

1994

# Eukaryotic Transcriptional Activation Mechanism: Protein-Protein Interactions

Tae Kook Kim

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Eukaryotic Transcriptional Activation Mechanism:  
Protein-Protein Interactions

A dissertation presented to  
The Rockefeller University  
in partial fulfillment of the requirements  
for the degree of Doctor of Philosophy

by  
Tae Kook Kim

April, 1994  
The Rockefeller University  
New York, N.Y.



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## LIST OF ABBREVIATIONS

Ad2ML	adenovirus serotype 2 major late
ADH	alcohol dehydrogenase
BSA	bovine serum albumin
CTD	carboxy-terminal domain
D	glutamic acid
DTT	1, 4-dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol bis- $\beta$ -aminoethylether-N,N,N,N'- tetraacetic acid
FOA	5-fluoroorotic acid
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HIV	human immunodeficiency virus
K	lysine
L	leucine
NTP	nucleotide triphosphates
P	proline
PAGE	polyacrylamide gel electrophoresis
PMSF	phenylmethanesulfonylfluoride
S	serine
SDS	sodium dodecyl sulfate
T	threonine
TAFs	TBP-associated factors
TBP	TATA-binding protein
Tris	tri(hydroxymethyl)aminomethane
Y	tyrosine

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## ABSTRACT

Various *in vivo* and *in vitro* assays have been employed to analyze how activators communicate with the general transcription machinery to stimulate transcription. As a first step, I analyzed the function of distinct kinds of activation domains in yeast and human. The results showed that the proline-rich activation domain of CTF1 can, like acidic activation domains, activate transcription in yeast and human. Based on this, I compared the activation pathways by acidic and proline-rich activation domains in yeast and human. These detailed comparative approaches yielded clues to the fundamental aspects of transcriptional activation mechanism in eukaryotes: activators target TFIID(TBP)-TFIIB-promoter complex formation in the preinitiation complex assembly process by inducing (or stabilizing) qualitative or quantitative alterations within TFIID(TBP)-TFIIB-promoter complexes that consequently enhance recruitment of downstream initiation factors. Consistent with this view, various activation domains have been demonstrated to have physical and functional interactions with TBP and/or TFIIB. Given the central role of TBP in transcriptional regulation, I also identified distinct TBP domains (or residues) important for different regulatory interactions including those with acidic activators, TFIIB, Dr1 (NC2), Pol I- and Pol III-specific factors. These mutational analyses have provided an insight into how the interplay of many regulatory factors occurs on the surface of a target factor, TBP, to specify and regulate transcriptional activity. Furthermore, I investigated the

essential features of the CTF1 proline-rich activation domain and showed that CTD-like sequences (Ser-Pro motifs) are important for activation, possibly by forming a  $\beta$ -turned omega ( $\Omega$ ) loop structure. Thus, the  $\beta$ -turn structure is likely to be a salient secondary structure in the activation domains in addition to a  $\beta$ -sheet structure which was previously proposed for acidic activation domains.



## ACKNOWLEDGEMENTS

I would like to thank my research advisor, Dr. Robert G. Roeder for his support during my studies. His hard work has paid off in creating a scientific environment which has been a great benefit to me, as well as many other apprenticed investigators. I am also thankful to many of members of the laboratory for their friendship and willingness to share their expertise with me. Many names come to mind, but those most helpful to the development of my project included Dr. Masami Horikoshi.

I thank all professors in the Department, especially my previous advisor Dr. Hyune Mo Rho, for unfailing enthusiasm in this study and for confidence in my ability to carry it out. I also thank them for many valuable lessons which have served me throughout my scientific career.

I thank my parents, brother, sister and parents-in law for their continued patience and confidence in me through many, many years both past and present. I hope that I have not disappointed them. In addition, I must recognize my family whose love and support helped make my dreams a reality. Thank my wife, Jina Ryu and my daughter, YaeSol Kim! It is of untold happiness to me that they have been with me and witnessed the completion of this endeavor.

Finally, I must thank the Jesus Christ who has helped me in so many ways. Now I must start another long journey with him again.

## CHAPTER I

### General introduction

A central problem in eukaryotic molecular biology is to understand the mechanism by which specific genes are expressed in a temporal or tissue-specific manner or are activated in response to extracellular inducers.

Transcription initiation is an early and critical event in eukaryotic gene expression. Ultimately, changes in the rate and specificity of transcription initiation play key roles in controlling cellular differentiation and development.

The development of the methods for cloning and characterizing individual genes in vivo and in vitro has provided the opportunity to study gene regulation mechanisms at the molecular level. The mutation of cloned genes and analysis of their effects on expression have identified DNA sequence elements required for the regulation of initiation of mRNA synthesis in eukaryotes, a minimal or core promoter element and enhancers or upstream promoter elements. Proteins that specifically bind to these DNA sequences have been identified and in some cases purified: general initiation factors, which are essential for initiation and which are sufficient to direct a basal level of transcription from a core promoter; and gene- or cell-specific regulatory factors, which stimulate the ability of general initiation factors to activate transcription initiation by interacting with upstream promoter elements.

The current challenges are to understand how specific protein-protein interactions of activators with general initiation factors regulate transcription and how these interactions are finally integrated into the overall pattern of gene regulation for cellular functions. In this introductory section, I summarize and discuss the information obtained to date regarding the nature of activators, function and regulation of general initiation factors in the preinitiation complex assembly, and then I raise relevant and specific questions which have been addressed during my thesis.

### Activators

Studies on a variety of eukaryotic activators have shown that they have a modular structure in which distinct regions of the protein mediate particular functions such as DNA binding and transcriptional activation. Therefore, a specific region of each individual activators will be involved in its ability to activate transcription following DNA binding. Such activation domains have been identified by so called 'domain-swap' experiments in which various region of one factor are linked to the DNA binding domain of another factor and the ability to activate transcription assessed. Following the identification of activation domains in different activators, it is likely that they can be classified into several distinct families according to their different chemical characters; acidic, proline-rich, glutamine-rich, and metal-

binding cysteine-containing activation domains (reviewed in Mitchell and Tjian, 1989). Although further structure-function studies are necessary to establish the relevance of this classification, these distinct activation domains are, at least, likely to function by contact with different targets in the general transcription machinery.

### 1. Acidic activation domain

Comparison of several activation domains including those of the yeast GAL4 and GCN4 and the activation domain of VP16 indicated that, although they do not show any strong amino acid sequence homology to each other, they all have a large proportion of negatively charged amino acids (reviewed in Ptashne, 1988; Struhl, 1988). Recent studies have shown that, in some cases, acidic residues are not the sole determinant of activator function since it is possible to decrease the activity of the GAL4 (Leuther et al., 1993) and VP16 (Cress and Triezenberg, 1991) activation domains without reducing the number of negatively charged residues. These studies suggested that hydrophobic amino acid residues are critical for activator function. Moreover, studies of GAL4 acidic activation domains have shown that these regions might consist of regular structural motifs such as a  $\beta$ -sheet structure (Leuther et al., 1993; Van Hoy et al., 1993). But it failed to explain the important structural features of other acidic activation domains, including that of VP16.

## 2. Proline-rich activation domain

Analysis of CTF1, which binds to the CCAAAT box sequences, revealed that the activation domain contains numerous proline residues (Mermod et al., 1989). As with the other classes of activation domains, this region is capable of activating transcription when linked to the DNA binding domains of other transcription factors. Similar proline-rich domains have been defined in several other mammalian and yeast transcription factors such as AP2, JUN, OCT2, SRF and SNF5. Like acidic activators, there is no obvious sequence similarity between them. Because proline favors turns or kinks for steric reasons, proline-rich activation domains are likely to form an irregular loop structure containing several reverse turns. It is possible that the prolines may form a framework that enables other residues (e.g. hydrophobic residues) to interact with the basic transcription machinery for activated transcription.

## 3. Glutamine-rich activation domain

Studies on Sp1 (Courey and Tjian, 1988) defined a third type of activation domain that two most potent activation domains contained approximately 25 % glutamine residues. Similar glutamine-rich regions have been defined in other transcription factors including the Drosophila proteins Antennapedia, Ultrabithorax, the mammalian proteins OCT1, OCT2, JUN, AP2, SRF and the yeast HAP1, HAP2, GAL11 factors. It was proposed that the amide moieties of the glutamine side

chains might be involved in hydrogen bonding to some component of the basic transcription machinery.

#### 4. Metal-binding cysteine-containing activation domain

A fourth type of activation domain was identified in the adenovirus Ela transcription factor (Lillie and Green, 1990). The central region, which contains a metal-binding domain with several cysteine residues, was shown to activate transcription when linked to GAL4 DNA binding domain. Importantly, in vivo competition experiments with distinct kinds of activation domains demonstrated that Ela utilized targets different from those employed by other activation domains such as acidic activation domains for activated transcription (Lillie and Green, 1990).

#### General initiation factors and cofactors: targets

To study activation mechanisms, it is critical to understand the structure and function of the general initiation factors which mediate preinitiation complex assembly through the core promoter elements and which serve as the direct or indirect targets for activators.

The general factors from human cells (and yeast cells) implicated in core promoter transcription include TFIIA (TFIIA), TFIIB (e), TFIID (d), TFIIE (a), TFIIF (g), TFIIH (b) and RNA polymerase II. These general factors are assembled in an ordered fashion at the core promoter to form a stable preinitiation complex (Buratowski et al., 1989; Van

Dyke et al., 1988; reviewed in Roeder, 1991; Zawel and Reinberg, 1992; Conaway and Conaway, 1993). The first component recruited to the promoter is TFIID, a large multiprotein complex which binds to the TATA box in a reaction that may be facilitated by TFIIA. Once stable TFIID-promoter interactions result in a template-committed complex, TFIIB can enter to form the so called DB or DAB complexes. This provides an landing site for RNA polymerase II which is escorted to the promoter by TFIIF. TFIIF has been proposed to prevent RNA polymerase II from binding non-specifically to DNA. Then TFIIE, TFIIH and TFIIJ, in that order, assembled to the complexes to complete the formation of the preinitiation complex. Activators have been thought to enhance one or more steps in the preinitiation complex assembly process (reviewed in Roeder, 1991; Greenblatt, 1991). In principle, any rate-limiting step in the assembly could be regulated by direct or indirect interactions between activators and some component of the basic transcription machinery. Although the exact activation mechanism is not known, several general factors are proposed as targets of activators: TBP (TATA binding subunit of TFIID); TAFs (TBP-associated factors in TFIID complexes); TFIIB; and the largest subunit of RNA polymerase II.

#### 1. TFIID (TBP and TAFs)

The first general factor to be identified as a target of activators is TFIID, consistent with its fundamental role in



preinitiation complex assembly. A viral activator, the pseudorabies virus IE protein, was shown to facilitate the association of TFIID with promoter--preincubation of the DNA template with purified TFIID obviates the need for the viral activator (Abmayr et al., 1988). This stimulation of transcription was particularly strong when examined on nucleosome-repressed promoters (Workman et al., 1988). TFIID also reduces the off-rate of a cellular activator, USF (Sawadogo et al., 1985). Importantly, two cellular activators, mammalian ATF and yeast GAL4, alter the DNase I footprinting of TFIID and increase protection the downstream of the TATA box (Horikoshi et al., 1988a; 1988b). Such qualitative changes in TFIID may facilitate assembly of the downstream general initiation factors.

A direct demonstration of TBP (TATA-binding protein) as a target for activators was performed by protein affinity chromatography methods. The herpes simplex virus VP16 acidic activation domain was shown to bind directly to cloned human and yeast TBPs (Stringer et al., 1990). Consistently, mutants in a VP16 activation subdomain which decrease its affinity for TBP also decrease activation in vivo (Ingles et al., 1991). The Epstein-Barr virus Zta activator was also shown to interact with TBP and to stabilize the binding of TBP to the promoter (Lieberman et al., 1991). In addition, many other regulators including adenovirus Ela (Lee et al., 1991; Horikoshi et al., 1991) have recently been shown to interact

directly with TBP. On the basis of these studies, it is likely that activation occurs, at least in some part, via interactions with TBP.

Recent studies comparing the activity of partially purified TFIID with cloned TBP (Hoffmann et al., 1990) indicate that TFIID contains additional factors, called coactivators, that are required to mediate activation but not basal transcription (reviewed in Roeder, 1991; Pugh and Tjian, 1992). Coactivators in human cells are found to be tightly associated with the TBP as an array of polypeptides (TAFs) (Tanese et al., 1991; Zhou et al., 1992; Chiang et al., 1993). Fractionation in the presence of denaturant was used to separate TBP and coactivators (TAFs), and subsequent reconstitution experiments showed that they are essential for stimulation by an activator bearing either the acidic, proline-rich or glutamine-rich activation domain in higher eukaryotic systems (Tanese et al., 1991; reviewed in Pugh and Tjian, 1992). Consistent with their ability to support activation, TAFs are also required for generation of extended downstream footprints on the promoter DNA by GAL4 and ATF1 (Chiang et al., 1993) which was observed with partially purified TFIID in earlier studies (Horikoshi et al., 1988a; 1988b). These TAFs are thought to be required for the realization of the functional consequences of interactions between activators and general factor(s) such as TBP. It remains to be determined how these TAFs and TBP function for

transcriptional activation, whether all of these TAFs are essential for activation, and whether individual TAFs are specific for distinct types of activator.

Similar coactivator activities have been described in the yeast transcription system (Flanagan et al., 1991; Berger et al., 1990; 1992). In particular, there is recent evidence for multi-component TBP-containing complexes similar to human TFIID (Thompson et al., 1993; Poon et al., 1993) and some of these complexes have been found to mediate activation by VP16 in a yeast in vitro transcription system. Thus, it will be interesting to know whether yeast coactivators function in a manner similar to human coactivators (TAFs).

## 2. TFIIB

Several experiments have demonstrated that the VP16 acidic domain interacts with TFIIB in addition to TBP (Lin and Green, 1991). Furthermore TFIIB mutants unable to interact with the VP16 acidic domain are specifically defective in supporting transcription directed by acidic activators and a VP16 mutant which cannot activate transcription is correspondingly defective for interaction with TFIIB (Roberts et al., 1993). In immobilized template assays that monitor preinitiation complex assembly, the TFIIB recruitment step is enhanced by activators (Choy and Green, 1993). Taken together, these data have indicated that TFIIB is another

pivotal component in the mechanism by which activators function.

### 3. RNA polymerase II

The carboxy-terminus of the largest subunit of RNA polymerase II contains multiple copies of the amino acid sequence SPTSPSY and this sequence is highly conserved in eukaryotes. Yeast mutants containing a reduced number of heptapeptide repeats in RNA polymerase II was shown to be specifically defective in their response to activators such as GAL4 (Scafe et al., 1990). However, no direct evidence for interaction of activators with these sequences has been obtained.

#### Activation mechanism and related problems

Studies of transcription factors have led to the identification and characterization of many regulatory factors and general initiation factors. However, as discussed above, the actual mechanisms by which activators function through the set of general factors are largely unknown. Thus our experiments have been aimed at understanding, at the biochemical level, how different regulatory factors activate initiation and which steps and events they affect in order to accomplish this. A more complete understanding of this regulatory process requires in vitro approaches directed toward the dissection of multi-step initiation complex assembly process. We have been using various in vivo and in vitro systems to analyze how activators communicate with the

general transcription machinery to stimulate transcription, addressing the following specific questions:

- (1) What step (or steps) in the transcription process do activators affect?
- (2) Which general transcription factor (or factors) is the direct target of activators?
- (3) Do different classes of activators work by similar or different mechanisms?
- (4) Does a given activator work by similar or different mechanisms in different species (e.g. yeast vs human)?  
Such comparative approaches have been employed to gain insights into the fundamental aspects of transcriptional activation mechanism in eukaryotes.
- (5) How the interplay of many transcription factors occurs on the surface of a target factor (e.g. TBP) to regulate transcription?

## CHAPTER II

Transcriptional activation by the proline-rich activation  
domain of CTF1 and the acidic activation domain of VP16  
in yeast and human

## ABSTRACT

Among eukaryotic transcriptional activators, those with acidic activation domains have been shown to stimulate transcription in organisms ranging from yeast to human. Functional *in vivo* and *in vitro* assays have been employed to test transcriptional enhancement in yeast by distinct kinds of human activation domains: the proline-rich activation domain of CTF1; the glutamine-rich activation domain of Sp1; and the metal-binding cysteine-containing activation domain of adenovirus Ela. The results demonstrate that the proline-rich activation domain of human CTF1 can activate transcription in the yeast *in vivo* and *in vitro*. Thus proline-rich activation domains, like typical acidic activation domains, function universally in yeast and human. Furthermore, we investigate the essential features of the CTF1 activation domain by deletion analyses. The results suggest that CTD-like sequences (SP motifs) in CTF1 proline-rich region are important for transcriptional activation and that motifs are likely to form a  $\beta$ -turned omega ( $\Omega$ ) loop structure.

## INTRODUCTION

Despite detailed information on the structure of several eukaryotic activator proteins, the mechanism by which these factors stimulate transcription is still unclear. In principle, a DNA-bound transcriptional activator must interact directly or indirectly with some target protein in the general transcriptional machinery, thereby facilitating the formation or function of a preinitiation complex containing general initiation factors and RNA polymerase II. Studies with in vitro reconstituted transcription systems containing purified or partially purified factors have identified several general factors as direct targets and implicated additional cofactors in the activation mechanism (reviewed in Ptashne and Gann, 1990; Greenblatt, 1991; Roeder, 1991; Pugh and Tjian, 1992; Zawel and Reinberg, 1992; Hernandez, 1993).

Acidic activation domains have been shown to function in eukaryotic organisms ranging from yeast to human (reviewed in Ptashne, 1988; Struhl, 1988). This universal functionality suggests a conserved mechanism of transcriptional enhancement by the acidic activation domain in eukaryotes. However, at least three apparently distinct types of activation domains have been identified in higher eukaryotes on the basis of distinct amino acid compositions within activation domains (reviewed in Mitchell and Tjian, 1989). The first is the



glutamine-rich activation domain represented by Sp1 (Courey and Tjian, 1988), the second is the metal binding cysteine-containing activation domain of adenovirus Ela protein (Lillie and Green, 1990), and the third is the proline-rich activation domain represented by CTF1 (Mermoud et al., 1989). Several lines of evidence suggest that these distinct activation domains may employ different transcriptional activation pathways, for example, by interacting with different target proteins or different adaptors impinging upon a common target (reviewed in Ptashne and Gann, 1990; Greenblatt, 1991; Roeder, 1991; Pugh and Tjian, 1992; Zawel and Reinberg, 1992; Hernandez, 1993).

As a first step to gain insights into potentially distinct activation pathways by various activation domains, and to compare these activation pathways in different species, we have analyzed the function of different activation domains in yeast. Here we have employed *in vivo* and *in vitro* transcription systems to demonstrate that the proline-rich activation domain of CTF1, previously characterized only in higher eukaryotes, is also functional in yeast (Kim and Roeder, 1993a). In addition, we have analyzed several deletion derivatives in the CTF1 activation domain in yeast in order to know the important structural features of the CTF1 proline-rich activation domain. The results show that CTD-like sequences (SP; Ser-Pro motifs) in proline-rich

region are important for activation by CTF1 (Kim and Roeder, 1994a).

## EXPERIMENTAL PROCEDURES

### Strains and $\beta$ -galactosidase assays

Yeast strain used for in vivo studies was YPH252 $\Delta$  (ura3, lys2, ade2, trp1, his3, leu2, gal4 $\Delta$ ). Yeast cells were made competent for transformation by treatment with lithium acetate (Ito et al., 1983).  $\beta$ -galactosidase assays were performed as described previously (Ma and Ptashne, 1987). For in vitro studies, whole cell extracts were prepared from yeast strain YPH252 $\Delta$ . All plasmid constructions were performed using E.coli strain DH5 $\alpha$  and bacteria were grown in LB medium. E.coli strains XA90 and BL21(DE3)pLysS were used for purification of GAL4(1-94) and GAL4(1-94)-CTF1, respectively.

### Plasmids

Plasmid pRSY was used to express various GAL4 fusion proteins in yeast. It was constructed by inserting an Sph I fragment containing the expression cassette (ADH I promoter-GAL4 DNA binding domain (1-147)-ADH I terminator) from pMA424 into pRS313 (Ma and Ptashne, 1987). For this cloning, Sal I and Bam HI-generated ends of plasmid pRS313 were filled with Klenow enzyme and an Sph I linker was added. Plasmids expressing GAL4 fusion with various activation domains in yeast were constructed by inserting appropriate fragments into the pRSY. The reporter gene was made by inserting the GAL4 or CTF1 binding sites into the Bam HI site of pCZ (Lue

et al., 1989). Templates for in vitro transcription were pGAL4X6 and pSal $\Delta$ CG- (Wootner et al., 1991). The activation template pGAL4X6 was made by inserting a fragment containing six GAL4 binding sites into pSal $\Delta$ CG-.

### Immunoblot analysis

In vitro expression of GAL4-fusion proteins was determined as follows. Exponentially growing yeast cultures (30 ml) were harvested and the cell pellets were resuspended in 400  $\mu$ l sample buffer for SDS-PAGE. Extracts were prepared by vortexing the cell suspension in the presence of glass beads and fractionated by SDS-PAGE. Western blotting was done following the manufacturer's instructions (Amersham).

### Purification of GAL4-fusion proteins

Purification of GAL4(1-94) was as described (Workman et al., 1991). GAL4(1-94)-CTF1 was expressed under control of the T7 promoter in E.coli strain BL21(DE3)pLysS. Cultures were grown at 30 °C to an A<sub>600</sub> of 0.7 and expression of the fusion protein was induced by adding IPTG to a final concentration of 0.4 mM. After 3 hr, cells were harvested and washed once in 20 mM HEPES (pH 7.5)-0.2 M NaCl. The cell pellet was resuspended in buffer A (20 mM HEPES (pH 7.5), 10 mM 2-mercaptoethanol, 25  $\mu$ M zinc sulfate, 1 mM PMSF, and 20  $\mu$ g/ml each of leupeptin and pepstatin) containing 200 mM NaCl. Lysozyme was added to a final concentration of 0.5 mg/ml and the cell suspension was incubated on ice for 15 min and then

lysed by sonication at 0 °C. All subsequent operations were performed at 0 ° to 4 °C. A crude extract was derived by centrifugation of the lysate for 20 min at 10,000 X g. Solid ammonium sulfate was added to the supernatant to a final concentration of 33 % and centrifuged. The pellet was suspended in buffer A containing 200 mM NaCl and passed over a DEAE-Sepharose CL-6B column. The flowthrough was dialyzed for 5 hr against buffer A containing 200 mM NaCl and then loaded onto a Heparin-Sepharose CL-6B column. The column was washed with 10 column volumes of buffer A containing 200 mM NaCl and GAL4(1-94)-CTF1 was step-eluted with 3 column volumes of buffer A containing 600 mM NaCl.

#### Preparation of whole cell extracts

Yeast cells were grown at 30 °C in YEPD media to an A<sub>600</sub> of 0.7, harvested and washed with cold sterile distilled water. All further steps were carried out at 0° to 4 °C. The pellet was resuspended in extraction buffer (0.2 M Tris base, 0.39 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 10 mM MgSO<sub>4</sub>, 20 % (v/v) glycerol, 1 mM EDTA, 1 mM DTT) containing 1 mM PMSF, 2 mM benzamidine hydrochloride, and 0.4 ug/ml pepstatin. The cell paste was loaded into a syringe, extruded into liquid nitrogen and then stored at -70 °C. Cells were ground continuously in liquid nitrogen with a ceramic mortar and pestle until the liquid nitrogen disappeared and the powder started to become sticky. Grinding was repeated following addition of more liquid nitrogen. Breakage was monitored by comparison of the protein

concentration in relation to that of unbroken cells. After grinding, the powder was mixed with 1 volume of extraction buffer plus protease inhibitors. The thawed lysate was centrifuged for 2 hr at 100,000 rpm. The supernatant was collected and  $(\text{NH}_4)_2\text{SO}_4$  added to 40 %-50 % saturation over the course of one hour. The suspension was stirred on ice for an additional 30 min, and then was centrifuged at 40,000 rpm for 30 min. The pellet was resuspended in a minimal volume of suspension buffer (20 mM HEPES (pH 7.5), 20 % (v/v) glycerol, 10 mM  $\text{MgSO}_4$ , 10 mM EGTA, 5 mM DTT) with the protease inhibitors listed above. The sample was dialyzed against suspension buffer containing 1 mM PMSF. The dialysate was centrifuged at 10,000 rpm for 10 min. The supernatant was stored in aliquots at -70 °C.

#### In vitro transcription reactions

The reactions were performed as described by Wootner et al. (1991). The reaction mixture (30  $\mu\text{l}$ ) contained 10 % (v/v) glycerol, 50 mM HEPES (pH 7.5), 90 mM potassium glutamate, 0.75 % (w/v) PEG, 5 mM EGTA, 10 mM magnesium acetate, 2.5 mM DTT, 30 mM creatine phosphate, 1.4 unit/ml creatine kinase, 0.5 unit of inhibit-ACE (5'-3', Inc), 0.4 mM CTP, 0.4 mM ATP, 20  $\mu\text{Ci}$  of  $[\alpha\text{-P}^{32}]$  UTP (600 Ci/mmol), template DNA as well as an optimal amount of GAL4-fusion protein and extract. The template was preincubated with GAL4-fusion proteins for 15 min and the reaction was initiated by addition of whole cell extract and nucleotides. After 30 min at room temperature,

transcription was stopped and the reaction incubated for an additional 10 minutes at 25 °C in RNase T1 solution, which contains 240 mM NaCl, 8 mM HEPES, 0.8 mM EDTA and 10 units RNase T1. RNase digestion was terminated by addition of SDS to 0.5 % and proteinase K to 0.25 mg/ml. After 20 min at 37 °C, the sample was precipitated with ethanol and carrier tRNA for 20 min on dry ice. The precipitate was separated on urea/polyacrylamide gel and analyzed by autoradiography.

#### Oligonucleotide-directed mutagenesis

Mutagenesis was carried out by the methods of Kunkel et al. (1987) to create various CTF1 deletion mutants. The plasmid containing CTF1 activation domain was transfected into E. coli strain (BW313: *dut*<sup>-</sup>, *ung*<sup>-</sup>), and single-stranded DNA (minus strand) was prepared after superinfection of the cells with helper phage M13K07. The appropriate mutagenic oligonucleotide was treated with T4 polynucleotide kinase and annealed in excess to the single-stranded DNA. The mixture was then treated with Sequenase version 2.0 (T7 DNA polymerase) to elongate DNA from the mutagenic primer, and the products were ligated with T4 DNA ligase. The resulting DNA was transfected into the E. coli strain DH5 $\alpha$  (*dut*<sup>+</sup>, *dug*<sup>+</sup>) cells to select for the mutated plasmid. Finally, mutated plasmid was isolated and sequenced to confirm the altered DNA.

### Characterization of SP sequences

Many of the published primary structures of transcription factors have been determined by the analysis of nucleotide sequences of cloned genes. The numbers of SP sequences were counted (Suzuki, 1989). The relative frequency of SP sequences was calculated from dividing the numbers of SP by the number of amino acid residues in each protein. In addition to these observed frequencies, the frequencies of SP expected when amino acids were arranged randomly were calculated by multiplying the ratio of S by that of P. From comparison of observed frequency of SP with calculated frequency of SP expected from a random arrangement of amino acid residues, it is clear that observed frequency of occurrence of SP in gene regulatory proteins is higher than the value from a random arrangement of amino acid residues. In general factors, the former value is a little less than the latter value.



## RESULTS

### Transcriptional activation by GAL4-CTF1 in the yeast in vivo

To determine whether distinct kinds of human activation domains are functional in yeast, we fused acidic (VP16-derived), proline-rich (CTF1-derived), glutamine-rich (Sp1-derived), and metal-binding cysteine-containing (E1a-derived) activation domains to the coding sequences of the DNA-binding domain of GAL4 (amino acids 1-147) (Fig. 1). We introduced these plasmids into a yeast strain with a reporter containing a CYC1-lacZ fusion gene with the upstream activation sequence (DNA binding site) for GAL4. The GAL4 fusion proteins were expressed from the yeast ADH (alcohol dehydrogenase) I promoter. Expression and integrity of the various GAL4-fusion proteins were assayed by immunoblotting of extracts from transformed strains (Fig. 2; data not shown). The activity of  $\beta$ -galactosidase, the product of the CYC1-lacZ fusion gene, was used to measure the transcriptional activation function of each GAL4 fusion protein. The acidic activation domain of VP16 stimulated expression of  $\beta$ -galactosidase dramatically, but significant stimulatory effects were also observed in response to the proline-rich activation domain of GAL4(1-147)-CTF1 (Fig. 3). As compared with a negative control containing no activation domain, expression was enhanced by GAL4(1-147)-CTF1 approximately 200 fold. When fused with the DNA-binding domain of GAL4, the activation domains of Sp1 (amino acids 82-500) and E1a (amino acids 121-223) failed to

activate transcription in yeast, in contrast to their effects in mammalian cells (Fig. 3; data not shown). Therefore, the proline-rich activation domain of CTF1 is, like acidic activation domains, functional in yeast, whereas the glutamine-rich activation domain of Sp1 and the metal binding cysteine-containing activation domain of E1a are not. This implies that there are species-specific transcriptional activation pathways that depend upon distinct activators. However, we cannot exclude the possibility that the inability to activate transcription in yeast by Sp1 or E1a may reflect certain intrinsic properties (e.g. instability) of these proteins. Thus we only consider our positive results to be clearly interpretable.

