TATA BINDING PROTEIN AND ASSOCIATED FACTORS IN MAMMALIAN TRANSCRIPTION: CHARACTERIZATION OF THE B-TFIID ACTIVITY

by

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Abstract

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The control of gene expression is of fundamental importance in both the normal development of organisms and the abnormal developments that accompany many disease states. This control is exerted at a number of steps along the path from DNA to protein, including the transcription, processing, transport and translation of RNA. Regulation of these steps involves the complicated interplay between nucleic acids and proteins.

This thesis describes a biochemical study of the mechanism of transcription focusing on one of the central protein components, the TATA-binding protein (TBP). As the only pol II transcription factor known to specifically recognize the promoter, TBP assembly on DNA is the prerequisite for the assembly of all the other factors required for transcription initiation.

The first part of this thesis describes the characterization of a novel complex, B-TFIID, which is comprised of the TBP and a 170 kd TBP-associated factor (TAF). B-TFIID is an abundant TBP-TAF complex that, like TBP, can promote initiation of transcription from a TATA-containing pol II promoter. Though competent to reconstitute basal transcription, neither B-TFIID nor TBP can respond to upstream activators in a stimulated transcription assay.

Recent experiments have implicated TBP in the transcription of genes controlled by pol I and pol III. The expanding role of TBP in transcription led us to consider new models for the function of B-TFIID. The second part of this thesis describes one such model and the experiments performed to test it. The model suggests that B-TFIID functions in pol III transcription as a component of the transcription factor TFIIIB. This model is based on the presence of TBP in both factors, and on the cofractionation of the B-TFIID complex with the TFIIIB activity. Using biochemical fractionations and pol III transcription reconstitutions we demonstrate that B-TFIID and TFIIIB are distinct TBP-TAF complexes, and that only the TAFs of the TFIIIB complex are competent to reconstitute pol III transcription. Finally, we demonstrate that multiple TBP-TAF complexes, including that of TFIIIB, can reconstitute pol II transcription from a TATA-containing promoter. This raises the possibility that all TBP-TAF complexes can function in the pol II *in vitro* assay and that B-TFIID may not be a pol II factor. The further analysis of TBP-TAF complexes and the transcription reactions they initiate, will shed light on the true *in vivo* function of B-TFIID.

Thesis Supervisor: Dr. Phillip A. Sharp
Title: Professor and Head of the Department of Biology

Table of Contents

Title Page....1

Abstract....2

Table of Contents....3

Dedication....7

Acknowledgments....8

Chapter I. Introduction

Pol I transcription....10

Promoter structure....10

Required factors....11

Pol III transcription....15

Promoter structure....15

Required factors....16

Pol II transcription....18

Promoter structure....18

General factors....19

TBP-TAFs across the spectrum....25

Common features of the three polymerase systems....25

Towards a new definition of TBP....30

Is yeast TBP decorated with TAFs?....31

Original definition of yeast TBP....31

Novel yeast TAFs....33

Previous data on B-TFIID....35

References....37

Chapter II. Purification and Characterization of Transcription Factor B-TFIID

Abstract....52

Introduction....53

Results....55

Purification of B-TFIID....55

Immunoprecipitation analysis of TFIID complexes....56

An ATPase activity copurifies with the B-TFIID complex....70

Discussion....78

Materials and Methods....83

Purification of B-TFIID....83

Purification of other transcription factors....84

In vitro transcription assay....84

Protein Analysis....84

ATPase Assay....85

References....87

Chapter III. TBP-TAFs in Pol III Transcription, is B-TFIID Involved?

Abstract....93

Introduction....94

Results....97

TBP from the phosphocellulose B fraction resolves into distinct complexes....97

TFIIIB activity separates into two peaks on anion exchange....107

B-TFIID cannot complement a TBP-depleted P-cell B fraction to restore pol III transcription....110

TBP-containing fractions function in a pol II assay....113

Discussion....120
Materials and Methods....123
Plasmids and RNA....123
Purification of B-TFIID....123
Purification of other transcription factors....124
In vitro transcription....125
Immunodepletion....126
References....127

Chapter IV. Conclusion/Discussion
Summary of results....134
What is the role of B-TFIID?....136
Specific TAF for a novel set of activators....136
Specific TAF for a novel promoter....143
Precursor to other TBP-TAF complexes....147
Endogenous basal transcription factor....148
Experimental approaches to further study B-TFIID....150
Cloning of p170....150
Mapping the site of interaction with TBP....151
Studying the effects of transcription modulators....152
What are the directions of the field?....153
References....156

Chapter V. Appendix

Abstract....162

Results....163

What are proteosomes....167

Materials and Methods....168

Purification of yeast TBP....168

Preparation of affinity columns....168

Preparation of yeast WCE and TFIIA....169

Affinity purification of TFIIA....169

N-terminal sequencing....170

References....171

Dedication

To David,
for always being there

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Chapter I. Introduction

Transcription initiation in eukaryotes is governed by the interaction of a seeming myriad of proteins with DNA promoter elements. The specific sequences, their relative locations, and the proteins that recognize them are different for the three classes of polymerases, (pols) I, II and III. Despite these differences, the three polymerase systems retain some common features. First, all three polymerase classes require a number of proteins, in addition to RNA pol, to initiate transcription at a specific site. Second, they all require one or more proteins that specifically bind to the promoter and nucleate the association of subsequent factors, including the correct polymerase, via protein:protein interactions. Third, though the transcription initiation complexes for each of the respective pol classes were believed to be distinct, they do share various components, including some RNA polymerase subunits, some upstream regulatory proteins (at least between pol II and III) and, most relevant to this discussion, TATA-binding protein (TBP).

Pol I transcription

Promoter structure

The three different polymerases use different DNA elements and distinct sets of proteins to mediate the initiation of transcription. Unlike pol II and pol III, which recognize a variety of promoters (see fig. 1), pol I recognizes a single type of promoter, that of the large rRNA gene. This recognition exhibits a strikingly stringent species specificity. Some pol I factors can be swapped experimentally without loss of function-but only between closely related species. Such exchangeability is not possible among more distantly related species (Grummt et al., 1982; Onishi et al., 1984). Consistent with these observations,

the rRNA promoter elements of distantly related species show very little sequence homology (Financsek et al., 1982). However, despite such sequence divergence, the overall promoter structure is maintained across most species and consists of 2 elements, a core region that overlaps the start site of transcription, and an upstream control element (UCE) located at around -150 (Reviewed, Geiduschek and Kassavetis, 1992).

Required factors

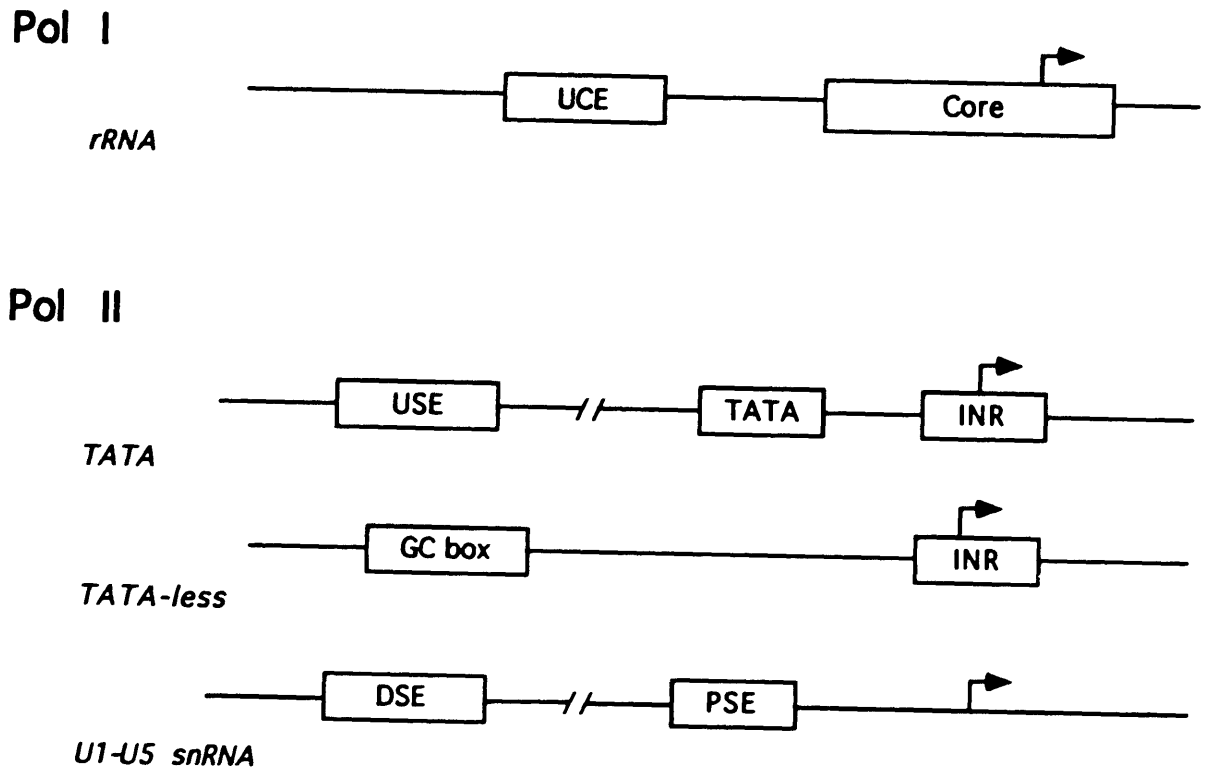
Two factors, upstream binding factor (UBF) and selectivity factor (SL1), are required in addition to RNA pol I to reconstitute accurate and promoter specific pol I transcription (Learned et al., 1985; Learned et al., 1986; Bell et al., 1988). UBF is a DNA binding protein that interacts with both the UCE and the core region of the rRNA promoter. UBF is the only sequence specific DNA binding protein in the pol I system and its assembly on the promoter is a prerequisite for the association of SL1 via protein:protein interactions (Learned et al., 1986). SL1 association leads to an extension of the footprint across the promoter and the subsequent assembly of RNA pol I to yield an initiation competent complex. The assembly of SL1 on the promoter occurs in a species specific fashion, and SL1 has therefore been labeled the species specific factor of the pol I system.

Although the UBF has been purified to homogeneity (Bell et al., 1988) and cloned (Jantzen et al., 1990), the nature of the SL1 factor remains to be completely elucidated. Because it is responsible for directing transcription solely from its cognate DNA template, it has become the focus of studies aimed at understanding pol I transcriptional specificity. To this end, Comai et al have purified the SL1 factor to apparent homogeneity and elegantly demonstrated the recapitulation of species specificity *in vitro* (Comai et al., 1992). Even more

interestingly, they determined that SL1 is a complex comprised of the TBP and three associated factors (TAFs). The sizes of these TAFs are 110, 63, and 48 kd (Comai et al., 1992).

On the heels of these biochemical results came genetic experiments that confirmed the presence of TBP in the pol I system by demonstrating that mutations in TBP altered the levels of pol I transcription (Cormack and Struhl, 1992; Schultz et al., 1992). Additionally, Schultz et al identified mutations in TBP that displayed polymerase specificity, one having effects on pol III and II, but not pol I transcription, and another affecting pol II, but not pol I or III transcription (Schultz et al., 1992). This suggested that the transcription factors for the three polymerase systems each interacted with distinct surfaces on TBP.

Figure 1. Structure of the promoters transcribed by RNA polymerase I, II, and III. All the pol I transcribed rRNA genes have a common promoter consisting of two consensus elements. The upstream consensus element (UCE) is upstream of the start site and the core consensus element surrounds the start site. Protein encoding genes are transcribed by pol II and are indicated as either TATA or TATA-less. They contain both upstream sequence elements (USEs) and proximal sequence element and may contain consensus elements surrounding the start site (INR). The pol II transcribed snRNA encoding genes U1-U5 contain both distal sequence elements (DSE) and proximal sequence elements (PSE) upstream of the start site. The pol III transcribed genes can be divided into three classes. Two of these classes, the 5S rRNA genes and the tRNA/VA genes, contain internal consensus elements, while the third class, the snRNA U6 genes, contains only upstream sequence elements.


Pol I
UCE
Core
rRNA
Pol II
USE
TATA
INR
TATA
GC box
INR
TATA-less
DSE
PSE
U1-U5 snRNA
Pol III
DSE
PSE
TATA
U6 snRNA
A
B
tRNA/VA
A
C
5S rRNA

Pol III transcription

Promoter structure

Pol III transcription appears to be more complicated than pol I transcription. Pol III promoters are more complex and require a greater diversity of factors (For review (Geiduschek and Kassavetis, 1992). Genes transcribed by pol III can be divided into three classes: the 5S rRNA genes (class 1), the tRNA and VA genes (class 2), and the U6 snRNA genes (class 3) (see fig. 1). Class 1 and 2 genes are similar in that all the promoter elements are located downstream of the transcription start site in the internal control region (ICR).

The prototypic ICR for 5S RNA genes contains both an A box and a C box. Additionally, all 5S RNA genes contain a binding site for the Zn finger protein TFIIIA (Miller et al., 1985). The requirement for TFIIIA distinguishes class 1 genes from those of classes 2 and 3. TFIIIA nucleates the assembly of an initiation complex by binding to the C box and protecting ~35 bp of DNA including sequences that extend outside the C box (Braun et al., 1989; Wang and Weil, 1989). The subsequent binding of TFIIIC to the TFIIIA/DNA complex extends the DNA protection and allows for the assembly of TFIIIB, further extending the protection upstream of the start site.

In a similar fashion, class 2 promoters contain two DNA elements, an A box and a B box, but, as previously mentioned, show no requirement for TFIIIA. At these promoters, TFIIIC binds directly to both of these sequence elements (Baker et al., 1987), displaying a slightly higher affinity for the B box. As with the class 1 genes, the association of TFIIIC with the promoter nucleates the

assembly of TFIIIB and the subsequent assembly of RNA pol III to form a transcription competent complex (Bartholomew et al., 1993). Though TFIIIB and pol III are clearly required for the transcription of the class 3 genes, these genes have a unique promoter structure and poorly understood "factorology" which will be addressed in a later section.

Required factors

Extensive purification and protein crosslinking experiments have aided in the dissection of the pol III factors. Yeast TFIIIC is a complex of 5 polypeptides ranging in size from 52 kd-140 kd (Gabrielson et al., 1989; Bartholomew et al., 1990), and the mammalian counterpart has polypeptides sizing between 60 kd-230 kd (Boulanger et al., 1987; Yoshinaga et al., 1989). Experiments in yeast have demonstrated that protein:DNA interactions extending from the B box through the transcription start site form a stable platform for the assembly of TFIIIB, originally defined in yeast as a dimer of a p70 and a p90 (Bartholomew et al., 1991; Kassavetis et al., 1991). The subunits of TFIIIB are chromatographically separable and bind sequentially to the TFIIIC/DNA complex, the 70 kd protein associating first (Kassavetis et al., 1991). Interestingly, once TFIIIB binds to the promoter it is quite stable and remains associated even after TFIIIC is stripped from the DNA (Kassavetis et al., 1990). This TFIIIB/DNA complex is competent to direct multiple rounds of pol III binding and transcription. For this reason, TFIIIB has been termed a true transcription factor, and TFIIIA and TFIIIC have been termed assembly factors (Kassavetis et al., 1990).

As the experiments proceeded in yeast, mounting evidence from studies in mammalian systems implicated a novel protein in some forms of pol III

transcription (Lobo et al., 1991; Margottin et al., 1991; Simmen et al., 1991). That protein, TBP, was first suggested to play a role in pol III transcription of the class 3 U6 snRNA genes because their promoters contained a TATA box upstream of the start site (see fig. 1). The promoters of U6 genes contain at least 3 defined cis elements, the distal sequence element (DSE), the proximal sequence element (PSE), located near bp -56, and the TATA box, located near bp -27. Like the TATA box which is found in many mRNA genes, both the PSE and the DSE are known to exist in pol II transcribed genes. Specifically, the DSE and the PSE are present in snRNA U1-U5 genes which are recognized by the pol II transcription machinery. Individually or in pairs, these elements (TATA, PSE, and DSE) are recognized by the pol II machinery, however, when present in triplicate, as in the U6 promoter, transcription is mediated by pol III. Experimental evidence suggests that the presence of the TATA box itself can convert a pol II promoter to a pol III promoter (Mattaj et al., 1988; Lobo and Hernandez, 1989).

Though TBP was first implicated in transcription of the class 3 genes, it has recently proven essential for the transcription of the more classical tRNA and VA genes. (For reviews (Sharp, 1992; White and Jackson, 1992; Hernandez, 1993). Both genetic (Buratowski and Zhou, 1992; Cormack and Struhl, 1992; Lopez-de-Leon et al., 1992; Schultz et al., 1992), and biochemical (Kassavetis et al., 1992; Lobo et al., 1992; Simmen et al., 1992; Taggert et al., 1992; White et al., 1992) experiments demonstrate that TBP is a component of the pol III transcription machinery and that TFIIIB is a TBP-TAF complex. The nature of the TFIIIB TBP-TAF complex will be discussed in a later section.

Pol II transcription

Promoter structure

Of the three polymerases, RNA pol II clearly has the most challenging job, that of transcribing all protein encoding genes. The diversity of the genes transcribed is mirrored by the diversity of promoter elements. Four upstream elements have been identified in pol II genes, though any given promoter may have as few as two and as many as four of them (see fig. 1). The initiator element (INR) spans the start site of transcription and is present in some but not all genes (Smale and Baltimore, 1989). Slightly upstream, centered at ~bp -30, is the TATA box, which is present in both INR-containing (INR+) and INR-lacking (INR-) promoters. Just upstream of TATA is the upstream stimulatory element (USE) containing the binding site for any number of upstream stimulators. The USE may or may not be present in TATA-containing promoters but is often present (usually in the form of a GC box), in TATA-less, INR + promoters (Smale and Baltimore, 1989). The fourth element found in pol II promoters is the enhancer, which can be located upstream or downstream of the transcription start site and is often many hundreds to thousands of nucleotides away (Breathnach and Chambon, 1981; Maniatis et al., 1987). These elements, like USEs, are the binding sites of transcription activators, but unlike USEs, they can function at great distances from the initiation complex in a mechanism believed to involve looping of the intervening DNA (Ptashne, 1988).

General factors

All of these promoter elements are believed to contain binding sites for some transcription factors, though the exact nature of all the proteins involved has not yet been elucidated. In particular, although several proteins that bind to the INR have been identified (Garfinkel et al., 1990; Roy et al., 1991; Shi et al., 1991), their exact role in transcription initiation remains controversial (Reviewed (Weis and Reinberg, 1992)). In contrast, over the last five years, significant progress has been made in our understanding of the factors that assemble over the TATA box, and their role in transcription. This group of factors, the general transcription factors, are believed to control basal transcription from all TATA-containing pol II promoters. The earliest descriptions of these factors came from fractionation of transcription competent HeLa whole cell (Samuels et al., 1982) and nuclear (Matsui et al., 1980; Davison et al., 1983; Dignam et al., 1983; Sawadogo and Roeder, 1985) extracts. Such fractionation led to the identification of partially purified fractions containing factors TFIIA, B, D, E/F, which when combined with RNA pol II *in vitro* could initiate specific transcription from the TATA-containing adeno major late promoter. Close examination of these fractions revealed the presence of multiple activities. To date the alphabet of factors is up to TFIIJ (for review: Buratowski and Sharp, 1992; Conaway and Conaway, 1993; Zawel and Reinberg, 1993).

Of all these factors, only one, TFIID (aka BTF1, D-TFIID, τ, holo-TFIID, DB) (Samuels et al., 1982; Davison et al., 1983; Conaway et al., 1990) is a sequence specific DNA binding factor which recognizes the TATA box. Early attempts to purify this factor were unsuccessful as a result of the apparent instability of the factor through multiple rounds of chromatography. A substantial advance came with the identification of a yeast protein which efficiently substituted for the

mammalian TFIID factor in an *in vitro* transcription reaction (Buratowski et al., 1988; Cavallini et al., 1988). Purification of the yeast factor revealed a single 27 kd polypeptide that could bind to the TATA box and support basal transcription (Hahn et al., 1989b; Horikoshi et al., 1989). This protein and its homologs in all species are referred to as TATA-binding protein (TBP) (Dynlacht et al., 1991). The only activity not supported by this protein was the reconstitution of upstream activator stimulated transcription (Hoey et al., 1990; Kambadur et al., 1990; Peterson et al., 1990). These results, combined with the observed difficulty in working with the purified mammalian factor, led to the suggestion that D-TFIID is a complex consisting of TBP and a number of associated proteins variously referred to as coactivators, mediators or accessory factors (Pugh and Tjian, 1990; Tanese et al., 1991). These associated proteins formed a large complex with TBP that sized at >700kd (Samuels et al., 1982).

With the purification of TBP from yeast it became possible to use a gel shift assay to study the assembly of the remaining transcription factors onto the DNA. Using such an assay the ordered assembly of the general factors on the TATA box was described (Buratowski et al., 1989). The first factor so implicated was TFIIA (aka AB, STF) (Egly et al., 1984; Samuels and Sharp, 1986; Reinberg et al., 1987; Hahn et al., 1989a) which assembles into a TFIIA/TBP/DNA complex via protein:protein interactions with TBP. This complex is believed to be more stably associated with DNA than TBP itself as evidenced by the ability to form gel shifts on a TATA-containing probe only in reactions that contained both the yeast TBP and TFIIA (Buratowski et al., 1989). Despite its stable association with TBP on and off (See appendix) the promoter, the actual role of TFIIA in transcription remains controversial. It is clearly not absolutely required to reconstitute basal transcription *in vitro* (Sawadogo and Roeder, 1985; Conaway et al., 1987; Wampler et al., 1990) but can have a stimulatory effect (Samuels

and Sharp, 1986; Reinberg et al., 1987). More recently it has been implicated in the stimulated transcription reaction, both as a protein whose activator enhanced association with the promoter is the rate limiting step (Wang et al., 1992), and as a protein that functions to dissociate inhibitory activities from the TFIID complex (Meisterernst and Roeder, 1991; Meisterernst et al., 1991; Inostroza et al., 1992; Merino et al., 1993). TFIIA has been purified to homogeneity from both yeast (Ranish and Hahn, 1991) and mammalian cells (Samuels and Sharp, 1986; Usuda et al., 1991; Cortes et al., 1992) and cloned in yeast (Ranish et al., 1992).

The next factor to join the initiation complex is TFIIB (aka a, e, BTF3) (Conaway et al., 1987; Ha et al., 1991; Malik et al., 1991; Moncollin et al., 1992; Tschochner et al., 1992) . While it can associate with a TBP/TFIIA/DNA complex it has also been shown to bind TBP in the absence of TFIIA (Buratowski and Sharp, 1990; Lin and Green, 1991). This 35 kd polypeptide is believed to bridge the initiation complex to pol II and aid in the selection of pol II over pol I or pol III. There is no doubt that TFIIB is an essential component of the pol II transcription machinery as even the barest minimal systems require it for function (Parvin and Sharp, 1993). It has been proposed that TFIIB may play a role in measuring the distance from the TATA box to the INR through interactions with both pol II and the initiation complex (Buratowski et al., 1989; Pinto et al., 1992; Tschochner et al., 1992). Evidence for this comes from both yeast and mammalian systems. In yeast, factor e has been demonstrated to stably associate with pol II (Tschochner et al., 1992) and mutations in the *SUA7* gene alter start site selection at a number of promoters (Pinto et al., 1992). In drosophila, TFIIB suppresses non-specific transcription initiation by pol II probably through direct interactions (Wampler and Kadonaga, 1992).

An even more provocative role for TFIIB has emerged from studies of activator dependent transcription from solid support tethered DNA. These experiments suggest that TFIIB plays a role in bridging the synthetic transcription activator GAL4 AH (Lin et al., 1988) to the initiation complex. Using protein affinity chromatography, a direct physical interaction between the activator and TFIIB was demonstrated (Lin et al., 1991). Additionally, a functional stimulation resulting from the recruitment of TFIIB onto the promoter was also observed (Lin and Green, 1991). This provides yet another model for the mechanism of transcription stimulation and undoubtedly represents only a portion of the story (Hahn, 1993). Perhaps, as Tjian and colleagues suggest, stimulation by an acidic activator requires the interplay of a tripartate complex of TFIIB, activator, and TAF 40 (Goodrich et al., 1993).

The factors described thus far represent what reconstitution experiments suggest to be the core set of essential transcription factors. In the presence of RNA pol II and a supercoiled template, these factors alone are competent to transcribe from certain promoters (Parvin and Sharp, 1993). This suggests that all of the information necessary for promoter recognition and initiation are contained within this core (See discussion chapter IV). It is also clear that transcription reconstituted with this core set of proteins represents a special case, and a number of promoters under a number of conditions are not functional with just this core set of proteins. In many cases, the additional protein required is TFIIF (Tyree et al., 1993).

TFIIF (aka factor 5, βγ, RAP 30/74 (Sopta et al., 1985; Flores et al., 1988; Conaway and Conaway, 1989; Price et al., 1989) was originally identified as a protein complex of 30 kd and 74 kd polypeptides that associated with RNA pol II (Sopta et al., 1985). This association is reminiscent of TFIIB and it is believed that the two factors act in concert to recruit RNA pol II to the correct site of

initiation (Conaway and Conaway, 1990; Killeen et al., 1992). This activity combined with a stretch of amino acid homology to E coli. σ^{70} led to the suggestion that TFIIF might function as a sigma factor to prevent non-selective binding of RNA pol II to DNA.

Though recombinant TFIIF (Sopta et al., 1989; Horikoshi et al., 1991; Finkelstein et al., 1992; Garrett et al., 1992) can replace the purified protein in reconstituted transcription, the native protein is known to be extensively phosphorylated *in vivo*. The role of this phosphorylation is yet to be understood and it is unclear whether the recombinant protein becomes phosphorylated during the assembly of the initiation complex. It remains a possibility that the response to an upstream activator requires the native TFIIF or simply the phosphorylated form, thereby explaining its *in vivo* phosphorylation. With TFIIF ends the clear data on the order of addition of factors in a gel shift assay. While several other factors are known to be involved in the transcription reaction, they can not be unambiguously placed in the hierarchy. One such additional factor, TFIIE (aka e,) is a heterotetramer consisting of 2 polypeptides, p34 and p56 (Ohkuma et al., 1990; Conaway et al., 1991; Inostroza et al., 1991). Cloned, E. coli produced TFIIE is fully functional in both basal and stimulated transcription (Ohkuma et al., 1991; Peterson et al., 1991; Sumimoto et al., 1991). Though initial experiments suggested that TFIIE might be a DNA-dependent ATPase (Sawadogo and Roeder, 1984; Reinberg and Roeder, 1987) neither the highly purified nor the recombinant TFIIE exhibits such an activity (Ohkuma et al., 1990; Conaway et al., 1991; Inostroza et al., 1991; Peterson et al., 1991). As was previously mentioned, it is not clearly required for all promoters (Parvin et al., 1992; Tyree et al., 1993). The particular features of the promoter and the reaction that dictate its necessity, remain to be discovered.

