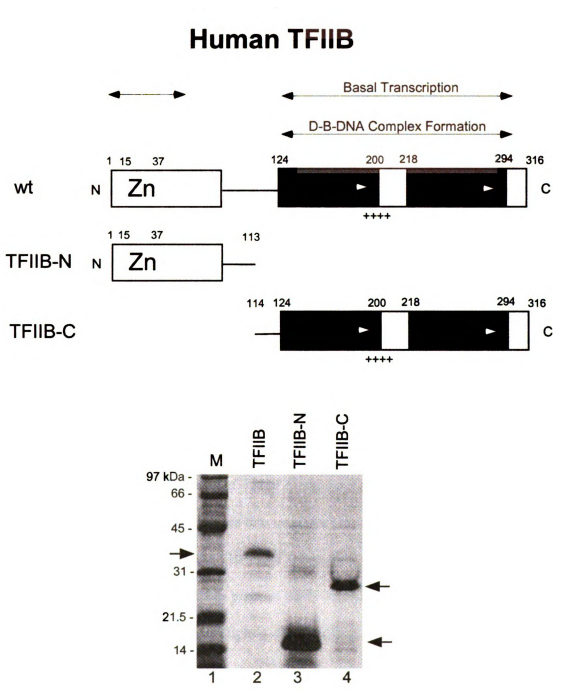

Human TFIIB
Basal Transcription
D-B-DNA Complex Formation
wt
N
Zn
1 15 37
124 200 218 294 316
C
++++
TFIIB-N
113
TFIIB-C
114 124 200 218 294 316
M
TFIIB
TFIIB-N
TFIIB-C
97 kDa
66
45
31
21.5
14
1 2 3 4

Figure 6

as a result of its association with RAP30 that allows it to bind TFIIB with much higher affinity, as is seen with the intact TFIIF complex. However, this scenario does not preclude RAP30's participation in this interaction.

*TFIIF binds the N-terminal domain of TFIIB (1-113)*

In addition, the dissociation constant for TFIIF binding to the N- and C- terminus of TFIIB was determined using TFIIB truncation mutants. Two TFIIB deletion mutants, TFIIB-N (1-113) and TFIIB-C (114-316), were used to determine which domain of TFIIB interacts with TFIIF (Figure 6). These mutants segregate the protein into two domains containing the Zinc finger and the direct repeat sequence responsible for interaction with the TBP-TATA complex. TFIIB bears two unique structural features that reinforce its importance in preinitiation assembly and function. TFIIB contains at its N-terminus a $Zn^{2+}$ finger domain that is dispensable for interaction with TBP, but is important for efficient recruitment of polymerase-TFIIF and to support basal transcription initiation. The N-terminal domain of TFIIB binds the RAP30 subunit of TFIIF and RNA polymerase II. At the C-terminus lies two imperfect direct repeats that are necessary and sufficient for interaction with the TBP-TATA complex. BIACORE analysis revealed that TFIIF interaction with TFIIB takes place at the N-terminus of TFIIB (Figure 7A). The TFIIF:TFIIB-N binding indicates slightly higher affinity than TFIIF:TFIIB with a $K_D$ of $1.25 \times 10^{-8}$ M. The C-terminal repeats of TFIIB do not appear to interact with TFIIF based on the flat sensorgram from the interaction between TFIIF:TFIIB-C (Figure 7B). The structure of the TATA -TBP-TFIIBc complex strongly indicated that the interaction between TFIIB and TFIIF might be through the N-terminal region of TFIIB (Nikolov et

al., 1995). The TFIIF:TFIIB-N BIACORE analysis corroborates the inference previously made based on the three dimensional structure of TFIIB-C. A summary of the dissociation binding constants ($K_D$) is presented in the table below :

| *Binding Interaction* | $K_D$ |
|---|---|
| TFIIF : TFIIB | $1.43 \times 10^{-8}$ M |
| TFIIF : TFIIB-N | $1.25 \times 10^{-8}$ M |
| TFIIF : TFIIB-C | No binding |
| RAP30 : TFIIB | $1.49 \times 10^{-7}$ M |
| RAP74 : TFIIB | $4.22 \times 10^{-7}$ M |

Table 1

*RAP74 Region 492-517 Is Required For TFIIB Interaction With TFIIF.*

In the absence of the RAP30 subunit, RAP74 C-terminal deletion mutants 1-492, 1-472, and 1-450 were all able to tightly bind TFIIB, although the response was slightly lower than full length RAP74(1-517) (Figure 8A). TFIIF complexes with wild type RAP30 and RAP74 mutants were also tested for TFIIB binding. When RAP74 mutants 1-492, 1-472, and 1-450 are complexed with RAP30, none , however, were able to bind TFIIB, as indicated by the flat sensorgram responses (Figure 8B). Truncation of the first C-terminal 25 amino acids significantly reduced or eliminated TFIIF binding to TFIIB. These results seem to indicate that, at least *in vitro*, TFIIB and TFIIF contact is mediated by the C-terminal region of the RAP74 subunit . The region between RAP74 a.a. 492 and 517 is required for this interaction. Even though RAP30 is able to bind TFIIB, RAP30 does not seem to contribute to TFIIB binding when it is associated with RAP74 as measured with BIACORE.

Figure 7. Kinetic analysis of TFIIF binding immobilized TFIIB-N (1-113) and TFIIB-C (114-316). Panel A) TFIIF interaction with TFIIB takes place at the N-terminus of TFIIB Panel B) The C-terminal repeats of TFIIB do not appear to interact with TFIIF, based on the flat sensorgram from the interaction between TFIIF:TFIIB-C . The structure of the TATA -TBP-TFIIBc complex strongly indicated that the interaction between TFIIB and TFIIF might be through the N-terminal region of TFIIB. The TFIIF:TFIIB-N BIACORE analysis corroborates the inference previously made based on the three dimensional structure of TFIIBc.

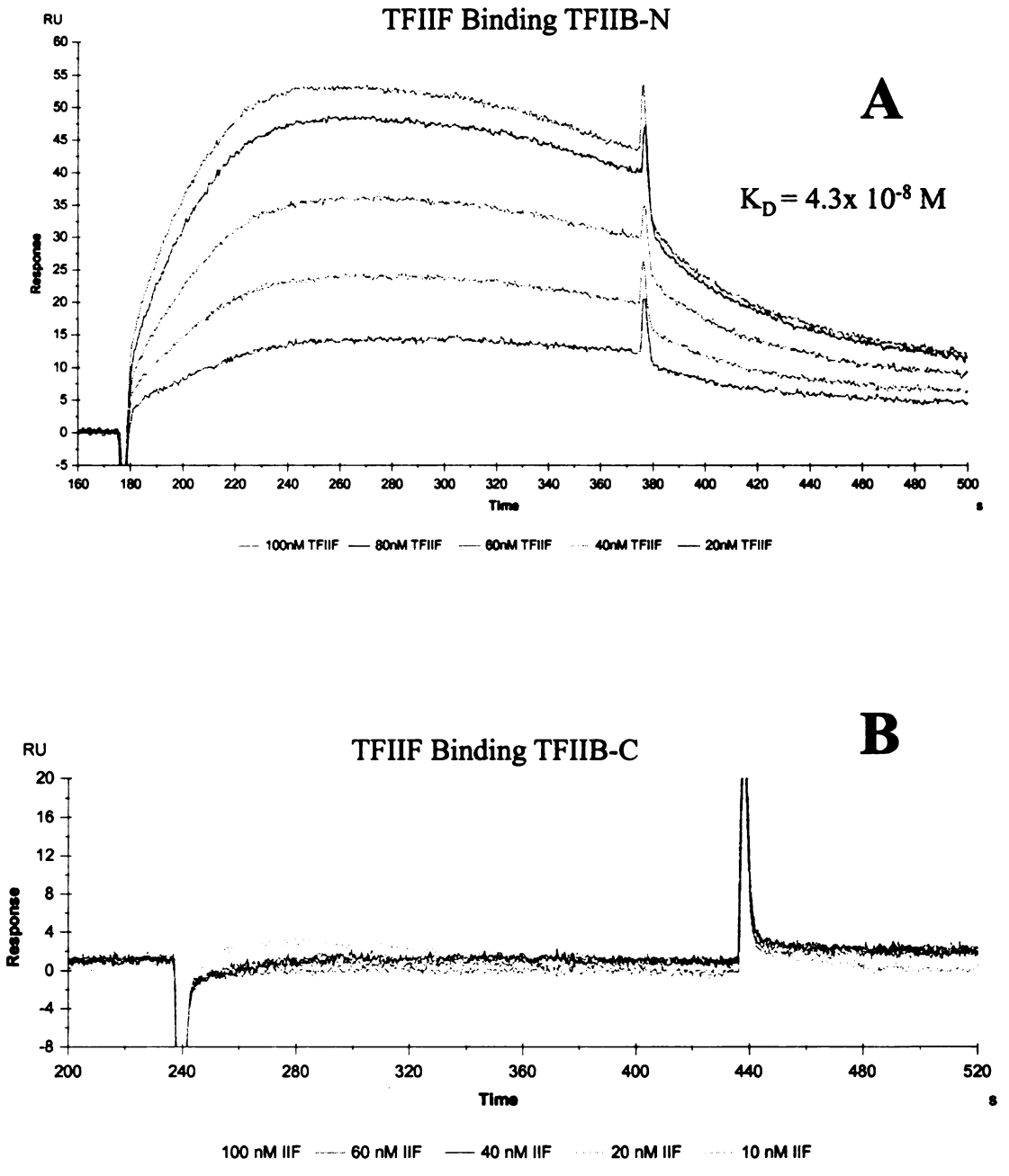

TFIIF Binding TFIIB-N
A
$K_D = 4.3x\ 10^{-8}$ M
RU
Response
Time
s
100nM TFIIF
80nM TFIIF
60nM TFIIF
40nM TFIIF
20nM TFIIF
TFIIF Binding TFIIB-C
B
RU
Response
Time
s
100 nM IIF
60 nM IIF
40 nM IIF
20 nM IIF
10 nM IIF

Figure 7

Figure 8. Qualitative BIACORE analysis of RAP74 and TFIIF truncation mutants complexes binding to immobilized TFIIB. Panel A) In the absence of the RAP30 subunit, RAP74 C-terminal deletion mutants 1-492, 1-472, and 1-450 were able to bind TFIIB with the same apparent affinity as wild type RAP74. Panel B) When complexed with RAP30, the RAP74 region between a.a. 492 and 517 is required for TFIIF-TFIIB interaction, as indicated by the flat sensogram responses. TFIIF mutants (1-492, 1-472, 1-450) were unable to bind TFIIB. These results seem to indicate that TFIIF and TFIIB contact is largely mediated through the RAP74 C-terminus.

