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THE UNIVERSITY OF OKLAHOMA HEALTH SCIENCES CENTER

GRADUATE COLLEGE

MOLECULAR INTERACTIONS IN RNA POLYMERASE II AND III TRANSCRIPTION SYSTEMS

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

In partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY
RODNEY J. MORELAND

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MOLECULAR INTERACTIONS IN RNA POLYMERASE II AND III TRANSCRIPTION SYSTEMS

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LIST OF ABBREVIATIONS

3'-O-MeGTP	3'-O-methylguanosine 5'-triphosphate
AdML	adenovirus type 2 major late promoter
BCIP	5-Bromo-4-Chloro-3-Indolyl Phosphate
BSA	Bovine Serum Albumin
Da	dalton
DEAE	diethylaminoethyl
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetate
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	high performance liquid chromatography
IPTG	isopropyl-β-D-thiogalactoside
NBT	Nitroblue tetrazolium
nt	nucleotide
NTPs	ribonucleoside 5'-triphosphates
PAGE	polyacrylamide gel electrophoresis
PMSF	phenylmethylsulfonyl flouride
Pol II	RNA polymerase II
Pol III	RNA polymerase III
RAP	RNA polymerase associated protein

Rpm	revolutions per minute
rRNA	ribosomal RNA
rTFIIIA	recombinant transcription factor IIIA
SDS	sodium dodecyl sulfate
SP	sulfopropyl
TAFs	TATA binding protein associated factors
TBE	tris/borate/EDTA
TBP	TATA-binding protein
TBP	TATA binding protein
TFIID	native TATA binding protein with associated factors
TFIIE	transcription factor IIE
TFIIF	transcription factor IIF
TFIIH	transcription factor IIH
TFIIIA	transcription factor IIIA
TFIIIA-IP	transcription factor IIIA interacting protein
TFIIIB	transcription factor IIIB
TFIIIC	transcription factor IIIC
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
tRNA	transfer RNA
WT	wild type

ABSTRACT

MOLECULAR INTERACTIONS IN RNA POLYMERASE II AND III TRANSCRIPTION SYSTEMS

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The elongation stage of eukaryotic messenger RNA synthesis is a major site for the regulation of gene expression. Elongation factor Elongin is a member of a class of transcription factors that stimulates the rate of elongation by RNA polymerase II *in vitro* by suppressing transient pausing at many sites along DNA templates. Mechanistic studies of Elongin in this dissertation have shown the following: (1) Elongin does not stimulate detectably the rate of promoter-specific transcription initiation by the fully-assembled preinitiation complex; (2) early RNA polymerase II elongation intermediates first become susceptible to stimulation by Elongin after synthesizing 8-9 nucleotide-long transcripts; and (3) the relative inability of Elongin to stimulate elongation of transcripts less than 8-9 nucleotides correlates not with the lengths of their associated transcripts but, instead, with the presence of the initiation factor TFIIF in transcription reactions.

Transcription factor IIIA (TFIIIA) is a RNA polymerase III factor that regulates eukaryotic 5S ribosomal RNA synthesis. This factor is the prototypical Cys_2His_2 zinc finger protein, and its amino acid sequence is highly divergent among frog species. *Xenopus* TFIIIA will promote transcription of the 5S RNA gene in mammalian cell extracts. This suggests that the transcription activation domain is conserved between amphibians and mammals. To investigate this relationship, the TFIIIA amino acid sequence from mouse was identified, and its sequence has provided important structural and functional insights. Sequence analysis of mouse TFIIIA revealed a 20 amino acid region in the C-terminus that is highly conserved between mouse and amphibians, conversely, the N-terminal region demarcating the oocyte form of TFIIIA in frogs and fish is not conserved in mammals.

The highly conserved C-terminal region of *Xenopus* TFIIIA was used in a yeast 2-hybrid screen to identify proteins that interact with this domain. This screen yielded a protein that interacts with *Xenopus* TFIIIA in yeast cells and in *in vitro* assays, including immunoprecipitations. The cDNA sequence of this protein revealed several motifs found in transcription factors such as a leucine zipper and a zinc finger. A function for this protein has not been defined, however, the protein has been localized to the nuclear membrane.

CHAPTER I

MECHANISM OF ACTION OF ELONGIN

INTRODUCTION

Eukaryotic messenger RNA synthesis is a complex biochemical process catalyzed by multisubunit RNA polymerase II and governed by the action of multiple classes of transcription factors. A preinitiation complex can be assembled on the Adenovirus Major Late (AdML) promoter *in vitro* with highly purified TFIIH, RNA polymerase II, and the following recombinant factors: TATA binding protein (TBP); TFIIB; TFIIF; and TFIIE. Assembly of the preinitiation complex is nucleated by sequence-specific binding of the native TATA factor (TFIID) to the core promoter. TBP is a subunit in native TATA factor and is believed to bind directly to the TATA box element located 30 base pairs upstream from the start site of transcription. Purification of native TATA factor has revealed that it is associated with at least seven TATA associated factors (TAFs) of ~250, 125, 95, 78, 50, 30, and 28 kDa mass (Zhou et.al., 1993). The current model is TAFs mediate interactions between the basal transcriptional apparatus and transcriptional regulatory proteins such as coactivators which may interact with DNA sometimes thousands of bases upstream or downstream from the start site of transcription. The TATA factor requires TFIIB for recruitment of RNA polymerase II to the promoter. TFIIB is composed of a single 35 kDa polypeptide and does not appear to bind DNA or

have any enzymatic activities (Ha et.al., 1991; Wampler et.al., 1992).

TFIIF functions in concert with TFIIB to promote stable binding of RNA polymerase II to the TATA factor at the promoter. TFIIF is often found associated with RNA polymerase II and prevents nonspecific binding of polymerase to DNA. How this is achieved has not been clearly established. TFIIF will stimulate elongation *in vitro* if concentrations of TFIIF are much greater than what is required for initiation. TFIIF is a heterodimer composed of RNA polymerase associated proteins (RAP) RAP30 and RAP74 kDa polypeptides (Aso et.al., 1992; Bradsher et.al., 1993). TFIIE enters the preinitiation complex along with TFIIH and together stabilize one another's interactions with the preinitiation complex. TFIIE is a heterodimer composed of 34 kDa and 56 kDa subunits (Sumimoto et.al., 1991; Ohkuma, et.al., 1991). TFIIH is a multifunctional initiation factor, possessing ATPase, DNA helicase, and kinase activities.

TFIIH is an ~400 kDa transcription factor composed of 8 polypeptides. TFIIH possesses a protein kinase activity that is capable of phosphorylating the C-terminal domain (CTD) of the largest subunit of RNA polymerase II. This polymerase subunit in mammals has 52 tandemly repeated copies of a heptapeptide with the consensus sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser. Phosphorylation of the CTD is not required for basal transcription *in vitro* (Serizawa et.al., 1993), however, it has been shown that nonphosphorylated CTDs preferentially enter the preinitiation complex, and CTDs of RNA polymerase II molecules that are actively engaged in RNA chain elongation are highly phosphorylated.

The largest subunit of TFIIH is the product of the ERCC-3 gene, which was originally isolated by its ability to correct a DNA repair defect in cells derived from patients with xeroderma pigmentosum (Weeda et.al., 1990). The ERCC-3 gene has a DNA helicase motif. It is not known if TFIIH functions in DNA repair. It is possible that the ERCC-3 gene product functions both as a component of TFIIH and as a separate entity required for DNA repair. TFIIH also has another subunit, ERCC2, that has been shown to have helicase activity. It is believed one or both of these helicases in TFIIH is responsible for the conversion to the "open" complex by the ATP dependent unwinding of the DNA at the start site of transcription. Once strand separation as occurred in the presence of ribonucleotide triphosphates, polymerase initiates transcription, clears the promoter, and enters the elongation phase.

The elongation phase is reached at approximately +30 base pairs downstream from the start site of transcription. By this point the initiation factors have been released and a ternary complex comprised of polymerase, DNA, and the nascent RNA transcript exists (Zawel et.al., 1995). This ternary complex is susceptible to regulation by elongation transcription factors. Elongin is an elongation transcription factor which functions by stimulating the overall rate of elongation by RNA polymerase II by suppressing transient pausing by polymerase at many sites along DNA templates (Bradsher et al. 1993; Bradsher et al. 1993a). In addition to elongin, this class includes ELL, ELL2, the Cockayne syndrome complementation group B protein (CSB), and transcription factor IIF (TFIIF), which is also required for transcription initiation by RNA

polymerase II (Shilatiford et al., 1997; Shilatifard et al., 1996; Price et al., 1989; Selby et al., 1997). General elongation factors that suppress pausing or prevent arrest of transcribing RNA polymerase II might be vital for expression of many eukaryotic genes, including extremely large genes such as dystrophin, which spans more than 2 megabases of chromosomal DNA and requires nearly 17 hours to be transcribed.

Elongin is a 3-subunit complex composed of an A subunit of ~770 amino acids and two small ~110 amino acid regulatory B and C subunits (Aso et al., 1995; Garrett et al., 1994; Garrett et al., 1995). Elongin B and C form a stable elongin BC complex that binds to elongin A and potently induces its transcriptional activity. Elongin C functions as the inducing ligand and activates transcription elongation through interactions with a conserved sequence motif (consensus sequence [T,S,P][L,M]xxx[C,S]xxx[V,L,I]) in the elongin A elongation activation domain (Aso et al., 1996; Conaway et al., 1998). Elongin B appears to perform a chaperone-like function by facilitating binding of elongin C to elongin A (Aso et al., 1995). Notably, elongins B and C are also found as integral components of a multiprotein complex containing the product of the von Hippel-Lindau (VHL) tumor suppressor gene (Duan et al., 1995; Kibel et al., 1995). The VHL protein and elongin A share the conserved elongin BC binding site motif, and, consistent with a role for the elongin BC complex in tumorigenesis, more than 70% of VHL mutations found in VHL families and sporadic clear cell renal carcinomas result in alteration or deletion of this site (Gnarra et al., 1996).

The mechanism of action of elongin is investigated in this dissertation. In a previous study exploiting oligo(dC)-tailed DNA templates, it was demonstrated that elongin is capable of stimulating the rate of elongation by RNA polymerase II in the absence of additional transcription factors, suggesting that elongin acts directly on the ternary elongation complex (Bradsher et al., 1993). Studies in this dissertation have yielded evidence that, during synthesis of promoter-specific transcripts, maximal stimulation of elongation by elongin requires prior conversion of the early RNA polymerase II elongation complex to an elongin-activatable form. These findings shed new light on the requirements for interaction of elongin with the RNA polymerase II elongation complex and suggest that, shortly after initiating transcription from a promoter, the RNA polymerase II transcription complex undergoes a structural transition that renders it maximally sensitive to stimulation by elongin.

EXPERIMENTAL PROCEDURES

Materials. Unlabeled ultrapure ribonucleoside 5'-triphosphates, dATP, and 3'-*O*-MeGTP[1] were purchased from Pharmacia Biotech, Inc. [α-^{32}P] CTP (>3000 Ci/mmol) was obtained from Amersham. Dinucleotides CpA and CpU, polyvinyl alcohol (type II), and α-amanitin were from Sigma. Acetylated bovine serum albumin and recombinant human placental ribonuclease inhibitor were purchased from Promega. Empty 4 ml spin columns were obtained from 5 Prime-3 Prime, Inc.

Buffers. TMSD is 10 mM Tris-HCl (pH 7.5), 1.5 mM $MgCl_2$, 0.25 M sucrose, 0.5 mM DTT, and 0.5 mM PMSF. Buffer A is 20 mM Hepes-NaOH (pH 7.9), 1 mM EDTA, 1 mM DTT, 10% (v/v) glycerol, and 0.5 mM PMSF. Buffer C is 40 mM Tris-HCl (pH 7.9), 0.5 mM EDTA, 1 mM DTT, and 10% (v/v) glycerol. Buffer D is 40 mM Hepes-NaOH (pH 7.9), 0.5 mM EDTA, 1 mM DTT, and 10% (v/v) glycerol. Buffer E is 25 mM Hepes (pH 7.6), 0.1 mM EDTA, 12.5 mM $MgCl_2$, 10% glycerol, 0.1% NP-40, 1 mM DTT, 0.1 mM PMSF, and 0.1 mM sodium metabisulfite. Buffer F is 20 mM Tris-HCl (pH 7.9, 500 mM NaCl, 0.2 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 10 mM imidazole (pH 7.9), 0.1 mg/ml lysozyme. Buffer Y is 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 10% (v/v) glycerol.

Preparation of DNA templates. DNA templates containing G-less AdML promoter derivatives Ad(G+13), Ad(G+11), Ad(G+9), Ad(G+7), and Ad(G+5) (Fig. 1) were constructed by oligonucleotide-directed mutagenesis of M13mp19-AdML DNA (Dvir et al., 1997) using the Muta-Gene M13 *in vitro* mutagenesis kit (Bio-Rad). DNA sequences of G-less AdML promoters were confirmed by dideoxy DNA sequencing using the fmol DNA Sequencing System (Promega). Ad(G+15) is the AdML promoter derivative in pDN-AdML (Conaway et al., 1988). To prepare premelted AdML promoter derivatives, single-stranded M13 DNA corresponding to the template strands of AdML promoter derivatives in M13mp19 was prepared as described (Sambrook et al., 1989). Oligonucleotides having the sequence of the non-template strand of each promoter from –

59 to +17 except for a 9 bp substitution (sequence AAGTAGAAG) from –9 to –1 relative to the *in vivo* AdML transcriptional start site were used as primers to direct synthesis of double-stranded DNA as described (Dvir et al., 1997). Plasmid or RF DNA for each of the G-less AdML promoter derivatives was purified by CsCl density gradient centrifugation and used to prepare duplex DNA templates for transcription. RF or premelted DNAs were digested with Kpn I and Ava II to generate ~410 bp fragments. pDN-AdML was digested with Eco RI and Nde I to generate an ~310 bp fragment. DNA fragments were purified by agarose gel electrophoresis and used as templates for transcription. The premelted template used in the experiment was prepared by annealing an oligonucleotide corresponding to the AdML template strand with sequence 3'-CCC CCG ATA TTT TCC CCC ACC CCC GCG CAA GCA GGA GTG AGA GAA GGA GAT CTC AGC TGG ACG TCC GTA CGT TCG AAC CG-5' to an oligonucleotide with sequence 5'-GGG GGC TAT AAA AGG GGG TGG GGG CGC GAA GTA GAA GAC TCT CTT CCT CTA GAG TCG ACC TGC AGG CAT GCA AGC TTG GC-3'; underlined bases correspond to the non-complementary DNA between -9 and -1 relative to the transcriptional start site.

Preparation of RNA Polymerase II and Initiation Factors. Fifty to sixty male Sprague-Dawley rats weighing 200-300 g each were fasted overnight. Rats were anesthetized with carbon dioxide and decapitated using a small rodent guillotine (Harvard apparatus). Livers were excised rapidly and immersed in ice-cold TMSD (10 mM Tris-HCl pH 7.5, 1.5 mM $MgCl_2$, 0.25 M sucrose, 0.5 mM DTT, 0.5 mM PMSF).

Fig. 1. Sequences of G-less AdML Promoter Derivatives Used in this Study. +1 indicates the position corresponding to the *in vivo* AdML transcriptional start site. The lengths of G-less transcripts initiated with either CpA or CpU are shown at the right of the figure. 3'-O-MeG is 3'-O-Methylguanosine 5'-triphosphate.

		Predicted length of 3'-O-MeG terminated transcripts initiated with:	
	+1 ▼	CpA	CpU
Ad(G+15)	CCTCACTCTCTTCCTCTAGAG	16	18
Ad(G+13)	CCTCACTCTCTTCCTCGAGAG	14	16
Ad(G+11)	CCTCACTCTCTTCCGCTAGAG	12	14
Ad(G+9)	CCTCACTCTCTTGCTCTAGAG	10	12
Ad(G+7)	CCTCACTCTCGTCCTCTAGAG	8	10
Ad(G+5)	CCTCACTCGCTTCCTCTAGAG	6	8

All further operations were carried out at 4°C in a cold room. Livers (~500 g total) were minced into small pieces using scalpels, suspended in TMSD to a final volume of 1400 ml, and homogenized by two passes through a Ziegler-Pettit continous flow homogenizer (11). The homogenate was distributed to six 250 ml conical bottom polypropylene bottles (Corning) and centrifuged for 10 min at 2000 rpm (800 x g) in a J-6 centrifuge. The supernatants (cytosol) were pooled and centrifuged for 90 min at 13,500 rpm (28,000 x g) in a JA-14 rotor. Solid $(NH_4)_2SO_4$ was then added slowly with stirring to the resulting post-lysosomal supernatant to 38% saturation (0.213 g $(NH_4)_2SO_4$ per ml). Thirty minutes after the ammonium sulfate dissolved, the pH was adjusted by additon of (1 µl of 1 N NaOH per g $(NH_4)_2SO_4$ added), and the suspension was centrifuged for 45 min at 9,500 rpm (16,000 x g) in a JA-10 rotor. The resulting pellets (cytosolic 0-38% ammonium sulfate fraction) were quick-frozen in liquid nitrogen and stored at –80°C. Solid $(NH_4)_2SO_4$ was then added slowly with stirring to the supernatant to 65% saturation (0.153 g $[NH_4]_2SO_4$ per ml). Thirty minutes after the ammonium sulfate dissolved, the pH was adjusted with NaOH and the suspension was centrifuged for 45 min at 9,500 rpm (16,000 x g) in a JA-10 rotor. The resulting pellets (cytosolic 40-65% ammonium sulfate fraction) were quick-frozen in liquid nitrogen.

The pellets (nuclei) of the first centrifugation step were washed twice more by resuspension in TMSD and centrifugation at 2000 rpm (800 x g) in the J-6 centrifuge. Crude nuclei were then resuspended in TMSD to a final volume of 2000 ml and extracted with 0.32 M $(NH_4)_2SO_4$ by dropwise addition of 175 ml of saturated $(NH_4)_2SO_4$ with

gentle stirring (13). Thirty minutes after the addition of ammonium sulfate, the extract was centrifuged for 90 min at 9,500 rpm (16,000 x g) in a JA-10 rotor. Solid $(NH_4)_2SO_4$ was then added slowly with stirring to the resulting post-nuclear supernatant to 40% saturation (0.186 g $(NH_4)_2SO_4$ per ml). Thirty minutes after the ammonium sulfate dissolved, the pH was adjusted with NaOH and the suspension was centrifuged for 45 min at 9,500 rpm (16,000 x g) in a JA-10 rotor. The resulting pellet (nuclear 0-40% ammonium sulfate fraction) was quick frozen in liquid nitrogen and stored at –80°C. The nuclear 0-40% ammonium sulfate pellet was resuspended with buffer B (50 mM Tris-HCl pH 7.9, 0.1 mM EDTA, 1mM DTT, 10% (v/v) glycerol) and dialyzed against buffer B until the conductivity is equal to 0.1 M $(NH_4)_2SO_4$ and loaded on DEAE cellulose (DE52 from Whatman) at 15 mg protein per ml of packed resin that had been prepared according to the manufacturer's instructions. The flow through of this column contains TFIIH.

RNA polymerase II purification

RNA polymerase II was eluted from the above DE52 column with 0.5 M $(NH_4)_2SO_4$ step and dialyzed against buffer A (20 mM Hepes-NaOH pH 7.9, 1 mM EDTA, 1 mM DTT, 10% glycerol) until the conductivity indicated that ~0.05 M $(NH_4)_2SO_4$ had been reached. The fraction was then loaded onto a phosphocellulose (P11) column at 20 mg protein per ml packed resin. Flow through fractions were collected, and bound proteins were successively eluted with buffer A containing either 0.5 M KCl or 1 M KCl. RNA polymerase II was in the 0.5 M KCl eluate. It was precipitated with ammonium sulfate

and applied to a TSK SW4000 sizing column run in Buffer A + 0.4 M KCl at pH 7.5. The active fractions were then pooled, diluted with Buffer A to a conductivity of ~0.1 M KCl and applied to a TSK DEAE-5PW column, and eluted with a 10 column volume gradient of Buffer A + 1M KCl. Aliquots of the active fractions were frozen and stored at –80 °C for transcription assays.

TFIIH purification

The flow through fractions from the above DE52 column were applied to a phoshocellulose (P11) column and eluted as above. TFIIH was recovered in the 0.5 M KCl eluate. The 0.5 M KCl step was then diluted with buffer A + 2.0 M $(NH_4)_2SO_4$ and centrifuged for 10 min at 7000 rpm (7,500 x g) in a JA-14 rotor. The supernatant was applied at 1-2 packed column volumes per hr to a Phenyl Sepharose 6FF column (~15 mg protein per ml packed column bed volume) that was equilibrated in buffer A + 1M $(NH_4)_2SO_4$ and that has the dimensions: height = 2-3X diameter. TFIIH was eluted at the same flow rate with a 10X column volume linear gradient from 1 M to 0 M $(NH_4)_2SO_4$ in Buffer A. One third column volume fractions were collected. Active fractions, which eluted from Phenyl Sepharose at ~0.1 M $(NH_4)_2SO_4$, were dialyzed against Buffer C to a conductivity equivalent to that of buffer C containing 50 mM KCl and centrifuged for 20 min at 30,000 rpm (100,000 x g) in a Beckman 45 Ti rotor. The resulting supernatant was applied at 5 ml per min to a TSK DEAE-5PW column (21.5 x 150 mm) equilibrated in buffer C containing 50 mM KCl. TFIIH was eluted at the same flow rate with a 500 ml linear gradient from 50 mM to 300 mM KCl in buffer C. Ten ml fractions were

collected. The active fractions, which eluted from TSK DEAE-5PW at ~0.2 M KCl, were dialyzed against buffer D to a conductivity equivalent to that of Buffer D containig 0.1 M KCl and centrifuged for 20 min at 30,000 rpm (100,000 x g) in a 45 Ti rotor. The resulting supernatant was applied at 1 ml per min to a TSK SP-5PW column (7.5-75mm) equilibrated in buffer D containing 0.1 M KCl. TFIIH was eluted at the same flow rate with a 30 ml linear gradient from 0.1 to 0.4 M KCl in buffer D. One ml fractions were collected. At this stage TFIIH, which elutes from TSK SP-5PW at ~0.23 M KCl, was >90% pure.

