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# Preinitiation Complex Assembly and General Transcription Factor IIA

Richard Alan Bernstein

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# Preinitiation Complex Assembly and General Transcription Factor IIA

Richard Alan Bernstein

A thesis submitted to the faculty of The Rockefeller University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.



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N.B.: The experiments shown in figures 13,19, and 25 result from collaborative efforts between Dr. Jeff DeJong and myself.



## **Dedication**

To my parents, Carol and Lawrence Bernstein;  
and to my wife, Marina Hoover





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## Abbreviations

ATP: adenosine triphosphate  
BSA: bovine serum albumin  
CTP: cytidine triphosphate  
DE: diethyl-aminoethyl  
DNA: deoxyribonucleic acid  
EDTA: ethylenediamine tetraacetate  
GTP: guanine triphosphate  
Hepes: 4-(2-hydroxymethyl)-1-piperazineethanesulfonic acid  
DTT: dithiothreitol  
IPTG: isopropyl- $\beta$ -D-thiogalactopyranoside  
kD: kilodalton  
MLP: major late promoter  
N3R-dUTP: 5-[N-(p-azidobenzoyl)-3-aminoallyl]-deoxyuridine triphosphate  
NP40: nonidet P 40  
PAGE: polyacrylamide gel electrophoresis  
PC: positive cofactor  
PIC: preinitiation complex  
PMSF: phenylmethylsulfonylfluoride  
pol: polymerase  
RNA: ribonucleic acid  
RNAP: RNA polymerase  
SDS: sodium dodecyl sulfate  
TCA: trichloroacetic acid  
TF: transcription factor  
Tris: tris[hydroxymethyl]aminomethane  
UTP: uridine triphosphate



## Note on Terminology

Any thesis representing several years work in a rapidly progressing field can not help being partially anachronistic. In particular, the names of reagents and factors may change during the time between the work represented in one chapter and that shown in the next. This is the case in this thesis with regard to the subunits of TFIIA. I have decided to mirror in this work the terminologic shift which took place in the literature .

Consequently, hTFIIA/ $\alpha$  is the designation for the 55 kD precursor to the 35 and 19 kD subunits of human TFIIA in Chapter IV, whereas this protein is known as hTFIIA/ $\alpha\beta$  in Chapter V, consistent with the change in nomenclature which took place in the field as a whole. Also, the drosophila TFIIA small subunit is designated dTFIIA-S, whereas the human TFIIA small subunit is known as hTFIIA/ $\gamma$ , consistent with usage in the literature.



**"Nothing alive can be understood."**

**--Franz Kafka**

**"Letter to his father"**





## Overview of the Thesis

The process of transcription is investigated biochemically from several perspectives.

First, a novel transcriptional activator, Gal4-IE, is shown to activate transcription in vitro from Gal4-DNA binding sites located upstream of two core promoters. The mechanism of activation is investigated by the development of a template competition assay. Using this assay, a partial preinitiation complex containing TFIIB is implicated as a target of Gal4-IE. These experiments, as well as others cited, suggest that activators affect early stages of PIC assembly, namely the formation of complexes including TFIID, TFIIA, and TFIIB. To further the study of this complex, attention is then turned to TFIIA.

A cDNA is isolated encoding the small subunit of TFIIA from *Drosophila melanogaster*. The protein encoded by this cDNA, dTFIIA-S, is highly homologous to the cognate protein from yeast, as well as a protein encoded by a previously uncharacterized cDNA from rice, which likely encodes that organism's TFIIA-S. Using an antibody raised against recombinant dTFIIA-S, I show that dTFIIA-S is present in *Drosophila*-derived nuclear extracts, and that the native dTFIIA-S migrates in SDS gels identically to recombinant dTFIIA-S, arguing against post-translational modification of the protein. In conjunction with a 55 kD precursor to the 35 and 19 kD subunits of human TFIIA, the functional properties of dTFIIA-S are investigated. These experiments show that dTFIIA-S is competent to form a bonafide DA promoter complex. Further, both the 55 kD precursor, and dTFIIA-S, are shown to be constituents of the DA



promoter complex. Finally, it is shown that dTFIIA-S can reconstitute TFIIA transcriptional activity in a reconstituted transcription system consisting of highly purified or recombinant factors including TFIID. Based on the above results, it is concluded that dTFIIA-S represents a functional TFIIA subunit, and that the combination of recombinant hTFIIA/ $\alpha$  and dTFIIA-S is sufficient to reconstitute functional TFIIA.

The protein-protein interactions of the recombinant TFIIA subunits are then investigated. First, both subunits of TFIIA are shown to independently interact with both human and yeast TBP. In the case of yeast TBP, this interaction is impaired by a non-conservative double mutation in the TBP "basic region". This mutant TBP, which is correspondingly impaired in TBP-TFIIA promoter complex formation activity, is used to analyze the functional significance of the TBP-TFIIA interaction. These experiments show that the TBP-TFIIA interaction is required specifically in physiologic transcription systems containing TFIIA, but not in a TFIIA-independent transcription system. Finally, interactions of TFIIA with preinitiation complex components and a novel cofactor are documented which suggest an intimate involvement of TFIIA in the process of transcriptional activation.



## Chapter I: Introduction



## 1.0 General Introduction

Cells regulate their gene expression largely at the level of transcription. The components of the eukaryotic cell which carry out transcription consist of two broad subclasses. One class, the gene specific factors, referred to throughout this thesis as "activators", transduce signals from the cell's various homeostatic and regulatory networks to the genes, which must be regulated in response to these signals. The other class, the general transcription factors, comprises the RNA-producing machinery of the cell. The general transcription factors are the ultimate functional target of regulatory factors.

Transcription of all genes in all organisms displays certain common motifs. Transcription of a gene must be preceded by the binding of the enzyme RNA polymerase to that gene's promoter. Promoters tend to be characterized by both stereotypical and unique DNA sequences. In many cases, RNA polymerase by itself does not bind promoter DNA. Recognition of promoters is accomplished by factors separable from the enzyme which bind to specific promoter DNA sequences and recruit the polymerase via protein-protein interactions. The final active complex assembled on the promoter is known as a "preinitiation complex" (PIC). Many transcriptional activators function by influencing the process of PIC formation.

During the assembly of the PIC on a promoter, several conformational changes in the complex take place. At a minimum, the DNA strands at the start site of transcription must melt to allow access of RNA polymerase to the nucleotide bases, the enzyme must initiate RNA synthesis, and the nascent transcript must be elongated





past the promoter. Transcription is thus at each step a potential target for regulation. As genomes (and organisms) became more complex during evolution, the interactions required to transcribe their genes increased in complexity. This increased complexity is reflected both in the number of components that carry out the above transactions, and the interactions between those components. However, the process itself is qualitatively similar from prokaryotes to man.

The major goals of the biochemical study of transcription in eukaryotes are 1) to define and isolate all of the involved cellular components; 2) to reconstitute active components with recombinant proteins; and 3) to determine the interactions in which these components take part. This thesis addresses general transcription factor IIA (TFIIA) with these goals in mind.

This introduction reviews what we know about the biochemistry of the general transcription factors and their regulation by activators. I begin with a brief discussion of bacterial transcription, which both evolutionarily and historically antedates transcription mechanisms in eukaryotes. I will then move to the main topic of the thesis, the transcription of genes by RNA polymerase II in eukaryotes.



## 2.0 Prokaryotic transcription mechanisms

### 2.1 Promoter specificity and sigma factor

*E. coli* RNA polymerase (RNAP) consists of a catalytic core of three subunits in an  $\alpha_2\beta\beta'$  configuration, in complex with various accessory factors. Core RNAP can transcribe DNA containing nicks or gaps, which serve as non-specific binding sites for the enzyme. However, the core enzyme can neither bind nor transcribe promoter DNA specifically (Burgess et al., 1969; Bautz and Bautz, 1971). In the cell, core exists bound to the specificity factor  $\sigma$ , which confers upon the resulting "holoenzyme" ( $E\sigma$ ) the ability to bind promoter DNA (Dunn and Bautz, 1969; Travers and Burgess, 1969). Differential gene transcription is accomplished at the level of RNAP promoter binding by two complementary mechanisms. First, there are multiple  $\sigma$  factors resulting in a population of holoenzymes with differing promoter specificities (for review see Gross et al., 1989). Second, promoter activity can be regulated by deviations from the optimum DNA sequence for binding a given class of holoenzyme. The affinity of holoenzyme for a given promoter is one factor which determines promoter activity (reviewed in von Hippel et al., 1992; see section 2.2).

$\sigma$  factors can be roughly divided into those related to *E. coli*  $\sigma^{70}$ , and those related to *E. coli*  $\sigma^{54}$ . Holoenzyme containing  $\sigma^{70}$ , the predominant  $\sigma$  in *E. coli*, binds promoters containing stereotypical sequences at -10 and -35 relative to the start site of transcription. In contrast, holoenzyme containing  $\sigma^{54}$  binds to promoters with radically different sequences located at -12 and -24 (Reznikoff et al., 1985; Doi and Wang, 1986). The ability of  $\sigma$  factors



to influence the promoter specificity of the otherwise untargeted core RNAP has obvious implications for the regulation of gene expression at the level of transcription. Synthesis by a cell of a  $\sigma$  factor with a given promoter specificity can result in the transcription of genes whose promoters are preferentially bound by holoenzyme containing that  $\sigma$ . If RNAP is limiting relative to  $\sigma$ , competition between classes of  $\sigma$  for binding to core can determine the relative levels of expression of genes recognized by these  $\sigma$ 's. The use of alternative  $\sigma$  factors as a mechanism of transcriptional control was first recognized in studies on gram-positive bacteria (Losick and Pero, 1981), but is now considered a general phenomenon in prokaryotes (Gross et al., 1992).

The canonical  $\sigma^{70}$  related sigma factor has been divided into four regions, some of which have been further subdivided, on the basis of inter-sigma amino acid sequence similarities. Regions 2.4 and 4.2 have been implicated in binding of the -10 and -35 regions of the promoter, respectively; region 2.1 has been implicated in binding of core RNAP (Helman and Chamberlin, 1988). These functions are recapitulated by multiple factors in eukaryotes, which often display sequence similarity to the functionally homologous regions of  $\sigma$  (McCracken and Greenblatt, 1991).

## **2.2 Formation of an open complex and production of a transcript**

$E\sigma^{70}$  binds to duplex promoter DNA in a temperature-independent step to form a "closed" complex, characterized by the formation of relatively short DNase I footprint over the promoter and the absence of any DNA melting (Kovacic, 1987; for review see



Gross et al 1992). A temperature dependent conformational change takes place within the holoenzyme resulting in a short-lived "intermediate complex" distinguished from the closed complex by an extended RNA polymerase footprint on the promoter (Mecbas et al., 1991). A further ATP-independent isomerization results in promoter melting between -5 and +5 to form the "open" complex (Sasse-Dwight and Gralla, 1988). The efficiency of isomerization to the open complex is the second step at which the overall transcriptional efficiency of a promoter can be regulated (see section 2.1). This type of regulation is carried to its logical extreme in the case of  $\sigma^{54}$ , which requires ATP and transcriptional activator proteins for open complex formation (Weiss et al., 1992). Protein-protein interactions between free core and  $\sigma^{70}$  are not as strong as those between  $\sigma^{70}$  and core within the open complex (Gill et al., 1991), indicating that the process of open complex formation results from changes both in protein-DNA and protein-protein interactions within the holoenzyme. Dynamic changes in the interactions of PIC components are also a hallmark of eukaryotic transcription.

Upon open complex formation, holoenzyme begins formation of 2-5 nucleotide abortive transcripts, 80-95% of which are not elongated, although polymerase remains bound to the promoter (Carpousis and Gralla, 1980; reviewed in von Hippel et al., 1992). In the case of transcripts which clear the promoter, elongation commences to approximately +8, at which point  $\sigma$  detaches from core and is replaced by the elongation and termination factor Nus A (Hansen and McClure, 1980). Both elongation and termination are highly regulated processes, albeit beyond the scope of this thesis.





In summary, transcription in bacteria takes place via a mechanism in which an active but non-specific polymerase is regulated by its interaction with a specificity factor. The recruitment of the enzyme complex to the promoter, and the dynamic changes within the complex, serve as points of regulation of the process. Although the components are more numerous in eukaryotes, the concepts are similar.



### **3. Transcription of Eukaryotic Genes**

#### **3.1 Three Polymerases**

Transcription in eukaryotes follows the same general pathway (promoter recognition, polymerase binding, initiation, promoter clearance) as it does in prokaryotes. However, certain differences are immediately apparent. Unlike prokaryotes, eukaryotes contain three RNA polymerases, designated pol I, II, and III. Pol I carries out the transcription of ribosomal RNA genes, while pol III is involved in the transcription of tRNA genes, short RNAs, and certain viral RNAs (reviewed in Roeder, 1976). RNA polymerase II carries out the transcription of all mRNA-encoding (class II) genes in the cell. The transcription of class II genes is the subject of the rest of this chapter.

#### **3.2 General Overview of Class II Gene Transcription**

Class II promoters consist of a relatively conserved core promoter and variable proximal and distal sequences. The core, comprised of a TATA box and/or an initiator element, serves as an assembly site for the general transcription factors, a collection of about 50 polypeptides whose function is to mediate transcription of all class II genes (reviewed in Roeder, 1991; Zawel and Reinberg, 1993). The variable proximal sites (and their distal counterparts, the enhancers) serve as binding sites for regulatory factors generically referred to here as "activators" but also including gene-specific repressors (Ptashne 1986). Since the general transcription factors comprise the RNA-making machinery of the cell, they must serve as the ultimate functional targets of activators.



The general transcription factors, including pol II, support core promoter transcription *in vitro*. They do this by binding core promoter DNA elements, assembling a multiprotein PIC through an ordered assembly process, and catalyzing further steps required for initiation and promoter clearance (reviewed in Roeder, 1991; Zawel and Reinberg, 1993). This thesis deals with the cloning (Chapter IV) and biochemical analysis (Chapters IV and V) of one general transcription factor, TFIIA. A brief analysis of activator function (Chapter III), which, together with other work, implicates TFIIA as a component of a subcomplex which serves as an activator target, precedes the work on TFIIA. The effort to characterize TFIIA derives from an ongoing effort to characterize eukaryotic PIC components and assembly pathways.

## **4.0 General Transcription Factors and Basal Transcription**

### **4.1 History: Multiple factors, Multiple steps**

Purified RNA polymerase II can neither bind nor transcribe promoter DNA. The development of a soluble extract capable of accurately transcribing purified promoter DNA-containing templates in the presence of exogenous pol II demonstrated that there were accessory factors required for promoter specificity, and suggested that these factors could be purified (Weil et al., 1979). The response of transcriptionally active extracts to exogenous pol II indicated that these factors could associate with the enzyme and form an active complex. These studies also established that the required factor(s) functioned at the initiation stage by regulating the specificity of the enzyme, rather than by influencing processing of a



randomly initiated RNA product (Weil et al., 1979). Fractionation of transcriptionally active extracts on Phosphocellulose (P11) resolved the required cofactors into four fractions, designated class II Transcription Factor A-D(TFIIA-TFIID) (Matusi et al, 1980). While TFIIC was shown not to be a true initiation factor (Slattery et al, 1983), the others proved relevant and ultimately were resolved into a number of factors including TFIIA, IIB, IID, IIE, IIF, IIG/J, and IIH (reviewed in Roeder, 1991; Zawel and Reinberg, 1993).

Concomitant with the recognition of a requirement for multiple biochemically separable factors was the delineation of multiple steps in the initiation pathway. The process of initiation could be functionally resolved into several steps based on differing sensitivities to the detergent sarkosyl at different stages of complex assembly (Hawley and Roeder, 1985; 1987). At least one step appeared to be ATP dependent (Bunick et al., 1982; Sawadogo and Roeder, 1984). The identification of multiple functionally resolvable steps was paralleled by the identification of multiple physically resolvable steps in PIC assembly. Partial PICs could be assembled from defined fractions, allowing the delineation of an order of addition of factors to form discrete complexes resolvable on native gels (Buratowski et al., 1989; Maldonado et al., 1990). The process of PIC assembly thereby came to be seen as the end result of a progression of discrete complexes assembled in an ordered process by the addition of multiple factors to core promoter DNA. This process, as well as the components which make up the PIC, is described in the sections that follow.

## **4.2 Assembly of the PIC and Core Promoter Transcription**





### 4.2.1 TFIID

The general factor TFIID binds to the TATA box, and nucleates the assembly of the PIC on the core promoter (Parker and Topol, 1984; Sawadogo and Roeder, 1985; Van Dyke et al, 1988; Nakajima et al., 1988). Initially identified as one of four P11 fractions required for specific transcription by pol II (Matsui et al., 1980), human and *Drosophila* TFIID has since been extensively purified by conventional (Nakajima et al., 1988) and affinity methods (Dynlacht et al, 1991; Tanese et al, 1991; Takada et al, 1992; Zhou et al., 1992; Chiang and Roeder, 1993; Chiang et al 1993; reviewed in Hoffmann, 1994). TFIID consists, in the case of human cells, of a 43 kD TATA binding subunit (TBP), the cDNA for which has been isolated (Hoffmann et al., 1990a; Peterson et al 1990; Kao et al., 1990), and a set of at least 10 TBP- associated factors (TAFs) ranging in molecular weight from 20 to 250 kD (Tanese et al., 1991; Takada et al., 1992; Zhou et al, 1992; Chiang et al., 1993). Isolated recombinant TBP mediates core promoter transcription *in vitro*. However, the full TFIID complex is required for activator function *in vitro* (see section 5.1; Hoffmann et al , 1990; Peterson et al., 1990; Hoey et al., 1990). The specific role of TAFs in core promoter transcription is not certain; some may mediate sequence specific DNA binding by TFIID on TATA-less promoters (Verrijzer et al., 1994; Martinez et al., 1994). Because of its ability to mediate class II core promoter transcription, and its role in transcription of class I and III genes (reviewed in Sharp, 1993), TBP has been extensively studied separate from the TAFs with which it is apparently always complexed *in vivo* (Hoffmann, 1994).



#### 4.2.1.1 TBP

Comparison of the predicted amino acid sequence of human TBP, and those of TBP from *Saccharomyces cerevisiae* (Schmidt et al., 1989; Cavallini et al., 1989; Horikoshi et al., 1989; Hahn et al., 1989b, Eisenman et al., 1989), *Drosophila melanogaster* (Hoey et al., 1990; Muhich et al., 1990), *Arabidopsis thaliana* (Gasch et al., 1990) and *S. pombe* (Hoffmann et al., 1990, Fiekes et al., 1990) reveals a variable N-terminus and a highly conserved (>80%) C-terminus containing two direct repeats (Hoffmann et al., 1990). Recombinant TBP initiates PIC assembly by binding to the TATA element, forming a protein-DNA complex which, under optimum conditions, is stable to electrophoresis (Horikoshi et al., 1989; Peterson et al., 1990; Meisterernst and Roeder, 1991). TBP interacts physically with at least 38 other proteins including general transcription factors, activators, viral proteins, and TAFs (reviewed in Hoffmann, 1994; also see section 5). The domains of TBP which mediate some of these interactions have been identified by mutagenesis (see eg. Lee et al., 1992; Buratowski and Zhou, 1992). However, the functional relevance of many of these interactions remain unproven.

The crystal structure of TBP bound to the minor groove of DNA has been solved, revealing a nearly symmetrical saddle-shaped protein bound to severely bent and partially unwound DNA (Nikolov et al., 1992; Kim et al., 1993a; Kim et al., 1993b). The bending, which was predicted by circular permutation analysis prior to the solution of the structure (Horikoshi et al., 1992), may facilitate the interaction of proteins bound upstream of TATA with those bound at the start site or further downstream. Furthermore, the crystal structure of



TBP reveals several surfaces that could conceivably be involved in protein-protein interactions. This multi-surface structure may reflect a role for TBP in nucleation of PIC assembly by both initial promoter recognition and multiple protein-protein interactions with other PIC components.

#### **4.2.2 TFIIA**

The binding of TFIID or TBP to the TATA element is facilitated by TFIIA (Reinberg et al., 1987; Buratowski et al., 1989), which interacts in solution both with TBP (Usuda et al., 1991; DeJong and Roeder, 1993) and TFIID (Ha et al., 1993; Yokomori et al., 1993). TFIIA, originally identified as an activity in the P11 flow-through fraction required for specific RNA polymerase II transcription (Matsui et al., 1980), stimulates core promoter transcription when purified TFIID is used as the source of TBP (Reinberg et al., 1987; Yokomori et al., 1994). It has been purified from yeast as a complex of 43 and 12 kD subunits (Ranish and Hahn, 1991), and from mammalian sources as heteromeric complexes of varying subunit compositions (Samuels and Sharp, 1986; Usuda et al., 1991; Waldschmidt and Seifart, 1992; Cortes et al 1992; Coulombe et al 1992; DeJong and Roeder, 1993; Ha et al., 1993). In part, the subunit heterogeneity of various TFIIA preparation may reflect the fact that the 19 kD subunit stains negatively with silver (DeJong and Roeder, 1993). Renaturation experiments have shown that the ability of human TFIIA to interact stably with the TBP-TATA complex is dependent upon three subunits of 35, 19, and 12 kD (Cortes et al 1992). Consistent with this, TFIIA eluted from an immunopurified



*Drosophila* TFIID complex displayed subunits of 30, 19 and 14 kD (Yokomori et al., 1993).

The requirement for TFIIA in core promoter transcription has been variable (reviewed in Conaway and Conaway, 1993), due both to TFIIA contamination of the TFIID fraction (Yokomori et al., 1993) and a conditional requirement for the factor (see below).

Interestingly, TFIIA is required for transcription using crude P11 fractions (Matsui et al, 1980), is not required in transcription systems reconstituted with partially purified fractions (Sawadogo and Roeder, 1985), but is required when highly pure TFIID was used (Reinberg et al, 1987). These results indicate that the TFIIA requirement is dependent upon the relative concentration of other general factors, and in addition is consistent with the idea that TFIIA is present as a contaminant in crude TFIID preparations.

It is now clear that TFIIA is not essential for transcription in highly purified systems reconstituted with TBP (Cortes et al., 1992; Sayre et al., 1992). In more physiologic (ie less purified) transcription systems reconstituted with TFIID, TFIIA may stimulate transcription by preventing inhibition of PIC assembly by TBP-interacting factors such as NC-1, NC-2, and TAFs (Meisterernst and Roeder, 1991; Meisterernst et al., 1991; Cortes et al., 1992; Inostroza et al., 1992; Kokubu et al., 1993). Consistent with this, high levels of induction by gene-specific activators in vitro requires both TFIIA and these inhibitory factors (Meisterernst et al., 1991; see section 5.2.2).

In contrast to the case for TFIIA from higher eukaryotes, yeast TFIIA (Hahn et al, 1989a) consists of two subunits of molecular weights 32 and 13.5, respectively (Ranish and Hahn, 1991). Genes





encoding both the large (TOA1) and small (TOA2) subunits of yeast TFIIA have been isolated and shown to be essential for viability (Ranish et al., 1992). The TOA1 and TOA 2 gene products, when corenated, reconstitute both TBP-dependent promoter complex formation and transcriptional activities of yeast TFIIA (Ranish et al., 1992). Because no cDNAs encoding TFIIA from higher eukaryotes have been isolated, reconstitution of active TFIIA from higher eukaryotes has not yet been possible.

TFIIA forms a TBP-dependent stable promoter complex (Maldonado et al., 1990) dependent upon the so-called "basic region" of TBP, which consists of basic residues in the conserved helix 2 of TBP (Lee et al., 1992; Buratowski and Zhou, 1992; Nikolov et al., 1992). TFIIA seems to stimulate TBP DNA binding by two complementary mechanisms: first TFIIA may induce structural changes in TBP which can be mimicked by removal of the non-conserved N-terminus in the case of yeast TBP; second, TFIIA relaxes the requirement of TBP for DNA sequences flanking the TATA element (Lee et al., 1992). The stimulation of TBP binding by TFIIA is most dramatic under conditions which are non-optimal for TBP DNA binding (Imbalzano et al., 1994).

TFIIA can enter the PIC at any stage of complex assembly (Cortes et al., 1992), suggesting that there may be protein-protein interactions of TFIIA with general transcription factors other than TBP. It has not been determined which subunit(s) of TFIIA mediate the interaction with TBP or with TFIID, nor have other protein-protein interactions of TFIIA been documented. Further analysis of TFIIA requires the isolation of cDNAs for the subunits of TFIIA and



the reconstitution of active TFIIA from recombinant components. This work is presented in chapters IV and V.

### **4.2.3 TFIIB**

TFIIB enters the nascent PIC by interacting directly with the TBP-DNA complex (Buratowski et al., 1989; Zawel and Reinberg, 1993). Human TFIIB consists of a single 33 kD polypeptide (Malik et al., 1991; Ha et al., 1991). The structural organization of TFIIB is similar to that of TBP in its possession of two direct repeats and homology to RNA-polymerase binding regions of  $\sigma$  factor (Malik et al., 1993). It functions to recruit pol II to the PIC via an interaction with the polymerase-associated 30 kD subunit of TFIIF (Flores et al., 1991). Consistent with this bridging function, TFIIB contains a protease-resistant C-terminal core which mediates TBP binding, and a protease sensitive N-terminal domain which mediates an interaction with pol II (Hisatake et al., 1993; Malik et al., 1993). Polymerase binding induces structural changes in promoter-bound TFIIB (Malik et al., 1993).

### **4.2.4 Pol II and TFIIF**

TFIID (or TBP), TFIIA and TFIIB can form a promoter-bound complex in vitro which is capable of recruiting RNA polymerase II (pol II). Pol II consists of over 10 subunits ranging in molecular weight from 240 to 12.6 kD (Roeder, 1976; Kolodziej et al., 1990; Sawadogo and Sentenac, 1990). The largest subunit contains a C-terminal domain (CTD) consisting of repeated heptamers (Allison, et al., 1984; Cadena and Dahmus, 1987; Corden, 1990) essential for cell viability (Allison et al., 1988; Bartolomei et al., 1988; Zehring et al., 1988). The CTD is found in vivo in either a hyperphosphorylated



(IIo) or unphosphorylated state (IIa). Direct physical analysis of promoter complex formation (Lu et al., 1991), chemical crosslinking of initiation and elongation complexes (Cadena, et al 1987; Bartholemew et al., 1986), and monoclonal antibody blocking experiments (Laybourn and Dahmus, 1990; Moyle et al., 1989) all indicate that it is the unphosphorylated form of the enzyme that associates preferentially with the nascent PIC; the phosphorylated form is found predominantly in elongation complexes. These observations suggest that the phosphorylation of the CTD is involved in the conversion of the initiation complex to an elongation complex.

Association of pol II with the PIC is dependent upon TFIIF (Flores et al., 1989). TFIIF consists of 30 and 74 kD subunits (also known as RAP 30 and RAP 74, respectively) which both bind directly to pol II (Flores et al 1989; Burton et al., 1988). The 30 kD subunit, which bears functional homology to the polymerase-interacting domain of *E. coli*  $\sigma$  70 (McCracken and Greenblatt, 1991) mediates the interaction of pol II with the DAB complex. RAP 74 has been variously implicated in transcription initiation, promoter clearance, and transcriptional activation by gene-specific factors such as SRF (Goodrich and Tjian, 1994; Zhu et al., 1994).

#### **4.2.5 Further Complex Assembly**

TBP, TFIIB, TFIIF and pol II constitute a "minimal complex" capable of mediating promoter-specific transcription initiation in a dinucleotide-primed abortive initiation assay (Goodrich and Tjian, 1994). Clearance of the promoter by pol II subsequent to initiation requires either negative supercoiling of the template or,



alternatively, the addition to the complex of TFIIE and TFIIH and the hydrolysis of ATP (Goodrich and Tjian, 1994). TFIIH (Flores et al., 1992), also known as BTF 2 (Gerard et al., 1991), factor  $\delta$  (Conaway and Conaway, 1989) or yeast factor b (Feaver et al., 1991), is a complex of greater than 5 polypeptides which contains helicase, ATPase and CTD-kinase activities (Fisher et al., 1992; Lu et al., 1992; Schaeffer et al., 1993; Serizawa et al., 1992, 1993). One of the polypeptides comprising TFIIH is ERCC3, a protein involved in DNA repair which has been implicated in the genetic skin disorder Xeroderma pigmentosum (Schaeffer et al., 1993; reviewed in Drapkin and Reinberg, 1993). In addition, a cDNA encoding the 62 kD subunit of human TFIIH has been isolated (Fischer et al., 1992). Phosphorylation of the pol II CTD by TFIIH may enhance promoter clearance by releasing the CTD from an interaction with promoter-bound TBP (Usheva et al., 1992). The TFIIH helicase activity is also likely to be involved in this process, given the fact that negatively supercoiled promoter DNA can serve as a substrate for promoter clearance in the absence of TFIIH. Promoter clearance may therefore require partial unwinding of the promoter, facilitated by either negative superhelical strain or TFIIH.