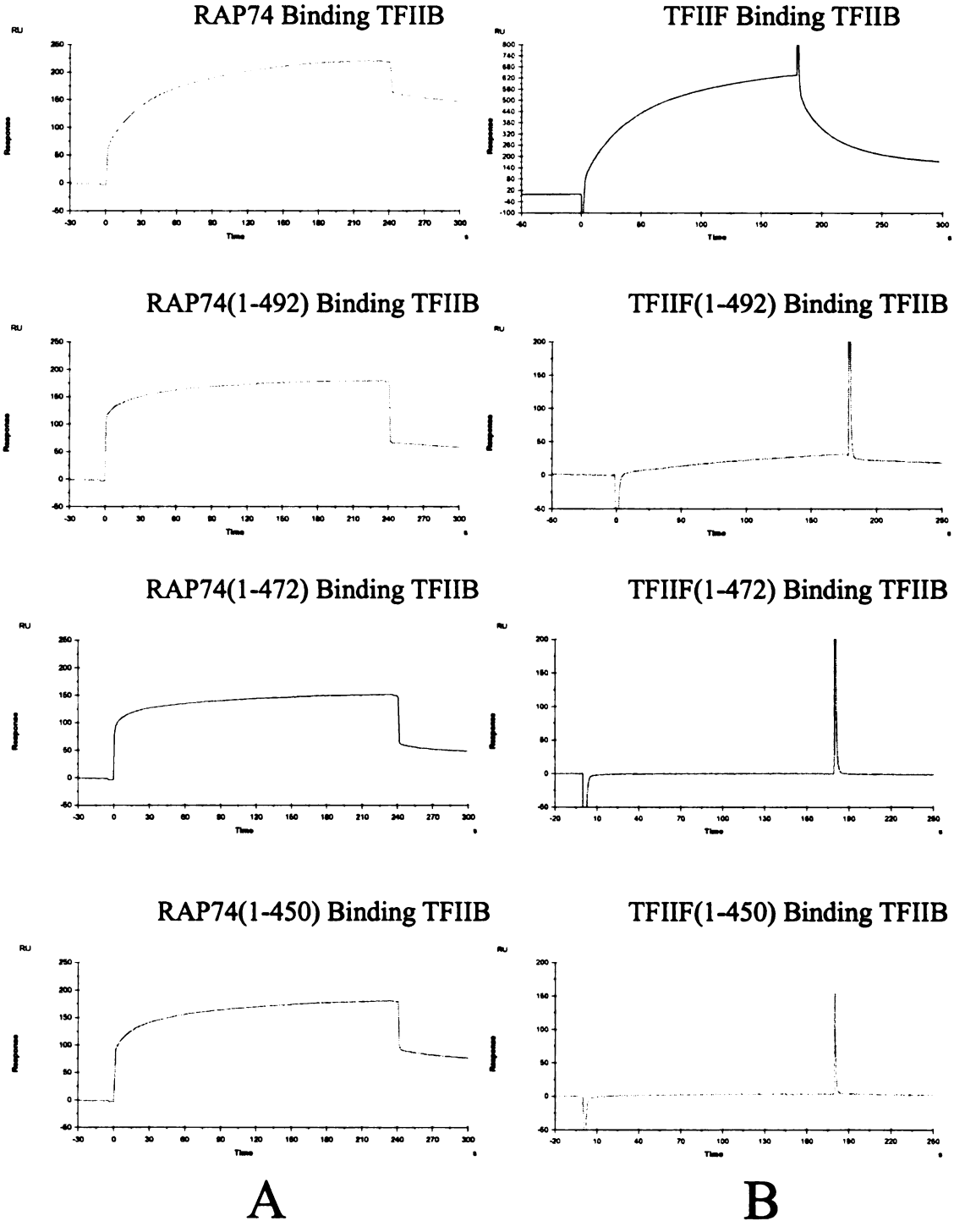

RAP74 Binding TFIIB
TFIIF Binding TFIIB
RAP74(1-492) Binding TFIIB
TFIIF(1-492) Binding TFIIB
RAP74(1-472) Binding TFIIB
TFIIF(1-472) Binding TFIIB
RAP74(1-450) Binding TFIIB
TFIIF(1-450) Binding TFIIB
A
B

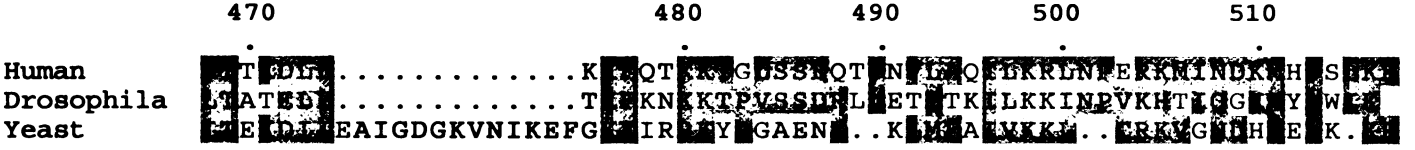
Figure 8

*RAP74 C-terminal Mutagenesis.*

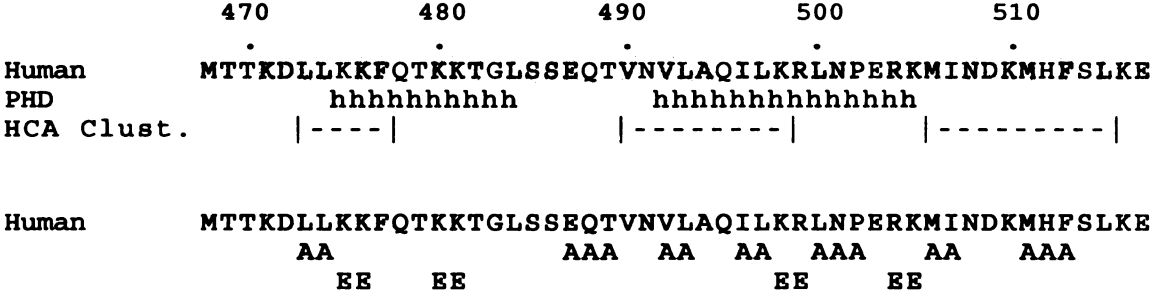
There is a clear indication that RAP74 sequence from 493 to 517 is important for multiple round transcription. Truncation of 25 amino acids at the C-terminal end reduced the total transcription level by more than 50 % (Figure 3B) and compromised TFIIF's ability to bind TFIIB. Detailed mutagenesis within this region was undertaken to reveal critical residues that are important for transcriptional function and TFIIB interaction. Alignment of the C-terminus of human RAP74 with homologues from *Drosophila* and yeast indicates that the region is very highly conserved among all three species (Figure 9). PHD secondary structure prediction analysis (Rost et al., 1994) indicates that the region encompassing a.a. 470 to 517 of human RAP74 may be comprised of two α-helices that are likely to be preserved in RAP74 homologues. Hydrophobic cluster analysis (HCA) is a method that relies upon a two-dimensional representation of protein sequences for comparison and analysis of distantly related proteins when no three-dimensional data is available (Gaboriaud et al., 1987). Hydrophobic cluster analysis (HCA) was used to indicate amino acid clusters of likely importance for C-terminus function of RAP74. Double, and triple amino acid substitutions are indicated beneath the sequence. Triple alanine and charge reversal mutagenesis in the 475 to 517 region was used as a screen to identify regions of importance in the C-terminal domain. 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. These mutants were subsequently assayed in transcription assays and monitored for TFIIB interaction.

Figure 9. Alignment of human, *Drosophila*, and *S. cerevisiae* of the C-terminal region of RAP74. Shaded residues indicate conserved amino acids. PHD secondary structure prediction analysis indicates that the region encompassing a.a. 470 to 517 of human RAP74 may be comprised of two α-helices that are likely to be preserved in RAP74 homologues. A combination of alanine substitutions and charge-reversal mutations in the 475 to 517 region was used to identify regions of importance in the C-terminal domain. The substitutions are indicated beneath the sequence and are summarized in the bottom table.

