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Purification of the Human Basal Transciption Factor TFIIE Produced in E.coli

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PURIFICATION OF THE HUMAN BASAL TRANSCRIPTION FACTOR TFIIE PRODUCED IN BACTERIA

By

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ABSTRACT

PURIFICATION OF THE
HUMAN BASAL TRANSCRIPTION FACTOR
TFIIE PRODUCED IN *E.COLI*

By

David P. Chavez

Overproduction and Purification of TFIIE, a basal transcription factor required for eukaryotic transcription initiation by RNA polymerase II, is described. Human TFIIE consists of two subunits of relative molecular mass 34,000 (TFIIE-β) and 56,000 (TFIIE-α) and enters the preinitiation complex after RNA polymerase and TFIIF. Here we report the expression of these cDNAs using a T7 RNA polymerase system in *E.coli*. Production of human TFIIE-β is efficient using the expression vector pET16b. TFIIE-β accumulates in inclusion bodies and is solubilzed using 6M urea. Approximately 15 mg of highly purified and soluble TFIIE-β can be obtained from one liter culture of cells. TFIIE-α was produced as a soluble protein in *E.coli* and did not require denaturant for purification. A yield of 6 mg TFIIE-α per liter of cultured bacteria was obtained. The pET16b vector is designed so that a stretch of 10 histidines is fused to the NH_2-terminus. This allows convenient purification using a Ni^{2+}-affinity resin. Polyclonal antibodies were raised against both recombinant subunits. In Western blot analyses, these antibodies react with the recombinant proteins as well as with RNA polymerase II - associating proteins of 34 and 56 kDa. The antiserum raised against the 34 kDa subunit inhibited transcription effectively (90%) in *in vitro* transcription assays, similar to serum raised against RAP30 (95%), while anti-serum against the 56 kd subunit inhibited slightly less effectively (70%). Recombinant TFIIE protein and antibodies raised against TFIIE subunits will be useful in future studies of accurate transcription *in vitro* by RNA polymerase II.

DEDICATION

To my parents, my wife, AnneMarie and

to God who made life.

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LIST OF ABBREVIATIONS

Abs	Antibodies
AdMLP	Adenovirus Major Late Promoter
ATP	Adenosine 5′-triphosphate
ATPase	Adenosine 5′-triphosphatase
BTF	Basal transcription factor
CTD	CH_2-terminal domain
cDNA	Complementary DNA
DNA	Deoxyribonucleic acid
DEAE	Diethylaminoethyl
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethyleneglycol-bis-(amino-ethyl ether) tetraacetic acid
E.coli	Escherichia coli
IPTG	isopropyl-Beta-D-thiogalactopyranoside

kDa	kilodalton
KCl	Potassium chloride
nt	nucleotide
PAGE	polyacryliamide gel electrophoresis
pol	Polymerase
ppt	precipitation
RNA	Ribonucleic acid
RNAP II	RNA polymerase II
RAP	RNA polymerase II-associating proteins
SDS	Sodium dodecyl sulfate
TAFs	Tata binding protein-associating factors
TBP	Tata binding protein
TF	Transcription Factor
Tris	Tris(hydroxylmethyl)aminomethane

1.0 INTRODUCTION

1.1 Eukaryotic Nuclear RNA Polymerases I, II, and III

In eukaryotic cells, there are three distinct nuclear RNA polymerases. RNA polymerases I, II, and III, respectively, transcribe genes encoding ribosomal RNA, pre-messenger RNA (or protein-coding RNA) and various short RNAs including transfer RNAs and 5S ribosomal RNA.

A common feature of all three eukaryotic RNA polymerases is the requirement for numerous auxiliary transcription factors in addition to the polymerase enzyme. Collectively, RNA polymerase and the basal transcription factors constitute the basal transcriptional apparatus. Basal factors required for *in vitro* transcription have been identified, purified and in some cases cloned for all three systems. RNA polymerase I requires at least the two basal factors, UBF and SL1, and additional peptide components that have not yet been described (Sollner-Webb and Mougey, 1991; Reeder 1992). There are at least seven basal RNA polymerase II factors (for review, see Zawel and Reinberg, 1993; Weinmann, 1992). Finally, RNA polymerase III requires at least three factors TFIIIA, TFIIIB, and TFIIIC (Gabrielson and Sentenac, 1991; Geiduschek and Kassavetis, 1992). The factors TFIIIB and TFIIIC are composed of multiple peptide components.

The three polymerase systems are not completely distinct. For example, five subunits are shared between the pol I, pol II, and pol III enzymes (for review, see Sentenac, 1985; Wolychik and Young, 1990). Second, at least one factor, the TATA-binding protein (TBP) is required for accurate transcription by all three polymerases (Comai et al. 1992; Cormack and Struhl 1992; Schultz et al. 1992, White et al. 1992). Biochemical studies using recombinant TFIID (TBP) have supported earlier observations (Reinberg et al. 1991) which had suggested that *in vivo* TFIID is part of a large molecular weight complex . It was later demonstrated (Dynlacht et al. 1991) that endogenous TFIID from Drosophila embryo extracts is tightly associated with at least six polypeptides ranging in size from 32 kDA to 150 kDa called TAFs (TATA binding protein-associating-factors). In the RNA polymerase III (pol III) system TBP has been shown to participate in transcription of vertebrate U6snRNA genes for which promoters are exclusively upstream of the coding sequence (Lobo et al. 1991). TBP is also required for pol III tRNA and 5SrRNA promoters, in which the promoters are located primarily within the transcribed sequence of the gene (White et al. 1992; Schultz et al. 1992). Since many of these promoters lack an identifiable TATA-box, their transcription appears analogous in some ways to TATA-less pol II promoters.

A second complex that contains TBP is the pol I basal factor SL1, which contains 3 TAFs and elutes from phosphocellulose columns in the high salt D fraction (PC-D; 0.6-1.0M KCL). There is evidence that shows a strong and specific interaction between the C-terminal domain of TBP and TFIIIB (white and Jackson, 1992). The precise polypeptide composition of TFIIIB has not been conclusively determined. It has been suggested that different TBP-TAF complexes are not only specific for different classes of RNA polymerases and that distinct sets of TAFs in each complex confer specificity, but the TAFs for one enzyme are antagonistic to the activities of the other enzymes. (Comai et al. 1992, White and Jackson 1992, Taggert et al. 1992).

A TFIIB homolog called PCF4 or TSD4 recently isolated from *S.cerevisiae* appears to function in RNA polymerase III-mediated transcription (Willis et al. 1992; Buratowski and Zhou, 1992). Since the SUA7 gene encodes the related RNA polymerase II factor, each polymerase may have its own TFIIB homolog to direct entry of the relevant RNA polymerase into the preinitiation complex. Since each polymerase mechanism requires TBP, different TAFs likely determine which TFIIB related function is chosen during assembly. The sequence similarity is strong (first 300 residues out of a 596 residue protein). These results suggest a strong conservation of fundamental transcription mechanisms for RNA polymerases I, II and III. Perhaps additional polymerase-specific basal factors

will be found to have homologs in other RNA polymerase systems.

The parallels between the pol II and pol III systems (and possibly pol I) indicate the possibility of a common initiation mechanism that was established before the divergence of the three eukaryotic polymerases. Detailed mechanistic comparisons of initiation by each of the eukaryotic RNA polymerases and their associated regulatory factors will allow definition of the very complex molecular systems that are key instruments in expressing the genome.

Clues to the functions of the eukaryotic RNA polymerase subunits are based on sequence similarities with prokaryotes and with each other. RNA polymerase I purifies into multiple polypeptides, at least five of which have been cloned from yeast (Reeder, 1992). The largest polypeptide is a 190 kDa subunit that shows homology with the largest subunits of RNA polymerases II and III, and with the β' subunit of *E.coli* RNA polymerase (Sollner-Webb and Mougey, 1991). Three of the thirteen polypeptides of yeast RNA polymerase I are shared by all three RNA pols and two others are shared by RNA polymerase I and pol III.

The amino acid sequences of the RNA polymerase II subunits reveal that the two largest subunits, RPB1 and RPB2, are clearly related to the two largest subunits of *E.coli* RNA polymerase, β and β'(Young et al. 1987). The sequence similarity occurs in eight or nine segments that span the two

large subunits. In addition, the two large subunits of prokaryotic and eukaryotic RNA polymerases are functional homologs. The β'(RPB1) subunit binds DNA, and the β subunit (RPB2) binds nucleoside triphosphate substrates and interacts with the transcription factor sigma.

In yeast the RPB1 subunit has a unique C-terminal domain (CTD) that is not shared with its prokaryotic homolog. This CTD contains 26 or 27 repeats, depending upon the strain, of the consensus sequence YSPTSPS. In mammals, the CTD consists of 52 repeats of the consensus heptamer sequence, YSPTSPS (Allison et al. 1985), which exists in phosphorylated and unphosphorylated forms that are distinguishable by apparent molecular mass on SDS-polyacrylamide gels (IIO - 240 kDa and IIA - 214 kDA respectively). A third form, IIB, arises from proteolytic cleavage of the C-terminal domain, resulting in the formation of a subunit with an apparent molecular weight of 180,000 (Allison et al. 1985). The CTD is required for cell viability, but the presence of this structure and its extensive modification by serine-threonine phosphorylation has yet to be correlated with a definite function (for review, see Dahmus and Dynan, 1992). Both large subunits contribute to the active site for catalysis.

Several points of evidence indicate that subunit RPB3 and *E.coli* α polymerase subunit are probably functional homologs. Both are similar in size and both occur twice in the RNA polymerase molecule. Also, mutations in both subunits affect

assembly of the enzyme (Kolodziej and Young, 1990). These identical polypeptides, together with the large subunits, constitute a core polymerase that may be the functional equivalent of the prokaryotic core enzyme. Detailed sequence comparisons between the RNA polymerases of archaebacteria, eubacteria and eukaryotes indicate that RNA polymerases II and III are more closely related to each other than to RNA polymerase I (Sentenac et al. 1988).

1.2 Organization of Class II promoters

DNA sequence elements within promoters for RNA polymerase II can be divided into two classes: 1) basal elements, which are recognized by the basal transcription factors, and 2) promoter-specific elements, which are recognized by promoter-specific regulatory proteins. Two basal promoter elements, the initiator (Inr) (Smale and Baltimore, 1989), which encompasses the transcription start site, and the TATA, which is located approximately 30 nucleotides upstream, constitute the most common minimal promoter elements required in order for specific transcription to occur. Both of these DNA elements position the assembly of the basic transcription machinery at the +1 position of a given gene. Promoter-specific elements are sequence elements which are recognized by DNA specific binding proteins. These may be proximal to the basal promoter or at distal sites. The promoter is required

for accurate and efficient initiation of transcription, whereas enhancers increase the rate of transcription from promoters. The distinctive characteristic of enhancers is that they can act on cis-linked promoters at great distances upstream in an orientation-independent manner and can also function downstream from the transcription unit.