TFIIH CTD-kinase activity is stimulated strongly by TFIIE (Lu et al., 1992; Ohkuma and Roeder, 1994; Goodich and Tjian, 1994), a tetrameric protein consisting of 34 and 56 kD subunits in an  $\alpha_2\beta_2$  configuration (Ohkuma et al., 1991; Sumimoto et al., 1991; Inostroza et al., 1991; Peterson et al., 1991). The TFIIE-regulated phosphorylation of the CTD by TFIIH may account for the ATP requirement for transcription initiation in vitro (Bunick et al., 1982;





Sawadogo et al., 1984), as ATP addition to the PIC causes a change in the mobility of the complex consistent with the dissociation of TFIIE and TFIIH (Goodrich and Tjian, 1994). The energy of ATP may also be required to mediate open complex formation (Wang et al., 1992a); while it is known that this step takes place after DAB complex formation (Wang et al., 1992b), the specific factor requirements for this process are unknown.

One general transcription factor that has not been well characterized beyond its original description is TFIIJ. Originally separated chromatographically from TFIIA (Cortes et al., 1992), TFIIJ has been reported to strongly stimulate TBP-mediated transcription, but only weakly stimulate TFIID mediated transcription (Cortes et al., 1992), raising the possibility that it may be weakly associated with TFIID (Cortes et al., 1992). TFIIJ may be identical to TFIIG (Sumimoto et al., 1990; Y. Ohkuma, personal communication). TFIIJ has not been fully characterized, and its role in transcription has not been definitively established.

#### **4.2.6. Recent Advances in Core Promoter Transcription**

An alternate pathway for PIC assembly dependent on the "initiator" DNA element has been described in which a 120 kD initiator(Inr)-specific DNA binding protein, TFII-I, facilitates TBP or TFIID binding to the TATA element and obviates the functional requirement for TFIIA (Roy et al., 1993). A TBP-TFII-I promoter complex can incorporate TFIIB to form a higher order transcription complex, suggesting the existence of multiple physical pathways for PIC assembly. Consistent with this, transcription of core promoters containing an Inr element was shown to be dependent upon either



TFII-I or TFIIA, whereas transcription of promoters lacking an initiator element could not be activated by TFII-I (Roy et al., 1993).

There is longstanding evidence that several general transcription factors, including RNA polymerase II, TFIIB, and TFIIE, can form complexes in the absence of promoter DNA, as measured by coelution from glycerol gradients (Reinberg and Roeder, 1987). Consistent with these early observations, a multi-subunit holoenzyme has been isolated from yeast cells (Koleske and Young, 1994). Holoenzyme consists of Pol II in complex with TFIIF, TFIIH, TFIIB, and SRB2,4,5, and 6 (Koleske and Young, 1994). This form of RNA Pol II is competent to mediate both basal and activated transcription, dependent only upon exogenous TBP and TFIIE (Koleske and Young, 1994). Whether the "holoenzyme" represents a PIC that had formed *in vivo* in a stepwise manner and then dissociated, or a true PIC precursor is not currently known. Furthermore, the existence in the human system of separable factors which reconstitute transcription indicates that the multiple factor/multiple step model of PIC assembly is valid. For these reasons, the stepwise assembly model of PIC formation will serve as the conceptual framework throughout this thesis.

The PIC assembly pathway described above results in core promoter transcription (also called basal transcription) *in vitro*. However, *in vivo*, basal transcription is regulated by a set of gene-specific activators (or repressors) which, directly and indirectly, regulate the factors and assembly steps described above. This



process is central to the success of organisms and has been the focus of considerable experimental attention; it is described in the sections below.

## **5.0 Activation of Transcription**

Gene-specific transcription is regulated by a large and diverse set of proteins which bind the variable (proximal and distal) sequence elements of their target genes in a sequence specific manner (reviewed in Mitchell and Tjian, 1989; Ptashne and Gann, 1991).

These activator proteins consist of separable DNA binding and transcriptional activation domains (Keegan et al., 1986). The genetic specificity of a transcriptional activator can be altered by fusion to a heterologous DNA binding domain (for review see Ptashne, 1988; Brent and Ptashne, 1985; Ma and Ptashne, 1987). In particular, the DNA binding region of the yeast activator Gal 4 can be fused to heterologous activation domains, resulting in hybrid activators which can stimulate transcription from multiple Gal4 binding sites located upstream of a core promoter (Keegan et al., 1986; Ma and Ptashne, 1987a; Ma and Ptashne, 1987b).

The ability to study transcription activation domains in isolation from variations in DNA binding specificity (Ma and Ptashne, 1987a) has allowed the delineation of several different structural families of activation domains (reviewed in Mitchell and Tjian, 1989). Acidic activation domains, found in the yeast proteins Gal4 (Brent and Ptashne, 1985), and GCN4 (Hope and Struhl, 1986), the Herpesvirus activator VP16 (Cress and Triezenberg, 1991), the pseudorabiesvirus IE protein (Martin et al., 1990) and several others, have been especially well studied mechanistically (see below).



Glutamine-rich activation domains have been found in the activator Sp1 (Courey and Tjian, 1988) and are presumed to exist in several other activators (Mitchell and Tjian, 1989). Finally, a proline-rich activation domain family has been defined in studies on CTF/NTF-1 (Mermoud et al., 1989). Similar regions have been noted in numerous other activators (Mitchell and Tjian, 1989).

## **5.1 Mechanism of Transcriptional Activation**

In vitro, activators increase the number or stability of functional PICs (White et al., 1992; Johnson and Krasnow, 1993) by interacting with a partial PIC (Hai et al., 1988; White et al., 1992) and increasing either factor recruitment, stability, or function. They do not increase the rate of complex formation in vitro (White et al., 1992). In the context of a cell with only one (or a few) copies of a target gene, activators must function by increasing the number of functional PIC's formed per unit time, or, equivalently, increasing the stability of a single PIC, allowing several rounds of transcription from a given PIC. Several PIC components have been implicated biochemically as functional targets of activation domains. We review below the evidence for the involvement of various PIC components in activation.

## **5.2 Factor requirements for activation suggest the roles for TFIID, TFIIA, and cofactors**

### **5.2.1 TFIID is a Target of Activators**

The general transcription factors which mediate core promoter transcription in vitro are insufficient to mediate stimulation by gene-specific regulatory proteins (Pugh and Tjian, 1990; Hoffmann et al., 1990). Several additional factors are required for this process





(Meisterernst et al., 1991). The finding that some of these factors are physically associated with TBP in the purified TFIID complex reinforced a body of evidence which had previously implicated TFIID as a target of regulatory factors

TFIID was implicated as an activator target by the finding that the adenovirus major late promoter transcription factor USF interacts physically with promoter-bound TFIID such that there is cooperative binding of these two DNA binding proteins to their respective sites on the Adenovirus 2 Major Late promoter (MLP) (Sawadogo and Roeder, 1985; Van Dyke et al., 1988). Consistent with the idea that activators have an effect on TFIID-promoter interactions, the activators ATF and Gal4-AH have both been shown to extend the DNA footprint pattern of a highly pure  $\omega$ -amino octyl TFIID fraction on the adenovirus E4 promoter (Horikoshi et al., 1988 a,b). This extended footprint persists even if the activator is subsequently competed off the template by an oligonucleotide (Horikoshi et al., 1988 a,b). The altered conformation of TFIID in the presence of an activator facilitated later steps of PIC assembly. These findings suggest that activators may work by increasing either the stability or activity of TFIID by causing a conformational change in TFIID resulting in stabilized binding of TFIID, or in a complex with increased affinity for other general transcription factors.

Studies of the pseudorabies virus immediate early (IE) protein also implicate TFIID as an activator target. IE stimulation of transcription from the Major Late Promoter is maximal at low template concentrations, suggesting that IE functions by increasing the extent or rate of some general factor's association with the



template (Abmayr et al., 1985). Pre-incubation of a crude TFIID fraction with the template increases basal transcription and obviates IE activation, implicating TFIID promoter binding as a functional target of IE (Abmayr et al., 1988). This observation was extended in studies which allowed competition for promoter DNA between histones and basal transcription factors. IE could stimulate transcription in this system only if it was allowed to interact with promoter-bound TFIID prior to nucleosome assembly (Workman et al., 1988). These studies suggested that IE functioned by increasing the rate of TFIID binding to the promoter, indicating that there is a functional interaction of IE and TFIID. This interaction results in a potentiated TFIID-promoter complex, preventing the inhibition of transcription by nucleosome assembly.

The study of TFIID as a target of activators was advanced by the cloning and functional analysis of the derived TATA-binding subunit of TFIID (TBP). In contrast to TFIID (Sawadogo and Roeder, 1985), the isolated TBP subunit was shown to be unable to mediate transcriptional activation (Pugh and Tjian, 1990; Hoffmann et al., 1990). This finding, together with the discrepancy in native size between TFIID and TBP, suggested that transcriptional activation required the presence of TBP-associated factors (TAFs), which have since been isolated from TBP-containing complexes (Dymlacht et al., 1991; Tanese et al., 1991; Zhou et al., 1992; Takada et al., 1992; Chiang et al., 1993). TAFs can, under some circumstances, reconstitute the TFIID activation function in conjunction with TBP (Weinzierl et al., 1993; Chen et al., 1994).



TAFs have been shown to serve as physical and functional targets of activators. Specifically, a *Drosophila* TAF with molecular weight 110 (dTAF<sub>II</sub>110) binds to the glutamine-rich activation domain of the activator Sp1 (Hoey et al., 1993); dTAF<sub>II</sub>40 binds the acidic activator VP16 (Goodrich et al., 1993); the Ile-rich activation domain of the activator NTF-1 interacts with dTAF<sub>II</sub>150 and dTAF<sub>II</sub>60 (Chen et al., 1994). As an indicator of the functional relevance of these interactions, reconstitution of functional TFIID subcomplexes with recombinant TAFs has demonstrated specific TAF requirements for specific activators which correlate with activator-TAF physical interactions (Chen et al., 1994). The specific mechanism by which TAFs contribute to the activation process remains unknown. Possibilities include mediation of direct protein-protein interactions between activators and the PIC via TFIID (Chen et al., 1994), participation in the assembly of TFIID-TFIIA-activator complexes (Lieberman and Berk, 1994), and facilitation of activator-stimulated late steps of PIC assembly (Choy and Green, 1993; Kim and Roeder, 1994b).

Numerous studies suggest that the TBP subunit itself may be a target of multiple activators including VP16, E1a, Zta, PU.1, and p53 (Stringer et al., 1990; Lee et al., 1991; Horikoshi et al., 1991; Lieberman and Berk, 1991; Hagemeyer et al., 1993; Truant et al., 1993; Boyer and Berk, 1993). For example, a VP16 activation domain affinity column binds TFIID (Stringer et al., 1990), as well as TBP (Ingles et al., 1991). Mutations in VP16 which inactivate this TBP-interaction correspondingly impair VP16 transcriptional activation (Ingles et al., 1991). Furthermore, a yeast TBP mutant deficient for



VP16 interaction correspondingly fails to mediate transcriptional activation in a reconstituted yeast transcription system (Kim et al., 1994b). These experiments suggest that the interaction between VP16 and TBP is necessary for activation, and raises the possibility that VP16 may activate transcription by increasing TBP recruitment to the promoter.

However, the physical interaction between TBP and VP16 is not sufficient for activation. This is indicated by studies of another yeast TBP mutant which interacts with VP16 as strongly as wild type, but which is impaired in mediation of VP16 transcriptional activation. This impairment seems to be due to an inability to mediate activator-dependent TFIIB recruitment, or to an inability to form an activator-stabilized TFIIB complex (Kim et al., 1994b). Therefore, the physical interaction between VP16 and TBP may reflect a role of VP16 in a later step of PIC assembly that is mediated through TBP (or TFIID).

To summarize, TFIID is the first core promoter factor to bind the promoter, and the binding, conformation, and subsequent activities of TFIID are all subject to regulation by activators. This regulation potentially takes place through direct physical interactions between activators and the TBP subunit and/or TAFs. These interactions may result in conformational changes of both TFIID and TBP which increase the stability of TFIID-promoter interactions, or may indirectly stabilize higher order complexes.

### **5.2.2 TFIIA and Negative Cofactors**

Like TAFs, TFIIA is required for high levels of transcriptional activation (Meisterernst et al., 1991; DeJong and Roeder, 1993) but has no effect on basal transcription reconstituted with TBP and





highly purified general factors (Sayre et al., 1992; Cortes et al., 1992). TFIIA may serve an anti-repression function in activation by overcoming the inhibitory effects of a set of negative cofactors which selectively inhibit basal transcription (Meisterernst and Roeder, 1991). These factors, including NC1, NC2, Dr1, Dr2, and MOT-1/ADI, prevent the assembly of TBP into functional PIC's in a manner overcome by TFIIA (Meisterernst and Roeder, 1991; Inostroza et al., 1992; Merino et al., 1993; Auble and Hahn, 1993; Ge and Roeder, 1994). The requirement for these negative cofactors and TFIIA for maximum levels of activation in vitro suggests that activators may work in part by preventing inhibition of PIC assembly by these factors (reviewed in Roeder, 1991). Since TFIIA can overcome the effects of these inhibitors, it is possible that activators favor the inclusion of TFIIA over that of inhibitors into the PIC.

A direct role of TFIIA as an activator target is suggested by experiments in which prior formation of a DA complex could substitute for, and partially obviate the effect of, the activators Sp1 (Meisterernst et al., 1991) and ZEBRA (or Zta) (Chi and Carey, 1993). Consistent with this, the activators Gal4-AH and Zta have both been shown to facilitate the formation of a promoter complex consisting of the activator, TFIIA and TFIID (Wang et al., 1992; Chi and Carey, 1993; Lieberman and Berk, 1994). These experiments all implicate TFIIA as a possible target of regulatory factors. This targeting may involve antirepression mechanisms (ie overcoming the effect of negative cofactors), and true activation (ie stabilization of the PIC). Consistent with the idea that TFIIA is a target of activation domains,



Ozer et al (1994) have shown that Zta directly interacts with human TFIIA.

### 5.2.3 Positive Cofactors

Physiologic levels of activation in vitro also require a set of Positive Cofactors (PC), which raise the absolute level of transcription in the presence of activators. These factors include PC 1, 2, 3, and 4 (Meisterernst et al., 1991; Kretzschmar et al., 1992; 1993; 1994; Merino et al., 1993; Ge and Roeder, 1994). One such factor, PC3 (Dr2) is identical to Topoisomerase I (Kretzschmar et al., 1993; Merino et al., 1993), although the topoisomerase activity is dispensable for its function in transcription (Merino et al., 1993). The precise mechanism by which positive cofactors function is unknown. However, PC4 has been shown to interact both with several activation domains and a TBP-TFIIA complex (Kretzschmar et al., 1994; Ge and Roeder, 1994). This observation suggests a role for PC4 in mediating an interaction between activators and the general factors via TFIIA.

An activity functionally analogous to the PC's, which confers upon a highly purified transcription system the ability to respond to the activators Gal4-VP16 and GCN4 has been isolated from yeast cells (Kim et al., 1994). Termed "mediator" (Kim et al, 1994c), this factor is a multisubunit complex consisting of TFIIF and at least nineteen other polypeptides, some of which had previously been implicated genetically in the process of transcriptional activation (Koleske et al., 1992; Thompson et al., 1993). In contrast to the positive cofactors isolated in the human system, mediator seems to increase both basal and activated transcription (Kim et al., 1994c).



Genetic studies in yeast have implicated several additional proteins as cofactors including Ada2 (Berger et al., 1992), Sug-1 (Swaffield et al., 1992), and Gal 11 (Himmelfarb et al., 1990; reviewed in Sakurai et al., 1993). Several of these proteins are components of mediator. Interestingly, physiologic levels of activation are observed with mediator in yeast transcription systems reconstituted without TFIIA. However, TFIIA depletion from yeast nuclear extract causes a 10-fold decrease in transcription (Kang et al, 1995). Therefore, there may be both TFIIA-dependent and TFIIA-independent activation pathways in yeast, raising the (as yet unanswered) question of whether transcriptional activation in the purified transcription system reflects a physiologically relevant TFIIA-independent process.

### **5.3 TFIIB is an Activator Target**

TFIIB was implicated as a potential target of acidic activators by the use of a solid-phase transcription assay, in which partial preinitiation complexes were assembled in the presence of an activator on a template linked covalently to an insoluble support (Lin and Green, 1991). These assays suggested that acidic activators functioned by increasing the recruitment or stability of TFIIB via a direct activator-TFIIB interaction (Lin and Green, 1991; Lin et al., 1991). Interestingly, activation-deficient TFIIB mutants have been isolated which have a correspondingly decreased affinity for VP16, strengthening the idea that TFIIB is a bonafide activator target (Roberts et al., 1993). Activator function via the step of TFIIB recruitment has also been demonstrated with the proline-rich activator CTF1 (Kim and Roeder, 1994), suggesting that this



mechanism is not restricted to acidic activators. Recent results suggest the possibility that the effect of activators on TFIIB incorporation into the PIC could be due to a conformational change within TFIID or TBP, or to an activator-dependent stabilization of a TBP-TFIIB complex (Kim et al., 1994b).

The effect of activators on TFIIB recruitment is not dependent upon TAFs. However, activation of transcription in the human system requires TAFs (Choy and Green, 1994). This suggests that the activator-TFIIB interaction is not sufficient for transcription activation, and that activation requires TAF function at some other step of PIC assembly. Consistent with this, TAFs have been shown to facilitate an as yet unknown activator-dependent step of PIC assembly which occurs (in this assay) subsequent to TFIIB incorporation (Choy and Green, 1994; Kim and Roeder, 1994).

## **6.0 Summary**

Transcriptional activators seem to interact with multiple targets within the PIC. These interactions lead, via an unknown mechanism, to an increase in the number of PICs on activated genes *in vitro*, and presumably to an increase in the number of PIC's formed per unit time on a single target gene *in vivo*. None of the mechanisms of activation described above are mutually exclusive. In fact, given the phenomenon of transcriptional synergy (Herschlag and Johnson, 1993), it is possible that one activator can function by several different mechanisms simultaneously.





When I entered the laboratory, I began my study of transcription by performing experiments designed to narrow down possible targets of the activator Gal4-IE. These experiments, which implicated TFIIB, and, indirectly, TFIIA and TFIID as important for activation, are described in Chapter III. Following those studies, I turned my efforts to cloning and reconstitution of TFIIA (Chapter IV), and finally to an analysis of the protein-protein interactions of TFIIA (Chapter V).



## **Chapter II: Materials and Methods**

Procedures used in the experiments described in this thesis are arranged here by Chapter. Except where noted, all molecular cloning techniques (ie restriction enzyme digestions, ligations, transformations) were performed according to standard protocols in Sambrook et al., (1989). Buffer C (BC) refers to 20 mM Tris (pH 7.9 @4<sup>o</sup> ), 0.2 mM EDTA, 20 % Glycerol (v/v), plus the indicated mM KCl.

## **Chapter III: Transcription Activation by Gal4-IE**

### **Purification of Gal4-IE**

The expression plasmid pG4-IE, which expresses Gal4 (1-147)-IE(1-34) from the bacterial pTAC promoter, was constructed by cloning the Hpa I-Xba I fragment of pGal4 (1-147)-IE (1-34) (Martin et al., 1990) into the large Hpa I-Sma I fragment of pTGH 21 (Ma and Ptashne, 1987a). This plasmid was transformed into *E. coli* strain XA90, and the resulting transformant used to overexpress Gal4-IE as described for Gal4-AH (Lin and Green, 1988). Gal4-IE was purified identically to Gal4-AH (Lin and Green, 1988), except that it was eluted from the DE-52 column with a NaCl gradient.

### **Gel Filtration of Nuclear Extracts**

HeLa nuclear extract (Dignam et al., 1983) was gel-filtered at 4<sup>o</sup>C on a Pharmacia PD-10 column according to the manufacturers instructions. The excluded volume from the column was aliquoted and stored at -70<sup>o</sup>C.

### **Transcription Assays**

Transcription of template G5E1bCAT in HeLa nuclear extract and detection of the transcript by primer extension was performed as



described (Lin et al., 1988). Run-off transcription using 100 ng of Sma I digested template G5HMC2AT (Ge and Roeder, 1994), or 200 ng pML(l) (Figures 9,11), was performed in 25  $\mu$ l reactions containing 75  $\mu$ g HeLa nuclear extract protein (Dignam et al., 1983) 20 mM Hepes (pH 8.6), 50-100 mM KCl, 12% Glycerol, 5 mM DTT, 6 mM MgCl<sub>2</sub>, 12 mM Tris (pH 7.9 @4<sup>0</sup>), 600  $\mu$ M ATP, CTP and O-methyl-GTP, 25  $\mu$ M UTP, 0.5  $\mu$ l  $\alpha$ P<sup>32</sup>-UTP, and 5 pmol Gal4-IE, where indicated. Proteins, template, and buffers were assembled on ice, mixed, preincubated at 30<sup>o</sup> for 15 minutes, then mixed with NTPs and radionucleotides, and further incubated at 30<sup>o</sup> C for 1 hour. Reactions were stopped by addition of 100  $\mu$ l STOP buffer (20 mM EDTA (ph 8.0), 0.2 M NaCl, 1% SDS, 1.25 mg/ml yeast tRNA) and 300  $\mu$ l 0.3 M sodium acetate, extracted with two volumes of Phenol:Chloroform:Isoamyl alcohol (Sambrook et al, 1989), precipitated with ethanol, and centrifuged. The pellets were washed with ice-temperature 70 % ethanol, resuspended in formamide loading buffer, and run on 50 % Urea / 5 % Polyacrylamide gels.

Activator-dependent stabilization experiments were performed under the same buffer conditions as above using gel-filtered nuclear extracts (Fig. 6,7), or gel-filtered and gel-filtered/depleted nuclear extracts (Fig. 8,9, 10, 11, 12). Indicated protein components and buffers were assembled with 100 ng G5HMC2AT and preincubated at 30<sup>o</sup>C, generally for 30 minutes. Reactions were then placed at room temperature, combined with 2  $\mu$ g (Fig. 6,7) or 500 ng (Fig. 8,10,12) of HinDIII-linearized pMLwt2 (Pognonec and Roeder,1991) , nucleotide triphosphates, and other



components as indicated in figure legends, placed at 30<sup>o</sup> C for one hour, and processed as above.

### **Immunodepletions of RAP 30 and TFIIB**

Appropriate anti-serum was combined with protein-A-agarose (Oncogene Science) at a 1:1 ratio (v/v) and rotated at 4<sup>o</sup> C for 2-3 hours, collected by centrifugation, and washed in 10 bed volumes BC 300. The resin was combined with 2 volumes of gel-filtered nuclear extract (made 300 mM in KCl), rotated 4-6 hours at 4<sup>o</sup>C, and centrifuged. The supernatant was dialyzed overnight at 4<sup>o</sup> C against BC 100.

### **Other procedures**

Purification of recombinant TFIIB (Malik et al., 1991), and preparation of TFII E/F/H fraction (Meisterernst et al., 1991), were performed as described.





## **Chapter IV: Characterization of the Highly Conserved TFIIA Small Subunit (TFIIA-S) from *Drosophila melanogaster***

### **Isolation of a cDNA encoding dTFIIA-12**

Nucleotides -672 through -743 of the genomic fragment of the *Drosophila virilis* rough gene (Heberlein and Rubin, 1990) were identified as probable TFIIA-S encoding sequences (see Chapter IV). A synthetic oligonucleotide identical in sequence to a part of this region (5'TATCAATTATATCGCAACACTACGCTTGGCAATACACTACAGGAG AGCCTTGACGAACTA3'), was used to screen one million plaques from an embryonic *Drosophila melanogaster* lambda-zapII cDNA library (Stratagene) at 47°C in 6X SSPE using standard cloning procedures (Sambrook et al., 1989). Clone D2-2 was obtained and sequenced on both strands using internal primers and the Sequenase system (USB).

### **Expression of dTFIIA-12**

The coding sequence of cDNA D2-2 was amplified with PCR primers to create a fragment containing a 5' NdeI site and a 3' BamHI site. This fragment was isolated by gel electrophoresis, purified using Gene-Clean (Bio101) and ligated into the NdeI-BamHI site of 6hisTpET11d (Hoffmann and Roeder, 1992). The derived plasmid, ph6dA12, expresses dTFIIA-S with six N-terminal histidines. pdA12 was created by introducing the same PCR fragment into the NdeI-BamHI site of pET11a (Rosenberg et al., 1987). The derived plasmid expresses dTFIIA-S without any extraneous sequences.



ph6dTFIIA-S was grown at 37°C in BL21(DE3) cells in LB medium (containing 4g/liter glucose and 50 µg/ml ampicillin) to an optical density of 0.5-0.6. The culture was then made 0.4 mM in IPTG, grown at 30° for three hours, harvested by centrifugation, and resuspended in 20 ml of 20 mM Tris (pH 7.9, 4°), 20% glycerol, 500 mM NaCl, 20 mg/ml Pepstatin and Leupeptin, 1% Aprotinin, 0.2 mM PMSF and 2 mM β-mercaptoethanol. Lysozyme was added to 0.5 mg/ml and the cells were incubated at 37°C for 3 minutes, and on ice for 20'. The mixture was sonicated until 90% lysis was achieved and then subjected to centrifugation. dTFIIA-S was found predominantly in the pellet, which was resuspended in Buffer µ (100 mM Na<sub>3</sub>PO<sub>4</sub>, 200 mM KCl, 2 mM β-mercaptoethanol) containing 6M guanidine HCl plus 0.025% NP40 at pH 8.0. This material was loaded onto a NTA column (Invitrogen) equilibrated in the same buffer and washed with Buffer µ containing 8M urea, first at pH 8.0 and then at pH 6.0. The protein was eluted with Buffer µ plus 8M urea at pH 5.0. The eluted material was renatured by gel-filtration into BC100 plus 5 mM β-mercaptoethanol and 0.2 mM PMSF on a Pharmacia PD-10 column according to the manufacturer's instructions.

### **Partial Purification of Drosophila TFIIA**

20 mg of *Drosophila melanogaster* embryo extract protein was diluted to 4 ml in Buffer A (20 mM Tris (pH 7.9, 4°C), 20% glycerol, 0.2 mM EDTA, 0.5 mM PMSF, 1 mM DTT) plus 100 mM KCl and loaded onto a preequilibrated 1.8 ml Q-sepharose column. After washing with the same buffer, proteins were eluted with Buffer A



plus 400 mM KCl. Peak fractions from the elution were pooled, adjusted to a final concentration of 100 mM Na<sub>3</sub>PO<sub>4</sub>, and loaded onto a 0.5 ml Hydroxyapatite column equilibrated in Buffer A plus 200 mM KCl and 100 mM Na<sub>3</sub>PO<sub>4</sub>. After collecting the flow-through fraction, proteins were eluted with Buffer A plus 400 mM Na<sub>3</sub>PO<sub>4</sub>. Peak fractions were pooled and dialyzed against two changes of Buffer A plus 100 mM KCl, and frozen at -70°C.

### **Antibody Production**

Polyclonal antiserum was raised in rabbits against dTFIIA-S by direct injection of the NTA-eluted material (Harlow and Lane, 1988). In general, third-bleed serum was used for supershifts and western blots. Western blots were performed using standard procedures as outlined in the instructions for the ECL kit (Amersham).

### **In vitro transcription**

Partially purified TFIID, TFIIA, pol II, USA (heparin sepharose 0.5 M KCl fraction), a fraction containing TFII E/F/H, and recombinant TFIIB were prepared and used to transcribe 100 ng of supercoiled pG5HMC2AT in the presence of GAL 4-AH (Lin et al., 1988) as described (Ge and Roeder, 1994). Transcriptionally active recombinant TFIIA was prepared by independent expression of human TFIIA/ $\alpha$  (DeJong and Roeder, 1993) and dTFIIA-S in *E. coli*, followed by dissolution of the insoluble pellets in 6M Guanidine-HCl, pH 8.0. The dissolved pellets were combined in roughly equimolar amounts at a total protein concentration of 0.375 mg/ml and dialyzed at 4°C for 18 hours against two changes of BC100 plus



0.1% NP40. The dialysate (100 µg/ml total protein) was clarified by centrifugation and frozen in liquid nitrogen in the presence of 0.5 mg/ml Bovine serum albumin.

### **Protein-DNA Crosslinking**

A DNA oligonucleotide consisting of the coding strand of the MLP from -51 to +1 was annealed to a complementary oligo spanning the region -16 to -35 of the MLP. 0.5 pmol of the annealed oligo was added to a labeling reaction (15 µl) containing 20 mM Tris (pH 7.8, 4<sup>o</sup>), 10 mM MgCl<sub>2</sub>, 50 mM NaCl, 100 µM dGTP, 100 µM N<sub>3</sub>R-dUTP (Bartholemew et al, 1991), 3 µM dCTP, 1.5 µl αP<sup>32</sup>-dCTP, and 6 units Klenow enzyme (GIBCO-BRL), and incubated at room temperature for 15 minutes. The volume was then raised to 50 µl with H<sub>2</sub>O, and the oligo was separated from unincorporated nucleotide triphosphates by gel filtration on a "Nick Column" (Pharmacia), followed by addition of 2 µg poly (dG-dC), ethanol precipitation, and resuspension at a concentration (assuming 100% yield) of 10 fmol/µl.

