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DISSERTATION

THE ROLE OF TATA BINDING PROTEIN AND OTHER FACTORS IN DETERMINING PROMOTER STRENGTH

Submitted by

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In partial fulfillment of the requirements

for the Degree of Doctor of Philosophy

Colorado State University

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COLORADO STATE UNIVERSITY

JANUARY 23, 2001

WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY JENNIFER J. STEWART ENTITLED THE ROLE OF TBP AND OTHER TRANSCRIPTION FACTORS IN DETERMINING PROMOTER STRENGTH BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

Committee on Graduate Work

Donald L. Mykles

Adviser

Department Head

ABSTRACT OF THE DISSERTATION

THE ROLE OF TATA BINDING PROTEIN AND OTHER FACTORS IN DETERMINING PROMOTER STRENGTH

Many eukaryotic RNA polymerase II core promoters contain a TATA box with the consensus sequence TATAAA, which is the recognition site for the TATA-binding protein (TBP). There are also a large number of functional core promoters that contain sequences that differ from the consensus element, but these promoters still depend on TBP for transcription initiation. To determine the mechanistic differences in transcription initiation between TATAAA and non-consensus sequences, we compared the functional activity of thirteen TATA elements, each with a systematic replacement of a cytosine or a guanine base at each of the six positions in the TATAAA sequence. Each of the single base substitutions in the consensus TATA sequence leads to severe transcriptional defects in vivo and disruption of various protein-DNA complexes in vitro, depending on the position involved. Initial binding of TBP, TFIIA-TBP and TFIIB-TBP to the different elements revealed that single base replacements in the first position (CATAAA) or the last position (TATAAG), did not affect the initial formation of any of these complexes, although transcription is severely compromised in vivo. Strikingly, the TFIIA-TBP-DNA complex was

significantly less stable on the CATAAA and TATAAG elements when compared to the TATAAA element. This loss of stability was specific to the higher order complex containing TFIIA since there was little difference in the stability of TBP-DNA or TFIIB-TBP-DNA complexes. However, when the N-terminal domain of TBP was removed, the TFIIA-TBP complex could no longer distinguish between TATAAA and CATAAA, suggesting a role for this domain of TBP in determining promoter strength. Taken together, these results suggest that TBP binding to the core promoter and interacting with other components of the general transcription machinery, especially TFIIA, are important considerations for RNA pol II transcription regulation.

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LIST OF ABBREVIATIONS AND ACRONYMS

AD	Activation Domain
DB	DNA Binding Domain
cTBP	Carboxyl-Terminal Domain of TATA Binding Protein
°C	Degree Celsius
DNA	Deoxyribonucleic Acid
EMSA	Electrophoretic Mobility Shift Assay
mM	Milli Molar
min	Minute
nM	Nano Molar
NOT	Negative On Transcription Complex
NTD	N (Amino)-Terminal Domain of TATA Binding Protein
PIC	Pre-initiation Complex
RNA	Ribonucleic Acid
RNA pol II	RNA Polymerase II
$T_{1/2}$	Half-Life
TAF	TBP Associated Factor
TBP:	TATA Binding Protein
TFIIA:	Transcription Factor IIA
TFIIB:	Transcription Factor IIB
µL	Micro Liter
URS:	Upstream Repressor Sequences
UAS:	Upstream Activator Sequences
v/v	Volume per Volume

CHAPTER 1

INTRODUCTION TO RNA POLYMERASE II TRANSCRIPTION INITIATION AT EUKARYOTIC PROMOTERS

1.1 OVERVIEW

The expression of a eukaryotic gene transcribed by RNA polymerase II (pol II) is dictated by a combination of cellular and environmental signals that control the activity of transcription regulatory proteins. How a gene responds to these incoming signals will depend on the physical state of the gene and the cellular machinery that transcribes it. The physical state of the gene can be categorized in three different ways. In a repressed gene, the chromatin is condensed and inaccessible to the transcription machinery, which renders it essentially off. All transcription in vivo is activated because activator proteins alter chromatin accessibility by recruiting one or more chromatin remodeling factors. Genes may reside within a less condensed state of chromatin and be accessible, but lack sufficient recruitment of the transcription machinery and be expressed at very low levels. Induced genes are also likely to reside in less condensed chromatin and be bound by activators, which efficiently assemble the transcription machinery and allow higher levels of gene expression.