The types of regulation observed with cellular enhancers indicate that enhancers can be divided into two categories: those that respond to changes in the environment (inducible enhancers) and those that are active only at specific times during development or only in specific tissues (temporal and tissue-specific enhancers). The general principle to emerge from the study of promoters is that both positive and negative regulation may be controlled by the modification of factors that interact with promoters and enhancers. Furthermore, if inducible or tissue-specific gene activation involves the modification of one or a few limiting factors that are required for the assembly of a transcription complex, it may be difficult to mimic *in vivo* conditions that may not occur *in vitro*.

1.3. Order-Of-Addition Model for Formation of a Preinitiation Complex

In contrast to prokaryotes, in which the RNA polymerase holoenzyme is often sufficient for promoter recognition and accurate initiation (for review see Goodrich and McClure, 1991), accurate initiation by RNA polymerase II requires assembly of a multiple factor complex. This entails the ordered interaction of an array of basal factors in addition to RNA polymerase II. The basal transcription factors that are required include: TFIIB, TBP, TFIIE, TFIIF, TFIIH and TFIIJ (see Table 1.3). The factor TFIIA is stimulatory to accurate initiation but not necessarily required. The direct involvement of these factors in the formation of preinitiation complexes has been defined using gel mobility shift assays and accurate transcription assays in which intermediates in complex formation can be observed using subsets of factors (Buratowski et al. 1989; Flores et al. 1991). These studies have helped define processes for assembly of the preinitiation complex. Some roles of individual factors have also been defined in these studies.

In the first step of the pre-initiation complex assembly TBP associates with the TATA element of the promoter (see figure 1.3, complex 1). Studies by Chambon's lab (1983) showed TFIID is necessary and sufficient for commitment of a particular template to the transcriptional process.

Name, alternate names	Molecular mass (Kd)	Properties	Species Cloned
TFIID, TBP, BTF1, Factor d	38 kd - humans 27 kd - yeast 22kd-Arabidopsis 38kd-drosophila	Interacts with the TATA-box	yeast, 1989 human,1990 arabidopsis, Drosph.,1990 , + others
TFIIA, STF	34,19,14 kd - humans	stabilizes TFIID/DNA interaction, removes repressor (Dr-2)	yeast, 1993
TFIIB, α Factor e, FA, SUA7	33kd - humans 38kd - yeast	associates with DA complex to form DAB complex	human, 1991 yeast, 1992
TFIIE, Factor a, ε	56 and 34 kd in humans	heterodimer, interacts with pol II, stimulates CTD kinase activity	human,1991
TFIIF,RAP30/RAP74, Factor 5, FC, β–γ, BTF4, Factor g	30 and 58 kd in humans	heterodimer interacts with pol II, recuits pol II to promoter, affects rate of elongation.	RAP30-human,rat, 1989,1992; RAP74-human,Drosophila, xenopus,1992,
TFIIH, δ, Factor b, BTF2	89,62,43,40 and 35kd	ATP-dependent DNA helicase, CTD kinase, binds to DAB pol FE	62kd subunit, 1992
TFIIJ	unknown	binds to DABpolFEH complex	none

Table 1. 3 Basal Transcription Factors

Similarly, binding of TFIID to the TATA motif was subsequently demonstrated by DNaseI footprinting (Parker and Topal, 1984; Sawadogo and Roeder, 1988;) and DNA-binding experiments (Horikoshi et al. 1989; Buratowski et al. 1989). TFIID protects a 20 nucleotide region centered around the TATA from -36 to -17 on the adenovirus major late promoter.

The transcription factor TFIID was first isolated and identified from human tissue culture cells as a 0.6-1.0M KCL eluate from a phosphocellulose column (Roeder et al., 1980). TFIID was found to stably associate with DNA templates containing a TATA motif (Davidson and Chambon, 1983). More recently, it was shown that TFIID activity associated with this fraction is made of TBP (for TATA-Binding-Protein) and a number of TBP-associated factors (TAFs). The TAFs account both for the large size of TFIID relative to TBP and for the ability of TFIID to respond to activators (Dynlacht et al. 1991). The gene encoding TBP activity has been cloned from yeast, Arabidopsis, Drosophila, and human cells (Hahn et al. 1989; Horikoshi et al. 1989; Hoey et al. 1990; Kao et al. 1990;). The human protein has been found to consist of a single polypeptide of 38 kDa.

Several studies have suggested that TFIIA appears to stabilize the binding of TFIID to the TATA motif, thereby generating the TFIID-TFIIA (DA-complex; see figure 1.3) (Fire et al. 1984; Reinberg et al. 1987). It is thought that stabilization occurs via direct contact with the conserved

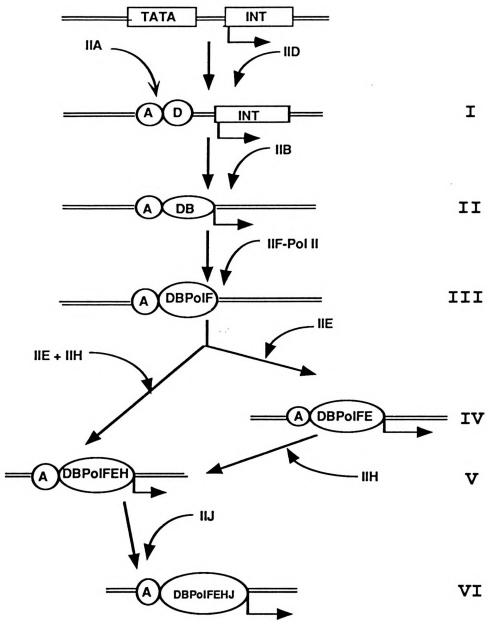


Figure 1.3 **Proposed model for the order of association of the basal transcription factors with the adenovirus major late promoter deduced using the mobilty shift assay.**

domain of TFIID since both yeast and human TFIID can interact with mammalian TFIIA protein (Cortes et al. 1992). The association of TFIIA with TFIID can occur in solution in the absence of DNA as well as with the TFIID bound to the TATA motif. It has also been shown that TFIIA stimulates basal transcription when native TFIID from Hela cells is used but is dispensable in a system reconstituted with bacterially produced TBP (Maldonaldo et al. 1990). It appears that TFIIA can associate with all of the preinitiation complex intermediates analyzed (Cortes et al. 1992). Previous studies have demonstrated that association of TFIIA with the preinitiation complex is dependent on the presence of complex I (As suggested in figure 1.3). TFIIA binds in a TFIID-dependent manner upstream from the TATA region from -35 to-40 relative to the transcription start site of the adenovirus-major late promoter (Maldonado et al. 1990). The binding of negative cofactors is thought to compete with TFIIA to bind the TFIID complex and repress transcription (Meisterernst et al. 1991). Thus, an experimental system that contains few negative cofactors would show a lesser requirement for TFIIA. The factor TFIIA has been purified using yeast TFIID affinity chromatography and shown to be composed of three polypeptides of 34, 19 and 14 kDa (Cortes et al. 1992).

TFIIB is the next factor to associate with the DA complex, resulting in the formation of the DAB complex (complex II), (Van Dyke et al. 1988; Buratowski et al. 1989).

When TFIID is bound to the TATA motif, TFIIB can stably associate to produce the DB complex. Thus the binding of TFIIA to the TFIID-DNA complex is not required for TFIIB binding; rather, the presence of TFIIA increases the overall stability of the complex. The association of TFIIB with bound TFIID is required for the association of the other remaining general factors and RNA polymerase II on the promoter sequences. In a model proposed by Buratowski (1992), TFIIB forms a bridge between TFIID and pol II. This suggests that TDS4 supplies an analogous activity for RNA polymerase III. It is possible that a TFIIB homolog will be found in the pol I system. Isolation of the clone encoding human TFIIB was reported by Ha et al. (1991). This cDNA encodes a polypeptide with a predicted molecular mass of 33 kDa.

At this point TFIIF (RAP30/74) physically interacts with RNA polymerase II and acts as a "chaperone", escorting the polymerase to the DAB complex to form the DABpolF complex (complex III). Without TFIIF the polymerase is unable to form any of the higher ordered complexes and is thus incapable of transcription. The human TFIIF is a heterodimer whose 74 kDa and 30 kDa subunits have been cloned (Finkelstein et al. 1992; Sopta et al. 1989). The small subunit of TFIIF, RAP30 has sequence homology with and is functionally analogous to the sigma 70 subunit of *E.coli* RNA polymerase II and can alone act as a chaperone without RAP74. The 30 kDa subunit of TFIIF can recruit pol II to the DAB complex independent of the large

subunit but the resulting DABpolRAP30 complex is less stable to electrophoresis than DABpolF. Addition of RAP74 stabilizes the complex (Flores et al. 1990). In reconstituted transcription systems, RAP30 alone is unable to functionally replace TFIIF (Finkelstein et al. 1992). RAP30 is required for initiation of transcription, but RAP74 is apparently dispensible for all initiation processes. RAP74, however, is essential for early elongation and appears to be required for promoter escape by polymerase (Chang et al. 1993 paper submitted for publication).

Once the DABpolF complex is formed, TFIIE and TFIIH associate to form the DABpolFE and DABpolFEH complexes, respectively (Flores et al. 1992). Studies by the Reinberg lab (Lu et al. 1992) have indicated that although entry of TFIIH into the transcription complex is dependent on complexes III and IV, the amounts of TFIIE required to form the DBPolFEH complex (complex V), in the presence of TFIIH, are approximately 5-fold lower than those required to obtain complex IV. It appears that the association of TFIIE and TFIIH with the preinitiation complex may be cooperative.

TFIIH consists of five subunits. A gene encoding one subunit of 62 kDa has been cloned (Fisher et al. 1992). A purified fraction of TFIIH is associated with a CTD kinase activity (Lu et al. 1992). The cloned 62kDa subunit showed no kinase activity, although a monoclonal antibody directed against the 62 kDa subunit inhibited both transcription and

CTD kinase activity (Fisher et al. 1992). In the presence of ATP, TFIIH binding is stimulated and not dependent on the presence of TFIIE in the complex. Recently, an 89 kd subunit was identified that shows an ATP-dependent DNA helicase activity (Schaeffer et al. 1993). Microsequencing of tryptic digests of the 89 kd subunit indicate that this polypeptide corresponds to the ERCC-3 gene product, a presumed helicase which has been shown to participate in the nucleotide excision repair process in humans, drosophila and yeast (Gulyas, K. and Donohue, T. 1992). Previously, it had been shown that an ATP-dependent DNA helicase activity was associated with the transcription factor TFIIF(RAP30/RAP74) and another polypeptide of molecular mass 90 kd when eluted from RNA polymerase-associated columns (Sopta et al. 1989). The finding of a TFIIH-associated helicase activity in transcription initiation has long been predicted and sheds light on the mechanistic events that occur during this process.