#### Transcriptional activation by the proline-rich activation domain of CTF1 in the yeast in vivo

We demonstrated above that GAL4(1-147)-CTF1 can activate expression from the CYC1-lacZ reporter gene under the control of the upstream activation sequence for GAL4 in vivo. One possible reason for activation is that a cryptic activation domain is generated in the GAL4(1-147)-CTF1 fusion protein. To show that the CTF1 proline-rich activation domain per se was active in yeast, we expressed both native CTF1 and proline-rich domain-truncated CTF1 in yeast cells and monitored activation of a reporter containing CTF1 binding sites upstream of a CYC1-lacZ gene (Fig. 4a). As shown in Fig. 4b, CTF1 stimulated transcription, whereas truncated

CTF1 missing the proline-rich activation domain did not. This result clearly indicates that the proline-rich domain of CTF1 can mediate activation in yeast.

#### Transcriptional activation by GAL4-CTF1 in the yeast in vitro

To more directly show transcriptional activation by CTF1, we employed a yeast-derived in vitro transcription system in conjunction with recombinant GAL4(1-94) and GAL4(1-94)-CTF1 proteins expressed in and purified from E.coli. For these analyses, sequences encoding amino acids 95-147 of the GAL4 DNA-binding domain were removed since they can stimulate transcription in vitro (Tanese et al., 1991; data not shown). The template employed to test activation by purified GAL4-fusion proteins was pGAL4X6, which contains GAL4 binding sites upstream of the CYC1 core promoter fused to the G-less cassette. Transcription was performed with a yeast whole cell extract supplemented with GAL4-fusion proteins. Addition of GAL4(1-94)-CTF1 to the whole cell extract significantly activated transcription from the pGAL4X6 template, consistent with the results of the in vivo study (Fig. 5). Under the same conditions, GAL4(1-94)-CTF1 did not activate transcription from a template (pSal $\Delta$ CG-) containing no GAL4 binding site (lane 2). To determine whether the activation by GAL4(1-94)-CTF1 was due to the proline-rich activation domain of CTF1, we checked the transcriptional activity of GAL4(1-94); this protein contains only the DNA binding domain of GAL4. Although some stimulation was observed by GAL4(1-94),

the amount of enhancement by GAL4(1-94)-CTF1 was significantly greater than that by GAL4(1-94) (Fig. 5). The activation by GAL4(1-94) was also observed in a human in vitro transcription system (data not shown) and might reflect the presence of some negatively charged residues between amino acids 75 and 94.

CTD-like sequences are important for transcriptional activation by the proline-rich activation domain of CTF1

We showed that like acidic activation domains, the proline-rich activation domain of CTF1 stimulates transcription in both yeast and human cells. To gain an understanding of relevant structural features of this proline-rich activation domain, the minimal region sufficient for an activation response was determined by deletion analysis (Fig. 6). Derivatives of the activation domain were fused to the DNA binding domain of GAL4 and assayed by introduction into a yeast strain containing a reporter CYC1-lacZ fusion gene with DNA binding sites for GAL4. The results show that a region from residues 460-479 is most important for activation since its deletion markedly reduced activation (5-fold) whereas deletion of N-terminal residues (399-459) had only a modest effect (Fig. 6). Interestingly, an amino acid sequence within the minimal CTF1 activating region (SPTSPSYSPDTSPT) is very similar to the consensus sequence (SPTSPSY) of the repeat unit of the C-terminal domain (CTD) of the largest subunit of RNA polymerase II (Corden et al., 1992). To more directly

show the involvement of this CTD-like sequence in activation, GAL4 (1-147) was fused to the yeast CTD containing 26 tandem repeats of the SPTSPSY sequence. This domain was remarkably active in activation of the reporter gene (Fig. 6). These results clearly show that SPTSPSY sequences in the CTD, as well as related sequences in the proline-rich domain of CTF1, can mediate activation in yeast.

#### SP motifs are frequently found in activation domains

The idea that the CTD-like sequences (SPTSPSY) are involved in activation is further strengthened by the results of a search for proteins containing SP motifs (Suzuki, 1989). This motif occurs frequently in transcriptional activators but less so in general proteins, suggesting its involvement in gene regulation. As summarized in Fig. 7, SP-containing transcription regulatory proteins include homeobox gene products (fushi tarazu, antenapedia, engrailed, deformed and Oct), Drosophila segmentation gene products (krüppel and hunchback), steroid hormone receptors, yeast transcription factors (ADR1, PPR1, GAL4, HSF and SWI5), and certain products of oncogenes (fos and myc). Furthermore, the location of SP motif is restricted; it is found neither in DNA-binding domains nor in regions responsible for ligand binding or dimerization, but mostly in regions important for activation or regulation. Therefore, many activating regions could contain SP motifs. Both functional and sequence

analyses suggest that the SP sequence motif is important for transcriptional activation.

## DISCUSSION

In vivo and in vitro transcriptional analyses of GAL4(1-94 or 1-147)-CTF1 have established that the proline-rich activation domain is, like acidic activation domains, functional in both yeast and human systems. Yeast TBP supports both basal and activated transcription by GAL4-VP16 in the exogenous TBP-dependent heat-treated nuclear extract from human (HeLa) cells (Kelleher et al., 1992), whereas yeast TBP apparently is not functional for CTF1-mediated activation using a similar human in vitro transcription system (Pugh and Tjian, 1990). In addition to general factors, coactivators are known to be required for transcriptional activation by VP16 or CTF1 (reviewed in Greenblatt, 1991; Roeder, 1991; Pugh and Tjian, 1992). It was reported that a partially purified coactivator fraction can support activation by the proline-rich activation domain of CTF1 in human, although it failed to mediate activation by the acidic activation domain of VP16 (Tanese et al., 1991). These results strongly suggest that different coactivators may be required for distinct transcriptional activators and that proline-rich and acidic activation domains may use different activation pathways to interact with components (e.g. TBP and TFIIB) of the basic transcription machinery (Kim and Roeder, 1994b; Kim et al., 1994d), even if they are universally active in heterologous organisms. It will be interesting to see whether CTF1

functions by similar processes in different species such as yeast and human.

Deletion analysis of the CTF1 activation domain reveals that CTD-like sequence (SP) motifs are important for activation. The SP motif is strongly predicted, by the method of Chou and Fasman (1978), to form a  $\beta$ -turn secondary structure. This is due to the fact that, of all 20 amino acid residues, proline has the highest tendency to become the second residue in the turn, and that serine or threonine is the residue found most frequently at the first position of a  $\beta$ -turn. Hence, the heptapeptide sequence of the CTD is predicted to have a strong tendency to form a  $\beta$ -turn structure but not an  $\alpha$ -helical or a  $\beta$ -sheet structure (Suzuki, 1990). The structure with serines one and four forming an internal hydrogen bond and the hydrophobic aromatic ring contained tyrosine residue on the surface for possible protein-protein interactions has been proposed (Suzuki, 1990; reviewed in Corden and Ingles, 1992). Several different approaches, including model building studies, two-dimensional NMR, and circular dichroism studies have also suggested that the SP motifs in the CTD are composed of turn structures (reviewed in Corden and Ingles, 1992). Additionally, we cannot exclude the possibility that CTD phosphorylation plays a role in activation by GAL4-CTD in the context of extended  $\beta$ -turn structures (Zhang and Corden, 1991). Consistent with the  $\beta$ -turn hypothesis, most strong activators identified from random *E. coli* genomic fragments



contained regions predicted to adopt  $\beta$ -turns (Ma and Ptashne, 1987). Two proline substitutions, which favor  $\beta$ -turns, in the activation domain of VP16 failed to affect function (Cress and Trienzenberg, 1991). Therefore, in addition to the primary sequence, the  $\beta$ -turn structure might be an important secondary structure in the activation domain. Recently, Johnston's laboratory has argued that the GAL4 activation domain contains antiparallel  $\beta$ -sheets (Leuther et al., 1993; Van Hoey et al., 1993). Their genetic analyses of the GAL4 acidic activation domain showed that negative charge was not essential for activation. In addition, they predicted that the secondary structure motif of this activating region was an antiparallel  $\beta$ -sheet connected by a short  $\beta$ -turn ( $\beta$ -hairpin). This  $\beta$ -sheet hypothesis was supported by biophysical analyses of peptides corresponding to GAL4 and GCN4 acidic activation domains. However, this motif fails to explain certain characteristics of other activation domains, raising the possibility that there are several classes of activators containing distinct secondary structures. Consistent with this view, the present results with the SP-containing motif suggest a  $\beta$ -turn structure as an additional secondary structure important for activation.

$\beta$ -turns are encountered many times in the guise of loop secondary structure designations. Leszczynski and Rose (1986) examined looped structure of which the  $\beta$ -turn is a member, in protein X-ray data and termed these 'omega ( $\Omega$ ) loops' (Fig.

8). This study has suggested that omega loop sequences form a regular secondary structure which were previously regarded as unstructured random coils. Ironically, acidic activation domains were also previously believed to have shapeless acidic blobs (Sigler, 1988). Some features of the  $\beta$ -turned omega loop structure as a regular secondary structure are very relevant to explain the properties of activation domains: (1) Omega loop functions as a highly compact substructure and has the potential for modular exchange between proteins. Similarly, activation domains can be swapped in transcription factors; (2) Loops are almost invariably located at the protein surface where they are poised to assume important roles in molecular function through protein-protein interactions. Therefore, the  $\beta$ -turned omega loop structure might provide the framework for activation, in which some important amino acids such as hydrophobic residues can interact with other factors in a proper way; and (3) Another general feature of activation domains is that progressive deletion of residues from the activation domain results in stepwise loss of activity. This can be explained if activation domain might contain several smaller embedded loops like the CTD. Interestingly, truncation of the CTD exhibited this kind of redundant loss of activity (Nonet et al., 1987).

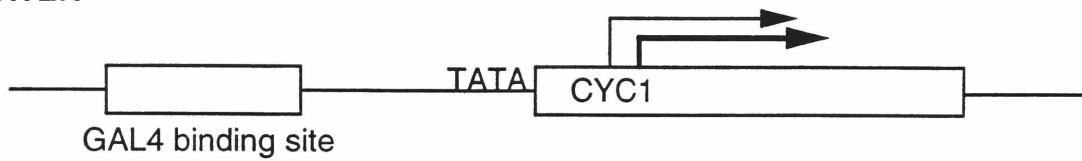
Apart from indicating that the proline-rich activation domain is function universally from yeast to human, the present

analysis indicates that CTD-like sequence (SP) motifs may be important for transcriptional activation by eukaryotic activators including CTF1, forming the  $\beta$ -turned omega ( $\Omega$ ) loop structure. Furthermore, the results have paved the way to analyze and compare the activation pathways by the acidic and proline-rich activation domains in yeast and human. These comparative approaches will be helpful to gain insights into the fundamental aspects of transcriptional activation mechanism in eukaryotes.

Fig. 1 Constructions used in this study.

The reporter gene is a CYC1-lacZ fusion, which is regulated by the GAL4 upstream activation sequence (UAS). Fusions of the amino-terminal 147 amino acids of GAL4 DNA-binding domain to the activation domains of VP16 (residues 413-490), CTF1 (residues 399-499), Sp1 (residues 82-500), and Ela (residues 121-223) were tested for activation.

## REPORTER



## EFFECTOR

GAL4DB	DNA binding domain	413	490
GAL4DB-VP16	DNA binding domain	399	499
GAL4DB-CTF1	DNA binding domain	82	500
GAL4DB-SP1	DNA binding domain	121	223
GAL4DB-E1a	DNA binding domain		

*Activation domain*

Fig. 2 Determination of GAL4-fusion protein levels in yeast by immunoblot analysis.

Equal amounts of whole cell extracts were fractionated by SDS-PAGE and analyzed by western blot using antiserum against the DNA binding domain of GAL4. The positions of the protein markers are indicated (kDa). Lane 1, GAL4(1-147)-VP16 yeast extract; lane 2, GAL4(1-147) yeast extract; lane 3, GAL4(1-147)-CTF1 yeast extract and lane 4, yeast extract containing no GAL4 fusion plasmid.

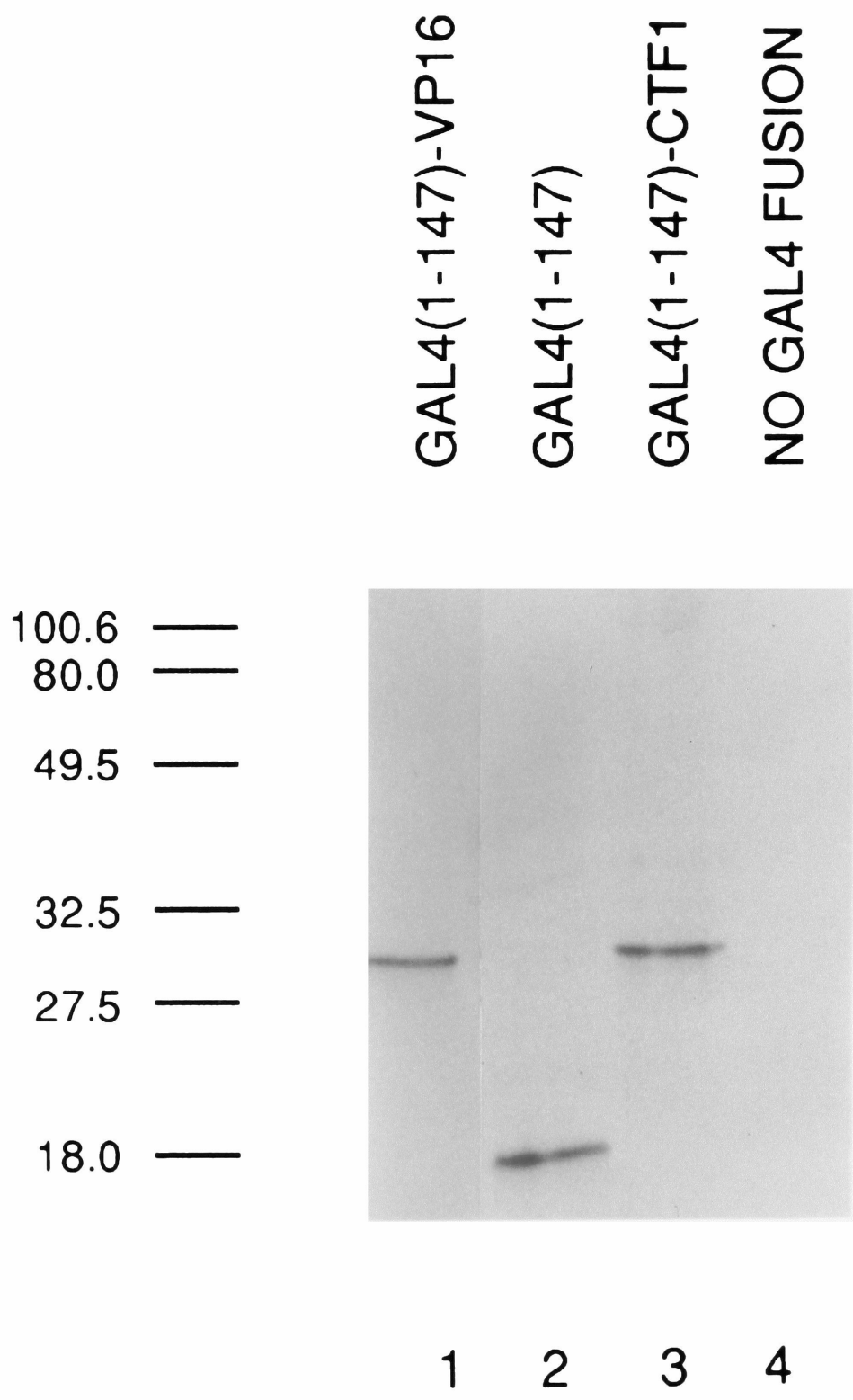


Fig. 3 Transcriptional activation by GAL4(1-147)-CTF1 in the yeast in vivo.

The single copy effector plasmids in Fig.1 were introduced into yeast strain YPH252 $\Delta$  which harbors the reporter gene.  $\beta$ -galactosidase activity was determined as described in Experimental Procedures. Shown is the average value of three independent samples, which varied by <20 %.



GAL4-fusion	Activation domain	$\beta$ -galactosidase activity
NONE	—	<1
GAL4(1-147)	—	<1
GAL4(1-147)-CTF1	Proline - rich	202
GAL4(1-147)-VP16	Acidic	2004
GAL4(1-147)-SP1	Glutamine - rich	<1
GAL4(1-147)-E1a	Metal binding Cysteine-containing	<1

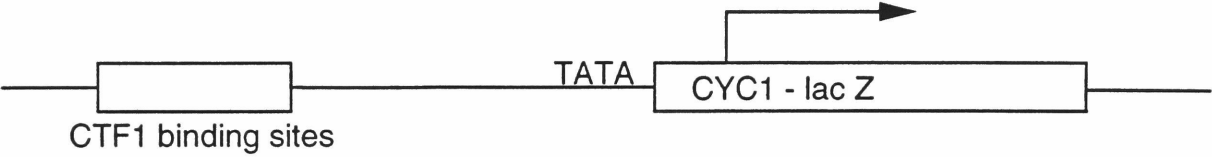
Fig. 4 The proline-rich domain of CTF1 is necessary for activation in yeast.

a, the reporter gene contains CTF1 binding sites upstream of the CYC1-lacZ fusion. Effectors include native CTF1 and truncated CTF1 missing the proline-rich domain.

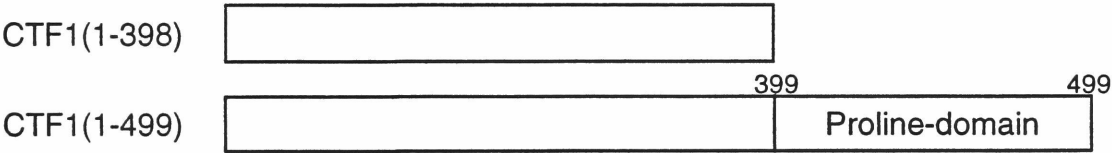
b, single copy effector plasmids were introduced into yeast strain YPH252 $\Delta$  which harbors the reporter gene. Transformants were grown and  $\beta$ -galactosidase activity was determined. Shown is the average value of three independent samples, which varied by <20 %.

**a**

REPORTER



EFFECTOR



**b**

Activator	Activation domain	$\beta$ -galactosidase activity
NONE	—	<1
CTF1(1-398)	—	5
CTF1(1-499)	Proline - rich	104

Fig. 5 Transcriptional activation by GAL4(1-94)-CTF1 in a yeast-derived in vitro system.

Templates (300 ng) containing the indicated GAL4-fusion proteins were transcribed in a yeast whole cell extract. The template DNAs contain a TATA box from the CYC1 promoter with or without six GAL4 binding sites. The chimeric GAL4-fusion proteins were expressed in and purified from *E. coli* for in vitro transcription analyses. Sequences encoding amino acids 95-147 of the GAL4 DNA-binding domain were removed since they contain acidic residues that can stimulate transcription in vitro. The dimerization and DNA binding properties of GAL4 are still retained in the truncated GAL4(1-94) protein. Transcriptions were done in the absence of GAL4-fusion protein (lane 1) and in the presence of increasing concentration of either GAL4(1-94) (50 ng in lane 3, 100 ng in lane 4, and 200 ng in lane 5) or GAL4(1-94)-CTF1 (200 ng in lane 2, 50 ng in lane 6, 100 ng in lane 7, 200 ng in lane 8, and 400 ng in lane 9). The specific transcripts are indicated by an arrow. Relative transcription activities were measured from the radioactivity in specific transcripts.

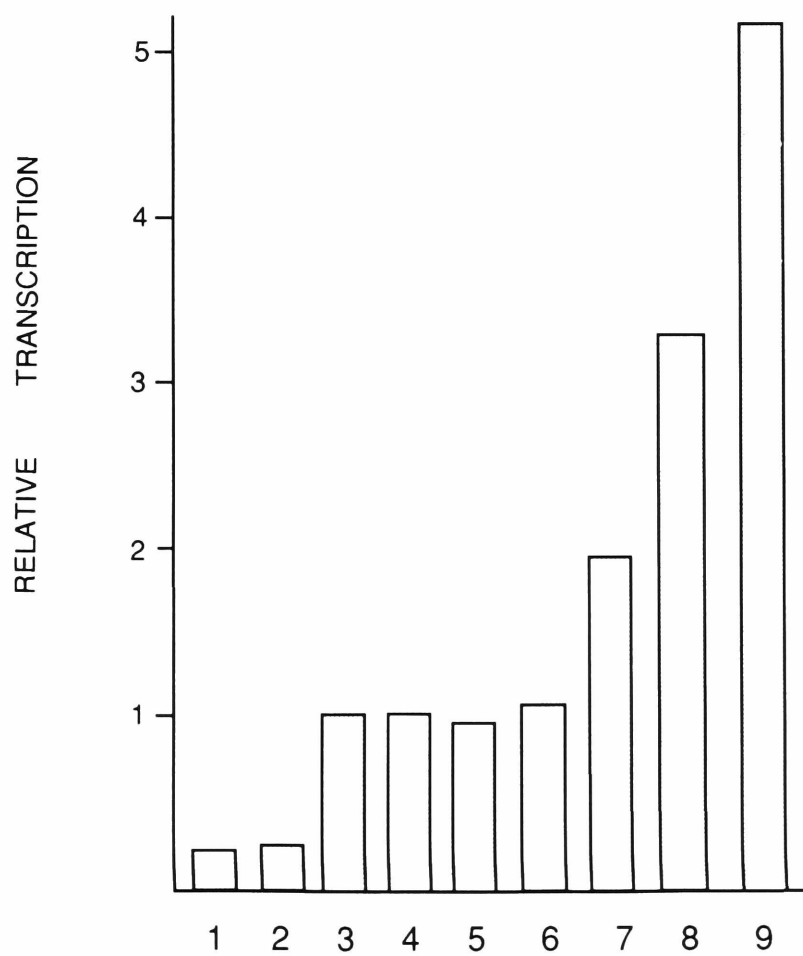
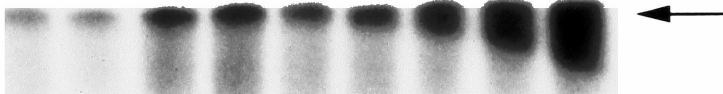


Fig. 6 An SP-containing motif is important for activation by the proline-rich activation domain of CTF1.

The reporter gene is a CYC1-lacZ fusion which is regulated by the binding site for the GAL4. The GAL4 DNA binding domain (amino acids 1-147) is fused to the various deletion derivatives of the CTF1 proline-rich activation domain and to the 26-heptapeptide (SPTSPSY) repeat of the C-terminal domain (CTD) of the largest subunit of yeast RNA polymerase II.

Deletions within the CTF1 proline-rich activation domain were made by an oligonucleotide-mediated mutagenesis method. Yeast transformants were grown and assayed for  $\beta$ -galactosidase activity. Numbers refer to the average units of  $\beta$ -galactosidase activity measured in three independent cultures, which varied by <20 %.

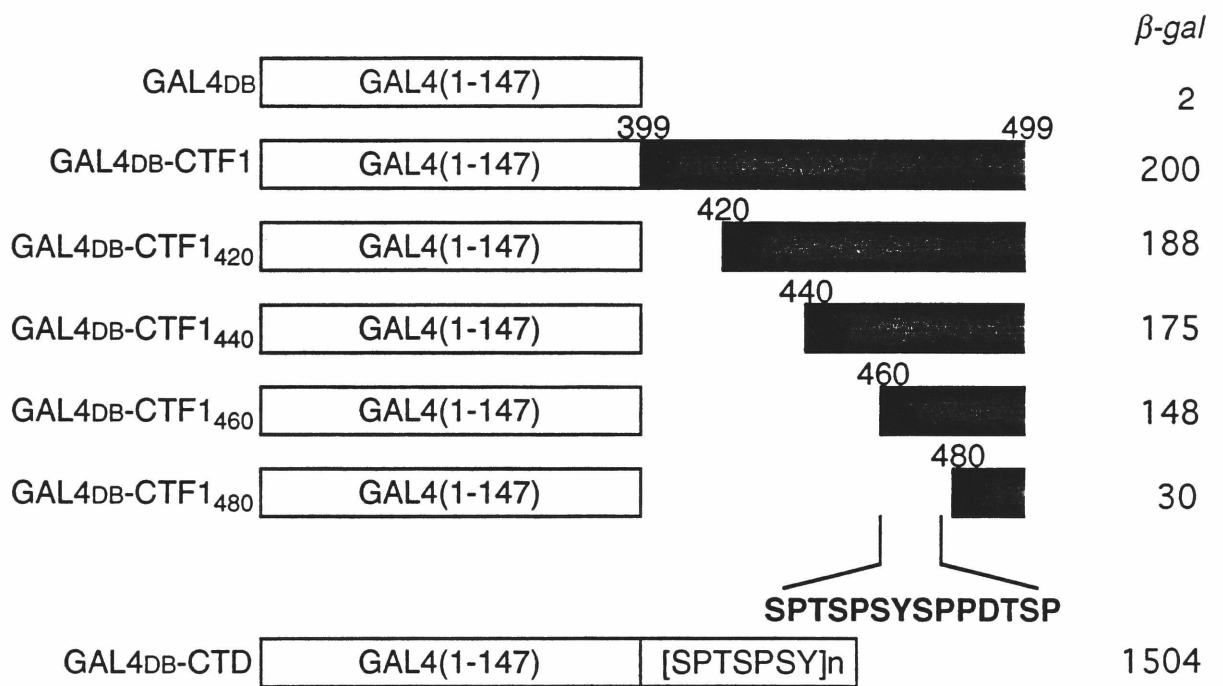


Fig. 7 SP motifs are frequently found in gene regulatory factors.

Many of the published primary structures of transcription factors have been determined by the analysis of nucleotide sequences of cloned genes. The relative frequency of SP sequences was calculated from dividing the numbers of SP by the number of amino acid residues in each protein. Shown are the example proteins whose activation domains SP motifs are frequently found in.



Homeobox gene products (fushi tarazu, antennapedia, engrailed, deformed and Oct)

Drosophila segmentation gene products (krüppel and hunchback)

Steroid hormone receptors

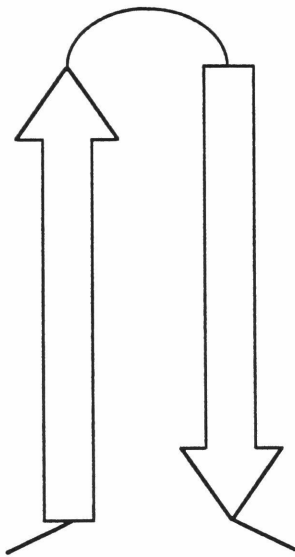
Yeast transcription factors (ADR1, PPR1, GAL4, GCN4, HSF and SWI5)

Oncogene products (jun, fos and myc)

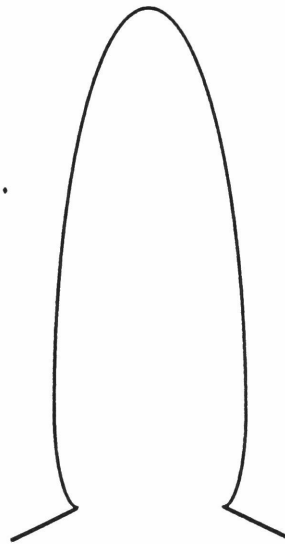
Fig. 8 Schematic drawing of  $\beta$ -hairpin and  $\beta$ -turned  $\Omega$ -loop structures which might be involved in transcriptional activation.

The  $\beta$ -hairpin structure was consist of a  $\beta$ -sheet connected by a tight turn. The  $\beta$ -turned  $\Omega$ -loop structure was characterized by the longer loop containing more than six amino acids and often included multiple  $\beta$ -turns.

**$\beta$ -hairpin**



**$\beta$ -turned  $\Omega$ -loop**



### CHAPTER III

Transcriptional activation mechanism by acidic activators in yeast: Effects of activation-defective TBP mutations on VP16-TBP and TFIIB-TBP-promoter interactions and TFIIB recruitment

## ABSTRACT

The TATA-binding initiation factor TBP has been shown to be a target for various regulators that include acidic activators. In order to investigate mechanisms of transcriptional activation by acidic activators, functional in vitro analyses were carried out to identify yeast TBP mutants specifically defective in activation by GAL4(1-147)-VP16. Three individual point mutations (L114K, L189K and K211L) were found to selectively abolish transcriptional activation by GAL4(1-147)-VP16, while maintaining normal basal transcription by RNA polymerase II in a TBP-dependent yeast in vitro transcription system. These mutants also maintained the ability to support in vitro transcription by RNA polymerases I and III. Further analyses showed that mutations L114K and L189K each reduced activator-induced recruitment of TFIIB to the promoter, apparently by affecting the ability of TBP to interact directly with VP16 and with TFIIB respectively. These results suggest that GAL4(1-147)-VP16 induces (or stabilizes) activation-specific changes in TBP-TFIIB interactions on the promoter through interactions with TBP. Mutation K211L might affect some other activation function, such as presumptive coactivator interactions. Consistent with in vitro results, activation-defective TBP mutants showed impaired in vivo functions in a plasmid shuffle complementation assay. In the recently obtained three-dimensional structure of TBP, two mutations (L114K and K211L)

map to  $\beta$ -strand positions that either overlap or are closely interdigitated with putative DNA interaction surfaces, whereas the other mutation (L189K) is located in a loop region which might be more directly accessible for direct protein-protein interactions.