Recombinant Yeast TBP

Recombinant yeast TBP was expressed as described previously (Schmidt et al., 1989; Conaway et al., 1991). 2.5 liters of *E. coli* strain N5151 which contains plasmid pASY2D encoding yeast TFIID, were grown in Luria-Bertoni broth in a 5-liter fermeter at 32°C to an O.D. of 1. An equal volume of media preheated to 65°C was added, and the cultures were maintained at 42°C for 1hr. Cells were spun and resuspended in 2.5 ml buffer Y [50 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 1mM DTT, 1mM PMSF, 10% glycerol]. Lysozyme was added to 1 mg/ml, and cells were incubated at 4°C for 15 min. The cells were dirupted by sonication, and the lysate was centrifuged at 10,000 x g for 20 min. The supernatant fraction was decanted and passed through 250 ml DEAE-cellulose (Whatman; DE-52) column equilibrated in buffer Y. The flow through was applied directly to a 50 ml heparin agarose column prequilibrated in buffer Y. The column was then washed with buffer Y containing 0.25 M KCl. TBP was eluted from the

column with a 0.4 M KCl step and concentrated with 0.4 g of $(NH_4)_2SO_4$/ml of fraction and then applied to an Ultrogel AcA 44 column. Fractions with activity were stored in small aliquots at –80°C.

Recombinant TFIIE

Recombinant TFIIE was expressed as described previously (Peterson et.al., 1991). The 34 kDa and 56 kDa subunits were expressed independently in *E. coli*. The cells were grown at 37°C in 400 ml of 2x YT medium, 0.4% glucose, 200 μg/ml ampicillin, to an $O.D._{600}$ of 0.5 and induced with 0.4 mM IPTG and grown for an additional 30 min at 30 °C. The cells were lysed by treatment with lysozyme and sonication in 0.3 M KCl buffer E [25 mM Hepes pH 7.6, 0.1 mM EDTA, 12.5 mM $MgCl_2$, 10% glycerol, 0.1% NP-40, 1 mM DTT, 0.1 mM PMSF, and 0.1 mM sodium metabisulfite]. The lysates were centrifuged to remove cell debris at 15,000 rpm for 10 min. The supernatant was loaded onto a DEAE-Sepharose column at 100 mM KCl. Both the 34 kDa and 56 kDa proteins were eluted from DEAE-Sepharose with a 0.3 M KCl step. The 34 kDa subunit was then dialyzed to 100 mM KCl and loaded onto S-Sepharose and eluted with a 0.3M KCl step. The 56 kDa subunit that eluted in the 0.3 M KCl step from DEAE-Sepharose was applied directly to Q-Sepharose equilibrated with buffer E plus 300 mM KCl and eluted with 0.5 M KCl step. The 56 kDa subunit was dialyzed against 1.4 M ammonium sulphate in buffer E which specifically precipitates the 56 kDa subunit. Roughly equimolar amounts of purifed 56K and 34K subunits were mixed and loaded on a gel filtration column. Fractions with TFIIE activity were frozen in small aliquots at –80°C.

Recombinant TFIIF

Recombinant TFIIF was expressed as described previously (Tan et. al., 1994). A 100 ml culture of *E. coli* strain JM109(DE3) was grown to an OD_{600}= 0.6 in S.O.B medium (2% Bacto tryptone, 0.5% NaCl, 2.5 mM KCl, pH 7.0, 2.5 mM $MgCl_2$ at 37°C. Cells were then infected with M13 carrying an IPTG inducible his-tagged RAP30 and RAP74 (not his-tagged) subunits of TFIIF at a multiplicity of infection of 10-20. After an additional 2.5 hours, the infected cells were induced with 0.4 mM IPTG and the culture was incubated for an additional 2.5 hours. Cells were harvested by centrifugation at 2000 x g for 10 min. at 4°C. The cell pellet was resuspended in 10 mL of ice-cold buffer containing 30 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 20% (wt/vol) sucrose and left on ice for 10 min. Cells were pelleted by centrifugation at 6000 x g for 10 min. and resuspended in 10 ml of cold H_20 and left on ice for an additional 30 min. Osmotically shocked cells were collected by centrifugation at 6000 x g for 10 min and resuspended in 7 ml of buffer F [20 mM Tris-HCl pH 7.9, 500 mM NaCl, 0.2 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 10 mM imidazole pH 7.9, 0.1 mg/ml lysozyme] and kept on ice for 30 min. After 1 cycle of freeze thaw, the cell lysate was clarified by centrifugation at 100,000 x g for 30 min at 4 C. The complex was then purified by nickel chromatography on Probond Metal-Binding Resin (Invitrogen). The supenatant was applied to a 1 ml ProBond column equilibrated with buffer F. The column was washed and then TFIIF was eluted with buffer F plus 300 mM imidazole, pH 7.9.

Recombinant TFIIB

Recombinant rat TFIIB was expressed as described previously (Tsuboi et.al., 1992). The rat TFIIB cDNA (plasmid pRTα) was expressed in *E. coli* strain BL21(DE3) using a T7-expression vector, pET-11a (Novagen). After induction with IPTG, the cells were disrupted by sonication and the supernatant was loaded onto a DEAE Sepharose column. The flow through fraction was applied onto a phosphocellulose column. TFIIB eluted in a 0.3 to 0.6M KCl step and was frozen in small aliquots.

Preparation of Elongin Subunits and Elongin Complexes. Individual recombinant histidine-tagged elongin A, B, and C subunits were expressed in *E. coli*, purified from guanidine hydrochloride-solubilized inclusion bodies by Ni^{+2}-nitrilotriacetic acid-agarose chromatography, and renatured alone or in combination as previously described (Aso et al., 1995). The reconstituted elongin ABC complex was further purified as described (Bradsher et al., 1993) by chromatography on consecutive TSK SP 5-PW and TSK phenyl 5-PW HPLC columns. The reconstituted elongin BC complex was purified as described (Takagi et al., 1996) by TSK DEAE 5-PW HPLC. The elongin A subunit was purifed as described (Aso et al., 1995) by TSK SP 5-PW HPLC.

Assay of Transcription. Preinitiation complexes were assembled at the AdML promoter at 28°C by a 45-60 min preincubation of 30 μl reaction mixtures containing 20

mM Hepes-NaOH (pH 7.9), 20 mM Tris-HCl (pH 7.9), 50 mM KCl, 4 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.5 mg/ml bovine serum albumin, 2% (w/v) polyvinyl alcohol, 3% (v/v) glycerol, 6 units of recombinant placental ribonuclease inhibitor, ~10 ng of DNA template fragment, ~5 ng of recombinant yeast TBP, ~10 ng of recombinant TFIIB, ~10 ng of recombinant TFIIF, ~20 ng of recombinant TFIIE, ~10 ng of TFIIH, and ~0.01 units of RNA polymerase II. Where indicated, ~20 ng elongin ABC or ~20 ng elongin A and ~10 ng elongin BC were included in transcription reactions. Transcription was initiated by addition of 4 µl of a solution containing the nucleotides indicated in the figure legends. Reactions were stopped by addition of an equal volume of 9.0 M urea containing 0.025% (w/v) bromophenol and 0.025% (w/v) xylene cyanol FF. Transcripts were analyzed by electrophoresis through polyacrylamide gels containing 25% acrylamide, 3% bis-acrylamide, 5.0 M urea, 89 mM Tris base, 89 mM boric acid, and 2 mM EDTA. Transcription was quantified using a Molecular Dynamics PhosphorImager.

Purification of Paused Elongation Complexes. Transcription reactions were scaled up as needed for each of the experiments shown in Figs. 3, 4, and 5. Transcription was initiated by addition of nucleotides to the final concentrations indicated in the figure legends. After 10 min at 28°C, 200 to 250 µl of each reaction mix was applied to a 3 ml Sephadex G-50 (Sigma catalog number G-50-150) spin column. Columns were then spun for 5 min at 1100 x *g* in a swinging bucket rotor to elute elongation complexes. 30 µl of eluant were used in each transcription reaction. The spin columns were prepared by

packing 3 ml of Sephadex G-50, pre-equilibrated in buffer containing 20 mM Hepes-NaOH (pH 7.9), 20 mM Tris-HCl (pH 7.9), 50 mM KCl, 4 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.5 mg/ml bovine serum albumin, and 3% (v/v) glycerol, into a 4 ml empty spin column (5 Prime–3 Prime, Inc.).

RESULTS

Differential Effects of Elongin on RNA Polymerase II Initiation and Early Elongation Complexes. To begin to characterize the requirements for interaction of elongin with the RNA polymerase II elongation complex, experiments were performed which compared the effects of elongin on transcription initiation and on subsequent elongation of short transcripts initiated by RNA polymerase II at the AdML core promoter in a transcription system reconstituted with purified RNA polymerase II, TFIIH from rat liver, and recombinant initiation factors TBP, TFIIB, TFIIE, and TFIIF. In these experiments, preinitiation complexes were assembled by preincubation of RNA polymerase II and initiation factors with a DNA fragment containing a G-less AdML promoter derivative designated Ad(G+15) (Fig. 1). The effect of elongin on transcription initiation was assessed by measuring the ability of elongin to stimulate the rate of synthesis of abortively initiated, dinucleotide-primed trinucleotide transcripts, in reactions containing [α-^{32}P]CTP, ATP, and the initiating dinucleotide CpA, which directs transcription initiation at position -1 relative to the *in vivo* AdML transcriptional start site

(Fig. 1). The abortive initiation assay has been widely used to measure synthesis of the first phosphodiester bond of nascent transcripts by both prokaryotic and eukaryotic RNA polymerases (Carpousis et al., 1980; McClure et al., 1978; Luse et al., 1987; Jiang et al., 1995; Jacob et al., 1991; Dvir et al., 1996). As shown previously, RNA polymerase II will utilize dinucleotides to prime synthesis of AdML promoter-specific transcripts; depending on the dinucleotide primer provided, initiation can occur over an approximately 9 basepair region centered on the normal AdML transcriptional start site (Samuels et al., 1984). If only a dinucleotide primer and the next nucleotide encoded by the template are provided as substrates for transcription initiation, RNA polymerase II will efficiently synthesize abortively initiated, trinucleotide transcripts.

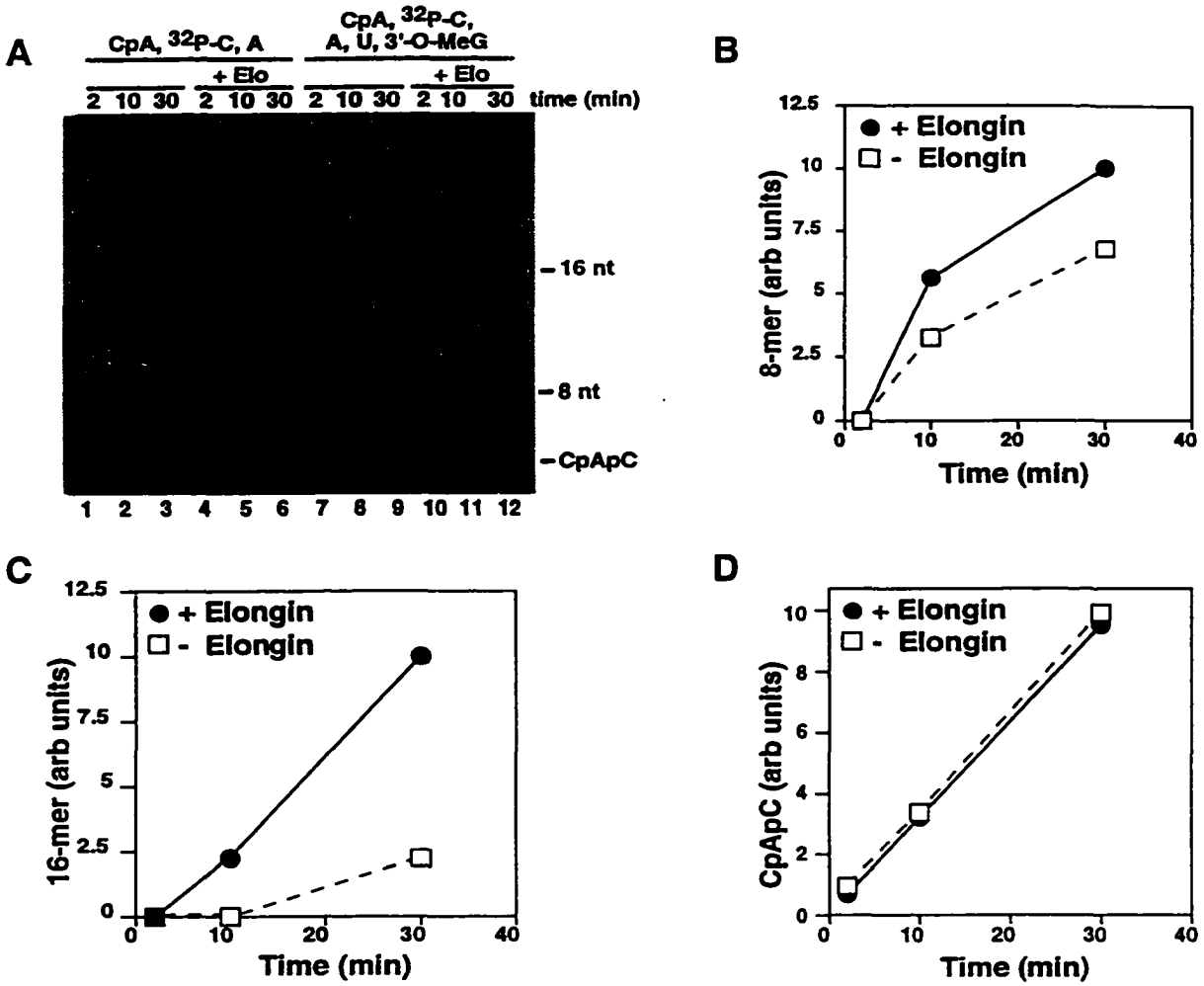
The effect of elongin on the rate of elongation by early RNA polymerase II elongation intermediates was assessed by measuring the ability of elongin to stimulate the rate of accumulation of 15 nucleotide long transcripts synthesized in the presence of [α-^{32}P]CTP, ATP, UTP, and the chain terminating nucleotide 3'-*O*-MeGTP, which prevents most transcription beyond the first G residue at position +15 in the Ad(G+15) transcript. Because we previously observed that elongin is capable of stimulating the rate of elongation by RNA polymerase II similarly at both low and near saturating ribonucleoside triphosphate concentrations (Bradsher et al., 1993), these reactions were carried out in the presence of low concentrations of UTP (400 nM) and [α-^{32}P]CTP (85 nM) to facilitate kinetic measurements.

As shown in Fig. 2, after 30 min of transcription, elongin induced an ~5-fold increase in accumulation of the 3'-*O*-MeG terminated 16 nucleotide long transcript and an ~1.5-fold increase in accumulation of an 8 nucleotide intermediate (panel A, lanes 7-12, and panels B and C). Elongin did not, however, measurably affect the rate of synthesis of abortively initiated trinucleotide transcripts (panel A, lanes 1-6, and panel D), suggesting that promoter-specific transcription initiation by RNA polymerase II on duplex DNA templates is relatively insensitive to elongin action.

The RNA Polymerase II Elongation Complex Becomes Susceptible to Stimulation by Elongin After Synthesizing 8-9 Nucleotide Transcripts. To determine when the RNA polymerase II elongation complex becomes susceptible to stimulation by elongin, the ability of elongin to stimulate the rate of incorporation of single nucleotides by early elongation intermediates paused at sites 14, 12, 10, 8, 6, and 4 basepairs downstream of the *in vivo* AdML transcriptional start site was investigated. To accomplish this, G-less AdML promoter derivatives were constructed that direct synthesis of transcripts with their first G-residues at 13, 11, 9, 7, and 5 basepairs downstream of the AdML transcriptional start site (Fig. 1).

In these experiments, preinitiation complexes were assembled by preincubation of RNA polymerase II and initiation factors with DNA fragments containing each of the G-less AdML promoter derivatives. Enriched populations of early RNA polymerase II elongation complexes paused at specific sites downstream of the AdML transcriptional

Fig. 2. Elongin Stimulates the Rate of Accumulation of Promoter-Specific 8 and 15 Nucleotide Transcripts but not of Abortive Trinucleotide Transcripts. Preinitiation complexes were assembled as described under *Experimental Procedures* at the Ad(G+15) promoter. *Panel A*, Synthesis of abortive trinucleotide transcripts was carried out at 28°C for the times indicated in the figure in the presence of 170 μM CpA, 5 μM ATP, and 15 μCi [α-^{32}P]CTP (lanes 1-6). Transcription was carried out for the times indicated in the figure in the presence of 170 μM CpA, 5 μM ATP, 0.1 μM UTP, 15 μCi [α-^{32}P]CTP, and 5 μM 3'-*O*-MeGTP (lanes 7-12). The transcription reactions in lanes 4-6 and 10-12 contained ~10 ng elongin. *Panels B and C*, Quantitation of 8 and 16 nucleotide long transcripts from transcription reactions in lanes 7-12 of panel A. *Panel D*, Quantitation of abortively initiated trinucleotide transcripts in lanes 1-6. 8-mer, 8 nucleotide transcript; 16-mer, 16 nucleotide transcript; arb units, arbitrary units determined by phosphorimager analysis; min, minutes; ^{32}P-C, [α-^{32}P]CTP; A, ATP; U, UTP; Elo, Elongin.


A
CpA, 32P-C, A
CpA, 32P-C, A, U, 3'-O-MeG
+ Elo
+ Elo
2 10 30 2 10 30 2 10 30 2 10 30 time (min)
16 nt
8 nt
CpApC
1 2 3 4 5 6 7 8 9 10 11 12
B
+ Elongin
- Elongin
8-mer (arb units)
Time (min)
C
+ Elongin
- Elongin
16-mer (arb units)
Time (min)
D
+ Elongin
- Elongin
CpApC (arb units)
Time (min)

start site were synthesized in the absence of GTP and in the presence of ATP, UTP, [α-^{32}P]CTP, and the initiating dinucleotide CpA or CpU. As shown in Fig. 1, CpA and CpU direct transcription initiation by RNA polymerase II at positions 1 and 3 basepairs upstream of the AdML transcriptional start site, respectively. Following transcription, RNA polymerase II elongation complexes paused one nucleotide before incorporating the first G residue in the transcript were purified by gel filtration to remove abortively initiated transcripts and unincorporated ribonucleotides.

Elongation complexes containing 15, 13, and 11 nucleotide long CpA-initiated transcripts were fully active following purification, since nearly all of these transcripts could be chased into longer RNAs following addition of high concentrations of the four ribonucleoside triphosphates (Figs. 3 and 4; lanes designated 4 NTPs). A fraction of elongation complexes containing 9 nucleotide CpA- or CpU- initiated transcripts and 7 nucleotide CpU-initiated transcripts was inactive after being purified and paused for up to 60 minutes. It is not known whether the 7 and 9 nucleotide transcripts that fail to chase are contained in arrested elongation complexes or whether they were terminated and released at some point after purification of the complexes. Elongation complexes containing 7 nucleotide long CpA-initiated transcripts were remarkably labile and became inactive if paused for only brief times (data not shown); these complexes could not, therefore, be used in assays for elongin activity.

To measure the ability of elongin to stimulate the rate of incorporation of single nucleotides by early RNA polymerase II elongation complexes, isolated complexes were

Fig. 3. Elongin Stimulates the Rate of Addition of Single Nucleotides to 9, 11, 13, and 15 Nucleotide Long Promoter-Specific Transcripts. Preinitiation complexes were assembled as described under *Experimental Procedures* at the Ad(G+15) (*Panel A*), Ad(G+13) (*Panel B*), Ad(G+11) (*Panel C*), and Ad(G+9) (*Panel D*) promoters. Elongation complexes paused just before incorporating the first G residue of transcripts were synthesized by a 10 min incubation at 28°C in the presence of 170 μM CpA, 5 μM ATP, 1 μM UTP, 0.8 μM nonradioactive CTP, and 10 μCi [α-^{32}P]CTP (*Panel A*) or 170 μM CpA, 5 μM ATP, 1 μM UTP, and 10 μCi [α-^{32}P]CTP (*Panels B-D*). Paused elongation complexes were purified by centrifugation through Sephadex G-50 spin columns as described under *Experimental Procedures*. Transcripts were chased at 28°C into 1 nucleotide longer RNAs by addition of 0.7 μM 3'-*O*-MeGTP (*Panel A*), 2.8 μM 3'-*O*-MeGTP (*Panels B and C*), or 1.4 μM 3'-*O*-MeGTP (*Panel D*). Quantitation of each set of reactions shown in the figure is shown in the graphs at the right. In this figure and in Figs. 4 and 5, *% converted* for each time point is equal to (n+1)/[n + (n+1)], where n is the level of transcript paused just before incorporating the first G residue in the transcript and (n+1) is the level of transcript 1 nucleotide longer. Purified elongation complexes in transcription reactions in lanes designated *4 NTPs* were incubated without 3'-*O*-MeGTP for 60 min (*Panels A and D*) or 120 min (*Panels B and C*) and then chased for 20 min with 50 μM ATP, 50 μM CTP, 50 μM UTP, and 50 μM GTP.