Finally, the last factor TFIIJ, a factor required for transcription when recombinant yeast or human TBP is used instead of endogenous TFIID, yielding complex VI. (Cortes et al. 1992). At this time little is known about TFIIJ.

At some time between the initial promoter binding and the formation of the first phosphodiester bond, the RNA polymerase II is converted to a highly phosphorylated form. It is thought that this phosphorylation event represents a critical step during initiation. The general factors TFIIE, TFIIH and TFIIJ

may all be involved in this step. Phosphorylation of the CTD by TFIIH may still provide a critical role in uncoupling the RNA polymerase from the preinitiation complex and initiating the elongation phase of transcription.

There are several lines of evidence that implicate the phosphorylation of the CTD as a requirement for the transition from transcription initiation to elongation. Photoaffinity labeling experiments demonstrated that nascent RNA transcripts crosslink almost exclusively to the phosphorylated IIO form *in vivo* and *in vitro*, suggesting that it is the IIO polymerase that elongates RNA chains (Cadena and Dahmus, 1987). In addition Lu et al. (1991) demonstrated that the nonphosphorylated form of RNA polymerase II (IIA), preferentially associates with the preinitiation complex, where it is phosphorylated by a protein kinase. More recently, it was shown that a chemically synthesized monomer of the CTD (heptamer repeat YSPTSPS) binds the human and yeast TBP. Also it was found that the nonphosphorylated form of RNA polymerase II, but not the phosphorylated form, binds to TBP (Usheva and Aloni, 1992). This may indicate that *in vivo* , binding of TBP to the CTD is one of the initial steps in the formation of a transcription-competent complex. In a model proposed recently by Koleske et. al. (1992) the CTD is one of the components that interacts with the DAB complex during recruitment of the enzyme to the promoter. This interaction is directly or indirectly influenced by SRB2,(a dominant suppressor of CTD

truncation mutations in yeast) and /or other factors that are associated with TFIID. Phosphorylation of the CTD may disrupt its association with the DAB complex, allowing RNA polymerase to exit the promoter complex.

1.4 RNA-Polymerase-II-Associating Proteins

To identify and purify proteins important for transcription by RNA polymerase II (RNAP II), Greenblatt and colleagues used calf thymus RNAP II as a ligand for protein-affinity chromatography (Sopta et al. 1985) . When calf thymus or HeLa cell-derived extracts were passed over these columns, three RNA pol II-associating proteins or (RAPs) were identified and designated RAP30, RAP38 and RAP74; the number indicates the molecular mass in kilodaltons . RAP38 appears to be the same factor as the elongation factor S-II (Natori et al. 1979). Using antibodies to RAP30, it was later shown that RAP30 and RAP74 interact with each other, and that both of these polypeptides are required to restore the transcriptional activity of a RAP-depleted nuclear extract (Burton et al. 1988). RAP30/RAP74 was also shown to be immunologically identical to the isolated factor TFIIF and $\beta\gamma$(Flores et al. (1988) and Conaway J. and Conaway R., 1989). Initially, it was thought that $\beta\gamma$ was the rat equivalent for the human factor TFIIE (Conaway, 1989). This was because in early transcription systems, TFIIF was present in crude TFIIE preparations (Sawadago and Roeder, 1985).

Other groups had taken different approaches for the isolation of proteins that interact and/or modify RNA polymerase II activity. For example, previous studies by Roeder's lab showed that the 0.5 M KCL phosphocellulose fraction contained at least two transcription factors (IIB and IIE) that could be separated from each other on a DEAE-cellulose column (Dignam et al. 1983). After purification of TFIIE, glycerol gradient studies showed that TFIIE specifically interacts with RNA polymerase II and TFIIB (Reinberg and Roeder, 1987a). These results suggested a weaker interaction between TFIIE and RNA polymerase II than between RNA polymerase II and TFIIF. TFIIB might enhance the interaction betwween TFIIE and RNA polymerase II. The purification of the previously described TFIIE protein fraction resulted in the identification of a new transcription factor TFIIF (Reinberg and Roeder, 1987b). It was then demonstrated that when the more purified TFIIF, TFIIE and RNA polymerase were independently sedimented through a glycerol gradient that both transcription factors can independently interact with and/or modify RNA polymerase II. Western blot analysis showed that the formation of a functional complex containing RNA polymerase II and TFIIF resulted from a direct physical interaction between the RAP 30 subunit of TFIIF and the polymerase (Flores et al. 1989). The fact that TFIIE was not found on RNA polymerase II columns was thought to be due to TFIIE having an unstable interaction with the polymerase

and/or an indirect interaction dependent on other factors. Since we now know that TFIIE enters the complex late after TFIIF and polymerase it would seem that both explanations are possible. In this thesis I will show evidence that TFIIE is indeed a RAP, by Western blot analysis of RAP fractions using anti-serum produced against recombinant TFIIE.

Using a genetic approach Young and colleagues have shown that RNA polymerase II CTD has a functional interaction with the TATA-binding factor. In order to understand the role of the RNAP II large subunit carboxy-terminal domain (CTD) in transcription initiation, they investigated the function of the SRB2 gene, which was isolated as a dominant suppressor of CTD truncation mutations. Template commitment assays indicate that SRB2 becomes associated with the transcription apparatus at the promoter, and binds specifically to TFIID as revealed by column chromatography studies. Biochemical studies by Usheva et al. (1992) indicate that the CTD interacts directly with the human and yeast TATA-binding factor (TBP). The phosphorylated form of the enzyme was unable to interact with TBP.

Recently the Kornberg lab reported the purification and characterization of another yeast basal initiation factor, Factor g, which consists of three polypeptides of 30, 54, and 105 kDa with a native molecular mass of 300 kd as judged by gel filtration (Henry et al. 1992). Factor g was required for promoter-directed transcription with purified factors a, b, e,

TFIID, and pol II. In addition, they showed that factor g can stably associate with RNA polymerase II by co-sedimentation in a glycerol gradient. Factor g is thought to be related to the human factors TFIIE or TFIIF since both of these also independently associate with RNA polymerase II. At this time it remains unclear whether factor g is homologous to any mammalian transcription factor. A possible experiment to test this is to see if antibodies against the human TFIIE will react with factor g. But due to expected sequence divergence between the two proteins this experiment seems unlikely to give positive results. The cloning and sequencing of the genes for factor g will resolve this issue.

1.5 Energy Requirements for Accurate Initiation of Transcription

Accurate transcription initiation by RNA polymerase II at various promoters requires the presence of either ATP or dATP (Bunick et al. 1982; Sawadago et al. 1984). Because nonhydrolyzable ATP analogues will not support accurate initiation, ATP hydrolysis of the β-γ phosphate bonds are utilized for energy during the initiation process prior to the formation of the first phosphodiester bond. The purpose of ATP hydrolysis is unknown at this time, but some possible roles are; (1) to provide energy for the formation of the first or first few phosphodiester bond(s) of the transcript; (2) to

phosphorylate one or more of the general transcription factors so that the complex becomes activated; (3) to convert the RNA polymerase II form (IIa) to the (IIo) form; (4) to establish the open complex, by the melting of the DNA template around the initiation site, to expose the template strand. It is not clear what happens inside the preinitiation complex during the ATP-dependent transition. Once the open complex is formed, the activated complex moves toward the elongation stage if other nucleosides triphosphates are present.

Previously, several authors had observed a DNA-dependent-ATPase activity associated with transcription factors TFIIE and RAP30/RAP74 (TFIIF) in partially purified fractions (Reinberg and Roeder, 1987; Sopta et al. 1989). These observations at present appear not relavant to a possible role of TFIIE or TFIIF in the hydrolysis of ATP during specific transcription initiation. This is because RAP74 is neither required for RNA polymerase II to initiate phosphodiester bond formation from a promoter (Chang et al. Paper submitted) nor for ATP hydrolysis in initiation, which precedes phosphodiester bond formation. The recently idenitifed 89 kd subunit of TFIIH appears to be the ATP-dependent DNA helicase required to melt the DNA template, to facilitate the onset of the transcription reaction. Neither RAP74 or RAP30 could be detected in the purest preparations of TFIIH as shown in immunoblotting results using two corresponding antibodies (Schaeffer et al. 1993).

Studies by Gralla et al. (1992) imply that transcription complexes generated by binding of TFIID, IIA, IIB, pol II, IIE/IIF, and TFIIH are all closed complexes, as they form in the absence of ATP. The closed complex containing the factors bound to the DNA is opened upon the addition of ATP. The opening or melting allows the polymerase access to the bases on the template and is thus a required event in gene activation. Transcriptional activators facilitate the assembly of these closed and open complexes by allowing them to form faster or more efficiently (for reviews, see Gralla 1990 and 1991). Gralla concludes that in the pol II and sigma 54 system, transcription can be stimulated by activators bound at close or distant positions and all promoters containing Intiator or TATA elements require ATP hydrolysis to form open complexes (Jiang et al. 1993). This differs from the pol III and sigma 70 systems which cannot be activated at a distance and do not require ATP hydrolysis to melt the DNA. This suggests there may be two distinct classes of mechanisms for transcription initiation present in eukaryotes and prokaryotes.

1.6 Structure and Properties of Transcription Factor TFIIE

In order to learn about the participation of the general transcription factors in the regulation of transcription initiation, the approach has been to isolate each factor in a pure form and to elucidate its function. Currently, seven basal transcription factors have been identified and extensively purified; TFIIA, -IID, -IIB, -IIE, -IIF, -IIH, and -IIJ.

As for most of these factors, the understanding of the functional properties of TFIIE has been hampered by the slow progress of its purification. Early studies with a partially purified TFIIE fraction indicated that it interacts with RNA polymerase II (Reinberg et al. 1987), that it acts at a late stage in preinitiation-complex assembly and function (Workman and Roeder, 1987; Buratowski et al. 1989) and that it may possess an ATPase activity (Sawadago and Roeder, 1984; Reinberg and Roeder, 1989). Further purification, however, resulted in the separation of TFIIE from TFIIF(RAP30/RAP74) (Reinberg et al. 1987; Burton et al. 1988). Homogenous TFIIE contained polypeptides of 34 kDa and 56 kDa that copurify with transcriptional activity (Ohkuma et al. 1990; Maldonaldo et al. 1990). In agreement with previous studies indicating an interaction between TFIIE and RNA polymerase II it was found that entry of TFIIE into the transcription cycle was subsequent to the entry of RNA polymerase II.