## INTRODUCTION

The TATA-binding protein (TBP) and associated components in TFIID are targets for various regulators including acidic activators (reviewed in Ptashne and Gann, 1990; Greenblatt, 1991; Roeder, 1991; Pugh and Tjian, 1992; Zawel and Reinberg, 1992; Hernandez, 1993). Consistent with its central role in transcriptional regulation, more than one TBP interaction have been suggested to be involved in activated transcription by activators. Yeast fractionation studies have shown that activation by acidic activators requires at least two factors (mediator or adaptor, fraction C) not required for basal activity (Flanagan et al., 1991; 1992; Berger et al., 1990). Genetic studies in yeast have also provided evidence for such cofactors (Berger et al., 1992), as well as cofactors that clearly interact with TBP (Eisenmann et al., 1992). In particular, there is recent evidence from several labs for multi-component TBP-containing complexes (Thompson et al., 1993; Poon et al., 1993) and, most importantly, some of these complexes have been found to mediate activation by GAL4(1-147)-VP16 in a yeast in vitro transcription system (R. Young, personal communication). In higher eukaryotes, fractionation studies have provided additional lines of evidence for essential coactivators that are associated with TBP in TFIID (reviewed in Hernandez, 1993) as well as distinct positive and negative cofactors (possibly analogous to fraction C in yeast), some of which interact with TBP, that are essential

for high level induction by activators but not for basal activity (Meisterernst et al., 1991a; 1991b; Inostroza et al., 1992; Merino et al., 1993). Moreover, the fact that TFIIB may serve as an independent or joint target for acidic activators (Lin and Green, 1991; 1992; Roberts et al., 1993; Choy and Green, 1993; Kim and Roeder, 1994b), and that this could require activation-specific interactions of TFIIB with TBP (Hahn, 1993; Goodrich et al., 1993), suggests that TFIIB-TBP interactions are important for activated transcription like cofactor-TBP interactions. Similar considerations apply to TFIIA, which is not essential for basal transcription but which is required for activator-dependent transcription (Meisterernst et al., 1991a; 1991b) and which is a TBP-interacting protein (Ranish and Hahn, 1992; Auble and Hahn, 1993).

Given the involvement of multiple interactions of TBP in transcriptional activation, it is very important to isolate distinct TBP mutations that affect some interactions described above in addition to those affect direct activator interactions in order to analyze the activation mechanisms in detail. Here, studies of yeast TBP mutants in a homologous cell free system have identified three individual point mutations (L114K, L189K, and K211L) which selectively abolish activation by GAL4(1-147)-VP16, while maintaining normal basal transcription by RNA polymerase II as well as RNA polymerase I and III. Mechanistic analyses showed that VP16



mediates enhanced TFIIB recruitment and, further, that mutations L114K and L189k independently reduce this activator-dependent TFIIB recruitment, apparently by altering TBP interactions with VP16 and with TFIIB, respectively. Therefore, VP16 function appears to involve both direct interactions with TBP and a corresponding induction (or stabilization) of an activation-specific TBP-TFIIB-promoter complex (Kim et al., 1994d).

## EXPERIMENTAL PROCEDURES

### Protein purifications

The fusion proteins, GAL4(1-147)-VP16 and GAL4(1-147) were expressed and purified in bacteria as described (Chasman et al., 1989). Wild-type and mutant TBPs were expressed as hexahistidine fusion proteins in bacteria and purified by nickel column chromatography to about 80 % purity (Hoffmann and Roeder, 1992). Two subunits of yeast TFIIA (TOA1 and TOA2) were purified from an insoluble fraction of lysed bacteria (Ranish et al., 1992). Yeast TFIIB was purified as a hexahistidine fusion protein by nickel chromatography (Hoffmann and Roeder, 1992).

### Site-directed mutagenesis

Mutagenesis was carried out by the methods of Kunkel et al. (1987) to create various TBP point mutants. The plasmid pGEM7Zf(+) containing the yeast TBP gene was transfected into E. coli strain (BW313: dut<sup>-</sup>, ung<sup>-</sup>), and single-stranded DNA (minus strand) was prepared after superinfection of the cells with helper phage M13K07. The appropriate mutagenic oligonucleotide was treated with T4 polynucleotide kinase and annealed in excess to the single-stranded DNA. The mixture was then treated with Sequenase version 2.0 (T7 DNA polymerase) to elongate DNA from the mutagenic primer, and the products were ligated with T4 DNA ligase. The resulting DNA was transfected into the E. coli strain DH5 $\alpha$  (dut<sup>+</sup>, dug<sup>+</sup>)

cells to select for the mutated plasmid. Finally, mutated plasmid was isolated and sequenced to confirm the altered DNA.

#### In vitro transcription with yeast reconstituted systems

The template DNAs for in vitro transcription contain a TATA box from the CYC1 promoter with or without six GAL4 binding sites. The basal template contains a G-less cassette of 400 base pairs and yields two major transcripts of 375 and 350 base pairs in vitro. The activation template with GAL4 binding sites has a 100 base pair-truncated G-less cassette. TBP-dependent yeast in vitro transcription system used in this study was reconstituted as described before (Flanagan et al., 1992). Yeast basal transcription factors and mediator were prepared by chromatography of yeast whole cell extracts on Bio-Rex 70 (Bio-Rad) and DE 52 (Whatman). Fraction C was prepared from yeast nuclear extracts. Each purified TBPs were assayed in these TBP-dependent reconstituted systems.

#### In vitro transcription with yeast whole cell extracts

Yeast strain with I143N TBP mutant was grown at the permissive temperature (24 °C) to an A<sub>600</sub> of 1.8. It was then grown for another one hour at the nonpermissive temperature (37 °C). Extracts were prepared using a mortar and pestle, and transcription reactions from yeast 35S rRNA gene promoter or yeast 5S rRNA gene promoter were performed with 20-40 ng

of TBP as described (Schultz et al., 1992; Kim and Roeder, 1994c).

#### Coimmunoprecipitation assays

The acidic activation domain of VP16 was expressed in bacteria as part of a fusion protein with the IgG-binding domain of *Staphylococcus aureus* Protein A and purified on IgG columns (Pharmacia) following the manufacturer's instructions. <sup>35</sup>S-methionine-labelled TBP was generated by in vitro transcription of corresponding cDNAs and translation of derived mRNAs. In vitro-translated yeast TBPs (5 ul) were mixed with Protein A or Protein A-VP16 (6 ug) in 30 ul buffer D (10% Glycerol, 100mM KCl, 20mM Tris-Cl (pH 7.9), 0.2 mM EDTA, 0.03% NP-40, 1mM DTT) containing BSA (20 ug/ml) and allowed to bind for 1 hr on ice. IgG beads were prepared as described before (Ingles et al., 1991) and preincubated with yeast TBP and BSA to prevent nonspecific interactions. After addition of IgG beads, reactions were incubated for 1 hr at 4 °C. The beads were then washed three times with 400 ul buffer D containing BSA (200 ug/ml) and centrifuged. Bound proteins present in the pellet were released by boiling in SDS buffer and separated, along with input proteins and unbound proteins in the supernatant, on a 15 % acrylamide gel.

#### Band-shift analyses

The formation of TBP-TFIIA-TFIIB complex was checked for the by band-shift analysis (Auble and Hahn, 1993). The DNA

fragment used for the band-shift assay contained the TATA box of adenoviral major late promoter. All DNA binding was carried out for 20 min at room temperature in 4 mM Tris (pH 8.0), 60 mM KCl, 5 mM MgCl<sub>2</sub>, 4 % glycerol, 100 ug/ml BSA, and 0.1 % Brij 58. The products were analyzed on a 6 % polyacrylamide gel containing 25 mM Tris (pH 8.3), 190 mM glycine and 0.5 mM DTT. The gel was cast in Tris-glycine running buffer containing no magnesium acetate (Auble and Hahn, 1993). Under this condition, TBP alone cannot form a complex unless DNA interaction is stabilized in the presence of other factors, TFIIA or TFIIB.

#### In vitro assays on the immobilized DNA templates

The DNA fragment containing six GAL4 binding sites upstream of the CYC1 TATA box and G-less cassette was isolated from pGAL4X6 template, biotin-labelled, and then coupled to streptavidin-agarose beads (Lin and Green, 1991; Kim and Roeder, 1994b). The beads with immobilized DNA templates were incubated with TBP and TFIIB in the presence (+) or absence (-) of GAL4(1-147)-VP16 for 90 min at 30 °C. After the first incubation, the beads were washed with transcription buffer. The beads were then subject to western blotting or in vitro transcription with complementation of other essential yeast transcription factors. Complementing fractions with essential factors were prepared by immunodepletion of yeast TFIIB from Bio-Rex 70-DE 52 fractions with anti-yeast TFIIB antisera.

### Plasmid-shuffling methods

The analysis employed yeast strain YTW22 containing a deleted version of the endogenous TBP gene and a URA3 plasmid expressing TBP gene for cell growth. The endogenous yeast TBP gene of the test strain was disrupted by replacing the 5' end of the gene, including amino acid 1-81, with the TRP1 gene. This yeast strain (YTW22) carries the wild-type yeast TBP gene on a single-copy URA3 plasmid. Mutated TBPs were introduced into these cells on a multicopy plasmid bearing the HIS3 gene as a selectable marker. The transformants were replica plated onto media containing 5-FOA to select against cells carrying wild-type TBP gene present on the URA3 plasmid. Thus the strain is only viable on 5-FOA plates if the TBP allele on the HIS3 plasmid functionally substitute for wild-type TBP.

## RESULTS

### Establishment of the TBP-dependent yeast in vitro transcription system

To identify TBP mutants defective in acidic activator-dependent transcription but not basal transcription, we used a reconstituted yeast system which is absolutely dependent upon exogenously added bacterially expressed TBP (Fig. 1). Templates with and without GAL4 DNA binding sites (Fig. 2a) were included in each reaction to ascertain that activation was mediated through sequence-specific DNA binding. The activator GAL4(1-147)-VP16, consisting of the DNA-binding domain (amino acids 1-147) of GAL4 fused to the acidic activation domain (carboxy-terminal 78 amino acids) of VP16 (Fig. 2a), markedly stimulated transcription when GAL4 sites were present (Fig. 2b, lanes 1 and 2). The DNA binding domain of GAL4 alone did not stimulate transcription with either template (Fig. 2b, lane 4), and no transcription was detected in the absence of TBP (Fig. 2b, lanes 3 and 5). Thus, transcription in this reconstituted assay system depends on the activation domain of VP16 in addition to exogenous TBP (Fig. 2b).

### Identification of TBP mutants defective in VP16-activated transcription but not basal transcription

A series of point mutants in the conserved C-terminal core of yeast TBP was used to define amino acid residues important

for basal and activated transcription, and to further test their separability. The distinguishing features noted from the crystal structure analysis (Nikolov et al., 1992) are two highly symmetric domains containing both the direct repeat regions and corresponding flanking sequences that include a central basic repeat region; the latter was suggested as a candidate for direct interactions with the acidic activation domains (Horikoshi et al., 1989). Because lysine and leucine residues are distributed extensively in these regions, we focused on a series of point mutants containing either lysine to leucine or leucine to lysine changes (Yamamoto et al., 1992). In transcription assays with the basal transcription activity normalized, all but three of 31 mutant TBPs showed either undetectable activities or both basal and activator-dependent transcription activities comparable to those displayed by the wild-type TBP (Fig. 3). In contrast, mutants L114K (leucine to lysine at position 114), L189K (leucine to lysine at position 189) and K211L (lysine to leucine at position 211) were defective with respect to their ability to effect a response to GAL4(1-147)-VP16, but nonetheless maintained the normal level of basal transcription. Somewhat surprisingly, point mutants (K133L, K138L, K145L, K151L and K156L) with changes in the lysine residues present on one face of the  $\alpha$ -helix in the central basic repeat region showed no defect in transcriptional activation. These results suggest that regions other than the basic repeat region in TBP might be more important in the mediation of



transcriptional enhancement by an acidic activator GAL4(1-147)-VP16.

#### Pol I and Pol III transcription by activation-defective TBP mutants

Recently, it has been shown that TBP is required not only for transcription by RNA polymerase II but also for transcription by RNA polymerases I and III (Cormack and Struhl, 1992; Schultz et al., 1992; Kim and Roeder, 1994c). To further assess the specificity of the mutant TBPs (L114K, L189K and K211L) for VP16 activated transcription by RNA polymerase II, possible effects in transcription by RNA polymerase I and III were analyzed. As the above-described TBP-dependent transcription system was found not to support pol I or pol III transcription well, we utilized extracts derived from yeast containing the I143N mutation of TBP (Schultz et al., 1992; Kim and Roeder, 1994c) to test the ability of activation-defective mutants to rescue pol I or pol III transcription. As shown in Fig. 4, these mutants restored transcription from both pol I (35S rRNA) and pol III (5S rRNA) gene promoters just as effectively as did wild-type TBP. Thus, in so far as can presently be tested in the yeast system, L114K, L189K and K211L are specifically defective in acidic activator-dependent transcription by RNA polymerase II.

### Direct interaction between activation-defective TBP mutants and the acidic activation domain of VP16

Transcriptional activation by acidic activators may involve a number of interactions including direct interactions between the activator and TBP. As a first step to explore the molecular basis of the activation defects in these three mutants, the interaction between yeast TBP and the activation domain of VP16 was analyzed by coimmunoprecipitation (Ingles et al., 1991). Consistent with previous results (Ingles et al., 1991), radiolabelled TBP bound to Protein A-VP16, but not to Protein A, in this assay (Fig. 5, lanes 1-6 ; I, P and S lanes reflect input, pellet and supernatant, respectively). We then tested interactions between the three activation-defective TBP mutants and the activation domain of VP16. As shown in Fig. 5, mutant L114K showed a severe loss of VP16 binding ability compared to wild type TBP (lanes 7-9), whereas mutants L189K and K211L retained most of their ability to interact with the acidic activation domain of VP16 (lanes 10-15). In fact, control mutants (e.g. K97L and K239L) which effected normal levels of basal and activated transcription, showed comparably small losses of VP16 binding activity in this assay (lanes 16-30; data not shown). For each of these mutants the small amount of unbound TBP (Fig. 5) might reflect a minor intrinsic population of conformationally inactive forms of TBP that cannot bind VP16 under these conditions. These results showed that one of activation-defective mutations (L114K) affected direct

interactions with the acidic activation domain which were important for activation.

#### Interaction of activation-defective TBP mutants with TFIIA and/or TFIIB

In addition to interactions with the acidic activation domain, regulated TBP interactions with TFIIA and TFIIB have been suggested to be important for transcriptional activation (Meisterernst and Roeder, 1991a; 1991b; Lin and Green, 1991; Wang et al., 1992; Hahn, 1993). To further address the molecular defects in other activation-defective TBP mutants, we checked their abilities to interact with TFIIA and/or TFIIB by band-shift analysis (Fig. 6). Consistent with the demonstrated defect in VP16 interactions, mutant L114K formed TBP-TFIIA, TBP-TFIIB, and TBP-TFIIA-TFIIB promoter complexes as efficiently as did wild-type TBP. In contrast, while normal with respect to TBP-TFIIA complex formation (lane 11), mutant L189K was markedly deficient in forming a TBP-TFIIB complex stable to electrophoresis (lane 12). However, the fact that mutant L189K is still active in TBP-TFIIA-TFIIB complex formation (lane 13) indicates that stabilizing interactions dependent upon TFIIA can compensate for the reduced TFIIB binding potential, consistent with the unimpaired capacity of L189K for basal transcription. Since L114K and K211L are both defective for independent DNA binding (observed under different ionic conditions) but not for basal transcription, one or more other basal factors must

be able to compensate for this loss of intrinsic DNA binding potential. This occurs at the level of TFIIA for L114K (lane 8) but must involve factors in addition to TFIIA and TFIIB for K211L (lane 16) (Fig. 6). This kind of compensation for defective DNA binding activities was also observed in other TBP mutants (e.g. K110L, K120L, K127L, K201L, and K218L) which showed normal basal and activated transcriptions (Lee et al., 1992).

VP16 enhances TFIIB recruitment with wild-type TBP but not with mutant TBPs defective for direct VP16 or TFIIB interactions

Taken together, these results suggested that the altered TFIIB interaction with mutant L189K might reflect a defect that is manifested only in acidic activator-dependent transcription. To test this possibility, we performed factor recruitment experiments with DNA templates fixed to agarose beads (Lin and Green, 1991; Kim and Roeder, 1994b). The immobilized promoter (CYC1) was incubated with TBP and TFIIB in the presence or absence of GAL4(1-147)-VP16. After washing, the amounts of TBP and TFIIB retained on the template were determined by western blotting. In addition, this complex was assayed for in vitro transcription potential by complementation with appropriate yeast extracts depleted of TBP and TFIIB but containing other essential factors. As shown in Fig. 7, in the absence of GAL4(1-147)-VP16, wild-type TBP and activation-defective mutants L114K and L189K

resulted in comparable levels of bound TBP and TFIIB, as well as comparable levels of basal transcription. (The L189K TBP-TFIIB promoter complex is obviously more stable in this assay than in the band-shift assay, presumably due to more stringent conditions in the latter case). However, while incubation with GAL4(1-147)-VP16 significantly increased the amount of TFIIB recruited into the promoter complex in the presence of wild-type TBP, the activation-defective mutants L114K and L189K both showed marked reductions in the levels of TFIIB recruited under the influence of the activator (Fig. 7, lanes 3-6). Results identical to those in Fig. 7 were obtained when TFIIA was present in the recruitment assay (data not shown). Therefore, consistent with results of the band-shift analysis showing a quantitative loss of intrinsic TFIIB binding potential in the L189K mutant (Fig. 6), the recruitment assay also suggests that the L189K mutation appears to affect direct TBP-TFIIB interactions but, interestingly, only those which are somehow important only for transcriptional activation by an acidic activator (and not for basal transcription). Importantly, the results of the L114K analyses (inability to bind GAL4(1-147)-VP16 or to mediate enhanced TFIIB recruitment) also suggests that GAL4(1-147)-VP16 can enhance the recruitment of TFIIB to the promoter through interactions with TBP. The importance for activation of the specific TBP-TFIIB interactions demonstrated here is supported by previous data (Lin and Green, 1991; Choy and Green, 1993) indicating activator-

mediated recruitment of human TFIIB to a promoter complex (an alternative possibility is an activator-induced alteration or stabilization of TFIIB binding that in turn enhances recruitment of downstream factors) and is especially relevant in view of a recent model (Hahn, 1993) which invokes qualitative changes in TBP-TFIIB interactions by acidic activators. In contrast to the situation with mammalian factors, however, we (data not shown) and others (S. Johnston; personal communication) have been unable to document direct interactions of GAL4(1-147)-VP16 with yeast TFIIB, in further support of models involving either indirect effects (via TBP) of GAL4(1-147)-VP16 on TFIIB or simultaneous interaction of GAL4(1-147)-VP16 with TBP and TFIIB.

#### Effects of activation-defective TBP mutations on yeast cell growth

To test in vivo functions, we further analyzed the effects of the three TBP mutants on the growth of yeast cells after replacing the wild-type yeast TBP gene by plasmid-shuffle methods (Figs. 8 and 9). As shown in Fig. 9, L114K exhibited an extreme temperature-sensitive phenotype and L189K resulted in a failure to complement a disrupted yeast TBP gene, consistent with in vitro results. Since mutants L114K, L189K and K211L can support pol I and pol III transcriptions efficiently (Fig. 4), the defective in vivo phenotype is most probably due to the failure to activate transcription of

certain class II genes. In contrast, mutant K211L grew almost normally at 30° and 37°C. We cannot discard the possibility that K211L mutation might affect some other minor in vivo functions. At the same time, the contrasting properties of K211L in vivo and in vitro may reflect some property (e.g. conformational instability) unique to the in vitro conditions.

## DISCUSSION

In these studies, we have succeeded in isolating TBP mutants (L114K, L189K and K211L) with selective defects in activator-dependent transcription and used these mutants to document specific interactions important for activation (summarized in Fig. 10): Thus, (1) Mutant L114K provides important confirmation of the functional relevance of the previously reported physical interactions between TBP and VP16 (Ingles et al., 1991); and (2) The other two mutants L189K and K211L provide strong evidence for the involvement of other kinds of interactions important solely for activation, including an activation-specific TFIIB interaction in the case of L189K. Importantly, these two conclusions have reconciled previous observation by Ingles et al. (1991) that VP16 binds TBP and by Lin and Green (1991) that VP16 causes the recruitment of TFIIB.

These studies also provide the first indication in yeast of an activation mechanism which involves enhanced TFIIB recruitment. Since we observed the involvement of two (or possibly three including K211L) different activation-defective TBP mutants (L114K, L189K) on activator-induced recruitment of TFIIB, TFIIB recruitment is an important mechanism for transcriptional activation in the eukaryotic systems. TFIIB recruitment may be induced through VP16 interactions with either TBP or TFIIB, or possibly through



simultaneous interactions with both in yeast. The consideration of TFIIB as a joint target with TBP is quite relevant since: (1) VP16 has been shown to interact with TFIIB and to induce TFIIB recruitment in a human system (Lin and Green, 1991; Roberts et al., 1993); (2) some VP16 coactivators (Berger et al., 1990; 1992; Goodrich et al., 1993; L. Guarente, personal communication) have been shown to interact with TFIIB in both yeast and human systems; and (3) the view involving multiple targets in basal transcription machinery can help to explain how eukaryotic activators stimulate transcription synergistically, especially given multiple activator binding sites in the template used in this study (Lin et al., 1990).

We showed that VP16 could induce activation-specific changes in TBP-TFIIB interactions through direct interactions with TBP. In direct DNA-binding studies, TBP has been shown to interact with reasonable affinity to DNA and the resultant TBP-DNA complex is relatively stable even in the absence of activators (Schmidt et al., 1989; Hahn et al., 1989; Sundseth and Hansen, 1992). Thus it is reasonable to expect that some activators affect other TBP interactions to increase the efficiency of transcriptional initiation. Consistent with this possibility, our data have suggested that VP16 induces qualitative changes in TFIIB interactions with TBP. This observation of an alteration of TFIIB binding potential of TBP is especially relevant in view of the recent model (Hahn,

1993) which invokes qualitative changes in TBP-TFIIB interactions associated with activator function and in view of previous data (Lin and Green, 1991; Roberts et al., 1993; Choy and Green, 1993) showing activator-TFIIB interactions and activator-induced recruitment of TFIIB to the TBP promoter complexes. Especially, recent data (Choy and Green, 1993) show that increased concentrations of TFIIB itself were unable to overcome the requirement for an activator. This result, along with present data, suggests that the activator does not simply increase the recruitment of TFIIB to the promoter complex, or even the stability of the resulting complexes, but rather that it induces qualitative changes in the structure of the TBP-TFIIB-DNA complexes.

In summary, our present data suggest that GAL4(1-147)-VP16 may either recognize a TBP-DNA complex and induce an activation-specific conformation that facilitates functional interactions (and increased recruitment) of TFIIB or, alternatively, that it may recognize and stabilize a conformation-specific TBP-TFIIB-DNA complex and, consequently, enhance TFIIB recruitment. The enhanced TFIIB recruitment mediated by GAL4(1-147)-VP16 in conjunction with TBP may nonetheless require additional coactivators for the functional consequences (enhanced transcription) to be realized (Flanagan et al., 1991; 1992; Berger et al., 1990; 1992). Thus it will be important to ascertain whether these coactivators have any effects on the GAL4(1-147)-VP16-TBP or

TBP-TFIIB interactions described here, or whether they act through other interactions. In this regard, the properties of the activation-defective K211L mutant (normal with respect to GAL4(1-147)-VP16 interactions) are consistent with the possibility of cofactor interactions with a specific domain of TBP. Clearly, the isolation of yeast TBP mutants showing selective effects on activator-dependent transcription relative to basal transcription will be helpful in further elucidating the activation mechanism.

The positions of the three altered amino acids in the three-dimensional structure of TBP are illustrated in the figure of the Appendix (Nikolov et al., 1992). They are found within strand S4 of the antiparallel  $\beta$ -sheet (L114), within the loop connecting strands S2' and S3' (L189) and at the beginning of strand S5' (K211). The loop region between S2' and S3' (site of L189) is particularly interesting because of its probable conformational flexibility and its potential, as a result of its exposed position, for direct protein-protein interactions. The other two mutations (L114K and K211L) map to strands in the concave surface known to be involved in interactions with DNA in a TBP-DNA complex (Kim et al., 1993). This raises the possibility that TBP has overlapping or closely interdigitated DNA binding and activation-specific domains (Ptashne, 1986; Schena et al., 1989; Keaveney et al., 1993), whose positions also might change during preinitiation complex assembly. The case of class III promoter activation

in yeast provides a relevant example of dynamic changes in the primary DNA-protein interactions during preinitiation complex assembly (Kassavetis et al., 1992). Related to the above, there are several lines of evidence for conformational changes in TBP-promoter complex structure. Recent studies have shown that TFIIA induces conformational changes in TBP, inducing altered contacts in the TATA region (Lee et al., 1992), and the altered DNA binding activity of the L114K mutant is known to be rescued by TFIIA (Lee et al., 1992). So concomitant or subsequent conformational changes in TBP induced by TFIIA (Lee et al., 1992) or by other initiation factors (e.g. TFIIB) or regulatory factors might allow activator-specific interactions of TBP (e.g. VP16 interactions with or near residue L114). At the same time, the activation defect might result from subtle conformational changes in mutant TBP-DNA complexes (e.g. hinge bending of TBP and/or kinking of DNA; S. Burley, personal communication) that affect only activator-specific transcription functions. Ultimately an understanding of dynamic structural changes in TBP-DNA complexes during preinitiation complex assembly will be important for understanding transcriptional regulatory mechanisms.

Fig. 1 Set up the TBP-dependent yeast in vitro transcription system.

Yeast basal transcription factors and mediator were prepared by chromatography of yeast whole cell extracts on Bio-Rex 70 (Bio-Rad) and DE 52 (Whatman) (Flanagan et al., 1992).

Fraction C was prepared from yeast nuclear extracts as described (Flanagan et al., 1992).

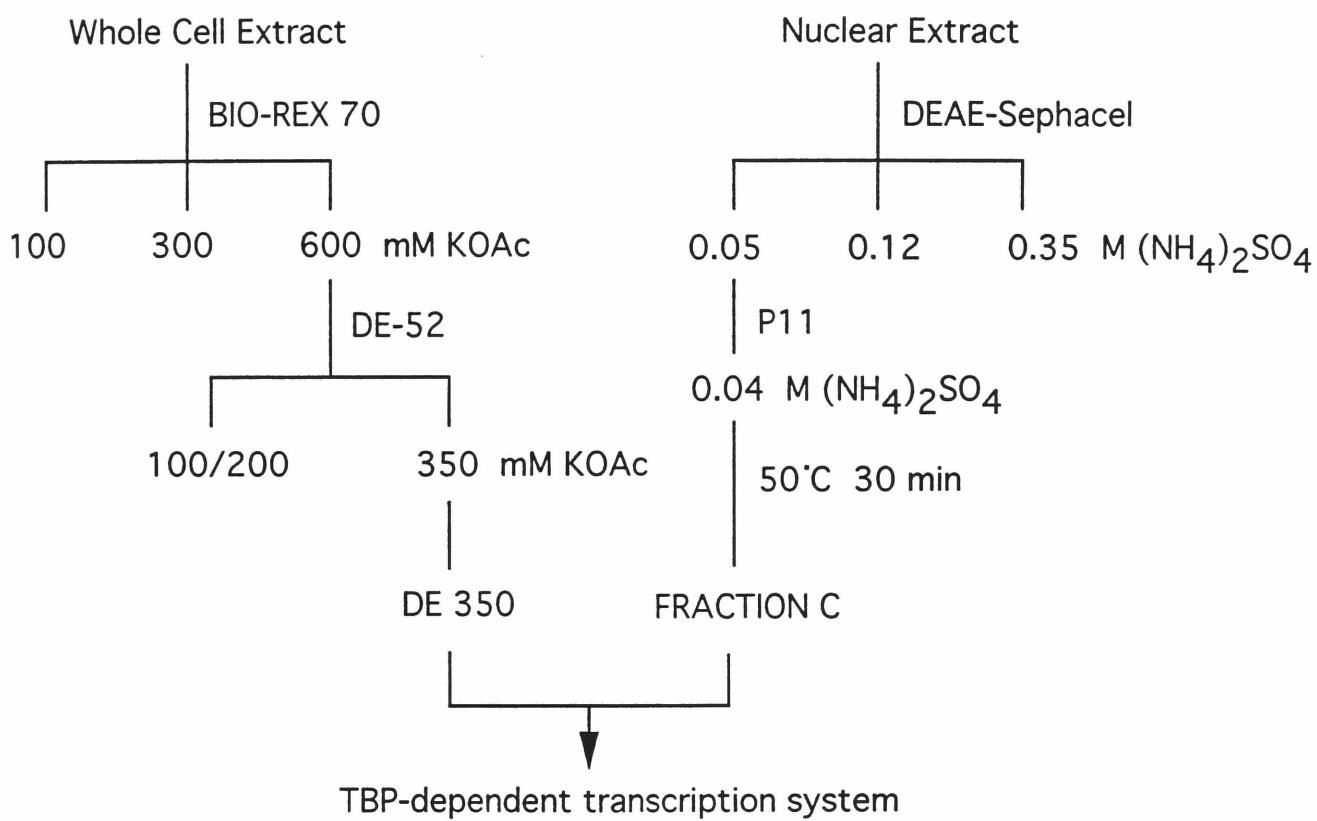
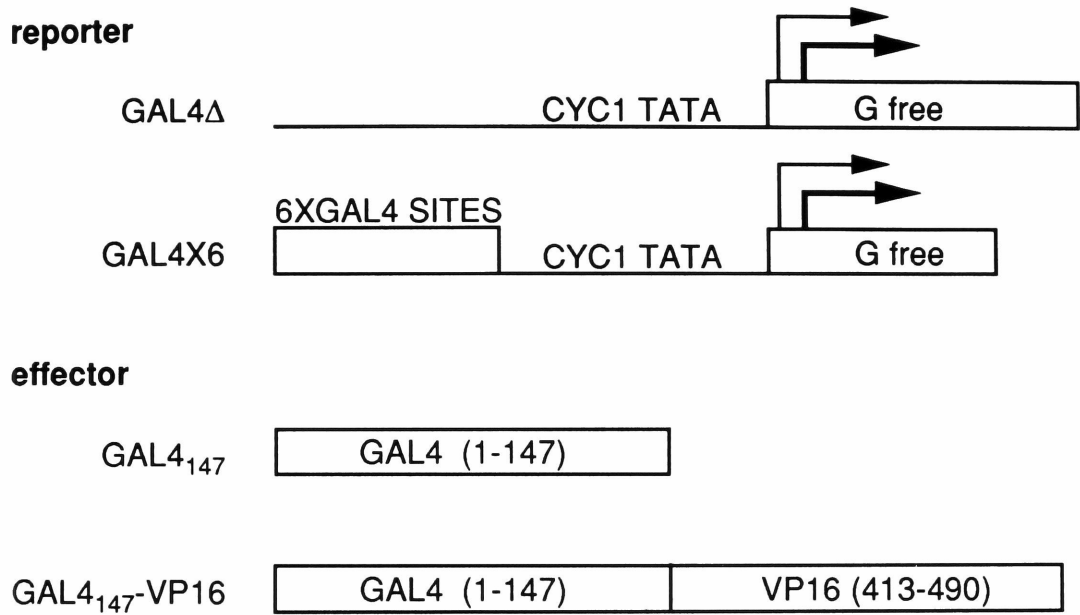


Fig. 2 TBP-dependent transcription system.

a, Constructions used in this study. The template DNAs for in vitro transcription contain a TATA box from the CYC1 promoter with or without six GAL4 binding sites. The basal template contains a G-less cassette of 400 base pairs and yields two major transcripts of 375 and 350 base pairs in vitro. The activation template with GAL4 binding sites has a 100 base pair-truncated G-less cassette. The chimeric GAL4(1-147)-VP16 protein is a fusion between the amino-terminal 147 amino acids of GAL4 and the carboxy-terminal 78 amino acids of VP16. The GAL4(1-147)-VP16 was expressed and purified in bacteria (Chasman et al., 1989).

b, In vitro transcription analysis. Transcription reactions were performed in the reconstituted yeast system in the presence of no activator (lane 1), 100 ng of GAL4(1-147)-VP16 fusion protein (lanes 2 and 3), 100 ng of control GAL4 protein containing the first 147 amino acids of GAL4 (lanes 4 and 5) or no bacterially expressed yeast TBP (lanes 3 and 5). In vitro transcription system used in this study was reconstituted as described in Fig. 1. The specific transcripts from basal (GA) and activation-specific (G6X) templates are indicated by arrows.