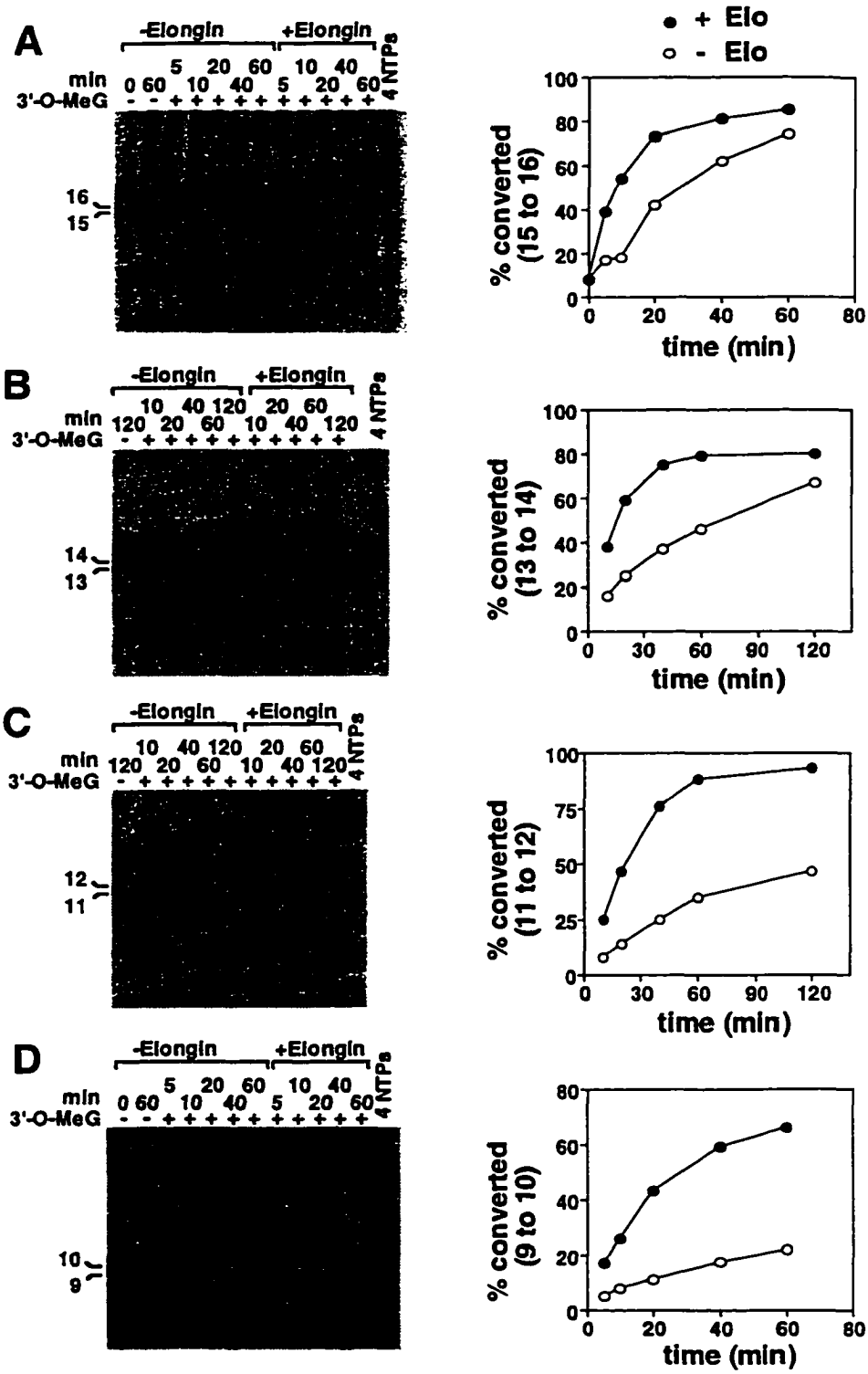
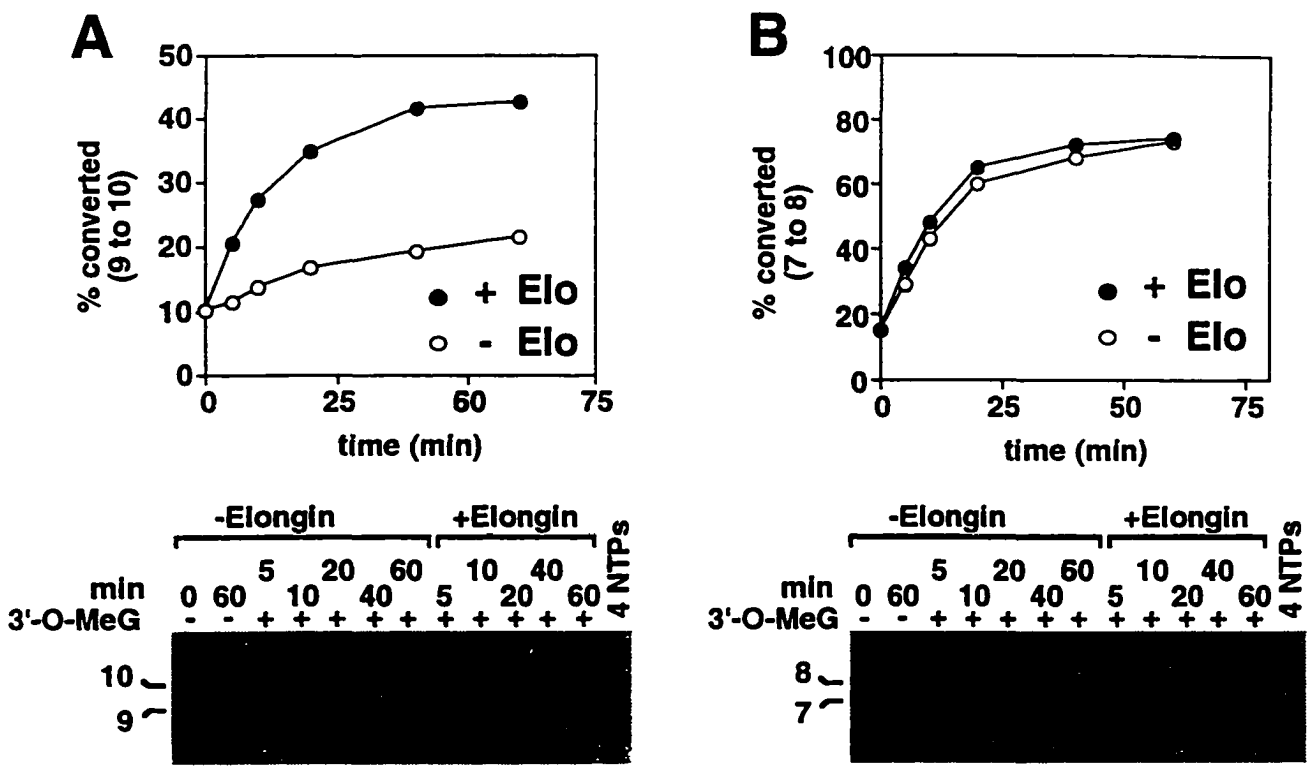

A
-Elongin
+Elongin
min
3'-O-MeG
4 NTPs
16
15
B
14
13
C
12
11
D
10
9
● + Elo
○ - Elo
% converted (15 to 16)
% converted (13 to 14)
% converted (11 to 12)
% converted (9 to 10)
time (min)

Fig. 4. RNA Polymerase II Elongation Complexes Containing 7 Nucleotide Long Transcripts are Relatively Insensitive to Stimulation by Elongin. Preinitiation complexes were assembled as described under *Experimental Procedures* at the Ad(G+7) (*Panel A*) and Ad(G+5) (*Panel B*) promoters. Elongation complexes paused just before incorporating the first G residue of transcripts were synthesized by a 10 min incubation at 28°C in the presence of 170 μM CpU, 5 μM ATP, 1 μM UTP, and 10 μCi [α-^{32}P]CTP. Paused elongation complexes were purified by centrifugation through Sephadex G-50 spin columns as described under *Experimental Procedures*. Transcripts were chased at 28°C into 1 nucleotide longer RNAs by addition of 1.4 μM 3'-*O*-MeGTP (*Panel A*) or 2.8 μM 3'-*O*-MeGTP (*Panel B*). Quantitation of each set of reactions shown in the figure is shown in the graphs at the top of each panel. Purified elongation complexes in transcription reactions in lanes designated *4 NTPs* were incubated without 3'-*O*-MeGTP for 60 min and then chased for 20 min with 50 μM ATP, 50 μM CTP, 50 μM UTP, and 50 μM GTP.


A
% converted (9 to 10)
50
40
30
20
10
0
0
25
60
75
time (min)
+ Elo
- Elo
-Elongin
+Elongin
min
0 60 5 10 20 40 60 5 10 20 40 60
3'-O-MeG
- - + + + + + + + + + +
4 NTPs
10
9
B
% converted (7 to 8)
100
80
60
40
20
0
0
25
50
75
time (min)
+ Elo
- Elo
-Elongin
+Elongin
min
0 60 5 10 20 40 60 5 10 20 40 60
3'-O-MeG
- - + + + + + + + + + +
4 NTPs
8
7

chased in the presence or absence of elongin by addition of 3'-*O*-MeGTP to reactions. At high concentrations of 3'-*O*-MeGTP, the reaction was complete within 1 or 2 minutes. Therefore low concentrations of 3'-*O*-MeGTP (1.4-2.8 μM) were used to allow accurate measurement of the rates of nucleotide addition. Each experiment was performed and quantitated 3 to 5 times; representative data is shown in Figs. 3 and 4.

The results can be summarized as follows. Elongin is capable of stimulating the rate of addition of a single 3'-*O*-MeG residue to CpA-initiated transcripts of 15, 13, 11, and 9 nucleotides. Elongin is also able to stimulate the rate of addition of a single 3'-*O*-MeG residue to a CpU-initiated transcript of 9 nucleotides (Fig. 4). In contrast, the rate of addition of 3'-*O*-MeG to a CpU-initiated transcript of 7 nucleotides was relatively insensitive to stimulation by elongin (Fig. 4). For elongation complexes containing 9 and 7 nucleotide transcripts, the fraction of active elongation complexes was somewhat variable from experiment to experiment; however, elongin consistently stimulated the rate of addition of 3'-*O*-MeG to 9 but not 7 nucleotide transcripts. Taken together, these results suggest that the early RNA polymerase II elongation complex undergoes a transition to a form that is more susceptible to stimulation by elongin once it has synthesized 8-9 nucleotide transcripts. More readthrough products are seen in the presence of elongin in some experiments. It is likely the increase in readthrough products is because elongin not only stimulates the rate of addition of 3'-*O*-MeG to transcripts but also stimulates the rate of addition of contaminating GTP in our nucleotide preparations; however, the possibility that elongin also stimulates misincorporation

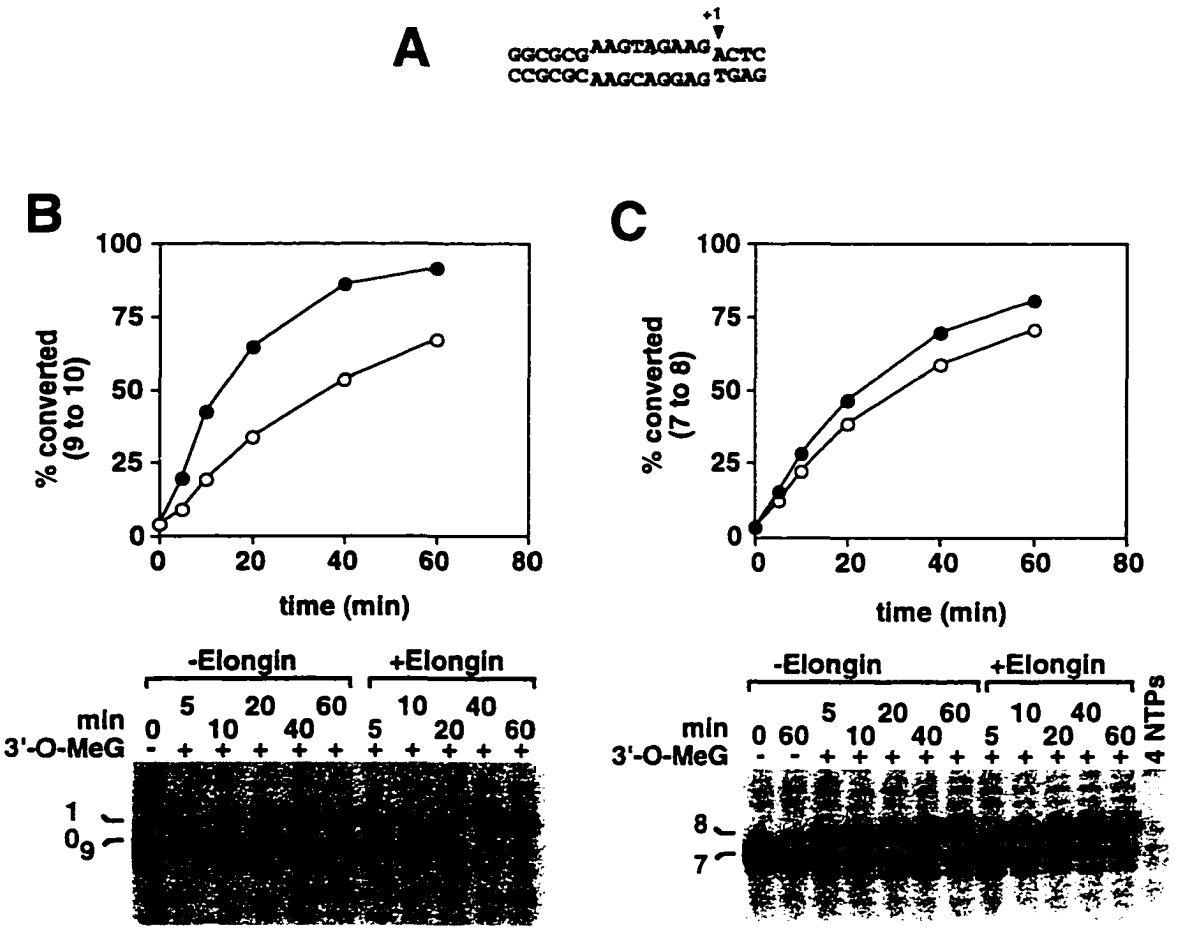
cannot be ruled out.

Synthesis of Abortive Trinucleotide Transcripts Can Be Stimulated by Elongin in the Absence of TFIIF. In a previous study, it was observed that elongin is capable of stimulating the rate of elongation by RNA polymerase II on oligo(dC)-tailed DNA templates in the absence of additional transcription factors (Bradsher et al., 1993), suggesting that elongin can stimulate elongation through a direct interaction with polymerase, the nascent transcript, the DNA template, or some combination of these components of the ternary elongation complex. As they emerge from the promoter, early RNA polymerase II elongation complexes undergo several dramatic structural changes, which could be prerequisites for functional interaction of elongin with the ternary complex. These changes include (i) appearance and growth of the nascent transcript, (ii) disassembly of the initiation complex and loss of initiation factors (Reines et al., 1991; Zawel et al., 1995), and perhaps (iii) alterations in the conformation of polymerase itself. In an effort to determine how the early RNA polymerase II elongation complex is converted to an elongin-activatable form, experiments were performed to determine whether susceptibility of early elongation intermediates to stimulation by elongin correlates with the presence of an extended (> 7 nucleotide) nascent transcript or with the absence of one or more initiation factors. To investigate these possibilities, premelted AdML promoter derivatives with non-complementary DNA between positions –9 and –1 upstream of the transcriptional start site were used (Fig. 5A). Synthesis of short

transcripts by RNA polymerase II from these promoters has been shown to depend on TBP and TFIIB but not on TFIIE and TFIIH (Dvir et al., 1997; Holstege et al., 1995; Timmers et al., 1994) and is reported to occur at substantially reduced but measurable levels in the absence of TFIIF (Pan et al., 1994).

To determine whether TFIIE and TFIIH are responsible for the relative insensitivity of early elongation intermediates to stimulation by elongin, preinitiation intermediates were assembled at the premelted Ad(G+7) and Ad(G+5) promoters in the presence of RNA polymerase II, TBP, TFIIB, and TFIIF but in the absence of TFIIE and TFIIH. RNA polymerase II elongation complexes that contained CpU-initiated transcripts of 7 or 9 nucleotides were synthesized in the absence of GTP. Following transcription, RNA polymerase II elongation complexes were purifed by gel filtration to remove abortively initiated transcripts and unincorporated ribonucleotides. Isolated elongation complexes were chased in the presence or absence of elongin by addition of 3'-*O*-MeGTP to transcription reactions. Similar to the results obtained in reactions containing TFIIE and TFIIH (Fig. 4), elongin is capable of stimulating the rate of addition of a single 3'-*O*-MeG residue to the CpU-initiated 9 nucleotide transcript (Fig. 5B), but has little effect on the rate of addition of a single 3'-*O*-MeG residue to the CpU-initiated 7 nucleotide transcript (Fig. 5C). These results suggest that the association of TFIIE and TFIIH with early elongation intermediates cannot account for their relative insensitivity to stimulation by elongin.

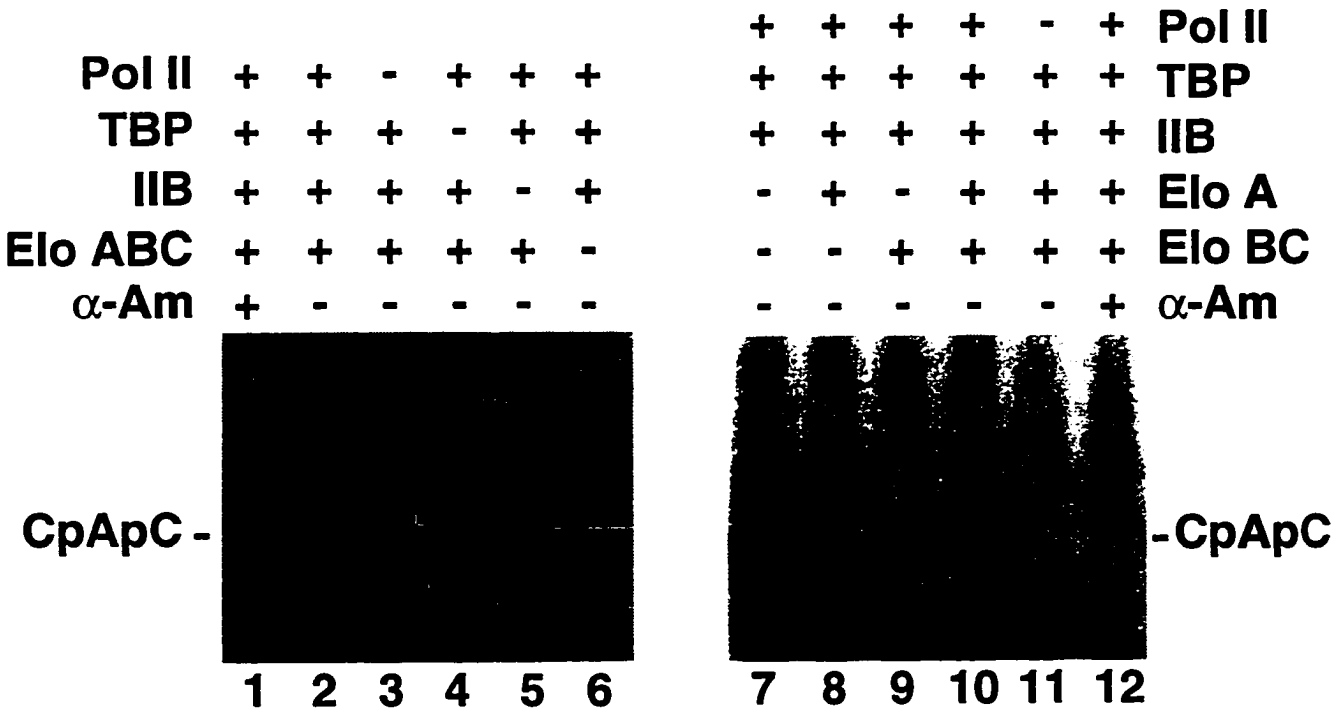
Fig. 5. TFIIE and TFIIH are not Responsible for the Relative Insensitivity of Early RNA Polymerase II Elongation Intermediates to Stimulation by Elongin. *Panel A*, DNA sequence surrounding the transcriptional start sites of the premelted Ad(G+7) and Ad(G+5) promoters. Preinitiation complexes were assembled as described under *Experimental Procedures* at the premelted Ad(G+7) (panel A) and Ad(G+5) (*Panel B*) promoters. Elongation complexes paused just before incorporating the first G residue of transcripts were synthesized by a 10 min incubation at 28°C in the presence of 170 μM CpU, 5 μM ATP, 1 μM UTP, and 10 μCi [α-^{32}P]CTP. Paused elongation complexes were purified by centrifugation through Sephadex G-50 spin columns as described under *Experimental Procedures*. Transcripts were chased at 28°C into 1 nucleotide longer RNAs by addition of 2.8 μM 3'-*O*-MeGTP. Quantitation of each set of reactions is shown in the graphs at the top of each panel. Purified elongation complexes in transcription reactions in lanes designated *4 NTPs* were incubated without 3'-*O*-MeGTP for 60 min and then chased for 20 min with 50 μM ATP, 50 μM CTP, 50 μM UTP, and 50 μM GTP.