Following the purification of TFIIE to apparent homogeneity, the Roeder and Reinberg laboratories made the following observations: (1) TFIIE is required for accurate *in vitro* transcription of several class II promoters; (2) the native molecular mass of TFIIE as determined by gel filtration was estimated at 200 kDa; (3) TFIIE exists in solution as a tetramer composed of two 34 kd and two 56 kd polypeptides; (4) the 56 kd subunit was sufficient for low levels of transcription activity using a reconstituted transcription assay depleted of TFIIE, (5) the addition of 34 kd subunit resulted in stimulation of transcription, (6) TFIIE failed to show any DNA-dependent or independent ATPase activity, helicase, kinase, or topoisomerase activity.

Both subunits of TFIIE have been cloned from HeLa cDNA libraries using reverse genetics (Peterson et al. 1991; Roeder et al. 1991). The predicted molecular mass of the two subunits are 49.5 kd (493 amino acids) and 33 kd (291 amino acids) respectively. The recombinant TFIIE (rTFIIE) behaves identically to native TFIIE in gel mobility shift assays and reconstitution assays in basal and activated transcription. In contrast to an earlier observation concerning the 34 kd subunit, neither TFIIE subunit alone was sufficient to reconstitute accurate transcriptional activity. It is likely that the earlier experiments were performed in an assay system contaminated with the 56 kd subunit of TFIIE.

Amino acid comparisons of both subunits of TFIIE revealed

no close similarities with any of the other known transcription factors or any other proteins in GENBANK and EMBL databases. Both subunits did, however, reveal several interesting motifs as well as subregion similarities with other transcription factors that may provide clues to functional roles in transcription initiation.

TFIIE-β (33 kd) is a highly basic protein of pI 9.5 with several basic amino-acid clusters through which it could complex with the TFIIE-α (49.5 kd) subunit, a very acidic protein with a pI of 4.5, suggesting that interaction of the two subunits may involve ionic interactions. A potential protein interaction site in TFIIE-β is a putative leucine repeat from residues 154 to 176 (figure 1.6A and 1.6C). Other interesting regions of TFIIE-β are the serine rich region (21-71) and the basic region sequence (251-273) which show similarity to part of the basic region-helix-loop-helix (BR-HLH) domain of the c-myc-related family of enhancer binding proteins (such as Myo-D1 and E12)(Davis and Weintraub, 1987). This basic region may be involved in protein-protein interactions or DNA-binding.

The large subunit TFIIE-α, also contains three potential structural motifs (leucine repeat, zinc finger, and helix-turn-helix) that are characteristic of many transcription factors (see figure 1.6B-1.6D). There is also a highly acidic region (378-389), a kinase consensus region and a predicted amphipathic alpha helix (12-41). Perhaps the most interesting

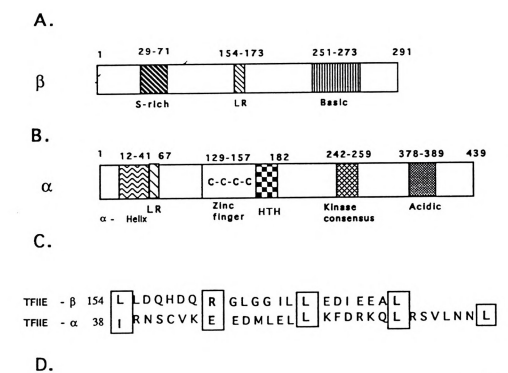


Figure 1.6 Structural features of TFIIE subunits β (56kDa) and α (34kDa). **A)** S-rich, region rich in serine residues; LR, Leucine repeat; Basic, region rich in basic residues; **B)** α–Helix, region predicted to form amphiphatic alpha helix; Leucine repeat region; Zinc finger, sequence that could potentially form a zinc finger; HTH, helix-turn-helix; Kinase consensus, region similar to proteins with known kinase activity ; Acidic, region rich in acidic residues. **C)** Leucine repeat. The putative leucine repeat of TFIIE-β is aligned with that of TFIIE-α. Revelant residues are boxed. Oppositely charged residues at the same position may also interact. **D)** Zinc finger in TFIIE-α. Similarities to Transcription elongation factor S-II(boxed C_2C_2 motifs). TFIIIA, and the *Drosophila* developmental protein Kruppel (boxed C_2H_2 motifs).

finding is a sequence similar to the zinc-finger motif containing a cluster of cysteine residues arranged in the C_2C_2(two cysteine pair, figure 1.6D) class. The structure and function of TFIIE-α putative zinc-finger is analogous to that of the single zinc-finger found in the transcription elongation factor TFIIS (Agrawal et al. 1991), which is thought to be involved in DNA-strand opening or protein-protein interactions. This region also shows sequence homology with the zinc finger motifs present in the UvrA and UvrB proteins found in *E.coli* (Husain et al. 1986).

Recently, work done in Phillip Sharp's lab (Parvin et al. 1992) questions the paradigm that all basal factors (in this particular case TFIIE) are essential for all polymerase II promoters. They suggest that not all basal factors are in fact general. A general factor is defined as a factor required by all polymerase II promoters, whereas, basal factors are required for unregulated transcription. Studies of basal factor requirements for transcription from the immunoglobulin heavy chain gene (IgH) showed no apparent requirement for TFIIE, whereas basal transcription from the adenovirus major late promoter is highly dependent upon TFIIE, in addition to the other basal factors. In these studies, reactions were reconstituted using partially purified basal factors from both HeLa cells and the human B cell line BJA-B.

The strongest evidence for the TFIIE requirement and possible role in transcription initiation was done in work

done in Reinberg's lab (Lu et al. 1992). In that study they tested whether TFIIH could phosphorylate RNA polymerase II in the absence of the other basal factors. In these experiments the addition of TFIIE to the assay resulted in stimulation of phosphorylation of RNA polymerase II. This suggested that TFIIE may stimulate the processivity of TFIIH-kinase activity. Because TFIIE is directly responsible for introducing TFIIH to the preinitiation complex, and also to RNA polymerase II, it is not surprising that it stimulates TFIIH activity. The availability of large quantities of purified recombinant TFIIE will facilitate studies of this protein's function in transcription and complex assembly.

1.7 Production of Human Protein in *E.coli*

The advanced knowledge of the genetics and physiology of *E.coli* has made this bacterium ideally suited as a host for protein overproduction. Additional characteristics that make it advantageous include rapid generation of biomass due to high rates of cell growth and availability of low-cost media. The disadvantages of this system are its limited capacity to secrete proteins and its inability to exert certain posttranslational modifications of proteins (such as disulfide bond formation, glycosylation and acetylation), which can affect the proper folding or function of the protein. Other problems more associated with human proteins include: the

high-level expression of many proteins can lead to the formation of "inclusion bodies", very dense aggregates of insoluble protein, which must be solubilized by denaturing reagents followed by careful renaturation. A problem with denaturation/renaturation is the yield of properly folded protein is variable and sometimes quite low; some proteins, especially large ones, cannot be properly refolded at all.

Translational efficiency problems are often encountered when foreign proteins, such as human proteins, are produced in *E.coli*. Translation initiation is mediated by the 5' untranslated mRNA region. There is now compelling evidence that not only the ribosome binding site (RBS) and the AUG determine the efficiency of the process, but also both the structure and sequence of this region affect the ribosome accessibility to the transcript. Therefore, this region may be manipulated to maximize translation initiation. Another potential problem is that of pre-mature translation termination due to *E.coli* codon preference. For example, the gene encoding human RAP74 appears to be translated inefficiently in *E.coli*. Interspersed throughout the central 1/3 of the gene are a number of rare *E.coli* codons, including two consecutive Arg codons (AGG) which have been reported to result in poor expression (Wang et al. 1993). This problem can be resolved by recoding the protein using PCR.

The last important parameter pertaining to protein yield is protein degradation. Proteolysis is a very selective and

carefully regulated process that influences the degree of protein accumulation in *E.coli*. Protein degradation has important practical consequences since many cloned proteins are recognized as abnormal by proteolytic systems of the cell, and are therefore rapidly hydrolyzed (Boyer et al. 1977). Despite these potential disadvantages, *E.coli* is a useful host for protein production.

The basic approach used to express foreign genes in *E.coli* starts with insertion of the gene into an expression vector. The minimal elements that an expression plasmid vector should supply are a well characterized origin of replication and a selection marker for plasmid propagation and maintenance, a strong regulatable promoter, a ribosome binding site, and appropriate cloning sites to clone the gene in the correct orientation within the vector.

In these studies the pET16b vector (see figure 1.7) was used to overproduce TFIIE-α and TFIIE-β. This plasmid carries the colicin E1 replication origin (ori) allowing maintenance of multiple copies in *E.coli*. The selectable marker is the gene encoding lactamase, which confers ampicillin resistance (amp). The strong inducible promoter is the bacteriophage T7 promoter. A very active enzyme, T7 RNA polymerase elongates chains about five times faster than does *E.coli* RNA polymerase. Background expression is minimal in the absence of T7 polymerase because the host enzyme does not initiate from T7 promoters. For gene expression from this promoter a

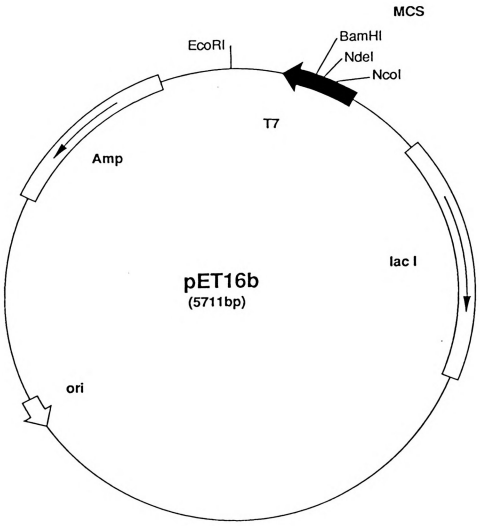


Figure 1.7 Schematic of Bacterial Expression Vector pET16b. The minimal features of the plasmid include: ori, orgin of replication; T7, regulatable promoter; amp, ampicillin resistance selective marker; mcs, multiple cloning site; lac, lac repressor gene.

specialized *E.coli* strain, BL21(DE3) is required that can supply T7 RNA polymerase from the defective DE3 lysogen. T7 pol is controlled by the lacUV5 promoter and lac operator in DE3. Addition of IPTG (isopropyl-Beta-D-thiogalactopyranoside) to a growing culture induces production of T7 RNA polymerase, which in turn induces production of the cloned gene encoded on the pET vector. Even in the absence of IPTG there is some expression of T7 RNA polymerase from the lacUV5 promoter in λDE3 lysogens. This may present a problem if the protein produced is toxic to *E.coli*. Studier and colleagues resolved this problem by designing vectors that contained a T7 lac promoter (Studier et al. 1990). When this type of vector is used for expression (pET16b and pET23a-d), the lac repressor acts both at the lac UV5 promoter in the host chromosome to repress transcription of the T7 RNA polymerase gene by the host polymerase and at the T7 lac promoter in the vector to block transcription by any T7 RNA polymerase that is made.