**a**



**b**

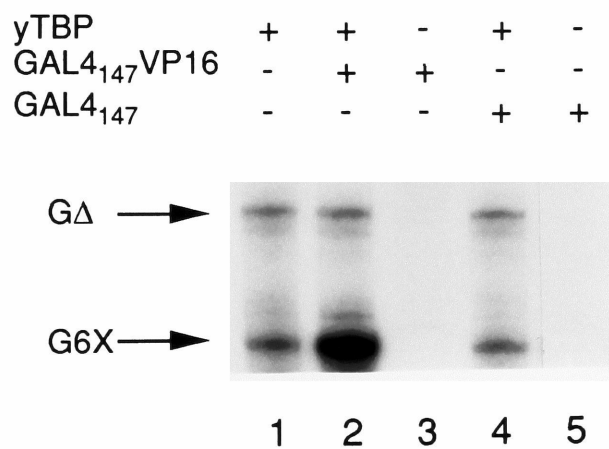


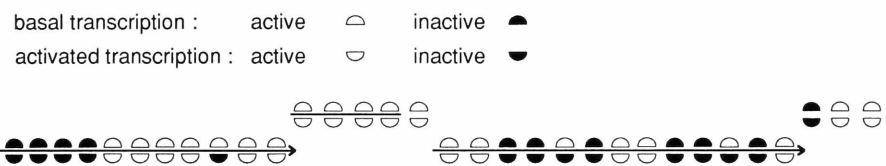


Fig. 3 Analysis of yeast TBP point mutants for activator-dependent versus basal transcription.

a, Schematic representation of structural motifs in TBP and summary of the effects of mutation on basal transcription and activation by GAL4(1-147)-VP16. The C-terminal conserved core domain of TBP contains two direct repeat domains (arrows) separated by a region rich in basic amino acids (solid line). The open and closed half circles indicate the basal versus activator-dependent transcription activities of the mutant TBPs indicated above the corresponding lanes in b. All of the mutants which failed to mediate basal or activated transcription also failed to show TATA binding in previous analyses (Yamamoto et al., 1992).

b, Transcription analysis with TBP mutants. Lanes 1-31 show analyses of responses to GAL4(1-147)-VP16 by TBP species mutated at the positions indicated above each lane (L67K indicates leucine changed to lysine at position 67, etc.). Lanes 33 and 34 contain, respectively, wild-type TBP without GAL4(1-147)-VP16 and GAL4(1-147)-VP16 without TBP. The arrows indicate specifically-initiated transcripts from basal (G $\Delta$ ) and activator-dependent (G6X) templates. Wild-type and mutant TBPs were expressed as hexa-histidine fusion proteins in bacteria and purified by nickel column chromatography to about 80 % purity (Hoffmann and Roeder, 1992). Each purified protein was assayed in the TBP-dependent reconstituted system. All transcription was done under conditions described in Fig. 2.

**a**



**b**

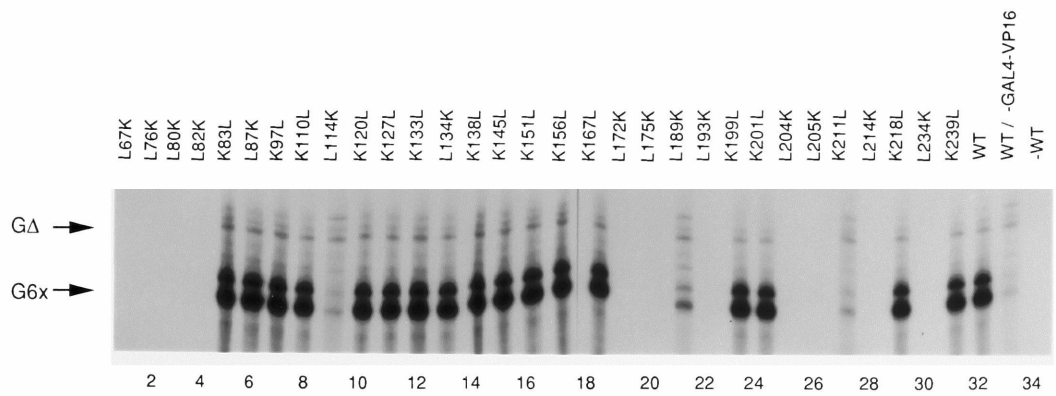


Fig. 4 Pol I and Pol III transcription by activation-defective TBP mutants.

The effects of mutations on the in vitro transcription activity of pol I or pol III were determined by using extracts from the temperature sensitive yeast strain containing TBP mutant (I143N) (Schultz et al., 1992; Kim and Roeder, 1994c). Yeast strain with I143N TBP mutant was grown at the permissive temperature (24 °C) to an A<sub>600</sub> of 1.8. It was then grown for another one hour at the nonpermissive temperature (37 °C). Extracts were prepared using a mortar and pestle, and transcription reactions from yeast 35S rRNA gene promoter or yeast 5S rRNA gene promoter were performed with 20-40 ng of TBP as described (Schultz et al., 1992; Kim and Roeder, 1994c).

**a**

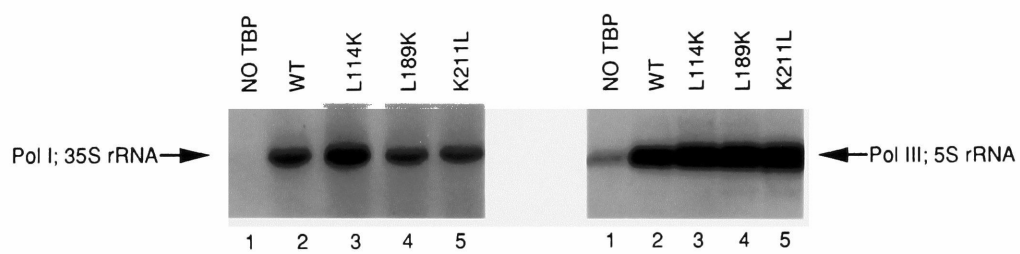


Fig. 5 Direct interaction between activation-defective TBP mutants and the acidic activation domain of VP16.

The ability of TBP mutants to interact with the acidic activation domain was analyzed by coimmunoprecipitation experiment (Ingles et al., 1991).  $^{35}\text{S}$ -labelled in vitro-translated TBPs were incubated with Protein A-VP16 or Protein A as a negative control. Bound TBPs were co-immunoprecipitated with agarose beads loaded with IgG. Bound proteins present in the pellet (P) were released by boiling in SDS buffer and separated, along with input proteins (I) and unbound proteins in the supernatant (S), on a 15 % acrylamide gel.

1 2 3 4 5                      1 2 3 4 5

**b**

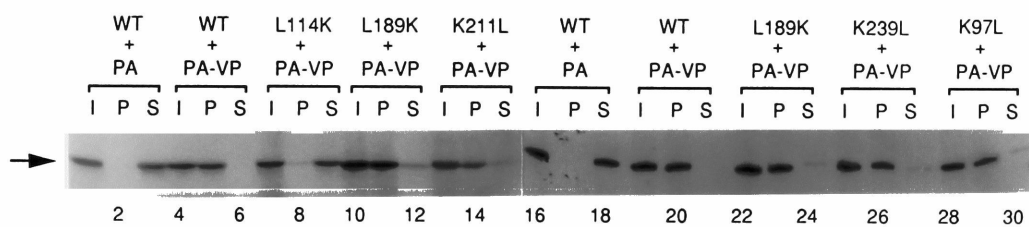


Fig. 6 Interaction of activation-defective TBP mutants with TFIIA and/or TFIIB.

The activation-defective mutants were checked for the formation of TBP-TFIIA-TFIIB complex by band-shift analysis (Auble and Hahn, 1993). Band-shift analysis was performed on a polyacrylamide gel containing no glycerol and magnesium acetate as described (Auble and Hahn, 1993). Under this condition, TBP alone cannot form a complex unless DNA interaction is stabilized in the presence of other factors, TFIIA or TFIIB. Two subunits of yeast TFIIA (TOA1 and TOA2) were purified from an insoluble fraction of lysed bacteria (Ranish et al., 1992). Yeast TFIIB was purified as a hexahistidine fusion protein by nickel chromatography (Hoffmann and Roeder, 1992).

**c**

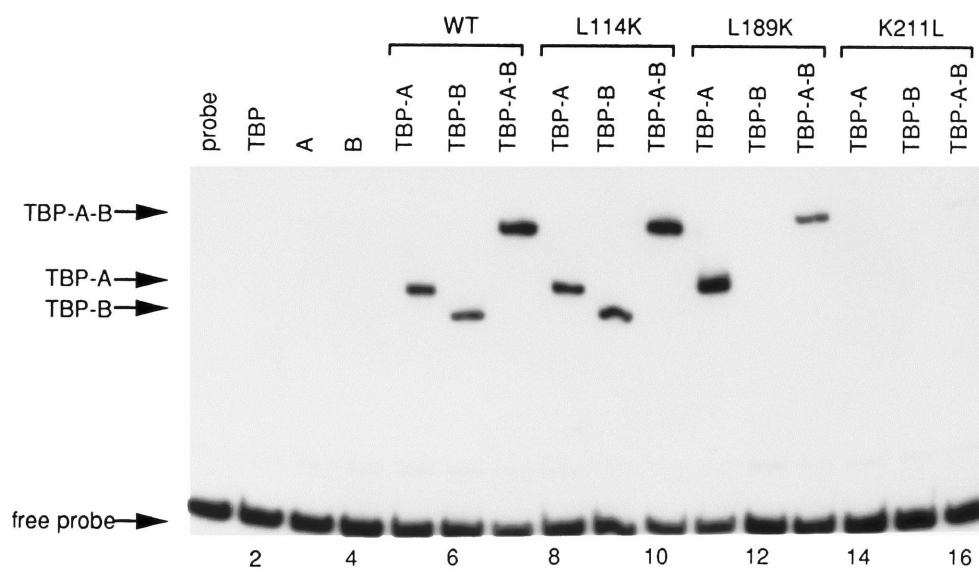




Fig. 7 Analysis of activation-defective mutants for TBP-TFIIB-promoter complex assembly in the immobilized template assay.

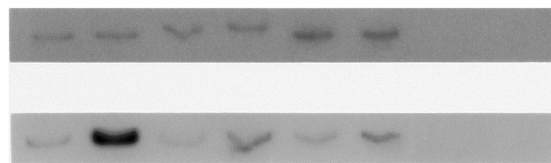
The effects of mutations on the TBP-TFIIB-promoter complex formation in transcription reactions were analyzed with templates immobilized to agarose beads (Lin and Green, 1991; Kim and Roeder, 1994b). The DNA fragment containing six GAL4 binding sites upstream of the CYC1 TATA box and G-less cassette was isolated from pGAL4X6 template, biotin-labelled, and then coupled to streptavidin-agarose beads. The beads with immobilized DNA templates were incubated with TBP and TFIIB in the presence (+) or absence (-) of GAL4(1-147)-VP16 for 90 min at 30 °C. After the first incubation, the beads were washed with transcription buffer. The beads were then subject to western blotting or in vitro transcription with complementation of other essential yeast transcription factors. Complementing factors were prepared by immunodepletion of yeast TFIIB from Bio-Rex 70-DE 52 fractions described in Fig. 1 with anti-yTFIIB antisera. In contrast to the results in western-blotting analyses, the failure to detect the L189K TBP-TFIIB interactions in the band-shift assay shown in Fig. 6 is most probably due to more stringent conditions.

***d***

TBP	WT		L114K		L189K		K211L	
IIB	+	+	+	+	+	+	+	+
GAL4-VP16	-	+	-	+	-	+	-	+

TBP →

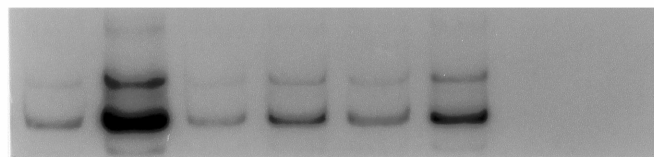
IIB →



1 2 3 4 5 6 7 8



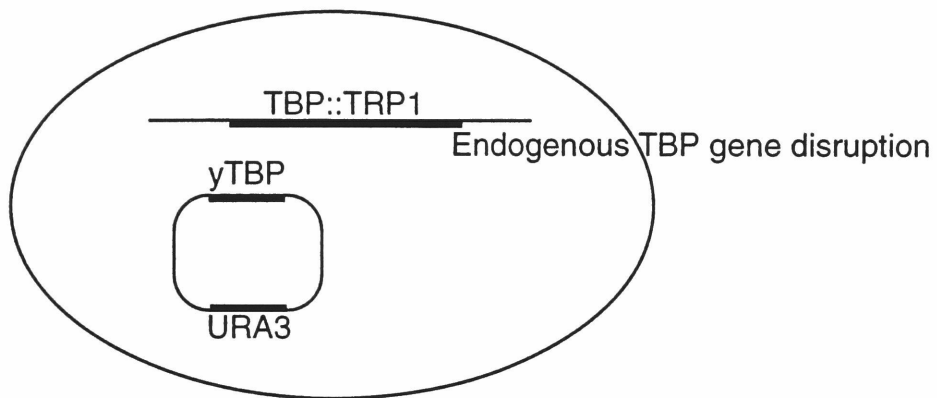
Missing Factors + NTPs  
RNA analysis



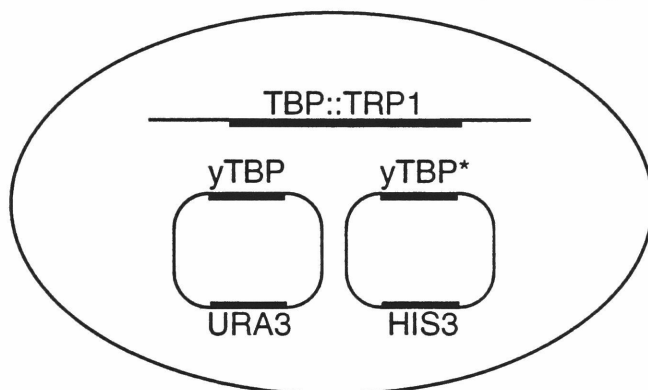
1 2 3 4 5 6 7 8

Fig. 8 Plasmid shuffling method to test the in vivo functions of activation-defective TBP mutants.

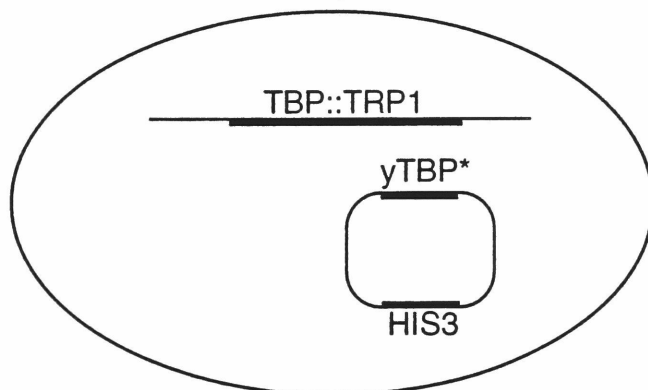
The plasmid shuffle complementation assay was used to determine the function of three yeast TBP mutants in yeast cells. The endogenous yeast TBP gene of the test strain was disrupted by replacing the 5' end of the gene, including amino acid 1-81, with the TRP1 gene. This yeast strain (YTW22) carries the wild-type yeast TBP gene on a single-copy URA3 plasmid. Mutated TBPs were introduced into these cells on a multicopy plasmid bearing the HIS3 gene as a selectable marker. The transformants were replica plated onto media containing 5-FOA to select against the URA3 plasmid containing the wild-type yeast TBP. Thus the strain is only viable on 5-FOA plates if the TBP allele on the HIS3 plasmid functionally substitute for wild-type TBP.



Transform yeast with **HIS3** plasmid expressing **yTBP\***



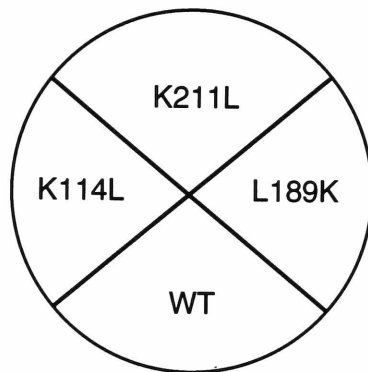
5-FOA plates to select for cells that have lost the **URA3** plasmid



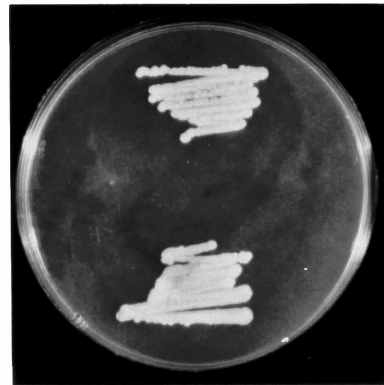
in vivo function analysis

Fig. 9 Effects of activation-defective TBP mutations on the growth of yeast cells.

The analysis employed yeast strain YTW22 containing a deleted version of the endogenous TBP gene and a URA3 plasmid expressing TBP gene for cell growth. The TBP mutants were introduced into this cell on the plasmid containing the HIS3 marker. The transformants were replica-plated onto media containing 5-FOA to select against cells carrying wild-type TBP gene present on the URA3 plasmid. Each set of replica plates was incubated at 30° or 37°C.



30 °C



37 °C

Fig. 10 Summary of properties of activation-defective TBP mutants.

See the text in details.

	L114K	L189K	K211L	WT
basal transcription in yeast	++++	++++	++++	++++
activation by VP16 in yeast	+/-	++	+/-	++++
Pol I/III transcription in yeast	++++	++++	++++	++++
direct interaction with VP16	-	+	+	+
interaction with DNA <sup>10</sup>	-	+	-	+
interaction with TFIIA on DNA	+	+	-	+
interaction with TFIIIB on DNA	+	-	-	+
interaction with TFIIA and TFIIIB on DNA	+	+	+	+
VP16-induced TFIIIB recruitment	-	-	?	+



## CHAPTER IV

Transcriptional activation mechanism by acidic activators in  
human: Different response to acidic activators by TBP mutants  
in the yeast and human in vitro transcription systems

## ABSTRACT

We previously identified three yeast TBP point mutants (L114K, L189K and K211L) which are defective in transcriptional activation by the acidic activator GAL4(1-147)-VP16 in a yeast-derived TBP-dependent in vitro transcription system (Kim et al., 1994d). Here, the transcriptional activities of these mutants are determined in a TBP-inactivated heat-treated human (HeLa) nuclear extracts. Surprisingly, TBP point mutants mediate almost normal activation by acidic activators in the human system, in contrast to the defective responses in the yeast system. This may reflect the involvement of different regulatory interactions (possibly of TBP) in the function of acidic activators in yeast and human.

## INTRODUCTION

TBP has been proposed to be the target, direct or indirect, of several upstream activators in addition to its crucial role in basal transcription (reviewed in Roeder, 1991; Greenblatt, 1991). Comparison of amino acid sequences of TBPs from a variety of organisms reveals a highly conserved carboxy-terminal domain of 180 amino acids and a variable amino-terminal domain. The conserved carboxy-terminal core is known to be sufficient for binding to the TATA box and for basal transcription in vitro (Horikoshi et al., 1990; reviewed in Hernandez, 1993). Despite striking structural and functional similarities among eukaryotic TBPs, human TBP (hTBP) fails to substitute in vivo for yeast TBP (yTBP) (Gill and Tjian, 1991; Poon et al., 1991; Cormack et al., 1991). One possibility is that TBP might be involved in species-specific interactions with other transcription factors.

However, recent studies have shown that yTBP can support both basal and activated transcription by acidic activators in a human in vitro system and that hTBP can be responsive to acidic activator in yeast cells (Strubin and Struhl, 1991; Kelleher et al., 1992). This interchangeability of y- and hTBPs argues against species-specific interactions at least for the response to acidic activators. Despite this functional interchangeability, hTBP is less active for the response to acidic activators than yTBP in yeast, determined

from the level of reporter gene expression in vivo and in vitro (Strubin and Struhl, 1991; Kelleher et al., 1992). Y- and hTBPs do differ in another respect: hTBP is found in a complex consisting of coactivators or associated factors (TAFs) (Goodrich et al., 1993), whereas yTBP is not stably associated with them in yeast cells (Flanagan et al., 1991; 1992; Berger et al., 1990; 1992). Since these factors are required to mediate transcriptional activation by acidic activators, one might expect that different activator interactions (possibly of TBP) are involved in the context of either the yeast or human transcription system. To assess possible species-specific interactions within the activation machinery and to compare the activation pathways for the acidic activators in yeast and human, we determined the ability of yTBP mutants, which are defective for activation in the context of the yeast system, to mediate transcriptional activation by acidic activators in the human system. The results showed normal activated transcription by those yTBP mutants, in sharp contrast to their defective responses to GAL4(1-147)-VP16 in yeast (Kim et al., 1993b).

## EXPERIMENTAL PROCEDURES

### Preparation of TBPs

Wild-type and mutant TBPs were expressed as hexahistidine fusion proteins in E.coli strain BL21(DE3)pLysS, using the T7 polymerase expression system. The expressed TBPs were purified from cell lysate by nickel affinity chromatography (Hoffmann and Roeder, 1992).

### In vitro transcription reaction

GAL4(1-147)-VP16 and GAL4(1-147)-AH were expressed in E.coli and purified as described before (Chasman et al., 1989). HeLa nuclear extract was prepared according to Dignam (1983) and was heated for 15 min at 47 °C to inactivate endogenous TBP activity (Nakajima et al., 1988). The transcription reactions were performed and analyzed as described (Nakajima et al., 1988). TBP-dependent yeast in vitro transcription system was derived from fraction C and BIO-REX-DE52 fractions and transcription was done as described (Kim et al., 1994d; Flanagan et al., 1992).

### HeLa reconstituted transcription systems

Transcription factors TFIIA, TFIIE, TFIIIF, TFIIH, TFIID as well as USA fraction were fractionated from HeLa nuclear extracts on a phosphocellulose column (P11) as described (Dignam et al., 1983). TFIIA was enriched on DEAE-cellulose and Mono S as reported before (Meisterernst et al., 1991a).

All subsequent chromatographic steps were performed in buffer A (Nakajima et al., 1988) with DTT (5 mM). The Mono S 0.10 M KCl flowthrough fraction was loaded on a Mono Q column, which was developed with a linear gradient from 0.10-0.50 M KCl. TFIIE, TFIIF, and TFIIH were copurified by application of the P11 0.5 M KCl fraction to a DEAE-cellulose column and elution with a linear gradient (0.10-0.50 M KCl). Fractions in the range of 0.13-0.19 M KCl were pooled and loaded without dialysis onto a Mono S column. Active fractions were applied to a DEAE-Sepharose column. Bound TFIIE and TFIIF were coeluted with 0.25 M KCl.

For TFIID and USA purification, the P11 0.85 fraction was loaded onto a DEAE-cellulose column. The column was developed in buffer BD (Meisterernst et al., 1991a). TFIID was eluted with 0.25 M KCl and loaded on a Mono S column. In linear gradient from 0.1-1.0 M potassium glutamate in buffer BD, TFIID eluted from 0.40-0.60 M potassium glutamate. The 0.10 M KCl flowthrough fraction of the DEAE-cellulose column, which contained USA activity, was loaded onto a Heparin-Sepharose column, eluted with 0.50 M KCl.

RNA polymerase II was purified from HeLa nuclear pellet extracts and chromatographed through Heparin-Sepharose, DEAE-cellulose and Mono Q columns as described before (Meisterernst et al., 1991a). In vitro transcription was performed in a 25 ul reaction mixture containing 100 ng pG5HMC2AT, 0.5 ug of TFIIA fraction, 10 ng of recombinant TFIIB fraction, 0.3 ug of TFIIE/TFIIF/TFIIH fraction and 0.3

ug of RNA polymerase II in the presence or absence of activators.

#### Coimmunoprecipitation assays

<sup>35</sup>S-methionine-labelled hTFIIB and yTFIIB were generated by in vitro translation of corresponding cDNA-derived mRNAs. In vitro labelled proteins were incubated with Protein A-VP16 and then coimmunoprecipitated with Sepharose beads loaded with IgG (Ingles et al., 1991; Kim et al., 1994d). The precipitated fractions were then separated by 15 % SDS-PAGE and detected by autoradiography.

## RESULTS

The mutations which selectively abolish VP16-activated transcription in yeast are located in the conserved carboxy-terminal domain and, furthermore, the mutated residues are identical at the corresponding positions of yeast TBP (yTBP) and human TBP (hTBP) (Kim et al., 1994d) (Fig. 1A). These TBP mutants were expressed in and purified from bacteria (Fig. 1B), and then employed in vitro transcription assays.

### The TBP mutants respond differently to GAL4-VP16 in the yeast and human transcription systems

In order to investigate the activities of activation-defective yTBP point mutants in the HeLa transcription system, the endogenous TBP activity in a HeLa nuclear extract was inactivated by heat treatment (Nakajima et al., 1988). Consistent with previous results (Kelleher, 1992), addition of wild-type yTBP to the heat-treated HeLa extract restored both VP16-activated transcription and basal transcription to levels similar those obtained with unheated extracts (Fig. 2). Under these conditions, we tested the response of activation-defective yTBP mutants to the acidic activators. Surprisingly, mutants L114K, L189K and K211L, which do not mediate normal activation in the yeast system, almost fully support the response to GAL4(1-147)-VP16 in the human in vitro transcription system (Fig. 3A). Similar results were



also obtained in the human in vitro reconstituted system (data not shown).

#### Promoter-specific effects are not the cause for contrasting behavior of TBP mutants

The contrasting activities of yTBP mutants in the yeast versus the human system might be due to the different promoters used in the respective assays: HIV-1/Ad2ML fusion promoter (five GAL4 binding sites preceding the HIV TATA box and the Ad2ML promoter initiator region) was used in the human whereas a CYC1 promoter was used in the previous yeast transcription assay. To rule out this possibility, we determined the transcription activities of the three TBP mutants in the yeast in vitro transcription system with templates containing the human promoter (HIV-1/Ad2ML). Using the human promoters, mutants (L114K, L189K and 211L) are defective for GAL4(1-147)-VP16 activation in the yeast system (Fig. 3B, lanes 5-10). Therefore, the differences in the activity of mutant TBPs for activation between the yeast and human systems are not due to the different promoters used to perform the assay. This result suggests that specific protein-protein interactions, rather than promoter-specific effects, are responsible for the difference in the response of yTBP mutants to GAL4(1-147)-VP16 in yeast and human.

#### Contrasting behavior of TBP mutants is also observed in activation by GAL4-AH in yeast and human

We also tested whether species-specific interactions are important for activation by other acidic activators, for example, GAL4(1-147)-AH. As with GAL4(1-147)-VP16, mutants (L114K, L189K and K211L) supported activated transcription in human as efficiently as did wild-type TBP and thus showed defective activation only in the yeast system (Fig. 4). Thus, the species-specific interactions that account for the difference in activity of yTBP mutants in the yeast and human systems may be involved in transcriptional activation by this class of activators, so called acidic activators (e.g. GAL4(1-147)-VP16 and GAL4(1-147)-AH).