A recently identified and particularly interesting factor is TFIIH (aka b, δ, BTF2) (Conaway and Conaway, 1989; Feaver et al., 1991b; Gerard et al., 1991). This factor consists of 3 polypeptide subunits in yeast and between 5-8 polypeptide subunits in mammals. The subunits range in size from 35kd-95 kd and, despite the differences in polypeptide composition between the yeast and mammalian factors, share many functional similarities. First, cDNAs encoding the yeast 75 kd polypeptide (Gileadi et al., 1992) are homologous to cDNAs encoding the p65 of mammals (Fischer et al., 1992). Second, both the rat d and the yeast b factors share DNA-dependent ATPase activity (Conaway and Conaway, 1989; Feaver et al., 1991b) and kinase activity with specificity for the carboxy terminal domain of RNA pol II (CTD) (Feaver et al., 1991b; Serizawa et al., 1992). Interestingly, though TFIIH was initially reported to be free of CTD-kinase activity (Lu et al., 1991), more recent experiments have identified an associated kinase with the similar CTD specificity (Lu et al., 1992). Although a number of experiments suggest that the state of phosphorylation of the CTD does change during the course of the transcription reaction, the role of CTD phosphorylation in transcription remains unclear. It is believed that the unphosphorylated form of CTD (II_a) preferentially enters the initiation complex and is converted to the phosphorylated form (II_o) prior to elongation (Laybourn and Dahmus, 1990; Lu et al., 1992). Consistent with this, only the II_o form of pol II was identified in crosslinking experiments on actively elongating initiation complexes (Cadena and Dahmus, 1987; Payne et al., 1989). Nonetheless, stimulation competent *in vitro* transcription can be reconstituted using RNA pol II lacking the CTD (Zehring et al., 1988; Thompson et al., 1989; Buratowski and Sharp, 1990)-although the relaxed requirements of the *in vitro* reaction cloud the significance of such reconstitution results. It is well established that such

deletions have deleterious effects *in vivo* (Nonet et al., 1987; Allison et al., 1988).

It is clear that the number of factors mediating pol II transcription is large and still growing. Not discussed are several of the recent additions, i.e. TFIIG (Sumimoto et al., 1990) and TFIIJ (Cortes et al., 1992), because their existence is still unconfirmed and their role in transcription is murky. Undoubtedly, more factors will be discovered whose effects on the *in vitro* reaction will be more subtle and condition dependent. Some of these will have modulatory effects that relate to the topology of the template, some will act as repressors, some as derepressors, some will act alone and others only in concert with each other or with an upstream activator.

TBP-TAFs across the spectrum

Common features of the three systems

In view of the preceding, it should be clear that several protein factors provide a mechanistic link between the three forms of polymerase. The first, which will not be elaborated on here, is RNA polymerase itself. Pols I, II, and III share several subunits which are presumably involved in a common function (Woychik et al., 1990; Carles et al., 1991; Young, 1991). The second, and less clearly linked factor is TFIIB and its homologues. In addition to its function in pol II transcription, its yeast homologue has been shown to be required in the pol III system where it bridges the initiation complex to pol III (Kassavetis et al., 1991). A TFIIB homologue probably exists in the pol I system as well, although this has not yet been demonstrated (Sharp, 1992; Rigby, 1993). One of the TAFs of the SL1 complex would be a likely candidate for a pol I TFIIB-like factor.

In each initiation complex the TFIIB like activity interacts directly with TBP and probably with its specific polymerase to assemble a promoter specific/polymerase specific complete initiation complex. Though its function has been confirmed in both pol II and pol III systems, the formal proof of its universality awaits the cloning of the SL1 TAFs.

The third factor that links these three pol systems, and the most relevant for this discussion, is TBP. It is clearly a component of the transcription complexes formed on all promoters and with all polymerases. In the case of pol I, TBP exists in the SL1 complex where it is associated with three TAFs (Comai et al., 1992). The SL1 complex does not specifically bind to DNA, and certainly not to a TATA box, as no such element exists in a pol I promoter (for review (Reeder, 1992)). SL1 binds DNA only indirectly through the action of a second factor, UBF, which is responsible for stabilizing the TBP-TAF complex on the promoter and forming the platform for pol I binding. Because SL1 is responsible for the species specificity observed in pol I transcription (Mishimi et al., 1982), it must evolve as the promoter evolves. That is, both UBF and pol I can be exchanged between species and still initiate accurate transcription, but the SL1 factor can only function on its cognate promoter. This implies that although SL1 requires UBF for tight binding to DNA it must be involved in specific promoter recognition.

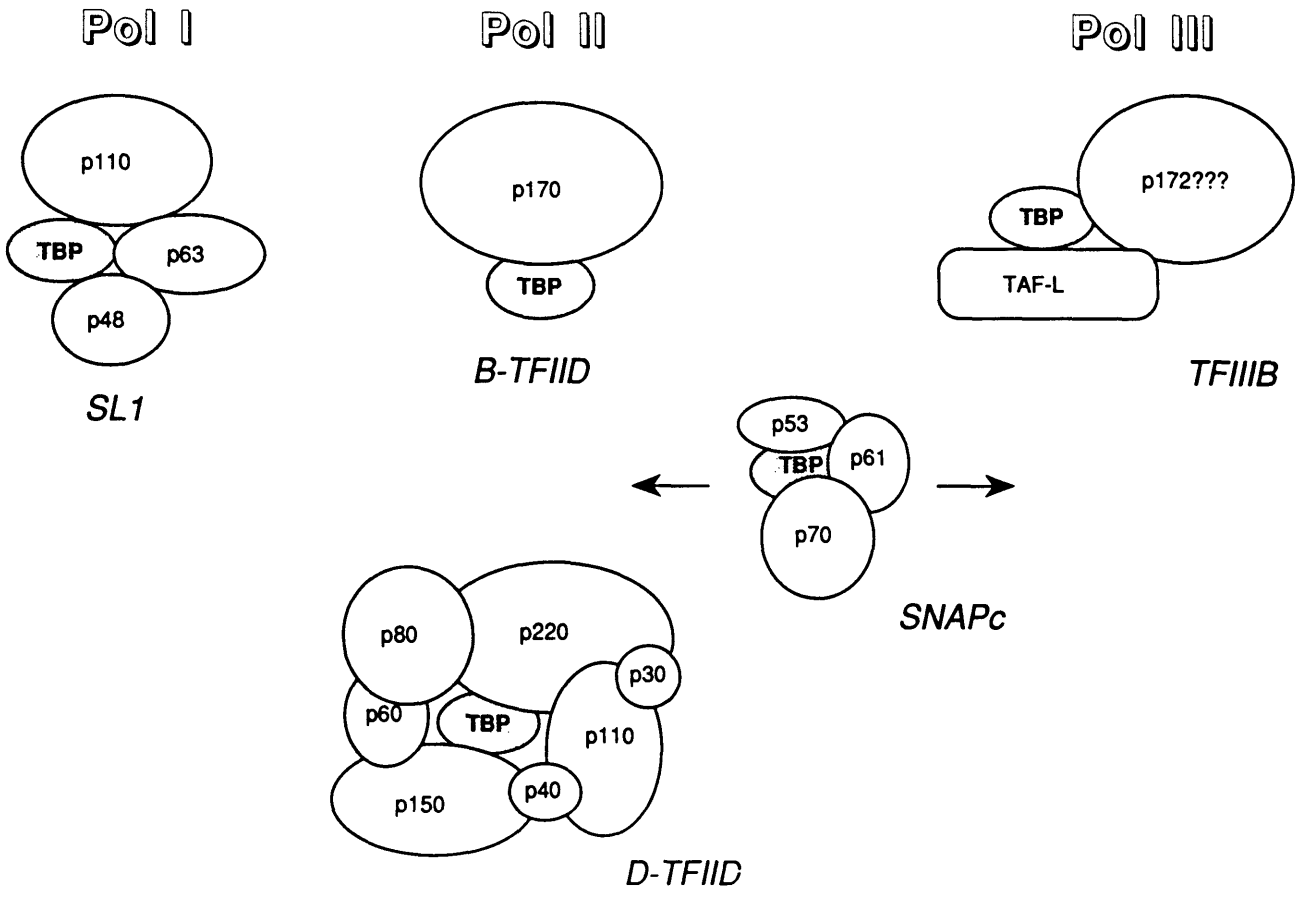
The requirement for another factor to mediate the binding of TBP-TAF complexes to their promoters is a common theme of all non-TATA box containing promoters. In the case of the TATA-less pol II promoters, a TBP-TAF complex is known to associate with the promoter only in the presence of an upstream binding factor, principally SP1 (Pugh and Tjian, 1990). The exact nature of the TAFs involved is not clear, but they appear to be contained within the holo-TFIID complex (Pugh and Tjian, 1991; Zhou et al., 1992). Once again,

a large component of the sequence recognition is presumably conferred by the interaction of these TAFs with the promoter.

Similarly, in the pol III reaction, the TBP-TAF complex of TFIIIB is essential for transcription initiation but can not associate with the DNA template in the absence of TFIIIC. TFIIIC acts as SP1 or UBF above to bind the promoter in a sequence specific fashion and properly position the TBP-TAF complex. These TAFs must confer promoter recognition because once assembled with the aid of TFIIIC, TFIIIB can remain stably associated with the DNA even after IIIC is stripped away (Kassavetis et al., 1990). In the absence of a TATA box, sequence recognition must be conferred by the TAFs.

Figure 2. TBP complexes in HeLa cells. Four of the five TBP-TAF complexes identified in HeLa cell extracts are believed to be specific for a specific polymerase. The SL1 complex is specific for pol I, the TFIIIB complex is specific for pol III, and the B-TFIID and D-TFIID complexes are specific for pol II. Only the SNAPc complex functions in both pol II and pol III transcription.

TBP Complexes in HeLa Cells



Towards a new definition of TBP

The promiscuity of TBP suggests that a reevaluation of its original characterization is in order. Perhaps the TATA-containing pol II transcription reaction represents the special case in which TBP or D-TFIID actually interacts with the promoter unaided. Because the common feature of many pol II promoters is a TATA box, it was obvious to look for a factor that bound there and played a role in pol II transcription. It is now clear that TBP is much more than a sequence specific DNA binding protein involved in pol II transcription and is more accurately described as a component of a diverse set of complexes involved in specific promoter recognition and polymerase selection.

In the most recently described TBP-TAF complex, SNAPc, TBP assumes a slightly different role, as part of a TAF complex with its own sequence specific DNA binding properties (Sadowski et al., 1993). In this case the TBP-TAF complex binds to the TATA-less snRNA U1 PSE and drives pol II transcription. The specifics of this reaction have not yet been worked out and it is not yet clear which other pol II factors are required. Nonetheless, it is clear that transcription of the U1 promoter represents a new class of TBP-requiring reactions, in which TBP is part of a sequence specific binding factor that can interact with its sequence element unaided. As suggested by the authors, perhaps other members of this class will emerge (Sadowski et al., 1993). As mentioned earlier, the PSE is common to all snRNA promoters, including the U6 promoter which contains a TATA box and is transcribed by pol III. The TBP-TAF complex SNAPc is also required for transcription of these genes (Sadowski et al., 1993). What then commits the U6 promoter to pol III instead of pol II? The answer must lie in the additional factor requirements of the U6 transcription

reaction. Sadowski et al. demonstrated that U6 transcription requires, in addition to SNAPc, a second TBP, the exact nature of which remains obscure (Sadowski et al., 1993). This suggests that the combination of SNAPc and a second TBP is a signal for the recruitment of pol III factors. In particular, at least a portion of TFIIIB must be assembled on the promoter to allow the subsequent assembly of pol III.

Is yeast TBP decorated with TAFs?

Clearly much has been learned from transcription experiments in yeast. Early experiments demonstrated that yeast activators could function in mammalian cells, and vice versa (Kakidani and Ptashne, 1988; Metzger et al., 1988; Schena and Yamamoto, 1988; Struhl, 1988; Webster et al., 1988), suggesting the existence of functional crosstalk between mammalian and yeast transcription systems. Those experiments indicated that the mechanism of transcription stimulation had been conserved from yeast to man. As outlined in this Chapter, many yeast homologues of mammalian transcription factors have been isolated since the identification of TBP.

Original identification of yeast TBP

Yeast TBP activity was originally identified by complementation of a mammalian *in vitro* transcription reaction (Buratowski et al., 1988). Such complementation supported the notion of functional relatedness. Further, sequence analysis of TBPs from various organisms indicated a high degree of conservation across the entire protein except for a completely divergent N-terminal domain (Greenblatt, 1991; Pugh and Tjian, 1992).

There is still no clear function ascribed to the N-terminus of TBP, though some experiments have suggested that it confers species specificity in a stimulated transcription assay (Hoey et al., 1990; Pugh and Tjian, 1990). As for the remaining general transcription factors, most of the functionally equivalent yeast activities have been isolated, principally by Kornberg and colleagues (Feaver et al., 1991a; Ranish and Hahn, 1991; Henry et al., 1992; Sayre et al., 1992a; Sayre et al., 1992b; Tschochner et al., 1992), but all the genes have not yet been cloned nor the degree of interspecies swapping fully assessed.

In any discussion of functional homology it is useful to recall a major difference between yeast and human TBP. While mammalian TBP clearly exists in complexes with a number of different TAFs (as described above), the yeast protein was initially isolated from cells as a single ~30 kd polypeptide (Cavallini et al., 1989; Hahn et al., 1989a; Horikoshi et al., 1989; Schmidt et al., 1989). In those experiments, basal transcription activity coeluted with the vast majority of the TBP and this TBP could bind a TATA-box in a gel shift assay and commit to a DNA template (Buratowski et al., 1988). This TBP could not, however, function in the mammalian stimulated transcription assay which, consistent with its small size, led to the suggestion that it was missing the associated proteins required for such activity.

Recent experiments by Zhou et al. have explored this difference between yeast and human TBP (personal communication). They have observed that the conserved C-terminal domain alone is sufficient to confer both tight regulation and complex formation to TBP expressed in HeLa cells (Zhou et al., 1992). Additionally, they have observed that fusion of the N-terminal domain of yeast TBP onto the conserved domain of the human protein yields a protein that is both highly expressed in HeLa cells, and uncomplexed (personal communication). This suggests that the yeast N-terminal domain inhibits

binding of TAFs and allows for the accumulation of monomeric TBP in mammalian cells.

Novel yeast TAFs

Although previous genetic experiments had identified yeast genes that suppressed mutations in TBP in an allele specific manner (Eisenmann et al., 1992), there was very little biochemical data to support the identification of these proteins as TAFs. Recently, though, several lines of experiments have demonstrated that yeast may in fact have TAFs. In these cases, TAFs are literally defined as TBP-associated factors and not as factors required for stimulated transcription. One set of such TAFs may be the SRB proteins of Young and colleagues (Koleske et al., 1992) isolated as supressors of CTD truncation mutations. Their experiments suggest that the SRBs exist in a complex with RNA pol II and ~5 % of the cellular TBP (Thompson et al., 1993). Though TBP is not present in stoichiometric amounts relative to either the SRBs or pol II, the extensive purification of this complex suggests an association between the subunits. The relatively low abundance of this TBP form could explain why initial experiments to islolate TBP did not identify the multisubunit SRB complex. Additional experiments are required to determine whether the SRBs are TAFs required for stimulated transcription, or a protein complex that mediates transcription from a variety of yeast promoters.

In an experiment designed to isolate the yeast TFIIIB factor that had previously been shown to contain TBP (Buratowski and Zhou, 1992; Colbert and Hahn, 1992; Kassavetis et al., 1992; Lopez-de-Leon et al., 1992) a second set of TAFs was isolated by Weil and colleagues (Poon et al., 1993). Purification over ion exchange, followed by immunoprecipitation (IP) with anti-TBP antibodies,

allowed the isolation of ~7 polypeptides (not including TBP) ranging in size from 60 kd-170 kd. The total complex could function with TFIIIC and pol III to reconstitute pol III transcription. Because this complex was not highly purified prior to immunoprecipitation it is not clear whether it represents a single TBP-TAF complex or a number of distinct complexes.

When these immunoprecipitated TAFs were cloned, it appeared that two of them were the previously identified p70 and p90 of TFIIIB (Bartholomew et al., 1991). A third TAF isolated in this precipitation, p170, was the previously identified protein MOT1 (Davis et al., 1992). MOT1, a member of a growing family of SWI2 homologues which includes the proteins RAD 54 and SNF 2 (Emery et al., 1991; Laurent et al., 1991), has been implicated in transcription regulation (Davis et al., 1992). Interestingly, the MOT1 protein surfaced in an independent set of experiments performed by Hahn and colleagues who were purifying an inhibitor of TBP. This inhibitor, which they called ADI, was purified and cloned based on its ability to release TBP from DNA in an ATP dependent fashion. The cloning and sequencing of ADI confirmed that it was homologous to MOT1. Thus, in separate experiments, two groups identified a protein that interacts with TBP and by definition qualifies as a TAF.

These experiments confirm the presence of TBP in multisubunit complexes in yeast cells. This suggests that the original identification of TBP as a monomeric protein in yeast probably reflects the affinity of TBP for these TAFs, the techniques used to disrupt the yeast cells and isolate proteins, and the assay used to detect TFIID activity. As genetic experiments support the idea that accessory proteins are required for stimulated transcription (Berger et al., 1992), the isolation of naked TBP in yeast may have been more a biochemical artifact than a functionally significant result.

Previous data on B-TFIID

The work described in this thesis involved the isolation and examination of a novel TBP-TAF complex, B-TFIID, which was initially discovered by Marc Timmers, a post doctoral fellow in the lab. When I joined this project, Marc had already begun to define some of the properties of B-TFIID. Using newly prepared polyclonal antibodies raised against the N-terminal conserved domain of human TBP fused to GST, he asked the following question: Does TBP comigrate over phosphocellulose with the well characterized TFIID activity identified in the high salt D fraction? The answer to this question, yes and no, was the foundation for all that followed. Marc observed that TBP not only chromatographed in the P-cell D fraction, but also in the P-cell B fraction, a previously uncharacterized protein pool (Timmers and Sharp, 1991).

There are several reasons why TBP was not previously discovered in the B fraction. First, an uncharacterized inhibitory activity also exists in the B fraction. Addition of the P-cell B fraction into a reconstituted transcription reaction therefore leads to a significant decrease in transcription. This in not true of the P-cell D fraction which can be supplied directly into a reconstituted reaction and support pol II transcription. Second, because the D fraction contained an activity that was required for transcription and interacted with a TATA box, there was no reason to look for such an activity elsewhere. The third reason that TBP in the P-cell B fraction went unnoticed was that RNA pol II was known to fractionate into the B fraction. A good purification of pol II had been previously developed (Hodo and Blatti, 1977) and transcription could be reconstituted with purified pol II and P-cell fractions A, C, and D eliminating any requirement for the B fraction.

With the discovery of TBP in the B fraction, Marc went on to compare it to the TBP in the D fraction, referred to as D-TFIID. He first compared the sizes of the two forms of TBP and quickly realized that like D-TFIID, a complex of ~700kd, B-TFIID was also a large complex, sizing at ~300 kd (Timmers and Sharp, 1991). He observed that all of the TBP in the WCE could be sized into complexes of >250 kd and that none existed in the monomeric 40 kd range. Marc developed a partial purification of B-TFIID and demonstrated that after a second column, the partially purified material could function as well as TBP or D-TFIID to reconstitute pol II transcription. This activity exhibited a lower salt optimum in transcription and in general appeared to bind less tightly to DNA. Thus B-TFIID was emerging as another TBP-TAF complex that could transcribe the adeno MLP but did not tightly bind a TATA box. The final observation Marc made was that B-TFIID could not support stimulation by either SP1 or GAL4 AH (Timmers and Sharp, 1991). This led us to rethink the role of TAFs as mediators of stimulation.

At this point I joined the project. Together Marc and I worked out a purification of B-TFIID and characterized its subunit composition. Along the way I discovered that B-TFIID was an ATPase, but the function of this ATP hydrolysis activity remains a mystery. This work is described in detail in chapter II. The work that followed grew out of our search for a function for B-TFIID. Several models were tested and proved to be incorrect. The most carefully explored model was that of B-TFIID as a pol III factor. That too turned out to be incorrect, as is elaborated in chapter III.

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Chapter II. Purification and Characterization of Transcription Factor B-TFIID

Abstract

Initiation of transcription by RNA polymerase II requires a TFIID factor which can recognize the TATA element common to many promoters. A number of distinct multi-subunit factors which contain the well characterized TATA-binding protein (TBP) can be resolved from extracts of mammalian cells. Two of these, B-TFIID and D-TFIID, were able to support pol II transcription in reconstituted reactions. The smaller complex, B-TFIID, was purified and its subunit composition was determined. The B-TFIID factor consists of two subunits: the TBP and a TBP-associated factor (TAF) of 170 kd. This TAF is specific for the B-TFIID factor and appears not to be present in the larger D-TFIID complex. Furthermore, it was found that the highly purified B-TFIID fractions contain an associated (d)ATPase activity.

Introduction

Initiation of transcription by eukaryotic RNA polymerase II is a complex process requiring multiple proteins (Mitchell and Tjian, 1989; Sawadoga and Sentenac, 1990; Sharp, 1992). Gene specific transcription factors regulate the initiation frequency by controlling formation of an initiation complex that contains both basal factors (TFIIA, -B, -D, -E, -F, -H) and RNA polymerase II (pol II). Assembly of this initiation complex occurs in an ordered fashion (Buratowski et al., 1989; Maldonado et al., 1990). The first step is the binding of the TFIID factor to a TATA-element and, subsequently, the other basal factors and pol II enter the complex in a sequential manner. Many of the basal factors have been purified to homogeneity by conventional chromatography allowing elucidation of their subunit composition and cloning of their corresponding cDNAs.

The TFIID factor has not been purified to homogeneity by conventional chromatographic methods. Recent experiments suggest that one of the difficulties in purification of the TFIID factor might be its heterogeneity (Pugh and Tjian, 1991; Tanese et al., 1991). Identification and cloning of a gene encoding a yeast protein that could substitute for the human TFIID factor in an *in vitro* transcription reaction led to the isolation of a human cDNA encoding a 38 kd protein (Buratowski et al., 1988; Cavallini et al., 1989; Hahn et al., 1989; Horikoshi et al., 1989; Schmidt et al., 1989; Hoffman et al., 1990; Kao et al., 1990; Peterson et al., 1990). This protein, TBP, (also known as TFIIDt) binds specifically to the TATA element and directs formation of the initiation complex. Antisera specific for human TBP were used to show that this protein does not exist as a free polypeptide in HeLa cell extracts (Timmers and Sharp, 1991). Instead, TBP is assembled into two predominant high molecular weight complexes: one of 300 kd (B-TFIID) and the other of >700 kd (D-TFIID).

Polypeptides associated with TBP in these complexes are referred to as TAFs, for TBP-associated factors (Dynlacht et al., 1991). Although the D-TFIID factor is the well-characterized TFIID activity in HeLa cell extracts, the B-TFIID factor contains the majority of the cellular TBP (Timmers and Sharp, 1991). These two TFIID complexes, as well as TBP itself, are equally efficient in supporting basal transcription in reconstituted reactions ((Timmers and Sharp, 1991)). However, the two activities are not equivalent since transcription reactions reconstituted with the traditional D-TFIID factor are responsive to gene specific transcription factors such as SP1, GAL4-AH (Timmers and Sharp, 1991), GAL4 TEF-1, (REM, data not shown) and Oct-1 (Jaesung Kim, personal communication) while reactions reconstituted with B-TFIID are not.

TBP has recently been shown to be a component of transcription factors for RNA polymerases I and III. For example, the SL1 factor which is necessary for transcription by pol I is composed of TBP and three TAFs ((Comai et al., 1992)). Genetic and physical evidence suggests that TBP is also essential for pol III activity ((Lobo and Hernandez, 1989; Margottin et al., 1991; Simmen et al., 1991; Cormack and Struhl, 1992; Schultz et al., 1992; White et al., 1992)). These TBP complexes are thought to be distinct from TFIID-type complexes in that the latter are specific for generation of an initiation complex containing pol II. While this may be true *in vivo*, more recent experiments described in chapter III suggest the this distinction is obscured *in vitro*.

The existence of the different forms of TFIID in cellular extracts has several implications for mechanisms of gene regulation. For example, B-TFIID could be a precursor in the assembly of more complex D-TFIID activities. Alternatively, B-TFIID and D-TFIID could be distinct, mature complexes, that respond to different regulators and serve different types of promoters. In this case, the sequence of the promoter and the nature of critical regulatory factors would

dictate the type of TFIID activity used to form the initiation complex. The latter model is supported by mutational analysis which provided evidence for functionally distinct processes recognizing TATA elements ((Chen and Struhl, 1988; Simon et al., 1988)). In order to elucidate the relationship between the B-TFIID and D-TFIID factors, we have purified the B-TFIID factor from HeLa cells both by conventional and immunoaffinity chromatography and have determined its subunit composition. Interestingly, our most highly purified B-TFIID fractions contain an (d)ATPase activity, suggesting a possible link between TAFs and the energy requiring step in the transcription initiation reaction.

Results

Purification of B-TFIID.