Purification of recombinant proteins from host organisms can be time-consuming. For many proteins removal of contaminants is critical to the characterization of a recombinant protein. One solution to this problem is to fuse the overproduced protein to a peptide for which an affinity purification is available. The newest pET vectors have the advantage of carrying a stretch of either 6 or 10 consecutive

histidine residues (H_6 or H_{10}-tag) that can be expressed at the N-terminal or C-terminal end of the protein. Some of these vectors allow removal of the His-Tag™ with proteases thrombin, enterokinase or Factor X_a. These modified vectors allow purification of proteins under gentle, physiological conditions if proteins are soluble. The His-Tag™ (available from Novagen), sequence binds to divalent cations (Ni^{2+}) and allows expressed proteins to be purified by metal chelation chromatography (Hochuli et al. 1987 and Smith et al. 1988). After washing of unbound proteins, the recombinant protein is recovered by elution with imidazole. The procedure is rapid and efficient, allowing purification to greater than 90% homogeneity in one step.

2.0 MATERIALS AND METHODS

2.1 Construction of recombinant clones pET16b/TFIIE-α and pET16b/TFIIE-β

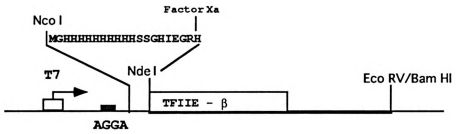
Isolation of cDNAs encoding TFIIE has been described (Peterson et al. 1991). The plasmids pAR34E and pAR56E containing the clones were the kind gift of Dr. Danny Reinberg. The cDNAs for both subunits were subcloned as an Eco RV to Nde I fragment between the NdeI and BamHI (the BamHI site was converted to a blunt end with Klenow DNA polymerase I) sites of pET16b by Janek Werel in our lab (see figure 2.1). When fused with the TFIIE cDNAs in frame this construct produces the 36 kd and 58 kd proteins with a short peptide extension at the NH_2-terminus: MGHHHHHHHHHHSSGHIEGRHM (H_{10}-TFIIE-β, H_{10}-TFIIE-α). This sequence contains a stretch of 10 histidines and a factor X_a cleavage sequence (IEGR/).

2.2 Growth and Lysis of cells

BL21(DE3) cells were transformed by each plasmid and the cells were grown at 37^0 C in 10 liters of LB medium containing 100 ug/ml ampicillin until A_{600} reached 0.6, at which time IPTG was added to a final concentration of 0.4 mM to induce synthesis of T7 RNA polymerase. After 3 hr induction, cells were harvested by centrifugation at 4,000 rpm for 15 min. in

A.

pET16b/TFIIE-β



B.

pET16b/TFIIE-α

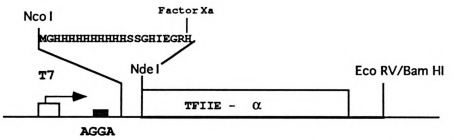


Figure 2.1 Schematic of TFIIE-α and TFIIE-β Overproduction Clones. **A)** pET 16b/TFIIE-β; this clone encodes H_{10}-TFIIE-β (34kDa subunit). **B)** pET16b/TFIIE-α; this clone encodes H_{10}-TFIIE-α (56kDa subunit). NH_2-terminal extensions are indicated by standard one letter aminoacid code. AGGA= ribosome attachment sequence. The dark line indicates human DNA.

a Sorvall RC3B centrifuge at 4^0 C. All subsequent steps were done at 4^0C except where noted. The methods for preparing the cell extract and for purification on the Ni^{2+} column were adapted from information provided by the manufacturer (Novagen, Madison, WI). Cells were resuspended using a dounce homogenizer in 100 ml of lysis buffer (20mM tris-HCL (pH7.9), 5 mM imidazole, and 500 mM NaCl containing 1 mg/ml lysozyme). Cells are then lysed by sonication (Ultrasonic Model 220-F, 5x for 30 seconds, with a 30 second rest in between at a setting of 5 using a microtip).

2.3 Purification of TFIIE-α (58 kd subunit)

Cells containing TFIIE-α were centrifuged at 5,000 rpm for 10 min. in a Sorvall SS-34 rotor to remove debris. The supernatant fraction containing soluble H_{10}-TFIIE-α was applied to a column made up with 10 ml of His-bind™ resin. The column was washed with 150 ml of 1x wash buffer (500 mM NaCl, 20 mM tris-HCL (pH 7.9) and 60 mM imidazole). The protein was then eluted into 60 ml of 1x elution buffer (1M imidazole, 500 mM NaCl and 10 mM tris-HCL,(pH 7.9). Prolonged incubation of TFIIE-α at 0.1 M KCL results in precipitation.

2.4 Purification of TFIIE-β (36 kd subunit)

Cells containing TFIIE-β were centrifuged at 5,000 rpm for 10 min. to remove debris. The cell pellet was then solubized by resuspending in 1x binding buffer (5 mM imidazole, 500 mM NaCl , 20 mM Tris-HCl, pH 7.9) containing 6M urea. For subsequent steps of washing and elution 6M urea was also used (1x wash and 1x elution buffer). The supernantant fraction containing denatured TFIIE-β was applied to the Ni^{2+} column containing 10 ml of resin followed by, 150 ml wash with 1x wash buffer and subsequent elution with 100 ml of 1x elution buffer. Prolonged incubation of TFIIE-β in low salt conditions (0.1M KCl) also results in precipitation.

2.5 Production of Anti-TFIIE-α and Anti-TFIIE-β Anti-serum and Western Blot Analysis

Polyclonal anti-serum was raised in rabbits against bacterially produced TFIIE-α and TFIIE-β proteins . Preimmune serum was taken from four New Zealand white female rabbits. Two were immunized with 150 μg of H_{10}-TFIIE-α and the other two with the same amount of H_{10}-TFIIE-β. Protein was emulsified in *Hunter's* Titre Max™ #HR-1 research adjuvant using 250 μl of adjuvant and aqueous solution containing protein. Rabbits were boosted on days 25 and 39 with 100 μg of protein. Rabbits were bled on days 46 and 53.

Western blots were performed essentially as in Harlow and Lane (1988). Proteins were separated by SDS-PAGE and transferred electrophoretically to nitrocellulose membranes (Bio-Rad). Nonspecific interaction of the antibody was prevented by incubating in blocking solution for 30 minutes in 50 mM tris-HCL (pH 8.0), 150 mM NaCl and (1X TBS), plus 3% gelatin followed by washing 2x for 5 min in 1X TBS . The immunoreaction was performed using a 1:500 dilution of antiserum. The membrane was washed 2X for 5 minutes in 1X TTBS, before incubating with the second antibody 1:3000 diluted goat anti-rabbit IgG (Bio-Rad). The immunoreactive bands were visualized with 20 ml of color development solution (20 ml of 1X carbonate buffer (100 mM Na HCO_3,1 mM $MgCl_2$),6 mg of NBT (p-nitro blue tetrazolium chloride) and 3 mg of BCIP (5-bromo-4-chloro-3-indoyl phosphate p-toluidine salt), (Bio-Rad). The reaction was stopped by rinsing with TBS. Pre-stained protein low and high molecular weight markers (Bio-Rad) were used to monitor electroblotting and as a size marker.

2.6 Preparation of protein-affinity fractions for Western analysis

Eluates from an RNA polymerase II affinity column and anti-CTD affinity column were collected from 250 ml of whole cell nuclear extract in 100 mM KCl, followed by wash in 100 mM

KCl and eluted at 500 mM KCl. The control column was Affi-gel (Bio-Rad) containing no protein. Eluates were tested for the presence of TFIIE. Proteins that bind to the RAP column and not the control column are considered RNA polymerase II-associating-proteins or RAPs. The protein fraction that binds to the anti-CTD column should also contain RAPs.

2.7 In vitro transcription Assay

The template used for runoff transcription was the adenovirus major late promoter (Ad-MLP) subcloned as an Xho I and Hind III fragment (coordinates -256 to +196) relative to the Ad-MLP cap site) between the XhoI and Hind III sites of the vector pBluescript II KS (+)(Stratagene). The DNA has a single Sma I site located at +217 relative to the AdMLP cap site. This template after digestion with Sma I was used to produce a runoff transcript of 217 nucleotides (see figure 2.6). The methods for in vitro transcription have been previously described (Burton et al. 1986). Reactions were done at 30°C. Whole nuclear extract and antibodies were combined with AdMLP DNA (60 μg/ml) and preincubated for 60 min. The preincubation reaction volume was 20 μl; reactions at 30°C for 30 min. Transcription buffer contained 12 mM Hepes pH 7.9, 12% glycerol, 60 mM KCL, 8 mM $MgCl_2$, 3.12 mM EGTA, 0.12 mM EDTA and 1.2 mM DTT. 600 μM ATP, CTP, UTP and 25 μM GTP(5 μCi/ reaction -γ^{32}P GTP) were added in 5 μl aliquots. Reactions were