## RAP74 C-Terminal Domain Alignment




## RAP74 C-Terminal Mutagenesis Targets




| Charge Reversal | Double Alanine | Triple Alanine |
|---|---|---|
| KK475EE | LL473AA | EQT487AAA |
| KK480EE | VL492AA | LNP500AAA |
| KR498EE | IL496AA | MHF511AAA |
| RK504EE | MI506AA | |

Figure 9

*TFIIB-TFIIF interaction Is Not Important for Multiple-Round Transcription.*

The objective for doing the site-directed mutagenesis was to find specific RAP74 mutants that would be severely compromised for initiation or would at least mimic the multiple round transcription defect seen with the RAP74 C-terminal deletion mutants. This would establish a relationship or correlation between TFIIF mutants compromised for TFIIB interaction and a specific defect in multiple round transcription (Figure 3B), thereby assigning a functional assay for TFIIF C-terminal mutants. RAP74 C-terminal deletion mutant (1-492) supports a single round of transcription approximately ≈ 60 % of native TFIIF (Figure 3A). All of the C-terminal point mutants were able to support a single round of transcription within a range of 60-80 % of native TFIIF, roughly similar to 1-492 (Figure 10 A). We had hoped that detailed mutagenesis within RAP74 sequence 493 to 517 (important for TFIIF:TFIIB interaction) would reveal a few critical residues that are important for supporting multiple round transcription. Surprisingly, most of the point mutants affect multiple round transcription to varying degrees (Figure 10 A). Many of the mutants were moderately defective in both single and multiple round transcription, however the pattern was not clear which made the interpretation of these results complicated. None of the defects in multiple round transcription seen with these mutants was as drastic as transcription levels reported previously (less than 50%) for the C-terminal deletion mutants (1-493 and 1-472). Two mutants, KK480EE and EQT487AAA, supported maximal or near maximal levels of transcription compared to native TFIIF (Figure 10 A).

Based on the premise that defects in multiple round transcription may be due to compromised TFIIB:TFIIF binding, the assay reveals that TFIIB:TFIIF interaction is not

Figure 10. Single and Multiple Round Transcription with TFIIF C-terminal point mutants. *In vitro* transcription was done with a TFIIF depleted Hela nuclear extract (DE). Transcription was initiated from the adenovirus major late promoter digested with restriction endonuclease SmaI to produce a +217 base runoff transcript. 5 pmol of TFIIF complex was used for all reactions. Preinitiation complexes were allowed to form for 60 min at 30 °C. Panel A) For the single-round sarkosyl block assay, ATP, CTP, and radiolabeled UTP were added and incubated for 1 min. GTP and 0.25 % sarkosyl were added and transcription continued for 60 min. Reinitiation was blocked by sarkosyl. Panel B) For the multiple-round assay, ATP, CTP, and radiolabeled UTP were added. ATP, CTP, GTP and radiolabeled UTP were added and transcription continued for 60 minutes. 0.25 % sarkosyl was added afterwards to block new initiation, and transcription was continued for an additional 30 min to complete all previously initiated chains. F517 and F217 represent wild type TFIIF and TFIIF(1-217), respectively. Samples were electrophoresed in a 6% polyacrylamide gel containing 50 % urea (w/v). The accurately initiated transcript was quantitated using a phosphorimager.

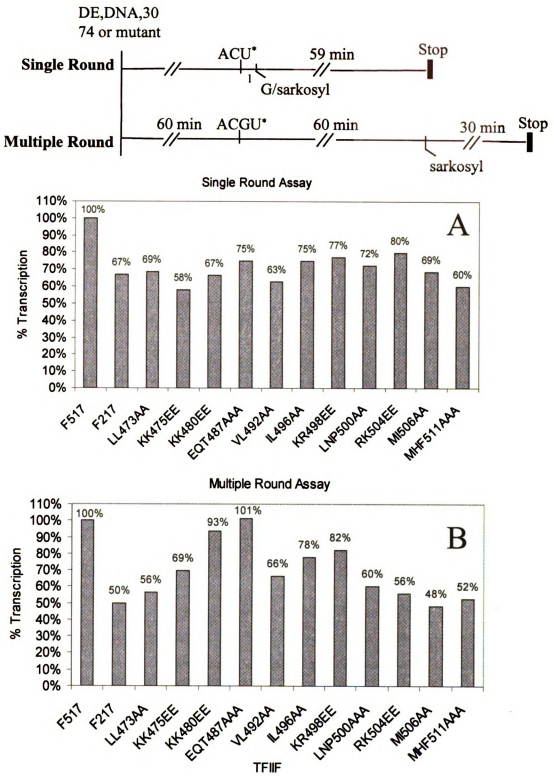

DE,DNA,30
74 or mutant
Single Round
ACU*
59 min
Stop
G/sarkosyl
Multiple Round
60 min
ACGU*
60 min
30 min
Stop
sarkosyl
Single Round Assay
% Transcription
A
100%
67%
69%
58%
67%
75%
63%
75%
77%
72%
80%
69%
60%
F517
F217
LL473AA
KK475EE
KK480EE
EQT487AAA
VL492AA
IL496AA
KR498EE
LNP500AA
RK504EE
MI506AA
MHF511AAA
Multiple Round Assay
% Transcription
B
100%
50%
56%
69%
93%
101%
66%
78%
82%
60%
56%
48%
52%
F517
F217
LL473AA
KK475EE
KK480EE
EQT487AAA
VL492AA
IL496AA
KR498EE
LNP500AAA
RK504EE
MI506AA
MHF511AAA
TFIIF

Figure 10

important for multiple round transcription. Most likely, the defects in multiple round transcription that we see involve more than simple disruption of TFIIB:TFIIF interaction. The C-terminus of RAP74 is also involved in RNA polymerase II binding (Wang and Burton, 1995) and CTD phosphatase stimulation (Chambers et al., 1995). Compromised TFIIF: CTD phosphatase interaction could affect the pool of competent RNAPII available for re-initiation (recycling) from a promoter. Changes in the affinity of TFIIF for RNA polymerase II could affect both initiation and elongation.

*Reconstituted Transcription Assay With Purified Components And RAP74 C-terminal and Point Mutants.*

The initiation transcription assay that we employ is complicated and is based on a depleted nuclear extract. Results obtained can be obscured by other steps in the transcription cycle such as elongation, termination, pausing, and recycling. Therefore, we postulated that a transcription system based on purified components could potentially be dependent on TFIIF, because TFIIF plays important roles both in initiation, isomerization, elongation, and recycling. We were particularly interested in how TFIIF mutants would behave in such an assay. A transcription assay based on purified components was adapted and developed (Malik et al., 1998; Parvin and Sharp, 1993) . This system consisted of purified calf thymus RNA polymerase II, recombinant human TFIIA, TFIIB, TFIIE, TBPc (core TATA Binding Protein), TFIIF, and negatively supercoiled DNA template. The use of a negatively supercoiled template obviated the requirement for the general initiation factor TFIIH (Holstege et al., 1996), which is necessary for promoter melting using relaxed DNA.

Figure 11. TFIIF C-teminal deletion and point mutants in a purified transcription assay system. Recombinant TBPc, TFIIB, TFIIA, TFIIE, and TFIIF and calf thymus RNA polymerase II were used in a defined transcription system with supercoiled pML(C2AT)Δ71 DNA that contains a G-less cassette downstream from the transcriptional start site. Both bands (denoted with the arrows) represented specific transcription products because they were sensitive to α-amanitin (lane 1), dependent on RNAPII and general factors, and resistant to digestion with RNase T1 (data not shown). TFIIF C-teminal deletion and the point mutants tested do not have any visible transcriptional defects in this assay.

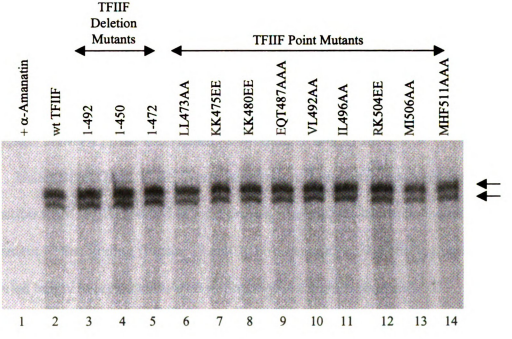

TFIIF
Deletion
Mutants
TFIIF Point Mutants
+ α-Amanatin
wt TFIIF
1-492
1-450
1-472
LL473AA
KK475EE
KK480EE
EQT487AAA
VL492AA
IL496AA
RK504EE
MI506AA
MHF511AAA
1
2
3
4
5
6
7
8
9
10
11
12
13
14

Figure 11

The results from the above experiment revealed that TFIIF C-terminal deletion and point mutants spanning from a.a. 472 to 513 do not have any discernable transcriptional defects (Figure 11). Transcription in this assay does not depend on RAP74 C-terminal sequence. TFIIF (1-217) mutant, which represents a truncation of 300 amino acids from the C-terminus, supports the same level of activity (100%) as native TFIIF (data not shown). It is possible that RNA polymerase II and some of the other general transcription factors can compensate for any binding defects between TFIIB and TFIIF. Alternatively, the nuclear extract system may contain factors that make the interaction between TFIIB and TFIIF more important for transcription.

*Interaction between TFIIF C-terminal Point Mutants and TFIIB*

Using BIACORE, TFIIF C-terminal point mutants were measured for their interaction with TFIIB. 200 nM of each protein was used to determine a qualitative assessment of the binding (Figure 12 and 13). A summary of the binding results is presented below in conjunction with multiple round transcription data:

| *TFIIF Mutant* | *TFIIB Binding* | *Transcription* * |
|---|---|---|
| LL473AA | + | 56 % (Moderate Defect) |
| KK475EE | - | 69 % (Moderate Defect) |
| KK480EE | - | 93 % (No defect) |
| EQT487AAA | - | 101 % (No defect) |
| VL492AA | - | 66 %(Moderate Defect) |
| IL496AA | - | 78 % (Mild to No Defect) |
| KR498EE | - | 82 %(Mild to No defect) |
| LNP500AAA | + | 60 %(Moderate Defect) |
| RK504EE | - | 56 % (Moderate Defect) |
| MI506AA | + | 48 % (Defect) |
| MHF511AAA | + | 52 % (Defect) |

Table 2

* Relative to wild type TFIIF

Figure 12. BIACORE qualitative analysis of TFIIF point mutation complexes capable of binding to immobilized TFIIB. 200 nM of each protein was used to determine a qualitative assessment of the binding.