Photocrosslinking assays were performed under standard conditions (Yokomori et al, 1993) with 30 ng yeast TBP, and 1 µl each of renatured hTFIIA/α (DeJong and Roeder, 1993) and 1 µl renatured dTFIIA-S (see above). After the 37<sup>o</sup> incubation, the tubes were opened and subjected to UV irradiation at 312 nm for 7 minutes at 5 cm distance. Micrococcal nuclease and DNase I were added and the mixture was incubated at 37<sup>o</sup> for 15 minutes. 2 µl 250 mM EDTA (pH 8.0) and 2.5 µl 100 % TCA were added, the mixture was stored on ice for 10 minutes, centrifuged, and the pellet was washed with 80 %





acetone, resuspended in SDS buffer loading buffer, and run on a 10 % SDS-Polyacrylamide gel.

### **Other Procedures**

Gel mobility shift assays (Maldonado et al., 1990), purification of recombinant yeast TBP (DeJong and Roeder, 1993), recombinant human TFIIA/ $\alpha$  (DeJong and Roeder, 1993), recombinant flag-tagged human TFIIB (Chiang and Roeder, 1993), native human TFIIA (NTA method, DeJong and Roeder, 1993), Drosophila nuclear extract (Wampler et al., 1990), and recombinant Gal4-AH (Lin et al., 1988) were performed as described.

## **Chapter V: Protein-Protein Interactions of TFIIA**

### **Plasmids**

Flag-tagged hTFIIA-12 and hTFIIA-55 expression plasmids were created by cloning the Nde I-Bam HI fragments from pRSET-hp12 (DeJong et al., 1994) and pRSET-hp55 (DeJong and Roeder, 1993) into the large Nde I-Bam HI fragment of f:hTBP (Chiang and Roeder, 1992). GST-12 and GST-55 expression plasmids were created by cloning the respective Nde I-Bam HI fragments from the pRSET vectors into the large Nde I-Bam HI fragment of the GST expression vector pGEX-2tL(t) (Hoffmann, 1994).

### **Preparation of Affinity Resins**

Bacterial strain BL21(DE3) pLysS containing expression plasmids for GST-55, GST-12 or GST was grown in LB plus 4 gram/liter glucose at 37<sup>0</sup> to an OD<sub>600</sub> of approximately 0.3, at which point IPTG was added to 1 mM. The cultures were further incubated for 2.5-3 hours and harvested by centrifugation. The bacterial pellet



was washed with 20 mM Tris (pH 7.9 @ 4<sup>0</sup>) plus 200 mM NaCl, centrifuged again, and resuspended in 10 ml BC 1000. The bacteria were lysed by sonication on ice for 5 minutes, and the resulting lysate was clarified by centrifugation at 20 Krpm for 20 minutes in a Ti45 rotor. 0.001 volume (compared to the volume of bacterial culture) of Glutathione-sepharose (Pharmacia) preequilibrated in BC 1000 was added to the extract, and the mixture was rotated at 4<sup>0</sup> for one hour. The resin was then collected by centrifugation, washed five times with 100 volumes of BC 1000, then washed three times with BC 100. The resin was stored as a 1:1 slurry in BC 100 at 4<sup>0</sup>.

### **Protein-Protein Interaction Assays**

Glutathione sepharose resin containing one microgram of fusion protein (as assayed by SDS-PAGE and Coomassie blue staining) was mixed with approximately 0.5 µg/ml of the target protein plus 1 mg/ml BSA in 100-200 µl BC100 (or as otherwise indicated), rotated for one hour at 4<sup>0</sup>, washed twice with 50 volumes of the same buffer (except where indicated in figure legends), resuspended in 15 µl protein loading buffer, and subjected to SDS-PAGE and western blotting as indicated in figure legends.

### **Transcription Assays**

Heat treated nuclear extract (Nakajima et al, 1988) was prepared by incubating 200-300 µl of HeLa nuclear extract (10 mg/ml) at 47<sup>0</sup> for 11 minutes in a 1.5 ml eppendorf tube. It was used in transcription assays as described in Chapter 3 and figure legends. Transcription reconstituted with highly purified or recombinant factors (Figure 29) was performed with recombinant TFIIB,



recombinant TFIIE, purified TFII F/H, and purified pol II as described in Ohkuma et al (1991).

### **Depleted Extracts**

TBP-depleted HeLa nuclear extract (Figure 30B) was prepared by preincubating 500  $\mu$ l anti-human TBP serum with 250  $\mu$ l protein-A agarose for 2 hours at 4 $^{\circ}$ . The resin was washed with 20 volumes of BC 400 five times, mixed with 1 ml HeLa nuclear extract (made 400 mM in KCl), and rotated 2 hours at 4 $^{\circ}$ . The mixture was then centrifuged, and the supernatant combined with 50  $\mu$ l protein-A sepharose (preequilibrated in BC 400) to absorb any free antibody, rotated for a further 45 minutes, and collected by centrifugation.

TFIIA depletion was performed using anti-hTFIIA/ $\alpha\beta$  serum as described in DeJong et al (1993).

### **Other Procedures**

Recombinant human TFIIA was prepared identically to the corenated human/drosophila TFIIA described for Chapter IV. Flag-tagged human TFIIA/ $\alpha\beta$  and TFIIA/ $\gamma$  were prepared from the soluble bacterial extract as described for Flag-tagged human TBP (Chiang and Roeder, 1993). In vitro translated hTAFII136 fragment was prepared by Sean Stevens using a TnT kit (Promega) according to the manufacturer's instructions.

Purification of TFIIF (HPLC-DEAE TFIIF fraction; Sumimoto et al., 1990), DA complex formation assay (Yokomori et al., 1993), purification of yeast TBP and yeast TBP mutant K/L 138,145 (Hoffmann and Roeder, 1992), purification and phosphorylation of recombinant PC4 (Ge and Roeder, 1994), and purification of GST-VP16 (Ge and Roeder, 1994) were all performed as described.



## **Chapter III: Activation of Transcription by Gal4-IE**





The pseudorabiesvirus immediate early protein (IE) was the first isolated class II transcriptional activator shown to stimulate transcription *in vitro* (Abmayr et al., 1985). Early functional studies revealed that the only DNA sequence element which was required for IE activation was a TATA box; IE was shown to be able to activate almost every class II gene on which it was tested (reviewed in Martin and Green, 1992). However, IE has been shown to have a promoter-targeting domain (Martin et al., 1990) and to bind the hsp 70 promoter with sequence specificity (Cromlish et al., 1990). The promiscuous promoter specificity of IE activation suggests that IE functions largely by protein-protein interactions with the general transcription factors which assemble on the core promoter.

Investigation of the mechanism of transcriptional activation by IE has suggested that it functions by increasing the binding of TFIID to the core promoter. For example, preincubation of the major-late promoter with either nuclear extract or a crude TFIID fraction raises the basal level of transcription and correspondingly obviates IE stimulation (Abmayr et al., 1988). Furthermore, if PIC assembly occurs concomitantly with chromatin assembly, IE stimulation of transcription depends upon an interaction of IE with TFIID at the time of the template's incorporation into chromatin (Workman et al., 1988). The above studies suggest that IE interacts functionally with some set of PIC components, perhaps only TFIID, to activate transcription.

To map the transcriptional activation domain of IE without the complication of its vague promoter specificity, the full-length IE protein was fused to the DNA binding region of Gal4 (amino acids 1-



147) (Martin et al., 1990). The resulting protein activates transcription from a promoter containing Gal4-binding sites, as measured in vivo by transfection assays. Serial deletions from the C-terminus of IE revealed that the N-terminal 34 amino acids, when linked to Gal4, were sufficient to mediate a 700 fold activation of transcription in vivo from such a promoter (Martin et al., 1990). The activation domain (amino acids 1-34) has a net charge of -8, a feature typical of Gal4-VP16 and other activators whose acidity has been shown to be important for activation (Mitchell and Tjian, 1989; Martin and Green, 1992). Clearly, based on the studies of Martin et al (1990), Gal4(1-147)-IE(1-34) (referred to below as Gal4-IE) is a potent activator in vivo. It has not been studied in vitro.

The usual transcription activation assay in vitro involves determining the ability of an activator to increase the transcription of a single promoter. However, an activator must function in vivo by increasing the transcription of a single promoter in the context of a steep competition between many promoters for limiting general factors. This suggests that activators may function by stabilizing either full or partial preinitiation complexes on their target promoters to the otherwise inhibitory effect of competition from other promoters. The underlying hypothesis in the studies in this chapter is that activators stabilize the preinitiation complex by interacting with some subset of the general transcription factors which assemble a PIC on the core promoter. This hypothesis is supported by numerous studies (e.g. Sawadogo and Roeder, 1985; Horikoshi et al, 1988a,b; Lieberman and Berk, 1994; Lieberman, 1995) which document stabilized binding of TFIID, a TFIID-TFIIA



complex, or a TFIID-TFIIA-TFIIB complex, to a given promoter in response to various activators (see Chapter 1, section 5).

The role of activators in mediating preinitiation complex stabilization is well established in the Pol III system, where TFIIA and TFIIC have been shown to promote the assembly of a highly stable TFIIB-promoter complex (Lassar et al, 1983; Kassavetis et al., 1990). Similar work in the pol II system has shown that the activator ATF renders a partial preinitiation complex consisting of TFIID, TFIIB and RNA Polymerase II stable to oligonucleotide challenge (Hai et al., 1988). The ATF study utilized an ATF binding site-containing oligonucleotide to destabilize the nascent preinitiation complex. To date, no study in the pol II system has used a competing core promoter to accomplish destabilization. However, this would seem to be a more physiological situation; in vivo, core promoters represent the true challenge for limiting general factors.

In this chapter I describe experiments in which Gal4-IE activates transcription in the presence of an excess of a competing core promoter. Using this assay system, I show that Gal4-IE functions by stabilizing a partial preinitiation complex dependent upon the general factor TFIIB.



## **Results**

### **Purification of Gal4-IE**

To study Gal4-IE in vitro it was first necessary to purify functionally active Gal4-IE. This was accomplished by conventional chromatography. As figure 1 shows, IPTG addition to cultures of bacteria containing the expression vector pG4-IE results in strong expression of a protein of 23 kD molecular weight, equivalent to that predicted for Gal4-IE (compare lanes 1 and 2). This protein is soluble (lanes 3 and 4), remains soluble in the presence of the DNA precipitant polyethyleneimine (lanes 5 and 6), and can be precipitated nearly quantitatively by ammonium sulfate (lanes 9 and 10). This behavior is identical to that of other acidic Gal4 fusion proteins (Lin et al., 1988) and indicates that Gal4-IE is well expressed in *E. coli*.

The resuspended ammonium sulfate pellet was loaded onto DE-52 (Fig. 2). It was eluted with a salt gradient, and the fractions shown in lanes 3-6 were pooled, dialyzed, and loaded onto Heparin-sepharose. Gal4-IE bound to this column and was eluted at nearly homogenous purity with 600 mM NaCl (Fig. 3). The indicated fractions were pooled, dialyzed, and stored in aliquots at -70.

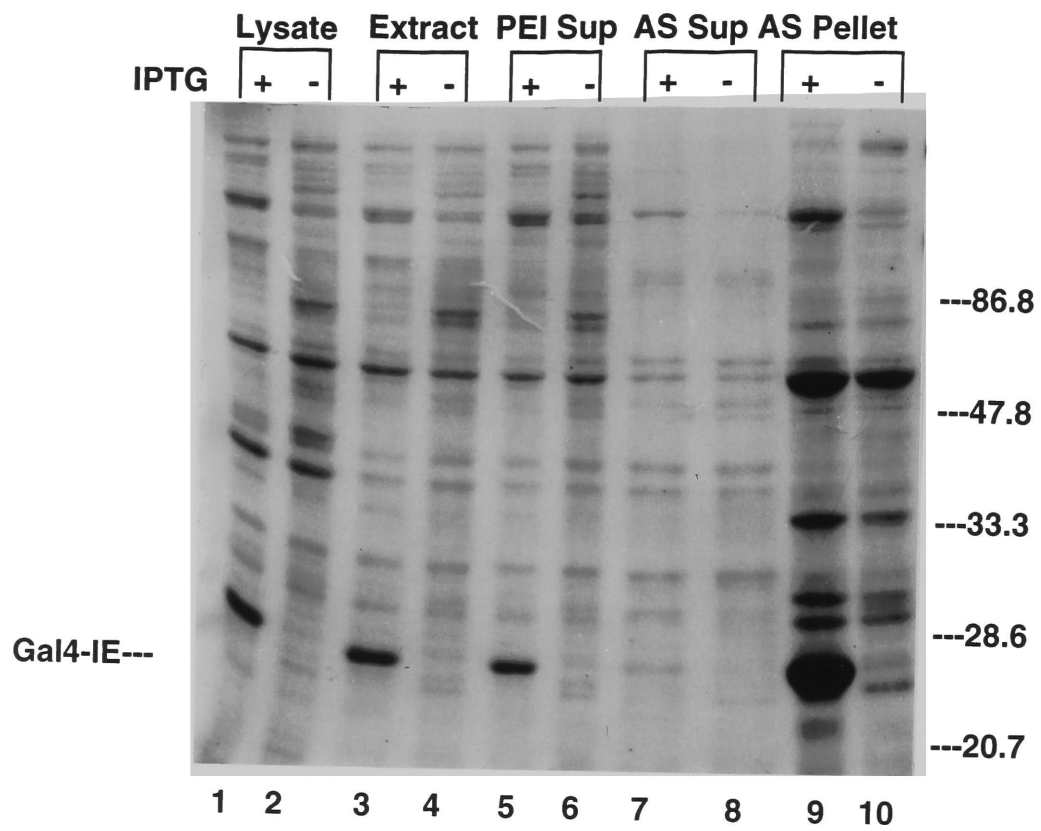
### **Recombinant Gal4-IE activates transcription in vitro from Gal4 DNA-binding sites**

Based on the in vivo behavior of Gal4-IE (Martin et al., 1990) it was expected that recombinant Gal4-IE would activate transcription from a template containing Gal4 DNA binding sites, but not from one lacking such sites. This function was investigated by assaying the ability of Gal4-IE to activate

### **Figure 1: Overexpression and Partial Purification of Gal4-IE**

Plasmid pGal4-IE was used to overexpress Gal4(1-147)-IE (1-34) [referred to as Gal4-IE] in bacterial strain XA90 as described in Chapter 2. Bacterial lysate (lanes 1,2), soluble extract (lanes 3,4), Polyethyleneimine supernatant (lanes 5,6), ammonium sulfate supernatant (lanes 7,8) and ammonium sulfate pellet (lanes 9,10) were run on a 15% SDS-Polyacrylamide gel and stained with Coomassie Blue. An IPTG-induced band with the predicted molecular weight of Gal4-IE is indicated to the left of the gel. Position of molecular weight markers are indicated to the right of the gel.



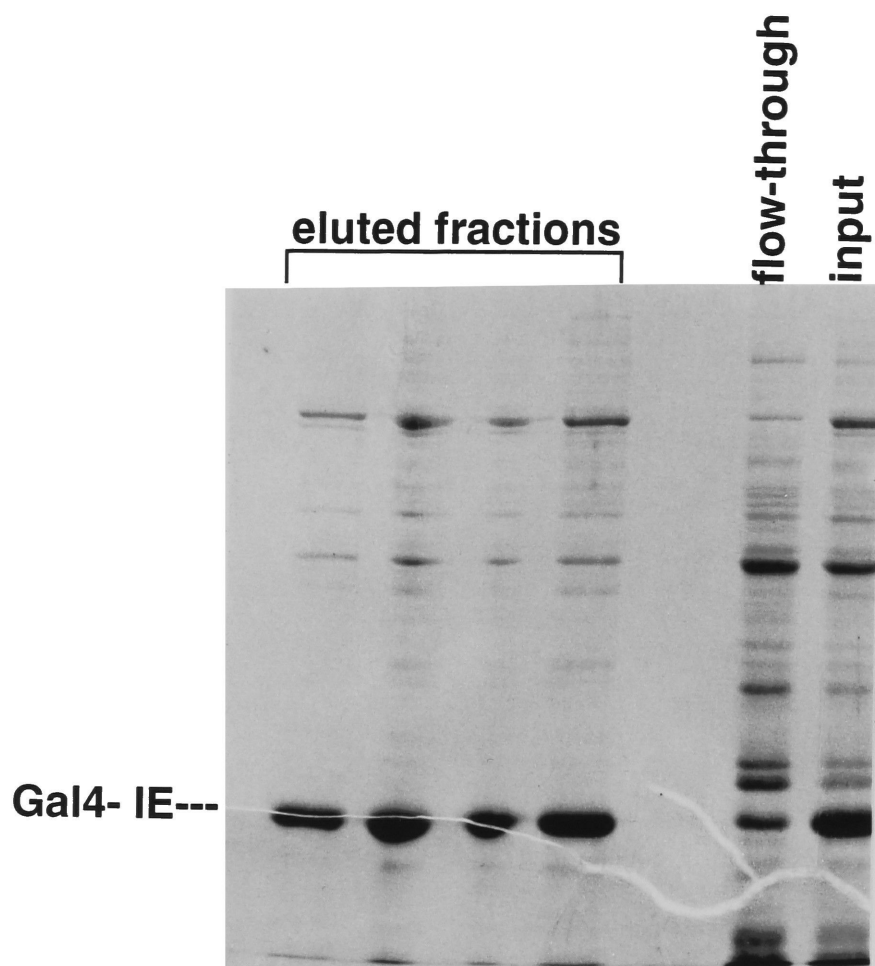






### **Figure 2: Purification of Gal4-IE by DE-52 Chromatography**

Ammonium Sulfate pellet (Figure 1) was chromatographed on DE-52 (Whatman). Gal4-IE (indicated to the left of the gel) was eluted from DE-52 column with a salt gradient between 200 and 500 mM NaCl. Input, flow-through, and Gal4-IE-containing eluted fractions were run on a 15% SDS Polyacrylamide gel and stained with Coomassie Blue.



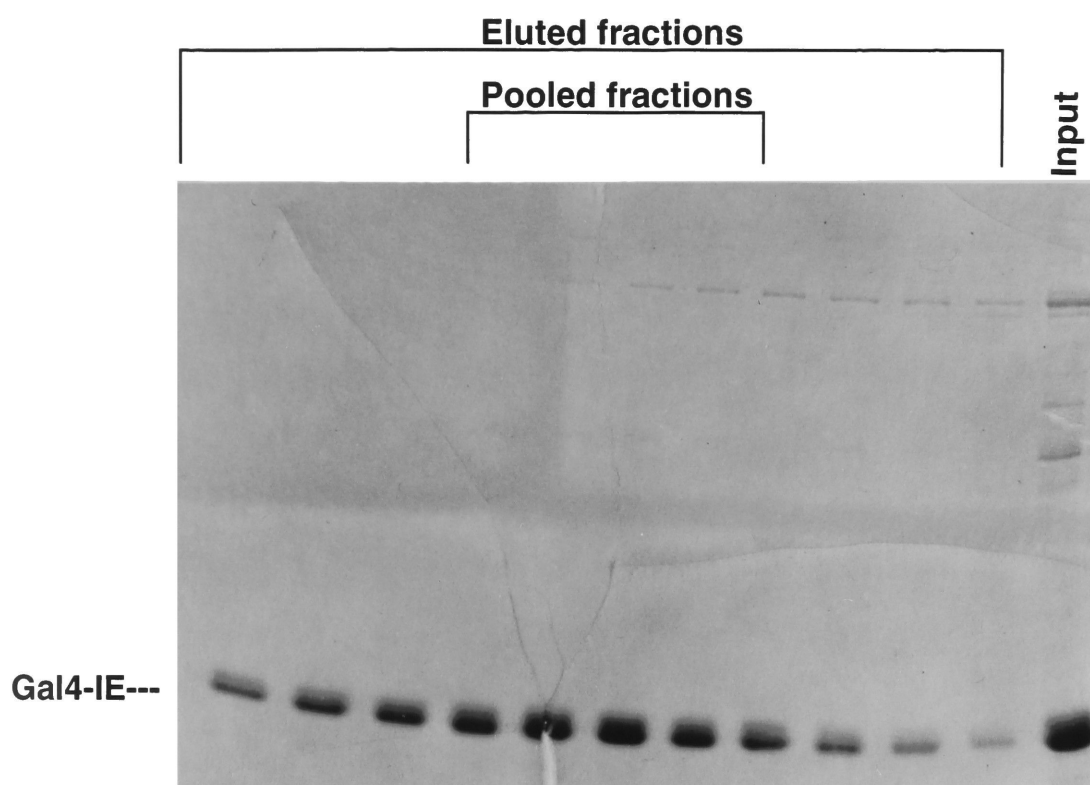




### **Figure 3: Purification of Gal4-IE on Heparin-Sepharose**

Gradient-eluted fractions from containing Gal4-IE were pooled and chromatographed over Heparin Sepharose (Pharmacia) as described in Chapter 2. Gal4-IE is indicated to the left of the gel. Input and eluted fractions were run on a 15% polyacrylamide gel and stained with Coomassie Blue.







transcription in a HeLa nuclear extract (Dignam et al., 1983) from two promoters which either contained, or did not contain, Gal4 sites. As expected, Gal4-IE does not activate a TATA-CAT promoter which contains no Gal4-DNA binding sites (Fig. 4; lanes 1 and 2). However, it clearly activates transcription of the same core promoter when 5 Gal4 sites are cloned 30 base pairs upstream of the TATA box (lanes 3,4). This indicates that the IE activation domain is active in vitro and that the Gal4 DNA binding domain determines the promoter specificity of the fusion protein. Because Gal4-IE could be purified in functionally active form from *E. coli*, it was deemed suitable for further in vitro analysis.

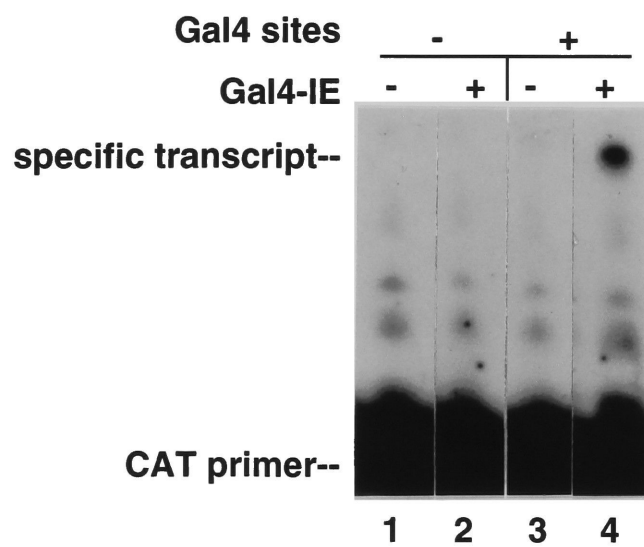
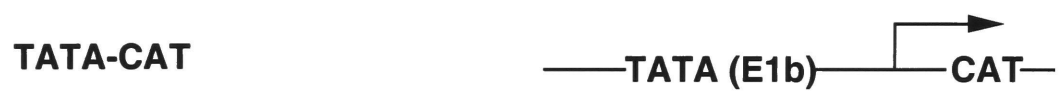
As figure 4 shows, the E1B promoter derivatives do not show a detectable level of basal transcription in HeLa nuclear extract. Further, because they are weak promoters, they must be assayed by the laborious method of primer extension. To increase the basal level of transcription and allow assay of Gal4-IE by the simpler run-off method, the template G5HMC2AT (Ge and Roeder, 1994) was employed. This promoter (Figure 5A) contains 5 Gal4 DNA binding sites upstream of the HIV TATA box and the Major Late Promoter initiator element. The transcribed region is the G-free cassette, which can be cleaved at +380 to result in a 380 bp run-off transcript. As figure 5 shows, this promoter has a detectable basal level of transcription in HeLa nuclear extract (lane 1) and can be activated by Gal4-IE (lane 2).

### **Mechanism of Activation of Transcription by Gal4-IE**

Transcriptional activation in the cell requires the facilitation of the transcription of a specific target promoter in the context of a vast

#### **Figure 4: Activation of Transcription by Gal4-IE**

Transcription reaction in HeLa nuclear extract using 100 ng of template TATA-CAT (lanes 1,2) or G5E1BCAT (lanes 3,4) including 5 pmol of Gal4-IE (lanes 2,4). Transcripts (indicated to the left of the gel) were detected using primer extension as described in Chapter 2.





excess of other promoters which compete for limiting general transcription factors. I tried to assay the the ability of Gal4-IE to carry out this function by reproducing in vitro the competition between promoters that occurs in vivo. To do this, I sought to set up a transcription system in which Gal4-IE would stabilize a transcription complex on its target promoter against competition by an excess of a second template (Lassar et al., 1987). The function of the second template in this context is to sequester any factors which are not bound stably to the first template.

I first determined the concentration of second template which would inhibit Gal4-IE activated transcription in nuclear extract. Figure 6 shows that, as expected, in the absence of any competitor template (lanes 1 and 2), Gal4-IE activates transcription from the G5HMC2AT template. However, in the presence of increasing concentrations of a MLP-containing template (which encodes a shorter transcript), transcription of G5HMC2AT is inhibited (lanes 3 through 12). Figure 6 therefore shows that excess MLP addition can inhibit Gal4-IE dependent transcription. This experiment also shows that intermediate concentrations of MLP out-compete G5HMC2AT for general factors in the absence, but not in the presence, of Gal4-IE. I chose to use high concentrations of MLP to reproduce the situation in vivo, in which transcription of bulk chromatin is unaffected by the activation of a single specific gene.

Next I developed conditions in which preincubation of Gal4-IE with G5HMC2AT could overcome the inhibition caused by excess MLP addition. As figure 7 demonstrates, addition of MLP simultaneously with Gal4-IE inhibits transcription from





G5HMC2AT (lanes 1,2). However, preincubation of Gal4-IE with nuclear extract (NE) and G5HMC2AT prior to MLP addition makes transcription of G5HMC2AT resistant to competition by the excess MLP. This effect increases with preincubations up to 30 minutes, after which the activity of the extract decreases (data not shown). Therefore, in future experiments, preincubation times of thirty minutes are used to allow Gal4-IE to interact with NE on the template. The experiment in Figure 7 shows that, consistent with the ability of Gal4-IE to activate transcription *in vivo*, Gal4-IE can activate transcription on its cognate promoter in the presence of an otherwise inhibitory concentration of a second, competing template. This suggests that Gal4-IE functions by stabilizing a Pre-initiation complex (PIC) on G5HMC2AT, rendering it impervious to competition by excess MLP.

The experiment in Figure 7 suggests a model of Gal4-IE function in which the activator stabilizes the PIC on G5HMC2AT, rendering it resistant to otherwise inhibitory competition by MLP addition. Consistent with this model, if Gal4-IE is added to the reaction subsequent to preincubation and excess MLP addition, no Gal4-IE dependent activation takes place (Figure 8, compare lanes 2,3). Gal4-IE addition after MLP addition results in exactly the same level of transcription as if Gal4-IE were not added (compare lanes 1 and 3). This experiment shows that preincubation of NE with G5HMC2AT, and addition of Gal4-IE subsequent to second template addition, is not, by itself, sufficient to allow activation of transcription by Gal4-IE. Rather, a concerted interaction between

**Figure 5: Activation of Transcription on G5HMC<sub>2</sub>AT by Gal4-IE**

A) Diagram of template G5HMC<sub>2</sub>AT

B) Run-off transcription performed in HeLa nuclear extract on template G5HMC<sub>2</sub>AT as described in Chapter 2 in the absence (lane 1) or presence (lane 2) of Gal4-IE. Position of specific transcript is indicated to the left of the gel.