Purification of the B-TFIID factor from HeLa whole cell extracts was performed by chromatography on six successive columns (Fig. 1). TBP-containing fractions were identified by immunoblot analysis using a rabbit serum raised against the N-terminal 139 amino acids of TBP (Timmers and Sharp, 1991). The TFIID-dependent transcription activity of the different fractions across the purification was assayed in a reaction containing the adenovirus major-late core promoter (Fig. 2). This analysis showed that after the second column the TFIID activity strictly coeluted with TBP. Quantitation of these transcription reactions indicated that each step yielded ~50- 60% recovery (Table 1) though such quantitation was accurate only on the more purified fractions. As can be seen with both crude D-TFIID (lanes 5 and 6) and crude B-TFIID (lanes 7-9), transcription did not linearly increase with increasing protein. In accordance with our previous findings, TFIID activity could not be detected in

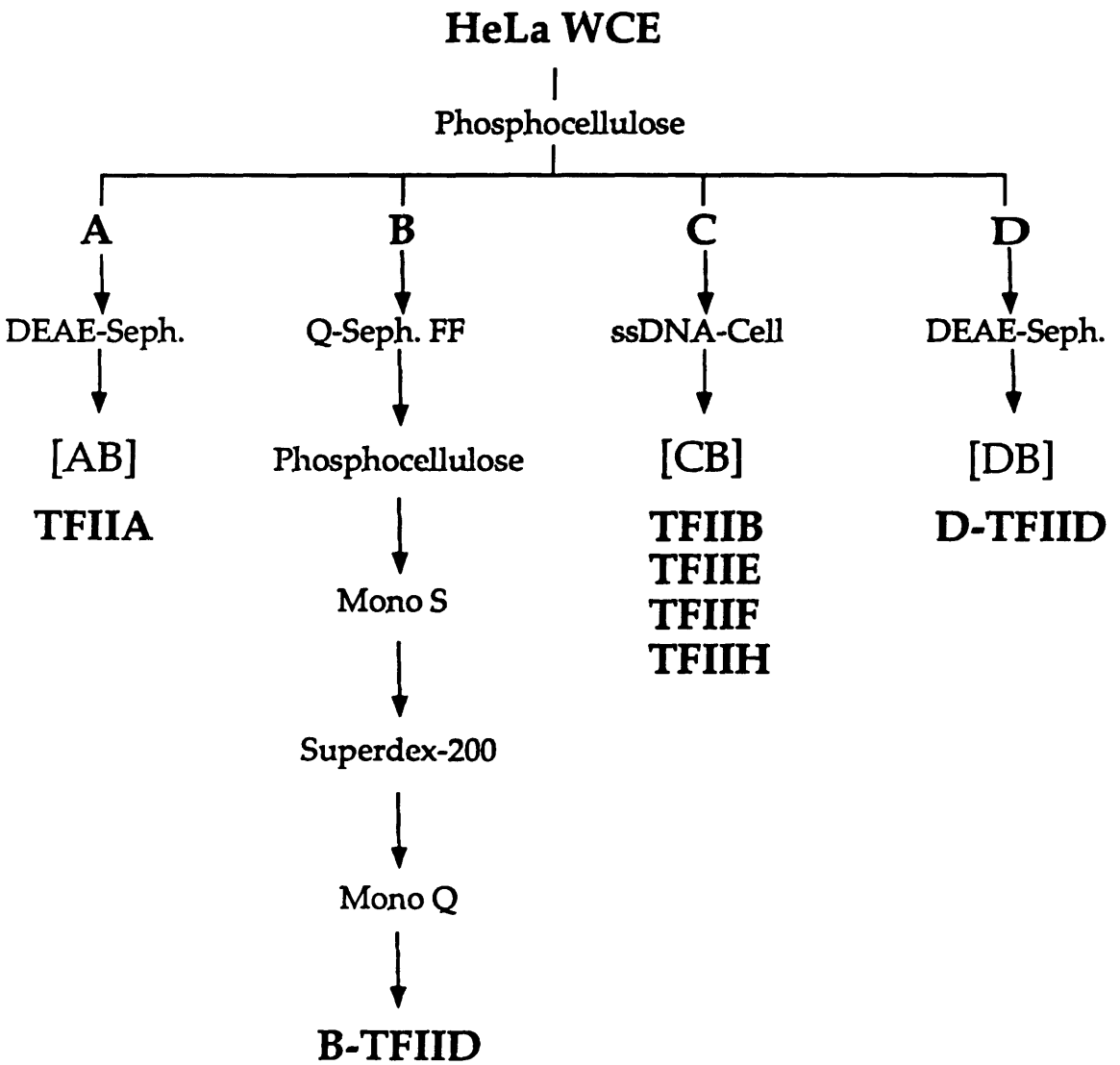
TBP-containing fractions from earlier columns (Timmers and Sharp, 1991). This might suggest the presence of an inhibitor specific for B-TFIID in early column fractions, however, such an inhibitor could not be detected in mixing experiments (data not shown).

Analysis of the fractions from the final Mono Q column are shown in Figure 3. Fractions 27 and 28 were peak fractions when assayed both for transcription activity (Fig. 3A) and for the concentration of TBP (Fig. 3B). Three polypeptides (43, 80 and 170 kd) coeluted with this B-TFIID activity as revealed by silver staining of an SDS/polyacrylamide gel (Fig. 3C). The 43 kd polypeptide corresponded to TBP, which migrated slightly slower than its predicted size of 38 kd (5, 15). The 80 kd and170 kd polypeptides were good candidates for components of the B-TFIID complex.

Immunoprecipitation Analysis of TFIID-complexes.

In order to determine the subunit composition of the B-TFIID complex, pools of the peak fractions of several columns were analyzed by immunoprecipitation using affinity-purified TBP-antibodies or control 12CA5 monoclonal antibodies directed against the hemagglutinin antigen of influenza virus (Niman et al., 1983). Antibodies were cross-linked to Sepharose beads and similar amounts of B-TFIID activity from different steps in the purification protocol were subjected to the immunoprecipitation analysis. The 170 kd polypeptide was specifically coimmunoprecipitated with the 43 kd TBP (Fig. 4B, lanes 3, 5, 7, and 9). The specificity of this is indicated by the fact that the 170 kd protein was not a major polypeptide in the PG-200 load used in the immunoprecipitation (Fig. 4A, lane 1). Comparison of lanes 3, 5, 7, and 9 indicated that the stoichiometry of the TBP polypeptide to the 170 kd protein was similar at different steps in the purification. The immunoprecipitation

Figure 1. Purification scheme for B-TFIID and the different general transcription factors from HeLa whole cell extracts. The (AB) fraction contains TFIIA as indicated, whereas (CB) provides TFIIB/E/F and possibly TFIIH. The (DB) fraction provides the traditional TFIID-activity here indicated as D-TFIID. RNA polymerase II was isolated from calf thymus.


HeLa WCE
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Q-Seph. FF
ssDNA-Cell
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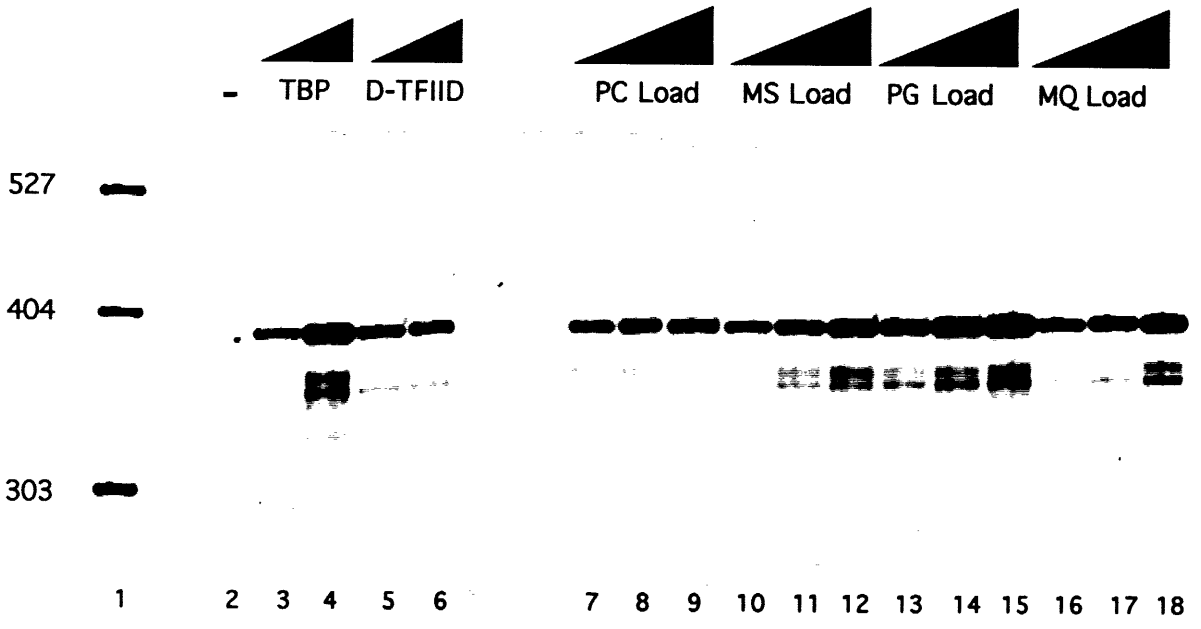
Phosphocellulose
[CB]
[DB]
TFIIA
TFIIB
TFIIE
TFIIF
TFIIH
D-TFIID
Mono S
Superdex-200
Mono Q
B-TFIID

Figure 2. Transcriptional analysis of B-TFIID across the purification. Five or 25 ng of His-tagged recombinant TBP (lanes 3 and 4 respectively), 5 or 7 μg of D-TFIID (lanes 5 and 6 respectively), and various B-TFIID fractions (lanes 7-18), were adjusted to buffer A plus 100 mM KCl and analyzed in a TFIID-dependent transcription reaction using pdML(C_2AT)19Δ-51 as the template (Buratowski et al., 1988; Sawadogo et al.,1985). This plasmid carries a 380 bp guanosine-less cassette under control of the adenovirus core major-late (ML) promoter (-53/+10). The reaction analyzed in lane 2 received only buffer A plus 100 mM KCl. Lanes 7-9 received 3, 5, or 7 μl of P-Cell load, respectively; lanes 10-12 received 2, 4, or 6 μl of Mono S load, respectively; lanes 13-15 received 0.3, 0.5, or 0.7 μl of PG 200 load, respectively, and lanes 16-18 received 3, 5, or 7, μl of Mono Q load, respectively. Lane 1 contains DNA molecular weight markers with sizes in bp indicated to the left.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TBP
D-TFIID
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MS Load
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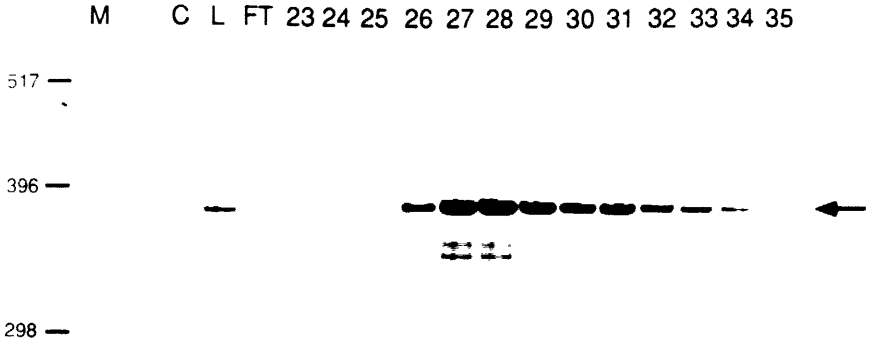
analysis also indicated that the 80 kd polypeptide was not stably associated with the B-TFIID complex.

Since it was possible that the binding of the TBP antibody might interfere with binding of the 80 kd protein and thus displace this protein from the complex, a B-TFIID fraction from the Superdex 200 column was further analyzed by chromatography on hydroxylapatite (HAP). Analysis of the peak fractions from this HAP column by silver staining of SDS/polyacrylamide gels and reactivity with anti- TBP serum in Western blots clearly showed that the 80 kd polypeptide did not elute in the fractions containing the 170 kd protein and TBP (Fig. 4C). While TBP and p170 eluted in fractions18-21, p80 eluted in fractions 24-27 (Fig. 4C). Further, immunoprecipitation of the peak fractions from this HAP column again yielded predominantly TBP and p170 (Fig. 4B lanes 13 and 14). This indicated that the 80 kd polypeptide coincidentally coeluted with the B-TFIID complex on the Mono Q column and is not a subunit of B-TFIID. Taken together, these data show that the B-TFIID factor consists of two subunits: a 170 kd polypeptide of unknown identity and a 43 kd polypeptide, which is TBP.

If the B-TFIID complex is simply a precursor of the larger D-TFIID complex then the 170 kd polypeptide should be present at a stoichiometric level to TBP in the D-TFIID preparation. This was tested by immunoprecipitation of a D-TFIID-containing protein fraction with anti-TBP antibodies (Fig.4B lanes 11 and 12). Close inspection of lanes 11 and 12 indicates that at least five polypeptides (>200 kd, 97 kd, 95 kd, 70 kd and 46 kd) specifically coimmunoprecipitate with the 43 kd TBP. The molecular weights of these polypeptides are very similar to the molecular weights present in the major D-TFIID complex characterized by others (Pugh and Tjian, 1991; Tanese et al., 1991; Zhou et al., 1992). However, none of the coimmunoprecipitated proteins

Figure 3. Analysis of chromatographic fractions of the last column in the purification of the B-TFIID factor. (A) Transcriptional analysis of the Mono Q fractions. Five µl of the load (L) and of the eluted fractions and six µl of the flow through (FT) were adjusted to buffer A plus 100 mM KCl and analyzed as in Fig. 2. The reactions analyzed in lane C received only buffer A plus 100 mM KCl. The arrow indicates the correctly initiated RNA product. (B) Immunoblot analysis of the Mono Q-fractions. Ten µl each of the load (L) and of the eluted fractions were analyzed with rabbit antiserum specific for human TBP. The positions of comigrated prestained markers are indicated to the left by their molecular weight in kd. The 43 kd band indicated by the arrow represents TBP. The other protein bands of slower mobility result from non-specific background staining (data not shown). (C) The different fractions of the Mono Q column were analyzed by silver-staining of a 12% SDS polyacrylamide gel. Fifteen µl of the eluted fractions and 25 µl of the load (L) were applied to the lanes. The positions of comigrated protein markers are indicated to the left of the gel by their molecular weight in kd. The arrows to the right indicate protein bands that comigrate with transcription activity.

A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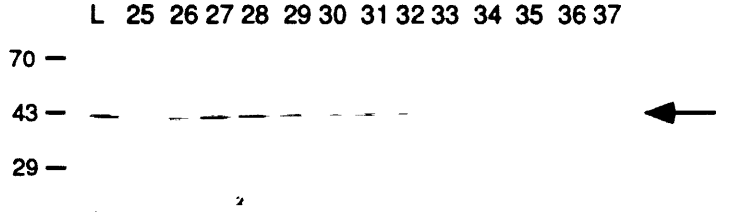
517
396
298

B


L 25 26 27 28 29 30 31 32 33 34 35 36 37
70
43
29

C

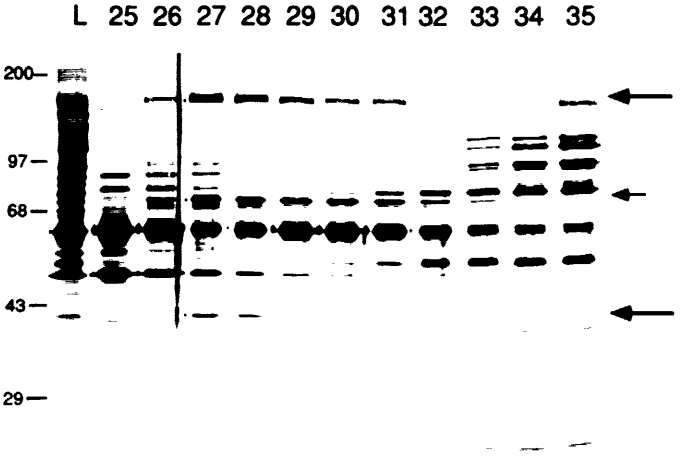

L 25 26 27 28 29 30 31 32 33 34 35
200
97
68
43
29

Figure 4A. Analysis of the immunopurified B-TFIID complex. Protein samples were separated by electrophoresis on a 10% SDS polyacrylamide gel and were visualized by silver staining. A) Silver staining of immunopurified TFIID fractions. Lane 1, 3.5 μg of the Superdex PG-200 load fraction; Lane 2, 0.5 μg of the Mono Q-fractions 27/28; Lane 3, 1.5 μg of a D-TFIID fraction. Lane 4, 200 ng His-tagged recombinant TBP, which is slightly larger than the endogenous TBP.

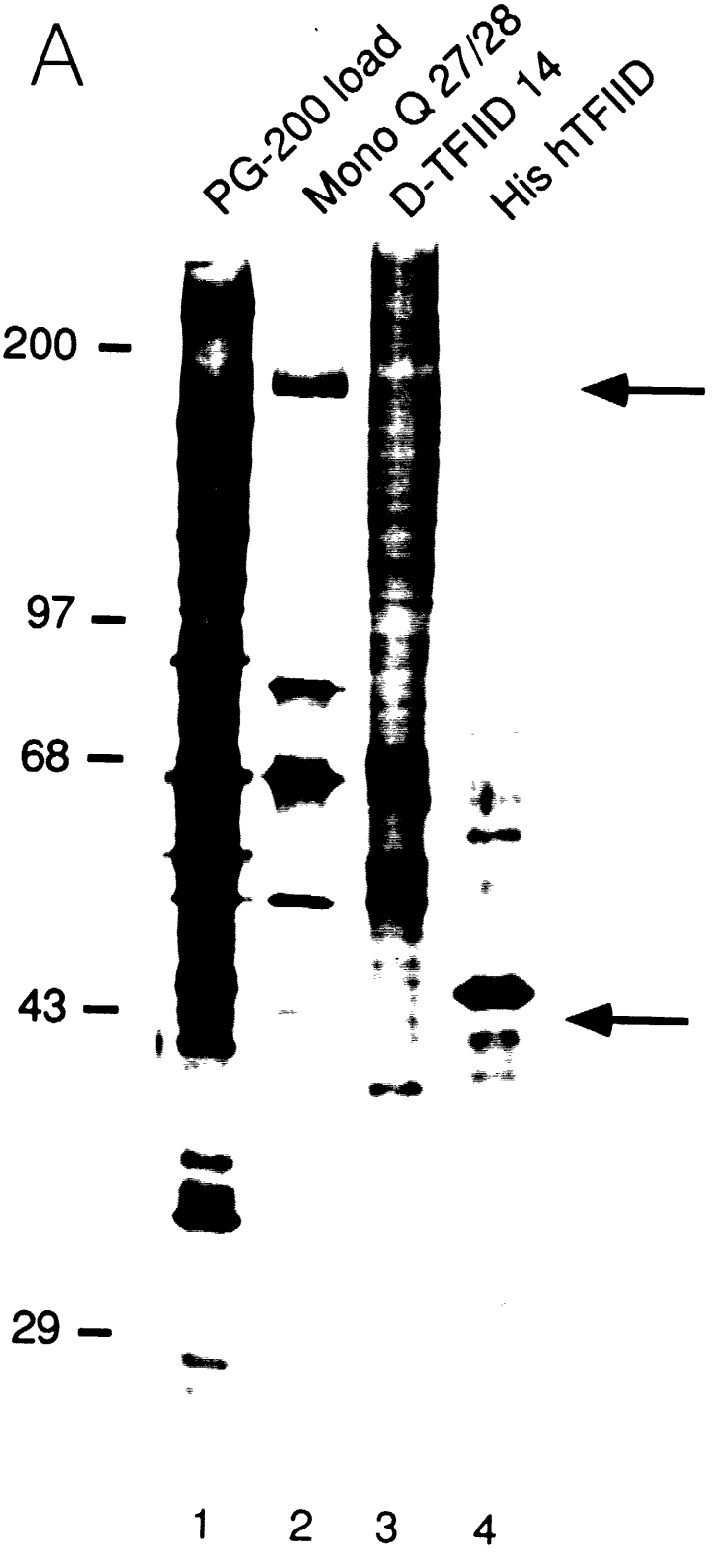

A
PG-200 load
Mono Q 27/28
D-TFIID 14
His hTFIID
200
97
68
43
29
1
2
3
4

Figure 4B. Immunoprecipitation across B-TFIID purification. Immunoprecipitations were performed using affinity-purified TFIID antibodies (+) (lanes 1, 3, 5, 7, 9, 11, and 13) or control 12CA5 monoclonal antibodies (-) (lanes 2, 4, 6, 8, 10, 12, and 14). The fractions used in the analysis are indicated above each lane: 100 ng his TBP, 480 µg P-Cell load, 75 µg Mono S load, 10 µg protein of the Superdex load, 1.4 µg of the Mono Q fractions 27/28, approximately 500 µg of D-TFIID containing fraction or 3 µg of HAP peak. Based on transcription activity about 5 fold more D-TFIID was used than B-TFIID. The arrows indicate the 43 kd TBP and the 170 kd component of B-TFIID. Bands migrating at about 50 kd represent both keratins and IgG heavy chains. The positions of protein markers are indicated to the left, by their molecular weight in kd. The large number of bands in the control precipitation in lane 6 must be due to either insufficient removal of the nonspecific proteins from the beads or to aggregation of protein during the IP procedure and, in either case, is an artifact of this lane.

B

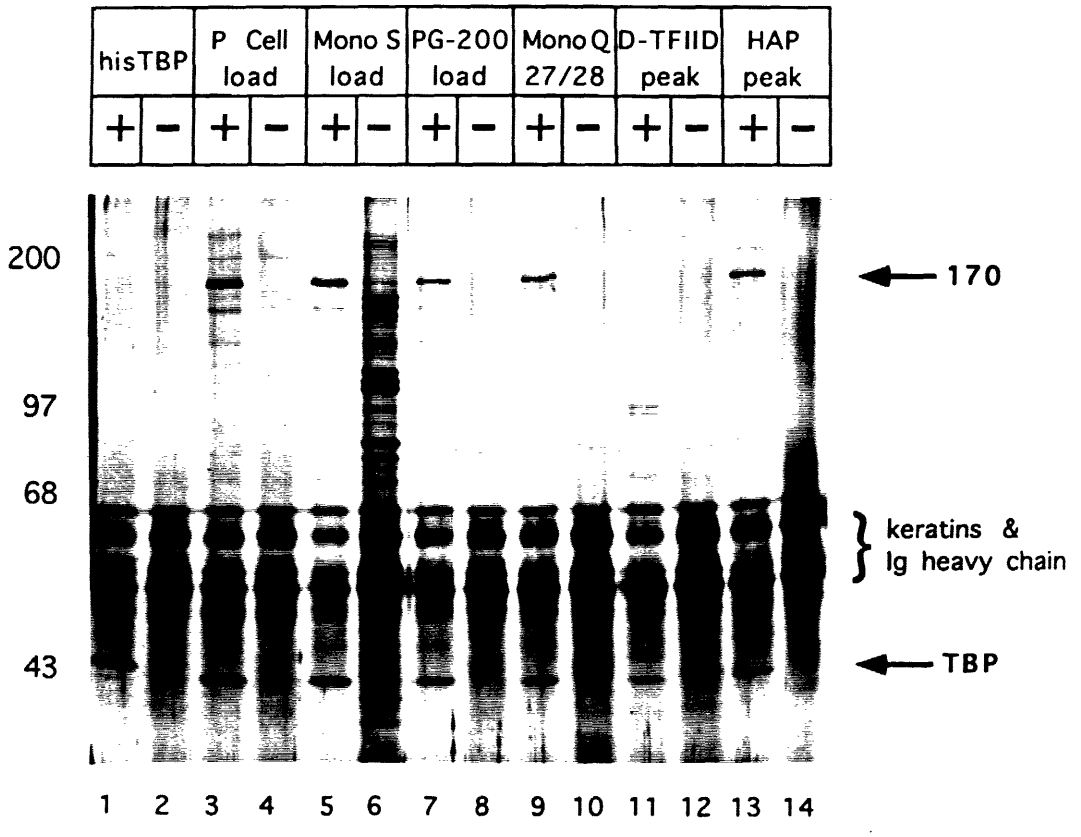
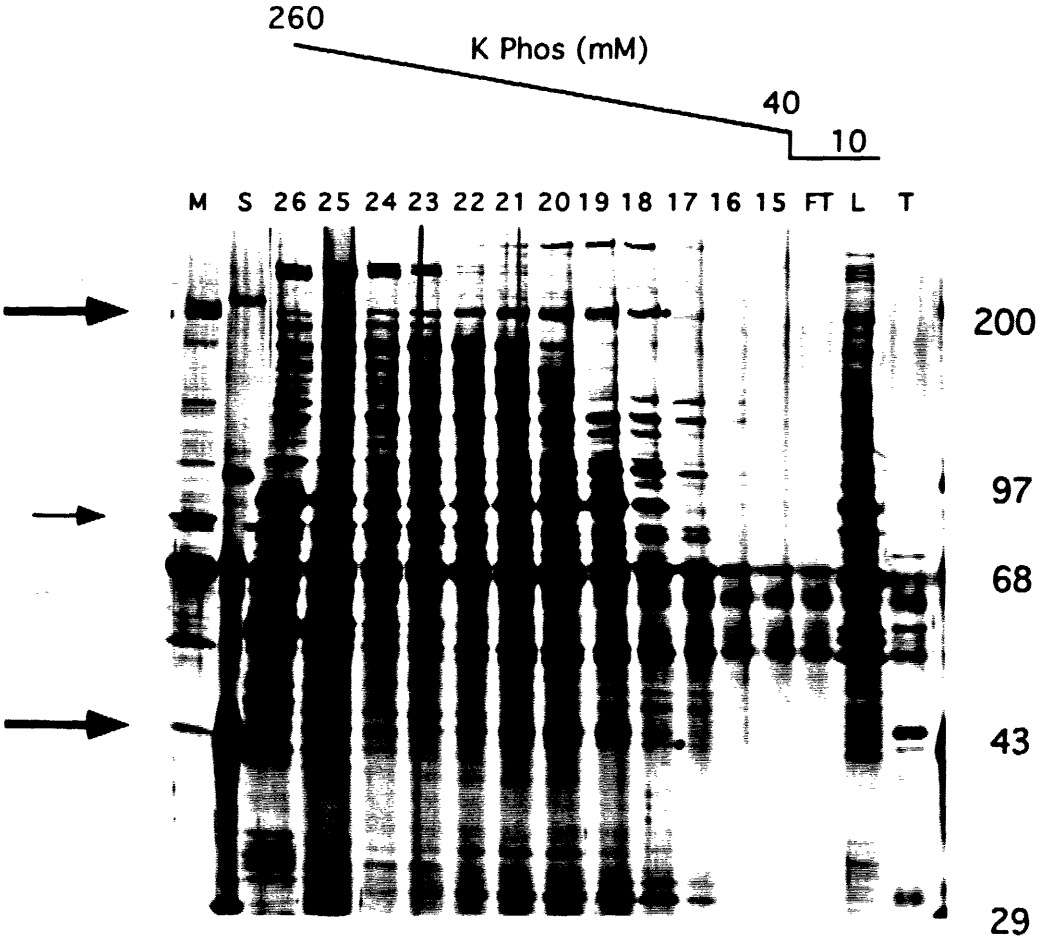

hisTBP
P Cell load
Mono S load
PG-200 load
Mono Q 27/28
D-TFIID peak
HAP peak
+ − + − + − + − + − + − + −
200
97
68
43
170
keratins & Ig heavy chain
TBP
1 2 3 4 5 6 7 8 9 10 11 12 13 14

Figure 4C. Silver stain analysis of HAP fractions. Forty µl of load (L) and 100 µl of flow through (FT) and indicated column fractions were microdialysed against buffer A plus 100 mM KCl and precipitated by adding 0.2 mg/ml insulin and 3 volumes acetone. Precipitated protein is resuspended in 40 µl SDS load buffer and subjected to PAGE on a 10 % gel. In addition to the indicated fractions the gel contains 50 ng TBP (T), 100 ng BRL mw standards (S), and 10 µl Mono Q fraction 28 (M).