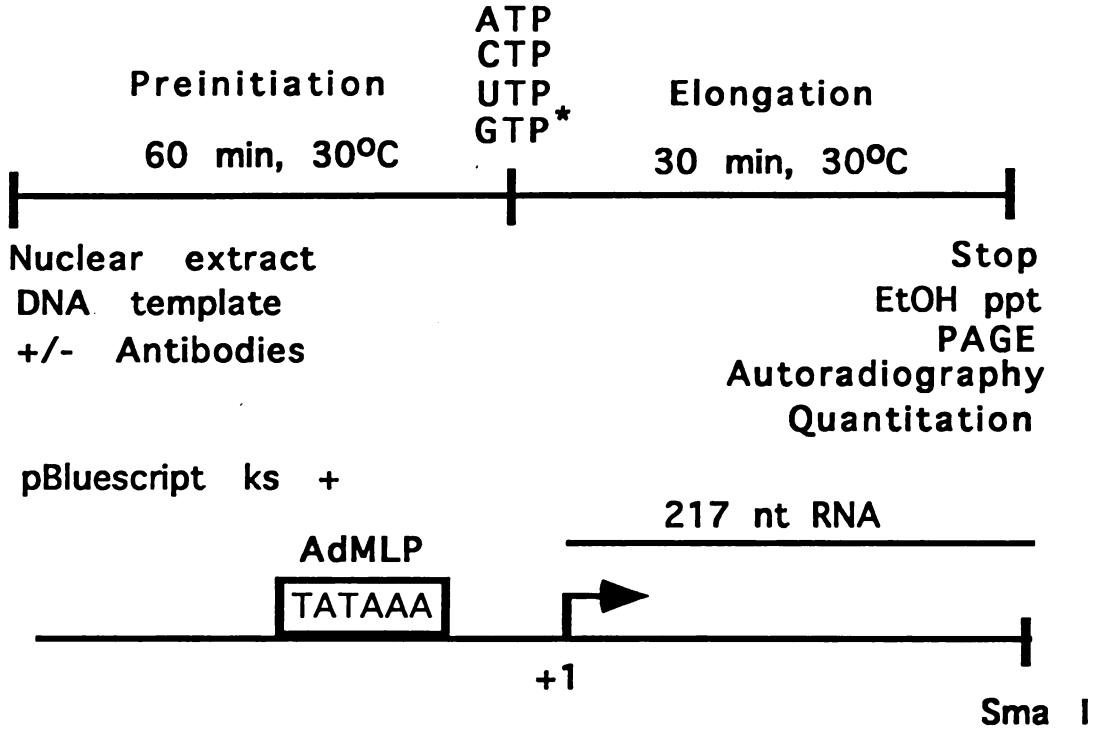


Figure 2.6 Schematic of *in vitro* transcription assay used in antibody inhibition studies. The top figure describes the protocol for a typical reaction. The bottom figure depicts the DNA template used (adenovirus major late promoter) and the RNA transcript produced during accurate transcription when the template is digested with Sma I .

stopped, phenol - chloroform extracted, ethanol precipitated, resolved on 6% PAGE containing 50% (w/v) urea and visualized by autoradiography. Quantitation of accurate transcription was done using a Molecular Dynamics Phosphorimager.

3.0 RESULTS

3.1 Production and Purification of TFIIE-α (58 kDa subunit)

TFIIE-α (58 kDa subunit) was isolated from *E.coli* BL21(DE3) containing the translation vector pET16b/TFIIE-α. pET16b/TFIIE-α produces H_{10}-TFIIE-α with the added sequence MGHHHHHHHHHHSSGHIEGRHMLD at the NH_2-terminal end of the protein. The whole cell protein is visualized by 10% SDS-PAGE and Coomassie blue stain. Production of TFIIE-α before and after induction with 0.4 mM IPTG is shown in Figure 3.1A. TFIIE-α produces a notable protein band on SDS PAGE of cells after IPTG induction although production is not as efficient as TFIIE-β (compare lanes 2,4 and 6 of figure 3.1A to lanes 3 and 4 of figure 3.1B). TFIIE-α produced in *E.coli* is soluble. TFIIE-α was purified to near homogeneity under native conditions using Ni^{2+} affinity chromatography (see figure 3.2, lanes 3 and 4). Lanes 3 and 4 contain approximately 2 ug and 5 ug of purified protein. The yield obtained of TFIIE-α from a liter of bacterial culture is approximately 6 mg based on compared observations of protein on Coomassie stained SDS-PAGE to known low protein marker amounts.

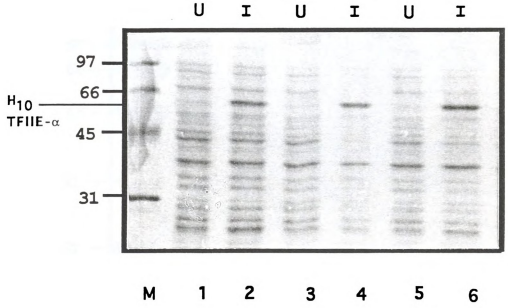


Figure 3.1A Overproduction of the large subunit of H_{10}-TFIIE-α in bacteria using the expression plasmid pET16b/TFIIE-α. The gel shown is a 10% polyacrylamide SDS-PAGE gel stained with Coomassie blue dye. Three separate colonies were grown and analyzed for efficiency of production (colony 1, lanes 1 and 2: colony 2 lanes 3 and 4: colony 3, lanes 5 and 6). Lanes 1, 3 and 5 - 10 µl of bacterial extract before induction (U: uninduced). Lanes 2,4 and 6 -10 µl of bacterial extract 3 hours after induction (I) with 0.4 mM IPTG. The apparent molecular weight of the recombinant protein is 58 kd (56 + 2).

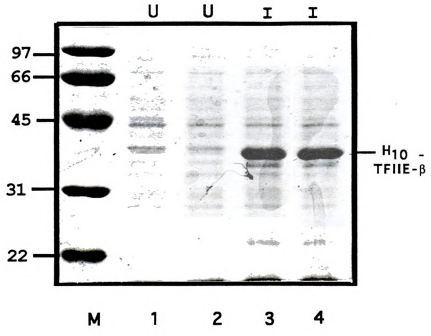


Figure 3.1B Overproduction of the small subunit of TFIIE (H_{10} - TFIIE - β) in bacteria using the expression plasmid pET16b/TFIIE-β. The figure shows a 10% SDS-Polyacrylamide gel stained with Coomassie blue dye. Two separate colonies were analyzed for growth. Colony 1, Lanes 1 and 3 - 20 ul of uninduced bacterial extract (U) and 3hrs after induction with 0.4 mM IPTG. Colony 2, Lanes 2 and 4 - 20 ul of uninduced and bacterial extract 3 hours after induction (I) with 0.4 mM IPTG. The apparent molecular weight of the recombinant protein is 36 kd (34 +2).

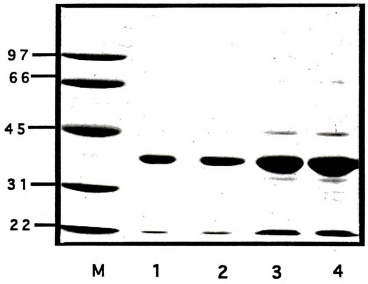


Figure 3.1C Purified and Partially purified TFIIE-β. The gel shown is a 10% polyacryiamide SDS-PAGE gel stained with coomassie blue dye. Lanes 1 and 2 contain approximately 5ug of purified H_{10}-TFIIE-β, lanes 3 and 4 contain approximately 15 ug of H_{10}-TFIIE-β with smaller and larger polypeptides of various molecular weights.

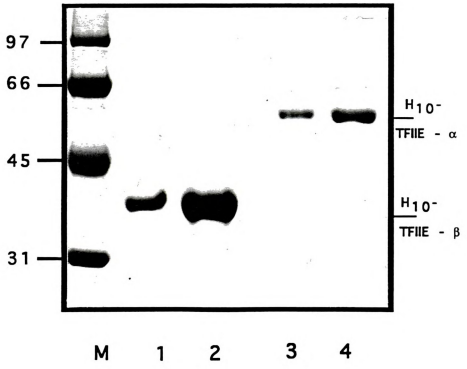


Figure 3.2 One step purification of recombinant proteins H_{10} - TFIIE - β and H_{10} - TFIIE - β using a Ni^{2+} affinity column (from Novagen). The figure shows a 10% polyacrylamide SDS - PAGE gel stained with Coomassie blue dye. M) low molecular weight marker (Bio-Rad) Lane 1- 5 ug of H_{10} - TFIIE-β ; Lane 2 - 15 ug of H_{10} - TFIIE-β ; Lane 3 - 2 ug of H_{10} - TFIIE - α; Lane 4 - 5 ug of H_{10} - TFIIE - α ; For purification of H_{10} -TFIIE - β, 1 x elution buffer containing 6M urea was used to solubilize the protein. H_{10} - TFIIE - α was purified under native conditions.

3.2 Production and Purification of TFIIE-β (36 kDa subunit)

TFIIE-β was purified from *E.coli* BL21(DE3) cells containing the translation vector pET16b/TFIIE-β. This vector produces H_{10}-TFIIE-β with the sequence MGH_{10}SSGHIEGRHLMD fused to the NH_2-terminal end of the protein. TFIIE-β accumulated within inclusion bodies as an insoluble aggregate. Purification was performed under denaturing conditions (6M urea) according to the pET-His protocols of Novagen. Figure 3.1B shows results of the whole cell extract before and after induction with IPTG as visualized on 10% SDS-PAGE with Coomassie blue stain. The small subunit migrated at a rate consistent with its molecular weight of 36 kd and appears to be the most abundant protein present in the extract. Figure 3.1C shows purified and partially purified TFIIE-β. Under native conditions only partial purification was possible and as a result smaller and larger polypeptides were also found after Ni^{2+}affinity purification(see lanes 3 and 4). Figure 3.2 shows results of purification using Ni^{2+} affinity chromatography using 6M urea for purification of TFIIE-β (see lanes 1 and 2). Approximately 5 and 15 ug of protein is shown in lanes 1 and 2. The yield of H_{10}-TFIIE-β from a liter of bacterial culture is approximately 15 mg. Based on compared observations of known protein amounts on Coomassie stained SDS-PAGE.

3.3 Western blot Analysis of recombinant TFIIE

Western blot analysis was performed using anti-serum against the bacterially produced proteins in *E.coli*. Each corresponding subunit of recombinant TFIIE (58 kDa and 36 kDa subunits) was detected. Experiments were performed to determine the sensitivity of detection of the antiserum generated or titre. Figure 3.3A shows an immunoblot containing the following amounts of H_{10}-TFIIE-α; 500 ng, 250 ng, 125 ng, 50 ng, 25 ng, 10 ng and 5 ng. Our antibody can be used to detect H_{10}-TFIIE-α to the low ng range, which is consistent with results obtained using RAP fractions (figure 3.3C, lane R2). The control lane (lane C) contained 125 ng of H_{10}-TFIIE-β. Figure 3.3B shows an immunoblot of H_{10}-TFIIE-β with decreasing amounts of protein; 500 ng, 250 ng, 125 ng, and 25 ng. The control lane contained 125 ng of H_{10}-TFIIE-α. A 1:500 dilution was used for the primary antibody for each blot. Immunoblots of RAP fractions (Figure 3.3C Lanes R1 and R2) provides immunological evidence that the recombinant proteins are derived from expression of cDNAs for each subunit of TFIIE. Results reveal that both subunits of TFIIE are present in RAP fractions in ng quantities (positive controls - 25 ng). Eluate from the anti-CTD affinity column gave a weak reaction for the TFIIE-α,(Lane C2), but TFIIE-β was below the level of detection in this experiment (Lane C1).