**G<sub>5</sub>HMC<sub>2</sub>AT**



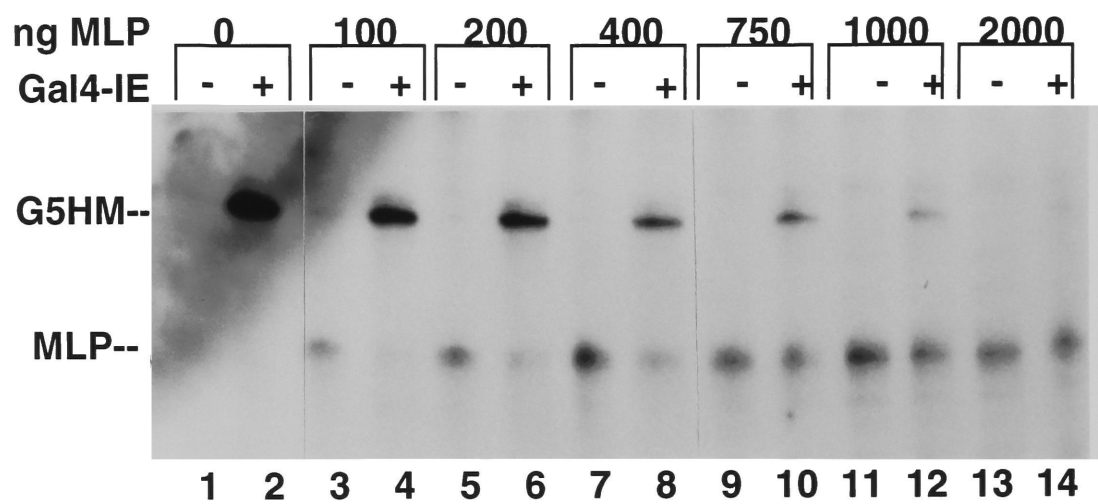




**Figure 6: Titration of MLP-containing plasmid into Gal4-IE  
Activation Reaction**

In vitro transcription reaction in HeLa nuclear extract performed as described in Chapter 2 with addition of 0 (lanes 1,2), 100 (lanes 3,4), 200 (lanes 5,6), 400 (lanes 7,8), 750 (lanes 9,10), 1000 (lanes 11,12) or 2000 (lanes 13,14) ng MLP-containing plasmid, and Gal4-IE where indicated. Run-off transcripts derived from G5HMC2AT and MLP are indicated to the left of the gel.

assemble all components on ice      1h transcription      STOP





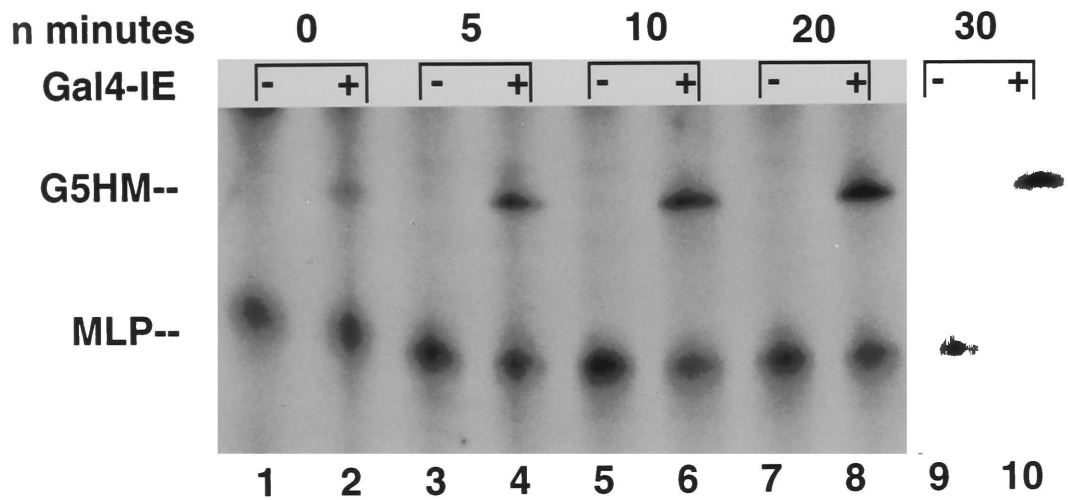




**Figure 7: Time-Course of Preincubation of G5HMC<sub>2</sub>AT with Gal4-IE and Nuclear Extract in Template Stabilization Assay**

Template stabilization reactions were performed as described in Chapter 2, with 2 µg MLP-containing plasmid added after 0 (lanes 1,2), 5 (lanes 3,4), 10 (lanes 5,6), 20 (lanes 7,8), or 30 (lanes 9,10) minutes of preincubation of Gal4-IE (lanes 2,4,6,8,10), nuclear extract, and G5HMC<sub>2</sub>AT. Positions of G5HMC<sub>2</sub>AT- and MLP-derived transcripts are indicated to the left of the gel.

NE  
 G5HM     Pre-inc.  
              n min.     x/s MLP     1 h     STOP  
 +/- Gal4-IE     NTP\*





NE components and Gal4-IE is required to allow activation in this context.

### **Gal4-IE Stabilizes Transcription by Interacting with a Partial Preinitiation Complex**

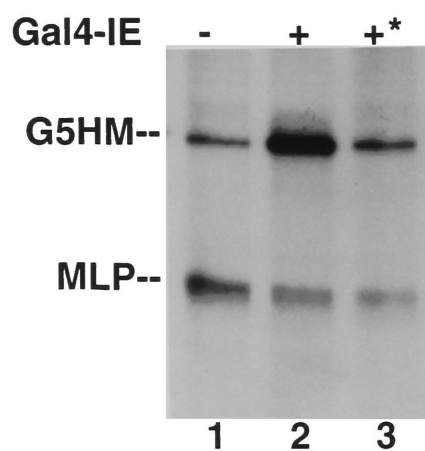
The experiments in Figures 6,7, and 8 showed that Gal4-IE can stabilize transcription of a promoter containing Gal4 sites if it is preincubated with that promoter and NE prior to addition of an otherwise inhibitory concentration of a second template. This finding suggests that there is an interaction between Gal4-IE and the general factors which renders the assembling PIC resistant to competition. However, the possibility exists that preincubation of Gal4-IE with complete NE and template allows transcription initiation prior to the addition of the second template, supported by contaminating nucleotide triphosphates in the NE. If this were the case, one would predict that the preincubation step would require all of the PIC components which are required for initiation (see Introduction). Alternatively, if Gal4-IE does in fact function by stabilizing a nascent PIC during the preincubation, those PIC components which are not targets of Gal4-IE would not be required during the preincubation. To test this possibility, preincubation experiments were carried out with extracts depleted general transcription factors which are required for the early steps of initiation (Buratowski et al, 1989).

I first used antibodies against the 30 kD subunit of TFIIF (RAP-30) to remove TFIIF from HeLa nuclear extract. Figure 9 shows that addition of a fraction containing TFIIF ( as well as TFIIE and TFIIH)

**Figure 8: Effect of Addition of Gal4-IE before or after Preincubation of G5HMC<sub>2</sub>AT and NE**

Template stabilization reaction was performed as described in Chapter 2, with Gal4-IE either not added (lane 1), added at the start of preincubation (lane 2), or after preincubation and MLP addition (lane 3). Positions of G5HMC<sub>2</sub>AT and MLP-derived transcripts are indicated to the left of the gel. (72 hour exposure).

NE                      30 min.                      x/s MLP                      1h                      STOP  
 -/+ Gal4-IE                      NTP (P32)  
 G<sub>5</sub>HM                      \*Gal4-IE



\*=Gal4-IE added after competitor





to Pre-immune serum depleted extract has no effect (lanes 1-3). However, extract depleted with antibody to RAP 30 is inactive in transcription (lane 4) but can be restored to control levels by the addition of the TFIIF-containing fraction (lanes 5 and 6). This result confirms that TFIIF is absolutely required for transcription, and that TFIIF-depleted extract can be complemented by a TFIIF fraction. According to the well-characterized order of assembly of the PIC described in Chapter 1 (Buratowski et al., 1989), no transcription complex beyond the DAB complex should be able to assemble in TFIIF-depleted extract. Most importantly, no initiation of transcription is expected to occur in TFIIF-depleted nuclear extract.

Figure 10 demonstrates the effects of TFIIF depletion on the ability of nuclear extract to support Gal4-IE activation in the context of competition from a second template. Consistent with the result in Figure 6, in the absence of preincubation, excess MLP addition inhibits G5HMC2AT transcription (lanes 1,2). Preincubation of Gal4-IE with G5HMC2AT in the presence of undepleted nuclear extract (lanes 3,4), or in the presence of TFIIF-depleted extract complemented with TFIIF (lanes 7,8) allows Gal4-IE dependent activation despite the presence of excess competitor template. Significantly, TFIIF depleted nuclear extract also mediates Gal4-IE dependent stabilization (lanes 5 and 6). [To satisfy the absolute requirement for TFIIF, it is added after MLP.] This experiment shows clearly that TFIIF is not required for the preincubation phase of the experiment during which Gal4-IE stabilizes transcription on G5HMC2AT. Furthermore, this result shows that activation of transcription caused by preincubation of Gal4-IE with NE and

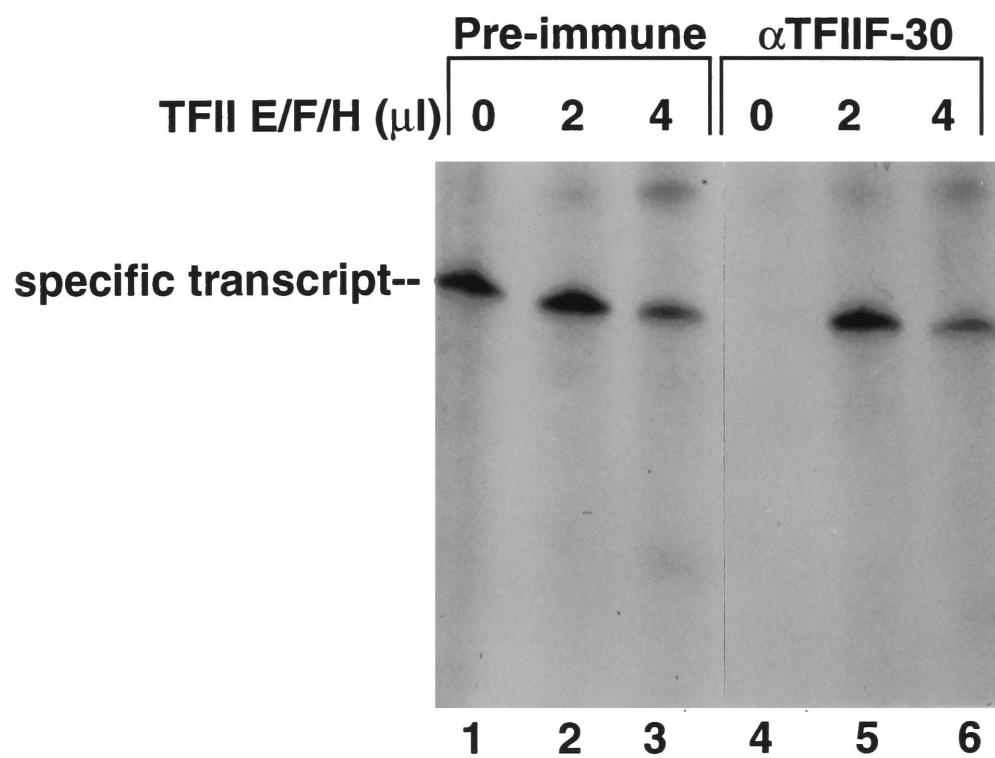


G5HMC2AT is not due to initiation of transcription during preincubation, since TFIIF is required for initiation (Goodrich and Tjian, 1994). Rather, Gal4-IE must function in this assay by stabilizing an uninitiated partial PIC that does not include TFIIF. Since TFIIF is required for progression of PIC assembly past the DAB complex (Buratowski et al., 1989), it follows that Gal4-IE must interact with the D, DA, or DAB complex, rendering it stable to competition from a second template.

The result of Figure 10 suggested that Gal4-IE could stabilize a partial PIC whose formation did not require TFIIF. However, it would be consistent with that result, and all of the previous ones, to argue that Gal4-IE was functioning in the preincubation assays by simply binding to G5HMC2AT and thereby sterically preventing occlusion of the template by non-specific DNA binding proteins such as histones, which are abundant in nuclear extract (Abmayr et al, 1988; Taylor et al., 1991; Laybourn and Kadonaga, 1991). If this were occurring, one would predict that no general factors would be required during the preincubation step, since Gal4-IE can bind to Gal4 sites efficiently on its own (Taylor et al., 1991). Alternatively, if one (or more) general transcription factors were required during preincubation, this would show that there was a true functional interaction between Gal4-IE and the general factors which rendered transcription stable to excess MLP competition. To

**Figure 9: Immunodepletion of TFIIF from HeLa Nuclear Extract**

In vitro transcription of 200 ng major late promoter in HeLa nuclear extract treated with Pre-immune (lanes 1-3) or anti-RAP-30 antiserum (lanes 4-6) as described in Chapter 2. TFII E/F/H fraction (Meisterernst et al., 1991) was added as indicated (lanes 2,3,5,6). Position of G5HMC<sub>2</sub>AT-derived run-off transcript is indicated to the left of the gel.







**Figure 10: Gal4-IE Template Stabilization Performed with TFIIF-Depleted Nuclear Extract**

Template stabilization reactions performed without (lanes 1,2) or with (lanes 3-8) preincubation of nuclear extracts with Gal4-IE, and G5HMC2AT. Preincubation was performed with Preimmune treated nuclear extract (lanes 3,4), or TFIIF-depleted nuclear extract (lanes 5-8) with TFIIF-containing fraction added after (lanes 5,6) or before (lanes 7,8) preincubation. Gal4-IE was added to reactions in lanes 2,4,6,8. Positions of G5HMC2AT and MLP transcripts are indicated to the left of the gel.



NE or  $\alpha$ IIFNE      pre-inc      x/s MLP      1 h.      STOP  
 +/- Gal4-IE       $\Delta$  factors      NTP\*

Pre-incubation	-		+					
factors present during pre-preinc	(PI)		PI		$\alpha$ IIF		$\alpha$ IIF E/F/H	
Gal4-IE	-	+	-	+	-	+	-	+
G5HM---								

MLP--



make this determination, I assayed the requirement for the presence of TFIIB during Gal4-IE preincubation.

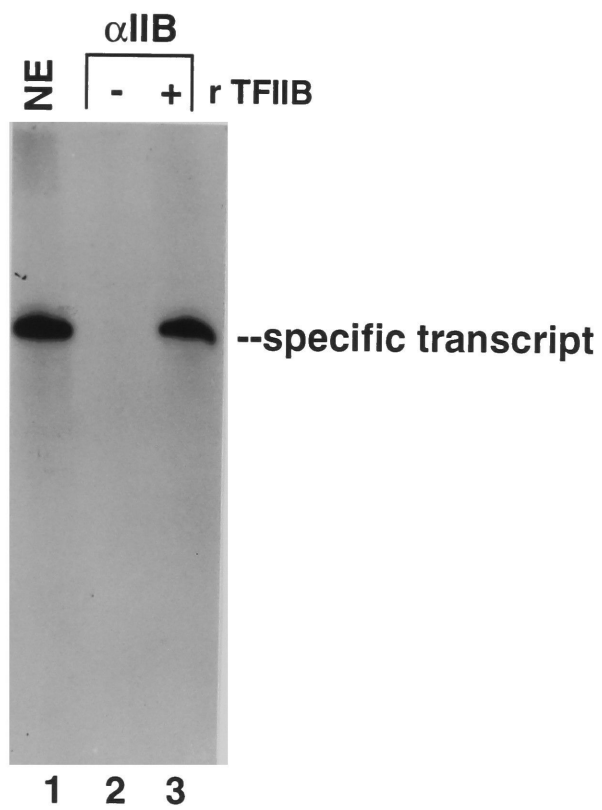
To determine whether TFIIB was required to be present during the preincubation of G5HMC2AT with Gal4-IE for Gal4-IE to mediate competition-resistant activation, TFIIB was immunodepleted from HeLa nuclear extract. Figure 11 shows that nuclear extract depleted of TFIIB is inactive in transcription (compare lanes 1 and 2). The extract can be complemented fully by addition of recombinant TFIIB (lane 3). This experiment demonstrates that TFIIB can be selectively immunodepleted from nuclear extract.

I tested whether TFIIB-depleted extract could mediate Gal4-IE dependent competition-resistant activation. As expected (Figure 12, lanes 1,2), in the absence of preincubation, addition of excess MLP to the reaction inhibits transcription of G5HMC2AT. Also as expected, preincubation of Gal4-IE and G5HMC2AT in the presence of either untreated nuclear extract (lanes 3,4) or TFIIB-depleted extract complemented with TFIIB prior to excess MLP addition (lanes 7,8) allows transcription of G5HMC2AT despite the addition of competitor. However, TFIIB-depleted extract minimally supports Gal4-IE dependent stable complex formation. This experiment shows that Gal4-IE does not promote competition-resistant transcription by simple steric "protection" of G5HMC2AT during preincubation. Rather, it must function by stabilizing a general factor complex whose formation requires TFIIB.

To summarize, the results presented in this chapter indicate that Gal4-IE can activate transcription from its target promoter in

### **Figure 11: Depletion of TFIIB from HeLa Nuclear Extract**

In vitro transcription reactions performed with 200 ng major late promoter in gel-filtered nuclear extract (lane 1) or anti-TFIIB-depleted/ gel-filtered nuclear extract without (lane 2) or with (lane 3) 30 ng of recombinant TFIIB (lane 3).







**Figure 12: Gal4-IE Template Stabilization Experiment Performed with TFIIB-Depleted Nuclear Extract**

Gal4-IE template stabilization reaction performed as described in Chapter 2 without (lanes 1,2) or with (lanes 3-8) preincubation. Preincubation was performed with nuclear extract (lanes 3,4), or TFIIB-depleted extract with TFIIB added after (lanes 5,6) or before (lanes 7,8) preincubation. Position of G5HMC2AT and MLP-derived transcripts are indicated to the left of the gel.



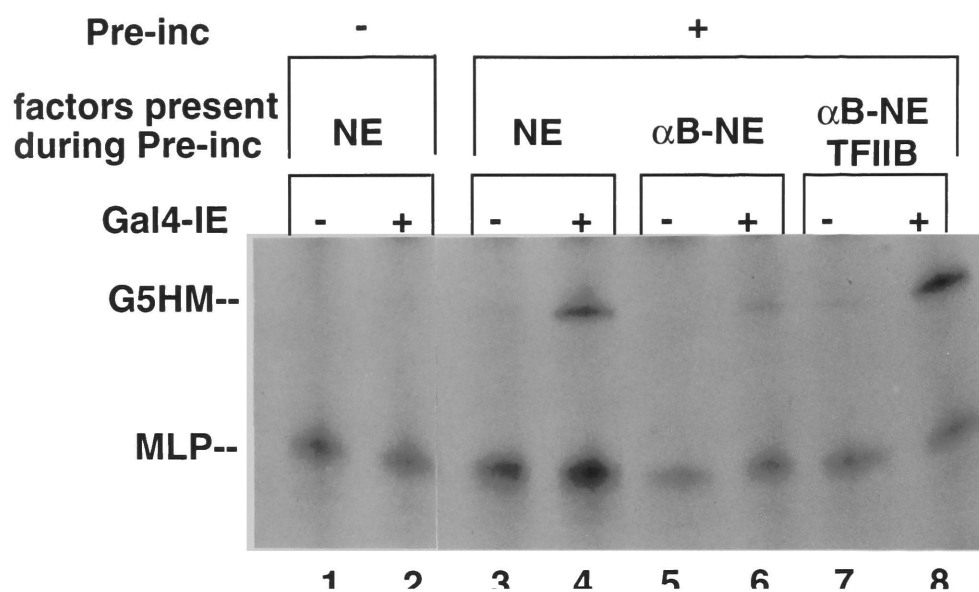
NE or  $\alpha$ IIB NE  
 +/- Gal4-IE  
 G5HM

pre-inc

x/s MLP  
 $\Delta$  Factors  
 NTP\*

1h

STOP





the context of competition from an excess of a second promoter by stabilizing a partial preinitiation complex. Gal4-IE does not carry out this function by simply preventing steric occlusion of the template by non-specific DNA binding proteins. Rather, it functions by interacting on DNA with a partial preinitiation complex whose formation requires TFIIB but not TFIIF.



## Discussion

In this chapter I have described transcription activation by Gal4-IE. Highly pure recombinant Gal4-IE (Fig. 3) was shown to activate transcription in HeLa nuclear extract from the E1B core promoter dependent upon the presence of Gal4 DNA binding sites (Fig. 4). These results mirror precisely the results of *in vivo* studies of Gal4-IE activation (Martin et al., 1990). To facilitate more rapid analysis of Gal4-IE *in vitro*, the template G5HMC2AT was utilized and shown to be responsive to Gal4-IE *in vitro* (Fig.5).

Since activators *in vivo* must facilitate transcription of their cognate promoters in the context of competition from other promoters, an assay was set up in which Gal4-IE would do this *in vitro*. This assay used the addition of an excess of MLP-containing template as an *in vitro* mimic of bulk genomic DNA. First, it was demonstrated that excess DNA containing the MLP could inhibit transcription of G5HMC2AT activated by Gal4-IE (Fig. 6). Then, it was shown that preincubation of G5HMC2AT with Gal4-IE and nuclear extract allowed the formation of PIC's on G5HMC2AT that were stable to competition (Fig. 7). Finally, I showed that Gal4-IE addition after preincubation of G5HMC2AT with nuclear extract had no effect (Fig. 8). The results of these preincubation experiments are consistent with those in which the role of the activator ATF was studied (Hai et al., 1988). In that study, preincubation of nuclear extract with ATF and template allowed the formation of an ATF-binding oligonucleotide-resistant PIC on the E4 promoter. These results are also consistent with studies of Gal4-AH, which showed that addition of an activator after formation of a



rate-limiting TFIID-TFIIA complex was insufficient to facilitate an activated level of full preinitiation complex assembly (Wang et al, 1992b).

There are several distinct models to explain these results. First, it is possible that during the preincubation step, transcription actually initiated, utilizing contaminating nucleotide triphosphates present in the nuclear extract. If this occurred, transcripts that appeared to be due to (partial) complexes which were stable to MLP addition might have actually been initiated prior to MLP addition. This would invalidate the conclusion that Gal4-IE functions by creating complexes stable to competition. This possibility was made unlikely, however, by the experiment of Figure 10, which showed that TFIIF was dispensible for Gal4-IE-dependent competition-resistant transcription. Since TFIIF is essential for transcription initiation (Goodrich and Tjian, 1994), the lack of a TFIIF requirement during preincubation rules out the possibility that initiation is taking place during that period. It also suggests that the Gal4-IE target complex does not require TFIIF to form.

A second explanation for the results of Figures 6, 7, 8, and 9 would be that Gal4-IE functions by binding to G5HMC2AT and prevents aggregation of nonspecific DNA binding proteins onto the template. If this were occurring, preincubation of Gal4-IE with G5HMC2AT would not require any general transcription factors to have an effect, since Gal4-IE can bind to DNA independently of general factors. However, Figure 12 shows that TFIIB is required during the preincubation step in order for Gal4-IE to mediate





competition-resistant activation. Therefore, a simple steric anti-repression model is not consistent with the data shown here.

The remaining model posits that Gal4-IE stabilizes a partial preinitiation complex on the template, rendering it resistant to competition. The partial complex most consistent with the factor requirements for stabilization described here is the DAB complex (Buratowski et al., 1989). There are several possible mechanisms by which such a stabilization could occur. First, a direct protein-protein interaction between promoter-bound Gal4-IE and TFIIB could account for the dependence upon TFIIB for Gal4-IE-mediated competition-resistance. Consistent with this idea, studies of Gal4-AH and Gal4-VP16, two proteins closely related structurally to Gal4-IE (Martin and Green, 1992), have indicated that acidic activation domains function by either recruiting TFIIB into the preinitiation complex, or by stabilizing its binding, via direct protein-protein interactions between the activator and TFIIB (Lin and Green, 1991; Roberts et al 1991). Given the acidity of the IE activation domain and the fact that TFIIB contains basic surfaces (Malik et al., 1991; Ha et al., 1991), such an interaction is possible.

It is also possible that Gal4-IE stabilizes the binding of TFIIA, resulting in a complex whose assembly into a productive PIC in turn is increased by TFIIB. Such a mechanism is consistent with studies of the activators Gal4-AH and Zta, which showed that these activators promoted the formation of a partial PIC consisting of TFIID and TFIIA (Wang et al., 1992; Chi and Carey, 1993; Lieberman and Berk, 1994). The stability of the Gal4-AH-TFIID-TFIIA complex is increased by the addition of TFIIB (Wang et al., 1992). If such a



mechanism is occurring with Gal4-IE, our assay must be stringent enough to require the additional stabilization caused by TFIIB addition (see below). Further investigation of TFIIA, including an examination of its possible role in activated transcription, is described in Chapters IV and V.

Finally, Gal4-IE may cause a change in TFIID stability (Sawadogo and Roeder, 1985; Van Dyke et al., 1988; Abmayr et al., 1988; Workman and Roeder, 1988) or conformation (Horikoshi et al., 1988 a,b) which promotes TFIIB recruitment (Kim et al., 1994b; Kim and Roeder, 1993). However, since TFIIB is required here during the preincubation step to generate a stable complex, the effects on complex stability of such a conformational change may be transient in the absence of TFIIB. Alternatively, Gal4-IE may stabilize a TFIID/TFIIB complex by interactions with both factors.

Recently, studies similar to the ones reported here have shown that the Epstein-Barr virus transactivator Zta promotes the formation of a stable complex consisting of TFIID, TFIIA and TFIIB (Lieberman, 1995). These general factors alone were required to render a Zta-dependent PIC stable to the detergent sarkosyl. Similarly, PIC stability to an oligonucleotide containing Zta binding sites required the further addition of the USA cofactor fraction (Meisterernst et al., 1991; Lieberman, 1995). It is not known which of these two situations most resemble the core-promoter competition assay used here. At a minimum, however, the results with Zta mirror precisely the TFIIB requirement in activator-dependent stabilization noted in this work.



Earlier studies have suggested that TFIID is a functional target of the native IE protein (Workman and Roeder; 1988; Abmayr et al 1988). How can those results be reconciled with those obtained here? The interaction between native IE and TFIID may potentiate TFIID interactions with the other general factors, including TFIIB, but may require TFIIB addition to cause an increase in complex stability. In fact, it is possible that Gal4-IE increases the number of PICs formed via an interaction with TFIID, but that any complexes formed require TFIIB to become stable to second template addition. In fact, ATF oligo competition experiments (Horikoshi et al, 1988a) have shown that the ATF-induced TFIID conformational change referred to above facilitates (and possibly stabilizes) interactions of other general factors with the promoter later in the PIC assembly pathway.

It is also possible that the factor requirements for stability to various disruptive regiments may be different. The studies on native IE assayed the ability of the activator to render transcription resistant to nucleosome assembly. In this assay, a TFIID-IE interaction took place which increased transcription in the context of competing nucleosome assembly (Workman et al., 1988). Thus, the factor requirements for chromatin anti-repression may be different than those for stability to a second template. Consistent with this idea, Zta-dependent PIC stability to a competing oligonucleotide requires TFIID, TFIIA, and TFIIB, whereas stability of such a complex to sarkosyl requires the addition of USA (Lieberman, 1995).

What are the implications of the results in this chapter for an understanding of activator function *in vivo*? First, these results



suggest that preinitiation complexes are likely not fully assembled on promoters whose transcription is regulated at the level of initiation. Rather, a concerted interaction during PIC assembly between activators and the general transcription factors is required each time the transcription of a gene is activated.

Given the inability of activators to stimulate transcription after PIC assembly (Wang et al., 1992), one would expect that *in vivo* a preformed PIC would also be refractory to stimulation (of initiation) by an activator (for an exception see Rougvie and Lis, 1988). This would imply that there exist mechanisms for clearing promoters of PIC's to allow regulation of initiation. Random dissociation of complexes and disruption by DNA replication may be two such mechanisms. In addition, complex activity and stability on promoter DNA may be regulated by phosphorylation and/or dephosphorylation of general factors.

The results presented in this Chapter, and others cited therein, suggest that activators target early steps of PIC assembly, specifically, the steps of TFIID, TFIIA, and TFIIB incorporation. While TFIIB and TFIID have received significant attention (see e.g. Hoffmann, 1994), relatively little is known about TFIIA. Therefore, in the next two chapters I turn to the cloning and reconstitution of TFIIA (Chapter IV), and an analysis of the protein-protein interactions of TFIIA (Chapter V).





## **Chapter IV: Characterization of the Highly Conserved TFIIA Small Subunit of *Drosophila melanogaster***



The experiments described in Chapter 3 indirectly implicated the DAB complex as a target of Gal4-IE mediated PIC stabilization. Several other lines of evidence also implicate TFIIA as having a central role in both basal and activated transcription (see Chapter 1, sections 4.2.2 and 5.2.2). To facilitate further study of TFIIA, it was necessary to clone the subunits of TFIIA and reconstitute transcriptionally active TFIIA from recombinant components.

Human TFIIA promoter complex formation activity was previously shown to be dependent upon three subunits of 35, 19 and 12 kD (Cortes et al., 1992). Similarly, TFIIA eluted from an immunopurified *Drosophila* TFIID displayed subunits of 30, 19 and 14 kD (Yokomori et al., 1993). At the time of the experiments described in this chapter, a cDNA isolated in the course of efforts to clone the 35 kD subunit of human TFIIA was found to encode a protein of 55 kD. The relationship between the 55 kD protein encoded by the cDNA (hTFIIA/ $\alpha$ ) and the 35 and 19 kD subunits of native TFIIA was unclear. No cDNA for the small subunit of TFIIA from any higher eukaryote had been isolated.

In contrast to human and *Drosophila* TFIIA, which consist of three subunits, yeast TFIIA consists of two subunits of molecular mass 32 and 13.5 kD (Ranish and Hahn, 1991). Genes encoding both the large and small subunits of yeast TFIIA (TOA 1 and TOA 2, respectively) have been isolated and shown to be essential for yeast viability (Ranish et al., 1992). The TOA1 and TOA2 gene products, when expressed separately in bacteria and corenated, reconstituted both promoter complex formation and transcriptional



activities of native TFIIA (Ranish et al., 1992). Unlike the case of hTFIIA/ $\alpha$ , the predicted molecular mass of the TOA1 gene product approximately matches the observed molecular mass of the large yeast TFIIA subunit. This chapter describes the cloning and functional analysis of the small subunit of *Drosophila* TFIIA.