1.2 RNA POLYMERASE II PROMOTER ARCHITECTURE

Promoters transcribed by eukaryotic RNA polymerase II consist of two distinct classes of elements, the core elements and the upstream regulatory elements (shown in Figure 1.1 and reviewed in 71). The core promoter elements are DNA sequences that are required for transcription initiation because they define the site of assembly of the components of the general transcription machinery. The most well characterized core element is the TATA element, which is located 25 bp (human) or 40-120 bp (yeast) upstream of the other prominent core element, the Inr or transcriptional start site (reviewed in 53, 71). The TATA element is the recognition site for the TATA-binding protein (TBP) and selection for functional elements by mutational analysis has defined TATAAA as the consensus sequence of yeast TATA elements (7, 11, 23, 25, 66, 77). The assembly of transcription factors at the core promoter is influenced by upstream activators and repressors.

Transcriptional repressors bind to the upstream repressor sequences (URS) of the gene. Repressors utilize a variety of mechanisms in order to impair transcription initiation. Repressors have been shown to directly counteract the stimulatory effects of activators or by recruiting other complexes with inhibitory effects (reviewed in 28). However, most of the known eukaryotic repressors act on the general transcription machinery, possibly by sterically blocking the addition of proteins in pre-initiation complex assembly or by preventing required conformational changes (reviewed in 28).

Upstream activator sequences (UAS) are DNA elements that function as binding sites for transcriptional activators. Activators typically contain at least two separable domains, a DNA-binding domain and an activation domain. DNA-binding domains are sequence-specific binding domains that interact with DNA sequences located upstream of the target gene. The activation domain is responsible for protein-protein interactions, which enable communication with general transcription machinery. Once associated with the UAS, activators are thought to function by recruiting chromatin remodeling factors or bringing limiting components of the transcription apparatus (9, 59, 74) to the promoter.

1.3 THE MAIN PLAYERS OF RNA POL II TRANSCRIPTION INITIATION

RNA polymerase II cannot recognize its target promoter directly without accessory proteins. An essential step in RNA pol II transcription initiation is the assembly of a pre-initiation complex (PIC) on and around the core promoter (Figure 1.1). The PIC consists of the pol II enzyme along with several general transcription factors including TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH (53). PIC formation is highly dependent upon core promoter sequences that direct the precise positioning of TFIID, a multi-subunit protein consisting of TBP and several TBP-associated factors (TAFs).

1.3a TATA-BINDING PROTEIN (TBP)