C


260
K Phos (mM)
40
10
M S 26 25 24 23 22 21 20 19 18 17 16 15 FT L T
200
97
68
43
29

corresponded to the 170 kd polypeptide present in the B-TFIID complex. This argues against a simple precursor/product relationship between B-TFIID and D-TFIID complexes.

The B-TFIID cofactor was purified about 250 fold in the last four chromatographic steps (Table 1). The degree of purification was determined relative to the Q-Sepharose B-TFIID fraction, since transcription activity could only be detected in B-TFIID-containing fractions after this second column (see also Timmers and Sharp, 1991). Assuming complete recovery on the first two columns, the maximal purification of B-TFIID would be about 2600 fold. The recovery of B-TFIID activity during conventional chromatography was much higher than that for the fractionation of D-TFIID activity (Table 1; unpublished observations; (Nakajima et al., 1988; Maldonado et al., 1990). This suggests that the B-TFIID complex is stable and homogenous and that TBP and the 170 kd TAF are tightly associated.

An ATPase Activity Copurifies with the B-TFIID Complex.

In rat liver extracts a factor, d, has been identified, which is required for the basal transcription reaction and has an intrinsic (d)ATPase activity ((Conaway and Conaway, 1988; Conaway and Conaway, 1989)). Interestingly, this ATPase activity is dependent on double-stranded DNA and, at least initially, was most efficiently stimulated by a TATA box-containing sequence. Similarities in the molecular weight, chromatographic profile and basal transcription activity suggested that the B-TFIID factor might be the human equivalent of the rat d factor. The most highly purified B-TFIID preparations were tested for dATPase activity in the presence of template DNA. Indeed, a (d)ATPase activity coeluted with the B-TFIID factor (Figure 5A). Whereas hydrolysis of ATP and dATP were efficient, the other ribonucleotides were essentially not used as substrates (Fig.

Table 1. Purification of B-TFIID.

Transcriptional activity was determined in reconstituted transcription reactions dependent on TFIID using the core adenovirus major late promoter. Activity is given in arbitrary units.

[a] Purification was determined relative to the Q-Sepharose fraction.

[b] The peak fraction of the Superdex 200 PG column contained 66% of the eluted activity and 83% of this was used for subsequent purification.

Purification of B-TFIID

	Protein	Volume	Activity	Specific activity	Purification	Yield
	mg	*ml*	*units*	*units/mg protein*		*%*
Whole-cell extract	3600	280	-	-		
Phosphocellulose	1200	280	-	-		
Q-Sepharose FF [a]	300	125	137,000	460	1	100
Phosphocellulose	45	60	92,000	2,000	4.3	67
Mono S-FPLC	8.8	3.4	90,000	10,200	22	66
Superdex 200 PG	1.0	12	25,000	25,000	54	18
Mono Q-FPLC [b]	0.17	2.1	11,500	67,600	150	8.4
Mono Q-fxn 27/28	0.05	0.6	5,400	108,000	235	

5B). Thus, the phosphatase activity is specific for adenosine. These features are similar to those of the ATPase of δ (Conaway and Conaway, 1989). However, in contrast to phosphatase activity of δ, the dATPase activity of fractions containing the B-TFIID factor was inhibited two- to three-fold by the addition of DNA (Figure 5C). This inhibitory effect was not dependent on the presence of a TATA box sequence (compare black bars to striped bars). This lack of DNA dependence suggested that B-TFIID was not the HeLa equivalent of the rat liver δ factor. Furthermore, the Km for dATP of this (d)ATPase was about 80 μM (Figure 6 and Materials and Methods), which is about 4 fold higher than that for δ (Conaway and Conaway, 1989). More recently the mammalian homologue of δ has been identified in a number of labs and is now referred to as TFIIH. In addition to ATPase activity, it also contains a CTD kinase (Lu et al., 1992) .

Figure 5. An (d)ATPase activity cofractionates with the B-TFIID complex. (A) Analysis of dATPase activity of the different Mono Q fractions. The indicated fractions, (3 µl each), were assayed for dATPase activity in the presence of 250 ng pML(C_2AT)19Δ-51 plasmid. (B) Nucleotide specificity of the phosphatase activity. Mono Q fraction 27, (3 ml), was assayed for hydrolysis of the indicated nucleotide triphosphates in the presence of 150 ng pML(C_2AT)19Δ-51 plasmid. (C) Effect of DNA on the (d)ATPase activity. Two µl of Mono Q fraction 26 was assayed for dATPase activity in the presence of the increasing amounts of different DNA fragments. The filled bars represent reactions containing the 51-bp polylinker of pUC19 and the striped bars represent reactions containing a 135-bp fragment carrying the -53/+33 sequences of the major-late promoter cloned into pUC19. (When normalized for moles of DNA added, the two fragments show equivalent levels of inhibition).

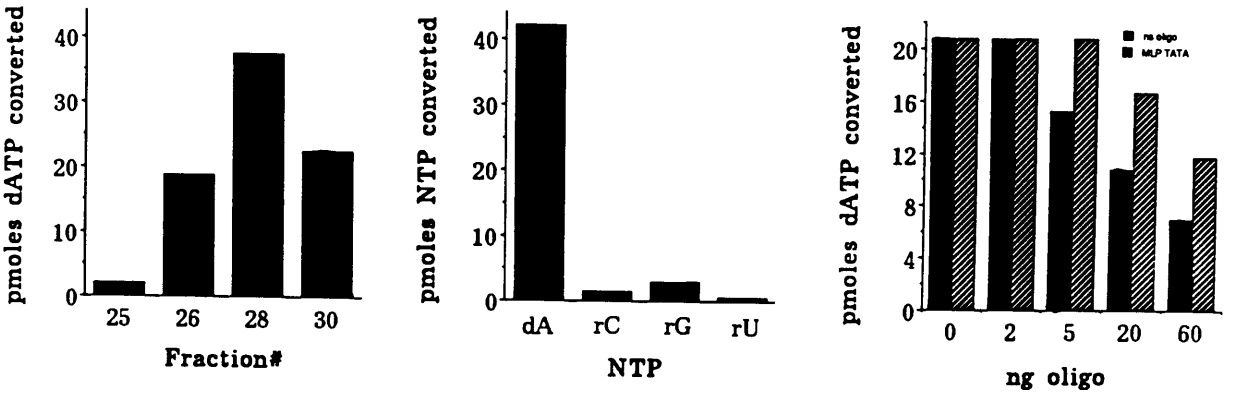
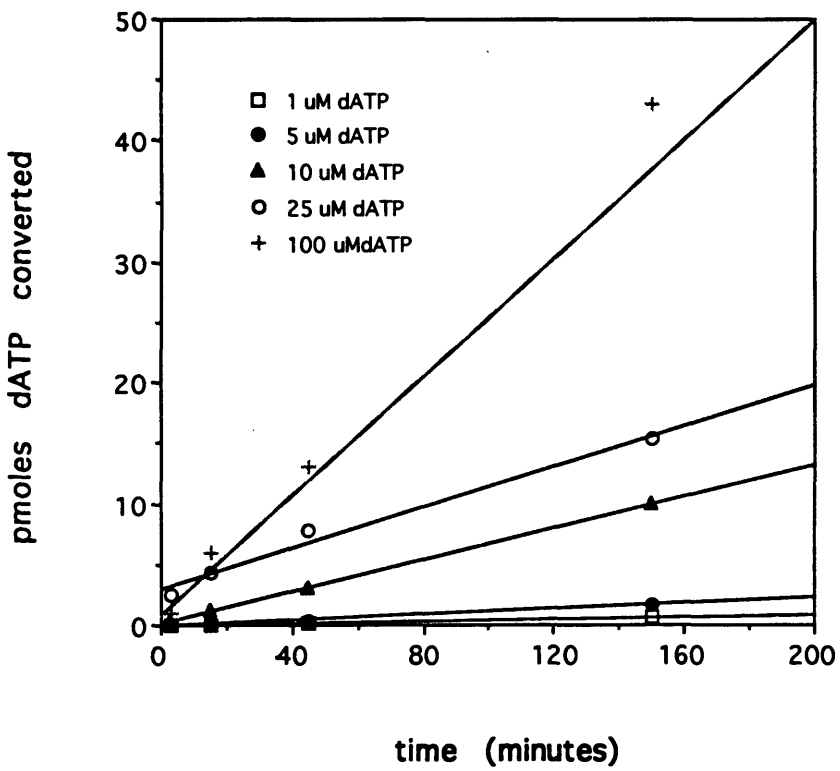

pmoles dATP converted
40
30
20
10
0
25
26
28
30
Fraction#
pmoles NTP converted
40
30
20
10
0
dA
rC
rG
rU
NTP
pmoles dATP converted
20
16
12
8
4
0
0
2
5
20
60
ng oligo

Figure 6. Rate curves for B-TFIID ATPase activity. Assays are performed as described in Materials and Methods at dATP concentrations of 1, 5, 10, 25, and 100 μM. Inset legend identifies the (substrate) corresponding to each line, and the solution for each curve is indicated to the right.

Rate curves for B-TFIID ATPase



y = 0.7742+ 0.281x R^2 = 0.99

y = 2.99 + 8.43e-2x R^2 = 0.98

y = 8.51e-2 + 6.60e-2x R^2 = 1.00

y = - 6.97e-2 + 1.16e-2x R^2 = 0.99

y = 1.19e-2 + 3.95e-3x R^2 = 0.99

Discussion

TBP is found in a variety of complexes in extracts of mammalian cells (reviewed (Pugh and Tjian, 1992; Sharp, 1992; Rigby, 1993; Hernandez, 1993). The predominant form of TBP-complex is the B-TFIID factor which is composed of a 170 kd polypeptide and the well characterized TBP. As noted previously, this complex is stable during purification and has a chromatographic profile on a gel filtration column consistent with a 300 kd factor (Timmers and Sharp, 1991). The discrepancy in molecular weight between the proposed 1:1 ratio of polypeptides and the gel filtration results may reflect an unusual shape for the complex.

The B-TFIID factor is both smaller and contains fewer subunit polypeptides than the D-TFIID factor (Timmers et al., 1992). The latter has a native molecular weight of approximately 700 kd and contains a set of associated polypeptides, TAFs, identified in these experiments as >200 kd, 97 kd, 95 kd, 70 kd, and 46 kd. This complex was not extensively characterized in this study and we assume that this subunit composition is equivalent to the previously described TFIID complex which contains eight TAFs (>200 kd, 150/125 kd, 110/95 kd, 78 kd, 70 kd, 50 kd, 40 kd and 30 kd (Tanese et al., 1991; Zhou et al., 1992). These studies suggest that the 170 kd TAF is unique to the B-TFIID complex and argue against a simple model in which the intact B-TFIID is the precursor of the D-TFIID factor. It remains possible that the B-TFIID complex is the precursor of the larger D-TFIID complex and that the 170 kd subunit is released upon formation of D-TFIID. We suggest that both the B-TFIID and D-TFIID complexes exist independently in the cell and are capable of supporting transcription by pol II (See chapter IV for a further discussion of these ideas).

A precedent for functionally and physically distinct TBP-TAF complexes comes from the analysis of the transcription factor SL1, which is specific for RNA

polymerase I. This factor is, in fact, a complex of TBP and three other proteins (110 kd, 63 kd, and 48 kd; (Comai et al., 1992). Analysis of RNA polymerase III transcription also suggests the involvement of TBP-TAF complexes as part of the TFIIIB factor (Lobo et al., 1991; Simmen et al., 1991; White et al., 1992). While it is likely that our crudest fractions (P-cell B) contained TFIIIB, its relatively low abundance and presumed removal in our next fractionation step (See chapter III) would have obscured its detection. Although TFIIIB has not been completely purified, most studies involving both biochemical fractionation and affinity purification have identified proteins with molecular weights significantly lower than 170 kd (Waldschmidt et al., 1988; Lobo et al., 1992). Further, complete characterization of the TFIIIB factor from yeast demonstrates that it contains proteins with molecular weights of < 100 kd (Bartholomew et al., 1991; Kassavetis et al., 1991). Thus, there is no evidence indicating that B-TFIID may be a transcription factor for polymerases other than RNA polymerase II. In fact, as indicated above, the observation that B-TFIID promotes initiation by polymerase II *in vitro* initially suggested an equivalent activity *in vivo*, though more recent experiments (see Chapter III), call this into question.

The B-TFIID factor is functionally distinct from the traditional D-TFIID factor (Timmers and Sharp, 1991). In contrast to transcription with the D-TFIID factor, reactions reconstituted with the B-TFIID factor do not respond to the transcriptional regulators SP1, GAL4-AH, Oct-1 or GAL4-TEF1 and only weakly to MLTF. It was also observed that the B-TFIID activity associates less stably with the adenovirus major-late promoter than both the D-TFIID factor and recombinant TBP (Timmers and Sharp, 1991). This latter finding was supported by the inability to detect specific B-TFIID/DNA complexes using highly purified fractions under a variety of gel mobility shift assay conditions and with different promoter fragments (data not shown). Obviously, these negative results might

reflect the limited range of promoters tested. Alternatively, it is possible that a partial function of the 170 kd TAF might be to prevent the stable association of the B-TFIID factor with the standard subgroup of promoters that are highly active *in vitro,* thereby redirecting the B-TFIID factor to a distinct class of core promoters or TATA elements. While this remains a formal possibility, experiments performed by Jeff Parvin, and described in Chapter IV, show that for a number of different promoters any differences in binding between TBP and B-TFIID are not reflected in differences in transcription activity.

The observation that an (d)ATPase activity is associated with the B-TFIID factor raises several interesting issues: Which of the two subunits is responsible for this (d)ATPase activity? Is such an activity associated with all TBP-TAF complexes? And how is this energy used in the transcription reaction? Experiments in our lab and others have failed to identify a (d)ATPase activity associated with TBP itself (unpublished observations), suggesting that the hydrolytic activity is contained within the 170 kd associated protein. Additionally, recent experiments of Taggert et al. have confirmed this by stating that highly purified p170 containing fractions, devoid of any detectable TBP, have an ATPase activity similar to that of B-TFIID (Taggert et al., 1992).

Early studies using crude transcription extracts showed that hydrolysis of the ß-γ bond of ATP or dATP is required for the initiation reaction (for review:(Sawadoga and Sentenac, 1990)). Although this (d)ATP requirement appears to be a late step in the formation of the initiation complex, it can not be attributed to the late-acting basal factors (like TFIIE and TFIIF) that have been purified to homogeneity and are now cloned. Therefore, the observation of a (d)ATPase activity associated with the B-TFIID factor raises the possibility that TBP-TAF complexes are responsible for the (d)ATP requiring step in the initiation of transcription.

Many labs have returned to the issue of the ATP hydrolysis requirement in transcription and several models have emerged. One model emerged from the observation that the recently identified basal transcription factor TFIIH is a pol II C-terminal domain (CTD) kinase (Feaver et al., 1991; Lu et al., 1992; Serizawa et al., 1992). This led to the suggestion that ATP hydrolysis was coupled to polymerase tail phosphorylation as a required step in the elongation reaction (Conaway and Conaway, 1993). This model was subsequently disproven in experiments demonstrating that the transcription reaction was refractory to kinase inhibiting drugs (Serizawa et al., 1993). As protein phosphorylation is an activity often associated with ATPases, the ability of B-TFIID to phosphorylate a number of substrates, including pol II CTD, TFIIA, B, E, F, and p170 itself, was tested. In no case was the transfer of phosphate to any added substrate observed.

A second model resulted from the cloning of the p89 subunit of TFIIH and the observation that it has an associated DNA helicase activity (Schaeffer et al., 1993). This led to the idea that ATP hydrolysis was required for the helicase activity necessary to unwind the DNA template and allow transcription. This model may indeed be true, but only under certain conditions. That is, it may be true that ATP hydrolysis is required for TFIIH mediated DNA unwinding, but not all transcription reactions require TFIIH, at least *in vitro*. It has been recently demonstrated that a minimal subset of basal transcription factors, excluding TFIIH, can function very efficiently in the initiation and elongation of transcripts from certain supercoiled DNA templates (Parvin et al., 1992; Parvin and Sharp, 1993; Tyree et al., 1993). For such reactions there is no observed ATP hydrolysis requirement (Jeff Parvin unpublished results, Marc Timmers submitted).

This still leaves open the possibility that B-TFIID functions in cells to open the DNA template, alter the conformation of a protein required for the initiation of transcription, or phosphorylate a novel substrate. The apparent DNA independence of our ATPase assay may simply reflect the conditions under which the experiment was performed.

Materials and Methods

Purification of B-TFIID.

Purification of the B-TFIID factor began with fractionation of 3.6 g of protein from HeLa whole cell extract (Manley et al., 1983) on a 500 ml phosphocellulose column (P11, Whatman) according to Samuels et al. (Samuels et al., 1982). The 350 mM KCl fraction B (1.2 g protein) was dialyzed against buffer A (20 mM Hepes-KOH pH 7.9, 20% glycerol, 1 mM EDTA, 1 mM DTT, 1 mM PMSF) plus 50 mM KCl and applied to a 190 ml Q-Sepharose FF column (Pharmacia). The column was washed extensively and developed by step elution, first with buffer A plus 200 mM KCl and then with buffer A plus 1 M KCl. The majority of the TBP (as judged by immunoblot analysis) eluted in the 200 mM KCl step. This fraction (320 mg protein) was adjusted to buffer A plus 40 mM KCl and applied to a 45 ml phosphocellulose column. The column was developed with a linear gradient of 40-400 mM KCl in buffer A, and B-TFIID eluted at 160 mM. Following this step, all buffers contained 1 µg/ml aprotinin and 1 µg/ml pepstatin A (Sigma). B-TFIID containing fractions (45 mg protein) were pooled, adjusted to buffer A plus 50 mM KCl and loaded on a Mono S FPLC column (Pharmacia). This column was developed with a linear gradient of 50-500 mM KCl in buffer A and B-TFIID eluted at 150 mM KCl. The corresponding fractions (8.8 mg protein) were pooled, adjusted to buffer A plus 400 mM KCl plus 0.01% Triton X-100 and chromatographed on a Superdex-200 PG column (Pharmacia). One hundred µg/ml insulin (Boehringer) was added to the eluted fractions as a carrier. Two fractions corresponding to 300 kd contained B-TFIID. The peak fraction contained 66% of the eluted B-TFIID activity and was adjusted to buffer A plus 50 mM KCl and loaded on a Mono Q FPLC column (Pharmacia). This column was developed with a linear gradient of 50-300 mM KCl in buffer A.

B-TFIID eluted in a broad peak from 100 to 170 mM KCl. In an alternative final step, the minor fraction from the gel filtration column was applied to a 1.5-ml hydroxylapatite (BioRad) column after addition of 10 mM K-phosphate pH 7.6. The column was washed with 50 mM KPi (50 mM K-phosphate pH 7.6, 10% glycerol, 5 mM DTT) and developed with a linear gradient of 50-500 mM KPi.

Purification of Other Transcription Factors.

Purification of RNA polymerase II and the basal transcription factor-containing fractions was exactly as described (Samuels et al., 1982). The expression and purification of histidine-tagged human TBP from *Escherichia coli* strain BL21 was performed as described (Parvin et al., 1992).

In vitro Transcription Assay.

A standard TFIID-dependent transcription reaction contained, in addition to the TFIID fraction, 0.3 µl calf thymus RNA pol II, 0.5 µl (AB), 1.5 µl (CB), 12 units RNAsin (Promega) and 200 ng pML(C_2AT)19Δ-51 (Sawadogo and Roeder, 1985; Buratowski et al., 1988) in a 20 µl volume. Incubation conditions were as described (Sawadogo and Roeder, 1985; Buratowski et al., 1988) with the addition of 0.1 mg/ml bovine serum albumin (Sigma) to stabilize proteins. RNA was extracted, processed and quantitated as described (Timmers and Sharp, 1991).

Protein Analysis.

Immunoblots were prepared and developed with anti-human TBP rabbit antiserum as described (Timmers and Sharp, 1991). For immunoprecipitation analysis, rabbit polyclonal anti-TBP sera were purified by affinity chromatography, according to Harlow and Lane (Harlow and Lane, 1988). This

involved coupling bacterially expressed his-tagged human TBP to cyanogen bromide-activated Sepharose (Pharmacia) at 3 mg protein/ml matrix, as recommended by the manufacturer. The affinity-purified anti-TBP and control 12CA5 antibodies (Niman et al., 1983) were cross-linked to protein A-Sepharose beads (Pharmacia) using dimethylpimelimidate (Harlow and Lane, 1988) and blocked prior to immunoprecipitation in incubation buffer (buffer A, 350 mM KCl, 0.1% NP-40, 1 μg/ml pepstatin A, 1 μg/ml leupeptin) plus 0.5 mg/ml insulin (Boehringer). The D-TFIID sample was preabsorbed using protein A-Sepharose to remove BSA from this fraction. All protein fractions were adjusted to the incubation buffer and incubated with the cross-linked antibodies for 3 hr at 4°C. The antigen-antibody complexes were washed four times with the incubation buffer and bound proteins were eluted by boiling in SDS sample buffer. The polypeptides were analyzed on a 10% polyacrylamide/SDS gel and detected by silver staining according to Blum et al. (Blum et al., 1987). Protein concentrations were determined using the BioRad Protein Assay with bovine gamma-globulin (Sigma) as the standard.

ATPase Assay.

ATPase activity was assayed by incubation of the indicated protein fractions (adjusted to buffer A plus 100 mM KCl) in a volume of 10-15 μl at 25 °C for 4.5 hr. Reactions contained 40 mM Tris-HCl pH 7.9, 50 mM KCl, 7 mM $MgCl_2$, 2 mM DTT, 0.1 mM EDTA, 0.5 mg/ml BSA, 8-10 μM α^{32}P-(d)NTP (25 Ci/mmol). DNA was included in the reactions where indicated. Reaction products were analyzed by thin-layer chromatography on polyethylenimine plates and developed with 1.5 M LiCl or 0.5 M LiCl plus 1 M HCO_2Na. Reaction products were quantitated using a Phosphorimager (Molecular Dynamics) and ImageQuant 3.0 software. For determination of Km, ATPase assays were

performed in a time course experiment at each of five substrate (dATP) concentrations. At each time point, 3 µl of reaction mixture was removed and reactions were terminated by adding EDTA to 50 mM and storing at 4°C. Data is plotted in Fig. 5 using Cricket Graph and the slope of each line is used to generate a rate at each substrate concentration tested. Rate values are plotted vs. substrate concentration using a modified Lineweaver-Burke plot developed in Joanne Stubbe's lab (personal communication) and Km values are obtained. Analysis of the data suggests that this value is accurate to within a factor of two.

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Chapter III. TBP-TAFs in pol III transcription, is B-TFIID involved?

Abstract

Transcription by RNA polymerases I, II and III requires the TATA-binding protein (TBP). This protein functions in association with distinct TBP-associated factors (TAFs) which may specify the nature of the polymerase selected for initiation at a promoter site. In the polymerase III (pol III) transcription system, the TBP-TAF complex is a component of the TFIIIB factor. This factor has been resolved into a TBP-TAF complex and another component both of which are required for reconstitution of transcription by pol III. Neither the TBP-TAF complexes B-TFIID and D-TFIID which were previously characterized as active for polymerase II transcription, nor TBP alone, can complement pol III transcription reactions that are dependent upon the TBP-TAF subcomponent of TFIIIB. Surprisingly, the TBP-TAF subcomponent of TFIIIB is active in reconstitution of polymerase II transcription.

Introduction

The mechanism of transcription in eukaryotes is a complicated process involving the interaction of a large number of protein factors (for review, see Sawadoga and Sentenac, 1990; Geiduschek and Kassavetis, 1992; Conaway and Conaway, 1993). Early results suggested that distinct sets of factors were required for the initiation of transcription by RNA polymerases (pols) I, II, and III. This view has been modified by the discovery that the three RNA polymerases share some common subunits (Woychik et al., 1990; Carles et al., 1991; Young, 1991). The original model was further refined with the discovery that another protein factor, the TATA-binding protein (TBP), is required for transcription by all three polymerases (Lobo et al., 1991; Margottin et al., 1991; Comai et al., 1992; Cormack and Struhl, 1992; Schultz et al., 1992; Simmen et al., 1992; White and Jackson, 1992). The obvious question of how the cell orchestrates the function of a single polypeptide in reactions involving three distinct polymerases, at distinct promoters, remains to be answered.

TBP was originally identified as a component of TFIID (D-TFIID), an activity required for pol II transcription from TATA box-containing promoters (Matsui et al., 1980; Samuels et al., 1982; Davison et al., 1983; Reinberg et al., 1987). It is distinguished from the large TBP-TAF complex D-TFIID, in that reactions reconstituted with TBP are refractory to stimulation by upstream activators while similar reactions reconstituted with D-TFIID are responsive (Hoey et al., 1990; Kambadur et al., 1990; Peterson et al., 1990; Pugh and Tjian, 1990; Meisterernst et al., 1991). This led to the suggestion that TAFs were functioning, at least in part, to bridge the basal transcription machinery to the upstream activator. Our studies have focused on the TBP-TAF complex B-TFIID,

which is composed of TBP and a polypeptide of 170 kd (See chapter II) (Timmers et al., 1992). The B-TFIID complex is similar to TBP in that reactions reconstituted with it are refractory to stimulation by upstream activators (Timmers and Sharp, 1991), suggesting that all TAFs do not function as mediators of transcriptional activation.