TFIIF Point Mutants That Bind TFIIB

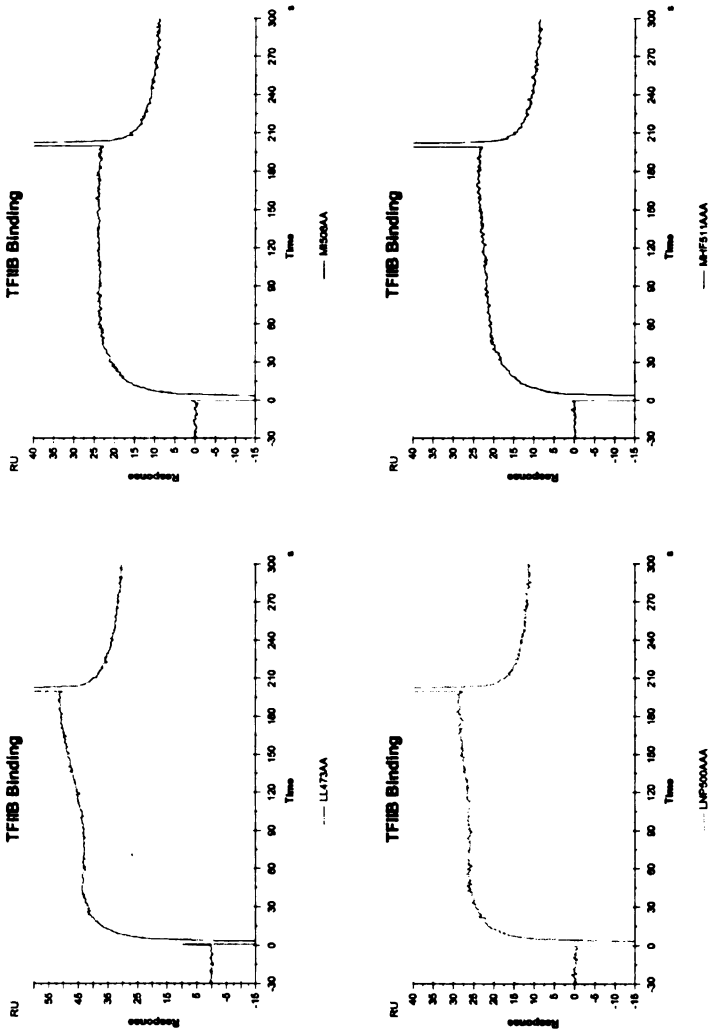



Figure 12

Figure 13. BIACORE qualitative analysis of TFIIF point mutation complexes compromised for TFIIB binding. 200 nM of each protein was used to determine a qualitative assessment of the binding.

TFIIF Point Mutants That Are Compromised For TFIIB Binding

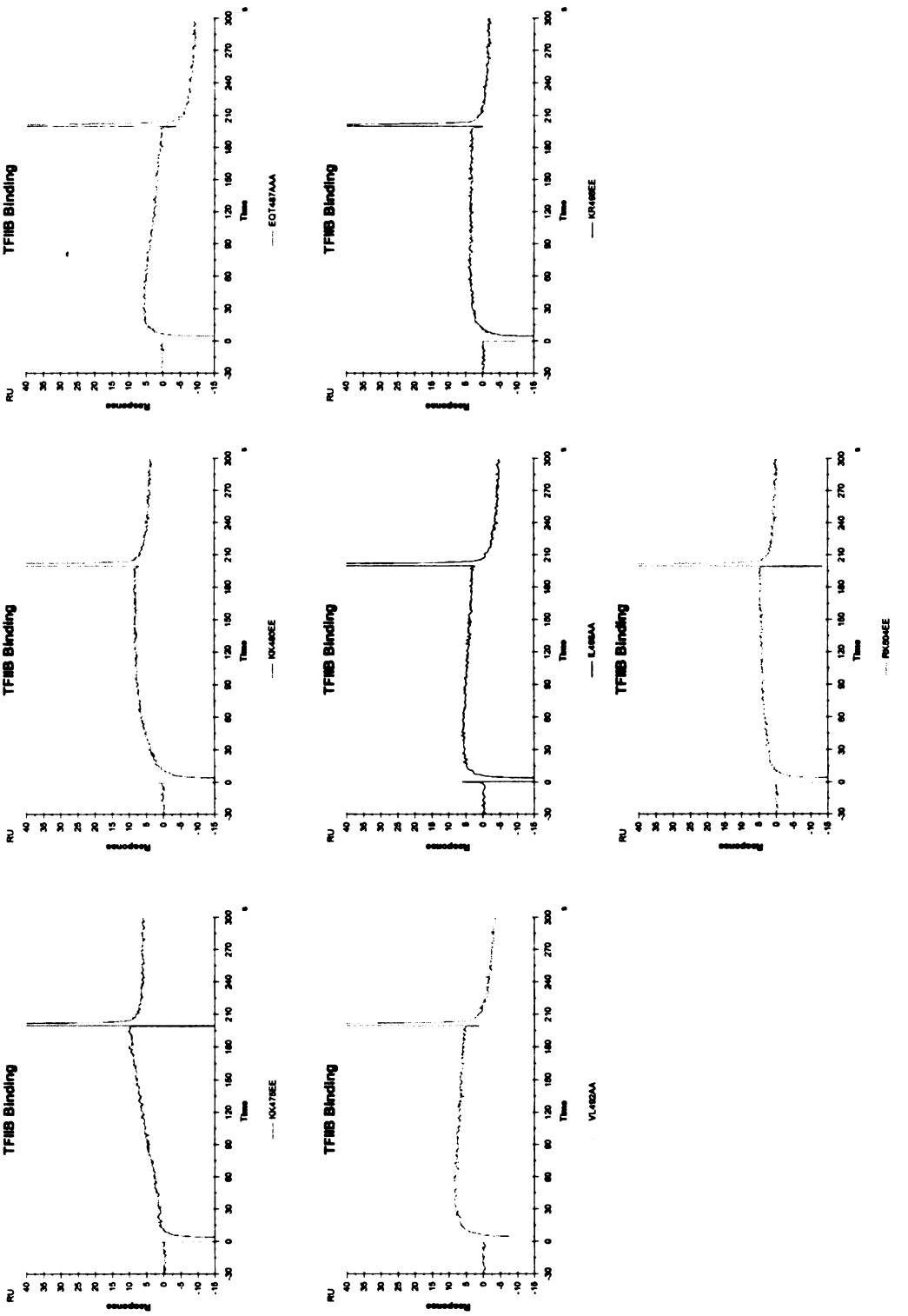
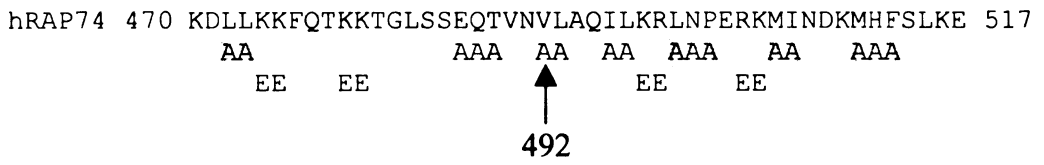



Figure 13




Indicates TFIIF mutants that are able to bind TFIIB.

It was previously reported that TFIIF mutants 1-450, 1-472, and 1-492 are unable to bind TFIIB. Based on those studies, we defined the region between amino acid 492 and 517 that was critical for TFIIB interaction. The BIACORE studies with the TFIIF point mutants further indicates that the region between 475 and 505 is important for TFIIB interaction. Surprisingly, the LNP500AAA substitution does not affect TFIIF-TFIIB interaction despite the drastic amino acid substitution; however reversing the charge on the residues adjacent to those amino acids disrupts TFIIF-TFIIB interaction. Interestingly, the defects in TFIIB:TFIIF interaction did not correlate with any significant transcriptional impairment, as evidenced by multiple and single round transcription in Figure 10.

*Expression And Purification Of TFIIB Deletion Mutants.*

To systematically localize the region(s) of TFIIB important for TFIIF interactions, 24 TFIIB mutants comprising C-terminal , N- terminal, and short internal deletions were expressed in and purified to near homogeneity from bacteria (Figures 14 and 15). Using gel mobility shift assays, these mutants were previously characterized for their ability to form TBP-TFIIB-DNA complexes (Hisatake et al., 1993) (Figure 14). We obtained these mutants to study the domains of TFIIB important for TFIIF interaction. These TFIIB mutants had certain solubility and expression problems that made them challenging to use in BIACORE. To compensate for these difficulties, we instead used gel mobility shift.

Figure 14. Effects of N- and C-terminal deletions of TFIIB on DB complex formation and basal transcription. Summary of mutant TFIIB proteins obtained from M. Horikoshi. Figure was adapted from published Nature 363:744-747 paper. Inner two right columns summarize previously published gel mobility shift data of DB complexes and basal transcription functional analyses. Outer two right columns summarize data obtained from our gel mobility shift data of DBPolF complexes and basal transcription functional analyses (see text for more details). Key: nt - not tested; Basal Txn – basal transcription.