TBP is an essential protein for transcription by all three eukaryotic RNA

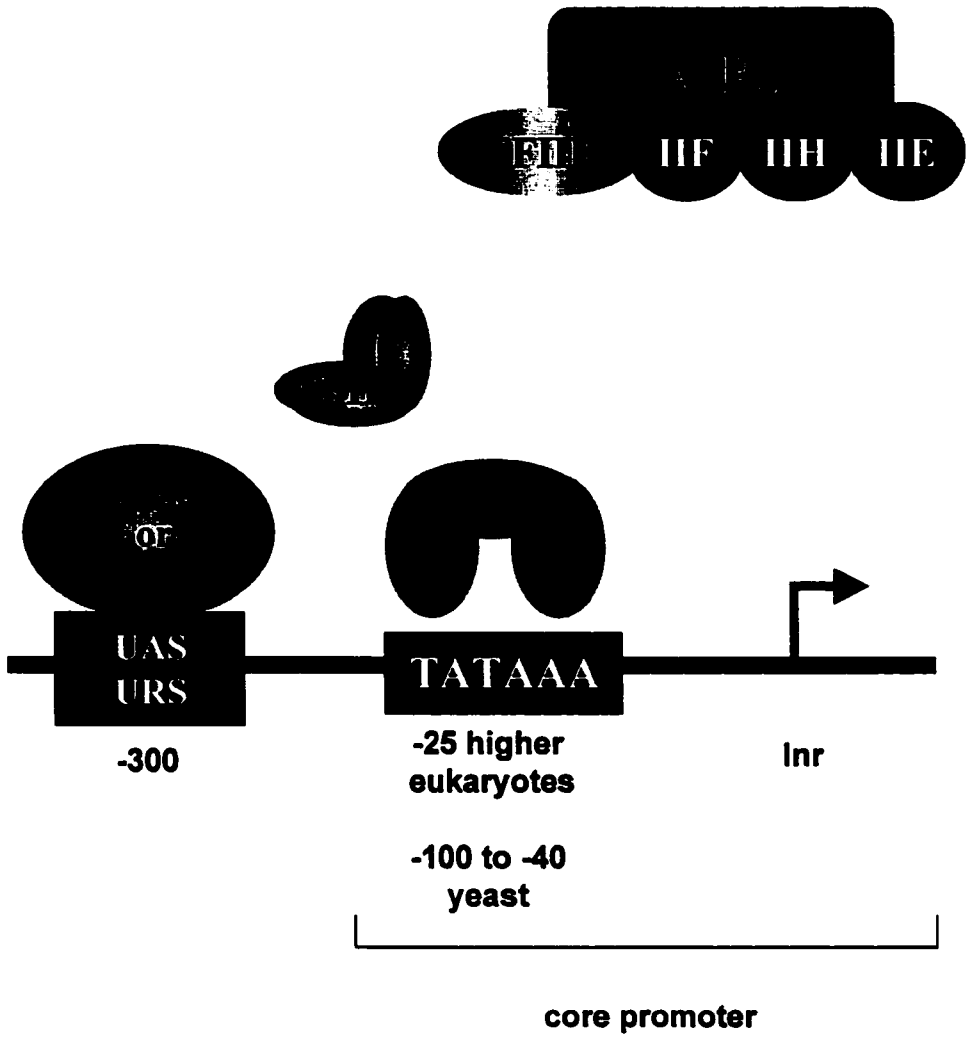

Figure 1.1 Schematic of a typical promoter transcribed by RNA polymerase II and several of the known protein complexes involved in pre-initiation complex formation. TATA-Binding protein (TBP), Transcription Factors (TFIIA, TFIIB, TFIIE, TFIIF, TFIIH), Upstream Activator (Repressor) Sequences (UAS, URS), Initiator (Inr) and RNA Polymerase II (RNA pol II).

Polymerases. In yeast, the gene for TBP (*SPT15*) was originally identified in a genetic screen looking for suppressors of the retrotransposon Ty insertion in the *HIS4* promoter (Supressor of TY) (17, 22). TBP binds to the TATA box present at many promoters transcribed by RNA pol II, and initiates the assembly of the pre-initiation complex (reviewed in 7, 23).

The *Saccharomyces cerevisiae* 27-kDa monomeric TBP consists of a protease resistant core domain (cTBP) and a 63 amino acid amino-terminal domain of unknown function. Human cTBP (29), yeast cTBP (19, 32, 33) and *Arabidopsis thaliana* cTBP (57) bound to TATA DNA have been crystallized. These structures show cTBP adopts a two-fold symmetric structure, resembling a molecular saddle complete with stirrups (Figure 1.2A). The concave underside of the saddle is a highly curved, 10-stranded, antiparallel β-sheet, containing the amino acids involved in DNA binding. The convex upper surface of the saddle consists of four α-helices, which interact with other transcription factors (for review, see 50). Once TBP recognizes and binds to the TATA box, an unusual protein–DNA complex is formed. The interaction is characterized by extensive hydrophobic interactions with the minor groove, unwinding the DNA by 120°, inducing an 80° bend toward the major groove with phenylalanine side chains kinking DNA by insertion between base pairs at the ends of the TATA box. TBP binds to the minor groove of DNA, which provides few functional groups to direct specificity of binding (41, 64, 70). It is believed that TBP exploits an induced-fit to recognize A-T rich sequences with a uniform minor-