Wild-type yTBP and hTBP respond differently to GAL4-VP16 in the yeast system, but not in the human system

Our results demonstrated that some yTBP point mutants could support almost normal activation by acidic activators in the human system, in contrast to the defective responses in the yeast system. This contrasting activity could be due to different protein-protein interactions in yeast and human transcriptional activation machinery. Such a difference might result in different efficiency of activation mediated by wild-type y- and hTBPs in addition to TBP mutants in each of the two transcription systems. To test this possibility, wild-type y- and hTBP were compared for the ability to support activated transcription in a TBP-dependent human and yeast in vitro transcription systems. Wild-type yTBP was indistinguishable from wild type hTBP in support of

activation by GAL4(1-147)-VP16 in the human in vitro system (Fig. 5). In contrast, wild-type hTBP supported the response to acidic activators less efficiently than wild-type yTBP in the yeast in vitro transcription system (Fig. 5). This is consistent with the defective response of TBP point mutants only in yeast. Apparently, the human transcription system is less sensitive to changes in the identity of TBP than is the yeast transcription system.

## DISCUSSION

Here we have shown that certain TBP point mutants (L114K, L189K and K211L) mediate normal acidic activator response in the human system, whereas they can not do so in the yeast system. This suggests that there are some interactions (possibly of TBP) within the transcriptional activation machinery which are not conserved from yeast to human. This species-specific behavior of TBP might be one of reasons for the failure of hTBP to substitute for yTBP in vivo and to support yeast cell growth despite striking primary structural homology in y- and hTBPs (Gill and Tjian, 1991; Poon et al., 1991; Cormack et al., 1991). The contrasting behavior of yTBP point mutants for the response to acidic activators in yeast and human could be explained by two models which are not exclusive to each other (Fig. 6).

It is known that stimulation of yeast RNA polymerase II transcription by acidic activators is dependent upon cofactors (mediator, adaptor, and fraction C) distinct from the general transcription factors (Kelleher et al., 1990; Berger et al., 1990; 1992; Flanagan et al., 1991; 1992). Similarly, fractions known as coactivator (TAFs; TBP-associated factors) and general cofactors (USA) have been described in the human system, which are required for transcriptional activation and are distinct from the general transcription factors (reviewed in Roeder 1991; Hernandez et

al., 1993). The action of acidic activators and these regulatory factors may involve interactions with general transcription factors, and TBP has been implicated as one of targets of such interactions. Notably, human coactivators (TAFs) are tightly associated with hTBP, whereas yeast regulatory factors (mediator, adaptor, fraction C) are readily separable from yTBP. Such cooperative interactions existing only within hTBP can be reflected by differences in the strength and specificity of the protein-protein interactions in the yeast and human transcription activation machineries. Given that certain interactions are disrupted, cooperative interactions of hTBP and TAFs could readily overcome this defect in the human transcription system. These regulatory interactions could be further stabilized by species-specific interactions with other basal transcription factors which transduce the activation signal from TBP to RNA polymerase II in the preinitiation complex. In particular, TFIIB, another target of acidic activators, might play an important role in stabilizing these interactions. Related to this, at least one of TAFs important for transcriptional activation by VP16 is shown to interact with TFIIB (Goodrich et al., 1993).

TFIIB is known to be a joint target with TBP of acidic activators (Lin and Green, 1991). Interestingly, we have observed that hTFIIB does bind to the acidic activation domain of VP16. By contrast, yTFIIB failed to interact with

VP16 (data not shown). Thus the contrasting activation functions of yTBP mutants could be due to the difference between y- and hTFIIB with respect to their binding to the acidic activation domain. Related to the differential interaction of VP16 with y- and hTFIIB, y- and hTFIIB show relatively low sequence homology and yTFIIB apparently fails to substitute for its human and rat homologs in a reconstituted in vitro transcription systems. It is possible that a direct contact between VP16 and TFIIB contributes to transcriptional activation by acidic activators in the context of the human machinery but not in yeast. Therefore, the yTBP mutant may have failed to support activated transcription by acidic activators by affecting activation specific interactions within TBP whereas these defective interactions could be compensated or overcome by interactions of TFIIB with activation machineries in the human system. The possible involvement of TFIIB for the contrasting behavior of yTBP is further supported by the observation that the proline-rich activator CTF1 interacts with TFIIB but not with TBP and can significantly stimulate transcription with yTBP mutants which are employed in this study (data not shown).

A final assessment of the importance of species-specific interactions in the view of proposed two models awaits complete isolation of components involved in transcriptional activation in yeast and human. Lastly, this kind of comparative studies in different species such as yeast and

human will be helpful to yield insights into the fundamental aspects of transcription mechanism in eukaryotes.

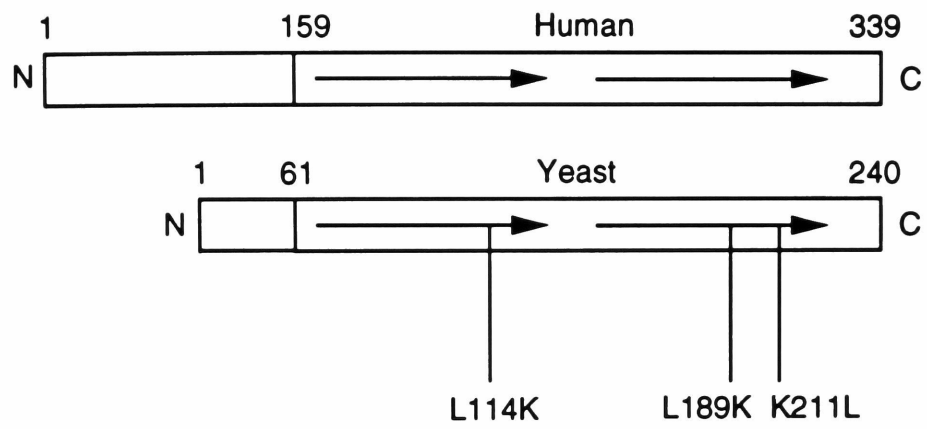
Fig. 1 Activation-defective yeast TBP point mutants used in this study.

(A) Comparison of the structure of yeast and human TBPs. The open boxed region indicates the carboxy-terminal domain of 180 amino acids that is strongly conserved among all eukaryotes (reviewed in Hernandez, 1993). Arrows represent almost perfect symmetric direct repeats present in the carboxy-terminal domain (Nikolov et al., 1992). The location of mutations used in the study are indicated with numbers. Abbreviations from amino acid residues are: K, Lys and L, Leu.

(B) SDS-PAGE analysis of the purified yeast TBP mutant proteins used in transcription assays. Wild type or mutant yeast TBPs are purified and indicated above of each lanes of SDS-PAGE.



**A**



**B**

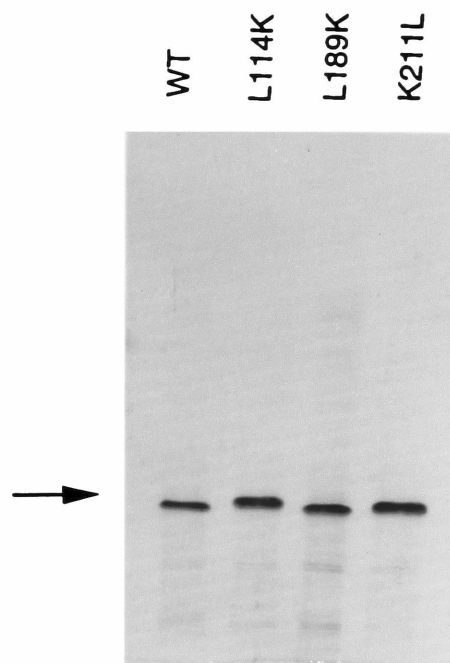


Fig. 2 Set up the TBP-dependent human in vitro transcription system.

Template was pG5HMC<sub>2</sub>AT which contains five GAL4 binding sites upstream of the HIV-1 core and the ML initiator promoter region fused to a G-less cassette. TBP was inactivated by heat-treatment of a HeLa nuclear extract and then transcription reactions were performed as described (Nakajima et al., 1988). The presense (+) or absence (-) of GAL4(1-147)-VP16 or TBP is indicated above each lane. The specific transcripts are indicated by arrows.

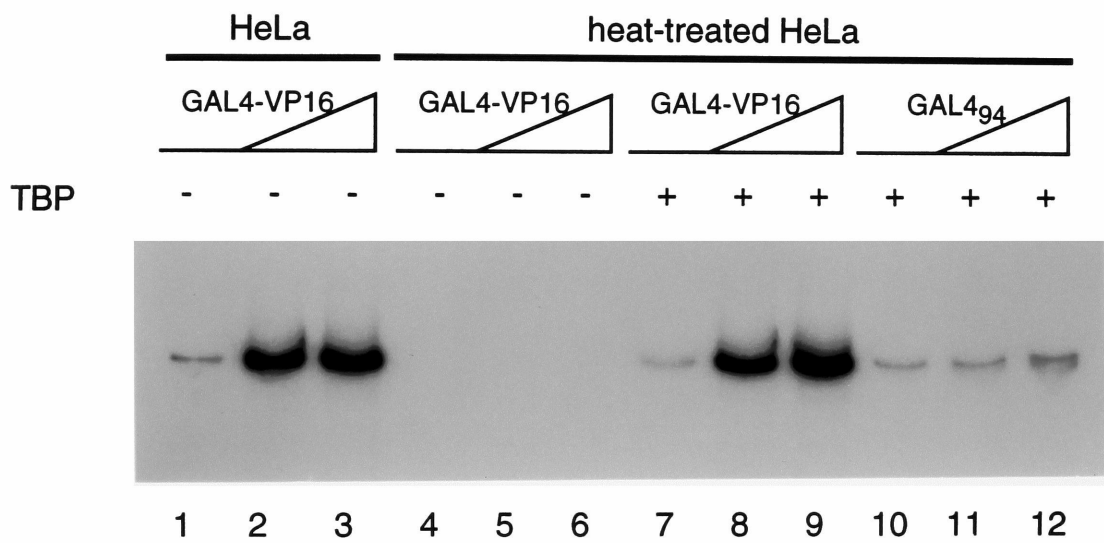


Fig. 3 Response of TBP mutants to GAL4(1-147)-VP16 in the human and the yeast in vitro transcription systems.

(A) Template was pG5HMC2AT which contains five GAL4(1-147) binding sites upstream of the HIV-1 core and the ML initiator promoter region fused to a G-less cassette. TBP was inactivated by heat-treatment of a HeLa nuclear extract and then transcription reactions were performed as described (Nakajima et al., 1988). The basal transcriptions were normalized by using 75 ng of TBPs. The presense (+) or absence (-) of GAL4(1-147)-VP16 or TBP mutants is indicated above each lane. The specific transcripts are indicated by arrows.

(B) In vitro transcription from the pG5HMC2AT was done in the TBP-dependent yeast in vitro assay system. By using TBP (50-100 ng), basal transcriptions were normalized. The presense (+) or absence (-) of GAL4(1-147)-VP16 or yTBP mutants is indicated above each lane. The arrows indicate the transcripts from template (pG5HMC2AT).

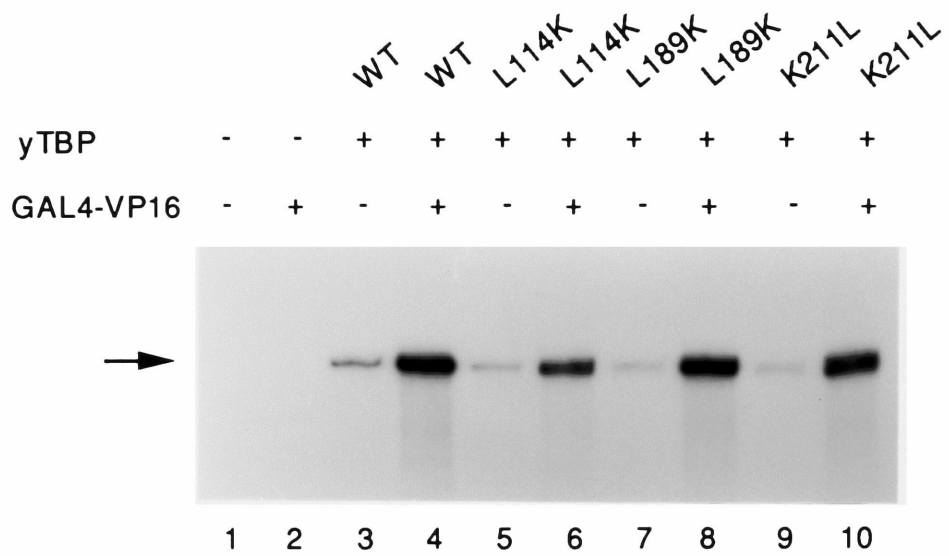
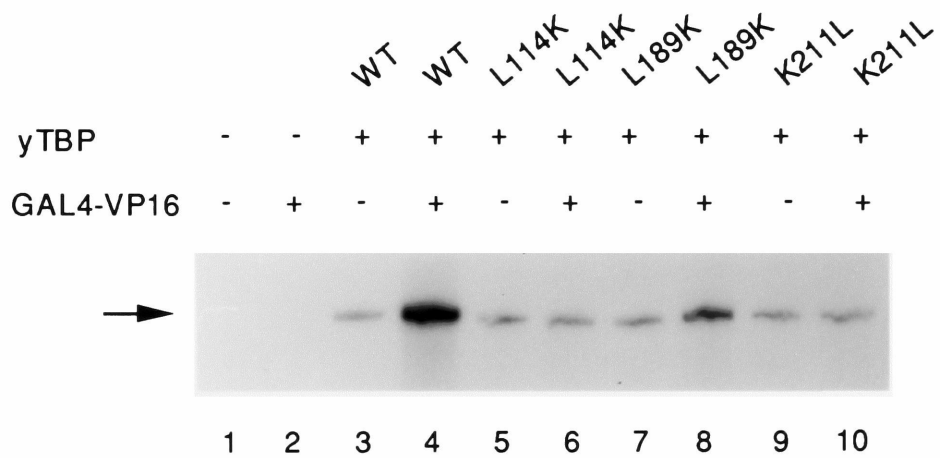
**A****B**

Fig. 4 Response of TBP mutants to GAL4(1-147)-AH in the human and the yeast in vitro transcription systems.

(A) Transcription under the human in vitro system.

Transcription in HeLa extract was as described in Fig. 3A from pG5HMC2AT. The presense (+) or absence (-) of GAL4(1-147)-VP16 or TBP mutants is indicated above each lane. The specific transcripts are indicated by arrows.

(B) Transcription under the yeast in vitro system.

Transcription reaction in yeast system was as in Fig. 3B. The template DNAs contain a TATA box from the CYC1 promoter with (CYCG6X) or without (CYCGΔ) six GAL4 binding sites.

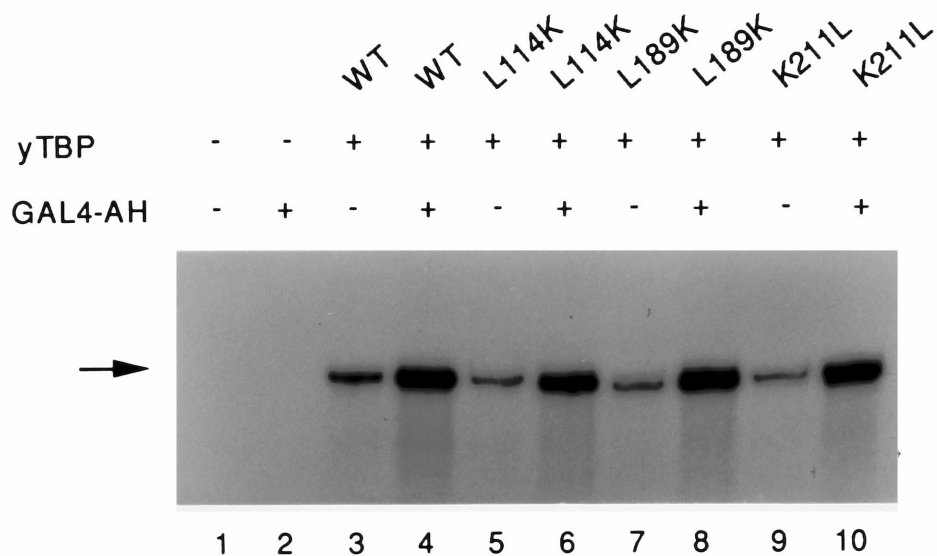
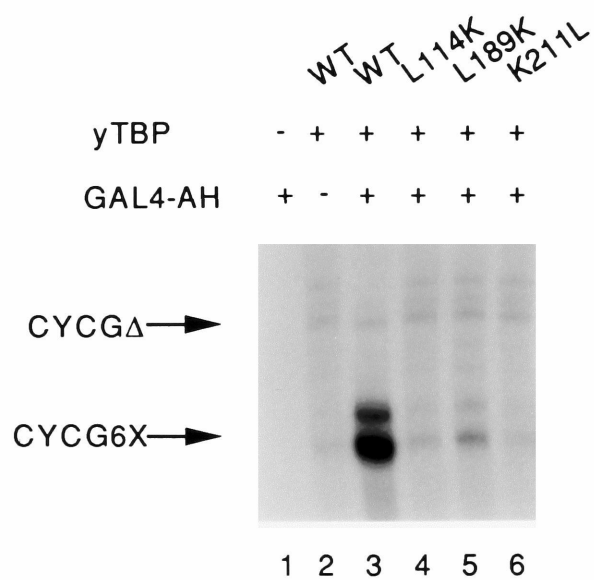
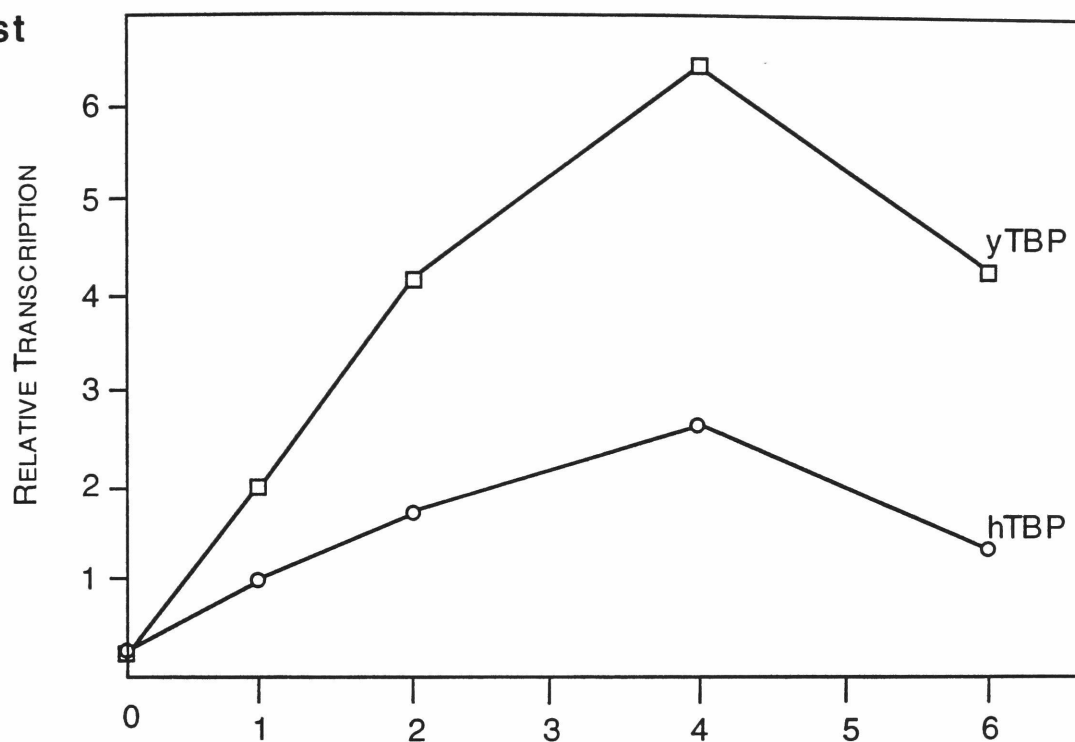
**A****B**

Fig. 5 Comparison of response to GAL4(1-147)-VP16 by wild-type yTBP and hTBP in the yeast and human in vitro transcription systems.

Wild-type y- and hTBP were tested for the ability to support activated transcription in a TBP-dependent yeast and human transcription systems. GAL4(1-147)-VP16 was added in increasing amount to transcription reactions. Relative amount of GAL4(1-147)-VP16 assayed are given in multiple of 30 ng. Reaction were performed as described (Nakajima et al., 1988). Radioactivity incorporated into specific transcripts is expressed as a relative transcription and plotted as a function of the GAL4(1-147)-VP16 concentration.



## Yeast



## Human

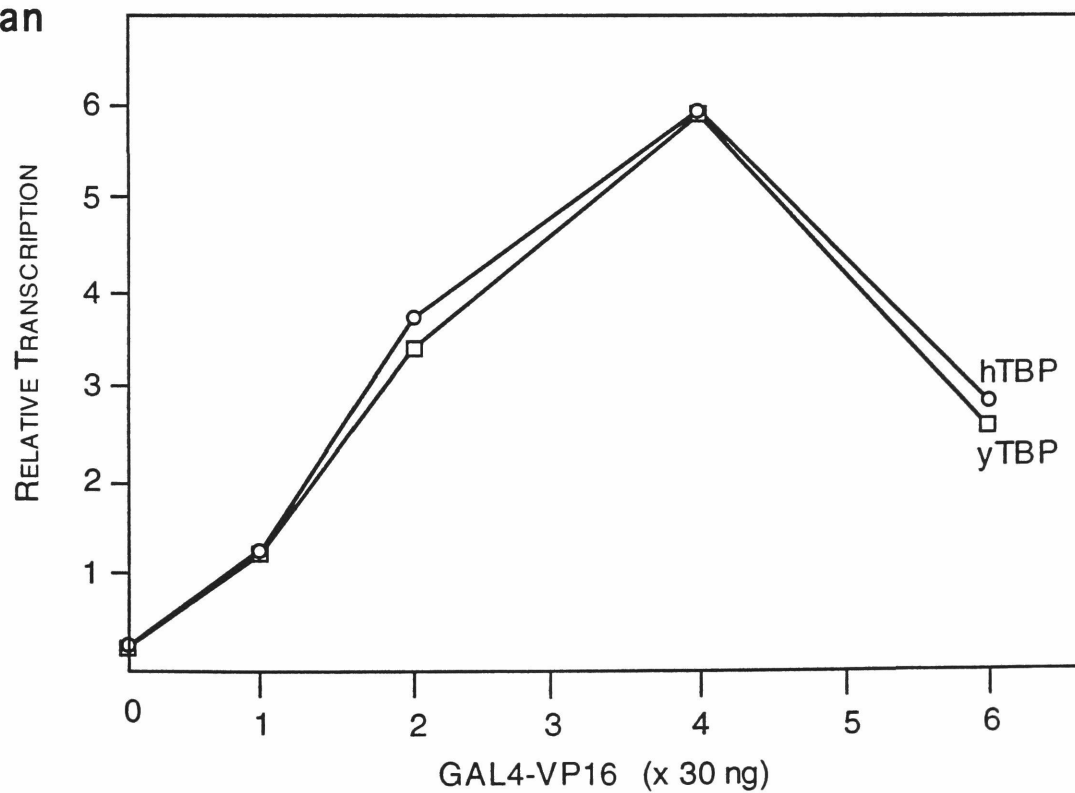
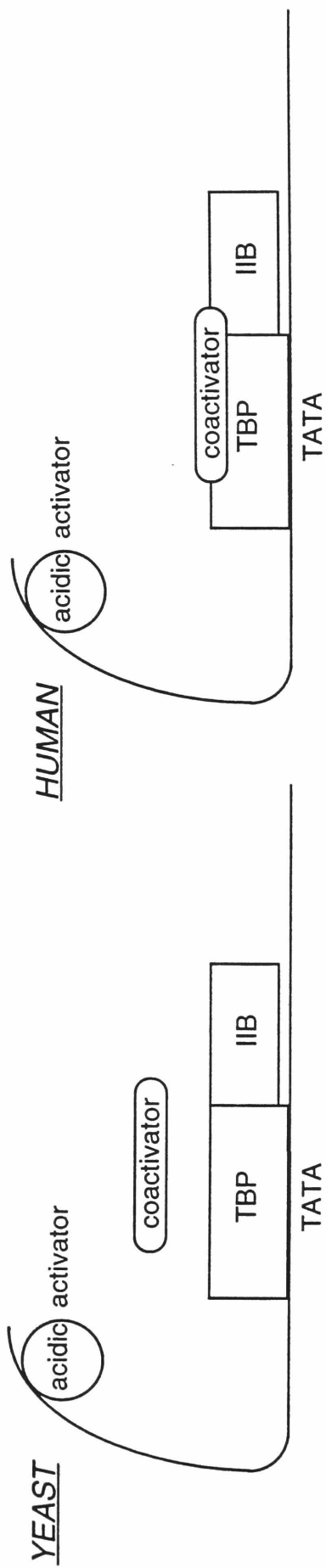


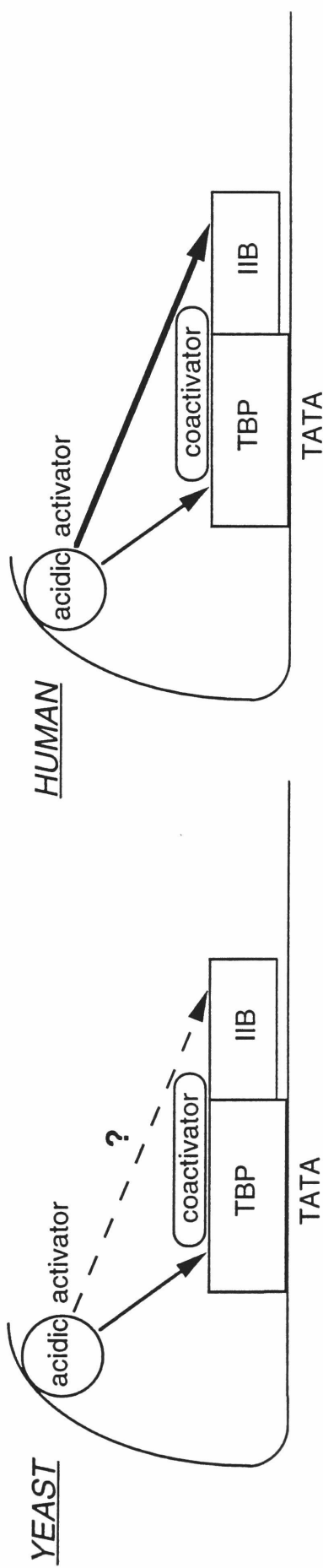
Fig. 6 Possible models for species-specific behaviors of TBP in transcription activation machinery.

Cooperative or specific protein-protein interactions only existed in human transcritpional activation machinery could overcome some defective interaction due to species-specific interactions. Explanations for this possibility are discussed in the text.

Stabilized interactions involving coactivators (TAFs) in human ?



Different target in yeast and human ?



## CHAPTER V

Transcriptional activation mechanism by proline-rich  
activators in yeast and human: The proline-rich activator  
CTF1 targets the TFIIB assembly step

## ABSTRACT

Activators can stimulate transcription through direct or indirect interactions with general initiation factors. We show here that the proline-rich activation domain of CTF1 selectively interacts with TFIIB but not with TBP, whereas previous studies have shown that the acidic activation domain of VP16 interacts directly with both TBP and TFIIB. In addition, consistent with studies of acidic activation domains, we demonstrate that the activation domain of CTF1 facilitates the recruitment (or stabilization) of TFIIB within TBP-DNA complexes during preinitiation complex assembly. CTF1-enhanced TFIIB recruitment was observed in both human and yeast systems. The results indicate that the proline-rich activation domain enhances transcription, at least in part, through direct interactions with TFIIB and, with previous observations, suggest models involving either quantitative or qualitative changes in TFIIB-TFIID-promoter interactions that lead to increased utilization of downstream initiation factors.

## INTRODUCTION

Transcription initiation on mRNA-encoding genes involves the assembly, on the promoter, of RNA polymerase II and other general initiation factors (TFIIA, -B, -D, -E, -F, -H and -J) to form a functional preinitiation complex (PIC) (reviewed in Roeder, 1991; Zawel and Reinberg, 1992; Conaway and Conaway, 1993). This process begins with recognition of the TATA element by TFIID, in a step facilitated by TFIIA, and continues with the ordered assembly of TFIIB, TFIIF/Pol II, TFIIIE, TFIIH and TFIIJ. The regulation of transcription initiation by gene-specific activators may involve facilitation of limiting steps in PIC formation (assembly) or function (initiation or elongation) through interactions with one or more of the general factors.

Consistent with its central role in recruiting other general factors to the promoter, TFIID was implicated as an activator target in the earliest studies of activation mechanisms (Sawadogo and Roeder, 1985; Horikoshi et al., 1988a; 1988b; Workman et al., 1988). In two cases (Horikoshi et al., 1988a; 1988b), activators were shown to effect qualitative (but not quantitative) changes in TFIID binding that correlated with increased recruitment of other general factors into the PIC. In accord with the model proposed, subsequent studies showed direct interactions of acidic (and other) activators with the TATA-binding subunit (TBP) of TFIID (Stringer et al., 1990;

Ingles et al., 1991; Lee et al., 1991). At the same time, other studies showed direct interactions of acidic activators with TFIIB (Lin and Green, 1991; Roberts et al., 1993; Choy and Green, 1993) that correlated with increased recruitment (or stabilized binding) of TFIIB to promoter complexes (Lin and Green, 1991; Choy and Green, 1993). While indicative of functions involving direct interactions of activators with TBP (in TFIID) and TFIIB, which also might also explain certain aspects of cooperativity between activators (Lin et al., 1990), these studies do not exclude interactions with other factors such as those required for activator-dependent transcription but not for basal transcription. These include USA-derived cofactors (reviewed in Roeder, 1991) and subunits (TAFs) associated with TBP in TFIID (reviewed in Hernandez et al., 1993). Indeed, recent studies have shown both that TAFs can interact directly with activators (Goodrich et al., 1993; Hoey et al., 1993) and that they are essential for activator-increased recruitment of factors that enter the PIC subsequent to TFIIB (but not TFIIB itself) (Choy and Green, 1993). These results support the earlier model of qualitative effects of activators on TFIID binding that effect recruitment of downstream factors (Horikoshi et al., 1988a; 1988b).