## Results

A computer homology search using the yeast TOA2 protein sequence as a query revealed that the 5' end of a genomic clone containing the *Drosophila virilis rough* gene (Heberlein and Rubin, 1992) contained dispersed sequences whose translation predicted a protein highly homologous to sections of TOA2. By positing splicing at consensus splice donor and acceptor sites in this genomic clone, approximately 85% of a presumptive *D. virilis* TFIIA-S gene could be reconstructed (Fig. 13).

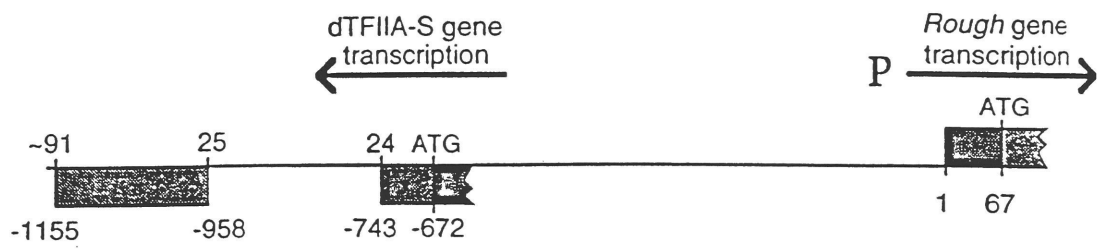
To isolate a full length cDNA corresponding to this gene, I screened a *D. melanogaster* embryonic cDNA library with an oligonucleotide homologous to sequences encoding the presumptive N-terminus of the *D. virilis* protein and isolated clone D2-2. This cDNA (Fig. 14) encodes a protein which has a predicted molecular weight of 12.5 kD (Fig 15) and differs by only one amino acid (A31 to G31) from the protein encoded by the partially reconstructed *D. virilis* gene.

The cDNA-encoded protein sequence is interesting in several respects. First, it displays 40% identity and 56% similarity to the yeast TOA2 gene product (Ranish et al., 1992). In particular, 22 out of 27 (81%) branched-chain hydrophobic amino acids are conserved between the two species (Fig. 16). In addition, the regions between residues 65 and 77 and between 87 and 98 show 70% and 85% sequence identity, respectively, to yeast TOA2. The former region is also highly conserved in a deduced rice protein sequence (Sesaki and Minobe, 1993) which was identified by a computer search and which may encode that organism's TFIIA-S, although it also contains

**Figure 13: Schematic Diagram of Genomic Fragment Containing Presumptive *Drosophila virilis* TFIIA-S gene**

*Rough* gene coding sequences (Heberlein and Rubin, 1992) are shown at right, presumptive TFIIA-S sequences are shown at left; numbers above the line refer to presumptive TFIIA-S amino acids, based on yeast TOA2 homology. Numbers below the line refer to nucleotides relative to the *rough* gene start site.





**Figure 14: Nucleotide sequence of dTFIIA-S cDNA .**

Both strands of the D2-2 cDNA were sequenced using the Sequenase system (USB).

```

      10          20          30          40          50
      *          *          *          *          *
GGCACGAGGT TCGTCGGAA TTAAAGCAGC CACTATGTGG TATCAACTGT
CCGTGCTCCA AGGCAGCCTT AATTCGGTGG GTGATACAGC ATAGTTGACA

      60          70          80          90          100
      *          *          *          *          *
ACCGCAACAC CACGCTCGGC AACACCCTGC AGGAGAGCCT CGATGAGCTG
TGGCGTTGTG GTGCGAGCCG TTGTGGGACG TCCTCTCGGA GCTACTCGAC

      110         120         130         140         150
      *          *          *          *          *
ATTCAGTACG GCCAGATTAC GCCCGGACTG GCTTTCAAGG TTCTGCTGCA
TAAGTCATGC CGGTCTAATG CGGGCCTGAC CGAAAGTTCC AAGACGACGT

      160         170         180         190         200
      *          *          *          *          *
ATTGACAAG AGCATCAACA ATGCCCTAAA CCAGCGGGTC AAGGCCCCGG
TAAGCTGTTT TCGTAGTTGT TACGGGATTT GGTGCCCCAG TTCCGGGGCC

      210         220         230         240         250
      *          *          *          *          *
TCACCTTCAA GGCTGGAAAA CTAACACCT ACCGCTTCTG CGACAATGTC
AGTGGAAGTT CCGACCTTTT GATTTGTGGA TGGCGAAGAC GCTGTTACAG

      260         270         280         290         300
      *          *          *          *          *
TGGACTCTCA TGCTTAACGA TGTAGAGTTC CGCGAAGTGC ACGAGATCGT
ACCTGAGAGT ACGAATTGCT ACATCTCAAG GCGCTTCACG TGCTCTAGCA

      310         320         330         340         350
      *          *          *          *          *
CAAGGTGGAC AAGGTGAAGA TCGTGGCCTG CGACGGCAAG AGCGGCGAGT
GTTCCACCTG TTCCACTTCT AGCACCGGAC GCTGCCGTTC TCGCCGCTCA

      360         370         380         390         400
      *          *          *          *          *
TCTGAACACC ACCACCCGAT CTGAACACCC AATGTAACCC CACTAAACAC
AGACTTGTGG TGGTGGGCTA GACTTGTGGG TTACATTGGG GTGATTTGTG

      410         420         430         440         450
      *          *          *          *          *
ACCATGTAAC CCCACAAAAC ACACCAATTA TAACCATTAC AAATAGTTGT
TGGTACATTG GGGTGTTTTG TGTGGTTAAT ATTGGTAATG TTTATCAACA

      460         470         480         490         500
      *          *          *          *          *
AAGATTCGTA GGACGATAAG CTGGGTTGGG AAACGTAAAA CCGACCGTGC
TTCTAAGCAT CCGCTATTG GACCCAACCC TTTGCATTTT GGCTGGCAGC

      510         520         530         540         550
      *          *          *          *          *
GATGCAGCAG GACTTGCAAG AAATACACTG TTAATTCAAA AAAAAAAAAA
CTACGTCGTC CTGAACGTTT TTTATGTGAC AATTAAGTTT TTTTTTTTTT

AAAAAAA
TTTTTTT

```

**Figure 15: Conceptual translation of dTFIIA-S cDNA**

MSYQLYRNTT LGNTLQESLD ELIQYGQITP  
GLAFKVLLQF DKSINNALNQ RVKARVTFKA  
GKLNTYRFCD NVWTLMLNDV EFREVHEIVK  
VDKVKIVACD GKSGEF



an additional 55 amino acid N-terminus lacking TFIIA-S homology. Thus, the small subunit of TFIIA is highly conserved between representatives of three kingdoms, suggesting that it plays an essential and evolutionarily invariant role in transcription.

In view of the highly conserved nature of TFIIA-S, it was surprising to find that a region corresponding to yeast amino acids 87-102 was missing in the *Drosophila* protein. However, PCR analysis on a *Drosophila* embryonic cDNA library using primers spanning the missing region produced only one product, whose size was identical to the product produced when clone D2-2 was used as template (data not shown). Recently, results of mutational analysis have revealed that amino acids 87-102 of yeast TFIIA-S are dispensible for TFIIA function (Kang et al, 1995). This is consistent with the deletion of this region in the evolutionary transition from yeast to *Drosophila*.

### **dTFIIA-S is Present in *Drosophila* Nuclear Extract**

Though suggested by immunoprecipitation experiments (Yokomori et al., 1993), the identification of a TFIIA small subunit in *Drosophila* has not been demonstrated unambiguously. To detect TFIIA-S directly, I used ph6dA12 (Chapter 2) to express N-terminal histidine-tagged dTFIIA-S in *E.coli*. The resulting 15 kD protein, which includes the 12.5 kD dTFIIA-S plus 2.5 kD of extraneous sequences, was purified from insoluble bacterial inclusion bodies to >95% purity (Fig. 17) and used to generate specific antisera in rabbits. As shown in Figure 18, preimmune and immune sera both reacted with a number of high molecular weight bands in a *Drosophila* embryo extract-derived P11 flow-through fraction.

**Figure 16: Amino acid homology between TFIIA-S proteins from multiple kingdoms**

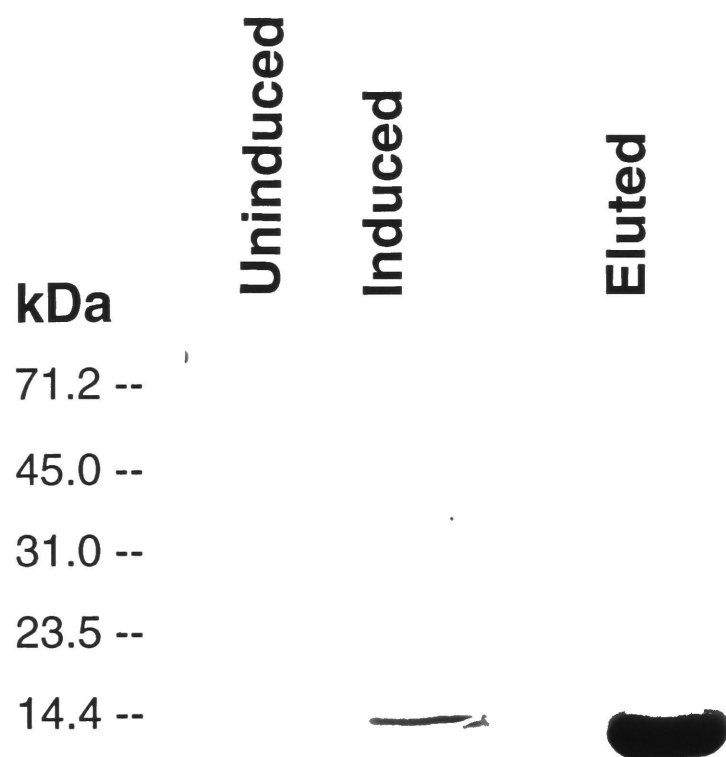
Amino acid sequences of TFIIA-S from *Drosophila melanogaster* (D.m.; top line), *Saccharomyces cerevisiae* (S.c., middle line) (Ranish et al., 1992), and Rice (R., lower line) (Sesaki and Minobe, 1993) aligned based on sequence similarity. Conserved amino acids are highlighted in black.



1	M	S	-	-	-	Y	Q	L	Y	R	N	T	T	L	G	N	T	L	Q	E	S	L	D	E	L	I	Q	Y	G	D.m.	
2	M	A	V	P	G	Y	Y	E	L	Y	R	R	S	T	I	G	N	S	L	V	D	A	L	D	T	L	I	S	D	G	S.c.
55	-	-	-	-	-	-	-	-	-	-	-	-	S	T	I	G	M	C	L	T	E	T	L	D	E	M	V	S	S	G	R.
27	Q	I	T	P	G	L	A	F	K	V	L	L	O	F	D	K	S	I	N	N	A	L	N	Q	R	V	K	A	R	V	D.m.
31	R	I	E	A	S	L	A	M	R	V	L	E	T	F	D	K	V	V	A	E	T	L	K	D	N	T	O	S	K	L	S.c.
74	T	L	S	P	E	L	A	I	Q	V	L	V	O	F	D	K	S	M	T	E	A	L	E	N	Q	V	K	S	K	V	R.
57	T	F	K	A	G	K	L	N	T	Y	R	F	C	D	N	V	W	T	L	M	L	N	D	V	E	F	R	-	-	-	D.m.
61	T	V	K	-	G	N	L	D	T	Y	G	F	C	D	D	V	W	T	F	I	V	K	N	C	Q	V	T	V	E	D	S.c.
104	S	I	K	-	G	H	L	H	T	Y	R	F	C	D	N	V	W	T	F	I	L	T	E	A	S	F	K				R
84	-	-	-	-	-	-	-	-	-	-	-	E	V	H	E	I	V	K	V	D	K	V	K	I	V	A	C	D	G	K	D.m.
90	S	H	R	D	A	S	Q	N	G	S	G	D	S	Q	S	V	I	S	V	D	K	L	R	I	V	A	C	N	S	K	S.c.
103	S	G	E	F																										D.m.	
120	K	S	E																										S.c.		

**Figure 17: Expression and purification of dTFIIA-S**

Total cell lysate from uninduced and IPTG-induced bacteria containing ph6dA12, and final eluted dTFIIA-S fraction, analyzed by SDS-PAGE and coomassie blue staining. Position of molecular weight standards are indicated to the left of the gel.

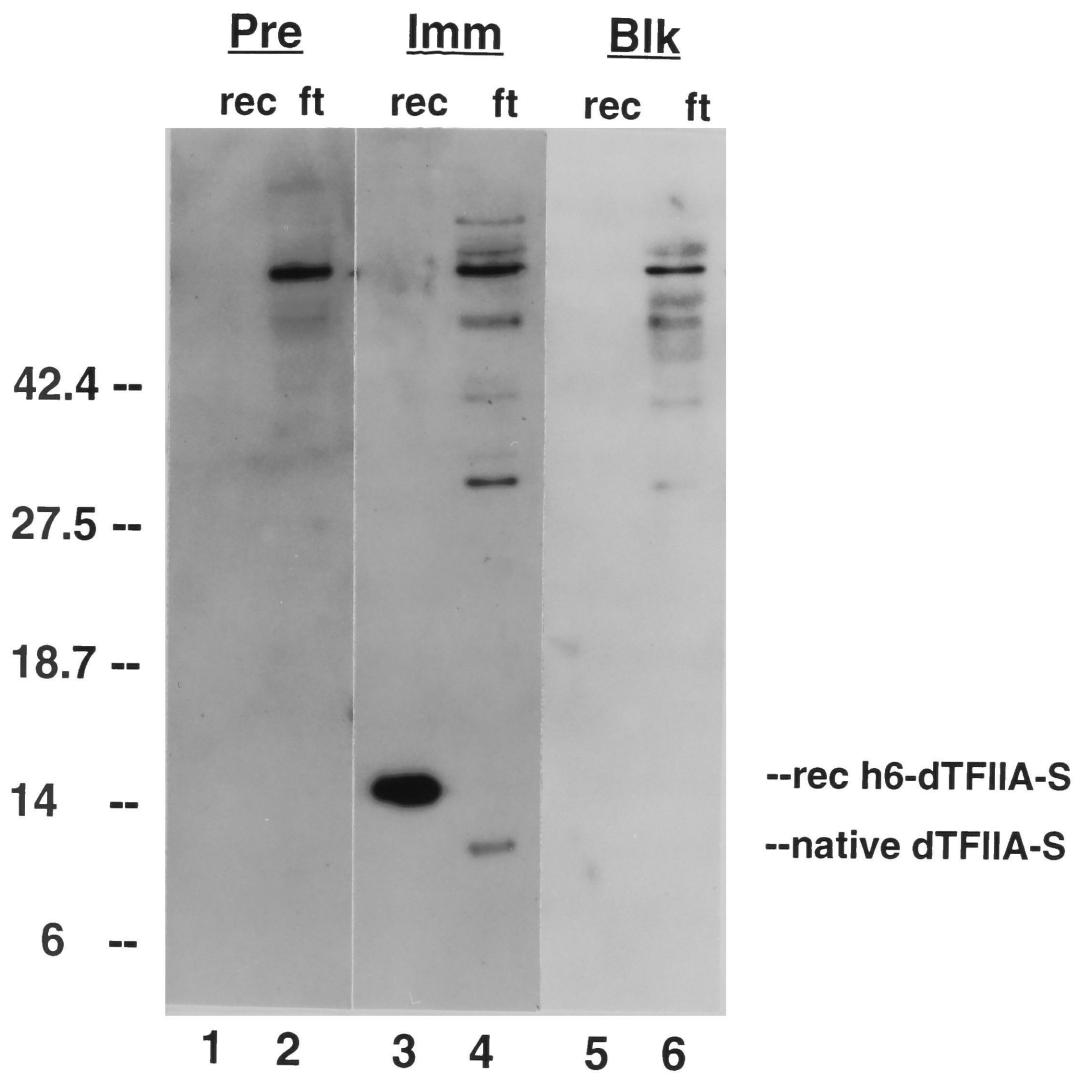






**Figure 18: Immunodetection of native dTFIIA-S**

Recombinant h<sub>6</sub>dTFIIA-S (rec; lanes 1,3,5) or *Drosophila* nuclear extract-derived P11 flow-through fraction (ft; lanes 2,4,6) were run on SDS-PAGE, blotted to nitrocellulose and probed with a 1:2000 dilution of preimmune serum (lanes 1,2), immune (anti-TFIIA-S) serum (lanes 3,4), or immune serum blocked by preincubation with recombinant dTFIIA-S (lanes 5,6). Positions of molecular weight markers and bands corresponding to native and recombinant TFIIA-S are indicated to the left and right of the gel, respectively.







However, immune serum additionally reacted with both recombinant dTFIIA-S (lane 3) and a 12.5kD protein in the P11 flow-through fraction (lane 4). As expected, preincubation of immune serum with recombinant dTFIIA-S blocked reactivity to this 12.5 kD band, but not to the high molecular weight bands (lanes 5,6).

Fractionation of *Drosophila* embryo nuclear extracts (Fig. 19) resulted in a significant concentration of dTFIIA in the 0.3M Na<sub>3</sub>PO<sub>4</sub> Hydroxyapatite fraction (data not shown) and eliminated most of the non-specific background bands observed in the previous Western analysis. Immunoblot analysis (Fig. 20) showed that native dTFIIA-S migrates identically to the recombinant protein (expressed without any extraneous sequences using plasmid pdA12), indicating that the dTFIIA-S cDNA encodes a full length protein. The two bands visible above the 42.4 kD marker were non-specific as judged by immunoblot with preimmune serum (data not shown). These results confirm the presence of dTFIIA-S in *Drosophila* nuclear extract and show no evidence for post-translational modification or processing, in contrast to the case for the large TFIIA subunits from human and *Drosophila* (DeJong and Roeder, 1993; Ma et al., 1993; Yokomori et al., 1993).

### **dTFIIA-S and hTFIIA/ $\alpha$ Reconstitute TFIIA Complex Formation Activity**

The 55 kD protein encoded by hTFIIA/ $\alpha$  had not been functionally analyzed, due to the assumption that it encoded only the 35 kD protein observed in native TFIIA preparations, and the observation (Cortes et al., 1992) that 35, 19 and 12 kD subunits were all required for TFIIA activity. To determine whether hTFIIA/ $\alpha$  could

**Figure 19: Partial purification scheme for Drosophila TFIIA.**

See Chapter 2 for detailed protocol. Drosophila embryo-derived nuclear extract was fractionated on Q-Sepharose and Hydroxyapatite columns as shown.

**Drosophila Embryo Nuclear Extract**

**Q-Sepharose**

**100**

**400**

**mM KCl**

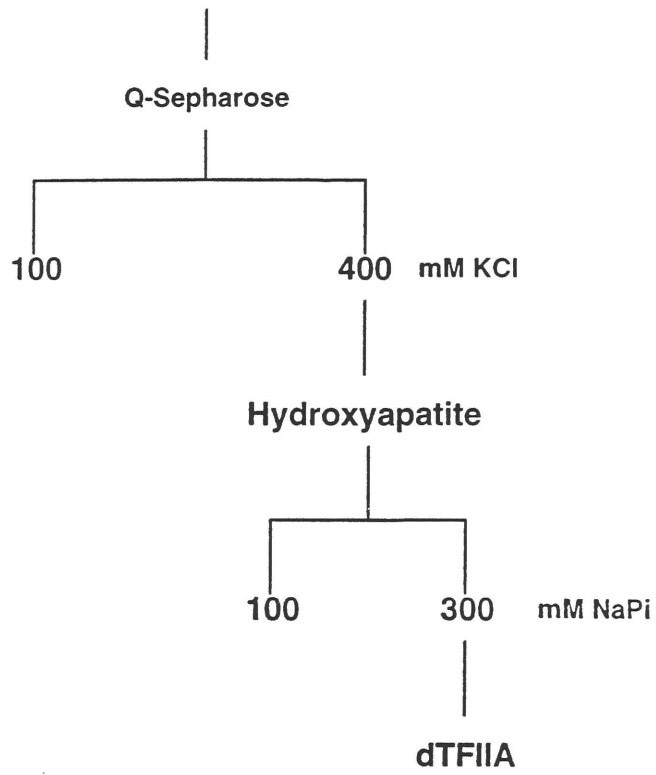
**Hydroxyapatite**

**100**

**300**

**mM NaPi**

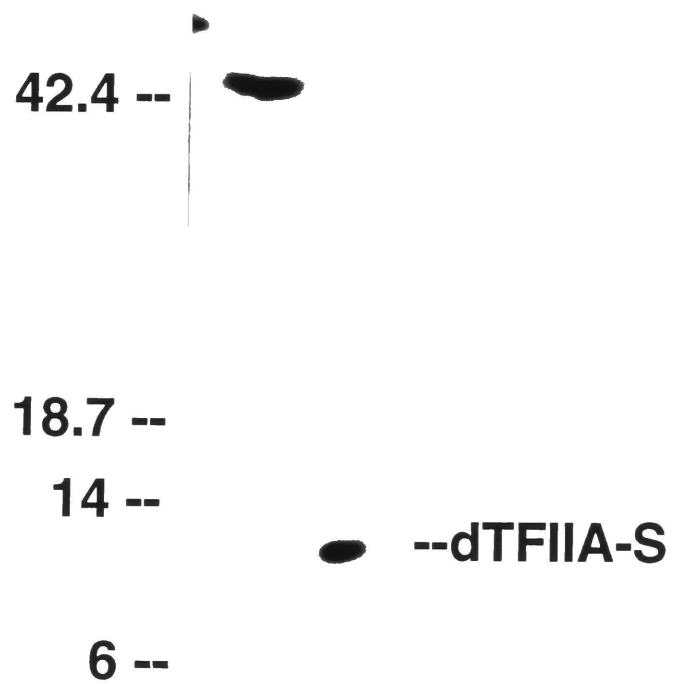
**dTFIIA**



**Figure 20: Immunodetection of native TFIIA-S**

Final hydroxyapatite eluted fraction (HAP 0.3; lane 1) and recombinant dTFIIA-S expressed without extraneous sequences (Rec.; lane 2) were immunoblotted with anti-TFIIA-S serum as in Figure 18. Positions of molecular weight markers are indicated to the left of the gel.

HAP 0.3  
Rec.



1 2



by itself substitute for both the 35 and 19 kD subunits, and to determine whether purified recombinant dTFIIA-S represented a functional TFIIA subunit the two proteins were assayed in a promoter complex formation assay. To assay this TFIIA activity, TBP and a TATA-box containing oligonucleotide were coincubated and run on a native gel under conditions which do not allow a detectable TBP-DNA complex to form in the absence of TFIIA (Fig. 21, lane 1). Addition of either recombinant human TFIIA/ $\alpha$  or dTFIIA-S failed to produce any TBP-TFIIA complex (lanes 3 and 4, respectively). However, the presence of both dTFIIA-S and hTFIIA/ $\alpha$  (lane 5) with TBP resulted in the formation of a DA promoter complex with a mobility identical to that of the complex formed with native human TFIIA and TBP (compare lanes 2 and 5). Formation of this complex was dependent upon TBP (lane 6). Human TFIIA/ $\alpha$  and dTFIIA-S could function in this assay with or without prior corenaturation, although in either case the specific activity was lower than that of native TFIIA (see below). This experiment demonstrates that dTFIIA-S is sufficiently conserved to allow formation of an active heteromeric complex with components from distantly related species.

Further, the experiment in Figure 21 suggested that hTFIIA/ $\alpha$  was capable of functionally replacing both the 35 and 19 kD subunits of native TFIIA. Subsequent to this experiment, it was shown conclusively by DeJong and Roeder (1993) that the 35 and 19 kD subunits of native TFIIA consist of N- and C-terminal fragments, respectively, of the protein encoded by the hTFIIA/ $\alpha$  cDNA.

**Figure 21: Effect of TFIIA on TBP-Promoter Complex**

Gel mobility shift assays performed with 2 ng yeast TBP (lanes 1-5), 2  $\mu$ l hTFIIA (DeJong and Roeder, 1993) fraction (lane 2), 100 ng hTFIIA/ $\alpha$  (lanes 3,5,6), and 100 ng dTFIIA-S (lanes 4-6). Positions of free probe and DA complex are indicated to the left of the gel.



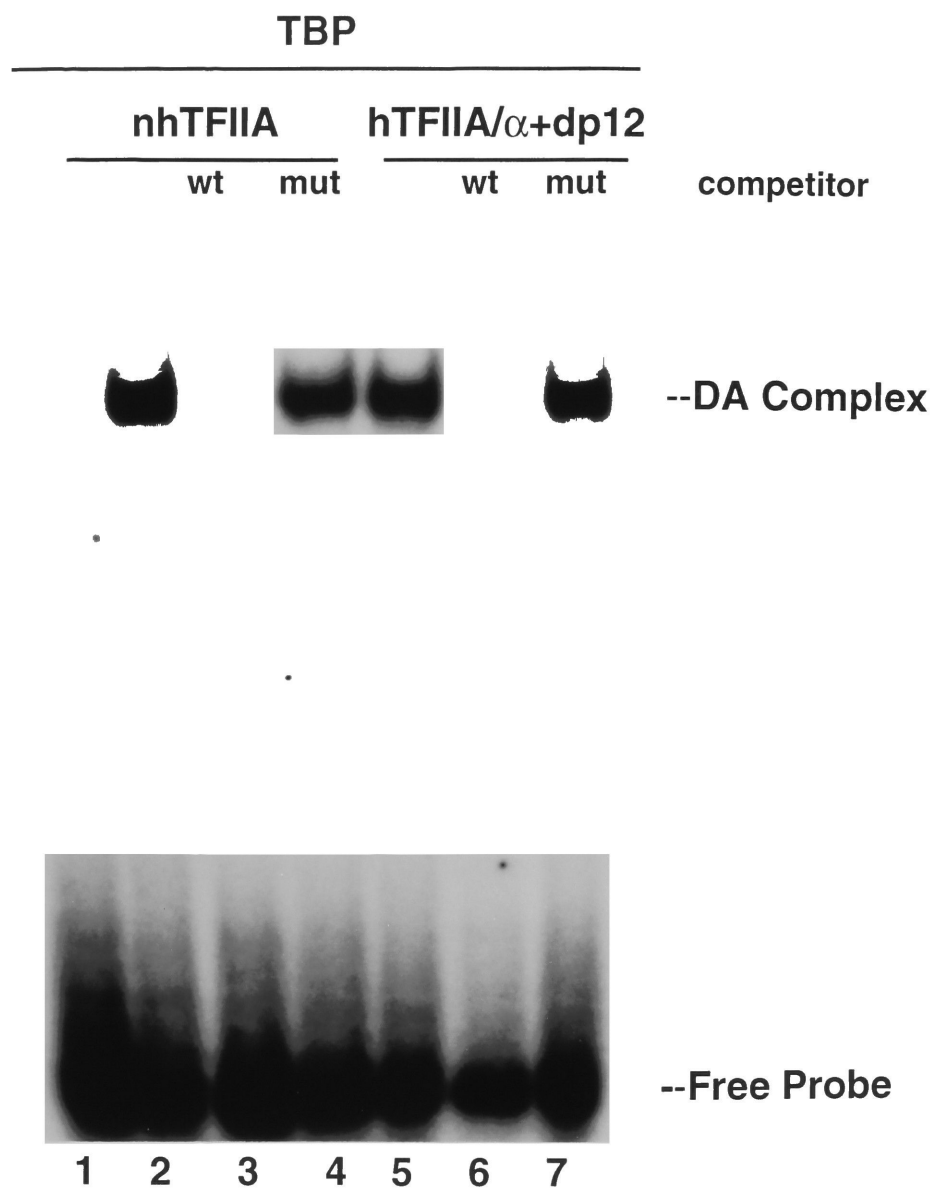
## ---free probe





### **Figure 22: DNA-binding specificity of DA Promoter Complexes**

Gel mobility shift assay performed as in Figure 21 with yeast TBP (all lanes) and purified human TFIIA (lanes 2-4) or hTFIIA/ $\alpha$  and dTFIIA-S (lanes 5-7). A ten-fold molar excess of cold competitor containing a wild-type TATA box (lanes 3,6) or mutated TATA box (lanes 4,7) was added. Positions of relevant complexes are indicated to the right of the gel.





To establish that the bandshift observed with dTFIIA-S and hTFIIA/ $\alpha$  had the characteristics of a natural TFIIA-containing TBP-TFIIA (DA) promoter complex, I performed experiments comparing the properties of these two complexes. As shown in Fig. 22, the formation of the native DA complex was blocked effectively by an unlabeled wild-type TATA oligonucleotide, but not by a mutant TATA oligonucleotide (compare lanes 3 and 4). The same pattern of competition was observed with the DA promoter complex formed with recombinant TFIIA components including dTFIIA-S (compare lanes 6 and 7).