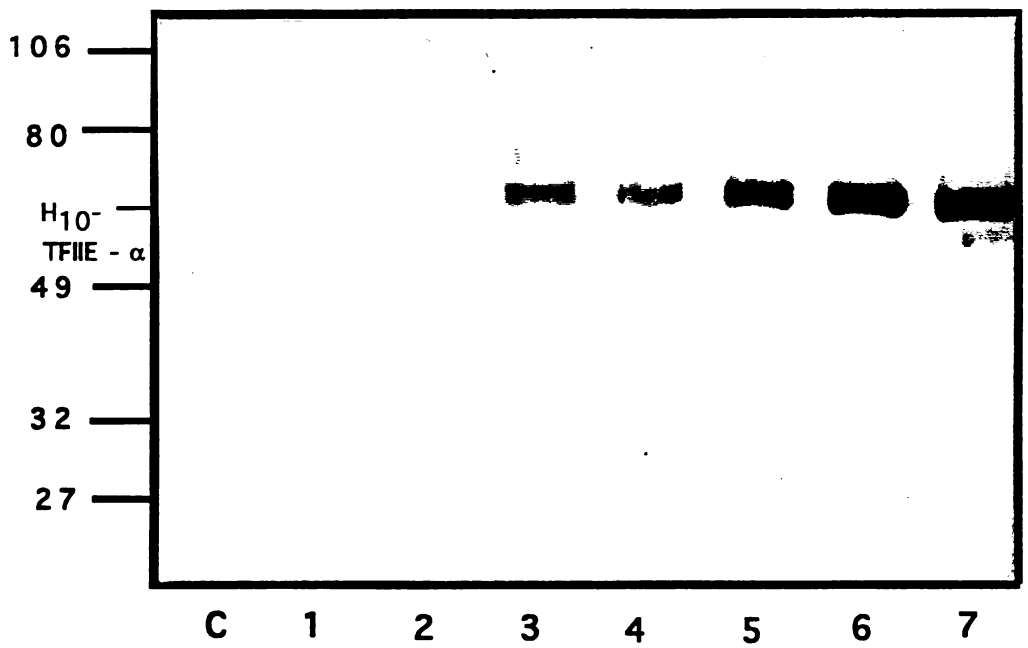


Figure 3.3A Western blot analysis for detection of the large subunit of TFIIE (TFIIE - α). Lanes 1 -7, H_{10} - TFIIE - α protein : 5, 10, 25, 50, 125, 250, and 500ng respectively. Lane C, H_{10}- TFIIE - β, 125 ng (negative control).

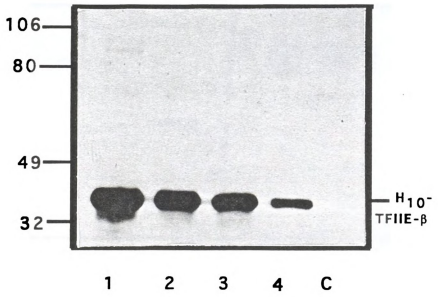


Figure 3.3B Western blot analysis for detection of small subunit of TFIIE (TFIIE - β). Lanes 1-4, H_{10} - TFIIE - β protein : 500ng, 250ng, 125ng and 25ng respectively. Lane C, H_{10} - TFIIE - α, 125ng (negative control).

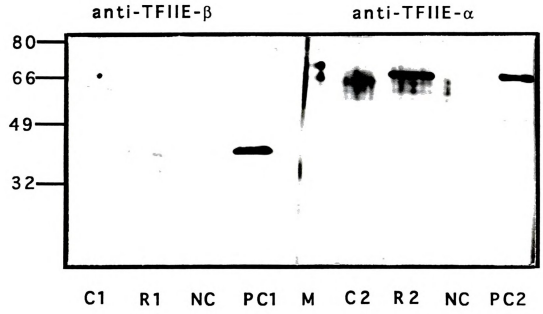


Figure 3.3C Western blot analysis of small and large subunit of TFIIE in RAP fractions from RNA polymerase II affinity column and anti-CTD affinty column. Lane C1 and C2, anti-CTD affinity column eluate, lane R1 and R2, RAP column eluate, lane NC, negative control column eluate, lane PC1, positive control, 25ng recombinant H_{10} -TFIIE- β, lane M, prestain molecular weight markers, lane PC2, positive control, 25ng recombinant H_{10}- TFIIE - α.

3.4 Inhibition of Accurate Transcription by Anti-TFIIE serum

Anti-serum against TFIIE subunits 36 kd and 58 kd were tested for their ability to inhibit accurate transcription from the AdMLP. The results are shown in figure 3.4. Both anti-TFIIE-α and anti-TFIIE-β show inhibition when added to *in* vitro transcription reactions of Hela nuclear extracts. This result supports previous evidence that TFIIE is required for basal transcription from the AdMLP. Anti-TFIIE-β inhibits transcription by more than 90%, while anti-TFIIE-α inhibits about 70%. Partial inhibition is also seen when anti-RAP30 serum is added to the reaction, inhibiting around 95%. In addition, anti-RAP74 serum is less effective, inhibiting about 50% (B.Q. Wang, unpublished results).

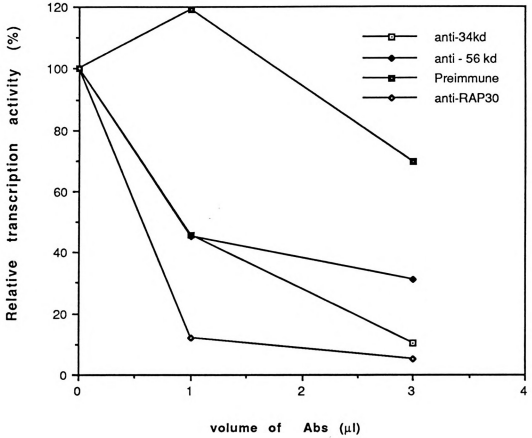


Figure 3.4 Inhibition of Accurate Transcription by Antiserum Raised Against Basal Subunits. Graph shows the effect of various antibodies raised against TFIIE subunits 34kd and 56 kd, RAP30, and preimmune serum. Total reaction volume is 20 µl.

4.0 DISCUSSION

An efficient method for the production and purification of human TFIIE subunits α and β in bacteria has been described. The method of Ni^{2+}- affinity chromatography provides a versatile, economic and convenient means of purification either under nondenaturing or denaturing conditions for a wide variety of proteins. Studies are now under way to test the activity of recombinant TFIIE in *in vitro* reconstitution assays.

TFIIE has been proposed to be a heterotetramer ($\alpha_2\beta_2$) structure during its formation of a preinitiation complex on a promoter (see figure 4.1A). This is based on gel filtration chromatography that suggests that the 36 kd and possibly the 58 kd subunit may form dimers in solution. When the recombinant proteins were mixed and subjected to gel filtration, they behaved as a complex of approximately 230 kd, which is similar in size to native TFIIE (Peterson et al. 1992). TFIIE, like many of the other basal factors contains many highly charged acidic, basic and hydrophobic regions that may be involved in protein-protein interactions. When individual subunits are isolated in the absense of their normal partners, binding domains may cause aggregation.

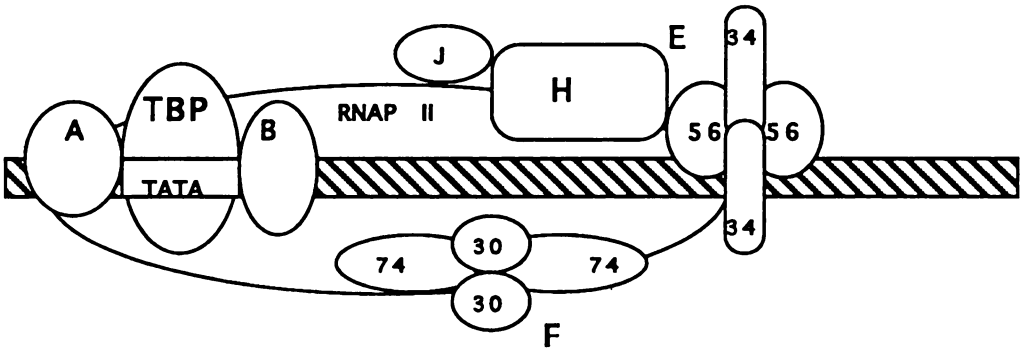


Figure 4.1A Current Model of the Preinitiation Complex. Factors D (TBP), A, and B associate first to form the DAB complex on the TATA box region of the promoter. Then RNA polymerase II (RNAP II) and TFIIF. Followed by factors E, H, and J which enter the complex late. Both TFIIE and TFIIF are depicted as heterodimers. TFIIH is a large complex with at least 5 subunits.

Multimerization of individual subunits may contribute to another problem that occurs during isolation of the proteins. That is, the separation of intact protein from protein fragments. Intact TFIIE-β copurifies with fragments of TFIIE-β, which may result from inefficient mRNA translation or proteolysis (see figure 3.1B, lanes 3 and 4). Dissociation of TFIIE-β from fragments is observed in 6 M urea.

Antiserum generated against recombinant subunits appears to be useful for detection of TFIIE subunits in RAP fractions. Detection of the large subunit of TFIIE was much stronger (around 25 ng when compared to positive control) then the detection of the small subunit which was barely visible. One would expect both TFIIE subunits to be present in equal molar amounts. One explanation may be that the large subunit binds to the RNA polymerase column more efficiently than the small subunit. Another is that the small subunit disassociates more readily during purification. This would be consistent with previous data by Inostroza et al. 1991 which shows the large subunit to be sufficient for low levels of transcription activity, while the small subunit is stimulatory when added. Perhaps the interaction with RNA polymerase is important for its activity and /or function.

Antiserum raised against TFIIE subunits shows specific inhibition of *in vitro* transcription assays. The antiserum may be a useful reagent in future studies in producing a TFIIE

depleted extract, immunoprecipitation studies of TFIIE, and for testing the quality of RAP fractions eluted from RNA polymerase II-affinity columns or anti-CTD affinity columns. The results provide additional support for the requirement of TFIIE in basal transcription from the AdMLP. Preimmune serum also gave moderate inhibition (25%), this may be due to the presence of RNases in serum. Purification of the antiserum on an affinity column prior to the *in vitro* transcription reaction may resolve this problem.