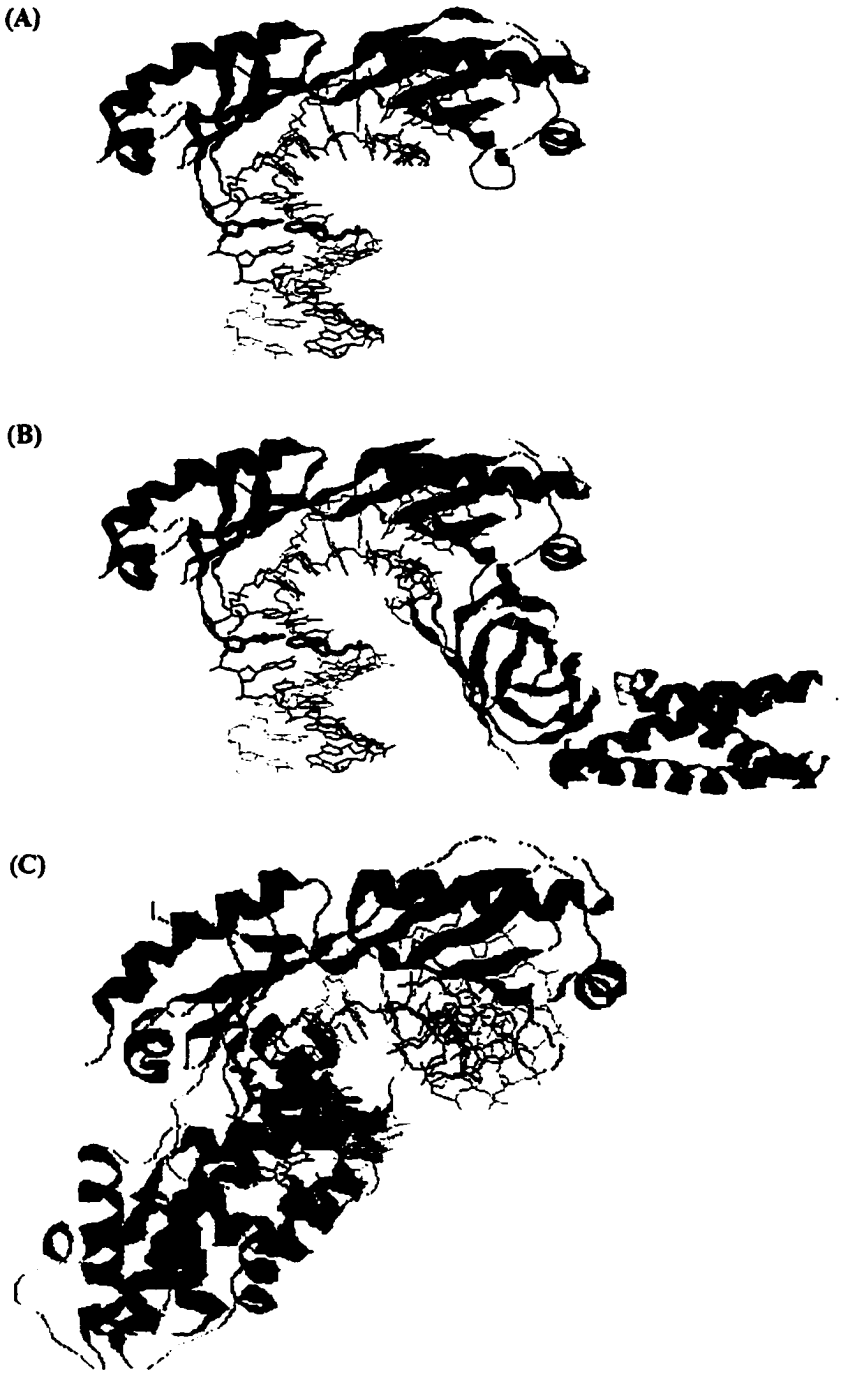

Figure 1.2 A. The crystal structure of the TBP-DNA complex. TBP is shown in blue, DNA in yellow and red. For orientation, the most downstream base pair of the TATA box is shown in black. **B.** The crystal structure of the TFIIA-TBP-DNA complex. The two subunits of TFIIA are shown in green (Toa1) and aqua (Toa2). **C.** The crystal structure of the TFIIB-TBP-DNA complex. TFIIB appears in gold.