In addition to the well studied acidic activation domains present in GAL4 and VP16, there are other classes of activation domains that include the glutamine-rich activation

domain found in Sp1 and the proline-rich activation domain found in CTF1 (reviewed in Mitchell and Tjian, 1989). We previously showed that the proline-rich activation domain of the mammalian activator CTF1, like acidic activation domains, can function in eukaryotes ranging from yeast and human (Kim and Roeder, 1993a; Kim and Roeder, 1994a). To investigate the possibility that both activation domains might target the same step in PIC assembly, we have extended our previous studies (Kim and Roeder, 1993a; Kim and Roeder, 1994a) on CTF1. Our results indicate that the proline-rich activation domain of CTF1 interacts directly with TFIIB and facilitates TFIIB recruitment during PIC assembly in both human and yeast systems, thus arguing for a more general role of such interactions in transcriptional activation (Kim and Roeder, 1994b).



## EXPERIMENTAL PROCEDURES

### Protein purifications

Protein A derivatives (Pharmarcia), GAL494 and GAL494-CTF1 were purified as described. Recombinant hTBP, hTFIIB, yTBP and yTFIIB were purified from bacteria as hexahistidine fusion proteins by nickel chromatography (Hoffmann and Roeder, 1992). For purification of  $^{35}\text{S}$ -labelled hTFIIB, E.coli BL21(DE3)pLysS cells expressing a hexahistidine-hTFIIB fusion protein (Hoffmann and Roeder, 1992) was grown in M9 medium containing  $10^{-4}$   $\text{Na}_2\text{SO}_4$  and 400 uCi  $\text{H}_2[^{35}\text{S}]\text{O}_4$  (Ingles et al., 1991).

### Coimmunoprecipitation assays

The acidic activation domain of VP16 and proline-rich activation domain of CTF1 were expressed in bacteria as part of a fusion protein with the IgG-binding domain of Staphylococcus aureus Protein A and purified on IgG columns (Pharmacia) following the manufacturer's instructions.  $^{35}\text{S}$ -methionine-labelled TBP and TFIIB were generated by in vitro transcription of corresponding cDNAs and translation of derived mRNAs. In vitro and in vivo (see above) labelled proteins were incubated with Protein A, Protein A-CTF1 or Protein A-VP16 in 30 ul buffer D (10% Glycerol, 100mM KCl, 20mM Tris-Cl (pH 7.9), 0.2 mM EDTA, 0.03% NP-40, 1mM DTT) containing BSA (20 ug/ml) and allowed to bind for 1 hr on

ice. IgG beads were prepared as described before (Ingles et al., 1991) and preincubated with BSA to prevent nonspecific interactions. After addition of IgG beads, reactions were incubated for 1 hr at 4 °C. The beads were then washed three times with 400 ul buffer D containing BSA (200 ug/ml) and centrifuged. Bound proteins present in the pellet were released by boiling in SDS buffer and separated, along with input proteins and unbound proteins in the supernatant, on a 15 % acrylamide gel.

#### Far-western blotting analysis

Crude soluble protein extracts from bacteria was separated on 15 % SDS-PAGE and transferred to nitrocellulose filters. The protein blots were then denatured in 6 M guanidine-HCl in renaturation buffer (20 mM HEPES (pH 7.9), 60 mM KCl, 10 % (v/v) glycerol, 6 mM MgCl<sub>2</sub>, 0.6 mM EDTA, 2 mM DTT) for 30 min and renatured in 100 mM guanidine-HCl in renaturation buffer supplemented with 0.02 % polyvinylpyrrolidone twice for 2 hr at room temperature. The blots were then blocked with 50 mg/ml of BSA in renaturation buffer for 1 hr. Protein probes were generated by in vitro transcription-translation in the rabbit reticulocyte lysates supplemented with <sup>35</sup>S-methionine. The lysates were incubated with blots in 10 ml renaturation buffer for 16 hr at room temperature with gentle shaking. The blots were then washed twice and exposed for autoradiography.

### Protein extracts for human and yeast in vitro transcriptions

Control and heat-treated (TBP-deficient) HeLa nuclear extracts were prepared as described (Nakajima et al., 1988). TFIIB-depleted extracts were prepared by incubation (1 hr at 4 °C) with anti-hTFIIB antisera in buffer D, followed by incubation (12 hr) with protein A-Sepharose beads (prewashed with buffer D) and subsequent centrifugation to remove beads. Yeast whole cell extracts deficient in TBP were prepared from a temperature-sensitive yeast strain carrying the I143N yTBP mutant (Schultz et al., 1992) as described (Kim and Roeder, 1994c). Yeast TFIIB-deficient extracts were prepared using anti-yTFIIB antisera as described above.

### In vitro analyses on the immobilized DNA templates

Immobilized DNA templates were prepared as described (Lin and Green, 1991; Kim and Roeder, 1994b). Briefly, DNA fragments containing GAL4 binding sites upstream of adenovirus E4 or yeast CYC1 TATA boxes were isolated from pG5E4T (Lin and Green, 1991) or pGAL4X6 (Kim and Roeder, 1993a) and attached to streptavidin-agarose beads via a biotin moiety. Standard in vitro transcription reactions contained 100-200 ng of DNA templates fixed to beads in a final volume of 50 ul. After an initial incubation with the appropriate transcription components at 30 °C for 1 hr, the beads were pelleted and extensively washed with transcription buffer containing no nucleoside triphosphates (NTPs). For in vitro transcription assays, washed beads were incubated (1 hr at 30 °C) in

transcription buffer with NTPs and indicated protein components. Transcripts were analyzed by primer extension (Lin and Green, 1991) and G-less cassette assays (Kim et al., 1994d) in the human and yeast systems, respectively. For immunoblot assays, the initial incubation reactions were scaled up 10-fold and proteins retained on washed beads were separated by 15 % SDS-PAGE, transferred to nitrocellulose membranes, and probed with either anti-TBP or anti-TFIIB antisera as described (Amersham).

## RESULTS

### CTF1 specifically interacts with hTFIIB, but not with hTBP

Given previous demonstrations of VP16 interactions with both TBP and TFIIB (Stringer et al., 1990; Ingles et al., 1991; Lin and Green, 1991; Roberts et al., 1993), we searched for similar interactions with the CTF1 activation domain by coimmunoprecipitation with radiolabelled human TBP (hTBP) and human TFIIB (hTFIIB). A purified Protein A-CTF1 fusion protein containing the proline-rich activation domain of CTF1 (amino acids 399-499) was incubated with  $^{35}\text{S}$ -labelled proteins translated in reticulocyte lysates and the protein complexes were then precipitated with IgG-Sepharose beads (Fig. 1a). hTFIIB was precipitated with Protein A-CTF1 but not with Protein A (right panel, lane 3 versus lane 2). In contrast, hTBP failed to show any detectable interaction with Protein A-CTF1 (left panel, lane 3). Under the same conditions and consistent with previous results (Stringer et al., 1990; Ingles et al., 1991; Lin and Green, 1991; Roberts et al., 1993), the acidic activation domain of VP16 was able to interact with both hTBP and hTFIIB, although the hTFIIB interaction was relatively weak (lane 2 versus lane 4 in left and right panels). Consistently, far-western blotting analyses showed physical interactions between CTF1 and hTFIIB (Fig. 2). These results indicate that the proline-rich activation domain of CTF1 can specifically interact with hTFIIB, but apparently not with hTBP.

### The proline-rich activation domain of CTF1 directly interacts with hTFIIIB

To investigate the possibility that unidentified reticulocyte lysate components might have been involved in the CTF1-hTFIIIB interactions described above, a hexahistidine-hTFIIIB fusion protein was expressed and labelled with  $^{35}\text{S}$ -methionine in E.coli and then purified by nickel chromatography. As shown in Fig. 1b, the coimmunoprecipitation assay again showed an interaction of this purified hTFIIIB with Protein A-CTF1, but not with Protein A (lanes 2 and 3). Therefore, the interaction between hTFIIIB and CTF1 is direct and does not necessarily require any other polypeptides.

### GAL4-CTF1 acts in the preinitiation complex assembly process

To investigate whether the interaction between the CTF1 activation domain and hTFIIIB might be an important event during PIC assembly, we employed an immobilized templated assay (Lin and Green, 1991; Kim and Roeder, 1994b). First, we tested whether a GAL494-CTF1 fusion protein (CTF1 amino acids 399-499) acts before or after PIC assembly (Fig. 3).

Biotinylated promoter fragments containing GAL4-binding sites upstream of the adenovirus E4 TATA box were coupled to streptavidin-agarose beads. The DNA beads were then incubated in a HeLa nuclear extract to allow PIC formation in the absence or presence of an activator (GAL494-CTF1 or GAL494). Factors not stably associated with DNA templates were removed by extensively washing the beads with transcription buffer.

GAL494-CTF1 or GAL494 was subsequently added to the purified PIC as indicated and transcription was initiated by addition of NTPs. Consistent with previous results (Tanese et al., 1991), GAL494-CTF1 showed a significant stimulation of transcription when incubated with a HeLa nuclear extract and assayed directly (Fig. 3, lane 2 versus lane 1). In contrast, GAL494-CTF1 did not stimulate transcription above the basal level when added to purified complexes (lane 4 versus lane 3). However, transcription from purified complexes was enhanced by GAL494-CTF1 when included in the preincubation with DNA plus nuclear extract (lane 5 versus lane 3 and lane 6 versus lane 4). Under these conditions, the DNA binding domain of GAL4 itself (GAL4 amino acids 1-94; GAL494) did not activate transcription (lanes 7 versus lane 3 and lane 8 versus lane 4). Moreover, the level of activation observed in these analyses with washed complexes was comparable to that observed when the immobilized templates with GAL494-CTF1 were analyzed in nuclear extracts without isolation of the complexes (lanes 5 and 6 versus lane 2). These results indicate that the proline-rich activation domain of GAL494-CTF1 stimulates transcription by facilitating PIC assembly, rather than by increasing the activity of a preassembled PIC.

#### The TFIIB assembly step is facilitated by GAL4-CTF1

To identify the PIC assembly step which can be promoted by GAL494-CTF1, we performed the experiments shown in Fig. 4. As diagrammed in Fig. 4b, PICs were assembled in a HeLa nuclear

extract in the absence of GAL494-CTF1 and purified. GAL494-CTF1 was then added to these purified complexes along with a nuclear extract (NE) depleted either of hTBP or of hTFIIB or of both. The hTBP activity in the TFIID complex was specifically inactivated by a mild heat-treatment of a HeLa nuclear extract (Nakajima et al., 1988) while immunoaffinity methods were used to prepare an hTFIIB-depleted HeLa nuclear extract. The depleted extracts showed no transcriptional activity alone but addition of recombinant hTBP and/or hTFIIB restored both basal (Fig. 4a) and activator-dependent transcription (data not shown). The fact that activator-dependent transcription can be restored by addition of hTBP to heat-treated nuclear extracts indicates that TBP is the most labile component of the TFIID complex (TBP plus TAFs) and that exogenous TBP can restore function (at least in part) by association with an endogenous TBP-free TAF complex.

The transcription data of Fig. 4b show that addition of an hTBP-depleted extract with GAL494-CTF1 to the purified complexes markedly stimulated transcription (lane 4) whereas GAL494-CTF1 alone had no effect (lane 3). In contrast, transcription remained at the basal level after addition of a joint hTBP/hTFIIB-depleted extract with GAL494-CTF1 (lane 5), whereas addition of hTFIIB along with the hTBP/hTFIIB-depleted extract restored the normal level of activation by GAL494-CTF1 (lane 6). The levels of activation (legend to Fig. 4) observed with the purified template complexes (lanes



4 and 6) were comparable to that observed with GAL494-CTF1 addition to non-fractionated extracts (lane 2; compare with lane 1). In addition, no enhanced transcription was observed when isolated complexes were incubated with nuclear extracts in the absence of GAL494-CTF1 (data not shown). These results indicate that the stable assembly of hTFIIB within the promoter complex is limiting in nuclear extracts in the absence of an activator and that it can be facilitated by GAL494-CTF1 containing the proline-rich activation domain. The data also show that TBP/TFIID binding is not limiting for activation by GAL494-CTF1, consistent with studies using purified TBP and TFIIB (following section) and with previous studies of activation by GAL4-VP16 in nuclear extracts (Choy and Green, 1993).

#### GAL4-CTF1 recruits TFIIB to TBP-DNA complexes in the human in vitro transcription

To further confirm a functional interaction between CTF1 and hTFIIB, we performed experiments that directly measure the recruitment of hTFIIB into the promoter complexes by GAL494-CTF1 (Fig. 5). Immobilized template DNA was incubated with recombinant hTBP and/or hTFIIB in the presence or absence of GAL494-CTF1. Complexes were isolated and analyzed for levels of bound TBP and TFIIB by immunoblot and for transcription potential by complementation with hTBP- and/or hTFIIB-depleted extracts containing other essential factors. A control analysis (preincubation of DNA with nuclear extracts)

showed a normal level of activation by GAL494-CTF1 when included in the preincubation (lanes 1 and 2). When the DNA template was preincubated with hTBP and complemented with an hTBP-depleted extract after washing, transcription was roughly the same whether GAL494-CTF1 was added during or after the preincubation (lanes 3 and 4). This is consistent with the ability of TBP or GAL494-CTF1 alone to associate stably with DNA (see also figure legend). When the template was preincubated with both hTBP and hTFIIB and then complemented with hTBP/hTFIIB-depleted extracts after washing, the presence of GAL494-CTF1 during the preincubation significantly activated transcription (lane 6 versus lane 5). Moreover, in this case the increased transcription was correlated with an increased level of recruitment of hTFIIB on the promoter complex whereas the level of bound hTBP was constant (bottom panels). In contrast, preincubation of DNA with hTFIIB and complementation with an hTFIIB-depleted extract after washing led to no transcription with or without GAL494-CTF1 in the preincubation, indicating that TFIIB recruitment by the activator requires TBP to be bound concomitantly to the promoter (lanes 7 and 8).

These transcription and immunoblot analyses lead to the conclusion that GAL494-CTF1 facilitates hTFIIB recruitment to (or stabilization within, see Discussion) the DNA complex containing bound hTBP. Furthermore, since the recruitment of hTFIIB by GAL494-CTF1 was observed with purified recombinant

factors (TBP, TFIIB and GAL4<sub>94</sub>-CTF1) and since direct contacts between hTFIIB and GAL4<sub>94</sub>-CTF1 could be demonstrated, direct physical interactions between a proline-rich activation domain and hTFIIB appear to play an important role in the recruitment of hTFIIB during PIC assembly. However, this does not exclude the possible involvement of concomitant GAL4<sub>94</sub>-CTF1 interactions with hTBP, or associated TAFs, that result in a more stable interaction of hTFIIB with the activator-TFIID promoter complex (see Discussion).

The recruitment of TFIIB into TBP promoter complexes is also important for activation by GAL4-CTF1 in yeast

Because the proline-rich CTF1 can activate transcription in both yeast and human systems, we examined the possibility of CTF1-mediated recruitment of TFIIB in yeast to confirm and extend the results with human factors (Fig. 6). The yeast CYC1 promoter DNA fragment was immobilized on agarose beads and incubated with yTBP and/or yTFIIB in the presence or absence of GAL4<sub>94</sub>-CTF1. After washing, complexes were assayed for yTBP and yTFIIB levels by immunoblot and for transcription potential by complementation with appropriate yeast extracts depleted of yTBP and/or yTFIIB but containing other essential factors. Depleted yeast whole cell extracts (WCE) are totally dependent upon yTBP and/or yTFIIB (Fig. 6a). As shown in Fig. 6b, GAL4<sub>94</sub>-CTF1 specifically increased the amount of yTFIIB recruited into the promoter complex containing yTBP, and in a manner that paralleled the

increased transcription activity (lanes 5 and 6). Therefore, consistent with the recruitment results in the human system, CTF1 can affect a TFIIB assembly step that appears important for transcriptional activation in yeast.

## DISCUSSION

Here, we have employed physical and functional assays to demonstrate that the proline-rich activation domain of CTF1 does not enhance TBP/TFIID binding to target promoters, but rather that it enhances TFIIB recruitment to TBP/TFIID-DNA complexes in both human and yeast systems. Moreover, we have provided the first evidence that a proline-rich activation domain can interact directly, in the absence of TBP or other factors, with TFIIB and we have failed to see any direct interaction with TBP. Combined with previous studies showing that acidic activators can interact directly with TFIIB (and with TBP) (Stringer et al., 1990; Ingles et al., 1991; Lin and Green, 1991; Roberts et al., 1993) to effect TFIIB recruitment (Lin and Green, 1991; Choy and Green, 1993; Kim et al., 1994d), these results suggest an important and more general role for activator functions through TFIIB--effecting either recruitment per se and/or structural (conformational) changes that enhance subsequent steps, probably with other coactivators, in PIC assembly and function.

Activator-induced TFIIB recruitment could reflect an alteration (or stabilization) of interactions within the TFIID-TFIIB-promoter complex by activators. Related to this, it has been proposed that activators may effect qualitative changes in TFIIB-TFIID-promoter interactions during the activation process (Hahn, 1993). The activator could either

stabilize or effect the de novo assembly of an activation-specific TFIID-TFIIB-promoter complex, thereby enhancing the ability of TFIID-TFIIB-promoter complexes to interact with downstream factors (TFIIE, -F, -H, -J and RNA polymerase II) in PIC assembly. Several previous results are especially relevant to this model and invoke qualitative changes in the TFIID-TFIIB-promoter complex for activation. First, it was shown that GAL4 and ATF qualitatively alter the binding of hTFIID to target promoters, probably through conformational changes that effect the induced interactions of TFIID with downstream of the TATA box, and, consequently, facilitate subsequent interaction with TFIIB and other general initiation factors (Horikoshi et al., 1988a; 1988b). Second, increased concentrations of TFIIB itself were unable to overcome the requirement for an activator (Choy and Green, 1993), suggesting that the activator may not simply increase the recruitment of TFIIB to the promoter complex, or even the stability of the resulting complexes, but rather that it induces qualitative changes in the structure of the TFIID-TFIIB-DNA complexes that enhance subsequent factor interactions. Third, an analysis of yeast TBP mutants has demonstrated that activation-specific interactions of TBP with TFIIB are involved in activation by VP16 in yeast (Kim et al., 1994d) and, further, that VP16 function involves a direct contact with TBP (Kim et al., 1994d). Our present data suggest that any activation-specific interactions between TFIIB and TBP might also be facilitated, at least in part, by

direct contact of the proline-rich activation domain with TFIIB. Taken together, these results suggest that activators may induce qualitative or quantitative alterations in TFIIB binding to TBP (or TFIID) promoter complexes through interactions with either TFIID or TFIIB, or possibly through simultaneous interactions with both.

TBP-associated polypeptides (TAFs) within the TFIID complex (reviewed in Hernandez, 1993), as well as other cofactors (reviewed in Roeder, 1991), are required for activated transcription, but not for the basal transcription that is effected independently by TBP (reviewed in Roeder, 1991; Hernandez, 1993). It has been proposed that such coactivators somehow enhance, perhaps by direct contacts, functional interactions of upstream activators with general transcription factors. Although we have observed that CTF1 can interact with and recruit TFIIB in the absence of other factors, consistent with previous studies of acidic activators (Stringer et al., 1990; Ingles et al., 1991; Lin and Green, 1991; Roberts et al., 1993; Choy and Green, 1993), some degree of facilitated recruitment of TFIIB to TFIID-promoter complexes by coactivators might still be necessary for maximal levels of stimulated transcription in vitro. Consistent with this possibility, a higher level of activation was observed when DNA templates and GAL4<sub>94</sub>-CTF1 were incubated with intact nuclear extracts than when DNA templates were incubated first with TBP and TFIIB and then

(after washing) with a TBP- and TFIIB-depleted extract containing endogenous TAFs and other cofactors (Figs. 5 and 6; see figure legends for the relative activation folds).

On the other hand, TAFs might be involved in activator-induced alterations (Horikoshi et al., 1988a; 1988b; Kim et al., 1994d) within the resulting TFIID-TFIIB-DNA complexes that consequently enhance recruitment of downstream initiation factors or they might be induced by activators to interact directly with these factors. Consistent with these possibilities, recent reports have shown that TAFs (within the natural TFIID complex) are needed for the activator-dependent recruitment of downstream factors to the TFIID-TFIIB-promoter complex (Choy and Roeder, 1993) and that TAFs can interact directly with activators (Goodrich et al., 1993; Hoey et al., 1993). These results support the conclusions of earlier studies (Horikoshi et al., 1988a; 1988b) showing that activators can induce qualitative changes in TFIID-promoter interactions that result in enhanced recruitment of downstream factors (including TFIIB). Importantly, consistent with the studies of individual TFIID components (Stringer et al., 1990; Ingles et al., 1991; Lin and Green, 1991; Goodrich et al., 1993; Hoey et al., 1993), these studies (Horikoshi et al., 1988a; 1988b) also showed that activator-TFIID interactions can occur independently of TFIIB, just as activators can recruit TFIIB independently of the TAFs (Choy and Green, 1993, this study). Thus it will be important to



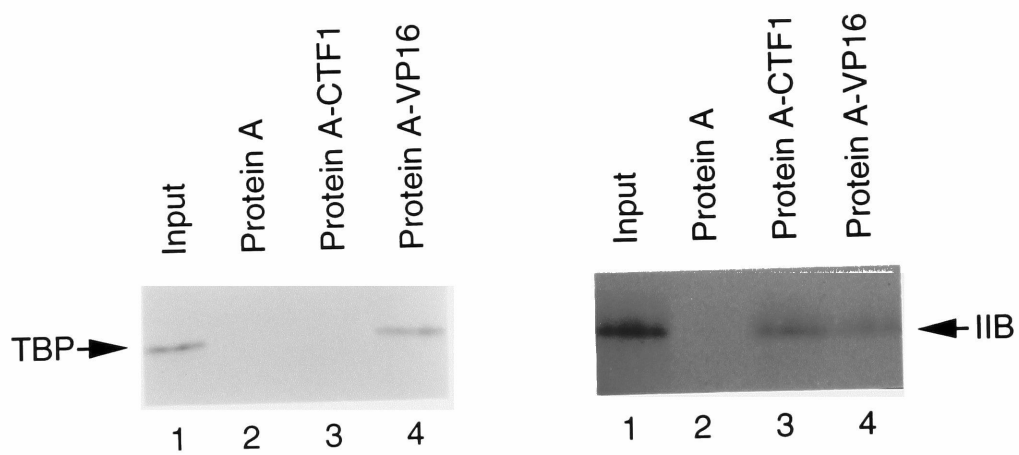
ascertain whether TAFs alter the TBP-TFIIB interactions described here and elsewhere (Choy and Green, 1993), to effect or stabilize an activation-specific TBP-TFIIB conformation (Kim et al., 1994d), or whether they act primarily through other interactions to stimulate transcription. Quite significantly, our results (Figs. 5 and 6) show directly that TAFs can function efficiently after the stable binding of activator, TBP and TFIIB-- presumably through reassociation with template-bound TBP. Ultimately an understanding of dynamic structural changes in promoter complexes during PIC assembly and function will be critical for understanding transcriptional regulatory mechanisms.

Fig. 1 Interaction of the proline-rich activation domain of CTF1 with hTFIIB, but not with hTBP.

a, The interactions of an acidic activation domain (VP16) or a proline-rich activation domain (CTF1) with  $^{35}\text{S}$ -labelled in vitro translated hTBP and hTFIIB were analyzed by coimmunoprecipitation.

b, Purified hTFIIB labelled with  $^{35}\text{S}$ -methionine in bacteria was used to show a direct interaction with the proline-rich activation domain of CTF1 by coimmunoprecipitation.

*a*



*b*

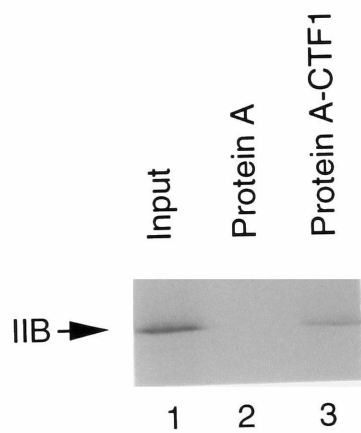


Fig. 2 Interaction of the CTF1 activation domain with hTFIIB by far-western blotting assay.

Crude bacterial extracts of Protein A and Protein A-CTF1 were fractionated in SDS-PAGE and stained with Coomassie brilliant blue (left panel) or transferred to a nitrocellulose membrane (right panel). Nitrocellulose blots were probed with  $^{35}\text{S}$ -methionine-labelled hTFIIB generated by in vitro translation in rabbit reticulocyte lysates.

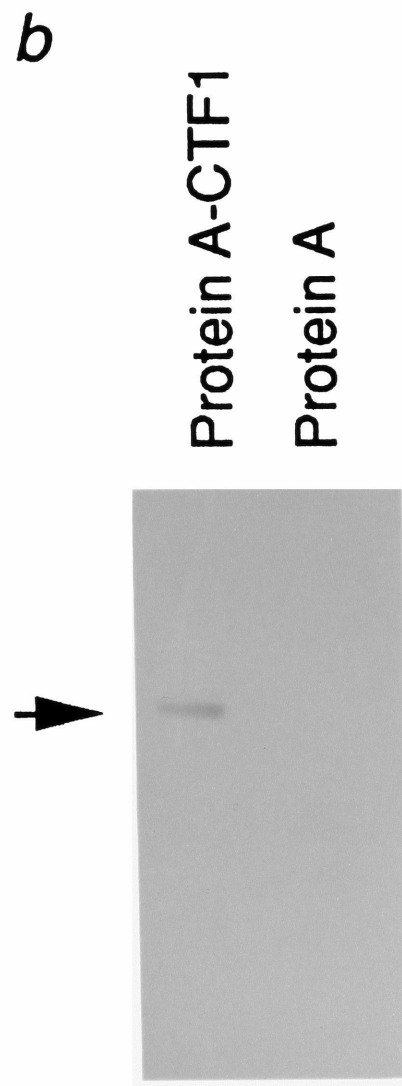
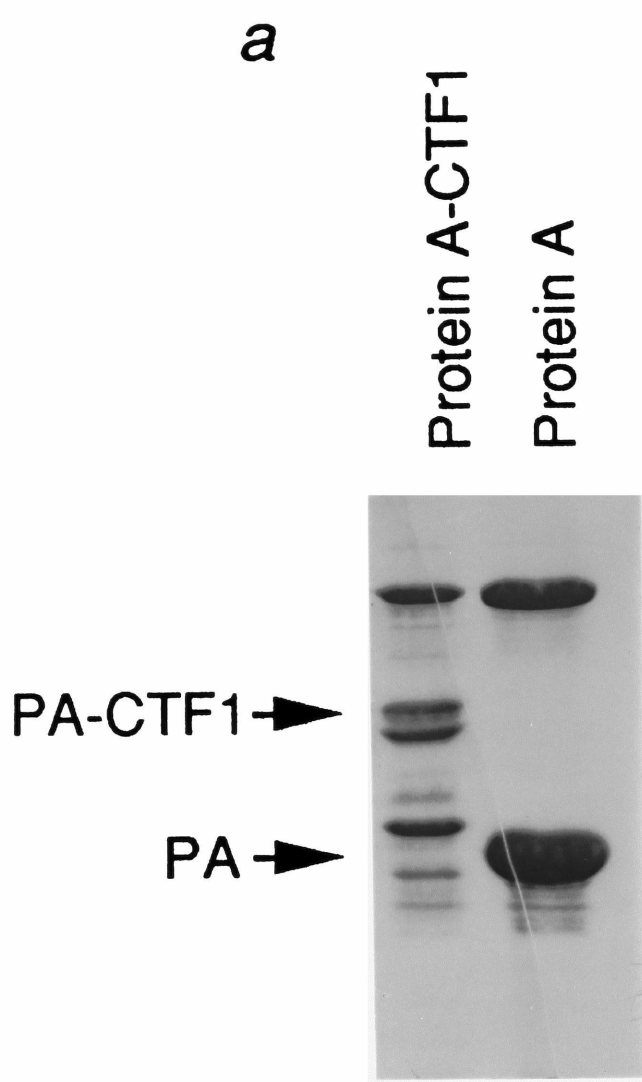
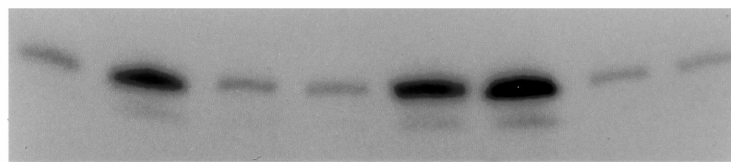


Fig. 3 GAL494-CTF1 acts in preinitiation complex assembly. HeLa nuclear extracts were incubated with pG5E4T DNA in the absence or presence of GAL494 or GAL494-CTF1. Immobilized preinitiation complexes were purified by washing and analyzed for transcription in the presence of NTPs and GAL494 or GAL494-CTF1 as indicated. The absence (-) or presence (+) of nuclear extracts (NE) in the initial incubation, of GAL494 or GAL494-CTF1 in the initial or final incubations, and of the washing step is indicated above each lane.