A
+1
GGCGCG AAGTAGAAG ACTC
CCGCGC AAGCAGGAG TGAG
B
% converted (9 to 10)
time (min)
-Elongin
+Elongin
min
3'-O-MeG
10
9
C
% converted (7 to 8)
time (min)
-Elongin
+Elongin
min
3'-O-MeG
4 NTPs
8
7

In further experiments investigating the effects of TFIIF and transcript length on the susceptibility of early elongation intermediates to stimulation by elongin, we observed that elongin is capable of stimulating synthesis of abortively initiated trinucleotide transcripts by RNA polymerase II at premelted promoters in the presence of only TBP and TFIIB (Fig. 6). As shown in Fig. 6, trinucleotide synthesis at the premelted AdML promoter is inhibited by low concentrations of α-amanitin and depends strongly on TBP and TFIIB, arguing (i) that it is catalyzed by RNA polymerase II and (ii) that it is promoter-specific and not carried out by polymerase bound non-specifically at the single-stranded region of the template. A low level of background trinucleotide synthesis in reactions that lack TBP or TFIIB was observed. It is well established that RNA polymerase II can initiate transcription on single stranded DNA (Lewis et al., 1982). Furthermore, previous experiments have shown that polymerase synthesizes low levels of abortive trinucleotide transcripts on single stranded, non-promoter DNA when primed with a dinucleotide in the absence of initiation factors (data not shown). Because the reactions in Fig. 6 were performed with promoters containing single stranded DNA between -9 and -1, this phenomenon is likely to be responsible for the observed levels of background trinucleotide synthesis. Consistent with previous results indicating that both elongin A and the regulatory elongin BC complex are needed for maximal stimulation of elongation by RNA polymerase II (Bradsher et al., 1993; Aso et al., 1995; Garrett et al., 1994; Garrett et al., 1995), maximal stimulation of synthesis of abortive trinucleotide transcripts by elongin depends on both elongin A and the elongin BC complex. Thus,

Fig. 6. Elongin Increases the Rate of Synthesis of Abortive Trinucleotide Transcripts from a Premelted AdML Promoter in the Absence of TFIIF.

Preinitiation complexes were assembled essentially as described under *Experimental Procedures* on the premelted AdML oligonucleotide template in the absence of TFIIE and TFIIH and in the presence of the indicated combinations of RNA polymerase II and transcription factors. Transcription reactions were carried out for 30 min at 28°C in the presence of 170 μM CpA, 5 μM dATP, and 15 μCi [α-^{32}P]CTP, with or without 3 μg/ml α-amanitin. Pol II, RNA polymerase II; TBP, TATA binding protein; IIB, TFIIB; Elo A, Elongin A; Elo BC, Elongin BC; α-am, α-amanitin.

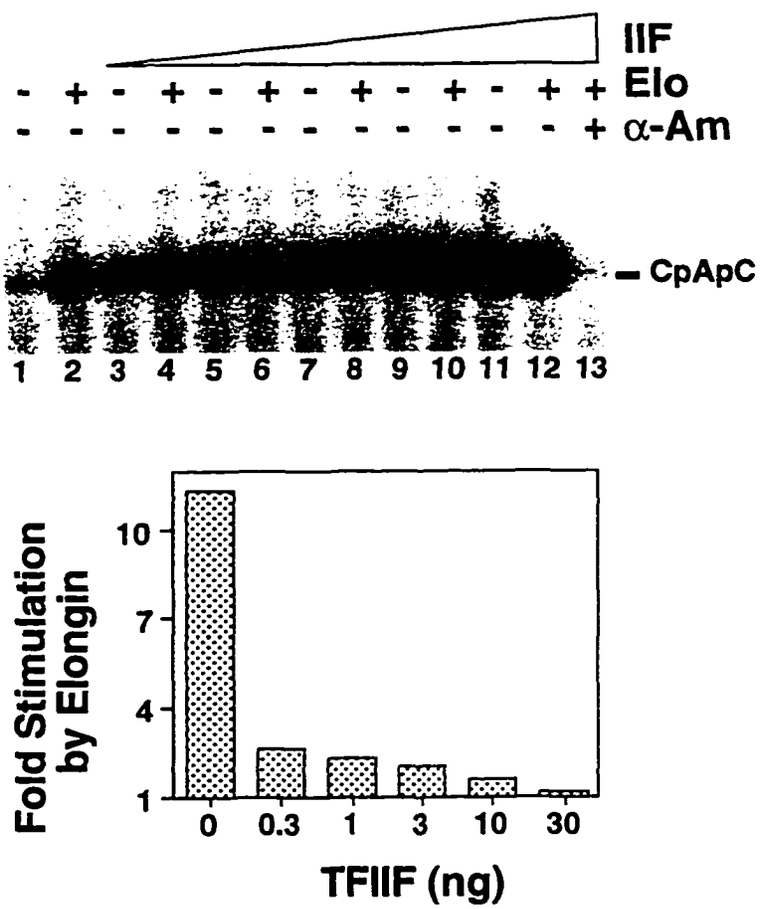

Pol II + + - + + +
TBP + + + - + +
IIB + + + + - +
Elo ABC + + + + + -
α-Am + - - - - -
CpApC -
1 2 3 4 5 6
+ + + + - + Pol II
+ + + + + + TBP
+ + + + + + IIB
- + - + + + Elo A
- - + + + + Elo BC
- - - - - + α-Am
-CpApC
7 8 9 10 11 12

although elongin does not detectably stimulate the rate of synthesis of abortive trinucleotide transcripts by RNA polymerase II in the complete initiation complex, elongin is capable of stimulating trinucleotide synthesis when TFIIF is absent. Similarly, we observe that elongin can stimulate trinucleotide synthesis in the absence of TFIIF when TFIIH and TFIIE are included in reaction mixtures (data not shown). Taken together, these results suggest that an extended nascent transcript is not essential for elongin function. In addition, they raise the possibility that TFIIF may be responsible for the observed insensitivity of the complete preinitiation complex and of very early elongation complexes to stimulation by elongin.

To investigate this possibility further, increasing concentrations of TFIIF were added back to abortive initiation reactions carried out in the presence of RNA polymerase II, TBP, and TFIIB (Fig. 7). Consistent with previous studies (Pan et al., 1994), TFIIF was capable of strongly stimulating the rate of synthesis of abortive trinucleotide transcripts under these conditions. Furthermore, as the concentration of TFIIF is increased in transcription reactions, the apparent stimulation of trinucleotide synthesis by elongin decreases dramatically from more than 10-fold in the absence of TFIIF to less than 1.5-fold in the presence of near-saturating TFIIF concentrations (Fig. 7, lower panel). These

results are consistent with the model that TFIIF, but not TBP or TFIIB, is responsible for the relative insensitivity of early elongation intermediates to stimulation by elongin.

Fig. 7. Addition of TFIIF Decreases the Fold Stimulation of the Rate of Synthesis of Abortive Trinucleotide Transcripts by Elongin. Preinitiation complexes were assembled essentially as described under *Experimental Procedures* at the premelted Ad(G+5) promoter in the presence or absence of 20 ng of elongin and in the presence of TBP, TFIIB, RNA polymerase II, and varying concentrations of TFIIF. Transcription reactions were carried out for 30 min at 28°C in the presence of 170 μM CpA, 5 μM dATP, and 15 μCi [α-^{32}P]CTP. Transcription reactions contained the following amounts of TFIIF: lanes 1 and 2, none; lanes 3 and 4, ~0.3 ng; lanes 5 and 6, ~1 ng; lanes 7 and 8, ~3 ng; lanes 9 and 10, ~10 ng; lanes 11 and 12, ~30 ng. The fold stimulation of the rate of synthesis of abortive trinucleotide transcripts by elongin is graphed in the lower panel and was calculated by dividing the level of CpApC synthesized in the presence of elongin by the level of CpApC synthesized in the absence of elongin. IIF, TFIIF; Elo, Elongin; α-am, 3 μg/ml α-amanitin.


IIF
Elo
α-Am
- + - + - + - + - + - + +
- - - - - - - - - - - - +
CpApC
1 2 3 4 5 6 7 8 9 10 11 12 13
Fold Stimulation by Elongin
10
7
4
1
0 0.3 1 3 10 30
TFIIF (ng)

DISCUSSION

Elongin is one of several transcription factors that are capable of increasing the overall rate of elongation by RNA polymerase II. In addition to elongin, these proteins include ELL (Shilatifard et al., 1996), ELL2 (Shilatifard et al., 1997), CSB (Selby et al., 1997), and TFIIF (Price et al., 1989; Bengal et al., 1991; Tan et al., 1994; Tan et al., 1995), which is also required for initiation by RNA polymerase II from most promoters. All of these proteins are capable of stimulating the rate of elongation by RNA polymerase II on oligo(dC)-tailed DNA templates in the absence of accessory transcription factors, suggesting that they stimulate elongation through a direct interaction with polymerase, the nascent transcript, the DNA template, or some combination of these components of the ternary elongation complex.

This study investigated the requirements for functional interaction of elongin with RNA polymerase II that has initiated transcription from a promoter in a basal transcription system reconstituted with the general initiation factors TBP, TFIIB, TFIIE, TFIIF, and TFIIH. The results of these studies indicated (i) that elongin is unable to stimulate the rate of synthesis of the first phosphodiester bond of nascent transcripts initiated in the presence of all five general initiation factors and (ii) that RNA polymerase II first becomes susceptible to maximal stimulation by elongin after synthesizing ~8-9 nucleotide transcripts. Taken together, these findings led to the hypothesis that, shortly

after initiating transcription, the early RNA polymerase II elongation complex undergoes conversion to an elongin-activatable form.

Efforts to understand the molecular basis of this conversion led us to consider two models. First, it was possible that elongin could not interact with RNA polymerase II until the enzyme had synthesized ~8-9 nucleotide transcripts, perhaps because elongin functions at least in part through a direct interaction with the nascent transcript or because elongin interacts optimally with those polymerases whose conformations have been altered by association with >~8-9 nucleotide transcripts. Second, it was possible that elongin could, in principle, stimulate the rate of elongation by RNA polymerase II that had synthesized transcripts shorter than ~8-9 nucleotides, but that its functional interaction with polymerase was either blocked or masked by association of one or more of the general initiation factors with early elongation intermediates. In particular, in light of evidence that TFIIE, TFIIF, and TFIIH are all capable of interacting with early RNA polymerase II elongation intermediates (Dvir et al., 1997; Zawel et al., 1995), functional interaction of elongin with RNA polymerase II might require prior dissociation of one or more of these general initiation factors from early elongation intermediates.

The findings are most consistent with the latter model and suggest that dissociation of TFIIF from the early RNA polymerase II elongation complex is required for maximal detectable stimulation of the rate of elongation by elongin. Elongin is capable of strongly stimulating the rate of synthesis of transcripts as short as 3 nucleotides from the premelted AdML promoter when TFIIE, TFIIF, and TFIIH are

absent from transcription reactions. This observation argues that elongin action requires neither the presence of an ~8-9 nucleotide nascent transcript nor a specific polymerase conformation dependent on prior synthesis of an ~8-9 nucleotide transcript. In addition, this observation suggests that elongin activity is unlikely to involve direct interactions with the nascent transcript, since evidence suggests that the 5'-end of growing transcripts do not emerge from RNA polymerase II until they are longer than ~8-9 nucleotides (Rice et al., 1991; Gu et al., 1996; Reeder et al., 1996). In this regard, elongin differs from several bacterial elongation factors, such as rho factor and nusG, which control the activity of bacterial RNA polymerase at least in part through direct interactions with the nascent transcript (Greenblatt et al., 1993; Platt et al., 1992).

Elongin activity is also not directly affected by TFIIE and TFIIH, since these factors do not affect the ability of elongin to stimulate the rate of synthesis of abortive trinucleotide transcripts from the premelted AdML promoter in the absence of TFIIF (R. Moreland, unpublished results) and, further, since they do not change the point at which early elongation complexes first become susceptible to stimulation by elongin when TFIIF is present. In contrast, inclusion of TFIIF in abortive transcription reactions on the premelted AdML template results in both a substantial increase in the rate of synthesis of abortive trinucleotide transcripts and a substantial decrease in the fold stimulation of abortive transcription by elongin. Based on these results, we propose that TFIIF is in large part responsible for the apparent inability of elongin to stimulate synthesis of the first phosphodiester bond of transcripts initiated in the presence of all five general

initiation factors, and they suggest that conversion of the RNA polymerase II elongation complex to a form that is susceptible to maximal stimulation by elongin requires dissociation of TFIIF from early elongation intermediates.

The results presented do not allow us to determine how interaction of TFIIF with early RNA polymerase elongation intermediates might render them relatively refractory to stimulation by elongin. Evidence from previous studies suggests that TFIIF remains stably associated with early RNA polymerase elongation intermediates until they have synthesized transcripts of ~10 nucleotides (Zawel et al., 1995). In contrast to its tight association with early transcription intermediates, however, TFIIF does not bind stably to RNA polymerase II elongation complexes that have synthesized longer transcripts (Price et al., 1989). Thus, one possibility is that TFIIF binds to early RNA polymerase II elongation intermediates and sterically prevents elongin from interacting with them. According to this model, elongin would gain access to RNA polymerase II only after TFIIF had dissociated from early elongation. Notably, such a mechanism controls the interaction of *E. coli* elongation factor nus A with *E. coli* RNA polymerase. The binding of Nus A and the initiation factor σ^{70} to *E. coli* RNA polymerase is mutually exclusive (Li and Greenblatt 1981); thus, Nus A is able to interact with polymerase only after dissociation of σ^{70} from early elongation complexes.

An alternative possibility is that elongin can physically interact with RNA polymerase II elongation complexes prior to synthesis of ~8-9 nucleotide transcripts, but does not alter the rate of transcript synthesis because polymerase activity is already

maximally stimulated by the elongation activity of TFIIF. TFIIF concentrations required for detectable stimulation of elongation are typically much greater than those used in our assays to saturate promoter-specific initiation (Tan et al., 1994), presumably because TFIIF does not bind stably to ternary elongation complexes. Because TFIIF is stably associated with very early elongation complexes, it is possible that it can stimulate elongation by these transcription intermediates and thereby mask elongin activity, even when present at the low concentration used in our assays.

Finally, our findings provide insight into the mechanism by which elongin stimulates elongation by RNA polymerase II. Substantial evidence argues that both prokaryotic and eukaryotic RNA polymerases can cycle between active and inactive conformations at each step of nucleotide addition during synthesis of a transcript (Rhodes et al., 1974; von Hippel et al., 1991; Erie et al., 1993; Krummel et al., 1992; Matsuzaki et al., 1994; Gu et al., 1995; Chamberlin et al., 1995). For each step of nucleotide addition, the average rate of nucleotide addition (the step time) often appears to be limited, not by the rate of phosphodiester bond formation (k_{cat}), but by the fraction of time polymerase spends in the inactive conformation. Sites of extended transcriptional pausing, then, would correspond to sites where polymerase has a high probability of falling into an inactive state or where, once inactivated, it does not readily cycle back into the active conformation.

An estimate of the maximum length of time needed for phosphodiester bond formation by RNA polymerase II is provided by measurements indicating that step times

at saturating nucleotide concentrations vary from milliseconds to seconds (Izban et al., 1992; Thummel et al., 1990; Ucker et al., 1984). Thus, our observation that elongin is capable of significantly decreasing the step time of RNA polymerase II, even when ribonucleoside triphosphate concentrations are extremely low and when a single nucleotide addition reaction requires 30 minutes to reach completion, suggests that elongin does not stimulate elongation simply by decreasing k_{cat}. Moreover, it is not likely that elongin has a major effect on the KM for ribonucleoside triphosphates, since we observed previously that elongin is capable of stimulating the rate of elongation by RNA polymerase II to a similar extent when reactions contain either low or near saturating concentrations of ribonucleoside triphosphates (Bradsher et al., 1993). Instead, as proposed previously for TFIIF (Gu et al., 1995; Buratowski et al., 1989), it seems more likely that elongin increases the rate of nucleotide addition by RNA polymerase II by increasing the fraction of polymerase molecules in an active conformation at any given time. Notably, evidence from previous studies suggests that elongin is capable of decreasing the frequency and/or duration of transient pausing by polymerase at many sites along the DNA template (Bradsher et al., 1993; Conaway et al., 1993). Elongin could accomplish this either by interacting specifically with polymerase in the inactive conformation and converting it to an active form or by interacting specifically with active polymerase and locking it into the active conformation until the incoming ribonucleoside triphosphate binds properly in the active site and a phosphodiester bond is formed. Although the nature of the structural transition(s) underlying conversion of RNA

polymerases from active to inactive conformations is presently unknown, studies of transcriptionally arrested polymerases have led to the proposal that at least some inactive polymerase conformations result from misalignment of the 3'-OH of the nascent transcript with catalytic site residues required for binding the incoming ribonucleoside triphosphate and catalyzing formation of the next phosphodiester bond (Gu et al., 1996; Gu et al., 1995; Chamberlin et al., 1995; Rudd et al., 1994; Izban et al., 1993; Borukhov et al., 1993; Reines et al., 1992). It was previously observed that elongin has the capacity to promote template-directed extension by RNA polymerase II of the 3'-OH termini of DNA molecules (Takagi et al., 1995). As suggested by Salzman and coworkers (Lavialle et al., 1982), the template-directed addition of ribonucleotides to 3'-OH termini of DNA by RNA polymerase II may occur in a reaction that mimics formation of the ternary elongation complex. In this case, the RNA polymerase II catalytic site for nucleotide addition would use the DNA 3'-OH as if it were the 3'-end of the nascent RNA transcript. In light of this model, elongin may promote extension of DNA molecules by RNA polymerase II by facilitating proper positioning of DNA 3'-OH termini with respect to the polymerase catalytic site and, by extension, might help to maintain polymerase in an active conformation by a similar mechanism at the RNA 3'-OH termini.

CHAPTER II

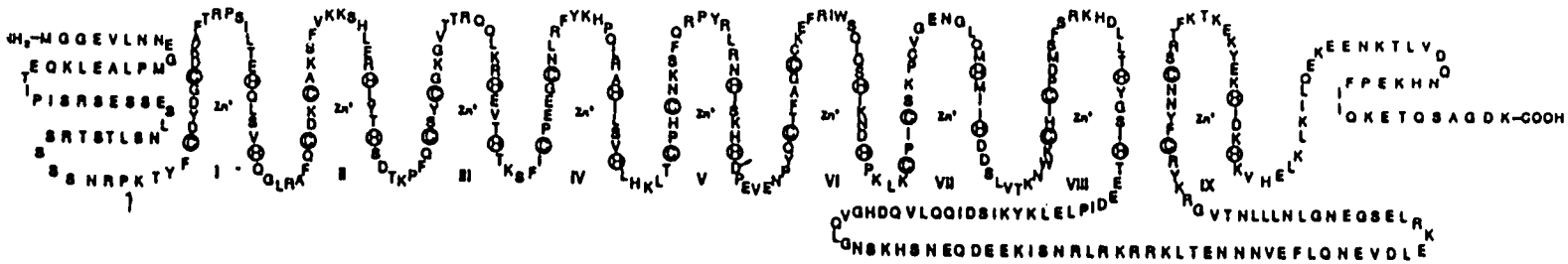
IDENTIFICATION OF CONSERVED REGIONS IN MAMMALIAN TRANSCRIPTION FACTOR IIIA

INTRODUCTION

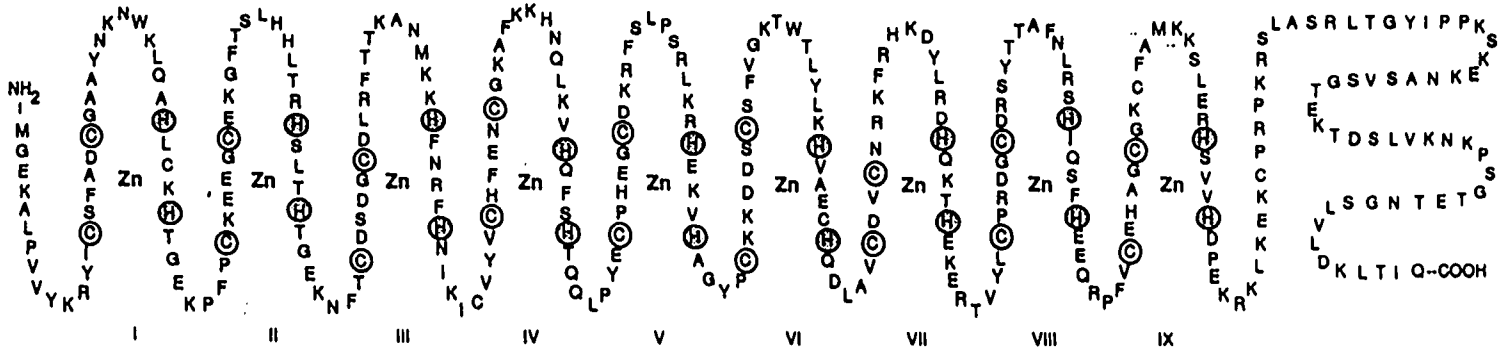
Transcription factor IIIA is the positive regulator of eukaryotic 5S ribosomal RNA synthesis. *Xenopus* TFIIIA (xTFIIIA) is a 39.9 kDa protein that is complexed with zinc and requires the metal for function (Hanas et al., 1983a). DNA sequence analysis shows *Xenopus* TFIIIA cDNA codes for a protein of 344 amino acids and contains nine zinc finger domains within the N-terminal three-quarters of the protein, Fig 8a. (Miller et.al., 1985; Ginsberg et.al., 1984). Each zinc finger is proposed to consist of approximately 30 amino acids in an imperfect repeat of (F,Y)-X-C-$X_{2\text{ or }4}$C-X_3-F-X_5-L-X_2-H-X_3-H-X_5 (Berg, 1986a; Berg, 1986b). Each finger contains approximately 30 amino acids and the primary structure reveals two Cys and two His residues invariantly present within the zinc fingers which are responsible for the coordination of the zinc atom. The Cys..Cys half of each finger folds into an antiparallel β-sheet and 12 amino acids on the His..His side comprise an α-helix (Pavletich and Pabo, 1991). The non-zinc finger C-terminal domain is not involved in DNA binding but instead contains the transcription activation domain (Vrana et al., 1988). This domain is responsible for the recruitment and interactions with other transcriptions factors involved in transcription of the 5S RNA gene. TFIIIA regulates transcription of the 5S RNA gene by binding to the internal

Figure 8. Primary sequence of *S. cerevisiae* TFIIIA and *Xenopus* TFIIIA folded according to the zince finger model. A. Yeast TFIIIA B. *Xenopus* TFIIIA

A



B



promoter from +40 to +80 such that the transcripiton activation domain is positioned near the start site for transcription.