Figure 14

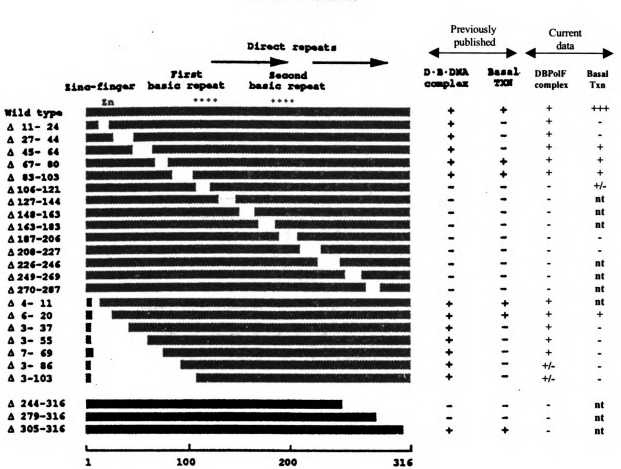

TFIIB Mutants
Previously published
Current data
Direct repeats
Zinc-finger
First basic repeat
Second basic repeat
Zn
D·B·DNA complex
Basal TXN
DBPolF complex
Basal Txn
Wild type + + + +++
Δ 11- 24 + - + -
Δ 27- 44 + - + -
Δ 45- 64 + - + +
Δ 67- 80 + + + +
Δ 83-103 + + + +
Δ106-121 - - - +/-
Δ127-144 - - - nt
Δ148-163 - - - nt
Δ163-183 - - - nt
Δ187-206 - - - -
Δ208-227 - - - -
Δ226-246 - - - nt
Δ249-269 - - - nt
Δ270-287 - - - nt
Δ 4- 11 + + + nt
Δ 6- 20 + + + +
Δ 3- 37 + - + -
Δ 3- 55 + - + -
Δ 7- 69 + - + -
Δ 3- 86 + - +/- -
Δ 3-103 + - +/- -
Δ 244-316 - - - nt
Δ 279-316 - - - nt
Δ 305-316 + + - nt
1
100
200
316

Figure 15. SDS-PAGE of TFIIB mutants stained with Coomassie blue. Panel A) and Panel B) represent TFIIB internal deletion mutants. Panel C) represents TFIIB N- and C-terminal truncation mutants. Histidine-tagged recombinant TFIIB were overexpressed in *E. coli* BL21(DE3) ArgU and purified to near homogeneity from inclusion bodies by affinity chromatography on $Ni^{2+}$-NTA resin. Key: M) Protein standard marker; wt) wild type TFIIB.

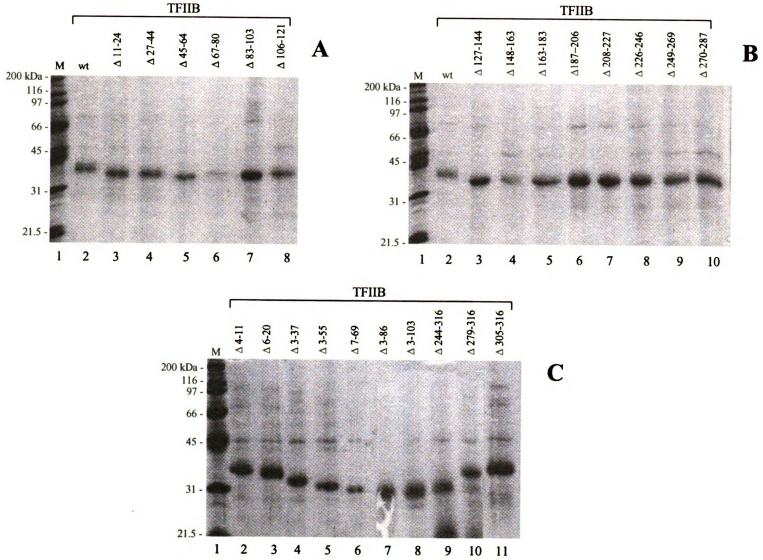

TFIIB
M
wt
Δ 11-24
Δ 27-44
Δ 45-64
Δ 67-80
Δ 83-103
Δ 106-121
A
200 kDa
116
97
66
45
31
21.5
1 2 3 4 5 6 7 8
TFIIB
M
wt
Δ 127-144
Δ 148-163
Δ 163-183
Δ187-206
Δ 208-227
Δ 226-246
Δ 249-269
Δ 270-287
B
200 kDa
116
97
66
45
31
21.5
1 2 3 4 5 6 7 8 9 10
TFIIB
M
Δ 4-11
Δ 6-20
Δ 3-37
Δ 3-55
Δ 7-69
Δ 3-86
Δ 3-103
Δ 244-316
Δ 279-316
Δ 305-316
C
200 kDa
116
97
66
45
31
21.5
1 2 3 4 5 6 7 8 9 10 11

Figure 15

assays to look at the recruitment of RNA polymerase II and TFIIF to the TBP-TFIIB-DNA complexes. The hypothesis is that if TFIIF:TFIIB interaction is compromised or completely abrogated, RNA Polymerase II and TFIIF will not be recruited to TBP-TFIIB-DNA preinitiation complex. It has long been speculated that the N-terminus of TFIIB is required for RNAPII recruitment (via TFIIF) to the preinitiation complex. It has previously been shown by gel mobility shift and basal transcription assays that disruption of the N-terminal domain of TFIIB, and in particular the Zinc finger, eliminates transcriptional activity and recruitment of RNAPII (Barberis et al., 1993; Buratowski and Zhou, 1993).

*TFIIB N-Terminal Deletions Support Recruitment Of RNA Polymerase II:TFIIF Wt Into The Preinitiation Complex.*

Using an electrophoretic mobility shift assay, N-terminal deletions of TFIIB Δ4-11, Δ6-20, Δ3-37, Δ3-55, and Δ7-69, were shown to be capable of supporting active recruitment of RNAPII:TFIIF into the preinitiation complex apparently with the same affinity as wild type TFIIB (Figure 16, compare lanes 5-9 with lane 4). TFIIB mutants Δ3-86 and Δ3–103 were slightly impaired for the formation of the DBPolF complex (Figure 16, lane 11). The results indicate that the N-terminal domain can be removed up to the first C-terminal direct repeat of TFIIB and still maintain recruitment of RNAPII and TFIIF. In this same assay, C-terminal deletions of TFIIB Δ244-316, Δ279-316, and Δ305-316 can support weak recruitment of the DBPolF complex, with TFIIB Δ244-316, Δ279-316 being slightly more disabled (Figure 16, lane 12-15) and collapsing into an unidentified complex (Figure 16, asterisk).

A gel mobility shift assay with the short TFIIB internal deletions corroborated the results above (Figure 17). Mutants removing segments of the N-terminus are able to recruit RNAPII:TFIIF into the preinitiation complex. TFIIB internal deletions Δ11-24, Δ27-44, Δ45-64, Δ67-80, Δ83-103 formed distinct DBPolF complexes (Figure 17, lanes 5-9). TFIIB internal deletion mutants within the C-terminal domain (Δ106-121, Δ127-144, Δ148-163, Δ163-183, Δ187-206, Δ208-227, Δ226-246, Δ249-269, Δ270–287) formed very unstable DBPolF complexes, also collapsing into an unidentified complex (Figure 17, lanes 10-18 asterisk). These mutants previously had been shown to be compromised for DB complex formation (Figure 14) (Hisatake et al., 1993), suggesting that the ability to form a stable DBPolF does not require TFIIF:TFIIB but rather requires a stable DB preinitiation complex. Taken together, these results suggest that no specific sequence between a.a. 11-103 is required for DBPolF assembly.

*TFIIB N-Terminal Deletions Support Recruitment Of RNA Polymerase II: TFIIF(1-217) Into The Preinitiation Complex.*

Previously, we showed that the C-terminus region of RAP74 spanning amino acids 492 to 517 is critical for TFIIF:TFIIB interaction. TFIIF complex mutants containing RAP74(1-217) are not able to bind TFIIB as shown by BIACORE analysis (data not shown). TFIIF(1-217) was used to test the importance of the C-terminus of RAP74 for the recruitment of RNAPII:TFIIF to the preinitiation complex with TFIIB mutants. Gel mobility shift assays with TFIIF(1-217) and TFIIB mutants mirrored the results obtained with wild type TFIIF (Figures 18 and 19). TFIIB N-terminal truncation and internal deletion mutants are fully able to recruit Polymerase:TFIIF(1-217) into the

DB complex (Figure 18, lanes 5-11 and Figure 19, lanes 5-9). Truncation mutants of TFIIF (1-492, 1-172, 1-158) were also capable of being recruited with RNAPII to preinitiation complexes containing wild type and TFIIB N-terminal mutants (data not shown).

C-terminal TFIIB deletion mutants are very unstable for the formation of DBPolF complexes containing truncation mutants of TFIIF (1-492, 1-217, 1-172, 1-158) (Figure 20, lanes 5-9). These TFIIB mutants had previously been shown to be incapacitated for DB complex formation, presumably because the mutations disrupt the direct repeat domains responsible for interacting with promoter bound TBP (Figure 14). Adding TFIIE can compensate for TFIIB mutants that do not have the capacity to stably recruit RNAPII:TFIIF . A representative gel mobility shift experiment with TFIIB(Δ208-227) showed that TFIIE allows the formation of DBPolFE complexes containing C-terminal truncated versions of TFIIF (Figure 20, lanes 10-14). TFIIE may work to stabilize the preinitiation complex (DB) allowing polymerase to dock.

The collective gel mobility data seems to indicate that TFIIF:TFIIB interaction through the C-terminus of RAP74 may not be important for the recruitment of RNAPII to the preinitiation complex *in vitro*. While TFIIF is absolutely required for the delivery of RNAPII to the preinitiation complex, that delivery does not appear to be mediated through TFIIB:TFIIF interaction, or interaction between TFIIB-N and RNAPII. It is possible that polymerase may have undescribed pathways for docking to the preinitiation complex .