NE	+	+	+	+	+	+	+	+
GAL4 <sub>94</sub>	-	-	-	-	-	-	+	+
GAL4 <sub>94</sub> -CTF1	-	+	-	-	+	+	-	-
↓								
WASH	-	-	+	+	+	+	+	+
↓								
GAL4 <sub>94</sub> -CTF1			-	+	-	+	-	-
GAL4 <sub>94</sub>			-	-	-	-	-	+



1 2 3 4 5 6 7 8

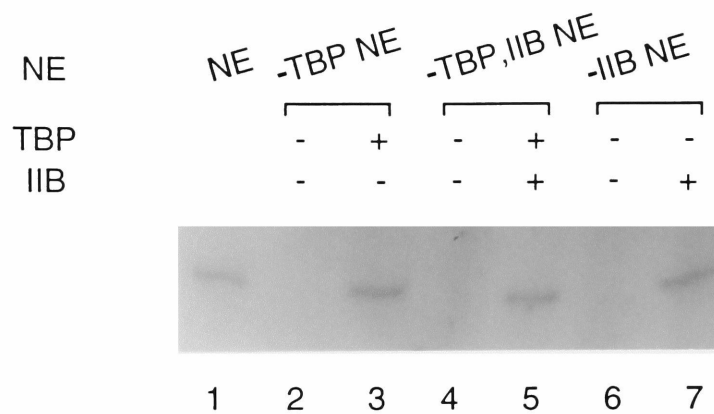
Fig. 4 The TFIIB assembly step is facilitated by GAL494-CTF1.

a, Activities of HeLa nuclear extracts depleted of hTBP (-TBP NE) or hTFIIB (-IIB NE) or both (-TBP,IIB NE), as indicated, were determined by in vitro transcription assays with immobilized DNA templates.

b, Assembly of TFIIB is facilitated by GAL494-CTF1. The addition and deletion of components are as explained in the legend for Fig. 3, except that the "factors" in the last incubation refer to depleted nuclear extracts and hTFIIB addition as indicated by the arrows to the right. The relative levels of transcription were quantitated by liquid scintillation counting: lane 1, 1.0; lane 2, 10.5; lane 3, 0.9; lane 4, 9.3; lane 5, 0.9 and lane 6, 8.9.



*a*



*b*

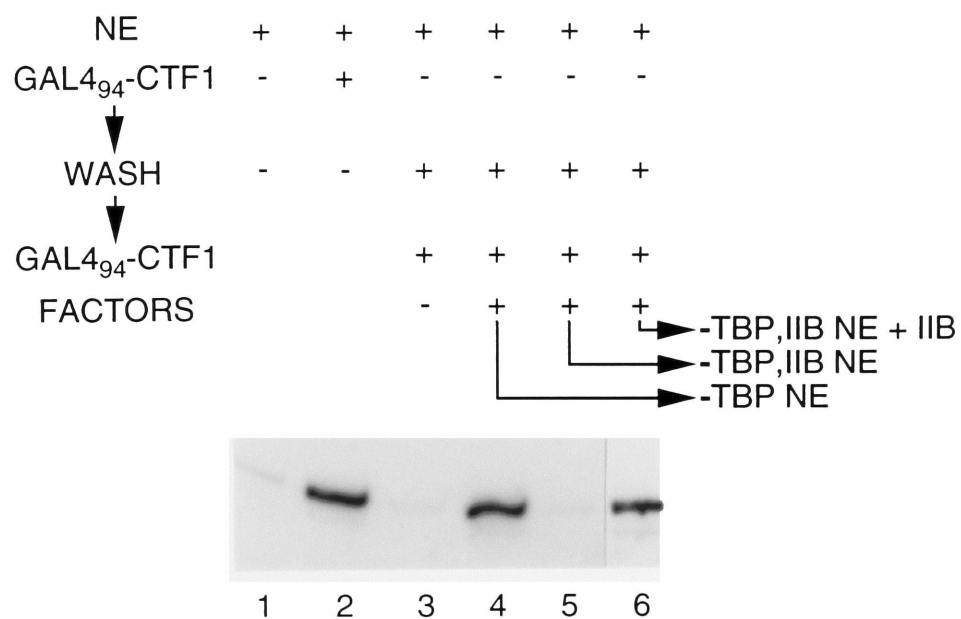


Fig. 5 GAL494-CTF1 facilitates recruitment of human TFIIB to TBP-DNA complexes.

The experimental protocol is diagrammed with additions as indicated and as explained in legends to Figs. 2 and 3. The promoter attached to agarose beads was incubated with a HeLa nuclear extract (lanes 1 and 2), purified recombinant hTBP (lanes 3-6), or purified recombinant hTFIIB (lanes 5-8) in the absence (lanes 1, 3, 5, 7) or presence (lanes 2, 4, 6, 8) of GAL494-CTF1. After preincubation, the agarose beads were washed and then subjected to immunoblot analyses or to transcription assays supplemented with hTBP- and/or hTFIIB-depleted HeLa nuclear extracts containing other essential factors. The relative levels of transcription were quantitated by liquid scintillation counting: lane 1, 1.0; lane 2, 12.1; lane 3, 5.1; lane 4, 4.7; lane 5, 0.6; lane 6, 4.9; and lanes 7 and 8, 0.0. The level of basal transcription in HeLa nuclear extracts (lane 1) is relatively higher than that observed with the purified complexes (lane 5), possibly due to stronger interactions of DNA templates with TFIID in nuclear extracts than with recombinant TBP under the assay conditions or to a partial inactivation of general factors in the -TBP NE. Hence the activated transcription levels in lanes 3, 4 and 6 should be compared to the basal level in lane 5 rather than that in lane 1. The amount of template-bound hTBP or hTFIIB determined by immunoblot is shown in the two bottom panels as indicated by arrows.

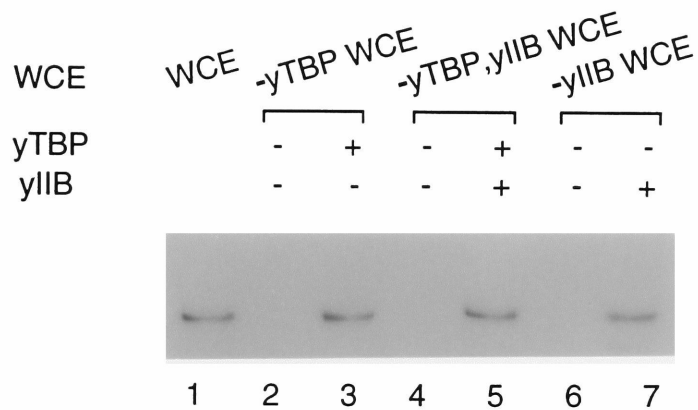


Fig. 6 GAL494-CTF1 recruits TFIIB to TBP-DNA complexes in the yeast system.

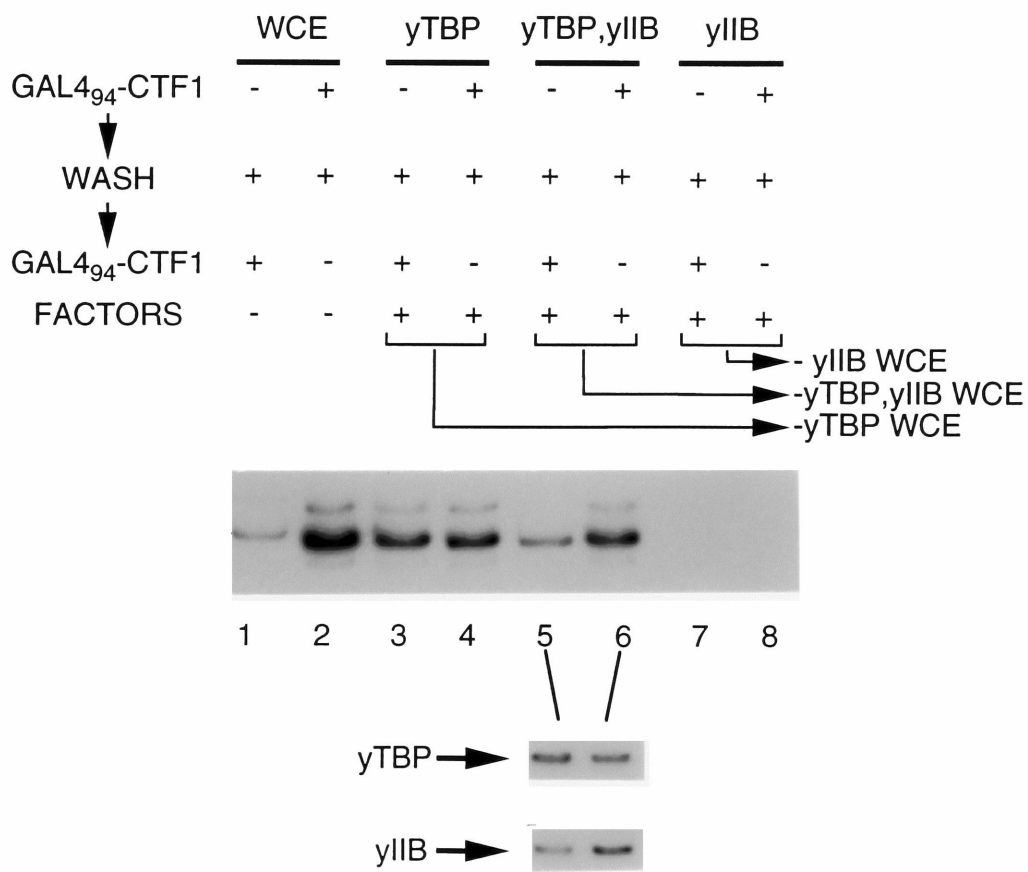
a, Activities of yeast whole cell extracts (WCE) depleted of yTBP and/or yTFIIB were determined by in vitro transcription assays with immobilized DNA templates.

b, GAL494-CTF1 recruits TFIIB to the TBP-DNA complex. The experiment was performed as described in Fig. 5 and diagrammed above the autoradiogram. The amount of template-bound yTBP and yTFIIB determined by immunoblot is shown in the two bottom panels. The relative levels of transcription were determined by liquid scintillation counting: lane 1, 1.0; lane 2, 4.2; lane 3, 2.5; lane 4, 2.6; lane 5, 1.1; lane 6, 2.5; and lanes 7 and 8, 0.0.

*a*



*b*



## CHAPTER VI

Interplay of several regulatory factors on the surface of  
TBP: Distinct interactions with Dr1 (NC2), TFIIB, Pol I-  
and Pol III-specific factors.

## ABSTRACT

The TATA-binding protein TBP plays a central role in transcription initiation by nuclear RNA polymerases I, II, and III. Consistent with its central role in preinitiation complex assembly, TBP is known to interact with many other general and regulatory factors. With knowledge of the three-dimensional structure of TBP, mutational analyses were focused on the exposed surfaces in TBP in order to identify amino acid residues important for each of its distinct interaction (and function). Using the band-shift assays, it is found that basic repeat region (K133L, K145L and K151L) is important for interaction with a negative cofactor Dr1 (NC2), whereas a distinct domain, the second stirrup of loop region (L189K) is important for interaction with TFIIB. In addition, transcriptional analyses have identified TBP mutations in the basic repeat region which could discriminate transcription functions of different RNA polymerases: one mutation (K156L) was found to specifically abolish transcription by RNA polymerase I and another mutation (K138L) specifically abolished transcription by RNA polymerase III, while each maintained the ability to support in vitro transcription by other two RNA polymerases. Along with previous studies, these results indicate that the basic repeat domain of TBP is important not only for transcription by RNA polymerase II but also for transcription by RNA polymerases I and III and, further, that TBP has distinct regions (or sites) for

interactions which are specific for RNA polymerases I, II and III regulatory factors.



## INTRODUCTION

The TATA-binding protein TBP was first identified as an initiation factor that is essential for transcription by RNA polymerase II, as a result of its key role in preinitiation complex assembly (reviewed in Roeder, 1991). In addition, TBP was recently shown to be necessary for transcription by RNA polymerases I and III (reviewed in Hernandez, 1993). The *in vivo* relevance of these biochemical studies is clear from genetic studies in yeast, which showed that transcription by each nuclear RNA polymerase (I, II, and III) is greatly reduced in TBP conditional mutants at the nonpermissive temperature (Cormack and Struhl, 1992). It is thus apparent that TBP must interact with a large number of other transcription factors. For transcription by RNA polymerase II, these interacting factors include: general initiation factors TFIIA and TFIIB; the carboxy-terminal heptapeptide repeat domain (CTD) of the largest subunit of RNA polymerase II; various negative regulatory factors (e.g. NC and Dr), activators and cofactors (e.g. USA fraction) and the TBP-associated factors (TAFs) in TFIID (reviewed in Roeder, 1991; Hernandez, 1993). At present, only interaction sites for TFIIA (Buratowski and Zhou, 1992a; Lee et al., 1992), an inhibitor of TBP binding to DNA (Auble and Hahn, 1993), and the activator E1A (Lee et al., 1991) on the TBP surface have been relatively well defined. Therefore, detailed mutational analyses of TBP, guided by the recently reported three

dimensional structure (Nikolov et al., 1992), will be important for understanding how the potentially competing interactions of different sets of transcription factors on the surfaces of TBP specify the function and regulation of the different RNA polymerases. To this end, our analysis has been directed toward identification of a structurally distinct yeast TBP domain whose residues are differentially required for discrimination between the different RNA polymerase transcription events (Kim and Roeder, 1994c) including interactions with a negative cofactor Dr1 (NC2) and a general factor TFIIB (Kim et al., in preparation).

## EXPERIMENTAL PROCEDURES

### Purification of recombinant proteins

Wild-type and mutant yeast TBPs were expressed from the T7 polymerase vector pET11d as hexahistidine fusion proteins in bacteria. Cells were broken by sonication and TBPs were purified by nickel and S-Sepharose column chromatographies. GAL4(1-147)-VP16 was expressed in and purified from bacteria as described (Chasman, 1989). Yeast and human TFIIBs were expressed as hexahistidine fusion proteins in bacteria and purified by nickel chromatography (Hoffmann and Roeder, 1992).

### Preparation of extracts and transcription factors

The HeLa nuclear extracts were prepared according to Dignam (1983). General transcription factors (TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH) and the USA cofactor fraction were purified from HeLa extracts as described before (Meisterernst, 1991a). Sp1 was separated from the general transcription factors upon phosphocellulose (P11) chromatography and eluted with 0.3 M KCl (Meisterernst, 1991a). Negative cofactors Dr1 (NC2) was purified from HeLa nuclear extracts as described (Inostroza et al., 1992; Meisterernst, 1991b).

### Band-shift analyses

The formation of TBP-TFIIB-DNA complex was checked by band-shift analyses (Auble and Hahn, 1993; Kim et al., 1994d). The DNA fragment used for the band-shift assay contained the TATA box of adenoviral major late promoter. All DNA binding was carried out for 20 min at room temperature in 4 mM Tris (pH 8.0), 60 mM KCl, 5 mM MgCl<sub>2</sub>, 4 % glycerol, 100 ug/ml BSA, and 0.1 % Brij 58. The products were analyzed on a 6 % polyacrylamide gel containing 25 mM Tris (pH 8.3), 190 mM glycine and 0.5 mM DTT. The gel was cast in Tris-glycine running buffer containing no magnesium acetate (Auble and Hahn, 1993). Under this condition, TBP alone cannot form a complex unless DNA interaction is stabilized in the presence of TFIIB. The formation of TBP-Dr1(NC2)-TBP was analyzed by band-shift assays as described before (Meisterernst et al., 1991b). Dephosphorylation reactions of purified Dr1 (NC2) were performed as described (Inostroza et al., 1992).

### Human in vitro transcription assays

Human in vitro reconstituted transcription was carried out in a 25 ul reaction mixture containing 1 ul TFIIA (0.5 mg/ml), 10 ng recombinant TFIIB, 1 ul TFIID (0.5 mg/ml), 1 ul TFIIE/F/H (0.3 mg/ml), 0.5 ul RNA polymerase II and DNA templates (pG5HMC2AT) in the presence or absence of other tested components and then analyzed as previously described (Meisterernst, 1991a; 1991b).

## Whole cell extract preparation and in vitro transcription assays

I143N mutant yeast cells were grown at the permissive temperature (24 °C) to an A<sub>600</sub> of 1.8-1.9, and then for a further hour at the nonpermissive temperature (37 °C) before harvest. Whole cell extracts were prepared using a mortar and pestle (Schultz et al., 1992; Kim and Roeder, 1994c). In vitro transcription assays for RNA polymerases I, II, or III were performed according to Schultz et al. (1992). All transcription reactions were performed in a final volume of 20 ul and included a preincubation in the appropriate transcription buffer of whole cell extracts with non-specific competitor (Pvu II-digested pBluescript) to titrate out a general inhibitor (Schultz et al., 1992).

## RNA polymerase I transcription

The pBYr11A containing the yeast 35S rRNA promoter was used for RNA polymerase I transcription (Schultz et al., 1992). Each reaction contained approximately 50 ug of whole cell extract. Where appropriate, recombinant TBP was added to whole cell extract and preincubated at 30 °C for 10 min prior to the addition of the template (5 ug/ml of pBYr11A) and nucleotides. The reactions were performed for 30 min at room temperature and transcription products were detected by S1 nuclease protection assays with an oligonucleotide spanning the promoter region from -15 to +35.

### RNA polymerase II transcription

Template DNAs used for RNA polymerase II transcription contain a TATA box from the CYC1 promoter with or without six GAL4 binding sites (Kim and Roeder, 1993a). The minimal template contains a G-less cassette of 400 base pairs and yields two major transcripts of 375 and 350 base pairs. The activator-responsive template with GAL4 binding sites has a 100 base pair-truncated G-less cassette. Templates with and without GAL4 binding sites were included in each reaction to ascertain that activation was mediated through sequence-specific DNA binding. The templates (5 ug/ml) were preincubated with GAL4(1-147)-AH for 5 min at room temperature and then mixed with whole cell extracts (80 ug) and recombinant TBP. After further preincubation for 5 min, reactions were initiated by addition of nucleotides (0.4 mM ATP, 0.4 mM CTP, 20 uCi of [ $\alpha$ -<sup>32</sup>P]UTP (600 Ci/mmol)) and stopped after 30 min at room temperature. The transcripts were precipitated with ethanol and carrier DNA. The precipitates were separated on urea/polyacrylamide gel and analyzed by autoradiography.

### RNA polymerase III transcription

Templates used for RNA polymerase III transcription were pUC5S containing the yeast 5S rRNA promoter or pYLEU3 containing the yeast tRNA<sup>Leu3</sup> promoter (Schultz et al., 1992). Whole cell extract (50 ug) was incubated for 5 min with recombinant TBP at room temperature. A further

incubation with 20 ug/ml of templates followed for 5 min. Then reactions were started by the addition of nucleotides (0.4 mM ATP, 0.4 mM CTP, 0.4 mM UTP, 20 uCi of [ $\alpha$ -<sup>32</sup>P]GTP (600 Ci/mmol)). The final transcription products were precipitated, separated and visualized by autoradiography.

## RESULTS

The conserved carboxy-terminal core domain of TBP folds into a highly compact and symmetrical structure (Nikolov et al., 1992). Since most deletion mutants of TBP and chimeras with TBP fragments have been suggested not to adopt the compact three-dimensional fold of TBP in a proper way, single point mutants were used to define specific amino acid residues (or domains) important for interactions with a negative cofactor Dr1 (NC2), a general factor TFIIB, Pol I- and Pol III-specific factors. Because lysine or leucine residues are distributed extensively in the conserved carboxy-terminal core region, we generated a series of TBP point mutants containing either leucine to lysine or lysine to leucine changes (Kim et al., 1994d). With the knowledge of three-dimensional structure of TBP (Nikolov et al., 1992), we focused on TBP mutants whose residues were in the exposed positions for possible direct protein-protein interactions. In addition, TBP mutants defective in both basal and activated transcription were excluded in this analysis, since they could simply reflect gross structural perturbations that disrupt all functions and indeed a number of these mutations map to the hydrophobic core of TBP and would be expected to disrupt the structure. Based on these criteria, selected mutant and wild-type TBPs were expressed as hexahistidine fusion proteins in bacteria and most of the mutations were found not significantly to affect the levels of proteins



produced in bacteria. TBPs were purified by nickel and S-Sepharose column chromatographies and each purified protein was then employed in various in vitro assays.

Basic repeat region of TBP is important for interaction with Dr1 (NC 2)

To determine the domain of TBP important for interaction with a negative cofactor Dr1 (NC2), we analyzed TBP-Dr1(NC2) complex formation on the promoter DNA by band-shift assays. Since recombinant Dr1 (NC2) purified from bacteria failed to interact with TBP under this condition (Inostroza et al., 1992), we purified Dr1 (NC2) from HeLa cell nuclear extracts as described (Inostroza et al., 1992) and analyzed its ability to interact with purified TBP mutants by band-shift assays. As shown in Fig. 1, three TBP mutants (K133L, K145L and K151L) were defective in the TBP-Dr1(NC2)-DNA complex formation but they were essentially active in the TBP-DNA complex formations. Thus three individual point mutations specifically abolished TBP interactions with Dr1 (NC2) on the promoter DNA. In the three-dimensional structure of TBP (Nikolov et al., 1992), the mutated residues map to the basic repeat region and, therefore, that domain of TBP may be important for direct interaction with Dr1 (NC2) (Figure in the Appendix).

Previous studies (Inostroza et al., 1992) have showed that phosphorylation of Dr1 (NC2) affected its interaction with

TBP on the DNA and that the interaction of Dr1 (NC2) with TBP precludes interactions of TFIIA with TBP. In the light of these properties, we next addressed the specificity of interaction of our purified Dr1 (NC2) with TBP. Consistent with previous results (Inostroza et al., 1992), Dr1 (NC2) dephosphorylated by calf intestine phosphatase (CIP) failed to interact with TBP under this condition (Fig. 2, lane 2), whereas treatment of Dr1 (NC2) with bovine serum albumin had no effect on its ability to interact with TBP on the DNA (lane 3). Furthermore, addition of increasing concentrations of Dr1 (NC2) to a preformed TBP-TFIIA-DNA complex transformed the latter into a TBP-Dr1(NC2)-DNA complex (Fig. 2, lanes 4-8), indicating that Dr1 (NC2) can compete for TBP interactions with TFIIA. All of these results were in good agreement with previously characterized properties of native Dr1 (NC2) (Inostroza et al., 1992). In particular, competition analyses suggest that Dr1 (NC2) and TFIIA interact with an identical or closely overlapping domain of TBP. Consistent with this possibility, we have found here that Dr1 (NC2) interacts with the basic repeat region of TBP like TFIIA. Interestingly, from analyses of a series of TBP point mutants employed in this study, some residues are found to be important specifically for interaction with Dr1 (NC2) (K133L and K151L) or TFIIA (K138L), whereas the residue (K145L) is involved in interactions with both Dr1 (NC2) and TFIIA.

We next analyzed the effects of Dr1 (NC2) on basal and activated transcription in yeast and human. As shown in Fig. 3, the addition of increasing amount of Dr1 (NC2) to the yeast whole cell extract resulted in inhibition of both basal and activator (VP16)-dependent transcriptions. Similar repression by Dr1 (NC2) was observed in basal and activated transcriptions by VP16 and Sp1 in the human in vitro reconstituted system (data not shown). Thus it seems likely that repression mechanism by Dr1 (NC2) is general, possibly through competing with TFIIA and/or TFIIB to inhibit formation of a functional preinitiation complex.

The second stirrup-like loop of TBP is important for interaction with TFIIB

To define the region of TBP important for interaction with TFIIB, we performed band-shift assays to analyze TBP-TFIIB-DNA complex formation with TBP mutants. These analyses demonstrated that all but one of tested TBP mutants showed TBP-yTFIIB-DNA complex formation activities comparable to that displayed by wild-type TBP (Fig. 4). In contrast, mutant L189K is defective in the TBP-yTFIIB-DNA complex formation (Fig. 4). Similar defective TBP interaction by mutant L189K was observed with hTFIIB on the promoter DNA by band-shift assays (data not shown). Since L189K mutant can interact with DNA and can form TBP-TFIIA-DNA and TBP-Dr1(NC2)-DNA complexes as efficiently as does wild-type TBP, this mutation specifically affect interactions with TFIIB. The failure to

interact with both  $\gamma$ - and hTFIIB by mutant L189K suggests that the same region of TBP containing residue L189 is involved in interaction with TFIIB. In the three-dimensional structure of TBP, a mutation (L189K) is found within the second stirrup of loop connecting strands S2' and S3' (Figure in the Appendix). This loop region is at the highly exposed position for direct protein-protein interactions. Despite the involvement of distinct TBP region in interaction with TFIIB and Dr1 (NC2), Dr1 (NC2) was known to dissociate TFIIB from the TBP complexes. This suggests that interaction of Dr1 (NC2) with TBP might result in conformational changes of TBP that prevent binding of TFIIB.

TBP mutants defective specifically in pol I or pol III transcription map to the basic repeat region

To examine the in vitro transcription activities of TBP mutants for RNA polymerases I, II, and III, we utilized a TBP-dependent extract derived from the temperature sensitive yeast strain (I143N) containing a mutation in TBP (Schultz et al., 1992). The extract from the I143N mutant strain was almost completely inactive in transcription for all three RNA polymerases (Fig. 5, lanes 1). Under these conditions, all mutants (K133L, K138L, K145L, K151L, and K156L) rescued both basal and activator-dependent transcription activities by RNA polymerase II as efficiently as did wild-type TBP (Fig. 5, panel 2). As further shown in Fig. 5, all but two TBP mutants (K138L and K156L) functioned well in transcription by either

RNA polymerase I or III. Mutant K138L was defective in RNA polymerase III transcription from the 5S rRNA and tRNA<sup>Leu3</sup> gene promoters, while efficiently supporting both RNA polymerase I transcription from the 35S rRNA gene promoter and RNA polymerase II transcription from the CYC1 gene promoter (Fig. 5, lanes 4). In sharp contrast, mutant K156L showed markedly reduced transcription by RNA polymerase I but was essentially normal for RNA polymerase II and III transcription (Fig. 5, lanes 7). These results suggested that K156L and K138L were defective specifically in RNA polymerase I and III transcription, respectively, while they were fully able to support transcription by other classes of RNA polymerase.

Residual transcription activities for RNA polymerases I and III were observed in defective TBP mutants K156L and K138L, respectively (Fig. 5). The possibility that this reflected smaller numbers of active molecules in those preparations relative to the wild-type TBP preparation can be ruled out by the fact that, at the protein concentration employed, mutants supported transcription by other RNA polymerases as well as did wild-type TBP. Another possibility is that the dose-response curves for transcription differ in the defective mutants. To further confirm the conclusions regarding the defective mutants (Fig. 5), effects of concentration on the transcriptional activities were analyzed. In the titration experiment shown in Fig. 6, the amount of extract was held

constant, and increasing amounts of recombinant TBPs were added to each extract. Wild-type TBP readily rescued transcription activities in the mutant extracts. However, mutants K156L and K138L were consistently less active for RNA polymerase I and III transcription at the varied amounts of recombinant TBP, when compared with wild-type TBP. These results clearly demonstrate that two individual mutations, K156L and K138L selectively inhibit transcription by RNA polymerases I and III, respectively.

## DISCUSSION

From the mutational analyses of exposed surface of TBP and with the knowledge of its three dimensional structure, we have identified residues important for interactions with Dr1 (NC2) (K133L, K145L and K151L) and TFIIB (L189K), as well as residues specific for RNA polymerase I and III transcription activities (K156L and K138L). They are found within the loop connecting strands S2' and S3' (L189) and within the convex surface of TBP that spans  $\alpha$ -helix H2 and  $\beta$ -strand S1' (K133, K138, K145, K151 and K156) in the TBP structure (Figure in the Appendix) (Nikolov et al., 1992). The basic repeat region is particularly interesting because it has been implicated in transcription by RNA polymerase II in addition to its importance for RNA polymerase I and III transcription events. In the former case, it has been shown to be important for interactions with the general factor TFIIA (Buratowski et al., 1992a; Lee et al., 1992), an inhibitor of TBP binding to DNA (Auble and Hahn, 1993), the adenovirus E1A activator (Lee et al., 1992) and a negative cofactor Dr1 (NC2) (Fig. 1). In the latter case, a recent report shows that the residue K138 interacts with a RNA polymerase III factor, TDS4 (Buratowski and Zhou, 1992b; Colbert and Hahn, 1992). Thus the basic repeat region of TBP is important for regulated interplay of different sets of transcription factors.

The universal role of TBP in transcription by all nuclear RNA polymerases can be explained by two simple models. One model involves TBP interaction with a factor(s) shared by the RNA polymerase I, II, and III transcriptional machineries. An alternative possibility is that TBP interacts with distinct factors which are specific for the different transcriptional machineries. Our results are most consistent with the second model, which is further supported by the existence of distinct TBP-containing multicomponent complexes in HeLa extracts (reviewed in Sharp, 1992; Hernandez, 1993); a 230 kDa complex (SL1) that is essential for function by RNA polymerase I; a 750 kDa complex (TFIID) that is essential for activator-dependent transcription by RNA polymerase II; and a complex (TFIIIB2) that is essential for transcription by RNA polymerase III and is probably equivalent to a 300 kDa complex designated B-TFIID. These observations on human factors suggest that SL1, TFIID, and TFIIIB are TBP-TAFs complexes that direct RNA polymerase I, II, or III transcription respectively and underscore the importance of TAFs in dictating the specificity for particular RNA polymerase transcription. Considering the high degree of conservation of transcription mechanisms and TBP sequences from yeast to human, there might be corresponding interactions of TBP with RNA polymerase-specific factors in yeast as well. Consistent with this, recent studies have shown that a major portion of yeast TBP, like human TBP, exists as distinct multiprotein complexes, while about 30 % of



TBP chromatographs as a 27 kDa monomer. In addition, some of the purified yeast TAFs have already been identified as subunits of TFIIB (Poon and Weil, 1993). These results indicate that yeast TBP, like its human counterpart, is stably associated with other factors, and raises the possibility that a unique set of TAFs associates with yeast TBP to form, at least transiently, a multisubunit complex specific for each of the nuclear RNA polymerases.