TBP-TAF complexes are also required for transcription from pol II promoters lacking a TATA box (Pugh and Tjian, 1990; Pugh and Tjian, 1991; Zhou et al., 1992). This suggests that TAFs might target TBP to the DNA and thus nucleate the assembly of the initiation complex (i.e. pol II and factors TFIIA-TFIIJ) (Pugh and Tjian, 1992; Sharp, 1992). The model of TAF function in promoter recognition was bolstered by the discovery that TBP-TAF complexes also play a critical role in the initiation of transcription by RNA pol I and pol III. Reconstitution of transcription by pol I *in vitro* is dependent upon the TBP-TAF complex SL1 (Comai et al., 1992; Jantzen et al., 1992). Genetic experiments in yeast have revealed that TBP is also critical for transcription from pol III promoters (Cormack and Struhl, 1992; Schultz et al., 1992) (Buratowski and Zhou, 1992; Colbert and Hahn, 1992). In mammalian extracts *in vitro*, TBP is required for transcription from the TATA box-containing pol III promoters of U6 snRNA genes (Lobo et al., 1991; Margottin et al., 1991; Simmen et al., 1992) and the TATA-less promoters of tRNA and VA RNA genes (Kassavetis et al., 1989; Lobo et al., 1992; Taggert et al., 1992; White and Jackson, 1992; White et al., 1992).

Fractionation of mammalian and yeast extracts led to the identification of three activities, TFIIIC, TFIIIB, and RNA polymerase III, which are required to reconstitute pol III transcription *in vitro* (Segall et al., 1980; Shastry et al., 1982). For the promoters of tRNA and VA RNA genes, TFIIIC binds first and nucleates the association of TFIIIB followed by RNA pol III (Lassar et al., 1983;

Braun et al., 1989; Gabrielson et al., 1989; Kassavetis et al., 1989; Kassavetis et al., 1990; Bartholomew et al., 1991; Geiduschek and Kassavetis, 1992; Bartholomew et al., 1993). Although the polypeptide composition of these factors in yeast and other organisms has been well established (Ginsberg et al., 1984; Bartholomew et al., 1990; Kassavetis et al., 1991; Kassavetis et al., 1992), their mammalian counterparts remain to be completely elucidated. Significant progress has been made in determining the polypeptide composition of the mammalian TFIIIC factor (Dean and Berk, 1987; Yoshinaga et al., 1987; Cromlish and Roeder, 1989; Yoshinaga et al., 1989). Additionally, several groups have identified polypeptides that are potential components of mammalian TFIIIB (Waldschmidt et al., 1988; Lobo et al., 1992; Taggert et al., 1992). In particular, Taggert et al. have suggested that in addition to TBP, and other loosely associated proteins, mammalian TFIIIB contains a 172 kd TAF (Taggert et al., 1992).

Since both B-TFIID and TFIIIB cofractionate during chromatography on a phosphocellulose matrix, and a polypeptide of approximately 170 kd has been suggested to be a component of both factors, it was conceivable that B-TFIID might function in pol III transcription as a component of TFIIIB. The confusion in the field as to the relationship between TFIIIB and B-TFIID has been discussed in a recent review by Rigby (Rigby, 1993). To clarify this confusion we have used a combination of conventional and immunoaffinity chromatography to resolve the TBP-containing component of TFIIIB from the previously identified B-TFIID factor.

Results

TBP from the Phosphocellulose B fraction resolves into distinct complexes.

The biochemical dissection of the factors important for transcription by pol II and pol III began with the fractionation of HeLa whole cell extracts (WCEs) and nuclear extracts by chromatography on a phosphocellulose (P-cell) column (Matsui et al., 1980; Samuels et al., 1982). Each of these P-cell fractions is known to contain a number of factors required for transcription by pol I, II, and III. In particular, the P-cell B fraction contains B-TFIID and a number of other basal transcription factors including TFIIIB, RNA polymerase III, and RNA polymerase II (Matsui et al., 1980; Segall et al., 1980). This and other cofractionations have led to the suggestion that the B-TFIID factor is a component of the TFIIIB activity (Simmen et al., 1991; Lobo et al., 1992; Taggert et al., 1992; Rigby, 1993).

The various TBP complexes and biochemical activities in the P-cell B fraction were separable by further fractionation on a Q Sepharose (QS) FF column. The B fraction was loaded at 50 mM KCl and eluted with sequential steps of 200 mM KCl (QS 200) and 1 M KCl (QS 1000), as shown in fig. 1. Western blot analysis using TBP polyclonal antisera showed that the majority (>80%) of TBP eluted in the 200 mM step with small amounts (<10-15%) in the 1 M fraction and even less in the 50 mM (QS 50) flow through (Fig. 2 and (Timmers and Sharp, 1991). The B-TFIID complex was previously purified from this QS 200 fraction (See chapter II (Timmers and Sharp, 1991; Timmers et al., 1992). Having identified TBP in more than one fraction from the QS FF column, and knowing that TFIIIB

Figure 1. Fractionation scheme for B-TFIID, TFIIIB, and the other pol III factors from HeLa whole cell extracts. Horizontal lines represent step elutions with the numbers indicating the molarity of KCl; diagonal lines represent gradient elutions. The C fraction contains TFIIIC and RNA polymerase III.

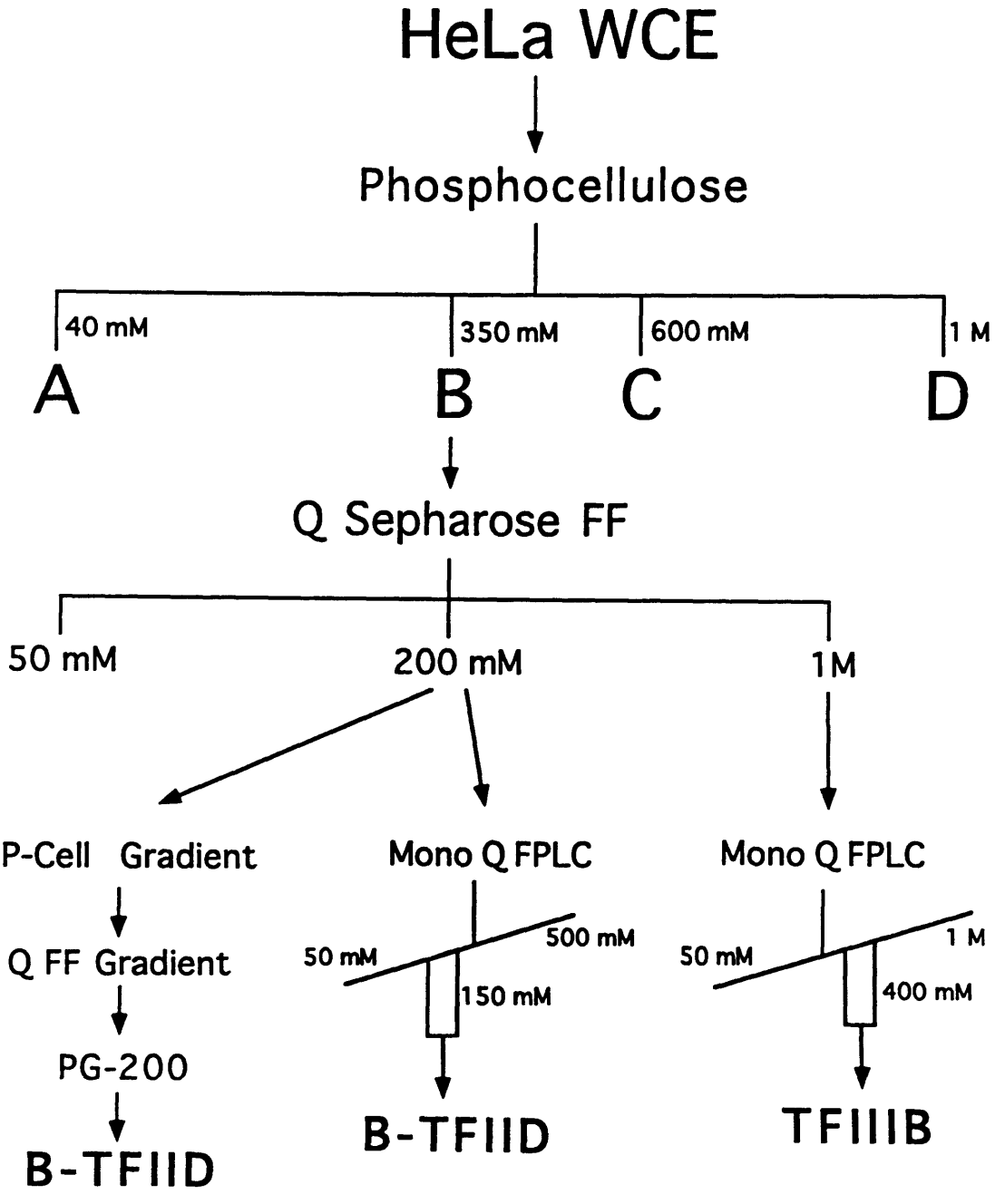
HeLa WCE
Phosphocellulose
40 mM
350 mM
600 mM
1 M
A
B
C
D
Q Sepharose FF
50 mM
200 mM
1M
P-Cell Gradient
Q FF Gradient
PG-200
B-TFIID
Mono Q FPLC
50 mM
500 mM
150 mM
B-TFIID
Mono Q FPLC
50 mM
1 M
400 mM
TFIIIB

Figure 2. Immunoblot analysis of the QS FF fractions. Ten microliters (µl) and 40 µl of load (lanes 1 and 2 respectively) and QS 50 flow through (lanes 3 and 4 respectively), and 4 µl and 16 µl of QS 200 (lanes 5 and 6 respectively) and QS 1000 (lanes 7 and 8 respectively) were resolved on a 10 % polyacrylamide SDS gel, transferred to nitrocellulose, and analyzed with rabbit antisera specific for TBP. The band corresponding to TBP is indicated by the arrow. The slower mobility bands are non-specific proteins that were recognized by the preimmune serum (data not shown).


Load
50mM
200mM
1M
1 2 3 4 5 6 7 8

is present in the starting P-cell B fraction, it was of interest to determine which of these fractions contained TFIIIB activity. To do so, these QS FF fractions were tested in a pol III transcription assay.

Pol III transcription reactions can be reconstituted with the P-cell B fraction, which supplies TFIIIB and some RNA polymerase III, and the P-cell C fraction, (350-600 mM KCl step), which supplies TFIIIC and the majority of the RNA pol III (Segall et al., 1980; Shastry et al., 1982). Using a DNA template encoding the adenovirus VA1 gene and the P-cell B and C fractions, pol III transcription was reconstituted. This transcription was promoter specific and sensitive to high concentrations of α-amanitin (Fig. 3A). Fractions from the Q Sepharose FF column were tested for their ability to substitute for the P-cell B fraction in the above assay. Surprisingly, complementing activity was observed in both the QS 50 flow through and the QS 1000 fractions but not in the QS 200 fraction, where most of the TBP eluted (Fig 3B lanes 4-6, fig 3C lanes 3-6 and 12). The activity in the QS 50 flow through fraction has not been further analyzed and may be aggregated components*, while the activity in the QS 1000 fraction contains the previously described TFIIIB factor.

Mixing experiments proved that the lack of TFIIIB activity in the QS 200 fraction was not due to the presence of non-specific inhibitors of transcription (Fig 3C). Adding the QS 200 fraction to reactions reconstituted with either the QS 50 fraction (lanes 7 and 8) or the QS 1000 fraction (lanes 13 and 14) did not result in inhibition except at very high concentrations of QS 200 (lanes 8 and 14). Furthermore, addition of more purified forms of B-TFIID to this assay

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*A similar fractionation of the P-cell B fraction over QS FF loaded at a lower protein concentration yielded < 5 % of the pol III transcription activity in the QS 50 and > 90 % in the QS 1000.

demonstrated that the B-TFIID present in the QS 200 fraction was not specifically inhibiting pol III transcription (lanes 9-11 and 15-17). Thus it is likely that TFIIIB activity did not elute in all the Q Sepharose fractions, but is specifically in the QS 1000 fraction. These initial results suggested that B-TFIID and TFIIIB were indeed distinct activities. As several labs have also shown that TFIIIB activity elutes at high salt from anion exchange columns (Lobo et al., 1991; Simmen et al., 1991; White et al., 1992), the complementing activity in the QS 1000 fraction was further analyzed. To do so, the QS 1000 fraction was purified over a second column and the elution profile of TBP contained within was compared to that of TBP from the QS 200 fraction that was similarly chromatographed.

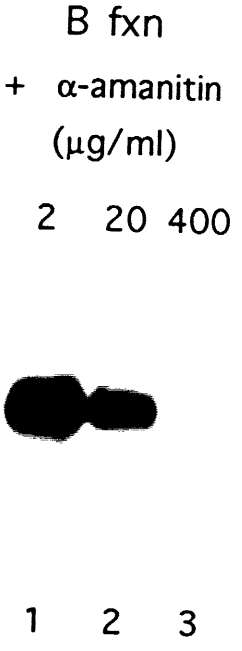
The QS 200 fraction was chromatographed on a Mono Q FPLC column developed with a linear gradient from 50-500 mM KCl. Fractions from this column were analyzed by Western blot and TBP was detected in fractions 16-19, eluting at ~150 mM KCl (Fig 4A). These results are consistent with our previous purification of B-TFIID (See chapter II and (Lobo et al., 1991; Simmen et al., 1991; White et al., 1992; Timmers, 1992) and with the results from a similar chromatography (i.e. Q Sepharose FF gradient elution) described here (See Materials and Methods and Fig 1).

The nature of the TBP complex in the QS 1000 fraction was similarly analyzed by chromatography on a Mono Q FPLC column developed with a linear gradient from 50 mM -1M KCl. Fractions from this column were assayed by Western blot and TBP was detected in a single peak in fractions 23-25 corresponding to a KCl concentration of ~350 mM (Fig. 4B).

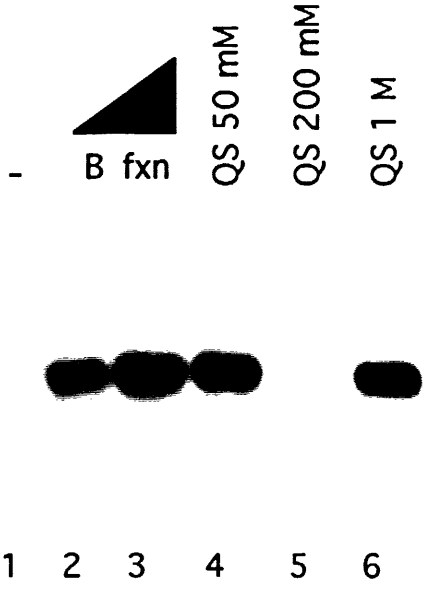
That the TBP-TAF complexes which originally separated on the Q Sepharose FF column, had different chromatographic properties on a

Figure 3. Reconstitution of pol III transcription. (A) Transcription is sensitive to high concentrations of a-amanitin. VA1 transcription is reconstituted by combining 4 µl of Q Sepharose load , (P-cell B fraction), and 3 µl of WCE-derived C fraction into reactions containing 2, 20, and 400 µg/ml α-amanitin. (B) Pol III transcription of QS FF fractions. Transcription was reconstituted by combining 1 or 4 µl of Q Sepharose load (P-cell B fraction); (lanes 2 and 3 respectively), 4 µl of QS 50 flow through (QS 50 mM; lane 4), 1 µl of QS 200 (lane 5), 1 µl of QS 1000 (lane 6), or Buffer A + 100 mM KCl (lane 1), to reactions containing 3 µl of WCE derived P-cell C fraction. (C) Neither QS 200 nor B-TFIID are inhibitors of pol III transcription. To reactions containing 3 µl of P-cell C fraction is first added 3 µl of P-cell B fraction (lane 2), 3 µl of QS 50 (lanes 3 and 6-11), 1 µl of QS 200 (lanes 4 and 5), or 1 µl of QS 1000 (lanes 12-17). In a second addition, reactions received 1 µl (lanes 7 and 13) or 4 µl (lanes 8 and 14) of QS 200, 4 µl of QG pool (lanes 9 and 15); or 0.75 µl (lanes 10 and 16) or 3 µl (lanes 11 and 17) of PG 200 fraction 15. All protein additions were performed on ice prior to the addition of NTPs and template. The second protein additions were normalized for TBP by quantitative western (data not shown). The 170 nt transcript generated by accurate initiation and termination is shown.

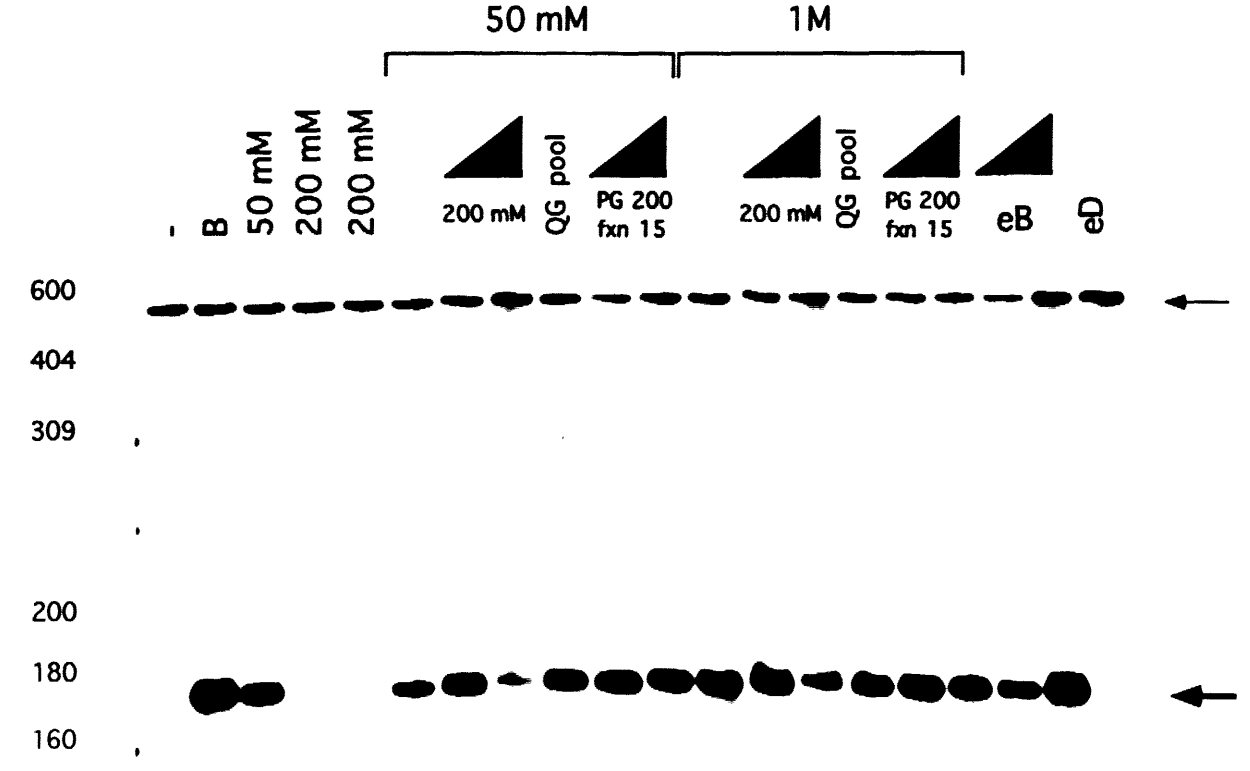
A


B fxn
+ α-amanitin
(μg/ml)
2 20 400
1 2 3

B


- B fxn QS 50 mM QS 200 mM QS 1 M
1 2 3 4 5 6

C


50 mM
1M
-
B
50 mM
200 mM
200 mM
200 mM
QG pool
PG 200 fxn 15
200 mM
QG pool
PG 200 fxn 15
eB
eD
600
404
309
200
180
160

second column further suggested that they are distinct complexes. The TBP in the QS 200 fraction is part of the TBP-TAF complex B-TFIID, and that in the QS 1000 fraction is probably bound by a different set of TAFs, as TBP alone would be expected to elute at a lower salt concentration on a Mono Q column (Hoey et al., 1990).

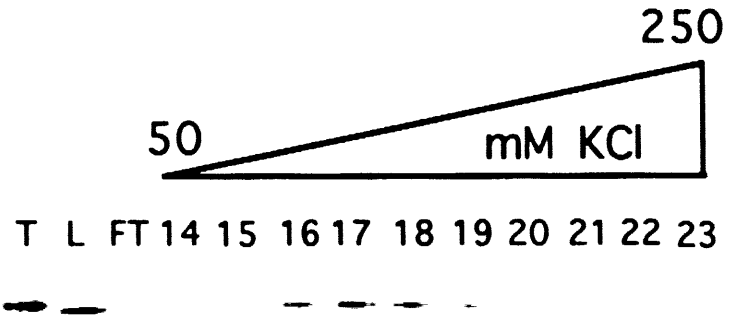
TFIIIB activity separates into two peaks over anion exchange.

The Mono Q fractions generated by chromatography of QS 1000 were assayed for TFIIIB activity by addition to a transcription reaction containing only the P-cell C fraction. TFIIIB activity was observed in fractions 24-26, eluting at ~ 400 mM KCl (Fig 5A), consistent with previous reports (Simmen et al., 1991; Lobo et al., 1992; White et al., 1992). Fractions 24-26 contained ~20% of the activity which was applied to the column (data not shown). Since Western blot analysis of these Mono Q fractions (Fig 4B) had identified TBP in fractions 23-25, the pol III complementing activity peak and the TBP peak were overlapping but non-identical. Specifically, the fraction containing the highest concentration of TBP (fraction 23) had no pol III complementing activity.

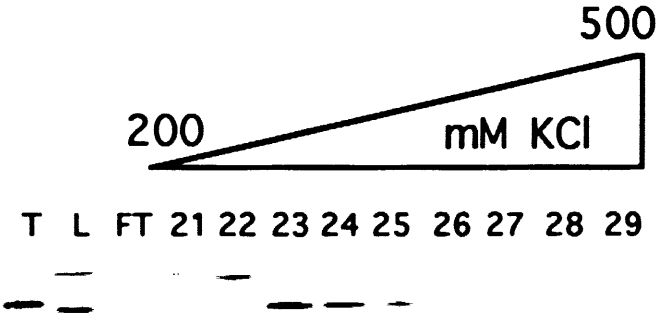
Previous experiments had suggested that TFIIIB could be resolved into a TBP-containing component and another component which does not contain TBP (Kassavetis et al., 1992; Lobo et al., 1992; Taggert et al., 1992). To test whether two overlapping activities were partially resolved by the Mono Q chromatography, fraction 23 was added to the remainder of the fractions from the Mono Q column. If the elution profile represents the splitting of two required activities, then the signal observed upon mixing fraction 23 with the later fractions should be enhanced. Indeed, both a stimulation of transcription in fractions 24-26 and an extension of the activity peak out to fraction 28 were observed in such mixing experiments (compare Fig 5A lanes 13-17 with Fig 5B

Figure 4. TBP from the phosphocellulose B fraction separates into distinct complexes. (A) Immunoblot analysis of Mono Q fractions from the chromatography of QS 200. Twenty µl of load (L) and flow through (FT), and 30 µl of fractions 14-23 were analyzed as in (Fig 2). Twenty ng of rTBP (T) was loaded as a positive control. The KCl concentration of each fraction is indicated above the lanes. (B) Immunoblot analysis of Mono Q fractions from the chromatography of QS 1000. Ten µl of load (L), flow through (FT), and fractions 21-29 were analyzed as in (A). Twenty ng of rTBP (T) was loaded and KCl concentrations are indicated as in (A). rTBP was extended with 6 histidines at its N-terminus and therefore has a slightly retarded mobility on SDS gels, as compared to the endogenous TBP.

A


250
50
mM KCl
T L FT 14 15 16 17 18 19 20 21 22 23

B


500
200
mM KCl
T L FT 21 22 23 24 25 26 27 28 29

lanes 1-5). The quantitation of the total activity in fractions 24-28 indicated that ~60% of the original activity had been recovered, instead of the 20% that had been previously observed. These results support the conclusion that there is an activity in fraction 23 that, in combination with a second activity in fractions 24-28, stimulates pol III transcription. The nature of the activity in fraction 23 was next explored.

B-TFIID cannot complement a TBP depleted P-cell B fraction to restore pol III transcription.

Although an activity that was associated with the TFIIIB factor cofractionated with TBP during chromatography on a Mono Q column, it was possible that TBP was not an integral component of the activity. To determine whether this coeluting TBP was required to restore pol III transcription, a pol III assay specifically depleted of an essential TBP component was developed. The P-cell B fraction was immunodepleted of TBP using anti-TBP rabbit polyclonal antibodies crosslinked to Protein A beads. As shown in figure 6, pol III transcription was almost completely abolished in reactions that were reconstituted with the TBP-depleted, but not the mock-depleted, B fraction (compare lanes 9 and 10). The slight amount of transcription retained in the TBP-depleted reactions was due to contaminating TBP from the P-cell C fraction and incomplete depletion of the P-cell B fraction.

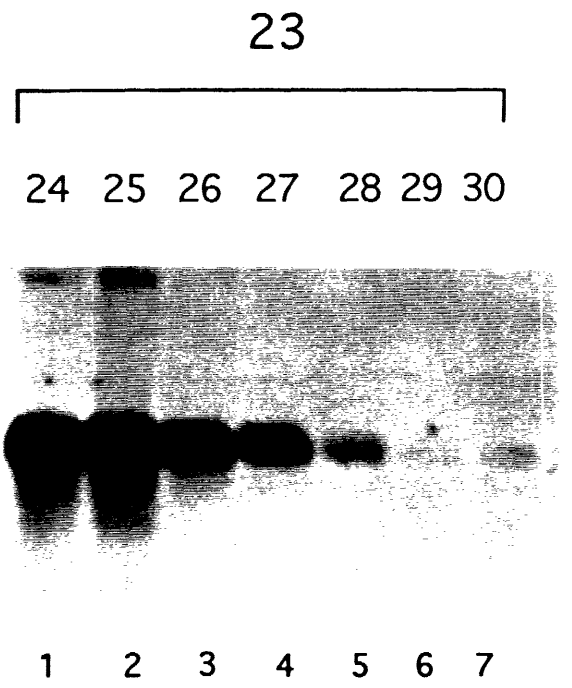
Various forms of TBP-containing complexes were added to reactions containing the P-cell C fraction and the TBP-depleted B fraction to test for their ability to restore pol III transcription. Only the TBP complex from the

Figure 5. Pol III transcription of Mono Q fractions from QS 1000.
(A) TFIIIB activity peaks at 400 mM KCl from a Mono Q column. Transcription reactions were reconstituted with 2 µl of the indicated fraction (lanes 2-22), or Buffer A + 100 mM KCl (lane 1), and 4 µl of the P-cell C fraction, as in Fig. 3. These fractions were eluted at [KCl] ranging from 50 mM, in the FT and in fractions 8-12, to a gradient from 200-700 mM, in fractions 19-33. (B) TBP-containing fractions stimulate a pol III assay. Reactions were reconstituted by combining 1 µl of Mono Q fraction 23, with 1 µl of Mono Q fractions 24-30 (lanes 1-7 respectively) and 4 µl of P-cell C fraction, as in (A). Transcripts were analyzed as in fig. 3.