TFIIIA makes up 10% of the soluble protein in a *Xenopus laevis* immature oocyte (Shastry et al., 1984). Because of its natural abundance, 2 x 10^7 molecules per 5S RNA gene in immature oocytes and fairly simple methods of purification, TFIIIA was the first eukaryotic transcription factor to be purified, cloned, and characterized (Hanas et al. 1983; Brown et al, 1985; Ginsberg et al., 1984; Miller et al., 1985). TFIIIA is capable of binding the 5S RNA gene as well as the 5S RNA itself. It exists in immature oocytes complexed to 5S RNA where it is believed to store the 5S RNA until ribosome synthesis begins later in oogenesis.

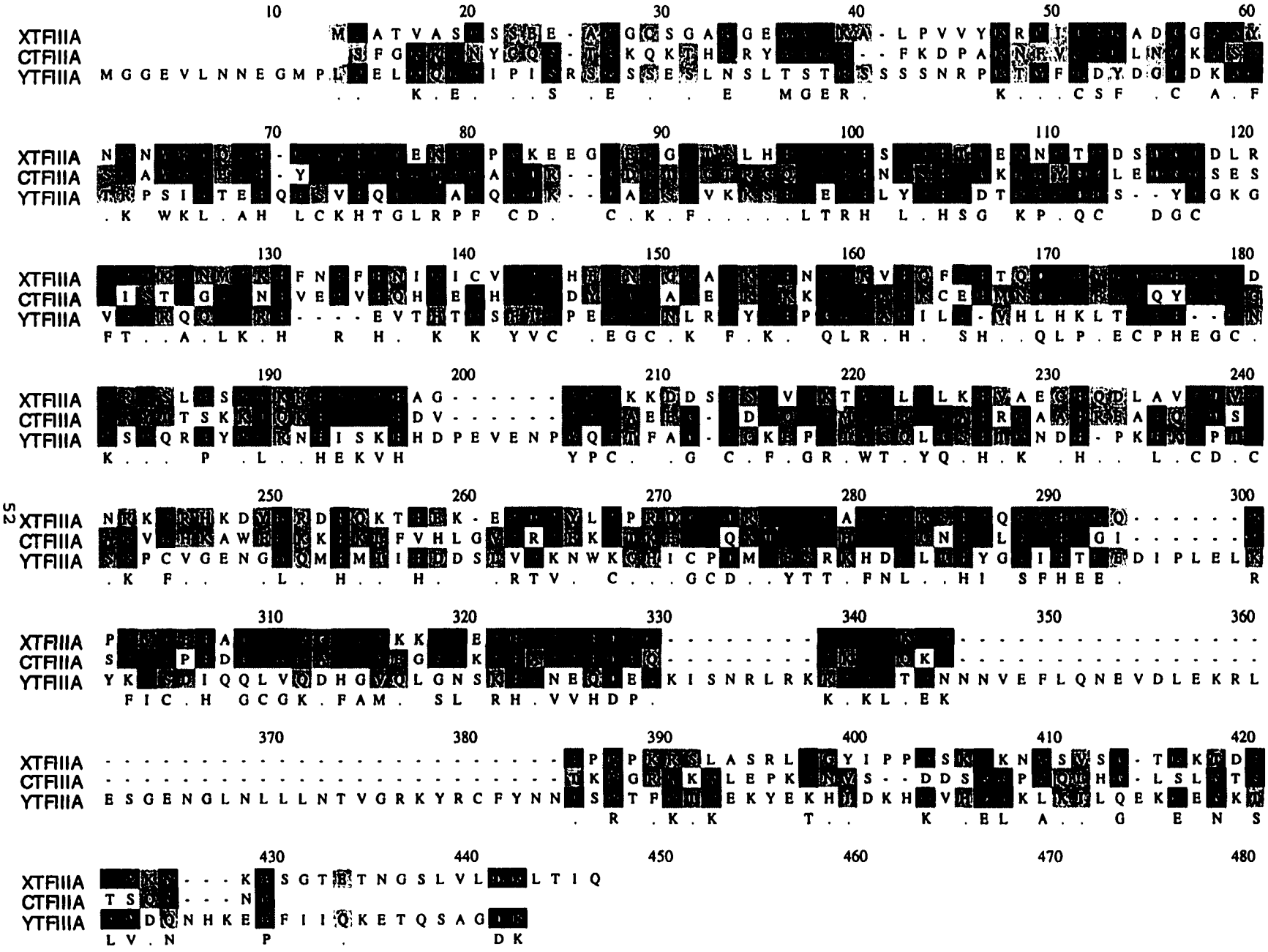
To date cDNAs have been isolated for TFIIIA from the following species, *Xenopus laevis*, *Xenopus borealis*, *Rana pipiens*, *Rana catesbeiana*, *Bufo americanus*, *Saccharomyces cerevisiae, Ictalurus punctatas* (catfish), and Human (Gaskins et.al, 1989; Gaskins et.al., 1992; Woychik et.al., 1992; Ogilvie et.al., 1997). The cloning of the many frog and toad cDNAs for TFIIIA has revealed considerable divergence. For example, the TFIIIA sequence identity between two different frog species, *Xenopus* and *Rana*, is only 63% yet the respective TFIIIA binding site in the 5S genes are 95% identical (Gaskins et al., 1989). One region of TFIIIA that has not diverged amongst frog species is a 15 amino acid domain in the C-terminal tail that was proposed to be the transcription activation domain (Gaskins et.al., 1992). On the basis of this conservation, this domain (KRSLASRLTGYIPPPK) was later shown to be essential for transcription activation,

using deletion and substitution mutants in a *Xenopus* transcription system (Mao and Darby, 1993). The activation domain in yeast is located between fingers VIII and IX and bears no resemblance to the activation domain in *Xenopus* (Fig. 8b) (Archambault et al., 1992, Woychik and Young, 1992, Milne and Segall, 1993). Yeast TFIIIA shares only 18% sequence identity with *Xenopus* TFIIIA and does not bind the *Xenopus* 5S gene. Furthermore the C-terminus of catfish TFIIIA shows very little homololgy to the *Xenopus* activation domain and the overall amino acid sequence identity between catfish and *Xenopus* is only 43%. The alignment of *Xenopus*, catfish, and yeast TFIIIAs are shown in Figure 9.

Human and *Xenopus* TFIIIA bind to and promote transcription of each other's 5S genes (Moorfield and Roeder, 1994, Waldschmidt et al., 1990). Human TFIIIA amino acid sequence is only 51% identical and 71% conserved to *Xenopus laevis* TFIIIA, but the C-terminal tail of human TFIIIA has a region KRSLAShLsGYIPPK (lower case letters are not identical) almost completely identical to the transcription activation domain in *Xenopus*.

Human TFIIIA is the only mammalian TFIIIA to have been sequenced. Some regions show very good conservation while others, like the N-terminus, show no homology at all to *Xenopus*. Another mammalian TFIIIA was needed to identify residues and domains that are conserved between amphibians and mammals. An effort was made to identify the mouse TFIIIA to determine which residues are conserved in mammals. By

Figure 9. Alignment of *Xenopus*, *S. cerevisiae*, and Catfish TFIIIAs. The shaded regions show identity and the bottom row is the consensus sequence. XTFIIIA is *Xenopus* TFIIIA, CTFIIIA is catfish TFIIIA, and YTFIIIA is yeast TFIIIA.


XTFIIIA
CTFIIIA
YTFIIIA
MGGEVLNNEGMPLELIPIRSSESLNSLTSTSSSSNRP
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
190
200
210
220
230
240
250
260
270
280
290
300
310
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330
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390
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410
420
430
440
450
460
470
480

doing so more could be learned about domains that are essential for the function of TFIIIA.

METHODS

cDNAs used for mouse TFIIIA. The polypeptide sequence of *Xenopus* TFIIIA was blasted (Altschul et.al., 1996) against the EST database in Genebank. Two expressed sequence tags (ESTs) from mouse with similarity to *Xenopus* TFIIIA were purchased from IMAGE Consortium. Accession number AA023081, clone id 455970 and AA020593 clone id 455490. Both clones came from a Soares mouse placenta library. 1st strand cDNA was primed with a NotI – Oligo (dT) primer [5' TGTTACCAATCTGAAGTGGGAGCGGCCGCGGAAATTTTTTTTTTTTTTTTTTT TTTTT 3']. Double-stranded cDNA was ligated to EcoRI adaptors 5'-d[AATTCGGCACGAGG]-3' and 3' (GCCGTGCTCC)p-5' (Pharmacia) and digested with NotI and cloned into the Not I and EcoRI sites of the modified pT7T3 vector (Pharmacia). The library went through one round of normalization and was constructed by Bento Soares and M. Fatima Bonaldo. Both clones were sequenced by primer walking.

RESULTS

The complete sequence and deduced amino acid sequence for mouse TFIIIA is shown in figure 10. The ESTs are different at their 5'ends because AA023081 is longer

Figure 10. Nucleotide and deduced amino acid sequences of mouse TFIIIA cDNA. The cDNA clone was obtained and analyzed as discussed in ***Materials and Methods.***

```
   1  GAATTCGGCACGGAAGTTCAAAGTCTCCGGGCGCCAGTGGCTTGCGTCCCGCGTTCCCGG
   1  E  F  G  T  E  V  Q  S  L  R  A  P  V  A  C  V  P  R  S  R

  61  GGACGGCACGTGGCTGCCCCGCGTCAGCCCGGGCCCGATCGTGTCGCCCTGGAGCCGCGG
  21  G  R  H  V  A  A  P  R  Q  P  G  P  D  R  V  A  L  E  P  R

 121  GTGTCAGTCGCGGAAGCGGTGTCGTCGCTGACCATCGCGGATGCGTTCGTCGGGGCCTGT
  41  V  S  V  A  E  A  V  S  S  L  T  I  A  D  A  F  V  G  A  C

 181  GAGGGCCCCGCGCCGCCGCGCCCCGCGCTGCCCAGCAGGTTCATCTGCTCCTTTCCCGAC
  61  E  G  P  A  P  P  R  P  A  L  P  S  R  F  I  C  S  F  P  D

  41  TGCAGCGCCAGTTACAACAAAGCCTGGAAGCTAGACGCGCACCTATGCAAACACACGGGG
  81  C  S  A  S  Y  N  K  A  W  K  L  D  A  H  L  C  K  H  T  G

 301  GAGAGGCCATTTGTTTGCGACTATGAGGGCTGTGGCAAGGCCTTCATCAGAGACTACCAT
 101  E  R  P  F  V  C  D  Y  E  G  C  G  K  A  F  I  R  D  Y  H

 361  CTGAGCCGGCATGTCCTGATTCACACCGGGGAAAAGCCGTTTGTTTGTGCAGATGATGGC
 121  L  S  R  H  V  L  I  H  T  G  E  K  P  F  V  C  A  D  D  G

 421  TGTAATCAGAAATTCAACACAAAATCAAACTTGAAGAAACACATTGAACGCAAACATGGA
 141  C  N  Q  K  F  N  T  K  S  N  L  K  K  H  I  E  R  K  H  G

 481  AACCCACAAAAACAGTATTTGTGCAGTTATGAGGGTTGCAAGAAGGCCTTTAAGAAGCAC
 161  N  P  Q  K  Q  Y  L  C  S  Y  E  G  C  K  K  A  F  K  K  H

 541  CAGCAGCTGAGAACCCATCAGTGCCAGCACACCAGCGAGCCGCTCTTCAGGTGTACCCAC
 181  Q  Q  L  R  T  H  Q  C  Q  H  T  S  E  P  L  F  R  C  T  H

 601  GAGGGATGCGGGAAGCACTTTGCCTCGCCCAGCAGGCTGAAACGGCATGGGAAAGTTCAC
 201  E  G  C  G  K  H  F  A  S  P  S  R  L  K  R  H  G  K  V  H

 661  GAGGGCTACCTGTGTCAAAAGGGATGTTCTTTCATGGGAAAAACGTGGACAGAGCTCCTG
 221  E  G  Y  L  C  Q  K  G  C  S  F  M  G  K  T  W  T  E  L  L
```

```
721  AAACACATGAGAGAAGCCCATAAAGAGGACATAACCTGCAATGTATGTCAGAGGATGTTC
241  K  H  M  R  E  A  H  K  E  D  I  T  C  N  V  C  Q  R  M  F

781  AAGCGCAGAGATTACCTTAAGCAGCACATGAAGACTCACGCCCCGGAAAGGGATGTGTAC
261  K  R  R  D  Y  L  K  Q  H  M  K  T  H  A  P  E  R  D  V  Y

841  CGCTGTCCGCGGCAAGGCTGCGGAAGAACCTACACAACCGTGTTCAACCTGCAGAGCCAC
281  R  C  P  R  Q  G  C  G  R  T  Y  T  T  V  F  N  L  Q  S  H

901  ATTCTCTCCTTCCACGAGGAAAAGCGCCCATTTGTGTGTGAGCACGCTGGCTGTGGCAAG
301  I  L  S  F  H  E  E  K  R  P  F  V  C  E  H  A  G  C  G  K

961  ACATTCGCAATGAAACAGAGTCTCATGAGGCACAGTGTCGTGCACGATCCCGACAAGAAG
321  T  F  A  M  K  Q  S  L  M  R  H  S  V  V  H  D  P  D  K  K

1021 AGGATGAAGCTCAAAGTAAGAGCCCCTCGGGAGAGACGCAGCTTGGCCTCTCGCCTCAGT
341  R  M  K  L  K  V  R  A  P  R  E  R  R  S  L  A  S  R  L  S

1081 GGGTACTTCCCTCCTAAGAGGAAACAAGAGCCCGACTACTCCTTGCCTAACGCCAGCGCA
361  G  Y  F  P  P  K  R  K  Q  E  P  D  Y  S  L  P  N  A  S  A

1141 GAGTCCAGCAGCAGCCCAGAGGCCCAGCTGCCCCCGCCAGCCGCCTTACTCACTGTCTGC
381  E  S  S  S  S  P  E  A  Q  L  P  P  P  A  A  L  L  T  V  C

1201 TAGGCGGGAAGACTTCAGGGCGGCCAGCAGAGGCTCCTGGCCTGCACTTTTCCTATTAAA
401  *

1261 GTCACTGACACACAAAAAAAAAAAAAAAAAAAAAAAAACTCTCGCGGCCGCAAGCTTATT

1321 CCCTTTAGT  1329
```

by 12 nucleotides. The seqeuence at the 5' ends is shown below with the EcoRI site in the adaptor in bold. The underlined sequence is homologous between the two clones.

AA023081 (RM108) is **GA ATT C**GG CAC GAG GGG GCC GCG TTC CCG GAA GTT CAA AGT CTC CGG GCG .....

AA020593 (RM109) is **GA ATT C**GG CAC GAG GCG GAA GTT CAA AGT CTC CGG GCG .....

The mouse TFIIIA cDNA was found to be 1300 nucleotides long. The open reading frame could code for a protein of 400 amino acids. The protein is likely not to be this long. The N-terminus of the protein could not be identified because there is not a methionine located anywhere upstream of the first highly conserved zinc finger. Mouse TFIIIA is 57% identical and 70% similar to *Xenopus* TFIIIA. More conservation is observed between mouse and human with 74% identity and 86% similarity. The alignment of Mouse TFIIIA, *Xenopus* TFIIIA, and Human TFIIIA is shown in figure 11.

DICUSSION

Evidence for mouse TFIIA

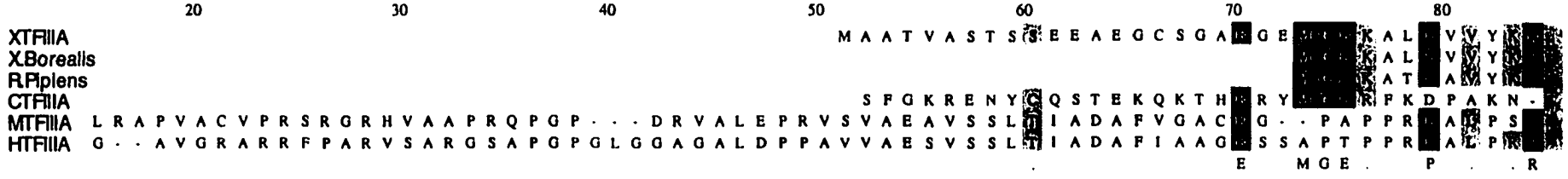
The evidence for identifying mouse TFIIIA is based solely on the deduced amino acid sequence comparison to *Xenopus* TFIIIA. The proposed mouse TFIIIA meets several important criteria to be considered TFIIIA (i) the overall identity is 57% and 70% similarity to *Xenopus* TFIIIA, (ii) nine contiguous zinc fingers exist before a C-terminal domain, (iii) the spacing between the fingers is identical to *Xenopus*, (iv) the C-terminal

Figure 11. Alignment of mouse, Xenopus, and human TFIIIAs. Shaded regions show identity. The bottom row is the consensus sequence. MTFIIIA is mouse TFIIIA, HTFIIIA is human TFIIIA, and XTFIIIA

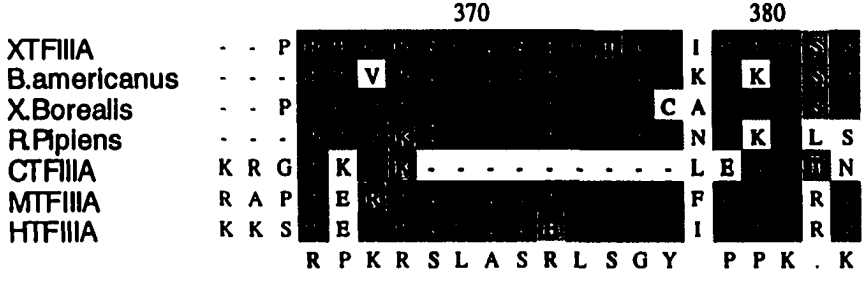

MTFIIIA
HTFIIIA
X TFIIIA
10 20 30 40 50 60
70 80 90 100 110 120
130 140 150 160 170 180
190 200 210 220 230 240
250 260 270 280 290 300
310 320 330 340 350 360
370 380 390 400 410 420

Figure 12. Comparison of the region required for transcription activation between *Xenopus*, mouse, and human. The shaded amino acids show identity. The bottom row gives the consensus sequence. A. alignment of the N-terminus of TFIIIA B. alignment of the transcription activation domain in the C-terminus. XTFIIIA, *Xenopus laevis* TFIIIA; X. Borealis, *Xenopus borealis*; B.americanus, *Bufo americanus*; R.pipiens, *Rana pipiens*; CTFIIIA, catfish TFIIIA; MTFIIIA, mouse TFIIIA; HTFIIIA, human TFIIIA

A


20
30
40
50
60
70
80
XTFIIIA
X.Borealis
R.Pipiens
CTFIIIA
MTFIIIA L R A P V A C V P R S R G R H V A A P R Q P G P - - - D R V A L E P R V S V A E A V S S L
HTFIIIA G - - A V G R A R R F P A R V S A R G S A P G P G L G G A G A L D P P A V V A E S V S S L

B


370
380
XTFIIIA
B.americanus
X.Borealis
R.Pipiens
CTFIIIA
MTFIIIA
HTFIIIA
R P K R S L A S R L S G Y P P K . K

domain contains a region of 15 amino acids that is conserved in all frog TFIIIAs (Fig. 12), (v) there is conservation of a tryptophan residue in the first zinc finger that has not been observed in other zinc finger proteins and most significantly (vi) the spacing between the zinc-binding residues is conserved with the exception of two less amino acids in finger VI between the coordinating cystidines. This is important because there are two families of zinc finger proteins that are distinguished by the number of amino acids between the zinc-coordinating Cys residues. The Kruppel-type family was named after the Drosophila developmental regulator and contains two invariant intervening residues throughout every zinc finger within the protein (Rosenberg et al., 1986). Another family represented by GLI, a regulatory zinc finger protein implicated in glioblastomas, contains four intervening residues (Kinzler et al., 1988). Excluding finger VI, the mouse TFIIIA has either 2, 4, or 5 amino acids between the zinc coordinating cystidines in complete agreement with *Xenopus* TFIIIA

The N-terminus is not conserved in mammalian TFIIIA

Two forms of TFIIIA are found in *Xenopus laevis* even though it was shown to contain a single gene for TFIIIA by southern blot analysis (Wolffe and Brown, 1988). This is because transcription of the TFIIIA gene in somatic cells initiates more than 200 bp upstream from the start site utilized in oocytes (Kim *et.* al, 1990). Somatic TFIIIA has an additional 22 amino acids at its N-terminus and begins with MAA. The oocyte form of TFIIIA begins with MGEK/R. The length of the N-terminus before the first zinc finger and the first three amino acids MGEK/R are conserved between catfish and

Xenopus. This MGEK/R is present in *Xenopus laevis*, *Xenopus borealis*, *Rana pipiens*, *Rana catesbeiana*, *Bufo americanus*, and, *Ictalurus punctatas* (catfish). It is not known if catfish has a somatic form or if there is a methionine 22 amino acids upstream as in *Xenopus*, but it is possible that catfish may have a somatic form of TFIIIA. Studies have shown that both forms are functionally indistinguishable *in vitro* in *Xenopus* (Kim *et* al., 1990). It is interesting that the mouse TFIIIA does not have conservation of the MGEK/R start site of translation observed in amphibians and catfish. Perhaps this oocyte form is not conserved in mouse or human because mammalian oogenesis is believed to be markedly different in amphibians and fish. Given the variation at the N-terminus of somatic and oocyte TFIIIA and the lack of conservation in mouse, this suggests the N-terminus of may not be involved in DNA binding or protein-protein interactions. This is supported by the ability of Xenopus TFIIIA to promote transcription in mammalian extracts (Moorfield and Roeder, 1994; Ogilvie et al., 1997).