In conclusion, our results demonstrate that a convex surface spanning the basic repeat domain of TBP is important for the interplay by factors important for transcription by RNA polymerase I, II and III and, further, that the region might have overlapped or distinct sites for interaction with factors or subunits specific to each nuclear RNA polymerase. Therefore, TBP itself can provide at least a certain part of the RNA polymerase specificity. In addition, the present analysis has defined the TBP domain (the second stirrup of the loop region) important for interaction with TFIIB. The RNA polymerase I, II or III-specific mutants described here will be useful for a future dissection of the functional interactions of TBP with the different RNA polymerase transcription machineries.

Fig. 1 TBP mutants defective interactions with a negative cofactor Dr1 (NC2).

Wild-type and mutant TBPs were expressed as hexahistidine fusion proteins in and purified from bacteria as described in Experimental Procedures. DNA binding reactions were performed as described (Inostroza et al., 1992) and DNA-protein complexes were analyzed by band-shift assays. The specific TBP-DNA and TBP-Dr1(NC2)-DNA complexes were indicated on the side of the panel.

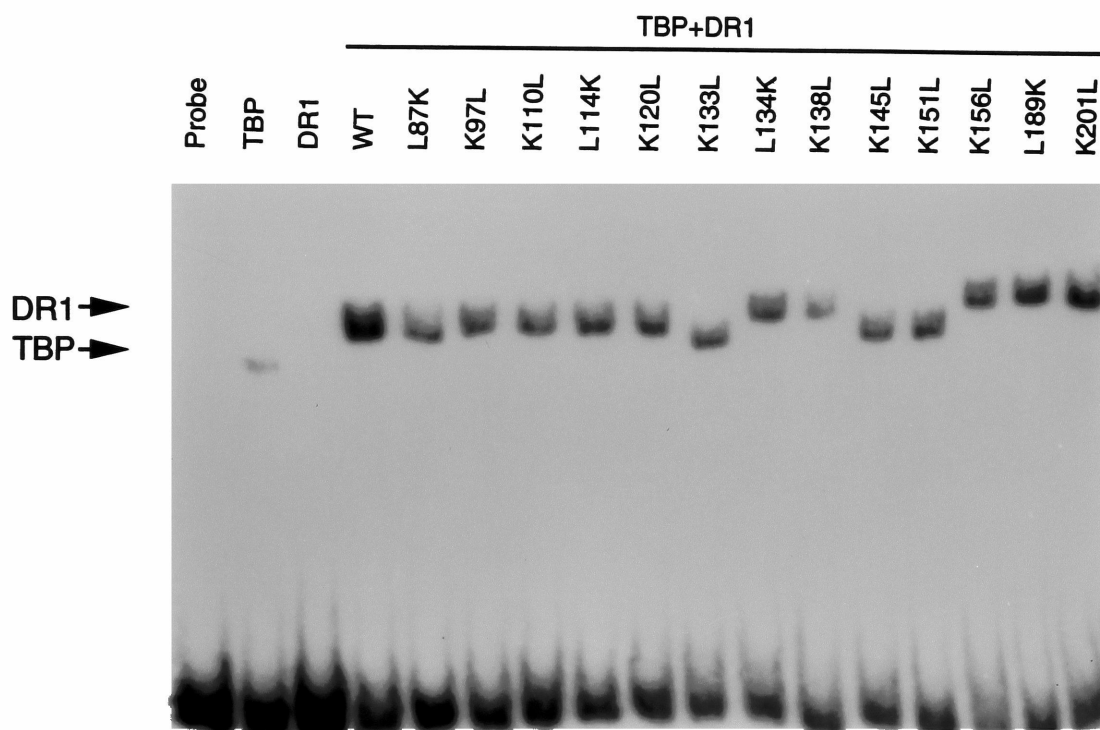
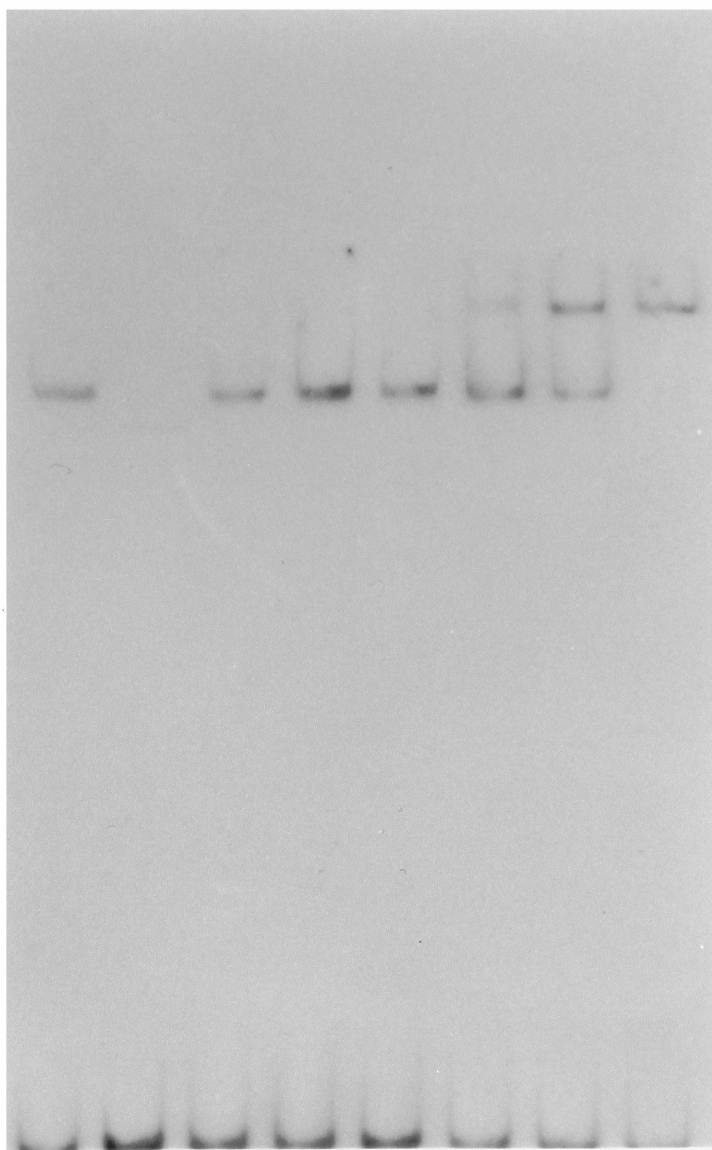
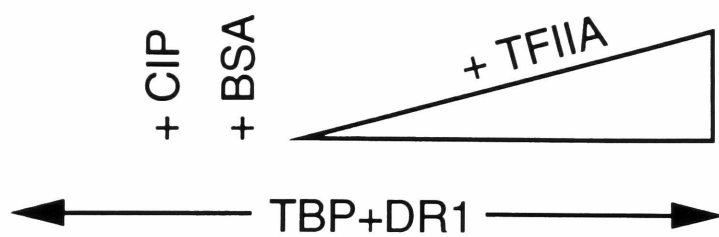


Fig. 2 Effect of CIP or TFIIA on TBP-Drl(NC2)-DNA complex formation.

Binding assays were performed as described (Inostroza et al., 1992). TBP and Dr1 (NC2) were incubated in the absence of CIP (lane 1) or in the presence of 2 U of CIP (lane 2), 20 ug of BSA (lane 3) and increasing concentration of TFIIA (lanes 4-8). Especially, for competition experiments, TFIIA was added after a preincubation of TBP and Dr1 (NC2) for 30 min. The position of the resulting DNA-protein complexes were indicated.



← TBP-IIA  
 ← TBP-DR1  
 ← TBP

1 2 3 4 5 6 7 8

Fig. 3 Repression of transcription by Dr1 in the yeast in vitro.

Transcription with increasing amount of Dr1 (NC2) was performed in the absence (lanes 1-3) or the presence (lanes 4-6) of GAL4(1-147)-VP16 as indicated. All reactions contained yeast whole cell extracts and pGAL4X6 under conditions described previously (Kim and Roeder, 1993b).

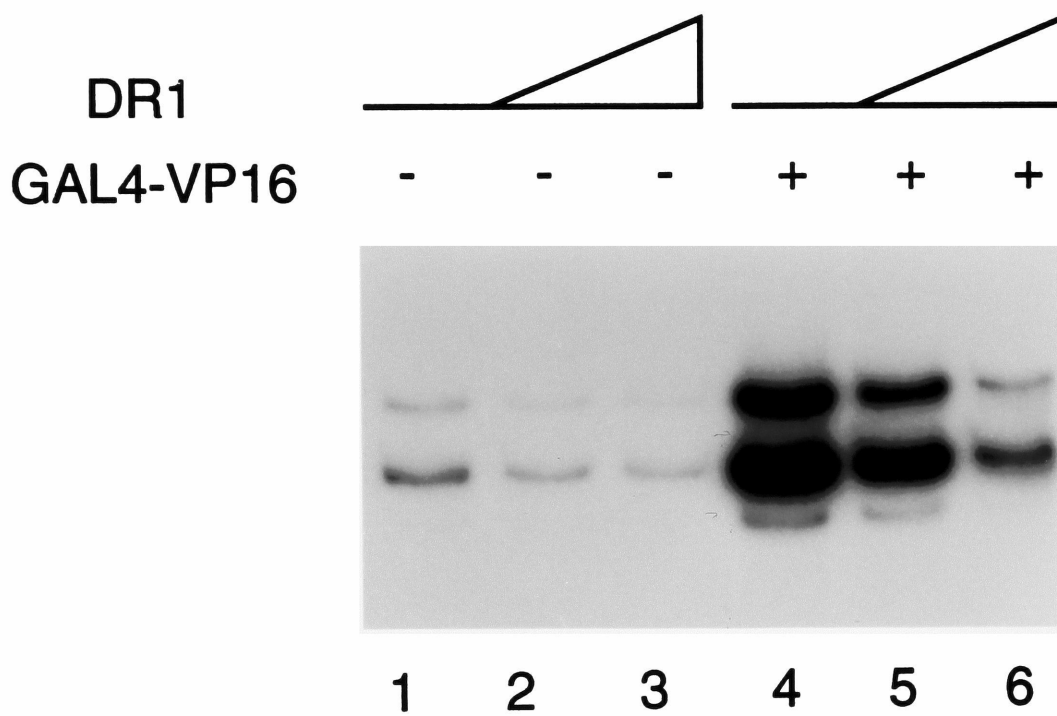


Fig. 4 TBP mutants defective in interaction with TFIIB. DNA binding reactions were performed as described (Auble and Hahn, 1993). Under these conditions, stable TBP binding requires the presence of other factors (e.g. TFIIB). The resulting DNA-protein complexes were analyzed by band-shift assays on the nondenaturing polyacrylamide gels. The specific TBP-TFIIB-DNA complexes were indicated on the side of the panel.



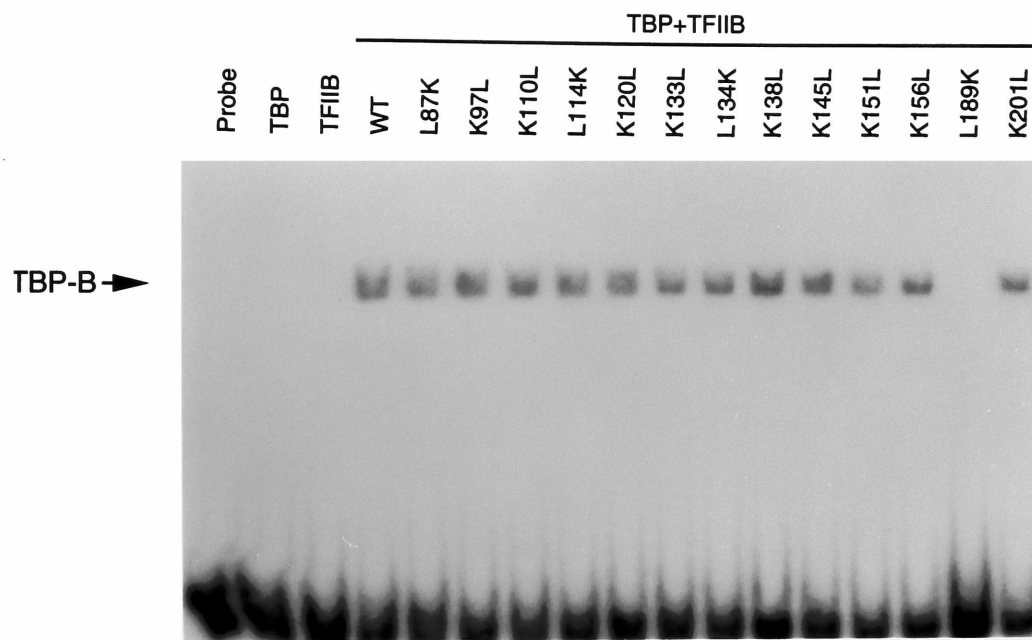


Fig. 5 TBP mutants important for transcription by RNA polymerase I, II, or III.

TBP-dependent extracts were prepared from the temperature-sensitive yeast strain containing a TBP mutant (I143N) and all nuclear RNA polymerase transcription reactions were performed as described in Experimental Procedures.

Especially, RNA polymerase II transcription reactions were performed in the presence of the same amount of minimal and activator-responsive templates. The minimal template for basal transcription contained a 100 base pair-longer G-less cassette than the activator-responsive template. The 20-30 ng of TBPs were used to rescue transcription for all three RNA polymerases. The specific transcripts are indicated by arrows.

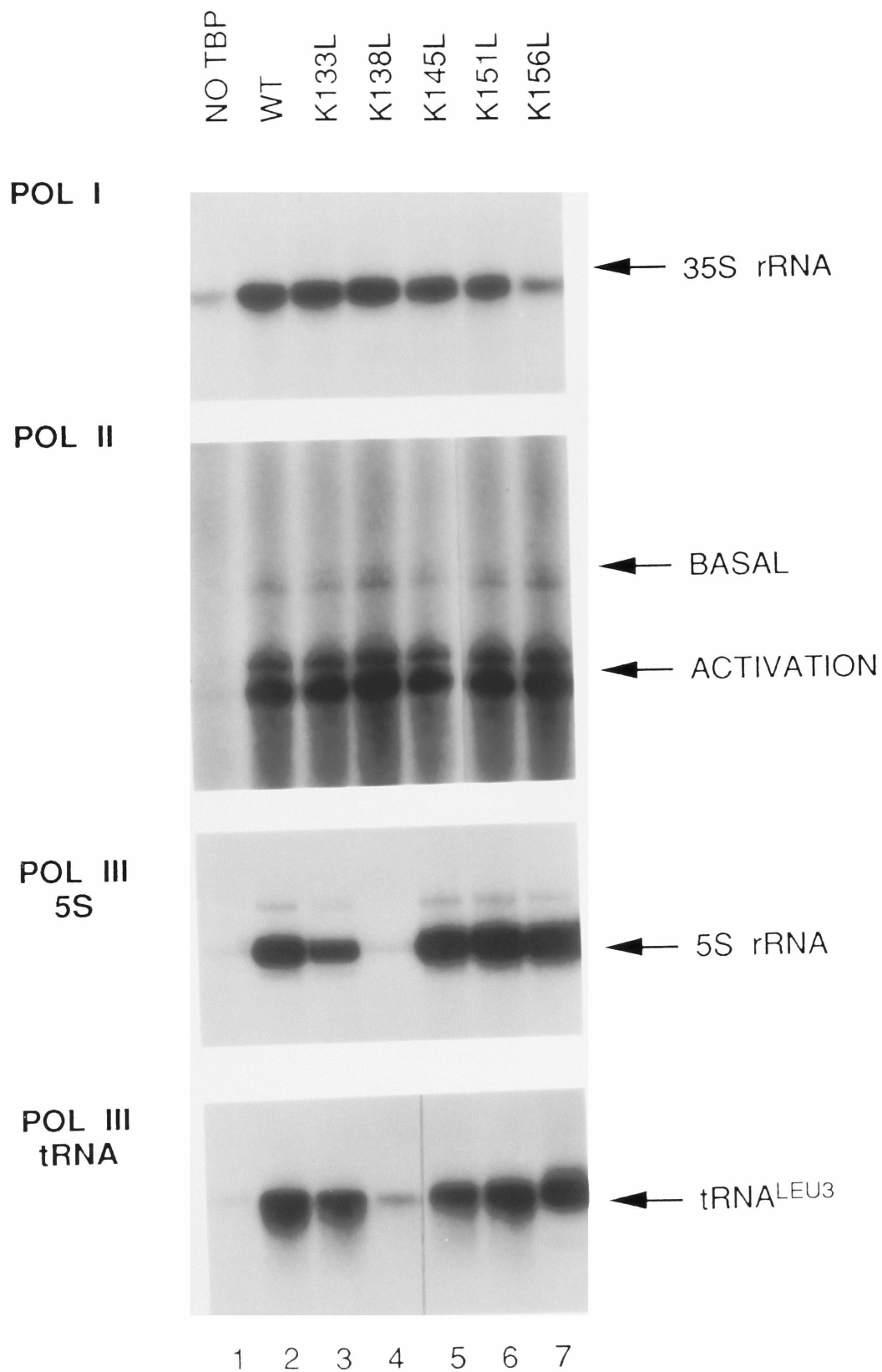


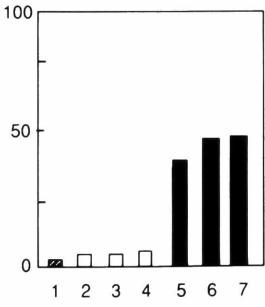
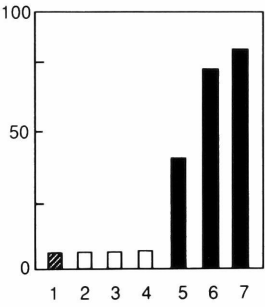
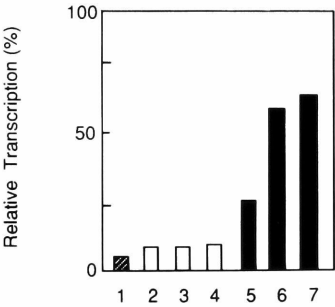
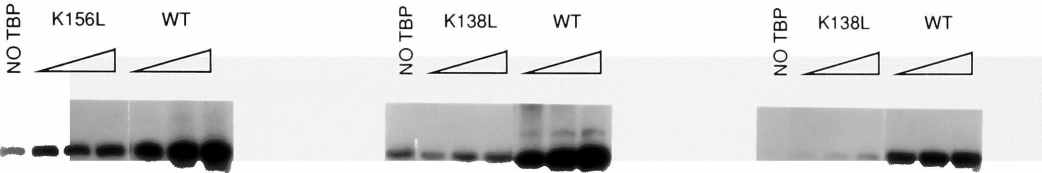
Fig. 6 Titration of defective mutants for RNA polymerase I or III transcription.

Transcription reactions were performed as described in Experimental Procedures. Transcriptions were done in the absence of TBP protein (lane 1) and in the presence of increasing amount of either mutant (10 ng in lane 2, 20 ng in lane 3, and 30 ng in lane 4) or wild-type TBP (10 ng in lane 5, 20 ng in lane 6, and 30 ng in lane 7). Relative transcription activities were measured from the radioactivity in transcripts and indicated as bars.

POL I

POL III  
5S

POL III  
tRNA



## CHAPTER VII

### Conclusions and perspectives

Here, studies of transcriptional activation mechanisms in vivo and in vitro have established the following main conclusions:

1. The proline-rich activation domain of CTF1, like acidic activation domains, can activate transcription in eukaryotes ranging from yeast to human, whereas the glutamine-rich activation domain of Sp1 and the metal binding cysteine-containing activation domain of E1a appear not to be functional in yeast (chapter II). This implies that there are species-specific transcriptional activation pathways that depend upon distinct activators.

2. Both acidic and proline-rich activation domains target TFIID(TBP)-TFIIB-promoter complex formation in the preinitiation complex assembly process (chapters III, IV and V). Importantly, activators induce (or stabilize) qualitative or quantitative alterations in TFIID(TBP)-TFIIB-promoter complexes, thereby enhancing the recruitment of downstream basal factors (TFIIE, TFIIF, TFIIH and RNA polymerase II) for stimulated transcription. These alterations within TFIID(TBP)-TFIIB-promoter complexes could be regulated by differential interactions of distinct activators with either TFIID(TBP) or TFIIB, or possibly through simultaneous interactions with both. For example, acidic activators interact directly with TBP but not efficiently with TFIIB in yeast whereas those activators can interact directly with

both TBP and TFIIB in human (chapters III and IV). In sharp contrast, the proline-rich activation domain of CTF1 can interact directly with TFIIB, but fails to show any direct interaction with TBP (chapter V). Consistent with this view invoking differential interactions, distinct activators have been similarly shown to interact with different TAF(s) (TBP associated factors) in the TFIID complex for activated transcription (Goodrich et al., 1993; Hoey et al., 1993).

3. CTD-like sequences (SP motifs) in the proline-rich activation domain are important for transcriptional activation, possibly forming a  $\beta$ -turned omega ( $\Omega$ ) loop structure (chapter II). Thus, the  $\beta$ -turn structure is likely to be a salient secondary structure in the activation domains in addition to the  $\beta$ -sheet structure, previously proposed for acidic activation domains.

4. TBP plays a key role in the interplay of general and regulatory factors including gene- and cell-specific activators. TBP provides distinct surfaces or sites for regulated interactions with specific factors; acidic activators (chapter III), TFIIB, a negative cofactor Dr1 (NC2), Pol I- and Pol III-specific factors (chapter IV). This information from detailed mutational analyses of TBP, along with its three-dimensional structure (Nikolov et al., 1992), will be important for understanding how the potentially competing interactions of different sets of transcription



factors on the surfaces of TBP specify the function and regulation of the different RNA polymerases.

Of greatest import is the concept of activation-specific alterations within TFIID(TBP)-TFIIB-promoter complex emerged from our studies. The existence of activation-specific interactions has been clearly demonstrated by the detailed analyses of TBP mutants with acidic activators (chapter III). Since TFIID(TBP)-TFIIB-promoter complex formation is consistently important for activation by various kinds of activators (chapters III and V), this complex formation seems to be the most critical step in regulating transcription initiation for eukaryotic activated transcriptions. The next important question is the role of coactivators (TAFs; TBP-associated factors) in activation-specific changes in TFIID(TBP)-TFIIB-promoter complexes. It has been proposed that such coactivators (TAFs) somehow enhance, perhaps by direct contacts, functional interactions of activators with general factors (reviewed in Roeder, 1991; Pugh and Tjian, 1992). Although we have observed that CTF1 can interact with and recruit TFIIB in the absence of other factors (chapter V), consistent with studies of acidic activators (chapters III and IV), some degree of facilitated recruitment of TFIIB to TFIID-promoter complexes by TAFs might still be necessary for maximal levels of stimulated transcription in vitro. Consistent with this possibility, a higher level of activation was observed when DNA templates and CTF1 were

incubated with intact nuclear extracts (containing TAFs) than when DNA templates were incubated first with TBP and TFIIB and then (after washing) with a TBP- and TFIIB-depleted extract containing endogenous TAFs and other cofactors (see Figs. 5 and 6 in chapter V).

On the other hand, TAFs might be involved in activator-induced alterations (Horikoshi et al., 1988a; 1988b; Kim et al., 1994d) within the resulting TFIID-TFIIB-DNA complexes that consequently enhance recruitment of downstream initiation factors or they might be induced by activators to interact directly with these factors. Consistent with these possibilities, recent reports have shown that TAFs (within the natural TFIID complex) are needed for the activator-dependent recruitment of downstream factors to the TFIID-TFIIB-promoter complex (Choy and Green, 1993) and that TAFs can interact directly with activators (Goodrich et al., 1993; Hoey et al., 1993). These results support the conclusions of earlier studies (Horikoshi et al., 1988a; 1988b) showing that activators can induce qualitative changes in TFIID-promoter interactions that result in enhanced recruitment of downstream factors (including TFIIB). Thus it will be important to ascertain whether TAFs alter the TBP-TFIIB interactions described here and elsewhere (Choy and Green, 1993), to effect or stabilize an activation-specific TBP-TFIIB conformation (Kim et al., 1994d), or whether they act primarily through other interactions to stimulate transcription. Related to this, an understanding of dynamic

structural changes in promoter complexes during preinitiation complex assembly will be critical for understanding transcriptional regulatory mechanisms.

What is required next is to link this detailed information with the information on the interplay of various gene- and cell-specific regulators. Such studies of the interactions between different activators (or repressors) are clearly more complex than the study of individual factors. These regulated factor-factor interactions are likely to play a crucial role in the complex regulatory networks which might allow a relatively small number of transcription factors to control highly complex processes such as cellular differentiation. Ultimately, therefore, the understanding of transcription factor function will require a knowledge of the interactions between different factors as well as more detailed structural studies of the domains within individual factors and the manner in which they interact with other domains before this is achieved.

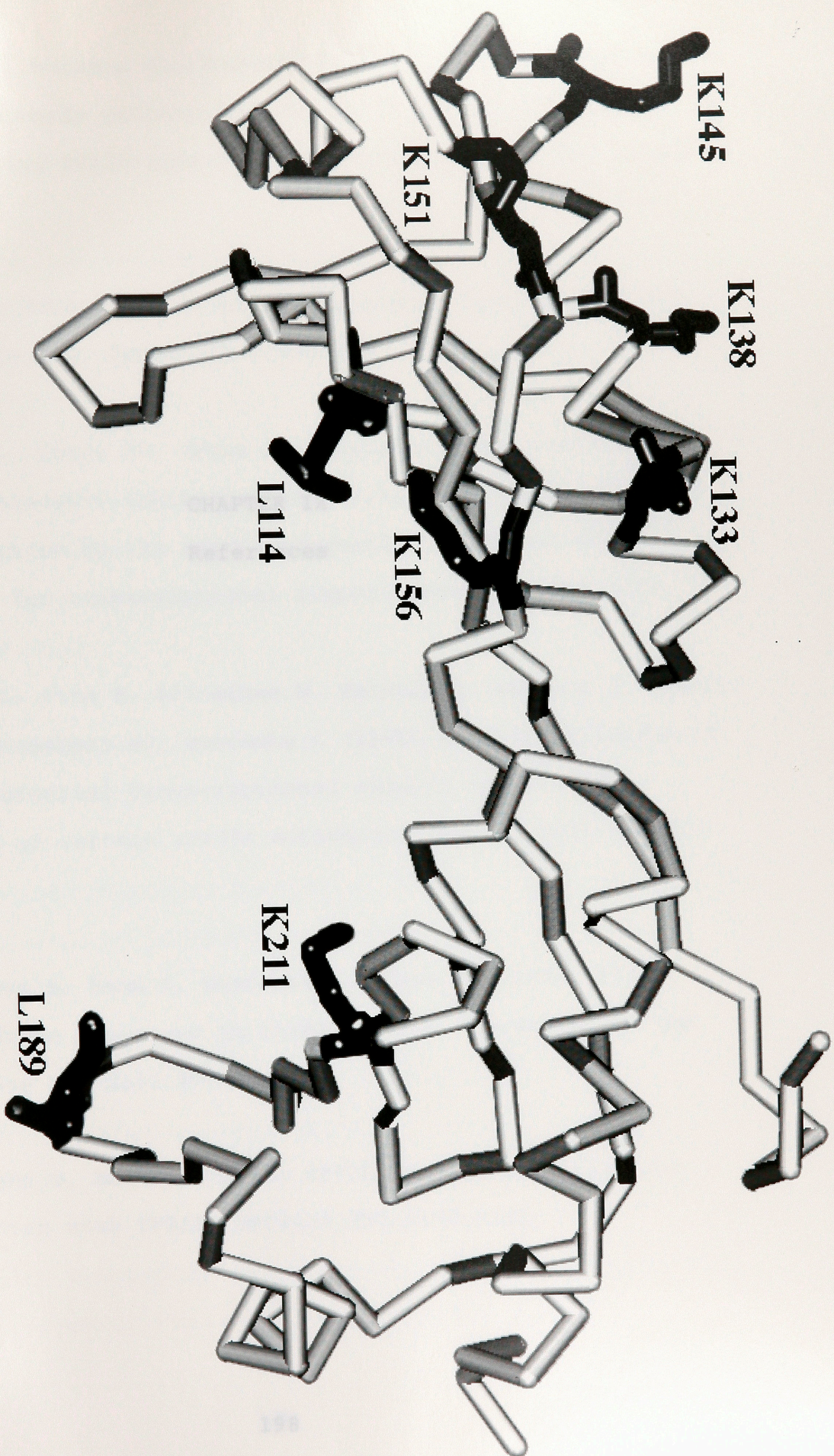
## CHAPTER VIII

### Appendix

Fig. Schematic drawing showing the positions of TBP mutations selectively affect acidic activator-dependent transcription by RNA polymerase II, transcription by RNA polymerase I or III as well as interactions with general factors TFIIB, TFIIA or a negative cofactor Dr1 (NC2).

The X-ray crystallographic analysis of the Arabidopsis homologue of yeast TBP has shown that the conserved core structure is comprised of two highly symmetric domains, each with two  $\alpha$ -helices and a five-stranded antiparallel  $\beta$ -sheet (Nikolov et al., 1992). The  $\alpha$ -carbon backbone is shown as a solid line. Also shown are side chains of defective TBP amino acid residues: L114, L189 and K211, which are defective in transcriptional activation by acidic activators in yeast; L189, which is defective in interaction with TFIIB; K133, K145L and K151, which are defective in interactions with a negative cofactor Dr1 (NC2); K138 and K145, which are defective in interactions with TFIIA; K156, which is defective in transcription by RNA polymerase I; K138, which is defective in transcription by RNA polymerase III.







## CHAPTER IX

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