A


- L FT 8 12 16 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22

B


23
24 25 26 27 28 29 30
1 2 3 4 5 6 7

Mono Q fractions of QS 1000 was able to restore pol III transcription (lanes 11-16). The peak of complementing activity followed exactly the peak of TBP as determined by Western blot analysis. Neither TBP alone, nor B-TFIID, at levels equal to or greater than those found in the Mono Q fractions, was able to restore pol III transcription in these reactions (lanes 11 and 12, respectively). Additionally, the TBP complex derived from the high salt (1M) fraction from a P-cell column, D-TFIID, was unable to supply a complementing activity in this assay (lane 13).

These transcription reactions required the P-cell C fraction (data not shown) which supplied both TFIIIC and RNA polymerase III, as well as two separable activities both of which could be supplied by the P-cell B fraction (compare lanes 1 and 2). Reactions containing only the P-cell C fraction and any of the TBP-containing fractions alone yielded no transcriptional activity (lanes 3-8). This suggested that the second activity required to reconstitute pol III transcription was not completely associated with TBP and was supplied by the TBP-depleted extract.

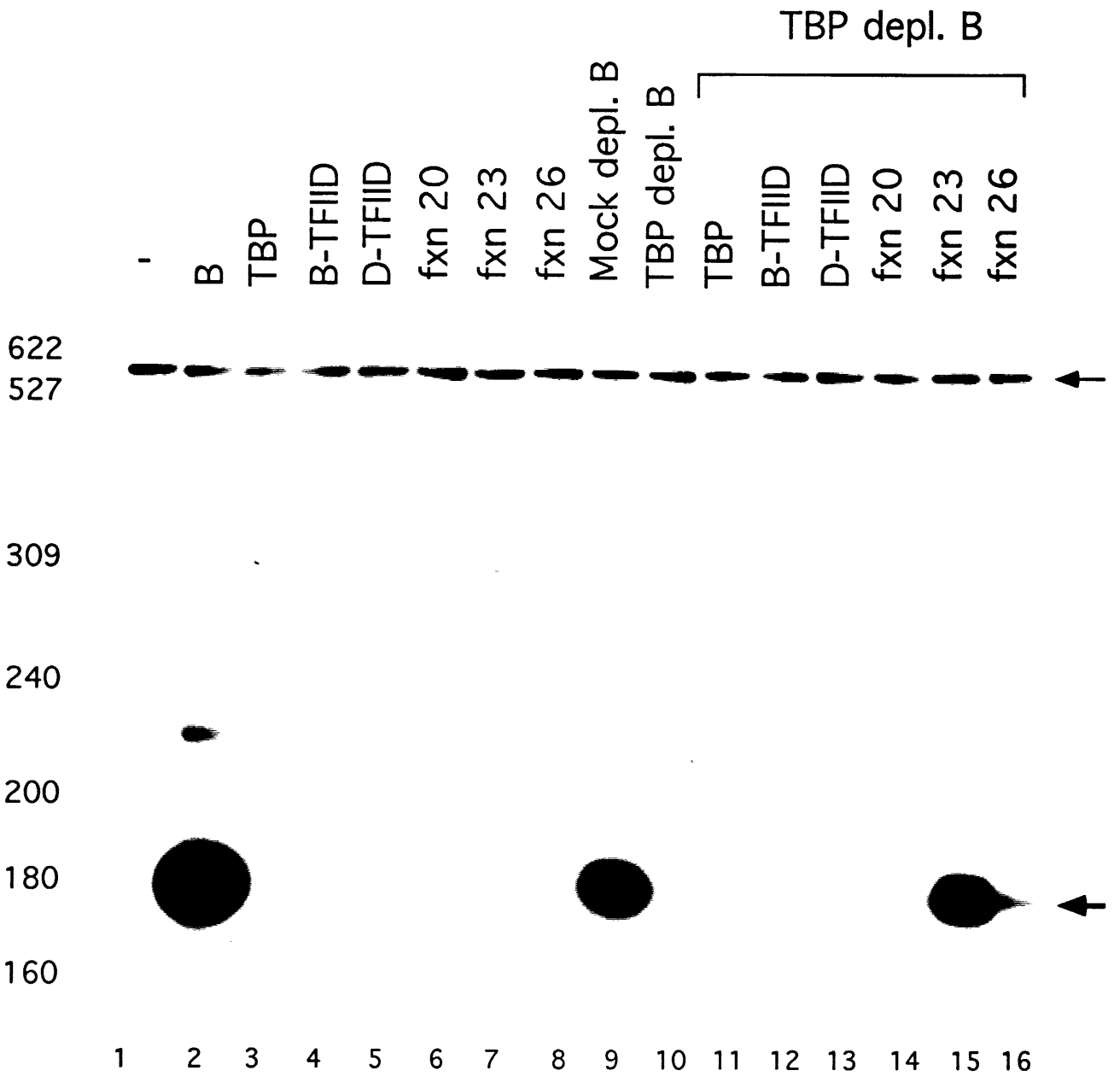
TBP-containing fractions function in a pol II assay.

Two TBP-TAF complexes, B-TFIID and D-TFIID, were unable to complement a pol III assay that was deficient in a TBP-associated activity. It was of interest to assay the activity of these complexes in pol II transcription and to test whether the TBP-TAF complex, which complements the pol III reaction, would also function in a pol II reaction. The basal pol II transcription reaction contained a DNA template with the minimal adenovirus major late promoter (MLP) fused to a G-less cassette (Sawadogo and Roeder, 1985), as well as recombinant TFIIB, recombinant TFIIE, a highly purified fraction containing HeLa TFIIF, TFIIH, and a small

Figure 6. TBP-depleted pol III transcription reactions can be reconstituted only with the TBP-TAF complex of TFIIIB. Reactions were reconstituted with 1 µl of nuclear extract-derived P-cell C fraction and the indicated protein fractions. Two µl of QS FF load (lane 2), 0.5 ng of rTBP (lanes 3 and 11), 1 µl of B-TFIID (lanes 4 and 12), 0.5 µl of D-TFIID (lanes 5 and 13), 0.05 µl of Mono Q fraction 20 (lanes 6 and 14), 0.05 µl of Mono Q fraction 23 (lanes 7 and 15), or 0.05 µl of Mono Q fraction 26 (lanes 8 and 16), were added to reactions that contained, in addition to P-cell C fraction, Buffer A + 100 mM KCl (lanes 1-8), 3 µl of mock-depleted B fraction (lane 9), or 3 µl of TBP-depleted B fraction (lanes 10-16). B-TFIID was from fraction 17 of the Mono Q chromatography of the QS 200 fraction and D-TFIID was from the P-cell D fraction. B-TFIID and D-TFIID contain ~0.5 ng/µl TBP as determined by quantitative Western blot (data not shown). Fractions 20, 23, and 26 were from Mono Q chromatography of the QS 1000 fraction. The positions of DNA markers are indicated to the left by their length in nucleotides. The thin arrow indicates the 550 nt recovery control RNA and the thick arrow indicates the 170 nt VA1 transcript.

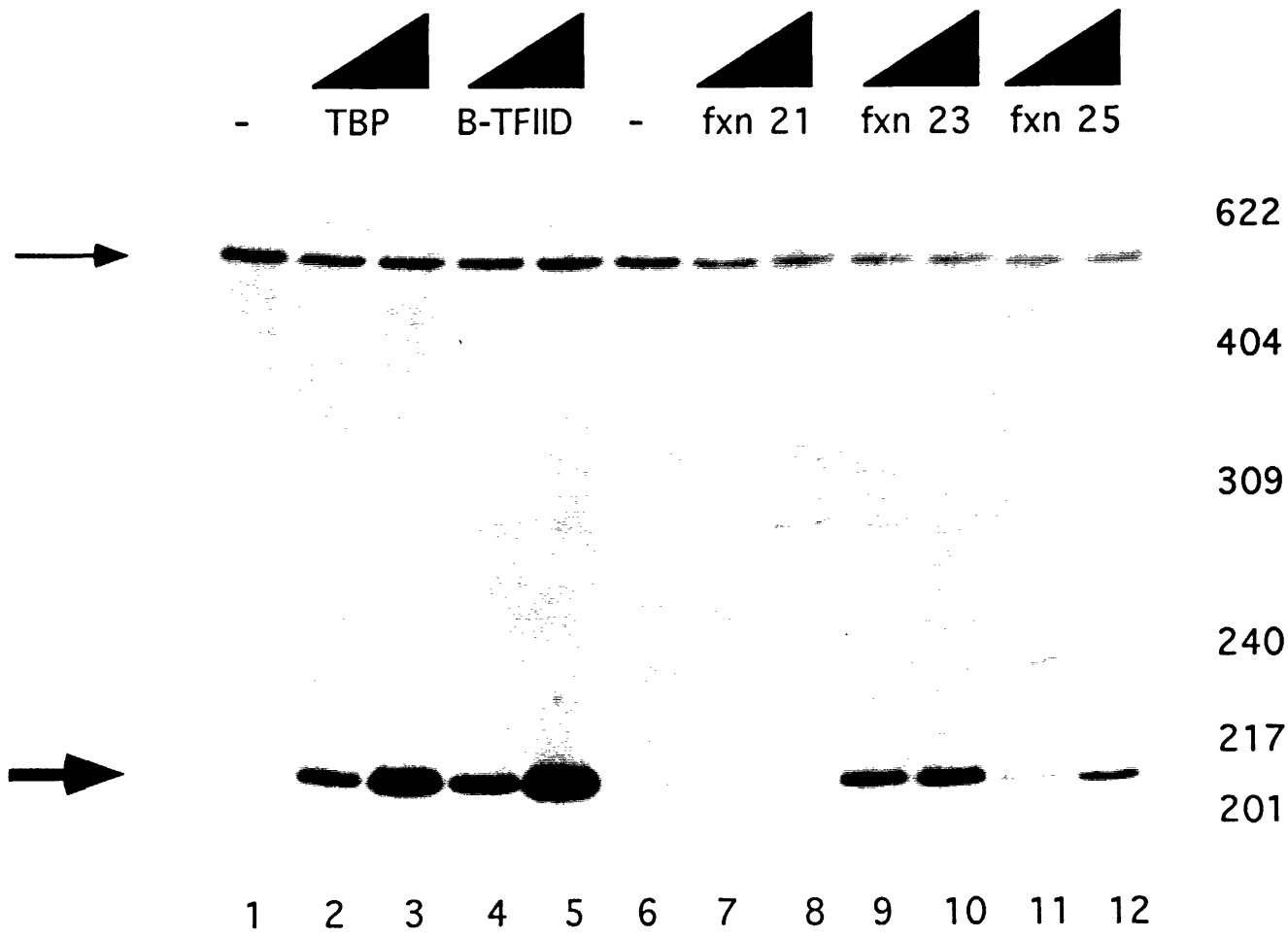
It should be noted that in the experiment shown in fig. 6, Mono Q fraction 26 did not generate a signal when assayed in the presence of the P-cell C fraction alone (lane 8). This is in contrast to the experiment shown in fig. 5A lane 15. The apparent discrepancy is explained by the fact that much lower concentrations of the Mono Q fractions were used in the experiments in fig. 6. The concentration of TBP in Mono Q fraction 23 is ~ 2.5 ng/µl and that in fraction 26 is ~0.4 ng/µl, as estimated by quantitative Western blot (data not shown). The experiments shown in figure 6 used 1/20 dilutions of these fractions, thereby adding 0.125 ng and 0.02 ng of TBP, respectively, to the assays containing fractions 23 and 26. The reduced level, 0.02 ng, of TBP-TAF

complex supplied by fraction 26 was not sufficient to restore pol III transcription activity in the presence of the P-cell C fraction alone.


TBP depl. B
-
B
TBP
B-TFIID
D-TFIID
fxn 20
fxn 23
fxn 26
Mock depl. B
TBP depl. B
TBP
B-TFIID
D-TFIID
fxn 20
fxn 23
fxn 26
622
527
309
240
200
180
160
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

amount of TFIIE, and highly purified RNA polymerase II. Reactions were reconstituted with the various TBP-containing fractions. As shown in figure 7, pol II transcription was restored by all forms of TBP assayed. In reactions normalized for the amount of TBP added, the level of transcription reconstituted with TBP or B-TFIID was almost identical (compare lanes 2 and 3 with lanes 4 and 5 and (Timmers and Sharp, 1991). Mono Q fractions containing the TBP-TAF component of TFIIIB from the QS 1000 fractionation were also able to restore pol II transcription (lanes 9-12), and the reconstitution activity strictly correlated with the presence of TBP as reactions reconstituted with Mono Q fraction 21 showed no pol II transcription (lanes 7 and 8) at a two fold lower efficiency per unit of TBP (lanes 7-12). Since the amount of TBP was normalized by quantitative Western blots and is only accurate to within a factor of 2, the decreased level of transcription observed with the TFIIIB fraction may be within experimental error. We conclude that fractions containing all three forms of TBP-TAF complexes are active in reconstitution of a pol II reaction containing purified basal transcription factors. This suggests that, for these reactions, TAF polypeptides do not interfere with promoter recognition by TBP within the complex and that the TBP-TAF complexes are able to associate with the pol II factors required to initiate transcription accurately.

Figure 7. TBP-containing fractions function in the pol II assay. The fractions indicated above each lane were titrated into a TBP-dependent transcription reaction using pΔML(200) as a template. The plasmid carries a 200 bp guanosine-less cassette under the control of the adeno core major late promoter (-53+10). Control reactions (lanes 1 and 6) received buffer A + 100 mM KCl, while experimental reactions received 2.5 or 7.5 ng of rTBP (lanes 2 and 3 respectively), 1 or 3 µl of B-TFIID (lanes 4 and 5 respectively), 1 or 3 µl of Mono Q fraction 21 (lanes 7 and 8 respectively), 1 or 3 µl of Mono Q fraction 23 (lanes 9 and 10 respectively), or, 1 or 3 µl of Mono Q fraction 25 (lanes 11 and 12 respectively). B-TFIID was from the Q Sepharose FF gradient chromatography (see Materials and Methods) and contains ~2.5 ng/µl TBP. Fractions 21, 23, and 25 were from the Mono Q chromatography of QS 1000. The position of DNA markers are indicated to the right by their length in nucleotides. The thin arrow indicates the 550 nt recovery control RNA as in figure 6, and the thick arrow indicates the correctly initiated 200 nt transcript from the G-less cassette.


-
TBP
B-TFIID
-
fxn 21
fxn 23
fxn 25
622
404
309
240
217
201
1
2
3
4
5
6
7
8
9
10
11
12

Discussion

The nature of the TAFs associated with TBP determines promoter specificity, as well as polymerase specificity, of the initiation complex. In the case of pol III genes, the TFIIIB complex, which binds upstream of the transcription start site and specifies the subsequent binding of pol III, almost certainly contains TBP. Since pol III promoters do not typically contain TATA sequences (Geiduschek and Kassavetis, 1992), the TAF polypeptides in this TBP complex must confer promoter specificity. Similarly, the TAF polypeptides in TFIIIB probably specify the selection of pol III over pol I or pol II. Interestingly, the TFIIIB factor is assembled on the DNA in a highly stable form by the action of the factor TFIIIC which recognizes sequences within the gene (Braun et al., 1989; Gabrielson et al., 1989; Kassavetis et al., 1989). After assembly, the TFIIIC factor can be stripped from the DNA and the TFIIIB complex will remain associated and direct transcription by pol III (Kassavetis et al., 1990). The only class of promoters where TBP alone is competent for both specific sequence recognition and polymerase selection are the TATA-containing promoters transcribed by pol II (Buratowski et al., 1988; Kao et al., 1990; Parvin et al., 1992). In this case, TBP directly binds to the promoter and interacts with TFIIB, which then specifies the association of pol II (Buratowski et al., 1989; Maldonado et al., 1990).

We have extended previous studies showing that the TFIIIB factor contains TBP. Additionally, we have demonstrated that this TBP represents a small fraction, 10-15%, of the total TBP in the P-cell B fraction and less than 10 % of the total TBP in extracts. Immunodepletion of a TFIIIB fraction with antisera specific for TBP removes activities critical for pol III transcription. Further, a TBP-TAF complex that has been resolved by chromatography over anion and

cation exchange resins will reconstitute the pol III reaction when added to TFIIIC, pol III, and the immunodepleted TFIIIB fraction. These results confirm previous chromatographic studies suggesting that TFIIIB can be separated into two mutually dependent factors, one containing TBP (Kassavetis et al., 1992; Lobo et al., 1992; Taggert et al., 1992).

Three TBP-TAF complexes have been tested for the ability to mediate initiation of pol III transcription: D-TFIID, B-TFIID, and the subcomponent of TFIIIB. Reassuringly, D-TFIID, B-TFIID, and TBP itself were not active in complementation of the pol III reaction. Only the TBP-TAF complex which cofractionated with the TFIIIB factor complemented this reaction. This suggests that the TAF polypeptide(s) in this complex were either directly or indirectly responsible for both sequence recognition and polymerase specificity.

The nature of the TAF polypeptides in TFIIIB has not been determined. Some experiments (Taggert et al., 1992), but not others (Waldschmidt et al., 1988; Lobo et al., 1992), have suggested that a TAF polypeptide in the TFIIIB factor has a molecular weight of 172 kd and may be identical to the TAF polypeptide, p170, in the more abundant B-TFIID complex (See chapter II (Timmers et al., 1992). However, we have clearly resolved the TBP-TAF complex responsible for pol III transcription from the previously characterized B-TFIID complex. Thus, the 170 kd TAF of B-TFIID is probably not a component of TFIIIB. In the experiments of Taggert et al., TFIIIB was isolated from the P-cell B fraction by direct immunoprecipitation using TBP antibodies (Taggert et al., 1992). We suggest that this immunoprecipitation resulted in the isolation of at least two TBP-TAF complexes, one that contained the p170 TAF of B-TFIID, and the other that yielded pol III transcription activity. Because B-TFIID is the most abundant TBP-TAF complex in the P-cell B fraction, the p170 TAF was the most abundant TAF identified by immunoprecipitation from the P-cell B fraction. It is

likely that other proteins in the precipitate, present at lower concentrations than p170 and therefore not observed by Taggert et al., are the true pol III TAFs. It is formally possible that the P-cell B fraction contains two distinct 170kd TAFs, one that is a component of B-TFIID, and the other that is a component of the pol III complex. However, the fact that the TAF 172 of Taggert et al. has DNA-independent ATPase activity, as was originally described for B-TFIID (See chapter II), is most consistent with the notion that the two large proteins are the same.

It is perhaps surprising that the TBP-TAF complex of TFIIIB is also active in reconstitution of transcription by pol II. This suggests that the surfaces of TBP necessary for interacting with both the TATA element and the TFIIB factor are accessible in the TFIIIB subcomponent. Alternatively, the TBP in the TFIIIB subcomponent could be released from the TAF polypeptides and thereby function in the pol II assay. This seems unlikely given the stability of the complex and the finding that per unit of TBP the TFIIIB subcomponent was almost as efficient as free TBP in restoring pol II transcription.

The activity of the TBP-TAF complex of TFIIIB in transcription reactions with pol II raises the possibility that the B-TFIID complex may not be a specific factor for pol II transcription. This TBP-p170 complex was originally identified as a factor which bound a TATA element with low affinity and facilitated initiation by pol II. Perhaps any TBP-TAF complex will have this activity. Thus, the abundant B-TFIID complex may be misnamed and could potentially be important for transcription of a subclass of pol I or pol III promoters. Alternatively, the original hypothesis may be correct and this complex could be specific for a subset of pol II genes. (See Chapter IV for a complete discussion of the models for B-TFIID function).

Materials and Methods

Plasmids and RNA

The VA1 template pVA1A, a gift from Noelle L'Etoile and Arnold Berk, contains a 250 bp Xba1-Ball fragment from the adenovirus type 2 genome map position 29.43% to 30.08%, cloned into pUC87. The MLP template pΔML(200) contains MLP sequences from -53-+10 fused to a G-less cassette, as described (Buratowski et al., 1988). Recovery control RNA was synthesized as a 550 nt run-off transcript by T7 RNA polymerase (Stratagene).

Purification of B-TFIID

HeLa WCEs were prepared and fractionated on P11 (Whatman) phosphocellulose columns as described (Matsui et al., 1980; Samuels et al., 1982; Manley et al., 1983). The P-cell B fractions (40-350 mM KCl step) from several WCE fractionations (3.2 gms protein) were adjusted to 50 mM KCl in Buffer A (20 mM HEPES-KOH pH 7.9, 2 mM DTT, 20 % glycerol, 1 mM EDTA, and 0.5 mM PMSF) and sequentially chromatographed over a Q Sepharose FF (Pharmacia) column and a phosphocellulose column, as previously described (Timmers and Sharp, 1991). Following this step, all buffers contained 1 μg/ml aprotinin and pepstatin A (Sigma). B-TFIID containing fractions from the P-cell gradient (160 mgs of protein) were pooled, dialyzed against buffer A + 50 mM KCl and loaded onto an 8 ml Q Sepharose FF column developed with a linear gradient of 50-250 mM KCl in Buffer A. B-TFIID eluted at approximately 130 mM KCl. The corresponding fractions were pooled (40 mgs protein, 23 mls) and precipitated with 6.7 grams of solid pulverized ammonium sulfate by mixing for 1 hour at 4 ^{0}C. The precipitated protein was recovered by ultracentrifugation at 30,000 rpm in a Vti 50 rotor (Beckman) for 20 minutes at 4 ^{0}C. The pelleted

protein (18 mgs) was resuspended in 5.6 mls of buffer A + 100 mM KCl + 0.01 % triton X-100 and adjusted to a conductivity equivalent to that of 400 mM KCl. Five mls (16 mgs) of protein was chromatographed on a Superdex 200 PG column (Pharmacia) as previously described (Timmers and Sharp, 1991; Timmers et al., 1992). B-TFIID eluted in fractions corresponding to ~300 kd.

In an alternative step, 1.2 mgs of protein from the 200 mM fraction of the first Q Sepharose FF column was adjusted to Buffer A + 50 mM KCl and chromatographed over a Mono Q FPLC column (Pharmacia). The column was developed with a linear gradient of 50-500 mM KCl in Buffer A.

Purification of other transcription factors

Recombinant human TBP was produced and purified as described (Parvin et al., 1992). Recombinant human TFIIB and recombinant human TFIIE were produced as described (Ha et al., 1991; Peterson et al., 1991; Parvin and Sharp, 1993). The RNA polymerase II used was a highly purified a-amanitin resistant form from the Ama 1 CHO cell line (Carthew et al., 1988). HeLa TFIIF, TFIIH, and low levels of TFIIE were contained within a highly purified fraction from a PG 200 gel filtration column as described (Parvin and Sharp, 1993). D-TFIID was prepared by adjusting 1 ml of protein from the P-cell D fraction to 80% saturation with solid pulverized ammonium sulfate by mixing for 1 hour at 4 ^{o}C. The precipitate was recovered by microcentrifugation for 15 minutes at 4 ^{o}C. The pelleted protein was resuspended in 150 µl of Buffer A + 100 mM KCl and dialyzed in the same. Pol III transcription factors were prepared by phosphocellulose chromatography of either HeLa whole cell (Manley et al., 1983) or nuclear (Dignam et al., 1983) extracts. The P-cell B fraction contained TFIIIB and some RNA polymerase III, and the P-cell C fraction contained TFIIIC and the majority of the RNA polymerase III. To prepare more highly purified

TFIIIB, the fractions eluted from the Q Sepharose FF column at 1 M KCl, as described above, were chromatographed over a Mono Q FPLC. Eighteen mgs of protein dialyzed into Buffer A + 50 mM KCl was loaded onto the Mono Q column and developed with a linear gradient of 50 mM - 1 M KCl in buffer A. Aliquots of each fraction were dialyzed against buffer A + 100 mM KCl for use in the assays described.

In vitro transcription

Pol II transcription reactions (20 µl) containing 12 mM HEPES (7.9), 0.6 mM EDTA, 60 mM KCl, 2.5 mM DTT, 12% glycerol, 5 mM $MgCl_2$, 60 µM ATP and UTP, 2.5 µM CTP, 40 µM 3'OMe GTP, 10 µCi [α^{32}P] CTP (800Ci/mmole; Dupont-NEN), 0.2 mg/ml BSA , 20 u rRNasin (Promega), 5 µg/ml pΔML(200) and basal transcription factors were assembled on ice. Each reaction contained 4 ng of rTFIIE, 35 ng rTFIIB, 1.5 µl TFIIF/E/H, and 0.5 µl RNA polymerase II. TBP containing fractions were titrated into each reaction as indicated. Reactions were incubated at 30^0 C for 60 minutes, terminated with 200 µl of stop mix containing 70 µg/ml t-RNA, 0.5% SDS, 1 M ammonium acetate, 10 mM Tris-HCl (7.9), 10 mM EDTA and ~500 cpms recovery control RNA, extracted once with phenol/chloroform (1:1), once with chloroform, and then precipitated with 2.5 volumes of ethanol. Products were resuspended in formamide dye mix and subjected to electrophoresis on an 8% polyacrylamide gel (19:1 acryl:bis), containing 8 M urea. Gels were directly exposed to film at -70^0 C for varying times.

Pol III transcription reactions (20 µl) containing 10 mM HEPES (7.9), 0.5 mM EDTA, 3 mM DTT, 10% glycerol, 4 mM $MgCl_2$, 500 µM ATP, GTP, and CTP, 10 µM UTP, 10 µCi[α^{32}P]UTP (3000Ci/mmole), 5 mM creatine phosphate, 85 mM KCl, 0.2 mg/ml BSA, 15 µg/ml poly(dG-dC)·poly(dG-dC), 20 U rRNasin, 15 µg/ml

pVA1A and protein factors were assembled on ice. All reactions contained 1-3 µl P-cell C fraction, from either WCE or nuclear extract, as indicated. Reactions were incubated at 25^0 C for 90 minutes, then terminated, processed, and analyzed as described for pol II reactions above.