groove face, because both A-T and T-A base pairing display similarly positioned hydrogen bond acceptors on the minor groove side (56). The importance of the TBP-TATA interaction has been demonstrated by studies showing that the binding of TBP to the TATA box is rate-limiting at a majority of TATA-containing promoters and therefore a likely target of transcription regulation (10, 14, 34, 35, 38, 43).

1.3b TRANSCRIPTION FACTOR IIB (TFIIB)

TFIIB enters the PIC upon TBP binding to form the requisite TFIIB-TBP-DNA complex for recruitment of RNA pol II (5). TFIIB interacts directly with TBP, RNA pol II and with other general transcription factors such as TFIIF (21). Footprinting (42) and cross-linking (39) experiments demonstrated that TFIIB binds both upstream and downstream of the TATA box, a result consistent with the crystal structure of the TATA-TBP-TFIIB ternary complex (51) (Figure 1.2C). Yeast TFIIB is a 38 kDa monomer encoded by the *SUA7* gene. Human and yeast TFIIB have two separate domains, a C-terminal region which folds into a protease-resistant core (cTFIIB) that binds TBP and an N-terminal region which interacts with RNA pol II-TFIIF (6, 21, 24, 78). The N- and C-terminal domains engage in an intramolecular interaction that undergoes an activator-induced conformational change, allowing assembly of the PIC (63). Since the N-terminal region was not included in the structural studies of TFIIB, little is known about the structure of this part of the protein. However, a solution structure for the

metal binding domain of *Pyrococcus* TFIIB suggests that this region forms a zinc ribbon (81). A basic region, comprised of the D1 and E1 helices of the first repeat of cTFIIB, interacts with the acidic C-terminal stirrup of TBP. Residues within the C2 and E2 helices of the second repeat contact DNA on both sides of the TATA box. cTFIIB consists of two similar structural domains, each containing five α-helices, corresponding to the two imperfect repeats.

TFIIB has also been implicated in start site selection. *SUA7*, the gene encoding TFIIB, was initially identified in a genetic selection for suppressors of a translational defect in the *CYC1* locus (58). Recessive *sua7* mutations shifted the transcription start site downstream on *CYC1* and *ADH1* genes (4, 58). Suppressor alleles were mutated in the N-terminal region, diminishing TFIIB's ability to interact with RNA pol II, correlating this interaction with start site selection. The presence of TFIIB in the PIC allows for recruitment of additional components of the general transcription machinery and the involvement of TFIIB in start site selection, show that this transcription factor is very important in RNA pol II transcription.

1.3c TRANSCRIPTION FACTOR IIA (TFIIA)

TFIIA has been shown to bind directly to TBP and stabilize its interaction with the DNA, especially under sub-optimal binding conditions (26, 30, 40). The crystal structure of this ternary complex in yeast shows that TFIIA binds TBP and makes contacts with DNA upstream of the TATA element (19, 73). Unlike

TFIIB, which binds both upstream and downstream of the TATA box, the portion of TFIIA present in the crystal structure interacts exclusively upstream of TATA (Figure 1.2B).

In *S. cerevisiae*, TFIIA is composed of two subunits, a 32-kDa subunit encoded by the *TOA1* gene and a 13.5-kDa sub-unit encoded by the *TOA2* gene (62). Both genes are essential for cell viability and show striking similarity to the TFIIA-encoding genes of higher eukaryotes. Crystal structures for two forms of yeast TFIIA-TBP-DNA ternary complexes have been solved (19, 73) (Figure 1.2B). In both cases, the structures were determined with the smallest form of TFIIA that retained biological function. The largest subunit of TFIIA had a large, dispensable central region of the polypeptide deleted. Two structural motifs were shown: a six-stranded β-domain and a four-helix bundle. The C terminus of both subunits contributed three strands to the β-domain, and the N terminus of each subunit contributed two helices to form the helical bundle.