Figure 16. Gel mobility shift assay of TFIIB N-terminal and C-terminal deletion mutants. DBPol complex formation is inefficient without TFIIF (lanes 3-4). N-terminal deletions of TFIIB Δ4-11, Δ6-20, Δ3-37, Δ3-55, Δ7-69, were all able to support active recruitment of RNAPII:TFIIF into the preinitiation complex apparently with the same affinity as wild type TFIIB (lanes 4-8). TFIIB mutants Δ3-86 and Δ3–103 were slightly unstable for the formation of DBPolF complex. C-terminal deletions of TFIIB Δ244-316, Δ279-316, and Δ305-316 can support weak recruitment of the DBPolF complex, with TFIIB Δ244-316, Δ279-316 being slightly more disabled (lanes 12-15) and collapsing into an unidentified complex (asterisk).

*
TFIIB Mutants
Probe
DB
DBPol
DBPolF
DB(Δ 4-11)PolF
DB(Δ 6-20)PolF
DB(Δ 3-37)PolF
DB(Δ 3-55)PolF
DB(Δ 7-69)PolF
DB(Δ 3-86)PolF
DB(Δ 3-103)PolF
DB(Δ 244-316)PolF
DB(Δ 279-316)PolF
DB(Δ 305-316)PolF
1 2 3 4 5 6 7 8 9 10 11 12 13 14
DBPolF
DBPol
DB
Free Probe

Figure 16

Figure 17. Gel mobility shift assay of TFIIB internal deletion mutants. TFIIB internal deletions Δ11-24, Δ27-44, Δ45-64, Δ67-80, Δ83-103 formed distinct DBPolF complexes just as wild type DBPolF complexes (lanes 4-9). Internal deletions within the C-terminal direct repeats of the protein do not form stable DBPolF complexes (lanes 10-18) and collapse into an unidentified complex (asterisk).

*
TFIIB Mutants
DB(Δ 270-287)PolF
DB(Δ 249-269)PolF
DB(Δ 226-246)PolF
DB(Δ 208-227)PolF
DB(Δ 187-206)PolF
DB(Δ 163-183)PolF
DB(Δ 148-163)PolF
DB(Δ 127-144)PolF
DB(Δ 106-121)PolF
DB(Δ 83-103)PolF
DB(Δ 67-80)PolF
DB(Δ 45-64)PolF
DB(Δ 27-44)PolF
DB(Δ 11-24)PolF
DBPolF
DBPol
DB
Probe
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
DBPolF
DBPol
DB
Free Probe

Figure 17

Figure 18. Gel mobility shift assay of TFIIB N-terminal, C-terminal deletion mutants using TFIIF (1-217). TFIIB N-terminal truncation mutants are able to recruit RNAPII:TFIIF(1-217) into the DB complex (lanes 5-11). Similar to result using wt TFIIF, TFIIB mutants Δ3-86 and Δ3–103 were slightly impaired for the formation of the DBPolF complex (lanes 10-11), while C-terminal deletions of TFIIB Δ244-316, Δ279-316, and Δ305-316 support very poor recruitment of the DBPolF complex (lanes 12-14).

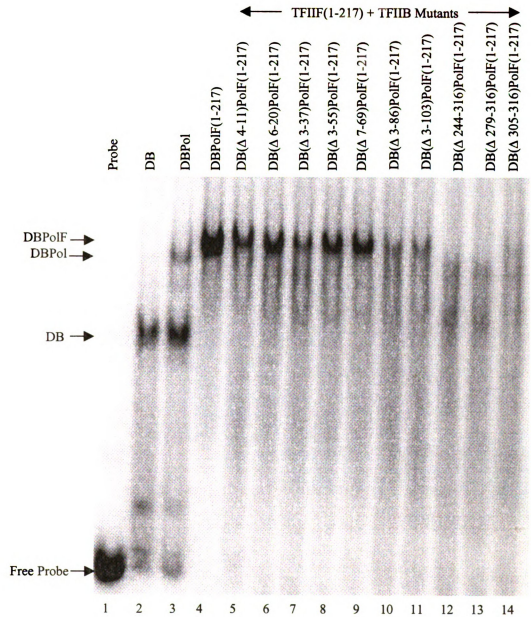

TFIIF(1-217) + TFIIB Mutants
Probe
DB
DBPol
DBPolF(1-217)
DB(Δ 4-11)PolF(1-217)
DB(Δ 6-20)PolF(1-217)
DB(Δ 3-37)PolF(1-217)
DB(Δ 3-55)PolF(1-217)
DB(Δ 7-69)PolF(1-217)
DB(Δ 3-86)PolF(1-217)
DB(Δ 3-103)PolF(1-217)
DB(Δ 244-316)PolF(1-217)
DB(Δ 279-316)PolF(1-217)
DB(Δ 305-316)PolF(1-217)
DBPolF
DBPol
DB
Free Probe
1 2 3 4 5 6 7 8 9 10 11 12 13 14

Figure 18

Figure 19. Gel mobility shift assay of TFIIB internal deletion mutants using TFIIF (1-217). Similar to experiment done with wild type TFIIF, TFIIB N-terminal internal deletion mutants Δ11-24, Δ27-44, Δ45-64, Δ67-80, Δ83-103 formed stable DBPolF complexes (lanes 4-9). Internal deletions at the C-terminal domain do not form stable DBPolF complexes (lanes 10-18).

Probe
DB
DBPol
DBPolF(1-217)
DB(Δ 11-24)PolF(1-217)
DB(Δ 27-44)PolF(1-217)
DB(Δ 45-64)PolF(1-217)
DB(Δ 67-80)PolF(1-217)
DB(Δ 83-103)PolF(1-217)
DB(Δ 106-121)PolF(1-217)
DB(Δ 127-144)PolF(1-217)
DB(Δ 148-163)PolF(1-217)
DB(Δ 163-183)PolF(1-217)
DB(Δ 187-206)PolF(1-217)
DB(Δ 208-227)PolF(1-217)
DB(Δ 226-246)PolF(1-217)
DB(Δ 249-269)PolF(1-217)
DB(Δ 270-287)PolF(1-217)
DBPolF
DBPol
DB
Free Probe
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

Figure 19

Figure 20. Compensation of the defective TFIIB internal deletion Δ208–227 with TFIIE as seen by gel mobility shift assay. TFIIB internal deletion Δ208–227 is not capable of stably recruiting RNAPII: TFIIF complexes (wt, 1-492,1-217,1-172,1-158). The addition of TFIIE stabilizes formation of the DBPolFE complex (lanes 10-14).

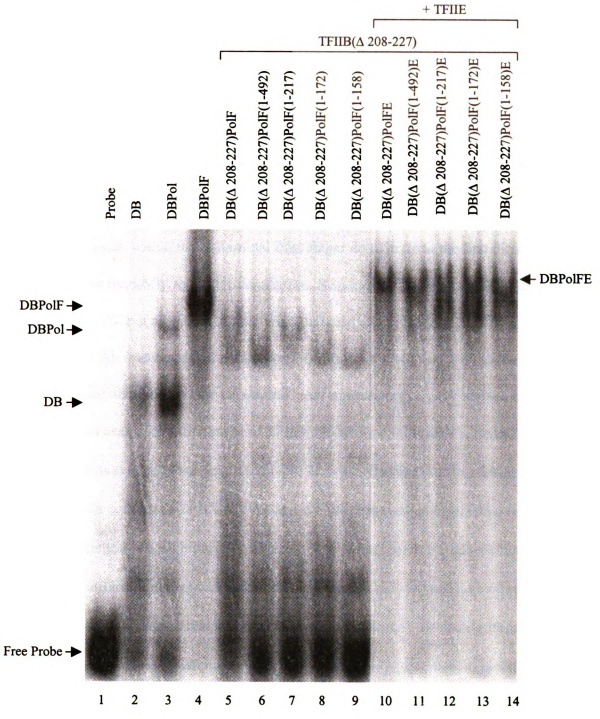

+ TFIIE
TFIIB(Δ 208-227)
Probe
DB
DBPol
DBPolF
DB(Δ 208-227)PolF
DB(Δ 208-227)PolF(1-492)
DB(Δ 208-227)PolF(1-217)
DB(Δ 208-227)PolF(1-172)
DB(Δ 208-227)PolF(1-158)
DB(Δ 208-227)PolFE
DB(Δ 208-227)PolF(1-492)E
DB(Δ 208-227)PolF(1-217)E
DB(Δ 208-227)PolF(1-172)E
DB(Δ 208-227)PolF(1-158)E
DBPolFE
DBPolF
DBPol
DB
Free Probe
1 2 3 4 5 6 7 8 9 10 11 12 13 14

Figure 20

*Reconstituted Transcription Assay With Purified Components And TFIIB Mutants.*

Using a reconstituted transcription assay with recombinant human TBPc, TFIIA, TFIIB, TFIIE, TFIIF, and a negatively supercoiled DNA template (Malik et al., 1998; Parvin and Sharp, 1993) the ability of the TFIIB mutants was assessed to support basal transcription. These mutants had previously been assayed for basal transcription, albeit in a cruder assay system using partially purified components such as TFIID/TBP and TFIIE/TFIIF fraction, RNAPII and recombinant TFIIB (Hisatake et al., 1993). Although, the N-terminal domain was dispensable for DB complex formation; in the transcription assay, disruption within the N-terminal Zinc finger domain up to the first direct repeat eliminated or markedly reduced transcription. Residues in this region whose removal reduced, but did not eliminate transcription included amino acids 6-20, 67-80, and 83-103 (Figure 14). All mutations that eliminated DB-promoter DNA complex formation (such as C-terminal disruptions) also eliminated basal transcription. In our revised basal transcription assay, total disruption of TFIIB's N-terminus (Figure 21, lanes 13-17) and specific deletion of the Zinc finger (Δ11-24, Δ27-44) eliminated transcription (Figure 21, lanes 4-5). In contrast to TFIIB Δ11-24 and Δ27-44, but similar to previous results, N-terminal deletion Δ6-20 (Figure 21, compare lane 12 to lanes 4-5) reduced but did not abrogate transcription. Regions within the N-terminus (Δ67-80, and Δ83-103) whose deletion did not eliminate transcription were also observed as previously characterized (Figure 21, lanes 7-8). Disruptions flanking both these regions (Δ45-64 and Δ106-121), however, indicated that our assay is slightly more sensitive than the one previously used, as deletions within those regions were also capable of supporting low level basal transcription. Though the TFIIB Δ106-121 was severely incapacitated for basal

transcription (Figure 21, lane 9). Mutations that eliminated DB complex formation by interfering with TFIIB's direct repeats (Δ187-206 and Δ208-227) also eliminated basal transcription (Figure 21, lanes 10-11). The above functional data indicate that the region between a.a. 11 to 45 is required for basal transcription. Because all the TFIIB mutants were purified under denaturing conditions, the presence of guanidine hydrochloride could have obscured the obtained results. However, as a control, TFIIB purified under native conditions was used to assess the effect of guanidine in this sensitive assay. TFIIB purified under denaturing conditions supported the same level of basal transcription as TFIIB purified under native conditions (Figure 21, compare lane 3 to 2).