Immunodepletion

Affinity purified TBP polyclonal antibodies and control 12CA5 antibodies (Niman et al., 1983) were crosslinked to Protein A Sepharose as previously described (Timmers et al., 1992). Beads were stored at 4 ^{0}C as a 10% slurry in PBS plus 0.01% thermisol (Sigma). Prior to use, beads were washed 3 times with Buffer A + 350 mM KCl and 0.1% NP-40. For TBP depletion of the P-cell B fraction, 100 µl of packed beads were transferred to a 0.1% NP-40 coated 500 µl eppendorf tube and mixed with 100 µl of the P-cell B fraction in buffer A + 100 mM KCl + 0.5 mM PMSF, and rotated end-over-end for 15 hours at 4 ^{0}C. The beads were pelleted by centrifugation in a microfuge and the supernatant was removed and used in transcription reactions. For mock depletion, 100 µl of packed 12CA5 beads were washed and mixed with P-cell B fraction exactly as described above.

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Chapter IV. Conclusions/Discussion

Summary of results

The discovery that TBP in mammalian cells exists in different complexes, each with a number of associated proteins (TAFs), and that it is involved in transcription from all three RNA polymerases (Sharp, 1992; White and Jackson, 1992; Hernandez, 1993), has profoundly altered our vision of the mechanism of transcription. It has led to the identification of a number of distinct TBP-TAF complexes that function on a variety of promoters. Using biochemical and immunoaffinity techniques, the novel TBP-TAF complex B-TFIID was isolated and characterized. This abundant complex was originally identified by probing fractions derived from HeLa WCEs with anti TBP antibodies (Timmers and Sharp, 1991) and was originally characterized as a pol II factor by virtue of its function in a TBP-dependent pol II transcription assay. In collaboration with Marc Timmers, we characterized the subunits of this complex and discovered an associated ATPase activity. That work is described in chapter II and represents a completely shared effort.

In a second set of experiments the function of this TBP-TAF complex in Pol III transcription was explored. The presence of TBP in both B-TFIID and TFIIIB combined with their common origin (the P-cell B fraction) led to the suggestion, by others, that B-TFIID was a subcomponent of TFIIIB (Reviewed (Rigby, 1993). Though it was previously demonstrated that B-TFIID functioned in the pol II assay (Timmers and Sharp, 1991), and it was believed that TBP-TAFs were specific for specific polymerases (Sharp, 1992; Weinzierl et al., 1993), I set out to test this idea, in experiments described in chapter III.

These experiments confirmed that we were partially correct. That is, while these data demonstrate the predicted result that B-TFIID is not involved in pol III

transcription, they also demonstrate that TBP-TAFs can function in transcription from multiple polymerases. Specifically, the TBP-TAF subcomponent of TFIIIB was shown to function in a TBP-dependent pol II transcription reaction. Though the pol I factor SL1 has not been tested in such an assay, it may too be functional. In fact, Comai et al. state that SL1 is "largely inactive" in pol II transcription, suggesting that it does function to some extent (Comai et al., 1992).

What about the converse experiment? Can pol II TBP-TAF complexes function in reconstituted pol I or pol III transcription reactions? At least for pol III transcription, the answer is no. Neither D-TFIID nor B-TFIID can substitute for the TBP-TAF component required for pol III transcription. One may then hypothesize that neither D-TFIID, B-TFIID, nor TFIIIB will function in pol I transcription.

It could be argued that the reconstitution of pol II basal transcription represents the special case because it is the only reaction that can be reconstituted with TBP itself. This obviates the need for any associated proteins and potentially relaxes the *in vitro* requirement for TBP. It is certainly possible that the pol III TBP-TAF can reconstitute pol II transcription because of the specific conditions used in the pol II reaction. The abundance of highly purified factors used in this pol II assay may drive the dissociation of the unnecessary TAFs, yielding naked yet functional TBP. If such a competition for TBP association were occurring, the most likely factor to be responsible for tipping the balance towards pol II transcription would be TFIIB. Because TFIIB is the first factor to associate with TBP, and is the bridge to pol II, it is the likely candidate for a factor to displace the TAFs from a TBP-TAF complex. To test this idea, transcription reactions were reconstituted with limiting amounts of TFIIB and each of the forms of TBP. Were there a simple competitive relationship

between TAF association on TBP and TFIIB association on TBP, limiting amounts of TFIIB might have yielded preferential pol II transcription in the reaction reconstituted with TBP alone. Unfortunately, these experiments showed no difference in the levels of transcription obtained when comparing the various TBP forms, and were therefore uninformative.

What is the role of B-TFIID?

This highly abundant TBP-TAF complex appears to be quite stable through chromatography and has been shown to function in the Pol II basal transcription reaction. We have also found that other TBP-TAF complexes can function in the pol II basal transcription reaction. This leads us to ask whether B-TFIID is truly a pol II specific factor.

Specific TAF for a novel set of activators?

Our data does not exclude the possibility that B-TFIID is a pol II factor that can function in stimulated transcription with an upstream activator that has yet to be defined (Fig 1A). Is there precedent for such specificity between activator and coactivator? Mounting data supports the idea that specific TAFs may function with specific activators. For example, experiments in yeast have identified the protein, ADA2, which appears to be required for transcription stimulation in response to GAL4-VP16, the prototypic acidic activator, but is not required for transcription stimulated by other acidic activators (Berger et al., 1992).

The ADA proteins were originally identified as suppressors of the lethal phenotype resulting from overexpression of GAL4-VP16. Overexpression of

such an activator was believed to titrate out an important coactivator necessary for stimulated transcription (Gill and Ptashne, 1988; Berger et al., 1990). A mutation in the ADA 2 gene conferred viability to cells overexpressing the GAL-VP16 fusion. The fact that ADA 2 does not function with all acidic activators suggests that there is something unique about the interaction between ADA 2 and VP16, whether it be direct or indirect. In this case, although direct interaction has not been proven, the data suggest that there is functional specificity between activator and coactivator.

In experiments using mammalian factors, Tjian and colleagues have begun to dissect the interaction between various transcription activators and the 8-10 TAFs that are components of the large D-TFIID complex (Tanese et al., 1991). Using immunoprecipitation, affinity chromatography, and *in vivo* binding assays, they have shown that the SP1 activator interacts with TAF 110 but not with any other TAF, and that TAF 110 does not interact with other glutamine rich transcription stimulators (Hoey et al., 1993). Similarly, Tjian and colleagues have reported the association between the proline rich activator NTF1 and TAF 150, as well as an interaction between VP16 and TAF 40 (Goodrich et al., 1993). Here again, seemingly prototypic activators are shown to exhibit specific, and non-generalizable interactions with coactivators. This suggests that the simple rules of class distinction used to describe transcription activators may not accurately reflect their diversity or their true class distinctions based on mechanism of action. Of course it must be noted that these mammalian examples only describe physical interactions between activator and coactivator and do not preclude the requirement for other TAFs in the stimulation process. It seems likely that additional activators displaying coactivator specificity will emerge and we have continued to explore the possibility that such a specific interaction exists between B-TFIID and an as yet untested activator.

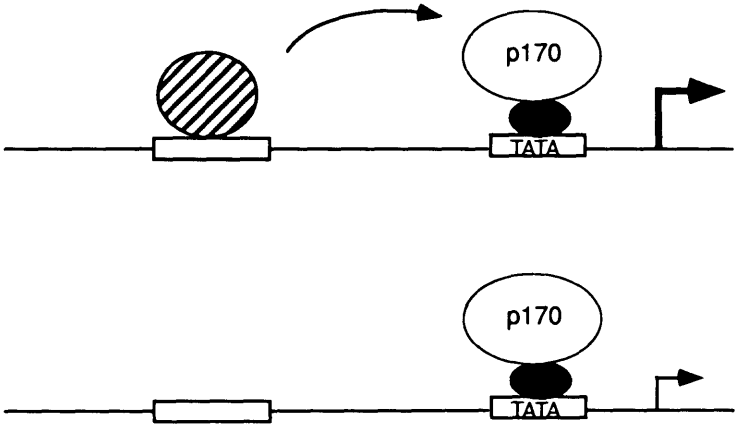
A reasonable candidate for such an activator was described by Chambon and colleagues. Their experiments identified a novel transcription factor, TEF-1, originally cloned by probing an expression library with a binding site oligonucleotide originating from the SV40 enhancer (Xiao et al., 1991). Northern analysis demonstrated that TEF-1 was not present in B cell lines and transfection experiments showed that overexpression of TEF-1 in HeLa, but not B cells, led to a decrease in transcription from templates containing TEF-1 binding sites (Xiao et al., 1991). This led to the hypothesis that not only do B cells not express TEF-1, they also do not contain the coactivator required for TEF-1 function. Further, the experiments suggested that HeLa cells do contain such a coactivator and this coactivator is being titrated out at the high activator concentrations used in the transfection assay (Xiao et al., 1991).

In vitro experiments in HeLa cell extracts reproduced the squelching effect, and fractionation of these extracts allowed the identification of two distinct TBP-associated activities that could cooperate with TEF-1 to stimulate transcription (Brou et al., 1993a). One of these activities is likely to be a component of D-TFIID, the TBP-TAF complex known to function with a large number of activators. The second and more interesting TBP-associated activity has chromatographic properties similar to those of B-TFIID, is specific for TEF-1, and does not function with VP16 or estrogen receptor (Brou et al., 1993a). This established TEF-1 as a reasonable candidate for a B-TFIID specific transcription stimulator. It was therefore of interest to test a GAL4 fusion of TEF-1 (kindly provided by P.Chambon), in an *in vitro* transcription assay reconstituted with various TBP-TAF complexes. Although stimulated transcription was observed in reactions containing D-TFIID and the GAL4-TEF-1 activator, we saw no stimulation when B-TFIID was supplied as the source of TBP-TAF complex (data not shown).

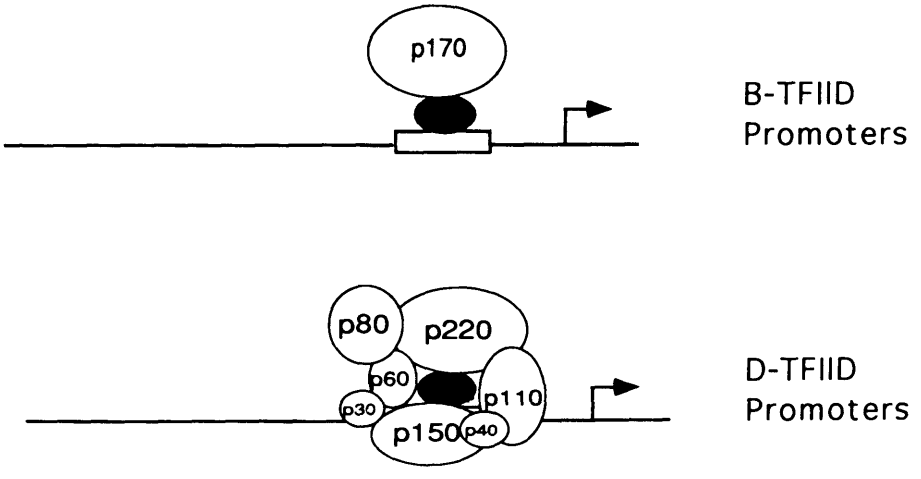
It was therefore concluded that the p170 TAF of B-TFIID cannot supply the coactivator function required by TEF-1, or any other activator tested thus far. As we have not tested all possible stimulators, we cannot rule out the possibility that stimulation mediated by B-TFIID is attainable. Consistent with this uncertainty, we have not yet developed an *in vitro* assay to specifically study the effect of repressors on stimulated transcription. It is possible that B-TFIID can mediate stimulated transcription in reactions regulated by transcriptional repressors-- i.e., perhaps B-TFIID can function to give activator dependent, binding site dependent release from basal repression that would functionally appear as stimulation (aka antirepression). Such experiments await the development of a clean assay to discriminate between stimulation through an increase above basal levels of transcription, and stimulation through the release from repression. (For a review of the processes controlling stimulation see: (Zawel and Reinberg, 1992; Zawel and Reinberg, 1993))

Figure 1. Models for B-TFIID function. (A) Upstream activator specificity. Stimulation (bold arrow) of transcription above a basal level (thin arrow) is conferred by a specific upstream activator. (B) Promoter specificity. A subset of pol II promoters, indicated here as TATA-less, are specifically transcribed by the B-TFIID complex. These promoters are distinct from the class that is recognized and transcribed by D-TFIID. (C) Precursor of TBP-TAFs. Newly translated TBP associates with p170 to form the B-TFIID complex. In a subsequent step, p170 dissociates as the other TAFs assemble on TBP to form the polymerase/promoter specific complexes.

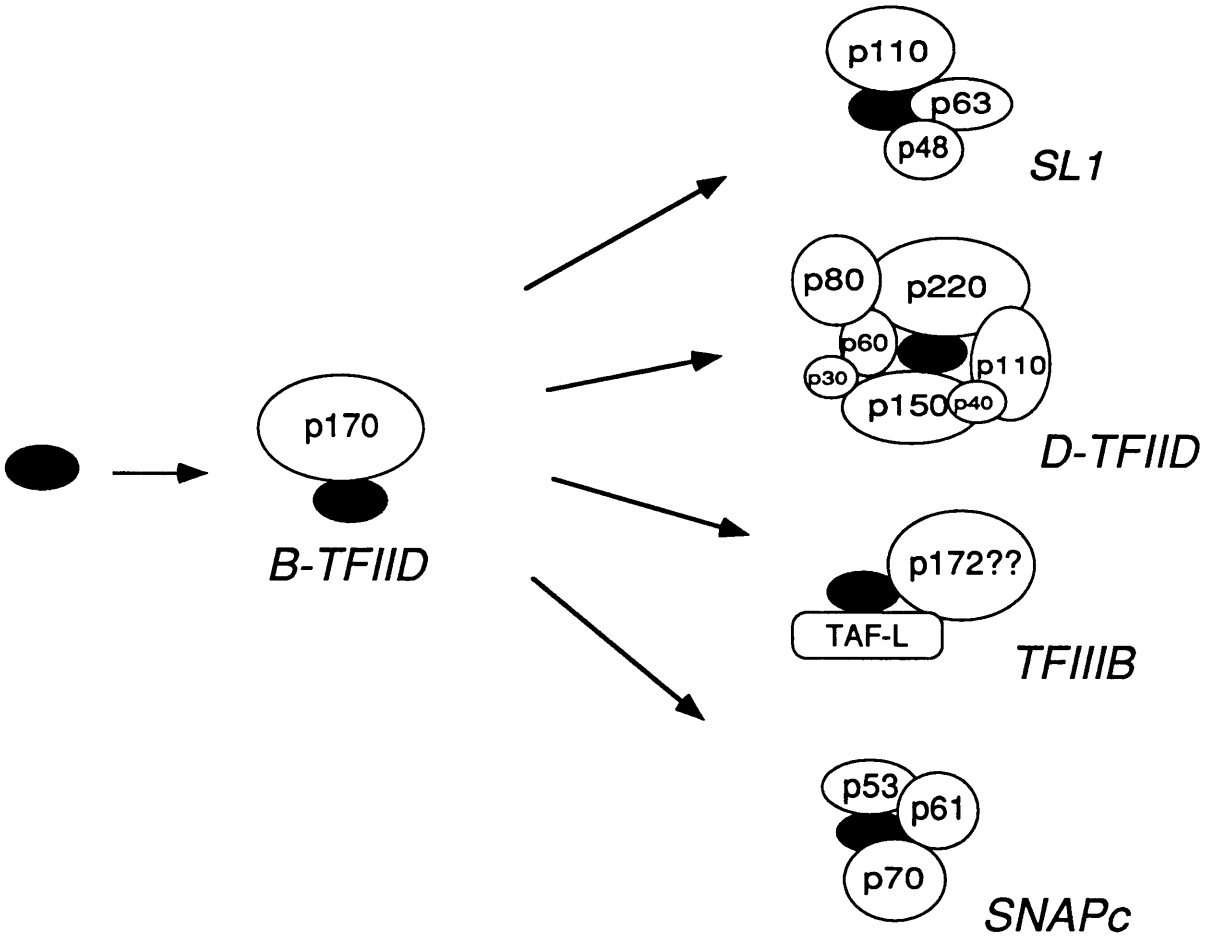
A. Upstream Activator Specificity



B. Promoter Specificity



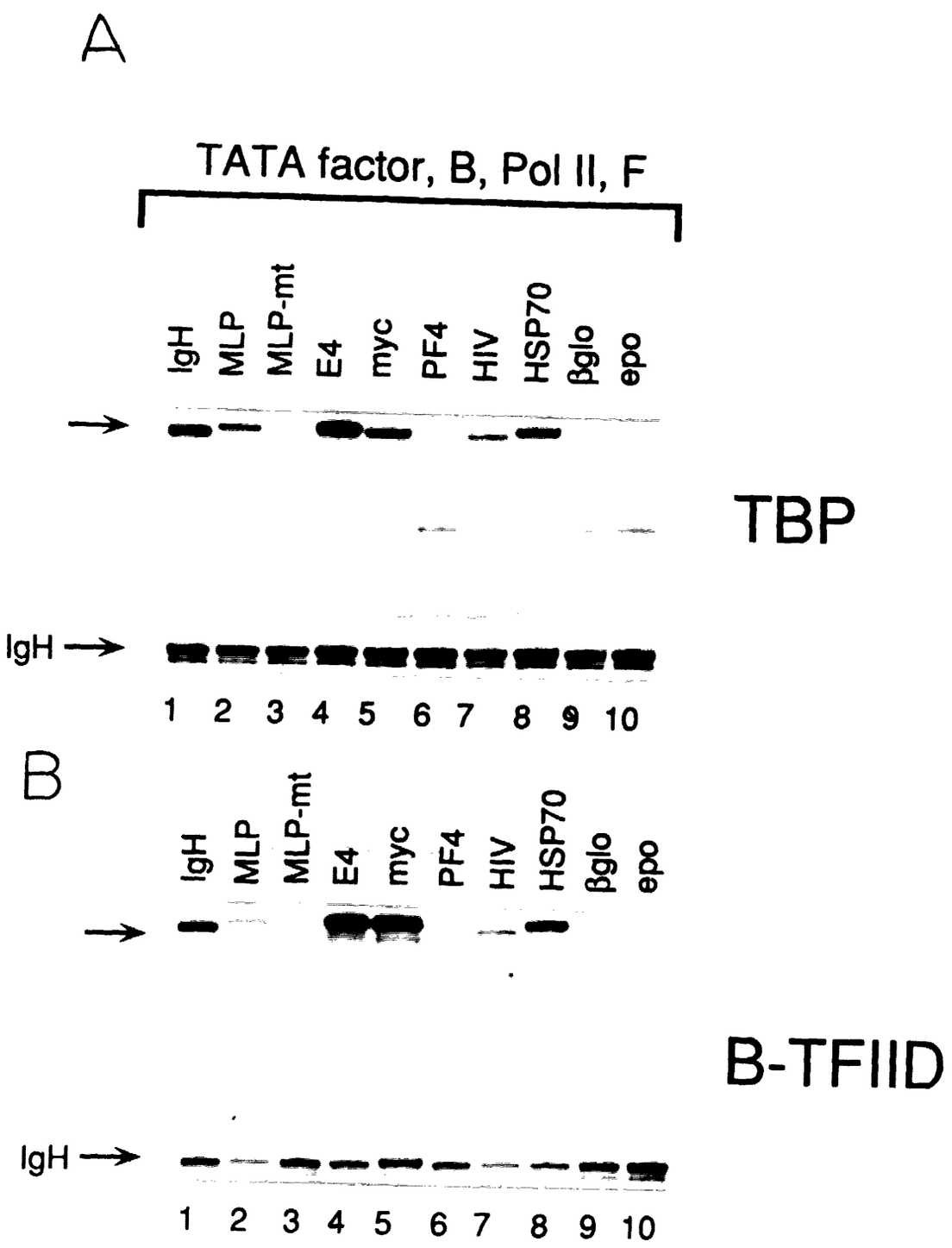
C. Precursor of TBP-TAFs



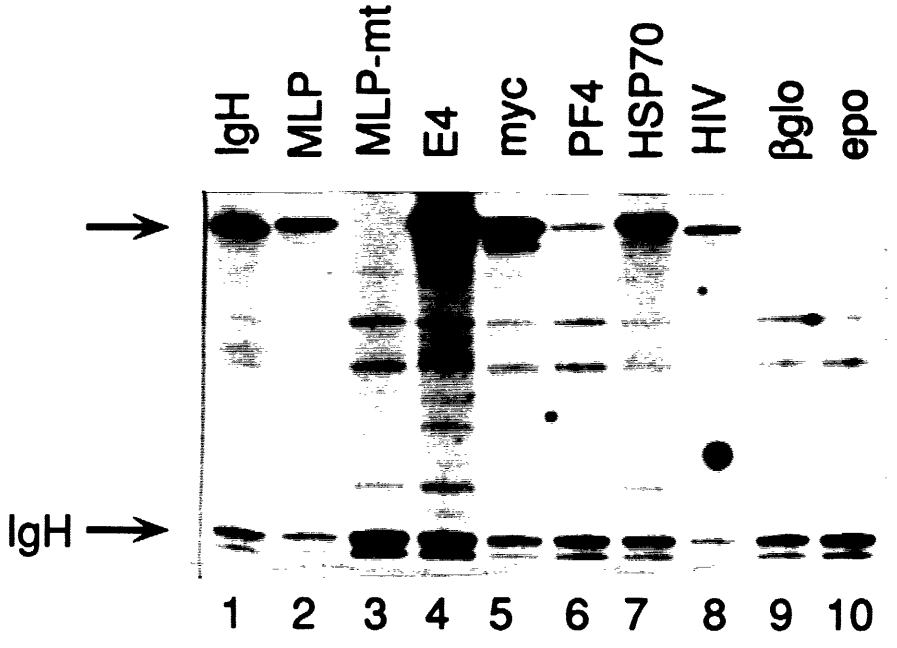
Specific TAF for a novel promoter.

It is possible that the p170 adds specificity to TBP on an as yet undefined promoter element or in an undefined protein context (Fig 1B). Thus far, many TATA-containing promoters tested can be transcribed by B-TFIID. In fact, an experiment performed by Jeff Parvin, a post doctoral fellow in the lab, demonstrated that the efficiency of transcription across a battery of promoters was nearly identical for both B-TFIID and TBP alone (Fig. 2 compare panels A and B). Furthermore, transcriptions reconstituted with the fully decorated TBP-TAF complex D-TFIID demonstrated the same promoter specificity (Fig. 2C). This experiment illustrates several points: First, it appears that the decreased binding specificity of B-TFIID, as compared to TBP, evidenced in gel shift assays does not translate into weaker transcription. Second, it appears that the TAFs bound to TBP do not alter its promoter specificity, at least for the promoters tested. This suggests that for TATA-containing promoters most of the DNA sequence recognition is conferred by TBP itself and is independent of the TAFs decorating it. If a novel B-TFIID promoter does exist it is likely to be of the TATA-less variety. A reasonable candidate appeared to be the TATA-less snRNA promoters of U1-U5 genes which are actively transcribed by pol II, but recent experiments described in chapter I have demonstrated that a TBP-TAF complex distinct from B-TFIID is responsible for driving their transcription (Sadowski et al., 1993).

Figure 2. Pol II transcription from a variety of promoters can be reconstituted with TBP, B-TFIID, or D-TFIID. (A) Pol II transcription with TBP. Transcription reactions are reconstituted essentially as described supplying rTBP as the source of TFIID activity. Thirty ngs of test template generating a 380 nucleotide transcript and 10 ngs of pμ(-47)-(G)-IV (Parvin et al., 1992) control template generating a 180 nucleotide transcript were added to each reaction. Test templates contain ~40 bp of promoter sequence (derived from the promoters indicated above each lane) extending from the TATA box through the start site, fused to the G-less cassette. (B) and (C) Pol II transcription with B-TFIID and D-TFIID. Reactions were reconstituted as in (A) by substituting either B-TFIID (B) or D-TFIID (C) for rTBP.


A
TATA factor, B, Pol II, F
IgH
MLP
MLP-mt
E4
myc
PF4
HIV
HSP70
βglo
epo
TBP
IgH
1 2 3 4 5 6 7 8 9 10
B
IgH
MLP
MLP-mt
E4
myc
PF4
HIV
HSP70
βglo
epo
B-TFIID
IgH
1 2 3 4 5 6 7 8 9 10

C



D-TFIID

Precursor to other TBP-TAF complexes

The level of TBP in mammalian cells is tightly controlled and efforts to overexpress TBP have been largely unsuccessful (Zhou et al., 1992). It appears that an increase in expression of TBP from a transfected plasmid occurs with the concomitant decrease in expression of the endogenous protein (Zhou et al., 1992). Additionally, as has been previously mentioned, all the TBP in HeLa cell extracts exists in complexes with associated proteins. In contrast to the TBP isolated from yeast extracts, that from HeLa extracts appears not to exist free in solution (Timmers and Sharp, 1991). Why is there no uncomplexed TBP in cells? A trivial possibility is that the TBP isolated from extracts does not accurately reflect the state of TBP in cells because the cell disruption procedure resulted in additional complex formation. Assuming that this unlikely possibility is not true, one wonders whether uncomplexed TBP is highly unstable in mammalian cells and is therefore subject to proteolysis.

It is possible that TBP in HeLa cells can be expressed to levels only as high as the levels of associated proteins. What's more, perhaps B-TFIID functions as the storage reservoir for TBP in cells-- i.e., upon translation TBP is first bound to p170 and thereby stabilized. This model would predict that signals within the cell would trigger the subsequent association of TBP with the TAFs that target it to a specific polymerase and promoter. In this way B-TFIID could be the precursor to all the TBP-TAF complexes in the cell (Fig 1C). Such a model would further predict that at early times post translation TBP be completely complexed to p170. Pulse chase experiments designed to examine the state of TBP in cells have been inconclusive due to the difficulty in identifying TAFs in a crude extract (Marc Timmers, unpublished observation). Additionally, the estimated steady state level of B-TFIID in cells is very high

relative to any other TBP-TAF complex (Timmers and Sharp, 1991), making the detection of even greater levels unreliable.

There is no data bearing on this question, but the following types of experiments may offer some clues. The ability of TBP to form complexes when exogenously added to extracts could be tested. This model would predict that no pool of uncomplexed TAFs exists in such extracts, and therefore exogenously added TBP would exist as a monomer. Such complexes could be detected by engineering an affinity handle onto the added TBP or by labeling the protein and following its chromatographic behavior. This model would also predict that subunit swapping experiments would unveil the exchange of TBP from B-TFIID with the TBP from D-TFIID, SL1, or TFIIIB. Again, such experiments would be facilitated by the use of a B-TFIID complex containing a tagged TBP that could be distinguished from endogenous TBP. A final prediction of this model is that overexpression of p170 in HeLa cells would allow for the overexpression of TBP in the same cells. Unfortunately, this experiment must wait the successful cloning of p170.