TFIIA plays important roles in core promoter functions (45, 54, 69), in activated transcription (12, 13, 16, 36, 44, 55), and in counteracting the effects of negative regulators (1, 20, 37). In addition, TFIIA induces a conformational change in TBP and a reorganization of the TFIID/promoter complex (12, 18, 39, 40, 52). How these diverse functions of TFIIA are mechanistically mediated and whether they are related to each other are topics of current interest. Clearly, recruitment of TBP and stabilization of the TBP-DNA complex are critical functions for TFIIA in the broader scheme of RNA pol II transcription

initiation.

1.4 TATA-LESS PROMOTERS

Despite the fact that TBP is required at all promoters transcribed by RNA pol II, many natural eukaryotic core promoters lack recognizable TATA elements. This non-canonical or "TATA-less" subclass includes the promoters of the ubiquitously expressed "housekeeping" genes, developmentally regulated genes (growth factors, growth factor receptors) and many oncogenes (reviewed in 67). In yeast, amino acid biosynthesis genes are often driven by promoters lacking the canonical TATA sequence. The *HIS3* promoter has been utilized to investigate the differences between TATA-containing and TATA-less core promoter transcriptional initiation (Figure 1.3). This promoter serves as an excellent model system to characterize transcription initiation from different types of core promoters because it contains two functionally distinct TATA elements (11, 48) that exhibit a clear pattern of differential utilization (27). One element, T_R, conforms to the canonical TATAAA sequence and is responsible for initiation at position +13. The other element, T_C, is made up of an extended sequence that does not resemble a consensus TBP binding site and directs initiation from position +1. T_C is preferentially utilized at low levels of transcription, T_C and T_R are utilized equally well at moderate levels of transcription, and T_R is preferentially utilized at high levels of transcription.