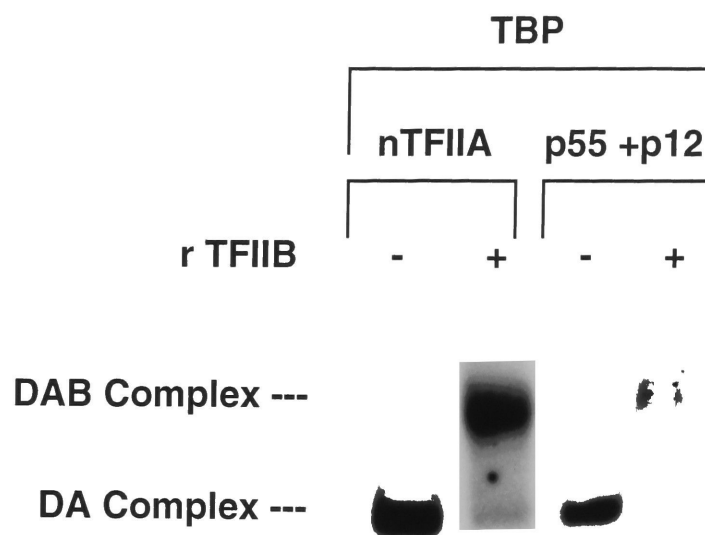
A second test of the functionality of the hybrid TFIIA was its ability to form a DA promoter complex that could support higher-order preinitiation complex formation. Addition of TFIIB to a native DA promoter complex results in a DAB promoter complex which migrates more slowly than the DA promoter complex (Buratowski et al., 1989; Maldonado et al., 1990). This was confirmed by bandshift analysis with native human TFIIA in the presence of TBP and purified recombinant human TFIIB (lanes 1 and 2 of Fig. 23). The DA promoter complex formed by recombinant hTFIIA/ $\alpha$  and dTFIIA-S was also converted into a higher order complex containing recombinant TFIIB (lanes 3 and 4), and with an efficiency similar to that observed with the native TFIIA-containing DA promoter complex. These experiments provide support for the equivalence of native TFIIA and the hybrid recombinant TFIIA.

The ability of the hTFIIA/ $\alpha$  gene product to support formation of a bonafide DA-promoter complex indicated that it contained all of the structural requirements of the native 35 and 19 kD subunits.

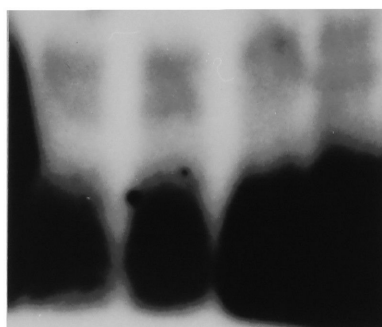
**Figure 23: Incorporation of DA complexes into DAB complex**

Gel mobility shift assay performed as in figure 21 with TBP (all lanes), purified human TFIIA (lanes 1,2) or hTFIIA/ $\alpha$  and dTFIIA-S (lanes 3,4), with 10 ng recombinant human TFIIB added (lanes 2,4). Positions of relevant complexes are indicated to the left of the gel.





Free Probe ---

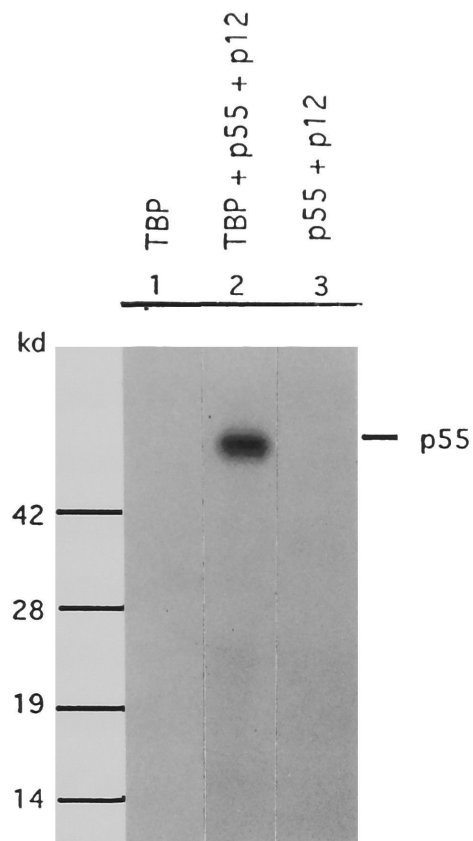






**Figure 24: Crosslinking of hTFIIA/ $\alpha$  within DA promoter complex**

Autoradiograph of SDS gel of reaction products after photocrosslinking and nuclease digestion as described in Chapter 2; reactions included TBP (lanes 1,2) and/or hTFIIA/ $\alpha$  and dTFIIA-S (lanes 2,3), and probe shown at bottom of figure (\*\* denotes position of incorporated N<sub>3</sub>RdUMP). Positions of molecular weight markers are indicated to the left of the gel.



-51  
GGGTGTTCTGAAGGGGGGCTATAAAAGGGGTGGGGGCGCGTTTCGTCCTCA +1  
CCGATATTTTCCCCACCC  
CCCACAAGGAC ••CCCC  
↑  
fill-in

Adenovirus  
Major Late  
Promoter



However, it was not known whether the recombinant protein functioned as a 55 kD moiety, or whether it was degraded into 35 and 19 kD fragments either during purification or upon assembly into a DA complex. To determine the form of hTFIIA/ $\alpha$  within the DA complex, an oligonucleotide probe containing human Major Late Promoter sequences between -51 and +1 was substituted at positions -39 and -40 with the photoactivatable crosslinker and TTP analog N3R-dUTP (Bartholemew et al., 1991; Coulombe et al, 1994) as well as with radioactive nucleotides adjacent to the crosslinking base. A DA complex was formed on this probe (data not shown) with recombinant TFIIA and TBP, the mixture was irradiated with UV light, digested extensively with nucleases, and the products displayed by SDS-PAGE and autoradiography. Consistent with the results of the bandshift experiments shown above, neither TBP alone nor recombinant TFIIA alone results in any protein-DNA crosslinking at this position. However, a 55 kD species is strongly crosslinked when TBP and recombinant TFIIA are allowed to form a DA complex on the probe. This experiment shows that hTFIIA/ $\alpha$  can functionally replace the 35 and 19 kD subunits of native TFIIA as an intact 55 kD moiety, and that cleavage into 35 and 19 kD subunits is neither required nor a consequence of promoter complex formation.

#### **Anti-dTFIIA-S Serum Recognizes the DA Promoter Complex**

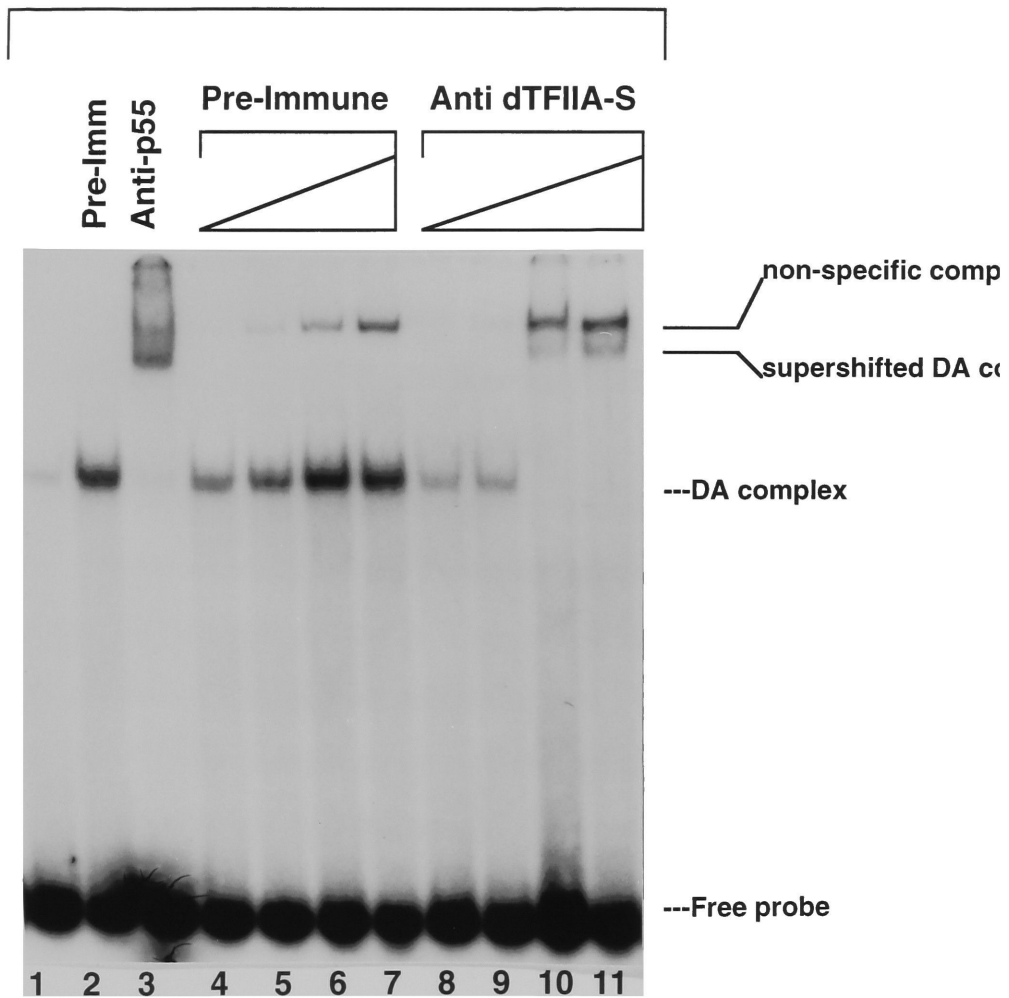
It has been shown that all three subunits of native human TFIIA are required to generate the DA promoter complex (Cortes et al., 1992), and that the large subunit is present in the complex (DeJong and Roeder, 1993; Yokomori et al., 1993). However, the stable association of the small subunit with the DA promoter complex has

### **Figure 25: Effect of anti-dTFIIA-S Antiserum on DA complex**

DNA binding reactions were carried out under the same conditions as Figure 21 using recombinant hTFIIA/ $\alpha$  (p55), and recombinant dTFIIA-S. 1  $\mu$ l preimmune and anti-hTFIIA/ $\alpha$  (DeJong and Roeder, 1993) were included in lanes 2 and 3, respectively. Preimmune and anti-dTFIIA-S antisera were included (0.2, 0.5, 1 and 2  $\mu$ l in lanes 4 and 5, 5 and 9, 6 and 10, and 7 and 11, respectively) as indicated. Products of DNA binding reactions were separated as in Figure 21.



TBP + dTFIIA-S + hTFIIA/ $\alpha$





not been demonstrated. As shown in Fig. 25, the DA complex was altered by immune serum directed against human TFIIA/ $\alpha$ , resulting in a complex of retarded mobility (lanes 2 and 3). Antiserum directed against dTFIIA-S yielded a similar result, although the DA promoter complex was super-shifted with lower efficiency (lanes 8-11). Preimmune serum did not super-shift the DA promoter complex (lanes 4-7). The DA promoter complex is enhanced (lanes 4-7) as a result of stabilization by serum proteins, which are also responsible for the uppermost complex in lanes 4-11 (labeled "nonspecific"). Based on the ability of a dTFIIA-S-specific antiserum to supershift the DA complex, I conclude that the small subunit of TFIIA stably associates with the DA complex.

### **dTFIIA-S and hTFIIA/ $\alpha$ Reconstitute TFIIA Transcriptional Activity**

Efforts to demonstrate transcriptional activity of recombinant TFIIA by simple mixing of the two recombinant proteins were unsuccessful (data not shown). This is likely due to the fact that the specific activity of the mixed recombinant TFIIA subunits was considerably lower than that of native TFIIA, as measured in the promoter complex formation assay. In an attempt to increase the specific activity of recombinant TFIIA, equimolar amounts of dTFIIA-S and hTFIIA/ $\alpha$  were dissolved in 8M Urea and 6M Guanidine HCL, respectively, combined, and subjected to dialysis to effect co-renaturation. This corenated recombinant TFIIA was active in promoter complex formation (data not shown).

To determine the transcriptional activity of this material, it was assayed in a transcription system composed of highly purified or



recombinant human transcription factors including TFIID.

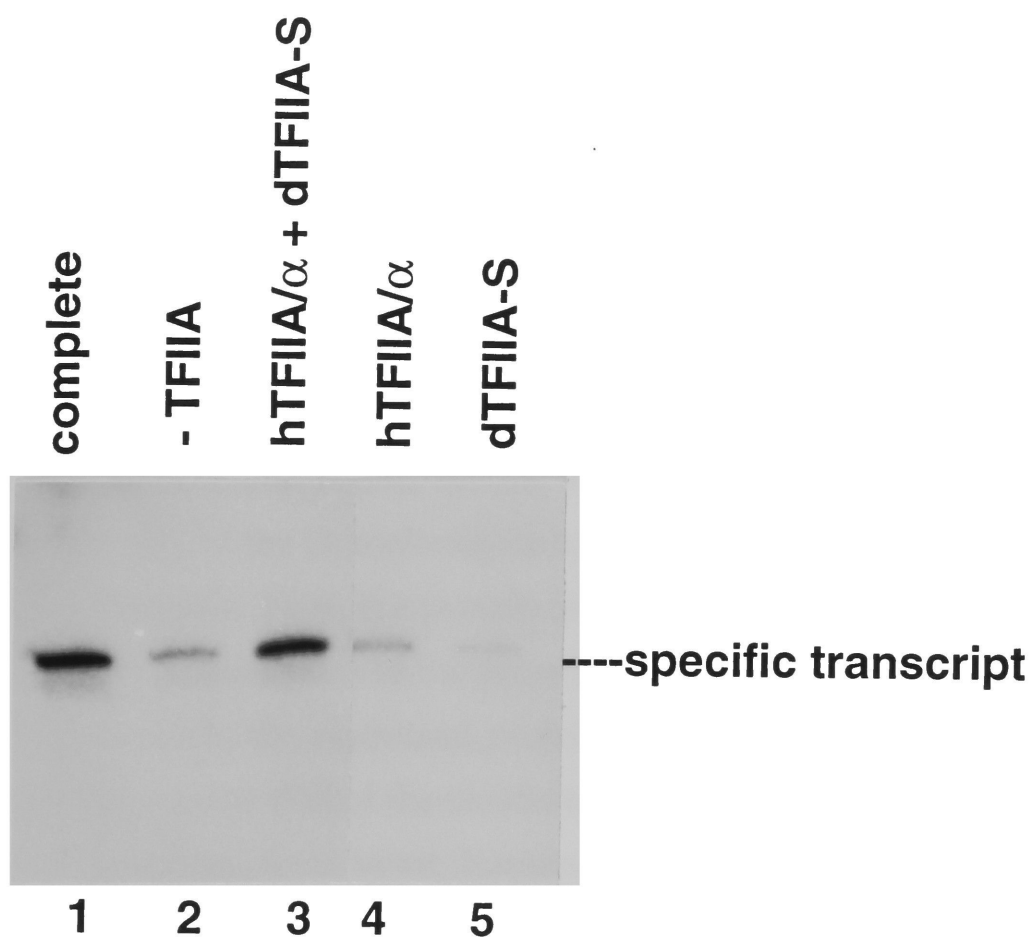
Transcription was performed in the presence of the activator Gal 4-AH because of the enhanced requirement for TFIIA in activated, as opposed to basal, transcription (see Chapter I).

As shown in Figure 27, the transcription system exhibits a partial (approximately 3-fold) dependence on the addition of TFIIA (compare lanes 1 and 2). The residual (ectopic TFIIA-independent) activity could reflect either basal transcription due to free TBP in one of the other factors or activator dependent transcription due to endogenous TFIIA in one of the other factors. However, it is also possible that TFIIA is not absolutely required for TFIID-mediated transcription in this reconstituted system (Roy et al., 1993 and references therein).

The requirement for TFIIA can be satisfied by addition of corenated TFIIA consisting of recombinant human TFIIA/ $\alpha$  and dTFIIA-S (lane 3). Independent additions of renatured TFIIA/ $\alpha$ , or of renatured dTFIIA-S, do not stimulate transcription, a result consistent with promoter complex formation assays. This experiment demonstrates that dTFIIA-S is a transcriptionally active subunit of TFIIA and is consistent with the observation (Yokomori et al., 1993) that native *Drosophila* TFIIA exhibits transcriptional activity in a system composed of human-derived components.

### **Figure 26: dTFIIA-S supports TFIIA transcriptional activity**

In vitro transcription using reconstituted human transcription system as described in Chapter 2 was carried out with 100 ng of G5HMC2AT in the presence of GAL4-AH. Complete reaction included natural human TFIIA (lane 1), no TFIIA (lane 2), 0.5  $\mu$ l of corenated hTFIIA/ $\alpha$  and dTFIIA-S (lane 3), renatured hTFIIA/ $\alpha$  alone, or dTFIIA-S alone (lane 5). The position of the specific transcript is indicated.







## Discussion

In this Chapter I have described the cloning of a cDNA encoding the small subunit of TFIIA from *Drosophila melanogaster*. This was accomplished using an oligonucleotide derived from a *D. virilis* TFIIA-S genomic DNA sequence (Heberlein and Rubin, 1990) identified in the upstream region of the *rough* gene. It is likely that the promoter for the *D. virilis* TFIIA-S gene is contained within this genomic clone as well, although precise identification of this promoter will require transcription start-site mapping. The possibility that shared regulatory elements drive both TFIIA-S and *rough* gene transcription merits further investigation.

The identity of the *D. melanogaster* cDNA is demonstrated by several observations. First, the protein encoded by the dTFIIA-S cDNA is highly homologous to the yeast TOA2 gene product (Ranish et al., 1992). Second, the expressed protein has a molecular size (12.5 kD) nearly identical to that of the small subunit of TFIIA identified in purified preparations from yeast, human, rat, and *Drosophila* (Conaway and Conaway, 1993; Yokomori et al., 1993). That I have isolated a full length cDNA is confirmed by the fact that native dTFIIA-S exhibits the same mobility on SDS gels as does the recombinant protein. Third, the protein acts in conjunction with the precursor to the large subunits of human TFIIA to form a TBP-TFIIA (DA) promoter complex. Fourth, the DA promoter complex formed by this reconstituted TFIIA behaves identically in all respects tested to the DA promoter complex formed with purified natural TFIIA. That the promoter complex formation activity of TFIIA/ $\alpha$  is not due to proteolysis into 35 and 19 kD fragments was determined by protein-



DNA crosslinking.. This observation reinforces the idea that the hTFIIA/ $\alpha$  gene product, like yeast TOA1, is competent for promoter complex formation without proteolytic processing into separate 35 and 19 kD subunits. Finally, the demonstration that dTFIIA-S (in conjunction with hTFIIA/ $\alpha$ ) can fully replace native TFIIA in a reconstituted human transcription system establishes that the gene we have isolated encodes a functional subunit of TFIIA.

The primary structure of dTFIIA-S is notable due to its high degree of sequence similarity to the small subunits of both yeast TFIIA and an apparent rice homologue (Fig. 16). In particular, approximately 80% of the branched chain hydrophobic amino acids are conserved between yeast and *Drosophila*. Although the exact function of TFIIA-S has not yet been determined, it is possible that this highly conserved subunit forms a core around which the more variable large subunit(s) of TFIIA are situated. Under this model, TFIIA-S would nucleate the assembly of the native complex by interacting with the conserved N-and C-terminal regions of the large subunits. This supposition is supported by the recent observation that the conserved N and C-terminal regions of yeast TOA1 mediate inter-subunit interactions within yeast TFIA (Kang et al, 1995). It is also possible that TFIIA-S interacts with conserved regions of other general transcription factors such as TBP. In any event, the high degree of homology between TFIIA-S gene products from yeast (Ranish et al., 1992), *Drosophila melanogaster*, *Drosophila virilis* and rice (Sesaki and Minobe, 1993), the ability of dTFIIA-S to support formation (with hTFIIA/ $\alpha$ ) of DA and DAB promoter complexes, and the ability of dTFIIA-S to function in a



human transcription system, suggest that the protein performs a highly specialized function which is invariant across three kingdoms.

The cloning of the large subunit of TFIIA from *Drosophila* represented the first step towards the analysis of this factor in the *Drosophila* transcription system (Yokomori et al., 1993). With the cloning of the small *Drosophila* TFIIA subunit reported here, we are now in the position of being able to undertake further analysis of TFIIA with defined components. Given the high degree of conservation between TFIIA-S genes from diverse species, it seems likely that insights gained into the function of this protein in any one system will be highly relevant to other transcription systems.

The cloning of dTFIIA-S facilitated the cloning by sequence similarity of the cognate gene from a human source. The cDNA isolated in that study (DeJong, Bernstein and Roeder, submitted) is almost identical to the *Drosophila* protein in amino acid sequence and biochemical activity. The availability of recombinant TFIIA subunits facilitated an investigation of the protein-protein interactions of TFIIA. Results from these studies are presented in Chapter V.



## **Chapter V : Protein-Protein Interactions of TFIIA**





The last chapter described the cloning and analysis of the gene for the small subunit of TFIIA from *Drosophila melanogaster* (Bernstein, et al., 1994; Yokomori et al., 1994). The isolation of this cDNA allowed the rapid cloning by sequence similarity of the cognate gene from humans, TFIIA/ $\gamma$  (DeJong et al., 1995; Ozer et al., 1994; Sun et al., 1994). In this chapter I examine the protein-protein interactions of hTFIIA/ $\gamma$ , as well as those of hTFIIA/ $\alpha\beta^1$ , the 55 kD precursor to the 35 and 19 kD subunits of native human TFIIA.

A well documented function of TFIIA is the facilitation of the binding of TFIID, or the derived TBP, to the TATA box to form the DA complex (Buratowski et al., 1988; Maldonado et al, 1989). This function correlates with the ability of native TFIIA to bind to TBP in solution (Usuda et al., 1991; DeJong et al., 1993; Ma et al., 1993). Formation of the DA complex depends upon basic residues in a conserved alpha-helical region of TBP (Lee et al., 1992; Buratowski and Zhou, 1992; Nikolov et al., 1992), which I will refer to as the "basic region". In particular, substitution of leucine for lysine residues 138 or 145 in the yeast TBP basic region impairs the ability of yeast TBP to support DA complex formation with both yeast and human TFIIA (Lee et al., 1992; Buratowski and Zhou, 1992). The effect of this mutation could be due to disruption of a direct protein-protein interaction with TFIIA, or to a deficiency in the ability of the mutant TBP to undergo a TFIIA-induced conformational change (Lee et al., 1992).

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<sup>1</sup> In this chapter, the 55 kD precursor to the 35 and 19 kD subunits of TFIIA is referred to as hTFIIA/ $\alpha\beta$ , and p12 is referred to as hTFIIA/ $\gamma$ , consistent with the shift in terminology which has taken place in the literature (see Ozer et al, 1994; DeJong et al, 1995; Sun et al, 1994). However, in Figure 28, GST-IIA/ $\beta$  refers to GST-12, and GST-IIA/ $\alpha$  refers to GST-55.



The importance of the TFIIA interaction to TBP function has not been determined. In part this is due to the fact that highly purified transcription systems complemented with TBP do not require TFIIA (Sayre et al., 1992; Cortes et al., 1992). This is likely due to the absence from these purified systems of repressors of TBP function which compete with TFIIA for inclusion into the PIC (Meisterernst and Roeder, 1991; Cortes et al., 1992; Inostroza et al., 1992; Ge and Roeder, 1994). These inhibitory factors are present in nuclear extract; correspondingly, TFIIA depletion renders nuclear extract transcriptionally inactive (DeJong and Roeder, 1993; Yokomori et al, 1994; Kang et al, 1995 ). To understand the importance of the TFIIA-TBP interaction to TBP function in a TFIIA-dependent system, in this chapter, I examine the effect of a non-conservative double mutation in the basic region on TBP function in nuclear extract-derived transcription systems.

TFIIA is required for high levels of transcriptional activation *in vitro* (Meisterernst et al, 1991; Meisterernst and Roeder, 1991; Sun et al., 1994; Ozer et al., 1994; Yokomori et al., 1994), and several studies suggest that either TFIIA or the DA promoter complex may be a functional target of activation domains. For example, formation of a TFIID-TFIIA promoter complex was shown to raise basal activity and concomittantly decrease the effect of the activators Sp1 and USF (Meisterernst et al., 1991). Consistent with this, the acidic activator Gal4-AH has been shown to promote the formation of a rate-limiting complex of DNA, TFIID and TFIIA (Wang et al., 1992b). Similarly, the Epstein-Barr virus transactivator Zta has been shown to promote the formation of a DA promoter complex (Chi



and Carey, 1993; Lieberman and Berk, 1994) possibly via a direct interaction with TFIIA/ $\gamma$  (Ozer et al., 1994). Finally, the role of TFIIA in activation is further suggested by studies of PC4, a cofactor derived from the USA fraction (Ge and Roeder, 1994; Kretzschmar et al., 1994). PC4 is required for high levels of transcriptional activation in purified transcription systems, and interacts in solution with both a TBP-TFIIA complex and with several transcriptional activation domains (Ge and Roeder, 1994). The above studies all suggest that diverse activators may function in part by promoting or stabilizing the interaction of TFIIA with the PIC.

The experiments in this chapter probe the physical interactions between TBP and TFIIA, providing evidence that these interactions are important for TBP function. Further experiments examine protein-protein interactions of TFIIA with other components of the PIC, which suggest multiple possible roles for TFIIA in transcription.



## Results

### Physical Interaction of TFIIA Subunits with TBP

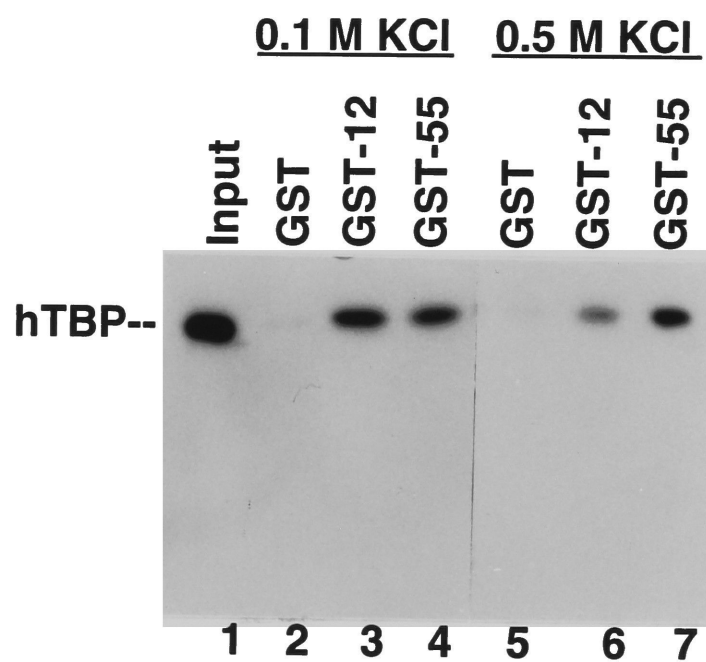
To determine which subunit(s) of human TFIIA interact with human TBP, fusion proteins consisting of GST linked to either hTFIIA/ $\alpha\beta$  (GST-55; DeJong and Roeder, 1993) or hTFIIA/ $\gamma$  (GST-12; DeJong et al., 1994) were expressed in *E. coli*, bound to glutathione-sepharose beads, and incubated with flag-tagged human TBP (f:hTBP; Chiang et al., 1993). As Figure 27 shows, a GST column does not retain any detectable f:hTBP. However, at 0.1 M KCl (lanes 3,4) or 0.5 M KCl (lanes 6,7), both GST-55 and GST-12 bind f:hTBP. This result shows that there are independent interactions between the individual subunits of TFIIA and TBP.

Previous results have implicated lysines 138 and 145 of yeast TBP as being important for the formation of a TBP-TFIIA promoter complex (Lee et al., 1992; Buratowski and Zhou, 1992). To determine if these residues are important for the interaction of the individual TFIIA subunits with TBP, a yeast TBP mutant in which both of these lysines were converted to leucines (mutant K138/145L) was analyzed for its ability to interact with GST-55 and GST-12. Figures 28A and 28B show that wild-type yeast TBP can bind to the individual TFIIA subunits, whereas TBP containing mutations in lysines 138 and 145 does not. (Yeast TBP is detected with an anti-peptide antiserum raised against the N-terminus of yeast TBP). This result reinforces the idea that the individual subunits of TFIIA bind TBP, and indicates that the

**Figure 27: Binding of Human TBP to GST-55 and GST-12**

50 ng of flag-tagged human TBP were incubated with GST (lanes 2,5), GST-12 (lanes 3,6) or GST-55 (lanes 4,7) as described in Chapter 2. The resin was washed with BC100 (lanes 2-4) or BC500 (lanes 5-7) as indicated, and the bound material was run on an SDS gel and immunoblotted with anti-Flag-tag monoclonal antibody. The position of human TBP is indicated to the left of the gel. Lane 1 represents approximately 20% of the input to the reaction.