Figure 21. TFIIB N- and C-terminal mutants in a purified transcription assay system. This assay is similar to the one described in Figure 11. Disruption of TFIIB's N-terminus (lanes 13-17) and specific deletion of the Zinc finger (Δ11-24, Δ27-44) eliminated transcription (lanes 4-5). Mutations compromising DB complex formation by interfering with TFIIB's direct repeats (Δ187-206 and Δ208-227) also eliminated basal transcription (lanes 10-11). N-terminal deletion Δ6-20 (lane 12) reduced but did not abrogate transcription. Regions within the N-terminus (Δ67-80, and Δ83-103) whose deletion did not eliminate transcription were also observed (lanes 7-8). In contrast to results published elsewhere, disruptions flanking both these regions (Δ45-64 and Δ106-121) (lanes 6 and 9) were also capable of supporting low level basal transcription. Although TFIIB Δ106-121 is significantly more impaired than TFIIB Δ45-64. Lane 1) α-Amanitin added to the complete reaction inhibiting RNAPII transcription. Lane 2) Complete reaction containing recombinant TBPc, TFIIB (not in guanidine HCl), TFIIA, TFIIE, wt TFIIF, and supercoiled pML(C2AT)Δ71 DNA . Lane 3) Identical to Lane 2 but with TFIIB diluted from guanidine HCl stock.. Lanes 4-17) TFIIB mutants.

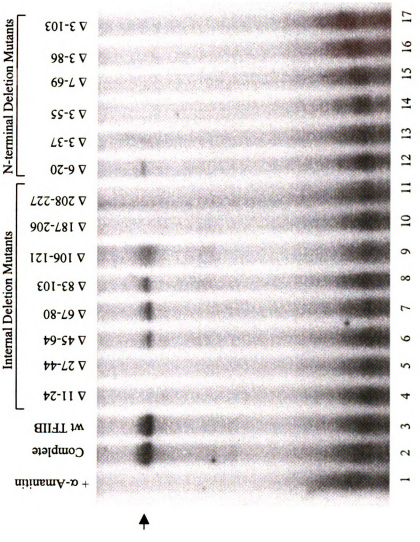

+ α-Amanitin
Complete
wt TFIIB
Δ 11-24
Δ 27-44
Δ 45-64
Δ 67-80
Δ 83-103
Δ 106-121
Δ 187-206
Δ 208-227
Internal Deletion Mutants
Δ 6-20
Δ 3-37
Δ 3-55
Δ 7-69
Δ 3-86
Δ 3-103
N-terminal Deletion Mutants
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

Figure 21

## DISCUSSION

Evidence suggests a functional interaction between TFIIF and TFIIB. These factors cooperate to bring RNAPII into the preinitiation complex (Buratowski et al., 1989; Conaway et al., 1991; Serizawa et al., 1994; Zawel and Reinberg, 1993). Furthermore, *in vivo* studies with yeast have established a genetic relationship between the RAP74 subunit of TFIIF and TFIIB that can affect transcription initiation start-site selection (Sun and Hampsey, 1995).

TFIIB contains at its N-terminus a Zinc finger domain that is dispensable for interaction with TBP, but is important for efficient recruitment of RNAPII-TFIIF, support of basal transcription initiation (Barberis et al., 1993; Buratowski and Zhou, 1993; Hisatake et al., 1993) and accurate start-site selection *in vivo* (Pardee et al., 1998; Pinto et al., 1994). Mutations at the N-terminus inhibit assembly of transcription intermediates presumably because they fail to interact with either TFIIF or RNAPII (Buratowski and Zhou, 1993; Ha et al., 1993; Hisatake et al., 1993; Malik et al., 1993). The N-terminal domain of TFIIB binds the RAP30 subunit of TFIIF and RNA polymerase II (Ha et al., 1993; Hisatake et al., 1993). At the C-terminus of TFIIB lies two imperfect direct repeats similar to the core domain of cell cycle regulatory proteins (Bagby et al., 1995; Gibson et al., 1994). The two cyclin-like C-terminal repeats of TFIIB (TFIIBc) are necessary and sufficient for interaction with the TBP-TATA complex (Hisatake et al., 1993). Based on the three-dimensional structure of TFIIBc (Bagby et al., 1995) and the TFIIBc-TBP-DNA ternary complex (Nikolov et al., 1995), the C-terminal region of TFIIB binds TBP,

activators (Roberts and Green, 1994) and DNA. The N-terminus is postulated to be a scaffold for the assembly of RNA polymerase II-TFIIF.

In addition to the functional interaction between TFIIB and TFIIF during transcription initiation, both factors may control the dephosphorylation of the Carboxy Terminal Domain of RNA polymerase II after transcription termination (Chambers et al., 1995) in a process known as recycling. Dephosphorylated RNAPII preferentially enters the preinitiation complex, and CTD kinases phosphorylate RNAPII within the preinitiation complex or shortly after initiation (Chambers and Dahmus, 1994; Laybourn and Dahmus, 1989). During elongation, RNAPII is hyperphosphorylated on the CTD (Chambers and Dahmus, 1994; Lu et al., 1991). The CTD phosphatase has been independently isolated by HeLa cell extract fractionation (Chambers and Dahmus, 1994) and a two-hybrid screen for RAP74 C-terminal interacting proteins (Archambault et al., 1997), hence its name FCP1 (TFII*F*-associating *C*TD *p*hosphatase). Despite its role in CTD dephosphorylation, Fcp1p does not resemble known eukaryotic protein phosphatases, and it is essential for most transcription by RNAPII *in vivo* (Kobor et al., 1999). The C-terminal domain of RAP74 stimulates a CTD phosphatase (Archambault et al., 1997; Archambault et al., 1998; Chambers et al., 1995). The C-terminal domain of RAP74 is also responsible for interaction with TFIIB (Fang and Burton, 1996) and RNAP II (Wang and Burton, 1995). TFIIB which can directly bind the CTD phosphatase (Jack Greenblatt, unpublished data), blocks stimulation of CTD phosphatase activity by RAP74 (Chambers et al., 1995). Because RAP74 stimulates and TFIIB blocks stimulation of CTD phosphatase activity (Chambers et al., 1995), TFIIB has been

suggested to be present in elongation complexes to block CTD dephosphorylation in order to prevent premature termination (Lei et al., 1998).

Multiple-round transcription in vitro using a Hela extract system appears to reflect a physiological recycling system, because this process involves activation of transcription complexes as a function of time (Lei et al., 1998). We originally initiated RAP74 C-terminal truncation mutagenesis to study the effects of that region in initiation and multiple round transcription. Three RAP74 C-terminal mutants (1-492, 1-472, and 1-450) were constructed and reconstituted with RAP30 to make TFIIF. All the RAP74 C-terminal truncation mutants were severely defective in the multiple-round transcription assay . In particular, RAP74(1-492) mutant, which represented a truncation of 25 amino acids, was significantly compromised for transcription . This truncation reduced the total transcription signal by more than 50 % (Figure 3B). Using biosensor surface plasmon resonance studies (BIACORE), we determined that these RAP74 C-terminal truncation mutants when complexed with RAP30 were defective for their interaction with the general initiation factor TFIIB (Figure 8). Similar to wild type RAP74, the RAP74 C-terminal truncation mutants by themselves were able to stably associate with TFIIB. It is possible that a conformational change occurs in RAP74 as a result of its association with RAP30 that modifies the C-terminus for TFIIB binding . This conformational change could enhance the overall affinity of TFIIF for TFIIB (3 to 10X higher affinity) as opposed to RAP74 or RAP30 interaction with TFIIB by themselves (Figures 5 and 4). Because TFIIF and TFIIB are intimately intertwined at the most crucial stages of the transcription cycle, we speculated that defects in transcription as seen with the C-terminal RAP74 mutants could be due to compromised binding with TFIIB.