Endogenous basal transcription factor

A fourth and related model for the function of B-TFIID is that of the true cellular basal transcription factor. Perhaps B-TFIID is the *in vivo* analogue of TBP and therefore performs all the transcription functions that TBP displays *in vitro*. The main difference between TBP and B-TFIID that we have observed is the inability of B-TFIID to form specific gel shift complexes on TATA-containing probes. This inability to form a gel shift complex has also been observed in experiments with the larger D-TFIID complex (unpublished observations). Although D-TFIID can bind to DNA and commit to a template (Samuels and Sharp, 1986; Nakajima et al., 1988), very few investigators have observed a

clear D-TFIID complex by gel shift (Zhou et al., 1992). This may simply be a consequence of the gel shift assay system and suggests that a gel shift is not necessarily a good measure of functional DNA association. Clearly B-TFIID can function in the pol II transcription reaction and therefore must be interacting with DNA. Experiments performed by Ben Shykind, a student in the lab, have confirmed that, like D-TFIID, B-TFIID can commit to a DNA template in a solid support based transcription assay (unpublished observation).

Is additional binding specificity conferred on B-TFIID in the context of the complete transcription system, or is p170 dissociated from TBP upon assembly of the preinitiation complex? At this point we cannot answer these questions, but the stability of B-TFIID through chromatography at least suggests that it remains as a complex during transcription. The addition of a subset of the general factors including TFIIA and TFIIB does not liberate TBP to the extent that it can form a specific gel shift complex, but it is possible that complete complex assembly-- i.e., TFIIA--H + pol II might liberate TBP from p170. It would, however, be possible to test for the presence of p170 throughout transcription using the solid support transcription assay mentioned above (See next section).

Are there basal promoters in cells whose transcription might be driven by B-TFIID? There is no evidence that basal promoters exist in cells, though genes without proximal stimulatory elements probably exist. Perhaps distal enhancer elements regulate the transcription of such genes through B-TFIID. Because there are very few examples of distal enhancers functioning *in vitro*, it is difficult to test such a model directly.

Experimental approaches to further study of B-TFIID

As I have alluded to already, we have identified a highly abundant TBP-TAF complex whose function is still largely unknown. There are a number of experiments that have been attempted or remain to be attempted that could shed light on its function. Some of these experiments are briefly described below.

Cloning of p170

In our effort to study B-TFIID, we attempted to purify and to obtain amino acid sequence from the p170 TAF using conventional techniques. The early efforts to use immunoaffinity chromatography were unsuccessful for large scale purification because the supply of highly reactive anti-TBP rabbit polyclonal serum was limited and the yields from such immunoprecipitations were low. We therefore chose a conventional chromatographic approach as outlined in Figure 1, Chapter III, to purify p170 for microsequencing. This, too, was ultimately unsuccessful due to an inability to transfer the 170 kd protein out of the preparative SDS gel and onto nitrocellulose. After extensive manipulations ~30 pmoles of protein remained in 3 SDS gel slices which were sent to the sequencing facility at Yale University for in-gel sequence analysis. Again, no sequence was obtained and the project was abandoned.

In repeating such a purification, several modifications would be made. First, at least one and possibly two additional steps of purification are required to allow the entire sample to be loaded into one lane of a preparative SDS gel. The search for additional columns included analytical fractionation on HAP and reverse phase HPLC, both of which were unsuccessful due to poor recovery. Purification on a heparin sepharose matrix has not been tested and remains a

possibility. Second, a more efficient method for transferring large amounts of p170 must be explored. The newly acquired semi-dry transfer apparatus may be useful in this regard because it appears to be more efficient in the transfer of high molecular weight proteins.

Upon obtaining protein sequence and ultimately cloning p170, homology searches might give the first clue as to B-TFIID function. Overexpression and purification of p170 would allow tests of its effect on transcription, on DNA binding, and on a variety of assays known to require TBP. Using antibodies we could follow its fate in fractionated extracts and determine whether it is entirely associated with TBP.

Mapping the site of interaction with TBP

Clone in hand, analysis of the domain of interaction between p170 and TBP could be explored. Affinity chromatography and far western assays have been successfully employed to identify TBP interacting domains in such proteins as E1a (Yamashita et al., 1993) and TFIIB (Ha et al., 1993). Further, competition binding experiments could identify which of the many TBP interacting proteins, such as TFIIB (Buratowski and Sharp, 1990; Ha et al., 1993), TFIIA (Buratowski et al., 1989) (Buratowski and Zhou, 1992), CTD (Usheva et al., 1992), can associate with TBP in the presence of p170. This again may give clues to the fate of p170 through the transcription reaction and begin to interrelate the myriad of TBP associating proteins.

The interesting question of how so many proteins can interact with TBP remains to be fully understood. By analogy to the interactions described for the SH2 and SH3 domains, it is likely that interaction domains consist of small numbers of amino acids. As the list of TBP interacting proteins grows (for a partial list see (Hernandez, 1993) and the associations begin to be observed *in*

vivo (e.g. c-rel (Kerr et al., 1993) and myc (Hateboer et al., 1993), it becomes more important to dissect these interactions and their role in regulating the function of TBP.

Studying the effects of transcription modulators

What other experiments would give clues to B-TFIID function? As has been mentioned earlier, large numbers of proteins that exhibit modulatory effects on the transcription reaction have been identified. A number of these proteins have been tested in transcription reactions reconstituted with B-TFIID and TBP in an effort to uncover a difference between the two that might suggest a function for the p170. No significant difference between B-TFIID and TBP was observed in these transcription reactions. The modulatory activities tested included transcription factor TFIIA (Samuels and Sharp, 1986), the inhibitor Dr 1 (Inostroza et al., 1992), and the poorly characterized stimulatory activity called 700k (Parvin et al., 1992). In addition, titrations of the basal transcription factors TFIIB, TFIIE, TFIIF, and pol II revealed no differences in the amount of transcript generated by TBP and B-TFIID . Though it is difficult to conclude anything from negative results, they are at least consistent with the possibility that the functional form of TBP in cells is B-TFIID and that TBP is a good *in vitro* model for its function.

The final regulatory protein tested was the tumor suppressor p53. In addition to its ability to stimulate transcription in a binding site dependent fashion, p53 is believed to function as a binding site independent inhibitor of basal transcription through the sequestration of TBP (Seto et al., 1992; Mack et al., 1993). *In vitro* transcription experiments correlated the inability of p53 to bind yeast TBP with an inability of p53 to inhibit transcription reactions reconstituted with the yeast activity. This supports the idea that direct

interaction leads to functional repression. It has been suggested that, while both D-TFIID and TBP can interact with and are inhibited by p53, the form of TBP in the P-cell B fraction does not interact with p53 (Anne Usheva, Shenk lab, personal communication). Extending this observation, it might suggest that B-TFIID driven transcription reactions are refractory to the inhibitory effects of p53, a potentially provocative result. It was therefore of interest to test the ability of p53 to inhibit transcription reactions reconstituted with B-TFIID. Unfortunately, we have been unable to develop a p53 responsive *in vitro* assay.

What are the directions of the field?

The depth and breadth of our knowledge about eukaryotic transcription has increased dramatically over the past 5 years. We have moved transcription from the somewhat descriptive to the molecular. A large number of the factors whose activities were defined in crude reconstitution experiments have now been purified, cloned, and overexpressed in a variety of systems. The complexity of the system continues to increase as the transcription reaction gets further dissected and our alphabet of factors expands. What was until recently TFIIA-F (Buratowski and Sharp, 1992) is now TFIIA-J and still growing (Conaway and Conaway, 1993; Zawel et al.,1993).

The ever increasing complexity of the transcription reaction has led to attempts to simplify it. The reaction has been reexamined with an eye towards minimizing the number of components that are absolutely required to obtain an RNA transcript. It is now believed that for certain promoters embedded in supercoiled DNA templates, transcription can be reconstituted using only TFIIB, TBP, RNA pol II, and in many cases TFIIF (Parvin and Sharp, 1993; Tyree et al.,

1993). This discovery has profoundly altered our understanding of transcription and has changed the vocabulary used to describe the myriad factors still being defined. It is clear that these core proteins can recognize the appropriate promoter region, initiate transcription, and elongate at least out to several hundred nucleotides. This suggests that the other general factors serve a more specialized role and are probably involved in increasing the efficiency and specificity of the various steps outlined above. As these additional factors become available in recombinant form, specific questions about their presence during various phases of the transcription reaction can be addressed.

In addition to the characterization of the modes of action of these basal factors, an active area of research has been the mechanism of stimulated transcription. It has long been believed that upstream stimulatory proteins act via modulation of the basal machinery. Early models proposed that activation occurred through stabilization of the TFIID activity (reviewed (Lillie and Green, 1989; Ptashne and Gann, 1990). It was later shown, principally with VP16 derivatives, that stimulation might occur through the stabilization of other basal factors, such as TFIIA (Wang et al., 1992), or TFIIB (Lin and Green, 1991). The identification of TAFs, and the observation that both TBP and TAFs interact with various stimulators, has led back to the model of activation mediated through TFIID (Pugh and Tjian, 1992; Boyer and Berk, 1993). It is undoubtedly the case that transcription stimulation is achieved through a variety of mechanisms. As described previously, at least two broad mechanisms, that of derepression of basal transcription (Croston et al., 1991; Kerrigan et al., 1991; Laybourn and Kadonaga, 1991; Meisterernst et al., 1991; Inostroza et al., 1992), and that of stimulation above an unaltered basal level (Dynlacht et al., 1991; Meisterernst and Roeder, 1991; Luo et al., 1992; Brou et al., 1993a; Brou et al., 1993b) are

at play. An understanding of how these processes are altered in various disease states will be the next major advance.

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Chapter V. Appendix

Abstract

This section describes experiments which were designed to identify yeast activities that associated with TBP. While not directed towards the identification or analysis of the B-TFIID factor, they do represent early attempts to study proteins that associate with TBP. These experiments ultimately did not lead to the homogeneous preparation of the transcription factors targeted, but they did yield an interesting result. Because these experiments are not closely related to the main body of this text, they are included in this appendix.

Results

Previous studies in the lab had identified yeast activities that could complement a mammalian transcription system to recapitulate accurate transcription initiation *in vitro* (Buratowski et al., 1988; Hahn et al., 1989). This observation, as well as a number of experiments demonstrating the functional integrity of many yeast/mammalian hybrid systems (Kakidani and Ptashne, 1988; Metzger et al., 1988; Schena and Yamamoto, 1988; Struhl, 1988; Webster et al., 1988), suggested that the identification of yeast transcription factors would be generalizable. It was therefore of interest to pursue the identification of additional yeast activities that could functionally substitute in the mammalian transcription reaction. By exploiting the favorable scale up properties of yeast we hoped to obtain large amounts of protein to use in studies of the mechanisms of transcription.

These studies began with an attempt to isolate the yeast transcription factor TFIIA, a protein believed to act early in the transcription initiation pathway and whose existence in yeast had been previously established (Hahn et al., 1989). Using a T7 overexpression system (Tabor and Richardson, 1985), several mgs of yeast TBP were prepared, purified, and covalently coupled to NHS-activated agarose (Affigel-10). Such protein production yielded TBP that was highly active in *in vitro* assays and, as an affinity matrix, offered the hope of great specificity.

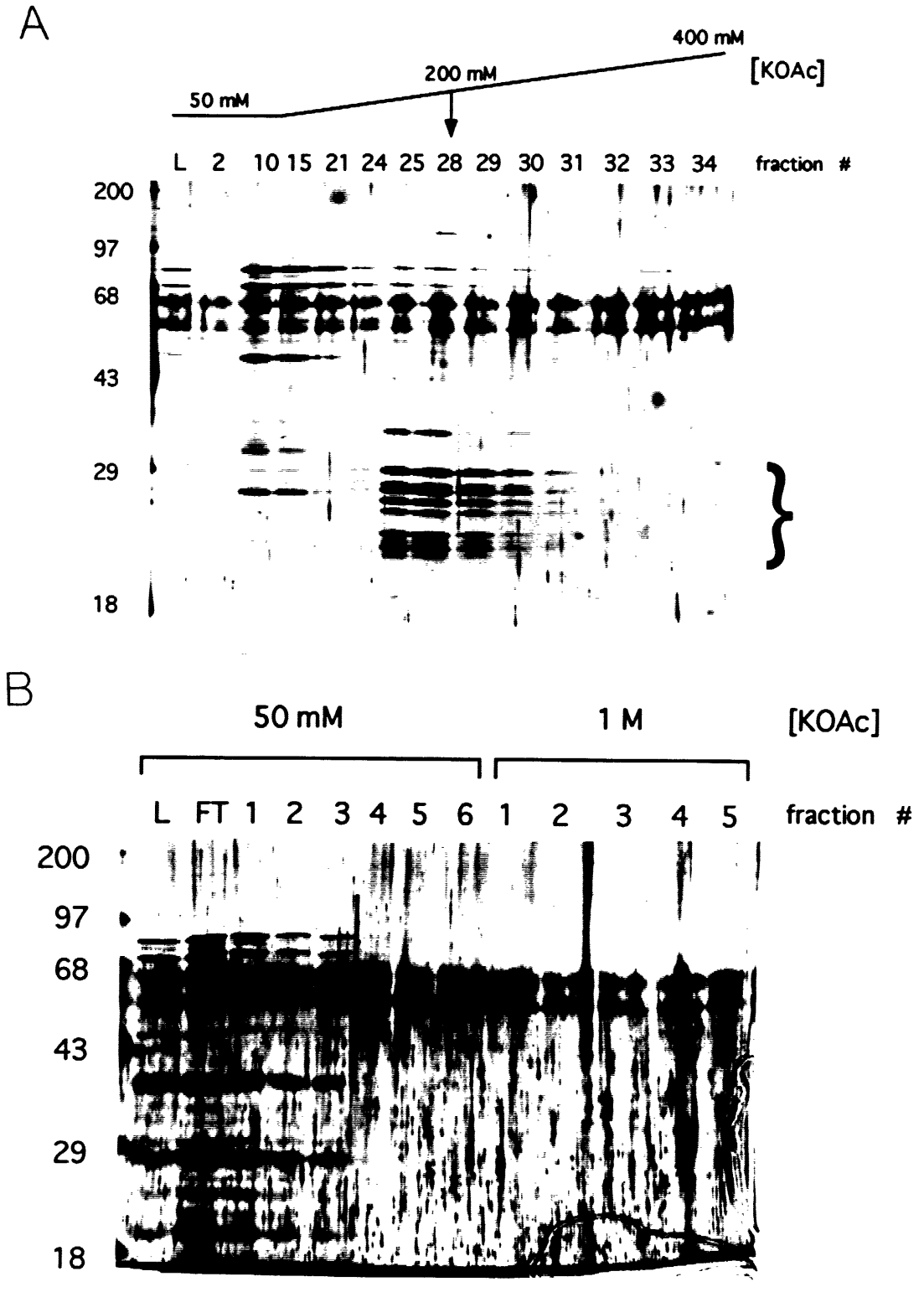
This column was then used to purify activities from partially purified yeast whole cell extracts (WCEs). To begin the purification of TFIIA, yeast WCE was conventionally chromatographed over a heparin sepharose column, as was previously described (Hahn et al., 1989). TFIIA-containing protein fractions were then loaded onto a DEAE column and eluted with a linear gradient from 50 mM -

1M potassium acetate (KOAc). TFIIA activity, identified using a TBP-dependent gel shift assay (Buratowski et al., 1989; Hahn et al., 1989), was adjusted to 2M KOAc and further purified over phenyl suparose developed with a linear gradient from 2M - 0M KOAc. Peak fractions were concentrated over a Mono Q FPLC column and chromatographed over the TBP affinity column. Fractions eluting from the affinity column were again assayed for gel shift activity and analyzed by silver staining of SDS polyacrlyamide gels (Fig 1A). To our surprise, no clear polypeptides were visible in the fractions that contained TFIIA activity (fractions 32-40), but a novel set of polypeptides was observed eluting from the column in fractions 25-28 (~200 mM KOAc). They eluted as a tight cluster of 7-9 polypeptides ranging in molecular weight (mw) from 20-30 kd. Repeated affinity chromatography beginning with cruder loads always yielded the same set of polypeptides (data not shown).

The specificity of this interaction was tested by repeating the affinity chromatography on a column prepared with insulin as the affinity ligand in place of TBP. Such columns bound neither the TFIIA activity nor the set of low mw polypeptides (Fig 1B).

To determine the nature of these polypeptides, the eluted fractions from several runs of the affinity column were pooled, precipitated with acetone, run on a preparative SDS PAGE gel, transferred to nitrocellulose, and subjected to N-terminal amino acid sequencing. The first three peptides sequenced were N-terminally blocked, but the next three tested yielded 5-8 amino acids each. Data base searches indicated that these

Figure 1. Affinity purification of TFIIA. (A) A TBP affinity column binds TFIIA and a set of small molecular weight proteins. Five μl of load and 30 μl of the indicated fractions were separated by electrophoresis on a 12 % SDS polyacrlyamide gel and were visualized by silver staining. The position of protein markers is indicated to the left by their molecular weight in kd. The potassium acetate (KOAc) concentration of each fraction is indicated above the lane The doublet at ~60 kd is contaminating keratin introduced with the load buffer. (B) TFIIA and small molecular weight proteins flow through a control insulin affinity matrix. Five μl of load and 40 μl of the indicated wash fractions are analyzed as in (A). The position of protein markers is indicated as above.


A
400 mM
200 mM
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[KOAc]
L 2 10 15 21 24 25 28 29 30 31 32 33 34
fraction #
200
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B
50 mM
1 M
[KOAc]
L FT 1 2 3 4 5 6 1 2 3 4 5
fraction #
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proteins might be related to the multicatalytic proteinases (reviewed: Bond and Butler, 1987; Rivett, 1989; Goldberg and Rock, 1992). Consistent with this, the purified polypeptides displayed chromatographic properties and a gel migration pattern that was very similar to a previously characterized set of proteins-the multicatalytic proteinases (proteosomes).

What are proteosomes?

Though a number of proteosomes have been cloned from a number of organisms, their function is largely unknown. The proteins are thought to be part of a non-lysosomal protease complex involved in the degradation of improperly folded or oxidized proteins (Rivett, 1985; Matthews et al., 1989). The complex nature of the proteosome made the determination of substrate specificity very difficult, and therefore a variety of peptides have been used for *in vitro* assays (Tanaka et al., 1986; Tanaka et al., 1988; Driscoll and Goldberg, 1990). This was the state of the field until very recently when several labs implicated these proteins in antigen processing in antigen presenting cells (Glynne et al., 1991; Driscoll et al., 1993; Gacznska et al., 1993; Howard and Seelig, 1993).

Having originally purified these proteins along with TFIIA on a TBP affinity column that contained a large excess of TBP, it was unclear whether these proteins had an effect on the ability of TBP to interact with TFIIA, or with any other proteins. To test the effect of proteosomes on various TBP activities, we obtained large quantities of proteosomes purified from rat, as a kind gift of Alfred Goldberg at H.M.S.. Addition of proteosomes to a variety of binding and transcription assays under a number of conditions was without effect (data not shown). Due to the absence of any assayable effect of these proteins on the function of TBP, this work has not been pursued any further. The association

between TBP and the proteosomes remains an interesting observation that lacks the proper context in which to understand it. Is there a relationship between the proteolytic function of the proteosomes and the association with TBP? Perhaps the proteosomes are involved in the tight regulation of TBP in cells (See Discussion).

Materials and Methods

Purification of yeast TBP

ryTBP was prepared essentially as described (Buratowski et al., 1991) with slight modifications. After purification on S-Sepharose the protein was loaded onto a heparin sepharose column developed with a linear gradient of 50-1000 mM KCl. The protein eluted at 400 mM KCl and was ~80% pure after this column.

Preparation of affinity columns

A TBP affinity matrix was prepared by spin concentrating 1.5 mgs of the above protein in a Centricon-30 (Millipore) micro concentrator at 4000 rpm in an SS-34 rotor. Concentrated protein was at 2 mg/ml in 500 µl of Buffer A (20 mM Hepes 7.9, 10 % glycerol, and 1 mM DTT) + 0.3M KCl. One ml of activated matrix (Affigel-10, Pharmacia) was washed with 10 mls of water and 3 mls of ice cold Buffer A + 100 mM KCl. One hundred and fifty (150 µl) of matrix was added to the TBP solution and rotated for 4 hours at 4 °C. After removing the unbound protein, unreacted sites were blocked by rotating the matrix with 500 µl of 0.5M Tris pH=8 overnight at 4 °C. The matrix was then washed 5x with 1 ml of Buffer A + 100 mM KCl and stored in the same.

The coupling efficiency was measured by comparing Bradford assays of the protein solution performed before and after coupling. The coupling efficiency was 82% yielding 150 µl of matrix containing 6 mg/ml TBP.

The control matrix prep. One mg of insulin (Boeringer Mannheim) was resuspended in Buffer A + 100mM KCl and coupled to 200 µl of Affigel-10 as indicated above. Protein assays indicated a coupling efficiency of 85% yielding 200 µl of matrix containing ~ 4 mg/ml insulin.

Preparation of yeast WCE and yeast TFIIA

Yeast WCE was prepared and chromatographed over heparin sepharose and DEAE sephadex, as previously described (Hahn et al., 1989). TFIIA activity was monitored using a TBP dependent gel shift (Buratowski et al., 1988) and the peak of activity was dialyzed against Buffer T (30 mM Tris pH=8, 2 mM EDTA, 20 % glycerol, 1 mM DTT) + 2M KOAc, and injected onto a phenyl suparose FPLC column. Protein was eluted with a linear gradient from 2M-0M KOAc and activity eluted at M KOAc. Peak fractions from several phenyl column runs were pooled and dialyzed against Buffer T + 200 mM KOAc and concentrated on a Mono Q FPLC loaded at 200 mM KOAc and eluted at 700 mM KOAc in Buffer T. Fractions were assayed for protein concentration and gel shift activity, and active fractions were used in affinity chromatography.

Affinity Purification of TFIIA

Thirty µg of TFIIA purified as indicated above was dialyzed against Buffer T + 50 mM KOAc and loaded onto the TBP affinity matrix which was packed into an HR5/2 column (Pharmacia) and attached to the FPLC. The column was developed with sequential salt steps of 150 mM, 300 mM, 500 mM, 750 mM

and 1M KOAc in Buffer T. Fractions of 400 µl were collected and analyzed by gel shift and SDS PAGE.

The insulin control matrix was mixed in batch with 10 µg of Mono Q concentrated TFIIA in 150 µl of Buffer T + 50 mM KOAc for 2.5 hours at 4 °C. The matrix was gently centrifuged, supernatant (Flow through, FT) was removed and then the matrix was washed in batch 6 x 10 minutes with 100 µl of Buffer T + 50 mM KOAc (washes 1-6, 50 mM). Bound proteins were eluted with 6 x 10 minute washes with 100 µl of Buffer T + 1 M KOAc (washes 1-6, 1M). Aliquots of each wash were analyzed by gel shift and SDS PAGE as indicated above.

N-terminal Sequencing

After multiple runs of the TBP affinity column, proteins eluting in fractions 25-30 were pooled, adjusted to a protein concentration of 0.15 mg/ml with insulin, and precipitated overnight in a 15 ml silanized Corex tube with 4 volumes of acetone at -20 °C. Protein was pelleted by spinning for 20 minutes at 9000 rpm in an SS34 rotor. Pelleted protein was resuspended in 1X SDS buffer and loaded onto a 0.75 mm 15 % acrylamide SDS gel and transferred to problot PVDF membrane (ABI) in 10 mM CAPS, 10 % MeOH, pH =11 for 45 minutes at 250 mAmps constant current. The proteins were visualized by staining the blot for 5 minutes in 0.1% comassie/ 50 % MeOH, then destaining in 50% MeOH. The polypeptides were subjected to N-terminal sequencing at the biopolymers facility in the CCR.

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Novel lymphokine cloning project: Total mRNA isolation; preparation and screening of cDNA libraries.
Supervisor: Dr. Gordon Wong

DNA based diagnostics kit project: Chemical modifications of DNA; developed methods for coupling modified DNA to enzymes and to solid supports; characterization of coupled DNA.
Supervisor: Dr. Gene Brown

9/83 -12/83 **Brandeis University**
Synthesis of organometallic compounds
Supervisor: Dr. M. Rosenblum

6/83 - 8/83 **Rockefeller University**
Purification of the M6 protein of group A S. pyogenes
Supervisor: Dr. V. Fischetti

9/82 - 5/83 **Brandeis University**
Synthesis of benzofuran natural product precursors
Supervisor: Dr. R. Stevenson

TEACHING EXPERIENCE

9/88-1/89 **MIT**
Graduate Biochemistry (7.31)
Professors: Dr. F. Solomon and Dr. R. Sauer

2/91-6/91 **MIT**
Undergraduate Intro. Biology (7.01)
Professors: Dr. P. Matsudaira and Dr. B. Cochran

PUBLICATIONS

Meyers and Sharp, (1993). *TATA-Binding Protein and Associated Factors in Polymerase II and Polymerase III Transcription.* Mol. Cell. Biol. **13**:7953.

Timmers, Meyers, and Sharp, (1992). *Composition of Transcription Factor B-TFIID.* Proc. Natl. Acad. Sci. USA **89**:8140.

Kremsky, Wooters, Dougherty, Meyers, Collins and Brown, (1987). *Immobilization of DNA via oligonucleotides containing an aldehyde or carboxylic acid group at the 5′ terminus.* NAR **15**:2891.

US Patent Application, *Novel Methods and Compositions for Attaching Nucleic Acids to Binding Components.*

ABSTRACTS

Meyers and Sharp, *B-TFIID is Distinct from the TBP-TAF Complex of TFIIIB.* Mechanism of Eucaryotic Transcription, Cold Spring Harbor Meeting on Cancer Cells (Sept 1-5, 1993).

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Meyers, Timmers and Sharp, *Functional and Structural Analysis of Mammalian TFIID Complexes: Subunit Composition of B-TFIID.* Fundamental Mechanisms of Transcription, Keystone Symposia on Molecular and Cellular Biology (Mar 28-Apr 3, 1992).

Meyers and Sharp, *Purification of Yeast TFIIA and other TFIID-Associated Proteins.* Regulation of Eucaryotic mRNA Transcription, Cold Spring Harbor Meeting on Cancer Cells (Aug 28-Sept 1, 1991).