The transcription activation domain is conserved in mammals

One of the goals of this study was to determine if the *Xenopus* transcription activation domain is conserved in mammals. It was found that this domain is nearly perfectly conserved (Fig. 12). This coincides with the observation that *Xenopus* and human TFIIIAs will function in transcription extracts from either species (Moorefield and Roeder, 1994). The conservation of this domain indicates that it is involved in the function of TFIIIA. This finding was very important to our studies involving TFIIIA because we then set out to identify mammalian proteins that interact with this domain

and this will be discussed further in Chapter 3.

CHAPTER III

IDENTIFICATION AND CLONING OF A TFIIIA INTERACTING PROTEIN

INTRODUCTION

Unlike RNA polymerase II transcribed genes, the genes encoded by RNA polymerase III are very short in length and show great diversity in promoter structures. Three types of genes are transcribed by RNA polymerase III (Pol III). The 5S RNA gene is referred to as type I. The promoter structure of this gene has been well characterized. Deletion analyses have demonstrated that only the internal sequences are essential to promote transcription (Sakonju et al., 1980; Bogenhagen et al., 1980). The internal control region (ICR) consists of three elements the A-block (+50 to +64), the intermediate element (+67 to +72), and the C-block (+80 to +97). Any disruption in these regions causes partial or complete loss of the abitlity to promote transcription. The bases separating these regions serve as spacers and do not influence transcription when different sequences are put in their place as long as the spacing is maintained (Pieler et al., 1987).

Type II genes include tRNA, VA, EBER, vRNA, 7SL, Alu, 4.5S, B1 and B2 genes. These genes have two internal domains, an A-block with the consensus sequence TGGCNNAGTGG and a B-block with the consensus sequence GGTTCGANNCC and

are generally separated by 30-40 bp. The A-blocks of types I and II are homologous and can substitute for one another in a *Xenopus* transcription system (Cilibreto et. al., 1983). A wide variety of separations between the A- and B-blocks are compatible with transcription in type II genes.

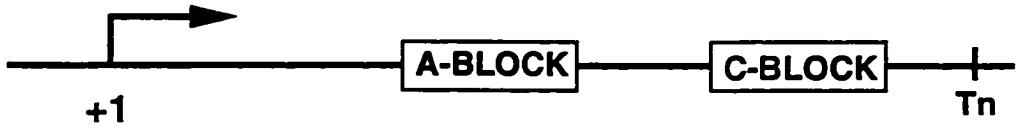
RNA polymerase III transcribed type III genes are represented by the U6 snRNA gene. Transcription of these genes in vertebrates is independent of intragenic elements and is solely determined by 5' flanking regions (Murphy et. al., 1987; Das, et.al., 1988). A schematic illustration of the three types of class III promoters is shown in figure 13. All known U6 promoters contain a TATA motif between –30 and –25 in *Xenopus*, mouse, and human. RNA polymerase III transcription of these genes is dependent on the TATA box and deletion of the TATA box results in Pol II transcription of the gene. Another promoter for U6 genes is the proximal sequence element (PSE) located between –66 and –46. Proximal element binding protein (PBP) binds to the PSE elements and promotes transcription of the type III genes. The composition of PBP is not known. The last promoter element in U6 genes is the distal sequence element (DSE) from –244 to –214. The factors that interact with the type III elements are not as well characterized as for the 5S RNA gene.

Three chromatographically separable human factors are required to reconstitute specific transcription of the 5S RNA gene. These general factors were designated TFIIIA, TFIIIB and TFIIIC, because they elute from phosphocellulose in steps in the PC-A (0.1M KCl flow through), PC-B (0.1-0.35M KCl), and PC-C (0.35-0.6M KCl)

Figure 13. Promoter structure of RNA polymerase III transcribed genes. A. The 5s RNA gene is an example of a type I promoter. It contains three functional domains shown by hatched regions. B. The tRNA genes are an example of type 2 promoters. The A box is functionally interchangeable between type I and type II genes. C. The U6 gene is an example of the type III genes.

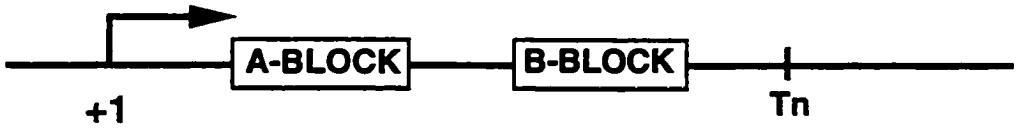
TYPE I PROMOTER

5S RNA Genes



TYPE II PROMOTER

e.g. tRNA Genes



TYPE III PROMOTER

e.g. Vertebrate U6 Genes



fractions, respectively (Segall et. al., 1980). The type II genes only require TFIIIB and TFIIIC for transcription. These fractions contain many proteins that are required for Pol III transcription.

The TATA-binding protein (TBP) is essential for transcription by pols I, II, and III. This was initially surprising because the pol III type I and type II genes do not have TATA boxes. This factor has been shown to exist as a complex in the TFIIIB fraction. One other member of this complex has been cloned in human and named TFIIIB 90 (Wang and Roeder, 1995). The N-terminal half of TFIIIB 90 shares homology with TFIIB which is a pol II factor that is known to associate with TBP. TBP exists in many different types of complexes, for example, TFIID is required for pol II transcription and it is believed there are ~10 TBP associated proteins (TAFs) in this complex. Another TBP-containing complex involved in pol I transcription is SLI. Both TFIID and SLI elute from phosphocellulose in the high salt PC-D (0.6-1.0M KCl) fraction. The current model is TBP achieves its polymerase specificity by associating with distinct sets of TAFs that are specific for each polymerase.

TFIIIC is one of the largest and most complex transcription factors identified to date. TFIIIC can be resolved into two components, TFIIIC1 and TFIIIC2, upon further fractionation. Highly purified TFIIIC2 will bind directly to the B-blocks in VA and tRNA genes and consists of 5 polypeptides, of 250, 110, 100, 80, and 60 kD. The 220 and 110 kD proteins have been cloned (L'Etoile et. al., 1994; Lagna G et. al., 1994; Sinn et. al., 1995). TFIIIC1 alone will not bind DNA, but it will extend the B-block footprint

generated by TFIIIC2 to include the A-block (Yoshinaga, et. al., 1987). It is not clear whether this reflects a direct contact between TFIIIC1 and DNA or a TFIIIC1-induced conformational change in TFIIIC2. Both components are required to reconstitute transcription of the 5S RNA gene. TFIIIC2 has been shown to interact directly with the 5S RNA gene through a low affinity interaction from −10 to +18. It is interesting that oniy a weak interaction was observed because purified TFIIIA and partially purified TFIIIC (containing both TFIIIC1 and TFIIIC2) results in the formation of a very stable preinitiation complex, whereas incubation with TFIIIA alone or TFIIIC alone does not (Hayes et. al., 1989; Setzer et.al., 1985; Bieker et. al., 1985; Carey et. al., 1986; Lassar et. al., 1983). This gives rise to a very interesting question involving preinitiation complex assembly, that is, what proteins are involved in formation of this stable complex and specifically which protein(s) interact with TFIIIA. TFIIIA was first identified 18 years ago and has become one of the most studied transcription factors. It's transcription activation domain as been defined (Chapter 2, Figure 12) but the transcriptional machinery that interacts with this domain is still a mystery. To identify proteins that interact with TFIIIA and more specifically the transcription activation domain in the C-terminal tail of TFIIIA the yeast 2-hybrid system was used (Fields and Song, 1989; Durfee et. al., 1993).

MATERIALS AND METHODS

Bacterial and Yeast Strains

E. coli TGI = supE hsdΔ5thi Δ(lac-proAB) F'[traD36 proAB$^+$ lacI^q lacZΔM15]

Yeast Y190 = Mata gal4 gal80 his3 trp1-901 ade2-101 ura3-52 leu2-3,-112 +

URA3::GAL→lacZ, LYS2::GAL→HIS3 cyhr

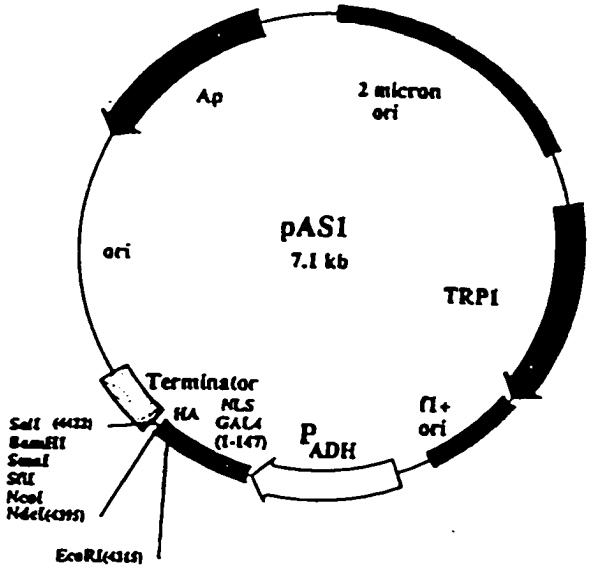
Construction of pAS1-TFIIIA fusions

The plasmid map of pAS1 is shown in figure 14. The last three zinc fingers and the C-terminal tail from amino acid 260 (QDLAVY...) to amino acid 366 of Xenopus TFIIIA was fused to the Gal4 binding domain in pAS1. To accomplish this an in-frame BamHI site was introduced at amino acid 260 and a downstream SalI at the 3' end by PCR. The same strategy was used for the yeast TFIIIA fusion to the Gal4 binding domain, the last 5 zinc fingers from amino acid 189 (DPEVEN...) to the C-terminus was cloned into pAS1.

Yeast transformations

A modification of the procedure originally reported by Schiestl and Gietz (1989) to transform yeast was used. The appropriate yeast were grown to ~1.5 x 10^7 cells/ml in 200 ml of the appropriate media and pelleted. The pellet was washed with 10 ml of sterile H_2O and spun. The pellet was resuspended in 1ml of 1xTE, LiAc (100ul 10xTE, 100ul 1M LiAc, 800 µl sterile H_2O. In a microcentrifuge tube, 200 µl of this cell suspension, 200 µg of carrier DNA and 2-4 µg of the appropriate plasmid were mixed. Next 1.2 ml

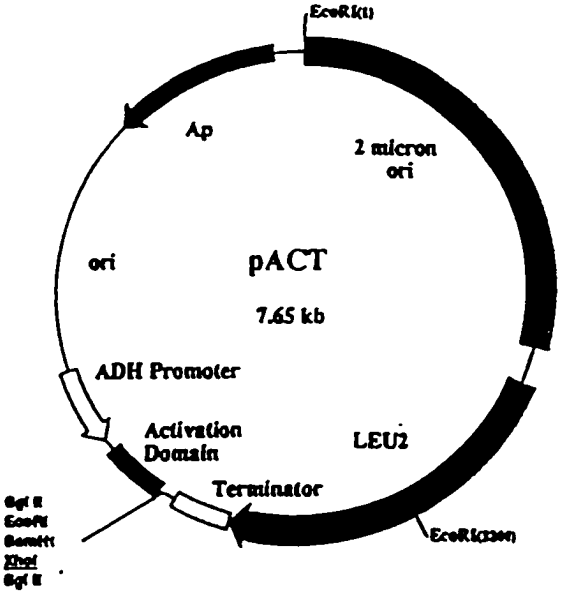
Figure 14. Plasmid map of pAS1 and pACT. PAS1 contains the sequences encoding Gal4 DNA-binding domain followed by a polylinker to facilitate construction of hybrids. pACT allows construction of cDNA libraries fused to sequences for the Gal4 transcriptional activation domain. Expression of the fusions in yeast is driven from the ADC1 promoter. Sequences for replication and selection in yeast and *E. coli* are indicated.



SfiI

CAT ATG GCC ATG GAG GCC CCG GGG ATC C

NdeI NcoI SmaI BamHI



BglII BamHI BglII

GAG ATC TGG AAT TCG GAT CCT CGA GAG ATC T

EcoRI XhoI

of 40% PEG solution (for 10ml; 8ml 50% PEG, 1.0 ml 10xTE, 1.0 ml 1M LiAc) was added mixed with a wide-bore pipet tip. The microcentrifuge tubes were sealed and shaken at 30°C in the bottom of a beaker at 300 rpm for 30 minutes. The tubes were then placed in a 42°C water bath for 15min and the cells were then pelleted in a microcentrifuge tube for 5 seconds. The cells were resuspended in sterile H_2O, using a wide-bore pipet and plated onto appropriate plates.

Library screening

Yeast strain Y190 was transformed to Trp prototrophy with pAS1-TFIIIA (Trp marker). A single colony was grown in SC – Trp medium and transformed with a rat brain cDNA library in pACT (Leu marker). This library was kindly provided by Ian Macara (Brondyk et.al, 1995). Aliquots were taken from each transformation mix and used to determine transformation efficiency by plating on SC media lacking tryptophan and leucine. The remaining transformation mix was plated on plates lacking tryptophan, leucine, and histidine but including 25 mM 3-aminotriazole, 3-AT (SIGMA, A8056) and incubated at 30C for 5 days. His^+ colonies were then screened for β-galactosidase activity using a filter lift assay. Approximately 12 x 10^6 transformants were analyzed in 4 independent screens and resulted in 4 identical positives which were designated TFIIIA interacting protein (TFIIIA-IP).

β-Galactosidase activity filter lift assay

Following 5 days of growth on plates with 3-AT and lacking His, Trp, and Leu, plates

with visible colonies were assayed for beta-galactosidase activity. Ahlstrom grade 95, 8.22 cm, circular filter paper with orientation marks were gently laid on the plates. The filter paper was then lifted and submerged in liquid nitrogen and then quickly thawed at room temperature. The filter paper was then laid (colony side up) in a clean petri dish that had a puddle of 1ml of Z buffer (8.5 g/L Na_2HPO_4, 5.5 g/L NaH_2PO_4, 0.75 g/L KCl, 0.25 g/L $MgSO_4$, 2.7 ml/L 2-mercaptoehtanol, final pH adjusted to 7.0) and 40 µl of X-Gal (20 mg/ml). The petri dishes were then placed in a 37°C incubator with the lid on to prevent the filter paper from drying out.

Recovery of library plasmid from yeast

Positive yeast colonies were grown in Leu$^-$ media in the presence of Trp. This causes loss of the bait plasmid (Trp selection marker) and maintenance of the library plasmid (Leu marker). A yeast cell pellet was obtained from a 5 ml culture and washed once with H_2O. The pellet was resuspended in 500 µl of GB lysis buffer (0.1M Tris-HCl pH 8.0, 50mM EDTA, 1% SDS). Glass beads beads (Sigma, #G1634 or 9268) were added until ~ 2 mm below the meniscus. The sample was then vortexed 2 times for 30s and briefly spun in a tabletop centrifuge to decrease the foam. The lysate was removed to a clean microcentrifuge tube and 25 µl of 5M NaCl was added. The lysate was then phenol/chloroform extracted and ETOH precipitated. The nucleic acid pellet was then resuspended in TE and used to electroporate *E. coli.* Large amounts of plasmid DNA was then propagated and characterized in *E. coli* using standard procedures for plasmid isolation and sequencing.

Preparation of yeast lysates for western blot analysis

A yeast cell pellet was collected from 10 ml of culture (~5 x 10^8 cells) and washed twice with 5 ml H_2O. The pellet was then resuspended in 200 µl of 16.6% prechilled trichloroacetic acid. Siliconized glass beads (Sigma, #G1634 or 9268). were added to the level of the supernatants and vortexed in the cold room 20 times for 15 second intervals, leaving samples on ice when not vortexing. The supernatant was removed to a clean tube with a yellow pipet tip that had the end crushed to prevent carryover of the glass beads. The glass beads were washed with 5% TCA to increase recovery. The supernatant was then spun in a microfuge for 10 min. at 4°C. The protein pellet was washed with 100% ETOH 2 times (MeOH can also be used). 100 µl of standard protein loading buffer for SDS PAGE was added and the pH was adjusted by the addition of 1M Tris-HCl pH 10.0 when necessary.

Western Blot Analysis

Transfer of proteins from a 12% SDS polyacrylamide gels was performed with an IDEA Scientific Trans-blot cell to 0.45 µM nitrocellulose (Nitrobind, MSI) in 0.096 M glycine/0.0125 M Tris base at 24 volts for 1.5 hours at room temperature. Following transfer, the filter was preblocked in 3% (w/v) nonfat dry milk in TBS (10 mM Tris-HCl, pH 7.4, 140 mM NaCl) for 20 minutes. The blot was then incubated with the appropriate antibody overnight in the 3% milk/TBS solution. The blot was incubated with secondary antibody the following day for 1hr. When the secondary antibody was conjugated to alkaline phosphatase the blot was developed with NBT (1µg/ml) and BCIP (0.5µg/ml) in

10 ml of TBS. Horse-radish-peroxidase conjugated secondary antibodies were developed with the reagents and directions supplied with the Renaissance Chemiluminescence Kit (NEN, #NEL102).

Expression constructs for partial rat TFIIIA-IP and full length human TFIIIA-IP

The junction of the rat pACT 2-hybrid positive designated TFIIIA-IP was seqeunced and gave the following sequence GAC(CTCGAG)CGGTGGGCATCGTCTCAGAA CTgcagctcaagctcaactccggcggctcctcagaagactccttcgtggagatcaggatgactgaaggagaggcggaaggg gcaatgaaggagatgaggaacagcgctggacccacaaggac and an open reading frame of RAVGIVSELQLKLNSGGSSEDSFVEIRMTEGEAEGAMKEMRNSAGPTRT. The partial rat cDNA insert was subcloned from pACT into the XhoI site of pBluescript. The orientation of the insert in pBluescript was determined by sequencing, and it was found the N-terminus of the cDNA was flanked by the BamHI site of pBluescript. The cDNA was then subcloned into TrcHis C (Invitrogen) by BamHI and KpnI for bacterial expression. The rat TFIIIA-IP sequence showed greater than 80% identity to a human EST from an infant brain library. The EST accession number is R36308, dBest ID is 206369, human clone Id is 38702. This same EST clone was also sequenced from the 3' end and given the accession number R49240, dBest ID 227281, human clone Id is 38702. Two years later another EST cDNA from human testis entered the database that gave us an additonal 1000 nucletotides and completed the full length cDNA. The 3' end of this clone was sequenced and given the accession number AA437216, dBest 1081510, human clone Id is 757436. The 5' end of this clone was given the

accession number AA442279, dBest ID 1086573, and the human clone Id was 757436. These two ESTs had a unique BbsI restriction site in the region where they overlapped. This restricition site was then used to splice the two ESTs together. This resulted in a cDNA of ~3 kb. This cDNA was then sequenced by the OMRF sequencing facility. The sequence is shown in Figure 18. We used the SacI and NotI sites to clone this full length cDNA into the expression vector pET28a(+). This expression construct was named RM121.

Expression

Recombinant Xenopus TFIIIA

2 liters of cells were grown in terrific broth until the O.D. was 0.6 and then the cells were induced by the addition of IPTG to 1mM. The cells were then incubated with shaking for 4 more hours and spun. The cells were resuspend in 80 ml buffer H (40mM Tris-HCl pH 7.4, 50 μM $Zn(SO)_4$, 1mM DTT, 10% glycerol, 100 mM KCl, 5M urea) and then broken at 1000 psi in a French pressure cell. The extract was then centrifuged at 30,000 rpm in a 45 Ti rotor. The supernatant was then loaded on a 20 ml column bed volume of SP Sepharose (Pharmacia cat # 17-0729-01) equilibrated in buffer H. Bound protein was step eluted in buffer A + 0.3M KCl, 0.6M KCl, and 1.0M KCl. TFIIIA elutes in the 0.3M KCl step and was then dialyzed against buffer H without urea and frozen in small aliquots.

Recombinant His-tagged TFIIIA-IP

The induction was the same as above but the cells were broken in buffer I (40mM Tris-

HCl pH 7.4, 0.5M NaCl, 10% glycerol, 20 mM imidazole pH 8.0) and centrifuged at 30,000 rpm in a 45 Ti rotor. The supernatant was then loaded on a 5 ml bed volume of ProBond Resin (Invitrogen). The bound protein was eluted with buffer I + 0.3M imidazole. The eluted protein was then dialyzed in buffer I without imidazole and frozen in small aliquots.

Production of affinity purified anti-TFIIIA-IP antibody

The partial rat cDNA was expressed as described above. Approximately 10 mg of purified protein was run on a 10% SDS PAGE gel and coomasie stained. The rat TFIIIA-IP band was excised and sent to Cocalico Biologicals (Reamstown, PA) as a gel slice for antibody production in rabbit. Thirty-four milligrams of soluble TFIIIA-IP protein in 15 ml was dialyzed against buffer G (0.2M $NaHCO_3$ pH 8.3, 0.5M NaCl). The protein was then coupled to a buffer G equilibrated 5 ml bed volume of Affi-Gel 10 resin (Bio-Rad) by light shaking overnight at 4°C. Unconjugated resin was blocked by the addition of 0.1 M ethanolamine for 30 minutes. The ethanolamine was washed from the resin and 8 ml (420 mg of total protein) of rabbit serum from a 2 month test bleed was then applied to the resin in buffer G. The column was washed until there was no detectable protein by bradford assay in the wash and the bound protein/antibody was eluted with 200 mM glycine at pH 2.5 with 0.1M NaCl. This step eluted 480 μg of protein from the column which was quickly neutralized with 1.0 M Tris-HCl pH 8.0. This fraction was tested by western blot analysis at a dilution of 1:5000 and gave a satisfactory signal. The composition of this fraction was not characterized further.