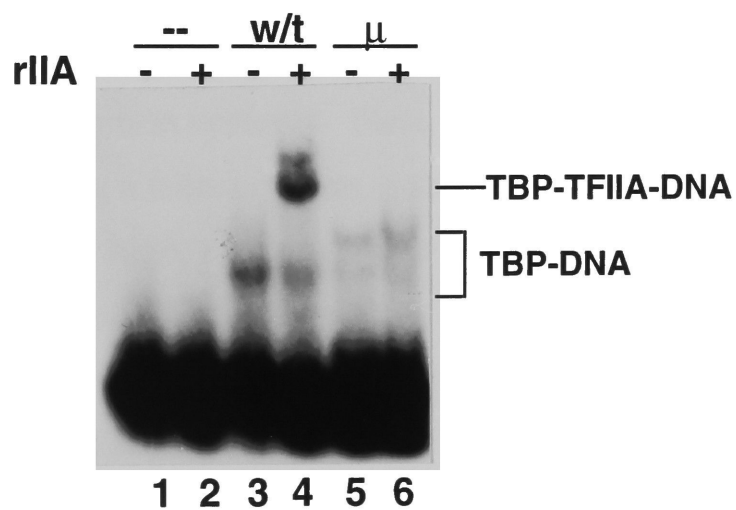
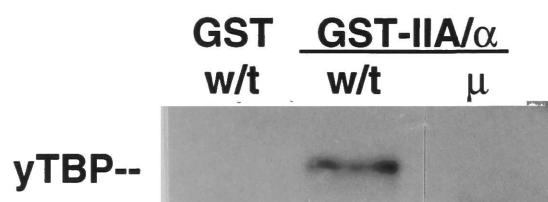
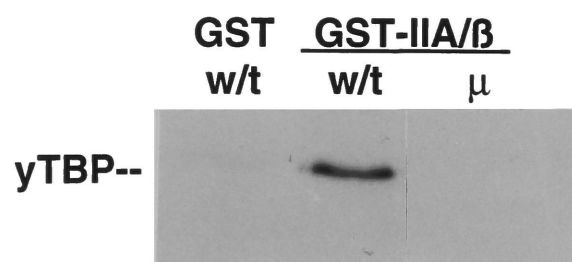


### **Figure 28: Interaction of Yeast TBP Mutant with TFIIA**

A) Wild-type yeast TBP (w/t), or TBP double mutant K138/145L ( $\mu$ ) was incubated with GST or GST-12 resin as described in Chapter 2. Bound material was analyzed by western analysis with anti-yeast TBP anti-serum.

B) Same as A, but with GST-55 resin instead of GST-12.

C) Gel mobility shift assay (Yokomori et al., 1993) comparing promoter complexes formed with wild-type TBP (w/t; lanes 3,4) and double mutant K138/145L ( $\mu$ ; lanes 5,6) in the absence (lanes 1,3,5) or presence (lanes 2,4,6) recombinant human TFIIA. Positions of relevant complexes are indicated to the left of the gel.





binding takes place through a domain of TBP important for DA complex formation (Lee et al., 1991; Buratowski and Zhou, 1991).

Since the TBP double-mutant does not interact physically with either of the TFIIA subunits, it would be predicted that this mutant TBP would not form a DA promoter complex with recombinant TFIIA (rTFIIA). Figure 28C shows that although this mutant can bind a TATA-containing oligonucleotide (compare lanes 3 and 5), it does not support DA complex formation (compare lanes 4,6). The impairment of the ability of TBP to support DA complex formation therefore correlates with the loss of TBP-TFIIA physical interaction. (The doublet formed by mutant TBP in this assay (lanes 5,6) is also noted with two-fold higher concentrations of wild-type yeast TBP. Both bands are specific as judged by oligonucleotide competition experiments; data not shown).

### **Functional Importance of the TBP-TFIIA Interaction**

To determine the relevance to TBP function of the interaction of TFIIA with the basic region of TBP, I compared the transcriptional activity of wild type TBP to that of the TFIIA-interaction-defective double mutant in several transcription systems. First, wild-type and mutant TBP were compared using a transcription system reconstituted with recombinant or highly purified general factors (Ohkuma et al., 1991). This transcription system does not contain any TFIIA, and is not stimulated by TFIIA addition, consistent with previous results (Cortes et al., 1992; Sayre et al, 1992; Y. Ohkuma, personal communication; data not shown). Transcription in this reconstituted system is stimulated equally by increasing amounts of wild-type or mutant TBP (Figure 29; compare lanes 2-6 with 7-11).





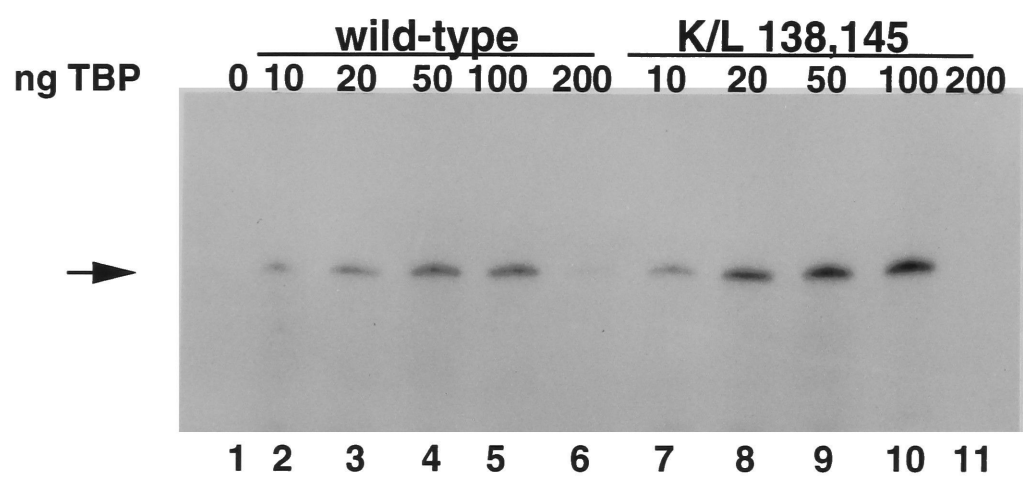
This result shows that the specific activities of wild-type and mutant TBP are the same in this transcription system. Therefore, any differences in transcriptional activity noted between wild-type and mutant TBP in a TFIIA-containing system can not be ascribed to a general functional defect of the mutant protein, such as global misfolding or impaired DNA binding.

The relative transcriptional activities of wild-type and mutant TBP were next compared in a crude nuclear extract-derived system containing functionally active TFIIA (DeJong et al., 1993). I employed a nuclear extract in which the endogenous TBP was selectively inactivated by mild heat-treatment (47°, 11 minutes) (Nakajima et al., 1988). This treatment denatures the endogenous TBP, transcriptionally inactivating it and releasing it from the TFIID complex. It is important to note that addition of a TFIIA fraction does not complement heat-treated nuclear extract, whereas readdition of a purified TFIID fraction fully complements heat-treated nuclear extract (Nakajima et al., 1988). Since TFIIA is required for TFIID-mediated transcription in nuclear extract (DeJong et al., 1993), it follows that the TFIIA in heat-treated nuclear extract is functionally active. Most importantly, the transcriptional activity of TFIIA from both human and calf thymus sources has been shown to be stable to a significantly more intense heat treatment (60°, 10 minutes) than that used here (Samuels and Sharp, 1986; Waldschmidt and Seifart, 1992).

Transcription in heat-treated nuclear extract is stimulated by increasing concentrations of wild-type TBP (Fig. 30A, lanes 2-4). However, the TBP basic region double mutant stimulates this extract

**Figure 29: Transcriptional activity of wild-type and TFIIA-interaction-defective TBP double mutant in a minimal transcription system.**

In vitro transcription reconstituted with recombinant TFIIB and TFIIE, highly purified TFII F/H, highly purified RNA Pol II, with 100 ng template G5HMC<sub>2</sub>AT. Indicated amounts of wild-type TBP (lanes 2-6) or K138/145L double mutant TBP (lanes 7-11) were included. Position of specific transcript is indicated by the arrow to the left of the gel.





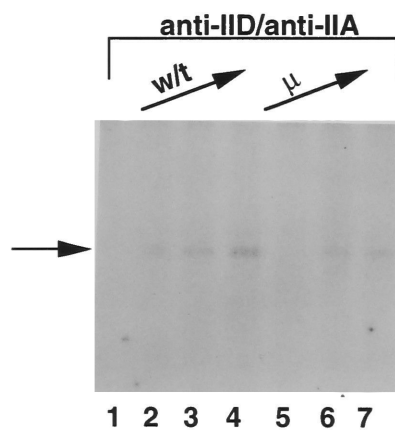
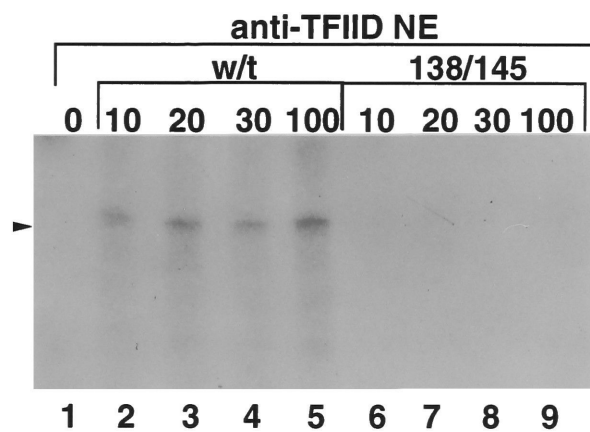
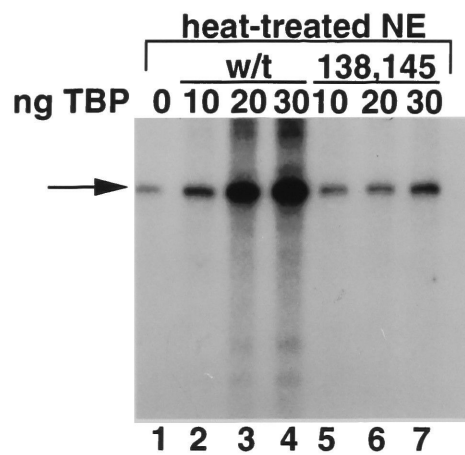


**Figure 30: Transcriptional Activity of Wild-type and TFIIA-Interaction-Defective TBP Double Mutant.**

A) In vitro transcription reaction carried out with heat-treated nuclear extract (Nakajima et al, 1988), 100 ng of G5HMC<sub>2</sub>AT, and indicated amounts of wild-type yeast TBP or K138/145L double mutant TBP.

B) Same as A, except transcription carried out with anti-TBP depleted nuclear extract and indicated ng of wild-type (lanes 2-5) or K 138/145 L (lanes 6-9) TBP (. (3 hour exposure).

C) Same as A, except transcription carried out with anti-TBP/anti-hTFIIA/ $\alpha\beta$  depleted nuclear extract (DeJong and Roeder, 1993) and 10 (lanes 2,5), 20 (lanes 3,6), or 30 (lanes 4,7) ng w/t or K 138/145 L mutant ( $\mu$ ) TBP, respectively. (16 hour exposure).







considerably less strongly (lanes 5-7). This defect in the transcriptional activity of the mutant indicates that the basic region of TBP is important for function in a TFIIA-containing transcription system. The equivalence of wild-type and mutant TBP in a TFIIA-independent transcription system, and the large difference in activity between wild-type and mutant TBP in heat-treated nuclear extract, raises the possibility that the difference between wild-type and mutant TBP is due to the presence of TFIIA. However, heat-treatment of nuclear extract releases TAFs from their association with endogenous TBP (T.K. Kim, unpublished results). These released TAFs may reassemble more favorably with wild-type TBP than with mutant TBP. While it is unclear what effect TAF association would have on TBP function in basal transcription, it is a formal possibility that such an association causes the difference in transcription activity between wild-type and mutant TBP noted in heat-treated nuclear extract.

To examine the possibility that the difference between wild-type and mutant TBP in heat-treated nuclear extract is due to a differential interaction of released TAFs, I compared wild-type and mutant TBP in a nuclear extract depleted of TFIID (and therefore of associated TAFs) by treatment with anti-TBP antiserum. Figure 30B shows that wild-type TBP stimulates transcription in this TFIID-depleted nuclear extract (lanes 2-5). However, mutant TBP stimulates this extract considerably less strongly (lanes 6-9). Longer exposure of the gel in figure 30B shows that the mutant does weakly stimulate transcription in this system, in a dose-dependent manner (data not shown). This experiment reinforces that idea that



mutation of the TFIIA-interaction domain of TBP impairs TBP functional activity in transcription systems containing TFIIA. It also suggests that the effect of the mutation is not due to a differential association of wild-type and mutant TBP with TAFs. Rather, this result furthers the possibility that the difference between wild-type and mutant TBP is due to TFIIA.

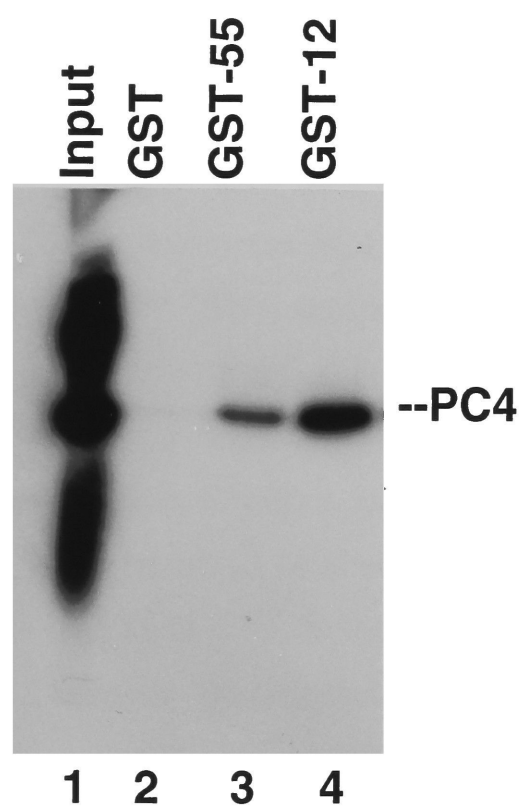
To determine if the functional defect of the mutant TBP is due to TFIIA, the transcription activities of wild-type and mutant TBP were compared in nuclear extract depleted of both TFIID and TFIIA. Figure 30C shows that in this TFIIA-depleted transcription system, the transcription activities of wild-type and mutant TBP are nearly identical (compare lanes 2-4 with lanes 5-7). This experiment shows that the difference in activity between wild-type and mutant TBP noted in TFIID-depleted nuclear extract is eliminated by depletion of TFIIA. This strongly suggests that the conditional defect in transcriptional activity of the basic region mutant is due to an inability to interact with TFIIA.

#### **Interaction of TFIIA with the Coactivator PC4**

Several lines of evidence implicate TFIIA as a functional target of transcriptional activators (see Introduction, section 5.2.2; Meisterernst et al., 1991; Wang et al., 1992; Chi and Carey, 1993; Lieberman and Berk, 1994 ). This evidence was strengthened by the isolation of the coactivator PC4, a 15 kD protein which mediates high levels of transcriptional activation by a variety of activators (Ge and Roeder, 1994; Kretzschmar et al., 1994). PC4 interacts physically both with activators (Ge and Roeder, 1994) and a complex consisting of TFIIA and TBP, but not with TBP alone (Ge and Roeder,

### **Figure 31: Interaction of PC4 with GST-55 and GST-12**

Radioactive PC4 (lane 1) was incubated with GST (lane 2), GST-55 (lane 3), or GST-12 (lane 4) in BC125, and the resin was washed with BC 225 as described in Chapter 2. Bound material was analyzed by SDS-PAGE followed by autoradiography of the gel.





1994; Kretzschmar et al., 1994). The above studies suggest that PC4 may mediate an interaction of activators with TFIIA. If such a model were correct, it would be possible that TFIIA might interact directly with PC4 directly.

To determine if there is a direct interaction between PC4 and TFIIA, recombinant PC4 was incubated with either GST, GST-55, or GST-12. Figure 31 shows that radiolabeled PC4 does not interact with GST, but binds to both GST-55 and GST-12. (The higher molecular weight band in the "input" lane may represent labeling of bacterial contaminants in the recombinant PC4 preparation.)

Since PC4 interacts with both TFIIA and activation domains such as that of VP16 (Ge and Roeder, 1994), it is possible that PC4 facilitates an interaction between an activation domain and TFIIA. To test this, GST-VP16 was incubated with a mixture of flag-tagged hTFIIA/ $\alpha\beta$  and hTFIIA/ $\gamma$  in the presence or absence of recombinant PC4. Neither TFIIA subunit binds to GST, in the presence or absence of PC4 (Fig. 32, lanes 2,3). Likewise, no detectable TFIIA binds to GST-VP16 (lane 4). However, in the presence of PC4, hTFIIA/ $\alpha\beta$  interacts with GST-VP16. This result indicates that PC4 facilitates an interaction between an activation domain and TFIIA/ $\alpha\beta$ , and is consistent with the idea that acidic activators like Gal4-VP16 promote the inclusion of TFIIA into the PIC via a cofactor-mediated physical interaction (Meisterernst et al., 1991; Wang et al., 1992; Chi and Carey, 1993; Lieberman and Berk, 1994; Lieberman, 1995).

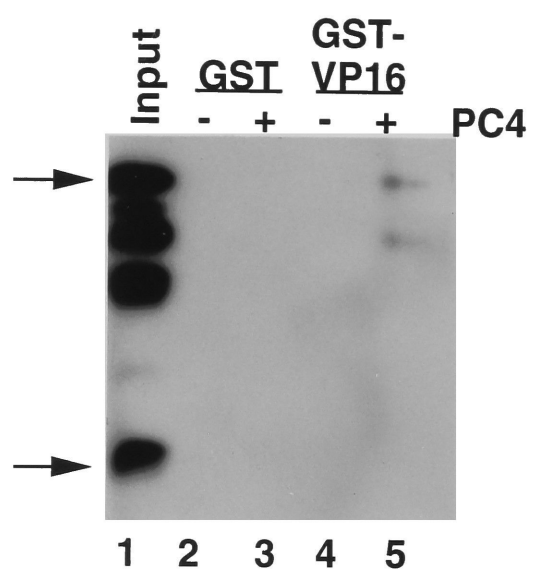
### **TFIIA Binds To a Conserved Region of TAF<sub>II</sub>136**

It has been shown that dTFIIA-L, the drosophila homolog of hTFIIA/ $\alpha\beta$ , interacts with dTAF<sub>II</sub>110 (Yokomori et al., 1993), a TFIID

### **Figure 32: Interaction of Recombinant TFIIA Subunits with GST-VP16**

Flag-tagged TFIIA/ $\alpha\beta$  and TFIIA/ $\gamma$  were mixed and incubated with glutathione-sepharose resin bound to GST (lane 2,3) or GST-VP16 (lane 4,5) in the absence (lanes 2, 4) or presence (lanes 3,5) of 500 ng recombinant PC4 in BC 125 and were then washed with BC 225. Bound materials were separated by SDS-PAGE and detected by immunoblotting with anti-Flag-tag monoclonal antibody. The position of full-length TFIIA/ $\alpha\beta$  (upper arrow) and TFIIA/ $\gamma$  (lower arrow) are indicated to the left of the gel.



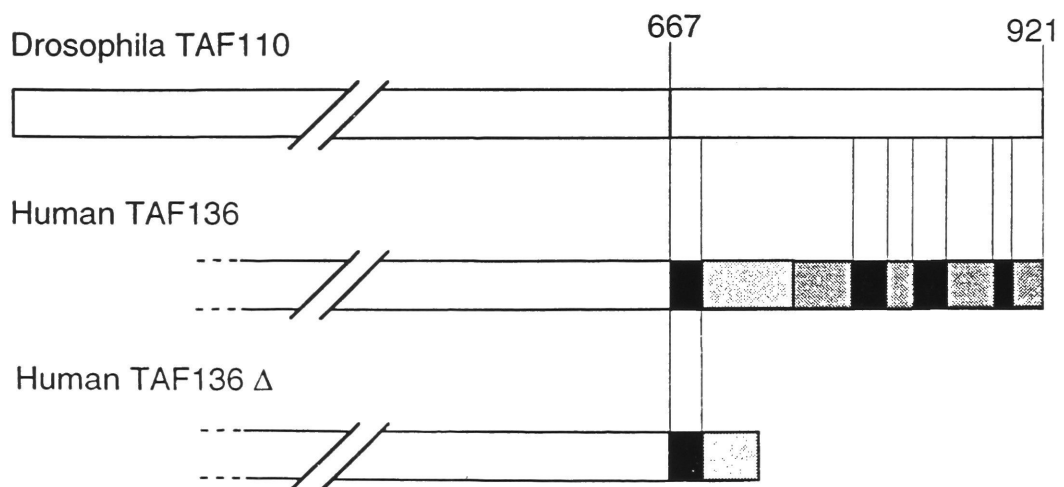
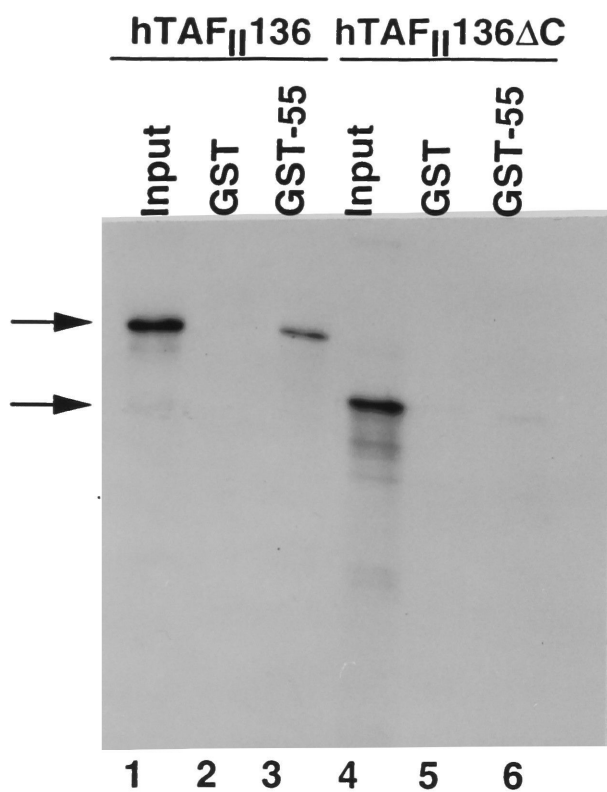






**Figure 33: Interaction of GST-55 with hTAFII136**

A) In-vitro translated partial clone of hTAFII136 (lanes 1-3) or hTAFII136 $\Delta$ C (lanes 4-6) were incubated with GST (lanes 2,5) or GST-55 (lanes 5,6) as described in Chapter 2. Position of hTAFII136 (upper arrow) and hTAFII136 $\Delta$ C (lower arrow) are indicated to the left of the gel. B) Schematic diagram of amino acid sequence similarity between dTAFII110, partial clone of hTAFII136, and hTAFII136 $\Delta$ C; regions of sequence identity are shown in solid black.





subunit which has also been shown to bind to the activator Sp1 in a functional interaction (Hoey et al., 1993). I was interested to see if the human homolog of dTAFII110, hTAFII136 ( R.G. Roeder, personal communication), interacted with hTFIIA/ $\alpha\beta$ . Figure 33A shows that there is an interaction between GST-55 and an in vitro translated partial fragment hTAFII136 (compare lanes 2 and 3). The C-terminal 254 amino acids of the TAFII 136 partial clone comprise a domain of nearly 100% amino acid similarity to dTAFII 110 (see Fig 33B). Truncation of three of the four most highly conserved domains of TAFII136 (TAFII136  $\Delta$ C; Fig 33B) renders the resulting protein unable to interact with GST-55 (Figure 33A, lanes 4-6). This shows that the interaction of TFIIA/ $\alpha\beta$  and TAFII136 takes place through a highly conserved domain of the TAF, and suggests that the interaction of TFIIA with this TFIID component may be a function which has been conserved throughout evolution.

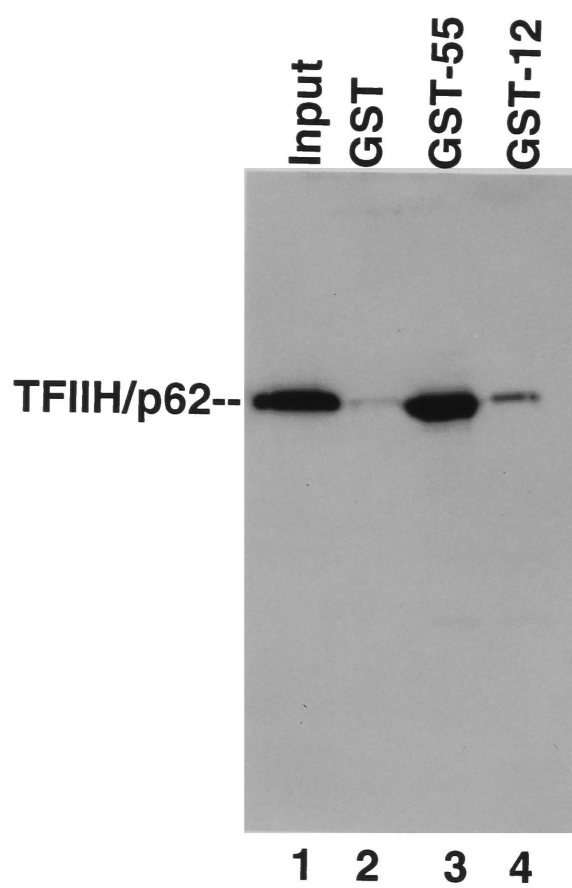
### **TFIIA Interacts With TFIIH**

TFIIH is a general transcription factor consisting of at least five subunits which is involved in promoter clearance by RNA Polymerase II (see Chapter I, section 4.2.5). The interaction of TFIIH with the individual subunits of TFIIA was tested by incubating highly purified TFIIH (see Chapter 2) with GST-55 and GST-12, running the bound material on an SDS gel and immunoblotting with a monoclonal antibody against the p62 subunit of TFIIH. As figure 34 shows, GST-55 quantitatively binds highly purified TFIIH, retaining greater than 50% of the input TFIIH on the column (lane 3). In contrast, GST-12 binds no more TFIIH than does GST alone. This result indicates that there is an interaction between TFIIH and

**Figure 34: Interaction of Purified TFIID with TFIIB**

10  $\mu$ l purified TFIID fraction (approximately 10 ngTFIID/ $\mu$ l; 50% of input shown in lane 1 ) were incubated with GST (lane 2), GST-55 (lane 3) or GST-12 (lane 4); binding and washing were both performed in BC 225 plus 0.1% NP40. Bound material was separated on SDS gel and displayed by immunoblotting with 1:2000 dilution of anti-TFIID/p62 monoclonal antibody. Position of p62 is indicated to left of the gel.



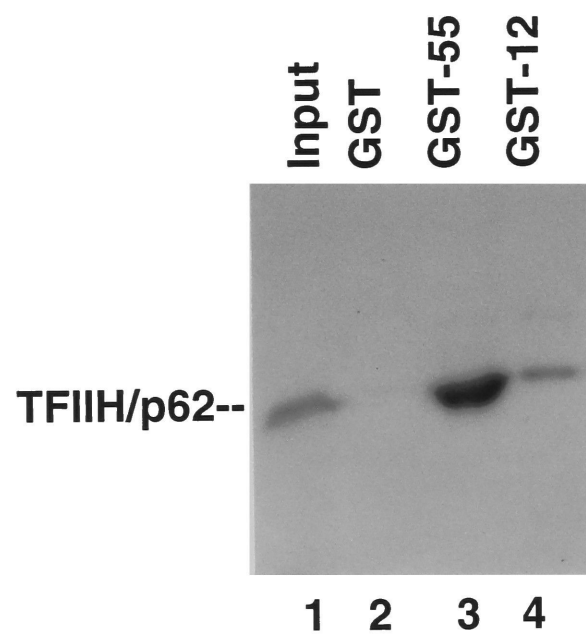






**Figure 35: Binding of TFIIH in HeLa NE by TFIIA**

Experiment performed identically to that in Figure 34, except 1 mg HeLa nuclear extract protein was substituted for purified TFIIH fraction. Lane 1: 20% of input; lane 2: GST-bound material; lane 3: GST-55 bound material; lane 4: GST-12 bound material.





TFIIA/ $\alpha\beta$ . It is consistent with the finding that recombinant TFIIA/ $\alpha\beta$  can be coimmunoprecipitated with TFIIH using a monoclonal antibody directed against the p62 subunit of TFIIH (Y. Ohkuma, personal communication).