As expected, both TFIIF subunits were capable of binding TFIIB individually, with tight binding affinities (Figure 4). TFIIF is thought to mediate the interaction between RNAPII and the DB complex and this function had long been attributed specifically to the RAP30 subunit (Flores et al., 1991). Consistent with this premise, TFIIB binds to RAP30, and this interaction has been mapped to the N-terminal domain of TFIIB (Fang and Burton, 1996; Ha et al., 1993). However, second site suppression genetic experiments in yeast have implicated RAP74 interaction with TFIIB (Sun and Hampsey, 1995). Both subunits of TFIIF, therefore are possibly involved in TFIIB binding. However, the N-terminus of RAP74 has been shown to block RAP30:TFIIB interaction by binding the N-terminal region of RAP30 (Fang and Burton, 1996). RAP30's TFIIB interaction domain overlaps its RAP74 binding domain. Also, by sequestering TFIIB, the C-terminus of RAP74 can compete for TFIIB binding and dissociate RAP30 :TFIIB interactions (Fang and Burton, 1996). This indicates that when TFIIF is intact, TFIIB:TFIIF contact would be maintained through the C-terminus of RAP74 and the N-terminus of TFIIB. If RAP30:TFIIB interaction is physiologically important, the TFIIF complex would have to dissociate within some complexes.

The TFIIF:TFIIB binding dissociation constant ($K_D$) was $1.43 \times 10^{-8}$ M, indicating a strong binding interaction (Figure 5). The TFIIF:TFIIB-N binding showed slightly higher affinity than TFIIF:TFIIB with a $K_D$ of $1.25 \times 10^{-8}$ M ( Figure 7). Roger Kornberg's lab has attempted to observe interactions between yeast transcription factors using a similar biosensor system, but has not been able to detect the TFIIF:TFIIB interaction (Bushnell et al., 1996). However, the concentration and purity of yeast TFIIF that was used was low and consequently the detection was near the low limit of the assay.

BIACORE binding studies with TFIIB and the adenovirus E1A 13 S transcriptional activator (Paal et al., 1997) have indicated that the dissociation constant is within the same order of magnitude ($10^{-8}$ M) as the value that has been calculated for TFIIF:TFIIB and TFIIF:TFIIB-N. The C-terminal repeats of TFIIB do not appear to interact with TFIIF, based on the flat sensorgram for the interaction (Figure 7). The solved 3D structure of the TATA -TBP-TFIIBc complex strongly indicated that the interaction between TFIIB and TFIIF might be facilitated by the N-terminal region of TFIIB (Nikolov et al., 1995). The TFIIF:TFIIB-N BIACORE analysis corroborates the inference previously made based on the crystallographic structure of TFIIB-C (Figure 7).

Based on multiple round transcription and BIACORE analysis, RAP74 sequence from 492 to 517 was deemed important for multiple round transcription and binding to TFIIB when RAP74 was complexed with RAP30. Point mutations in a.a. 470 to 517 region of full length RAP74 were made to identify regions of the C-terminal domain that are important for transcriptional function and TFIIB interaction. The BIACORE studies with the TFIIF point mutants indicated that the region between RAP74 a.a. 475 and 505 was critical for TFIIB interaction (Figures 12 and 13). Interestingly, the LNP500AAA substitution did not affect TFIIF-TFIIB interaction; however reversing the charge on the residues adjacent to those amino acids disrupted TFIIF-TFIIB interaction. Noticeably, charged residues K476, K480, K481, K498, K499, R504, K505 are conserved between human, *Drosophila*, and yeast RAP74 (Figure 9). It is possible that these residues may form a salt bridge with acidic amino acids on TFIIB and thereby form the basis for the TFIIF-TFIIB interaction. The N-terminus of TFIIB harbors several acidic amino acids that are conserved between human, *Drosophila*, and yeast TFIIB (data not shown). We

know from BIACORE data that TFIIF binds slightly more strongly to the N-terminus of TFIIB than to the full-length protein. It is plausible that the reason why TFIIF binds slightly better to the N-terminus of TFIIB is because the conserved acidic amino acid groups are unmasked as a result of the truncation of the C-terminus, allowing TFIIF easy access to the TFIIB region responsible for that interaction.

Transcriptional defects seen in multiple round transcription appear to be more complicated than simple TFIIB:TFIIF interaction disruptions. Using the RAP74 C-terminal point mutants, there did not seem to be a clear linkage between transcription function and TFIIB:TFIIF interaction (Figures 10, 12, 13, Table 2). Although, the TFIIF C-terminal point mutants described have some transcriptional defects, these cannot be attributed totally to compromised TFIIB:TFIIF, because some of the same C-terminal point mutant (TFIIF: LL473AA, LNP500AAA, MI506AA, MHF511AAA) could still maintain TFIIB:TFIIF interaction.

The above results may implicate the RAP74 C-terminus in CTD phosphatase interaction and stimulation rather than TFIIB interaction. Transcriptional defects may be due to disruptions in the functional complex responsible for polymerase recycling and CTD phosphorylation /dephosphorylation. Changes in the affinity of TFIIF for CTD phosphatase could affect elongation and polymerase's ability to reinitiate. The calf thymus RNAPII used in the reconstituted purified components transcription 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). Moreover, CTD kinases and phosphatases are not included in the purified components transcription system. Therefore, CTD phosphorylation and dephosphorylation are not relevant in the reconstituted

transcription system. This might explain why no transcriptional defect is seen with TFIIF C-terminal truncation (including TFIIF(1-217)) and point mutants in the purified transcription assay (Figure 11). TFIIF C-terminal mutations affecting CTD phosphatase interaction may, therefore, be the main reason for the presumed inhibition of RNAPII recycling in the HeLa TFIIF-depleted nuclear extract system .

Evidence suggests that TFIIB is conformationally pliable and that TFIIB N- and C- terminal regions are involved in an intramolecular interaction that potentially sequesters the N- terminus from associating with other factors (Roberts and Green, 1994). If TFIIF interacts with TFIIB's N terminal domain as a docking scaffold, then TFIIB must be conformationally unlocked where the N and C terminal intramolecular interactions are disrupted in order to recruit TFIIF interaction. This disruption can be facilitated by specific activators such as VP16 (Roberts and Green, 1994). This model is consistent with recent structural studies of human TFIIB showing that interaction of either VP16 or the N-terminus of TFIIB with the C-terminus of TFIIB induce distinct changes in the orientation of the two repeat domains of the C-terminal region of TFIIB relative to each other (Hayashi et al., 1998). The studies however did not address the potential role of TFIIB conformational changes on transcription. Recently, an activation-specific role for TFIIB *in vivo* was observed with an activator-induced TFIIB conformational change that may facilitate PIC assembly (Wu and Hampsey, 1999). The yeast activator Pho4's interaction with TFIIB induces a conformational change that might represent disruption of the nIIB-cIIB interaction which then stimulates *PHO5* transcription *in vivo*. Some activators may stimulate transcription by inducing a conformational change in TFIIB that drives preinitiation complex assembly forward.

We obtained C-terminal , N- terminal, and short internal deletion TFIIB mutants to localize regions of TFIIB important for TFIIF interactions (Hisatake et al., 1993) (Figure 14). These TFIIB mutants had certain solubility and expression problems which made them challenging to use in BIACORE. To compensate for these difficulties, we instead used gel mobility shift assays to look at the recruitment of RNA polymerase II and TFIIF to the TBP-TFIIB-DNA complexes.

The results of the gel mobility shift experiments indicated that removal of the N-terminal domain of TFIIB up to the first direct repeat of the protein was able to maintain recruitment of polymerase and TFIIF. TFIIB sequence from amino acid 11 to 103 is dispensable for the assembly of DBPolF complexes (Figure 16-19) but amino acid sequence between 11 to 45 is required for basal transcription (Figure 21). These results were in marked contrast to those previously published in which N-terminal disruptions that affected the Zinc finger domain could form a DB complex but could not recruit PolF to form DBPolF (Buratowski and Zhou, 1993). The reagents used previously were Hela fractionated components that might have harbored contaminants which could have obscured these results. In our assay we have used recombinant protein for all the general transcription factors and purified calf thymus RNAPII. Functional data indicated that the region between a.a. 11 to 45 is required for basal transcription. This region encompasses the Zinc finger domain, located between a.a. 15 to 37, and suggests that the region immediately flanking the Zinc finger is also important. Moreover, partial deletion of the Zinc binding domain (Δ6-20) severely compromises basal transcription but does not eliminate it. Deletion of region 45-103 can maintain moderate level of transcription, whereas further deletion beyond this region (affecting DB complex formation) does not

support basal transcription as expected. The Zinc finger and the region immediately flanking it are essential for basal transcription but are not required for recruitment of RNAPII and TFIIF (Figure 21).

It has long been thought that TFIIB's Zinc finger could recruit RNAPII through protein-protein interaction with TFIIF and/or polymerase. Zinc finger motifs can function as a metal-linked interaction domain. In fact, several RNA polymerase II subunits contain zinc-binding domains that are known to be functionally important (Treich et al., 1991). Our data show that the N-terminal region of TFIIB is not primarily required for the recruitment of polymerase and TFIIF but rather for another function in initiation. This finding does not diminish possible TFIIB:TFIIF interactions important downstream of the pathway, though recruitment of polymerase to the PIC may take place through undescribed docking interactions with the DB complex. TFIIB mutations that disrupt the C-terminal repeats form very unstable DBPolF complexes that collapse into a complex which, based on size, might be a DBF complex having lost polymerase (Figures 16 and 17, asterisk). These C-terminal mutants have been shown to be compromised for DB complex formation (Figure 14) (Hisatake et al., 1993). The inability to form a stable DB complex, a result of disruption of TFIIB domains that interact with TBP:DNA, is the cause for poor recruitment of RNAPII:TFIIF. The addition of TFIIE can partially compensate for TFIIB's inability to form a stable DB and recruit RNAPII:TFIIF to form a DBPolFE complex (Figure 20) but does not restore function (Figure 21). TFIIE contributes to DNA wrapping around RNA polymerase within the PIC (Robert et al., 1998; Robert et al., 1996) which may compensate for defective TBP:DNA:TFIIB complexes. TFIIE-α 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).

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