Immunoprecipitation of TFIIIA

An Immuno catcher kit (cat #C02-050) was obtained from CytoSignal and used according to their instructions. The following was added to two microfuge and allowed to bind for 30 minutes, 30 µl bed volume protein A/G resin (binding capacity of 30 mg/ml rabbit IgG) and 50 µl affinity purified antibody (3 µg of total protein). Unbound antibody was washed from the resin. Tube A then received ~10 µg of recombinant, human TFIIIA-IP protein; Tube B received buffer A. After 1.5 hours both tubes were washed 2x with buffer A to remove unbound human TFIIIA-IP protein in tube A. Then ~6 µg of recombinant *Xenopus* TFIIIA was added to both tubes and allowed to bind for 2 hours. Both tubes were then washed with buffer A + 0.3M KCl 6 times. The bound recombinant TFIIIA was eluted with SDS PAGE sample buffer and loaded on a 12% SDS PAGE gel.

RESULTS

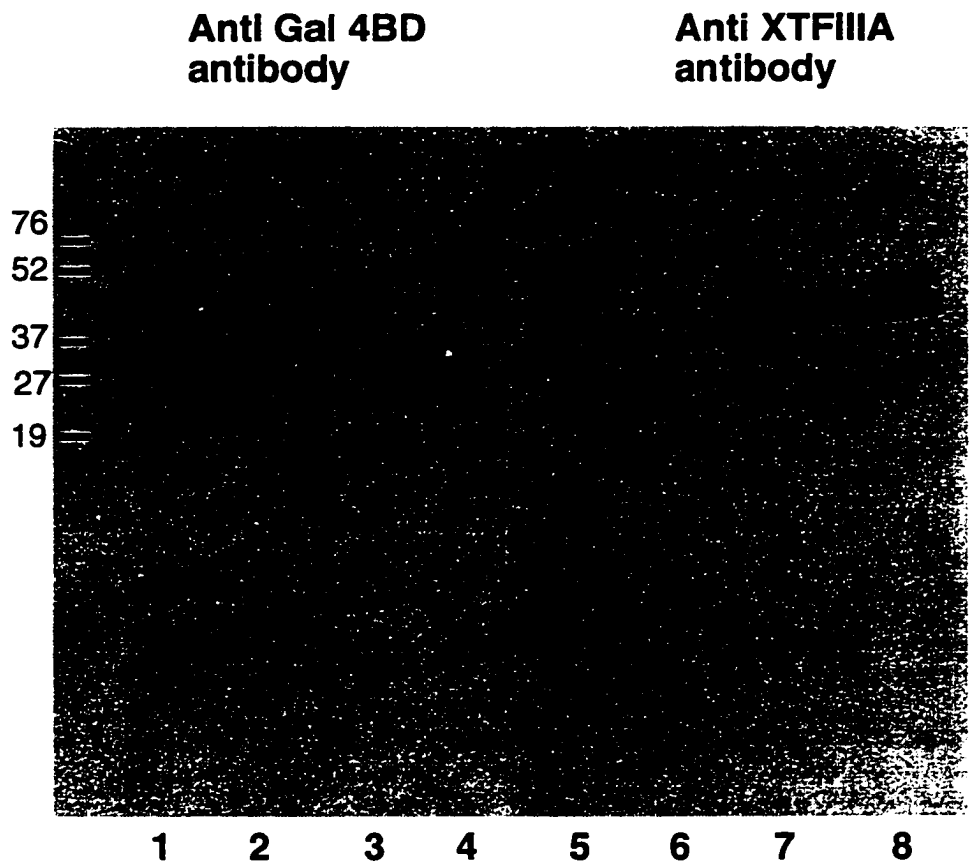
Experimental approach

Many transcription factors have been shown to be modular in function (Ma and Ptashne 1987). TFIIIA appears to have two modular domains—a DNA binding domain and transcription activation domain. The evidence for this is removal of the transcription activation domain does not affect the abilitiy of the protein to bind the 5S RNA gene. It seemed reasonable that the transcription activation domain alone may retain its ability to interact with the appropriate activators and thus be a good candidate for the yeast 2-hybrid system to identify proteins that interact with this region. The C-terminal tail with

fingers 7, 8, and 9 was fused to the Gal4 DNA binding domain as described in the Materials and Methods. Western blot analysis using antibody specific for *Xenopus* TFIIIA shows we were expressing the right size and immunologically correct species (Fig. 15). Yeast, carrying this plasmid, were then transformed with a second plasmid made up of a rat brain cDNA library fused to Gal4 activation domain. If a protein encoded by the rat brain cDNA library is able to interact with TFIIIA, the resulting complex will regain the ability to activate transcription from promoters containing Gal4-binding sites (upstream activating sequence from GAL1, UAS_G). The yeast strain Y190, which provides a dual selection system, was used. The strain carries two chromosomally located reporter genes whose expression is regulated by a Gal 4 binding site in their promoters. The reporters are the *E. coli* lacZ gene and the selectable HIS3 gene. HIS3 was chosen, because very low levels of its enzyme product [imidazole glycerol phosphate (IGP) dehydratase] are required for prototrophy. The gene for Gal80 has been deleted because it is a negative regulator of Gal4. Also the strain Y190 has had the Gal4 gene deleted so expression of the reporters is not due to endogenous Gal4. However, the HIS3 reporter is a little leaky in the absence of Gal4 and enough IGP dehydratase is made to allow growth without exogenous histidine. This was overcome by growing cells in the presence of 25 mM 3-aminotriazole (3-AT), a chemical inhibtor of IGP dehydratase.

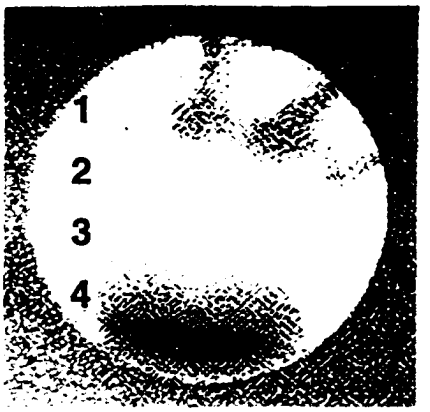
To screen for associated proteins, Y190 cells expressing the Gal4 binding domain fused to the C-terminus of *Xenopus* TFIIIA were transformed with a rat brain cDNA library fused to the Gal4 activation domain. Interacting hybrids are isolated by selecting

Figure 15. Western blot of yeast extracts from cells expressing TFIIIA fusions to the Gal4 DNA-binding domain. Lanes 1, 2, 7, and 8 are the C-terminal tail of Xenopus TFIIIA fused to the Gal4 DNA-binding domain as described in *Materials and Methods*. Lanes 3, 4, 5, and 6 is the C-terminal tail of yeast TFIIIA fused to the Gal4 DNA-binding domain. A rabbit polyclonal antibody to amino acids 1-147 of the Gal4 DNA-binding domain (Upstate, #06-262) was used in lanes 1-4. A rabbit polyclonal antibody to *Xenopus* TFIIIA was used in lanes 5-8. Molecular weight markers of 76, 52, 37, 27, and 19 kDa are shown to the left.

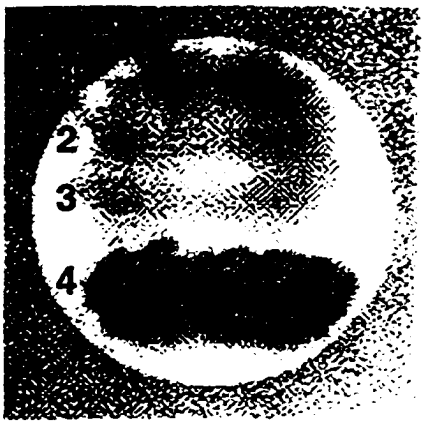

Anti Gal 4BD antibody
Anti XTFIIIA antibody
76
52
37
27
19
1
2
3
4
5
6
7
8

for His+ prototrophs and subsequently screened for β-galactosidase (β-gal) activity. This secondary screen eliminates His+ revertants and plasmids bearing the HIS3 gene from the rat cDNA library. Colonies that are His+ and blue are considered positives and analyzed further. After the initial identification of positive colonies the library plasmids were isolated and tested for false positives. First, the library plasmid was isolated retransformed and tested for β-gal activity alone (Materials and Methods). Figure 16 A. shows β-gal activity for 4 initially positive library plasmids without the presence of any Gal4 binding domain constructs. None of the library plasmids could stimulate the lacZ gene alone. Next the following fusion constructs laminin-Gal4, SNFI-Gal4, yeast TFIIIA-Gal4 were used as controls to test for specificity of the library plasmids for the original *Xenopus* TFIIIA used in the screen. Including yeast TFIIIA as a control was done because it has some similarity to *Xenopus* TFIIIA in regard to the zinc fingers but it cannot substitute for TFIIIA in transcription extracts. Therefore, it may help delineate any positives that are only recognizing the zinc finger structure rather than the acitvation domain. Figure 16 A,B,C,D,E show that while library plasmid #4 alone is not positive it will recognize any fusion to the Gal4 DNA binding domain. This positive was not pursued any further. The #1 library plasmid gave weak β-gal activity. This plasmid was seqeunced and the insert was found to be the gene for Signal Sequence Receptor α (SSRα). This plasmid may be pursued in the future. The #3 library plasmid showed strong β–gal activity only in the presence of the original *Xenopus* TFIIIA fusion to the Gal4 DNA binding domain. This plasmid was partially sequenced and a human homolog

Figure 16. Control lifts for detecting false positives in 2-hybrid screen. Four activation domain library plasmids that were originally identified as positives were isolated and tested for specificity to *Xenopus* TFIIIA with the β-gal filter assay. A. The four library plasmids in the absence of any Gal4 DNA-binding domain plasmid are not positive. B. Laminin fused to the Gal4 DNA-binding domain shows that plasmid #4 is a false positive. C. SNFI fused to the Gal4 DNA-binding domain . D. Yeast TFIIIA fused to the Gal4 DNA-binding domain as described in *Materials and Methods*. E. The original Gal4-TFIIIA construct used, *Xenopus* TFIIIA fused to the Gal4 DNA-binding domain, shows library plasmid #3 is specific for *Xenopus* TFIIIA.



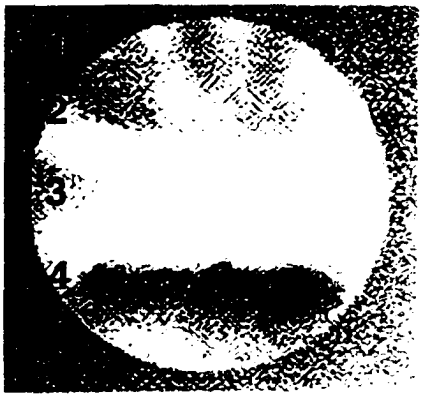
A



B Lamin



C SNFI



D Yeast TFIIIA



E Xenopus TFIIIA

with greater than 80% identity was observed in the EST databases. ESTs were used to clone the full length human gene (Fig. 18) (Materials and Methods). The identity between the original, partially sequenced rat gene identified in the screen and the human gene is shown in figure 17.

It is likely the full length human cDNA has been obtained because an open reading frame is not observed in the 5' or 3' ends of the transcript. This protein has been named TFIIIA interacting protein (TFIIIA-IP). The open reading frame codes for a protein of 617 amino acids with a molecular weight of 70,600 kDa. The most abundant amino acid is glutamate which comprises 14.7% of the protein. A search for motifs has shown a Cys2..His2 zinc finger from amino acids 423 to 437 and a leucine zipper from amino acids 143 to 164. Both of these domains are often associated with transcription factors (Klug, 1995a; Klug, 1995b).

Interaction of TFIIIA-IP with TFIIIA *in vitro*. To study a direct protein-protein interaction between TFIIIA and the TFIIIA-IP proteins, recombinant forms were expressed and purified as described in materials and methods. The interaction of the TFIIIA-IP protein with TFIIIA was shown *in vitro* immune absorption of both with an antibody to the TFIIIA-IP protein. Figure 19. Lane 1 is the rTFIIIA alone used in the experiment. The smaller band is believed to be a proteolytic fragment. It is interesting that a similar smaller band has been observed by others using the same expressin protocol (Del Rio, et.al., 1991). Del Rio et.al. believe the smaller band lacks the transcription

Figure 17. Protein comparison of rat and human TFIIIA-IP. The shaded regions show identity. The bottom row gives the consensus seqeunce.


human TFIIIA-IP
rat TFIIIA-IP

Figure 18. Nucleotide and deduced amino acid sequences of human TFIIIA-IP.