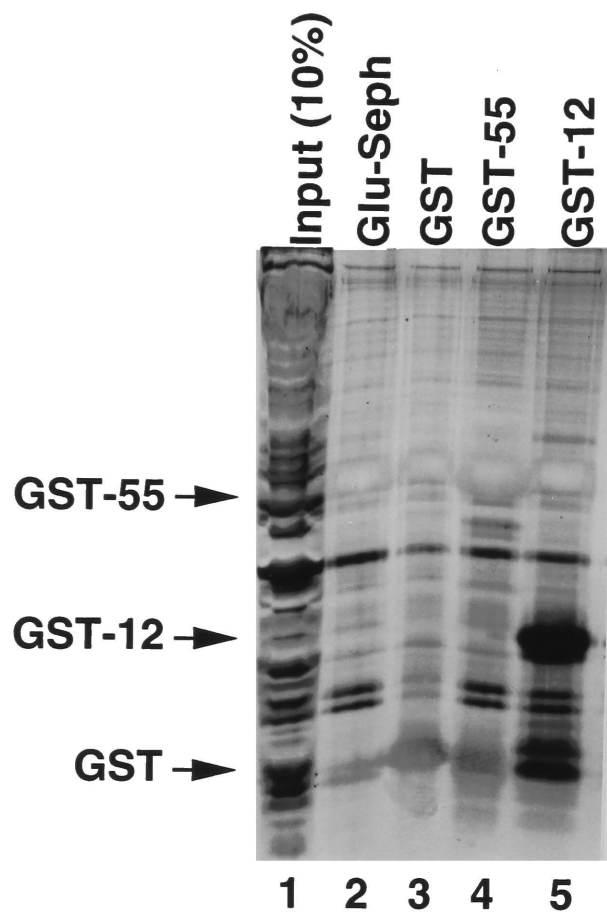
To determine whether the interaction between hTFIIA/ $\alpha\beta$  and TFIIH can take place in the presence of an excess of HeLa nuclear extract proteins, nuclear extract was incubated with the GST-TFIIA fusion proteins. The bound material was then analyzed by immunoblotting with anti-p62 antibody. Figure 35 shows that a GST-55 column quantitatively (compare lanes 1 and 3) binds the TFIIH present in HeLa nuclear extract with considerably higher affinity than GST or GST-12 (lanes 1-4). To determine whether this interaction is due to a non-specific general affinity of HeLa nuclear extract proteins for GST-55, HeLa nuclear extract was incubated with the GST fusion proteins under identical conditions to those used in Figure 35, with the bound material run on an SDS gel and visualized by silver-staining. Figure 36 shows that glutathione-sepharose resin alone (lane 2), resin with GST (lane 3), GST-55 (lane 4) or GST-12 (lane 5) bound, all retain the same amount of bulk HeLa nuclear extract proteins, approximately 1% of the input. This result should be interpreted in contrast to Figure 35, in which GST-55 bound approximately 100% of the TFIIH in the nuclear extract. This indicates that the binding of TFIIH to GST-55 is not due to non-specific protein binding by GST-55 relative to the other affinity resins. Rather, the specific interaction of TFIIH with GST-55 takes place in the presence of many non-specific proteins which bind equally to all of the affinity resins (compare lane 2 to lanes 3-5).

**Figure 36: Retention of Bulk HeLa NE Proteins by GST Fusion Proteins**

1 mg HeLa nuclear extract proteins (10% of input shown in lane 1) were incubated with Glutathione-Sepharose resin (lane 2), or resin pre-bound with GST (lane 3), GST-55 (lane 4), or GST-12 (lane 5) under identical conditions to those used in Figures 34 and 35.

Washed resin was boiled in sample buffer, run on an SDS-gel and silver stained. Positions of GST (lane 3), GST-55 (lane 4) and GST-12 (lane 5) are indicated to the left of the gel.







## Discussion

This chapter has examined the interaction of TFIIA with several other preinitiation complex components. I have shown that the individual TFIIA subunits interact with TBP dependent upon TBP residues required for TBP-TFIIA-promoter complex formation. I have provided evidence that this interaction is important for TBP function in TFIIA-containing transcription systems, but not for TBP function in TFIIA independent transcription systems. Second, I have shown that TFIIA binds the coactivator PC4, and that PC4 may facilitate a VP16-TFIIA interaction. Third, I have shown that TFIIA interacts with a conserved region of a TAF (TAF<sub>II</sub>136) implicated in activated transcription. Finally, I have described a novel interaction between TFIIA and the general transcription factor TFIIF.

The overall specificity of these interactions is indicated by several results. First, the interactions of both TFIIA subunits with TBP is dependent upon specific residues of TBP. Second, the interaction of GST-55 with hTAF<sub>II</sub>136 depends upon an evolutionarily conserved domain of the TAF. Third, neither GST-55 nor GST-12 displays a generalized affinity for bulk HeLa nuclear extract proteins relative to GST. The specificity of the GST-12 column is further indicated by its ability to retain hTFIIA/ $\alpha\beta$  in a manner dependent upon the N-terminal 50 amino acids of hTFIIA/ $\alpha\beta$  (Jeff DeJong and R.B; data not shown). This is consistent with genetic data obtained using yeast TFIIA (Kang et al, 1995). Likewise, the GST-55 column does not bind to several other transcription factors, including TFIIE/ $\alpha$  and the Thyroid Hormone



receptor (data not shown). Therefore, the GST-TFIIA affinity columns do not have non-specific affinity for most proteins

### **TFIIA Interacts Functionally with the Basic Region of TBP**

In this work, we have shown that both TFIIA/ $\alpha\beta$  and TFIIA/ $\gamma$  are able to interact in solution with TBP (Figs 27,28; independently confirmed by Ozer et al., 1994; Yokomori et al., 1994). The functional importance of the TBP-TFIIA interaction is suggested by recent results which show that mutations in the yeast TFIIA subunits which disrupt the TBP-TFIIA complex are lethal (Kang et al, 1995). The interaction of TBP with the TFIIA subunits is mediated by the basic region of TBP, as indicated by the finding that a non-conservative double mutation in this region impairs the interaction with both TFIIA/ $\gamma$  (Fig. 28A), and TFIIA/ $\alpha\beta$  (Fig 28B). Consistent with earlier data (Lee et al, 1992; Buratowski and Zhou, 1992), this mutation also prevents TBP from supporting DA promoter complex formation (Fig 28C).

It is puzzling that a double mutation in TBP eliminates its ability to bind to both individual subunits of TFIIA. One explanation for this could be that the two surface-exposed leucine residues present in the mutant protein cause TBP to dimerize at this position via hydrophobic interactions between four leucines in two separate mutant TBP molecules. Such an interaction could sterically occlude a greater area than that occupied by the two lysine residues alone, possibly masking the entire basic region and preventing the interaction of p55 and p12 with distinct sites. Consistent with the idea that the double mutation causes increased TBP dimerization, a mutant TBP-DNA complex migrates in native gels as a doublet (Fig.



28C, lane 5). Both bands observed in the mutant TBP-DNA gelshift are specific, as indicated by oligonucleotide competition (data not shown). Furthermore, wild-type yeast TBP forms similar complexes at two-fold higher concentrations in this assay. In the case of wild-type TBP, both the upper and lower bands are supershifted by the addition of TFIIA, indicating that the TBP in the upper complex is not denatured. Therefore, it is possible that the K138/145L double mutation accentuates dimerization by TBP at this region.

The role of TFIIA in TBP-mediated basal transcription has been observed to vary among different transcription systems (see Chapter 1, section 4.2.2). Most highly purified transcription systems display no responsiveness to TFIIA. This lack of a TFIIA requirement in highly purified systems may reflect the role of TFIIA in mediating anti-repression of TBP function in less purified systems, possibly by competing with TBP-binding inhibitors such as NC-1, NC-2, and HMG1 for inclusion into the preinitiation complex (Meisterernst and Roeder, 1991; Inostroza et al, 1992; Ge and Roeder, 1994b). Consistent with this, the double mutation in TBP discussed above, which impairs DA complex formation, is phenotypically silent in a transcription system reconstituted with highly purified general factors (Figure 29). This result indicates that the mutant TBP can productively interact with the general factors present in this reconstituted system, and that the specific activities of wild-type and mutant TBP are the same.

In contrast to a highly purified transcription system, transcription in nuclear extract is dependent upon endogenous TFIIA (DeJong and Roeder, 1993). In the context of both heat-treated





nuclear extract and TFIID-depleted nuclear extract, mutation of the TFIIA-interaction domain of TBP inactivates its ability to fully stimulate transcription (Figure 30A,B). That this deficiency is due to an inability of the mutant to mediate stimulation by the endogenous TFIIA present in the extract is indicated by the observation that depletion of TFIIA from the TFIID-depleted nuclear extract eliminates the difference between wild-type and mutant TBP (Fig. 30C). Thus, in nuclear extract-based transcription systems, TBP function is dependent upon the TFIIA interaction domain for basal transcription.

The importance of the TBP -TFIIA interaction for basal transcription documented here is surprising in light of the finding that single point mutations in the basic region which apparently disrupt TFIIA-promoter complex formation (Lee et al., 1992) are phenotypically silent in a crude transcription system containing TFIIA (Yamamoto et al., 1992). A possible resolution of this discrepancy lies in the finding that these single point mutants are in fact able to form DA complexes, albeit more weakly than wild-type TBP (data not shown). These partially disrupted mutant TBP-TFIIA interactions may be stabilized in transcription by other protein-protein interactions. Such interactions may not be sufficient to suppress the phenotype caused by the double mutation employed here.

In any event, mutation of the basic region of TBP impairs the ability of TBP to support DA complex formation, eliminates the ability of TBP to interact with the individual TFIIA subunits, and causes a specific defect in TBP function only in TFIIA -dependent transcription systems. These findings indicate that the interaction of



TFIIA with the basic region of TBP is important for core promoter transcription, consistent with genetic studies which have demonstrated similar results in vivo (Buratowski and Zhou, 1992; Kang et al, 1995). Future work on this topic should investigate the role of the TBP-TFIIA interaction in transcription mediated by the full TFIID complex.

### **TFIIA is a Target of Activation Domains**

The coactivator PC4 mediates physiological levels of transcriptional activation by several classes of activation domains in highly purified transcription systems (Ge and Roeder, 1994; Kretzschmar et al., 1994). In this chapter I have shown that PC4 can interact directly in solution with both recombinant subunits of TFIIA (Figure 31). Further, PC4 facilitates a physical interaction between the VP16 acidic activation domain and TFIIA/ $\alpha\beta$  (Figure 32). These results implicate TFIIA as a target of acidic transcriptional activation domains.

This result is consistent with several others that suggest that TFIIA is important for transcriptional activation. Studies of the USA cofactor fraction suggested that one mechanism of cofactor-mediated activation might be activator enhancement of TFIIA incorporation into the nascent PIC (reviewed in Roeder, 1991). Consistent with this, the activators Gal4-AH and Zta have been shown to promote the formation of a rate-limiting complex between promoter DNA, TFIID and TFIIA (Wang et al., 1992; Chi and Carey, 1993; Lieberman and Berk, 1994). Furthermore, TFIIA is required for transcriptional activation by Gal4-VP16 (Yokomori et al, 1994; Ozer et al., 1994; DeJong et al, 1995). Based on these results, it is possible



that diverse activators may interact with the PIC by a PC4- mediated protein-protein interaction between activation domains and TFIIA.

### **Interactions of TFIIA With PIC Components**

I have presented evidence, consistent with findings using *drosophila* TFIIA-L (Yokomori et al., 1993), that the large subunit of TFIIA interacts specifically with a partial fragment of hTAF<sub>II</sub>136 (Figure 33). This interaction is dependent upon conserved domains of the C-terminus of the TAF, suggesting that the interaction with TFIIA represents a TAF function that has been preserved through evolution. Given the fact that this experiment was performed with a partial clone, the possibility that there may be TFIIA interactions with residues in the as yet uncloned N-terminus of hTAF<sub>II</sub>136 can not be excluded.

The potential importance of the TAF<sub>II</sub>136-TFIIA result is two-fold. First, the *Drosophila* homolog of TAF<sub>II</sub>136, dTAF<sub>II</sub>110, has been implicated in the process of activation by virtue of its ability to physically interact with Sp1 (Hoey et al., 1993), and its selective requirement in Sp1 activation mediated by a partial TFIID complex (Chen et al., 1994). Second, the interaction of TFIIA and hTAF<sub>II</sub>136 indicates that TFIIA can interact with the TFIID complex in a manner not dependent upon the accessibility of TBP. It is not currently known whether the basic region of TBP is sterically accessible in the TFIID complex, although recent results showing that NC2 interacts with the TBP basic region but represses even TFIID-mediated transcription suggest that the basic region may be accessible (Kim et al, 1995). However, if it is not accessible, the interaction of TFIIA with TAF<sub>II</sub>136 could be crucial for TFIIA



function in TFIID dependent transcription. TFIIA has been shown to facilitate Zta-dependent downstream changes in the TFIID footprint (Ozer et al., 1994). Furthermore, the activator Gal4-VP16 has been shown to facilitate late steps of PIC assembly dependent upon the presence of TAFs (Choy and Green, 1993). Given the interaction of TFIIA with VP16 (via PC4), Zta (Ozer et al., 1994), and TAF<sub>II</sub>136, a role for TFIIA in activator and TAF-dependent interactions seems possible (see Chapter 6). Direct determination of the functional relevance of the TAF<sub>II</sub>136-TFIIA interaction awaits reconstitution of TFIID with truncated TAF<sub>II</sub>136 mutants deficient in TFIIA interaction, and the functional analysis of such TFIID variants in a TFIIA-dependent transcription system.

Finally, I have shown that there is an interaction between immobilized TFIIA/ $\alpha\beta$  and TFIIH (Figure 34). This interaction also occurs in the reciprocal situation: TFIIA/ $\alpha\beta$  is quantitatively coimmunoprecipitated with TFIIH using an anti-TFIIH-p62 monoclonal antibody column (Y. Ohkuma, personal communication). The interaction between TFIIH and TFIIA/ $\alpha\beta$  occurs nearly quantitatively even in the context of a large excess of HeLa nuclear extract components (Figure 35). This interaction is not a consequence of increased non-specific protein binding on the part of GST-55 relative to GST or GST-12 (Figure 36). It is possible that this interaction suggests a heretofore unknown role of TFIIA in TFIIH function.

TFIIH has been shown to be dispensible for initiation, but required for promoter clearance, in a minimal TBP-containing transcription system devoid of TFIIA (Goodrich and Tjian, 1994). It





is possible that promoter clearance assays reconstituted with more physiological mixtures of factors may reveal a role for TFIIA in promoter clearance. There is evidence that transcription initiation may take place through different pathways under different experimental conditions. Specifically, Wang et al. (1992a) have shown that in a crude transcription system, ATP hydrolysis is required for open complex formation (and therefore for initiation). This differs from results using a highly purified reconstituted transcription system, in which ATP hydrolysis was only required after initiation (Goodrich and Tjian, 1994). These differences indicate that it will be important to delineate roles of general transcription factors in initiation and/or promoter clearance in physiologic, as opposed to minimal, transcription systems.

In conclusion, TFIIA interacts with several other PIC components. These interactions link TFIIA to the processes of basal and activated transcription, and suggest that TFIIA may be a central player in both processes. A model for TFIIA function in transcription is presented and discussed in Chapter VI.



## **Chapter VI**

### **Perspective and Future Directions**



Understanding the biochemical mechanism of eukaryotic transcription depends upon progress in three complementary endeavors. First, the components of the cell which carry out transcription must be identified, cDNAs encoding these factors must be cloned, and mixtures of recombinant factors which together reconstitute transcription must be assembled. Second, the recombinant proteins must be characterized individually as to their structure, chemical properties, interactions with other cellular components, and function. Finally, factors which are required in vitro must be analyzed in the cell and ultimately in the organism. When genetics can not easily be used to discern important factors, as is the case for the human system, progress in the first endeavor is a prerequisite for progress in the second two.

In this thesis, the general transcription factor TFIIA has been examined. First, a cDNA encoding the small subunit of TFIIA from *Drosophila melanogaster* was isolated. The protein encoded by this cDNA was expressed in bacteria, purified, and used to reconstitute a recombinant factor with the same biochemical activities as native TFIIA. To begin to gain an understanding of the function(s) of TFIIA, the recombinant proteins which together reconstitute active TFIIA were studied as to their interactions with other general transcription factors. The results of this study allow one to make definitive conclusions about the makeup of the TFIIA activity as originally identified biochemically, and to posit models for TFIIA function in both basal and activated transcription.

### **Polypeptide composition of TFIIA**



TFIIA from higher eukaryotes has been variously reported to consist of 19 and 12 kD subunits (Samuels and Sharp, 1986), a single polypeptide of molecular weight 38 kD (Usuda et al., 1991; Waldschmidt and Seifart, 1992), and 35, 19 and 12 kD polypeptides (Cortes et al., 1992; DeJong et al., 1993; Yokomori et al., 1993; Aso et al., 1994). Earlier work demonstrated that gel-eluted and renatured polypeptides of 35, 19, and 12 kD were required for TFIIA promoter complex activity (Cortes et al., 1992). However, the corenated material failed to exhibit TFIIA transcriptional activity, raising the possibility that there were further components of TFIIA which were required for transcriptional activity. The uncertainty surrounding the subunit structure of TFIIA was underscored by the finding that, in contrast to mammalian and *Drosophila* TFIIA, yeast TFIIA consists of only two subunits (Ranish et al., 1992).

The cloning of a single cDNA encoding a 55 kD protein corresponding to the 35 and 19 kD subunits of human (DeJong et al., 1993; Ma et al., 1993) and *Drosophila* (Yokomori et al., 1993) TFIIA was the first step toward reconstituting functional TFIIA from higher eukaryotes with recombinant components. The fact that immunodepletion with antisera raised against this TFIIA subunit could specifically inactivate transcription in a HeLa NE (DeJong and Roeder, 1993) established the essentiality of TFIIA for transcription in this most physiological of *in vitro* transcription systems. This biochemical result, as well as the lethality of TFIIA gene deletion in yeast (Ranish et al., 1992) confirms the importance of TFIIA in transcription (see Chapter I).





The isolation of the TFIIA-S cDNA (this work; Yokomori et al., 1994; Ozer et al., 1994; Sun et al., 1994; DeJong et al., 1994) finalized the process of determining the components of TFIIA. The reconstitution of both promoter complex formation and transcriptional activities of TFIIA using recombinant hTFIIA/ $\alpha\beta$  and recombinant dTFIIA-S (Chapter IV) establishes definitively that TFIIA consists of only these polypeptides. Furthermore, the high degree of sequence homology of dTFIIA-S from yeast, rice, drosophila, and human suggests that this protein plays an evolutionarily invariant and important role in cell physiology.

### **Role of Processing in TFIIA Function**

The availability of reconstituted recombinant TFIIA allowed an opportunity to determine the functional importance of the processing of the large subunit of TFIIA. No qualitative biochemical difference has been detected between native (35, 19 and 12) and recombinant (55 + 12) TFIIA. It is clear that formation of a TBP-TFIIA-promoter complex neither requires nor causes a conversion of p55 to 35 and 19 kD moieties (Chapter IV), and that TFIIA/ $\alpha\beta$  does not require *in vivo* processing for functional activity. Therefore, the role of the presumed processing event which generates p35 and p19 from p55 remains unknown. It is possible that the *in vitro* assays used to compare the recombinant and native forms of TFIIA are not sensitive enough to detect a difference between the two forms. However, given the fact that the hTFIIA/ $\alpha\beta$  homolog in yeast, TOA1, functions as a single polypeptide *in vivo* (Ranish et al., 1992), it seems likely that the processing observed in human and *Drosophila* is not functionally relevant. Definitive resolution of the role of the

1. The first part of the document is a list of the names of the persons who have been appointed to the various offices of the Board of Directors of the Corporation.

processing awaits a precise determination of the cleavage site, and the functional analysis in vivo of mutant proteins which can not undergo processing.

### **The Role of TFIIA in TBP-Mediated Transcription**

TFIIA interacts with the basic region of TBP (Lee et al., 1992; Buratowski et al., 1992) in a manner which stimulates TBP binding (Maldonado et al., 1990; Cortes et al., 1990) . This stimulation of binding is especially dramatic when the conditions for TBP binding are rendered suboptimal by deviations in the concentration of divalent magnesium cation (Imbalzano et al., 1994). TFIIA has been shown to relax the requirement for sequences flanking the TATA region (Lee et al, 1992). Similarly, transcription mediated by TBP can be stimulated by TFIIA most strongly in reconstituted systems when the core promoter contains TATA elements to which the binding of TBP is suboptimal (Aso et al., 1994). Thus, the expansion of the conditions under which TBP can mediate high-affinity binding to core promoters would seem to be one mechanism by which TFIIA stimulates transcription through its interaction with TBP.

This work has shown that the domain of TBP required for TBP-TFIIA complex formation is essential for TBP function exclusively in TFIIA-dependent transcription systems. These systems contain factors which inhibit the assembly of TBP into functional preinitiation complexes (reviewed in Roeder, 1991). The inhibitors of TBP function present in nuclear extract, including NC1, (Meisterernst et al., 1991), HMG1 (Ge and Roeder, 1994a), and NC2/Dr1 (Meisterernst et al., 1991; Insotroza et al., 1992) bind to TBP and prevent its incorporation into productive preinitiation



complexes. TFIIA can overcome the effects of these inhibitors in promoter complex formation assays and, in some cases, in transcription assays (Inostroza et al., 1992; Merino et al., 1993; Ge and Roeder, 1994b). The mechanism by which TFIIA mediates this antirepression effect is not certain. Promoter complex formation assays have indicated that there may be direct competition for TBP interaction between TFIIA and some negative cofactors (Meisterernst and Roeder, 1991). Consistent with this, mutational analysis of TBP has revealed competitive interactions between NC2 and TFIIA for occupancy of the basic region of TBP, although TFIIA and NC2 have been shown to interact with distinct (but overlapping) sets of residues within the basic region (Kim et al, 1995). However, it is also possible that TFIIA may induce structural changes in TBP (Lee et al, 1992) which prevent the binding or function of negative cofactors. TFIIA therefore may activate TBP-mediated transcription by facilitating TBP DNA binding under suboptimal conditions, and by preventing the incorporation of TBP into non-productive complexes with inhibitors.

The role of the TBP-TFIIA interaction in TFIID-mediated transcription has not been investigated. It is not known whether the basic region of TBP is sterically accessible to TFIIA when TBP is assembled into the TFIID complex. Such studies will require the assembly of TFIID complexes containing TFIIA-interaction defective TBP mutants, and the functional analysis of these complexes in TFIIA-dependent transcription systems.

### **Role of TFIIA in Transcriptional Activation**



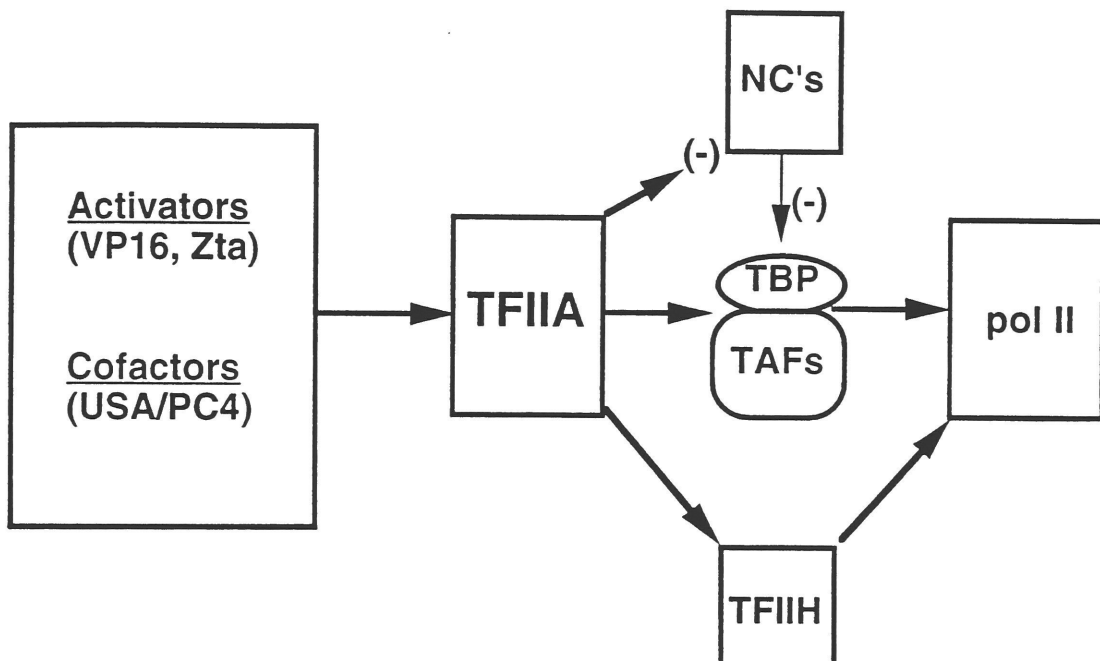
A model for the role of TFIIA in transcriptional activation is shown in Figure 37. According to this hypothesis, activators such as VP16 and Zta exert their effects by facilitating the incorporation of TFIIA into the PIC. This facilitation may take place by a direct physical interaction between activators and TFIIA, as is the case for the activator Zta, which has been shown to interact directly with human TFIIA/ $\gamma$  (Ozer et al, 1994). Results presented here suggest that an indirect connection between VP16 and TFIIA may be mediated by the cofactor PC4. Under the model shown in Figure 37, the recruitment of TFIIA into the PIC by activators results in increased transcription via several functional pathways which ultimately converge upon the enzyme RNA polymerase II.

First, TFIIA interacts with TBP, blocking the inhibitory effect of negative cofactors on PIC assembly and possibly stimulating TBP DNA binding (see above). It is possible that the interaction of TFIIA and TAF<sub>II</sub>136 may also serve an anti-repression function. In transcription systems reconstituted with highly purified general transcription factors, TFIIA stimulates TFIID-mediated transcription strongly, whereas it does so minimally in systems reconstituted with TBP (Cortes et al, 1992). This has led to the hypothesis that TAFs may inhibit TBP within TFIID in a manner overcome by TFIIA. Consistent with this model, the N-terminal domain of dTAF<sub>II</sub>230 has been shown to negatively regulate the DNA binding activity of TBP (Kokubu et al., 1993; 1994), although the ability of TFIIA to overcome this TAF-mediated inhibition has not yet been directly tested.

**Figure 37. Model for TFIIA function in activated transcription  
via converging functional pathways**

Arrows indicate physical interactions and/or functional effects.  
See text for details.







The interaction between TFIIA and TAFs may serve functions other than antirepression. TFIIA has been shown to be required for the formation of a stable promoter complex induced by the activator Zta (Lieberman and Berk, 1994). The interaction between TFIIA and one or more TAFs may be an important part of the Zta activation mechanism, as TFIIA facilitates a downstream promoter-TFIID interaction that seems to be dependent upon a TAF, as evidenced by the failure of TBP to mediate this downstream interaction (Lieberman and Berk, 1994). Activator-TFIIA-TAF interactions may also have a role in activator and TAF-dependent late steps of factor recruitment into the PIC (Choy and Green, 1993; Kim and Roeder, 1994).

Finally, the TFIIA-TFIIH interaction noted in this work may indicate a novel role for TFIIA in the regulation of RNA polymerase processivity. TFIIH has been shown to facilitate promoter clearance by pol II in a minimal transcription system reconstituted with recombinant basal transcription factors. Given the requirement for TFIIA in activated transcription, and the ability of several transcriptional activators to increase the processivity of pol II (Yankulov et al, 1994), the strong interaction between TFIIH and TFIIA may indicate one functional pathway by which activators exert an effect on polymerase processivity. However, it is important to note that direct TFIIH-activator interactions have been documented recently (Xiao et al, 1994).

Activators likely function via multiple targets and multiple steps of PIC assembly. There is strong evidence that direct protein-



protein interactions between activators and TBP, TAFs, TFIIB (see references in Chapter 1) and TFIIH (Xiao et al., 1994), may contribute to activation. Given the fact that multiple activators, or multiple copies of a single activator, activate transcription synergistically, it seems likely that activators function via several different converging mechanisms (Herschlag and Johnson, 1993). Therefore, the TFIIA-centered model of activation put forward here is not to be construed as mutually exclusive with other models of activation based on interactions of TFIIA with other PIC components.

### **Future Directions: Testing the model**

The reconstitution of TFIIA from recombinant proteins, and the development of TFIIA-depleted extracts which are complemented by recombinant TFIIA, allows testing of the model presented in Figure 37. The importance of the protein-protein interactions described in this work can be tested by creating a panel of mutated TFIIA subunits. Correlation of mutant TFIIA functional activity with the ability to interact with specific general transcription factors would suggest that such interactions are important. This has been partially accomplished in the yeast system, in which genetic analysis has revealed the essentiality of the interaction of the subunits of TFIIA with each other and TBP (Kang et al., 1995).

The role of activators in facilitating the incorporation of TFIIA into the PIC can be investigated directly by in vitro factor recruitment assays (Lin and Green, 1991; Choy and Green, 1993; Kim and Roeder, 1994; Kim et al, 1994b ). To date, such assays have not been used to probe the presence of TFIIA in template-bound complexes, due the unavailability of immunologic reagents which can sensitively detect



TFIIA. Given the cloning of the subunits of TFIIA, and the consequent availability of anti-TFIIA antibodies, such studies are now feasible.

The novel interaction of TFIIA and TFIID may represent a role for TFIIA in promoter clearance. To date, promoter clearance assays have not been performed in physiologic systems which require TFIIA. The development of such a system may become possible as TFIIA and TFIID become more readily available in recombinant form. If, using mixtures of factors which require TFIIA for transcription, it becomes possible to functionally distinguish initiation from promoter clearance, the staging of the requirement for TFIIA will be of extreme interest. Given the role of TFIID in DNA repair (Drapkin et al, 1994), the TFIID-TFIIA interaction suggests that there may be a role for TFIIA in this process as well. Given the feasibility of depleting TFIIA from in vitro repair systems, this possibility can be addressed.

The study of the general transcription factors is leaving the analytic stage. With the cloning and reconstitution of TFIIA, and the imminent completion of the isolation of cDNAs encoding TFIID and TAFs, functional transcription systems which support a physiological range of transcriptional effects will soon be assembled from recombinant components. This will allow the field to turn to more mechanistic questions, including the role of specific protein-protein interactions in PIC assembly and function.





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