Although structural motifs and sequence similarities give clues to possible functions of TFIIE, little is known about its role in transcription initiation. Previous studies showed that the purified HeLa cell 56 kd subunit of TFIIE alone has some TFIIE activity, whereas the 34 kd subunit is stimulatory (Inostroza et al. 1991). Reconstituted basal transcription from the AdMLP is dependent on both subunits to reconstitute transcriptional activity (Peterson et al. 1991). Various studies with a partially purified TFIIE have indicated that it interacts with RNA polymerase II (Reinberg et al 1987b; Flores et al 1989), that it assocites with the transcription complex at a late stage during complex assembly after pol II and TFIIF (Buratowski et al. 1989). It is still unclear whether the interaction with RNA polymerase II is direct or indirect, although results obtained with RNA polymerase II- affinity studies (figure 3.3C) suggest a direct interaction. Crude TFIIE fractions from HeLa cell nuclear extracts possess a DNA-

dependent ATPase activity, but no activity is associated with the highly purified TFIIE suggesting possible interactions with the 89 kd subunit of TFIIH, which was shown recently to possess an ATP-dependent-DNA helicase activity (Ohkuma et al. 1990; Schaeffer et al. 1993). The basal factor TFIIH also enters the preinitiation complex late after TFIIE and it may interact with TFIIE and RNA polymerase II. There is evidence that TFIIE may stimulate the processivity of TFIIH- kinase activity which also suggests a possible interaction between the two factors (Lu et al. 1992).

Other questions to be explored include: 1) do homologs of TFIIE exist in the RNA polymerase I and polymerase III systems?; 2) is there a TFIIE equivalent in yeast or other systems ? and, if so, how closely related are they in structure and function? 3) does TFIIE cycle off of polymerase during the transition from initiation to elongation? 4) what other factors interact with TFIIE and what are their functions? Antisera raised against TFIIE subunits may be useful in determing TFIIE equivalents and /or homologs in human or other systems.

As mentioned above it will be important to test whether the recombinant TFIIE subunits will function in a highly purified transcription system. The purification of TFIIE is an important step toward this goal. Work is under way in our laboratory to set up a reconstitution assay for TFIIE. Until then we are unable to demonstrate biological activity with the

cloned proteins. Another important goal to the transcription field is the development of a system of completely defined components that will accurately transcribe from promoters. This would include the following purified proteins reported to make up the system: TBP, TFIIB, TFIIF (RAP30/RAP74), TFIIE (34 and 56 kDa subunits), TFIIH (at least five subunits), TFIIJ (undefined) and RNA polymerase II (10 subunits). Since not all components of TFIIH and TFIIJ have yet been defined, as well as cloned and expressed, a completely defined system is not possible at present. Yet, the availability of large quantities of purified recombinant TFIIE is another step closer to this larger goal of obtaining a complete transcription system.

5.0 APPENDIX

This section includes information on the aminoacid sequence and structure of TFIIE subunits, calculated molecular weight, isoelectric point, and extinction coefficient. Also included is sequence data of recombinant TFIIE subunits with the additional 22 amino acid peptide. This can be compared with the wildtype human sequences. The 22 amino acid peptide adds an additional calculated molecular mass of 2520.7 daltons to each subunit of TFIIE.

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      51  SKQNSDHSNG SFNLKALSGS SGYKFGVLAK IVNYMKTRHQ RGDTHPLTLD

     101  EILDETQHLD IGLKQKQWLM TEALVNNPKI EVIDGKYAFK PKYNVRDKKA

     151  LLRLLDQHDQ RGLGGILLED IEEALPNSQK AVKALGDQIL FVNRPDKKKI

     201  LFFNDKSCQF SVDEEFQKLW RSVTVDSMDE EKIEEYLKRQ GISSMQESGP

     251  KKVAPIQRRK KPASQKKRRF KTHNEHLAGV LKDYSDITSS K*
```

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Tfiie56k.pep Length: 440 October 8, 1992 14:00 Type: P Check: 1543 ..

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      51  LLKFDRKQLR SVLNNLKGDK FIKCRMRVET AADGKTTRHN YYFINYRTLV

     101  NVVKYKLDHM RRRIETDERD STNRASFKCP VCSSTFTDLE ANQLFDPMTG

     151  TFRCTFCHTE VEEDESAMPK KDARTLLARF NEQIEPIYAL LRETEDVNLA

     201  YEILEPEPTE IPALKQSKDH AATTAGAASL AGGHHREAWA TKGPSYEDLY

     251  TQNVVINMDD QEDLHRASLE GKSAKERPIW LRESTVQGAY GSEDMKEGGI

     301  DMDAFQEREE GHAGPDDNEE VMRALLIHEK KTSSAMAGSV GAAAPVTAAN

     351  GSDSESETSE SDDDSPPRPA AVAVHKREED EEEDDEFEEV ADDPIVMVAG

     401  RPFSYSEVSQ RPELVAQMTP EEKEAYIAMG QRMFEDLFE*
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Session Name: clvax1

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TRANSLATE of: tfiie34k.txt check: 2239 from: 191 to: 1066
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 With Enzymes:

April 19, 1993 09:15 ..

Summary for whole sequence:

Molecular weight = 35564.15 Residues = 312
Average Residue Weight = 113.988 Charged = 16
Isoelectric point = 10.46
Extinction coefficient = 19120

Residue	Number	Mole Percent
A = Ala	13	4.167
B = Asx	0	0.000
C = Cys	1	0.321
D = Asp	19	6.090
E = Glu	21	6.731
F = Phe	10	3.205
G = Gly	20	6.410
H = His	20	6.410
I = Ile	14	4.487
K = Lys	39	12.500
L = Leu	29	9.295
M = Met	6	1.923
N = Asn	11	3.526
P = Pro	10	3.205
Q = Gln	15	4.808
R = Arg	17	5.449
S = Ser	34	10.897
T = Thr	10	3.205
V = Val	15	4.808
W = Trp	2	0.641
Y = Tyr	6	1.923
Z = Glx	0	0.000
A + G	33	10.577
S + T	44	14.103
D + E	40	12.821
D + E + N + Q	66	21.154
H + K + R	76	24.359
D + E + H + K + R	116	37.179
I + L + M + V	64	20.513
F + W + Y	18	5.769

Session Name: clvax1

$ TYPE TFIIE56KH10.PEPSORT
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TRANSLATE of: tfiie56k.txt check: 1285 from: 64 to: 1383
generated symbols 1 to: 440.

With Enzymes:

April 19, 1993 09:19 ..

Summary for whole sequence:

Molecular weight = 51972.35 Residues = 460
Average Residue Weight = 112.983 Charged = -35
Isoelectric point = 4.92
Extinction coefficient = 28380

Residue	Number	Mole Percent
A = Ala	45	9.783
B = Asx	0	0.000
C = Cys	6	1.304
D = Asp	35	7.609
E = Glu	54	11.739
F = Phe	15	3.261
G = Gly	24	5.217
H = His	23	5.000
I = Ile	18	3.913
K = Lys	24	5.217
L = Leu	32	6.957
M = Met	16	3.478
N = Asn	14	3.043
P = Pro	20	4.348
Q = Gln	11	2.391
R = Arg	30	6.522
S = Ser	28	6.087
T = Thr	25	5.435
V = Val	25	5.435
W = Trp	2	0.435
Y = Tyr	13	2.826
Z = Glx	0	0.000
A + G	69	15.000
S + T	53	11.522
D + E	89	19.348
D + E + N + Q	114	24.783
H + K + R	77	16.739
D + E + H + K + R	166	36.087
I + L + M + V	91	19.783
F + W + Y	30	6.522

Session Name: clvax1

$ type tfiie34k.pepsort
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TRANSLATE of: tfiie34k.txt check: 2239 from: 191 to: 1066
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 With Enzymes:

October 8, 1992 13:53 ..

Summary for whole sequence:

Molecular weight = 33043.45 Residues = 291
Average Residue Weight = 113.551 Charged = 16
Isoelectric point = 10.46
Extinction coefficient = 19120

Residue	Number	Mole Percent
A = Ala	13	4.467
B = Asx	0	0.000
C = Cys	1	0.344
D = Asp	19	6.529
E = Glu	20	6.873
F = Phe	10	3.436
G = Gly	17	5.842
H = His	8	2.749
I = Ile	13	4.467
K = Lys	39	13.402
L = Leu	29	9.966
M = Met	5	1.718
N = Asn	11	3.780
P = Pro	10	3.436
Q = Gln	15	5.155
R = Arg	16	5.498
S = Ser	32	10.997
T = Thr	10	3.436
V = Val	15	5.155
W = Trp	2	0.687
Y = Tyr	6	2.062
Z = Glx	0	0.000
A + G	30	10.309
S + T	42	14.433
D + E	39	13.402
D + E + N + Q	65	22.337
H + K + R	63	21.649
D + E + H + K + R	102	35.052
I + L + M + V	62	21.306
F + W + Y	18	6.186

Session Name: clvax1

$ type tfiie56k.pepsort
 PEPTIDESORT of: Tfiie56k.Pep check: 1543 from: 1 to: 440

TRANSLATE of: tfiie56k.txt check: 1285 from: 64 to: 1383
generated symbols 1 to: 440.

 With Enzymes:

October 8, 1992 14:01 ..

Summary for whole sequence:

Molecular weight = 49451.64 Residues = 439
Average Residue Weight = 112.646 Charged = -35
Isoelectric point = 4.55
Extinction coefficient = 28380

Residue	Number	Mole Percent
A = Ala	45	10.251
B = Asx	0	0.000
C = Cys	6	1.367
D = Asp	35	7.973
E = Glu	53	12.073
F = Phe	15	3.417
G = Gly	21	4.784
H = His	11	2.506
I = Ile	17	3.872
K = Lys	24	5.467
L = Leu	32	7.289
M = Met	15	3.417
N = Asn	14	3.189
P = Pro	20	4.556
Q = Gln	11	2.506
R = Arg	29	6.606
S = Ser	26	5.923
T = Thr	25	5.695
V = Val	25	5.695
W = Trp	2	0.456
Y = Tyr	13	2.961
Z = Glx	0	0.000
A + G	66	15.034
S + T	51	11.617
D + E	88	20.046
D + E + N + Q	113	25.740
H + K + R	64	14.579
D + E + H + K + R	152	34.624
I + L + M + V	89	20.273
F + W + Y	30	6.834

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