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   1 TTGGCCCTCGAGGCCAAGAATTCGGCACGAGGGCCAGGCCGCGCATCAGCCCCAGGCACC
  61 CCAGTCCCGGCTGCCCCCTCCGCCACCGCCGCCGCCCGCCGGCAGGTTCCCTGGTCAGCG
 121 TCCCATCCCGGTCGGGAGTTCTCTCCAGGCGGCACGATGCCGAGGAAACAGTGACCCTGA
 181 GCGAAGCCAAGCCGGGCGGCAGGTGTGGCTTTGATAGCTGGTGGTGCCACTTCCTGGCCT
 241 TGGATGAGCCGTACGCCTCTGTAAACCCAACTTCCTCACCTTTGAAACAGCTGCCTGGTT
 301 CAGCATTAATGAAGATTAGTCAGTGACAGGCCTGGTGTGCTGAGTCCGCACATAGAACTT
 361 CTGCAATGTCCCATCAACCTCTCAGCTGCCTCACTGAAAAGGAGGACAGCCCCAGTGAAA
   1     M  S  H  Q  P  L  S  C  L  T  E  K  E  D  S  P  S  E  S
 421 GCACAGGAAATGGACCCCCCCACCTGGCCCACCCAAACCTGGACACGTTTACCCCGGAGG
  20   T  G  N  G  P  P  H  L  A  H  P  N  L  D  T  F  T  P  E  E
 481 AGCTGCTGCAGCAGATGAAAGAGCTCCTGACCGAGAACCACCAGCTGAAAGAAGCCATGA
  40   L  L  Q  Q  M  K  E  L  L  T  E  N  H  Q  L  K  E  A  M  K
 541 AGCTAAATAATCAAGCCATGAAAGGGAGATTTGAGGAGCTTTCGGCCTGGACAGAGAAAC
  60   L  N  N  Q  A  M  K  G  R  F  E  E  L  S  A  W  T  E  K  Q
 601 AGAAGGAAGAACGCCAGTTTTTTTGAGATACAGAGCAAAGAAGCAAAAGAGCGTCTAATGG
  80   K  E  E  R  Q  F  F  E  I  Q  S  K  E  A  K  E  R  L  M  A
 661 CCTTGAGTCATGAGAATGAGAAATTGAAGGAAGAGCTTGGAAAACTAAAAGGGAAATCAG
 100   L  S  H  E  N  E  K  L  K  E  E  L  G  K  L  K  G  K  S  E
 721 AAAGGTCATCTGAGGACCCCACTGATGACTCCAGGCTTCCCAGGGCCGAAGCGGAGCAGG
 120   R  S  S  E  D  P  T  D  D  S  R  L  P  R  A  E  A  E  Q  E
 781 AAAAGGACCAGCTCAGGACCCAGGTGGTGAGGCTACAAGCAGAGAAGGCAGACCTGTTGG
 140   K  D  Q  L  R  T  Q  V  V  R  L  Q  A  E  K  A  D  L  L  G
 841 GCATCGTGTCTGAACTGCAGCTCAAGCTGAACTCCAGCGGCTCCTCAGAAGATTCCTTTG
 160   I  V  S  E  L  Q  L  K  L  N  S  S  G  S  S  E  D  S  F  V
 901 TTGAAATTAGGATGGCTGAAGGAGAAGCAGAAGGGTCAGTAAAAGAAATCAAGCATAGTC
 180   E  I  R  M  A  E  G  E  A  E  G  S  V  K  E  I  K  H  S  P
 961 CTGGGCCCACGAGAACAGTCTCCACTGGCACGGCATTGTCTAAATATAGGAGCAGATCTG
 200   G  P  T  R  T  V  S  T  G  T  A  L  S  K  Y  R  S  R  S  A
1021 CAGATGGGGCCAAGAATTACTTCGAACATGAGGAGTTAACTGTGAGCCAGCTCCTGCTGT
 220   D  G  A  K  N  Y  F  E  H  E  E  L  T  V  S  Q  L  L  L  C
1081 GCCTAAGGGAAGGGAATCAGAAGGTGGAGAGACTTGAAGTTGCACTCAAGGAGGCCAAAG
 240   L  R  E  G  N  Q  K  V  E  R  L  E  V  A  L  K  E  A  K  E
1141 AAAGAGTTTCAGATTTTGAAAAGAAAACAAGTAATCGTTCTGAGATTGAAACCCAGACAG
 260   R  V  S  D  F  E  K  K  T  S  N  R  S  E  I  E  T  Q  T  E
1201 AGGGGAGCACAGAGAAAGAGAATGATGAAGAGAAAGGCCCGGAGACTGTTGGAAGCGAAG
 280   G  S  T  E  K  E  N  D  E  E  K  G  P  E  T  V  G  S  E  V
1261 TGGAAGCACTGAACCTCCAGGTGACATCTCTGTTTAAGGAGCTTCAAGAGGCTCATACAA
 300   E  A  L  N  L  Q  V  T  S  L  F  K  E  L  Q  E  A  H  T  K
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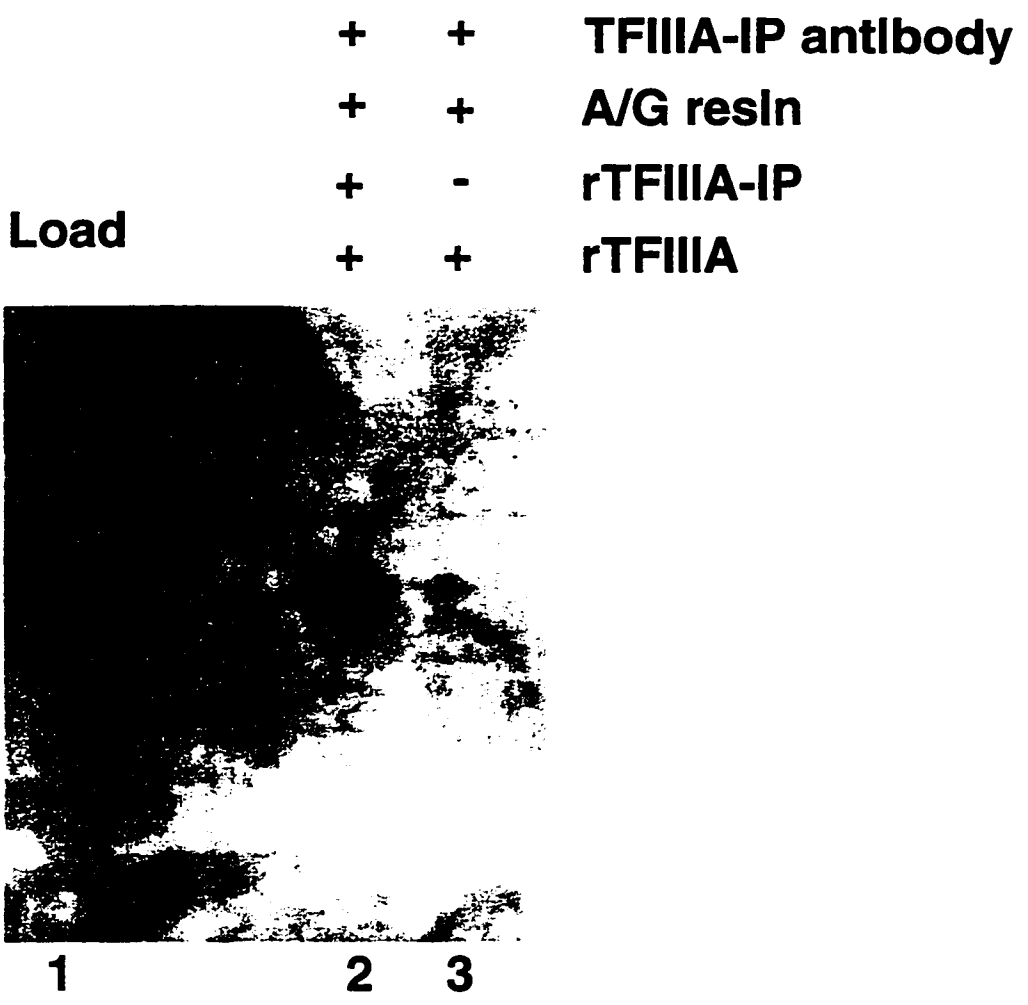
1321 AACTCAGCGAAGCTGAGCTAATGAAGAAGAGACTTCAAGAAAAGTGTCAGGCCCTTGAAA
 320   L  S  E  A  E  L  M  K  K  R  L  Q  E  K  C  Q  A  L  E  R
1381 GGAAAAATTCTGCAATTCCATCAGAGTTGAATGAAAAGCAAGAGCTTGTTTATACTAACA
 340   K  N  S  A  I  P  S  E  L  N  E  K  Q  E  L  V  Y  T  N  K
1441 AAAAGTTAGAGCTACAAGTGGAAAGCATGCTATCAGAAATCAAAATGGAACAGGCTAAAA
 360   K  L  E  L  Q  V  E  S  M  L  S  E  I  K  M  E  Q  A  K  T
1501 CAGAGGATGAAAAGTCCAAATTAACTGTGCTACAGATGACACACAACAAGCTTCTTCAAG
 380   E  D  E  K  S  K  L  T  V  L  Q  M  T  H  N  K  L  L  Q  E
1561 AACATAATAATGCATTGAAAACAATTGAGGAACTAACAAGAAAAGAGTCAGAAAAAGTGG
 400   H  N  N  A  L  K  T  I  E  E  L  T  R  K  E  S  E  K  V  D
1621 ACAGGGCAGTGCTGAAGGAACTGAGTGAAAAACTGGAACTGGCAGAGAAGGCTCTGGCTT
 420   R  A  V  L  K  E  L  S  E  K  L  E  L  A  E  K  A  L  A  S
1681 CCAAACAGCTGCAAATGGATGAAATGAAGCAAACCATTGCCAAGCAGGAAGAGGACCTGG
 440   K  Q  L  Q  M  D  E  M  K  Q  T  I  A  K  Q  E  E  D  L  E
1741 AAACCATGACCATCCTCAGGGCTCAGATGGAAGTTTACTGTTCTGATTTTCATGCTGAAA
 460   T  M  T  I  L  R  A  Q  M  E  V  Y  C  S  D  F  H  A  E  R
1801 GAGCAGCGAGAGAGAAAATTCATGAGGAAAAGGAGCAACTGGCATTGCAGCTGGCAGTTC
 480   A  A  R  E  K  I  H  E  E  K  E  Q  L  A  L  Q  L  A  V  L
1861 TGCTGAAAGAGAATGATGCTTTCGAAGACGGAGGCAGGCAGTCCTTGATGGAGATGCAGA
 500   L  K  E  N  D  A  F  E  D  G  G  R  Q  S  L  M  E  M  Q  S
1921 GTCGTCATGGGGCGAGAACAAGTGACTCTGACCAGCAGGCTTACCTTGTTCAAAGAGGAG
 520   R  H  G  A  R  T  S  D  S  D  Q  Q  A  Y  L  V  Q  R  G  A
1981 CTGAGGACAGGGACTGGCGGCAACAGCGGAATATTCCGATTCATTCCTGCCCCAAGTGTG
 540   E  D  R  D  W  R  Q  Q  R  N  I  P  I  H  S  C  P  K  C  G
2041 GAGAGGTTCTGCCTGACATAGACACGTTACAGATTCACGTGATGGATTGCAATCATTTAA
 560   E  V  L  P  D  I  D  T  L  Q  I  H  V  M  D  C  N  H  L  S
2101 GTGTTGATGTATCACCTCCCCAAAAACTGTTGGTAAATGTCAGATTTTTTCCTCCAAGAG
 580   V  D  V  S  P  P  Q  K  L  L  V  N  V  R  F  F  P  P  R  V
2161 TTGTGCTTTTGTGTTATTTGTTTTCACTCAAATATTTTGCCTCATTATTCTTGTTTTAAA
 600   V  L  L  C  Y  L  F  S  L  K  Y  F  A  S  L  F  L  F  *
2221 AGAAAGAAAACAGGCCGGGCACAGTGCTCATGCCTGTAATCCCAGCACTTTGGGAGGTCG
2281 AGGTGGGTGGATCACTTGGGGTCAGGGTTTGAGACCAGCCTGGCCAACATGGCGGAACCC
2341 TGTCTCTACCAAAATTACAAAAATTAGCCGAGCATGGTGCGCATGCCTGTAGTCGCAGCT
2401 ACTCGCGAGGTTGAGGCAGGAGAATTGCTTGAACCCAGGAAGTGGCAGTTGCAGTGAGCC
2461 GAGACGACACCACTGCACTCCAGCCTGGGGAGACAGAGGNGAAACTTTTGTTTNGAANNA
2521 AAGAANAGAANAAAANGGGANGGGAAGGANAAGGGATGGAAAGGAAAAAGAAAAGGCCCT
2581 TGTTCTACGTAAAAACNCCTTTTGGGGGGAGTTCCCATCAACTTCGCNAAGGTANAATCC
2641 TTTACCTACTAACTTNTTGGTGANAAAAAATCTNAAATATNTTTTTATGTTGGGGTNGAT
2701 GCAANCAANTTGCCCTTGGTCATGCANTTANCTACNNTGGTGNNTTTTTGTGACAAAAGT
```

2761 TTATTGTNTTTAGNTGGGAAAAAACCCTCCCTGGTTTAATTTTTAAAAACNTTTTNGGGG
2821 ATNCNAGAACCANTGAAGTGNCCNCANTTTGCGCTGGTTTAAGAANCTTTTTTAAAAACG
2881 AATTGTAGTTGTTTTTCTTCATTTAAAATGGATCTGTTGGAGGTTATGTGTGTATGTTGT
2941 AGTTTTATTGCAGCCACCAATAATTTTACCAAAGTTTTCACATAGGCAGTTAGCCTTTAC
3001 TTAATATCAAGACAAGTGAAAAAATATTGGCATCGATGAAACCGATAACATTGGCCTCAT
3061 TGGATTTCTTTACCCATTCACAGTGTAAAAAGTTACCTTCATGCTTTCATTGACCTGCAG
3121 GCCTGTGGGCTTGTACAGTAGATAATTAATTTCTAAAAAGAACAGCTGCCTATTTTCTTC
3181 CTAGGTTAGGTTATATCTTCATAATCACAAGAATTAGTGATGGCAAAATAAAATTTTGCT
3241 TATGAATCTTTTACATTGTTTATATATGATTAATATCATCATATATATTTTCTGTATTAA
3301 GCTCATTTGGCTTCATTTAAGCTGTATACTTAGTCATATATCTTTCATTAGTTCTATGGA
3361 TATGAGCAGATCCCTTTACTGGAGCCCAGTATGTGCTGTGTGAGTTAGAAGTCATTCTTG
3421 CTGAGAAGGTGAATAGGTAGGGATTTGCCTTGTTTTGTAAGTCTACAATTTGCCAAGAGT
3481 AAATAACACTGGACCAGCTGTAAAAGTAAACAGTGTGTTTATGCATTGAGATACTAAAGC
3541 ATTTAAGAAAAAATTAAAAGATCTCTTTTGTTTAAAAAAAAAAAAAAAAAAATTCCTGCGG
3601 CCGCAAGCTTATTCCCTTT

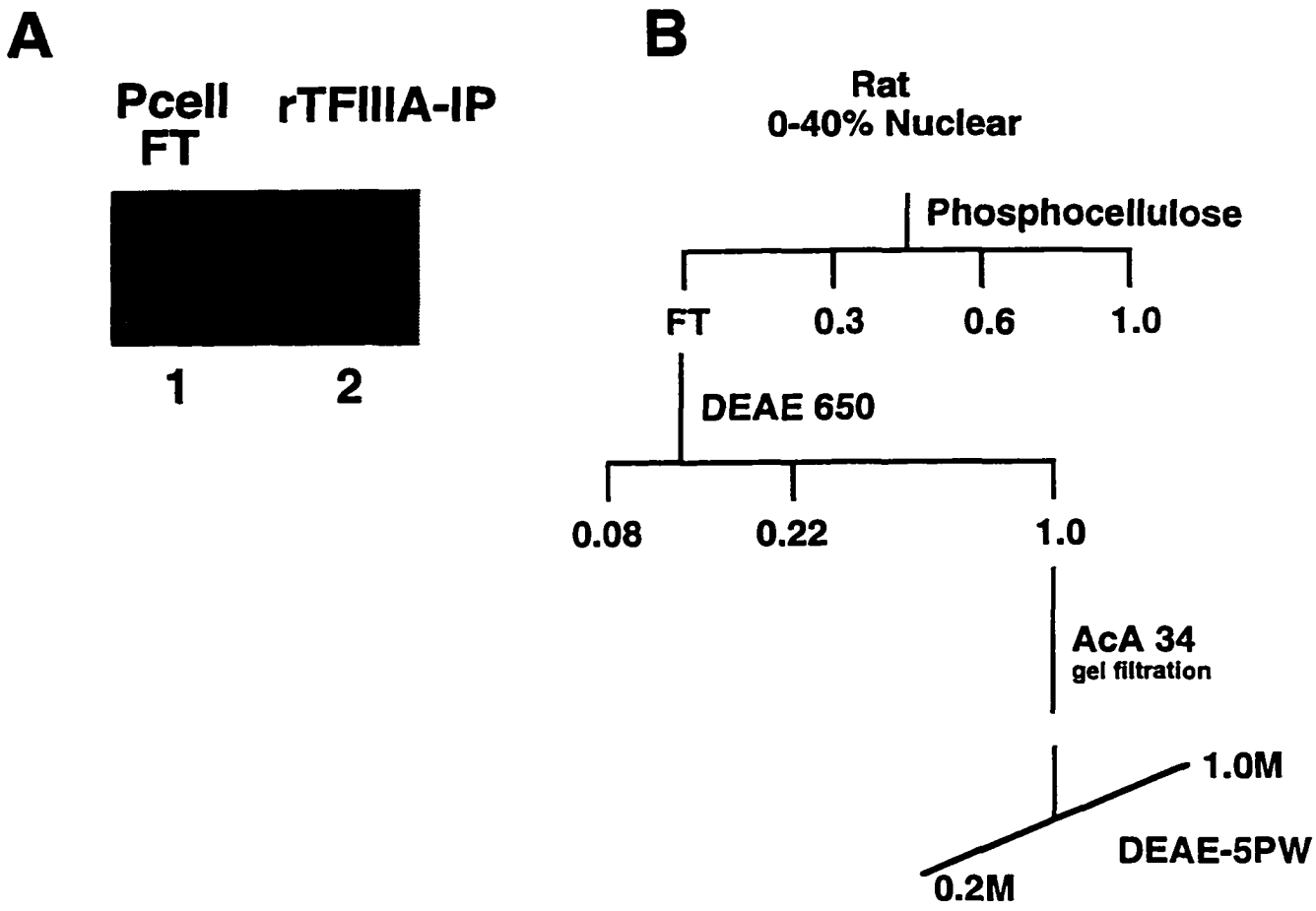
Figure 19. Immunoprecipitation of recombinant *Xenopus* TFIIIA using TFIIIA-IP. Recombinat TFIIIA, recombinant TFIIIA-IP, and affinity purified TFIIIA-IP antibody were prepared as described in *Materials and Methods*. Lane 1 is the recombinant TFIIIA used in the experiment. Lane 2 received TFIIIA, TFIIIA-IP, A/G resin, and affinity purified TFIIIA-IP antibody. Lane 3 received TFIIIA, A/G resin, and affinity purified TFIIIA-IP antibody.


+ + TFIIIA-IP antibody
+ + A/G resin
+ - rTFIIIA-IP
Load
+ + rTFIIIA
1 2 3

activation domain. The smaller band in our prep has not been analyzed but this would be consistent with the inability of TFIIIA-IP to immunprecipitate this species. This could be addressed by performing the experiment with C-terminal tail deletions of TFIIIA. Lane 2 is TFIIIA that was precipitated in the presence of the TFIIIA-IP protein, lane 3 shows very little TFIIIA is precipitated in the absence of the TFIIIA-IP protein.

Interaction of TFIIIA-IP and TFIIIA in rat liver nuclear extracts. To further analyze an interaction between TFIIIA and TFIIIA-IP, rat extracts were prepared and fractionated as described in Chapter I. A schematic diagram for the fractionation is shown in figure 20. The fractions were analyzed for cofractionation by western blot, using antibodies to the TFIIIA-IP and TFIIIA proteins. TFIIIA is known to flow through phosphocellulose (Segall et.al., 1980). The TFIIIA-IP antibody recognizes a protein in the phosphocellulose flow through that migrates very closely to the recombinant TFIIIA-IP (Fig. 20a). The recombinant TFIIIA-IP protein has an extra 4 kDa from the expression vector. Fractions across DEAE-5PW were probed with anti *Xenopus* TFIIIA and TFIIIA-IP antibodies (Fig. 20c). The protein recognized by the TFIIIA-IP antibody did not flow through DEAE and eluted in the same vicinity as the protein recognized by the monoclonal antibody to TFIIIA.

Figure 20. Detection of possible TFIIIA-IP in rat liver extracts. Fractionation and western blots were performed as described in *Materials and Methods*. A. western blot comparison of recombinant TFIIIA-IP and the flow through of rat 0-40% nuclear extract on phosphocellulose. B. Schematic diagram of the fraction following TFIIIA-IP. C. Fractions across the DEAE-5PW column were run on 10% SDS PAGE and transferred to Nitrocellulose. The blot was probed with a polyclonal antibody to *Xenopus* TFIIIA and then probed with a polyclonal antibody to TFIIIA-IP.


A
Pcell FT
rTFIIIA-IP
1
2
B
Rat
0-40% Nuclear
Phosphocellulose
FT
0.3
0.6
1.0
DEAE 650
0.08
0.22
1.0
AcA 34
gel filtration
1.0M
DEAE-5PW
0.2M
C
anti TFIIIA-IP
anti TFIIIA
rXTFIIIA
6
7
8
9
10
11
12
13
14
15
16

DISCUSSION

The yeast two-hybrid system was used to identify the TFIIIA-IP protein which interacts with *Xenopus* TFIIIA. The TFIIIA-IP protein was specific for *Xenopus* TFIIIA and did not interact with SNFI, laminin, or yeast TFIIIA. Experiments have revealed that recombinant TFIIIA-IP will immunoprecipitate recombinant TFIIIA *in vitro*. Initial experiments have shown TFIIIA-IP to be present in the phosphocellulose flow through where TFIIIA is known to elute, however, this fraction is very crude. The current model is that TFIIIC interacts with TFIIIA and TFIIIC is known to elute in the 0.3M-0.6M KCl fraction off phosphocellulose. Antibodies to the TFIIIA-IP protein have not recognized any proteins in this fraction. It is possible that TFIIIA interacts with TFIIIC through the TFIIIA-IP protein. It was considered that the TFIIIA-IP protein may not be involved in transcription. TFIIIA is found in large amounts as a 7S RNP particle, consisting of one TFIIIA and one 5S RNA molecule in the cytoplasm of oocytes. This interaction is important for the efficient nuclear export of 5S RNA in amphibian oocytes (Guddat et.al., 1990). This suggested TFIIIA might have a domain that is recognized by the nuclear RNA export machinery. This was recently confirmed by experiments involving the HIV-1 and HIV-2 encoded nuclear regulatory protein, termed Rev (Fridell et. al., 1996 and references therein).

Rev is essential for the expression of viral structural proteins. Rev induces the nuclear export of these viral mRNAs by binding to a cis-acting RNA target site termed

the Rev response element (RRE). In the absence of Rev these mRNAs are sequesterd in the cell nucleus and not translated. The ribonucleoprotein particle recruits a cellular cofactor that induces the entry of these viral mRNAs into a preexisting nuclear export pathway. The Rev activation domain responsible for this export has been mapped to these critical residues, LxxLTI/L. This domain is also present in the last 19 amino acids in the C-terminus of *Xenopus* TFIIIA which was included in our yeast 2-hybrid construct used in the screen. Microinjection of oocytes with peptides encoding the Rev activation domain resulted in specific inhibition of Rev-dependent RNA export and 5S RNA export, but mRNA and tRNA export was unaffected. Furthermore, deletion of the last 50 amino acids of Rev destroyed the nuclear export activity, but addition of the last 19 amino acids of TFIIIA restored the export activity. The activation domain in this chimeric protein was mutated with a leucine to an alanine mutation (LxxLTL→LxxATL). This change caused a loss of function in the export activity. This approach could be used to investigate the domain the TFIIIA-IP protein recognizes in the C-terminal tail of TFIIIA. Mutations like this in the Rev like domain and the upstream transcription activation domain in TFIIIA (Chapter 2) could be used to go back into the yeast 2-hybrid assay and check for loss of an interaction between TFIIIA and the TFIIIA-IP protein. A protein given two names Rab and hRIP has been shown to bind the Rev domain but Fridell et. al. 1996 were unable to demonstrate an interaction between this protein and TFIIIA.

Recently another group has identified the TFIIIA-IP protein in a two-hybrid screen, using E3-14.7K protein (Li et.al., 1998). They named the cDNA FIP-2 (for

14.7K-interacting protein). Their cDNA codes for a protein that is 41 amino acids shorter at the C-terminus and is missing 1300 nts of 3' untranslated seqeunce compared to our cDNA for TFIIIA-IP. Otherwise the sequences are identical. They used 5' and 3' rapid amplification of cDNA ends (RACE) to isolate their full length cDNA. It is likely that random annealing of the PCR primers to the 3' mRNA end resulted in a cDNA that is not full length. The E3-14.7K protein is a human Adenovirus encoded protein involved in inhibition of the cytolytic effects of tumor necrosis factor alpha (TNFα) upon viral infection. They have shown cells expressing E3-14.7K can coimmunoprecipitate transiently expressed FIP-2 protein that is tagged with a Flag epitope. They used immunohistochemistry to demonstrate a perinuclear localization of FIP-2. This is consistent with unpublished results that TFIIIA also has a perinuclear localization (Jay Hanas, personal communication). The authors could not identify a function for this protein and suggest it may be inolved in transcription because of its leucine zippers. It is interesting that transcription of RNA pol III genes is enhanced by adenovirus infection (Sinn et. al., 1995; Hoeffler et. al., 1988). The adenovirus protein E1A has been shown to be partly responsible for this stimulation. E1A has also been shown to stimulate TNFα which E3-14.7K inhibits. The future elucidation of the function for this TFIIIA-IP protein will provide much needed insight into these many different pathways.

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IMAGE EVALUATION
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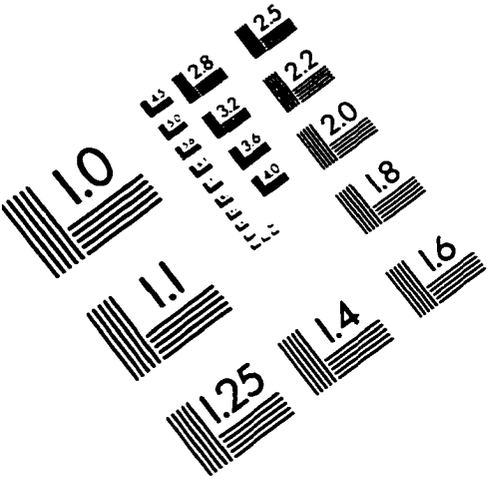

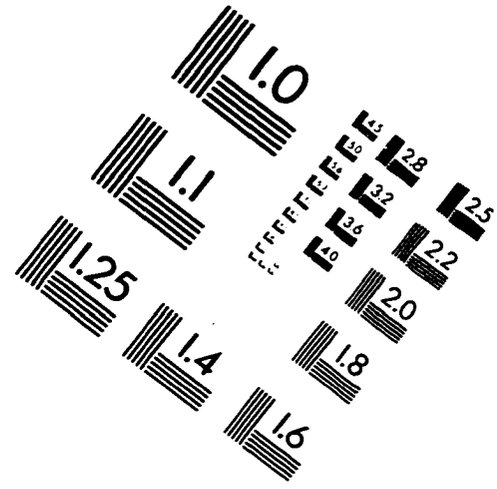

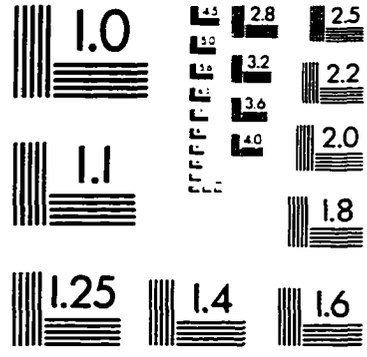

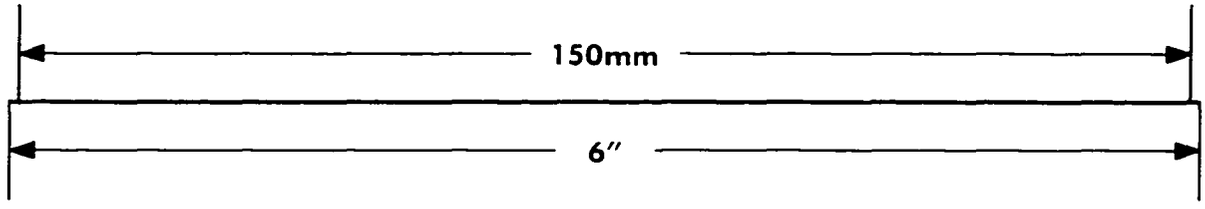

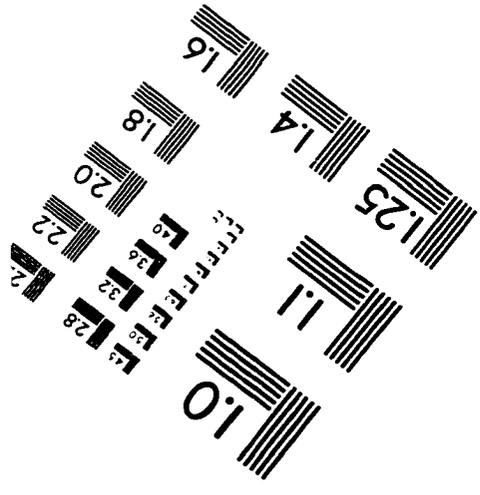

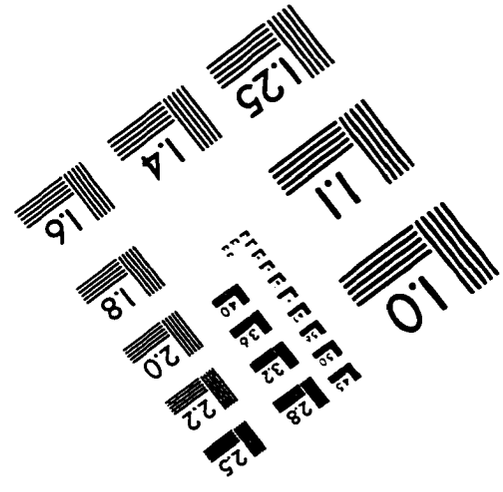

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