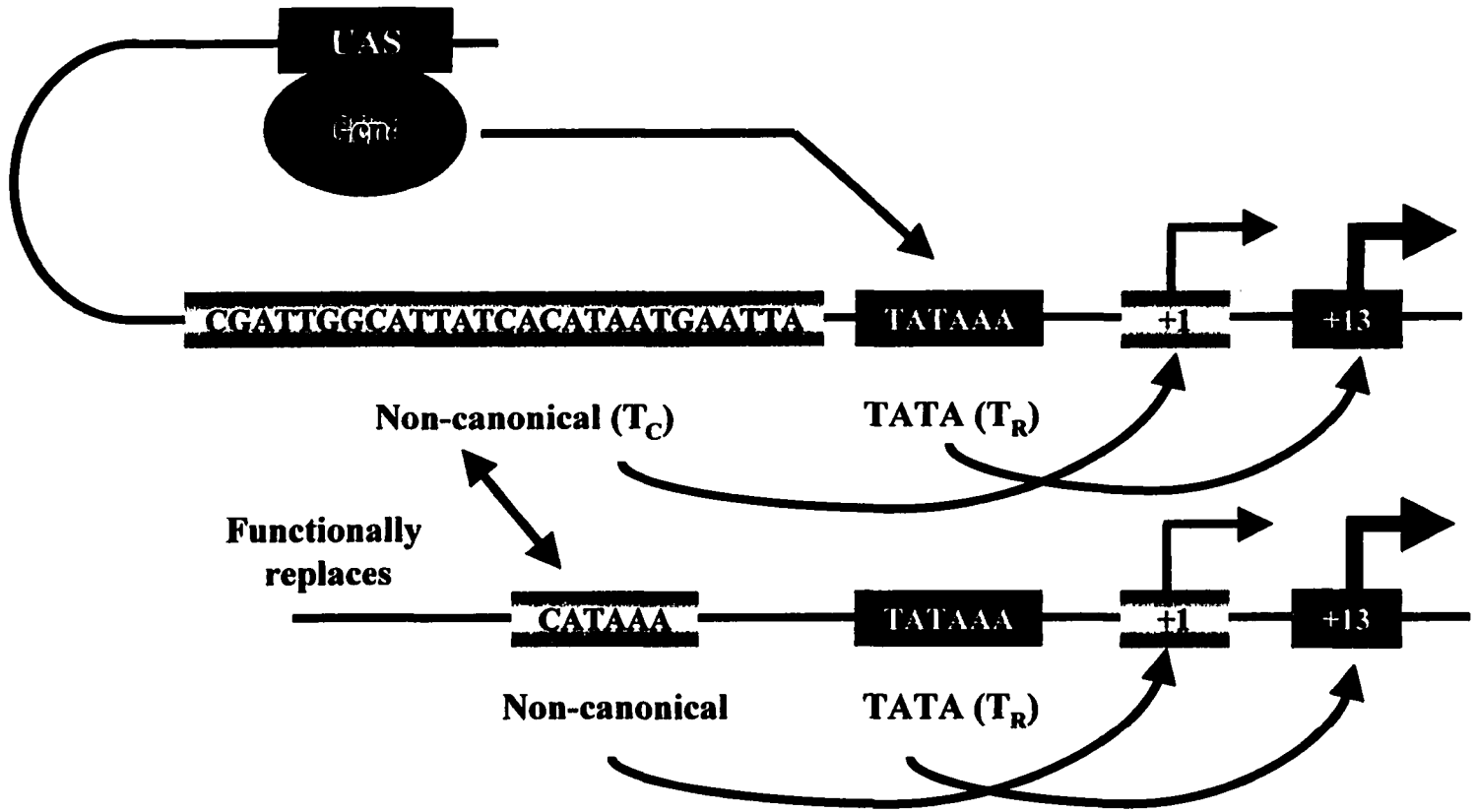

Figure 1.3 Schematic representation of the yeast *HIS3* promoter showing two functionally distinct core promoter elements. T_R, (canonical TATAAA) is responsible for initiation at position +13 while T_C (non-canonical) directs initiation from position +1. T_C is preferentially utilized at low levels of transcription, T_C and T_R are utilized equally well at moderate levels of transcription, and T_R is preferentially utilized at high levels of transcription. This clear pattern of differential utilization can be mimicked by substitution of T_C by CATAAA.

1.5 TBP IS REQUIRED AT "TATA-LESS" PROMOTERS.

TBP is required for transcription of class II genes that do not contain a consensus TATA box (3, 8, 60, 61, 68, 79, 80), for the TATA-less ribosomal genes transcribed by RNA polymerase I (15), and for TATA-containing and TATA-less genes transcribed by RNA polymerase III (31, 46, 47, 49, 65, 72, 75, 76). How is TBP recruited to TATA-less promoters? In the case of RNA polymerase I genes, TBP is part of the multisubunit selectivity factor SL1, which is recuited to the core ribosomal RNA promoter by protein-protein interactions with an activator, the upstream binding factor (UBF) (2, 15). TATA-less class III genes bring TBP to the promoter as part of the TFIIIB multisubunit complex, which is tethered to the promoter by protein-protein interactions with TFIIIC bound to the intragenic core promoter elements (31, 65, 72, 76). The mechanisms of TBP recruitment to RNA polymerase II promoters remain unclear. However, as is the case with RNA pol I and RNA pol III, it seems likely that other components of the RNA pol II machinery are involved.

1.6 IDENTIFICATION OF A NOVEL MECHANISM OF TRANSCRIPTION REGULATION FROM NON-CANONICAL PROMOTERS INVOLVING TFIIA.

For my dissertation research, I have chosen to investigate the mechanistic differences between transcription initiation at TATA-containing versus non-canonical RNA polymerase II promoters. I studied thirteen different core promoter sequences, each with a different base substitution of the six base

pair consensus TATAAA sequence. I found that all twelve single base changes in the consensus TATAAA sequence caused a severe decrease in transcription in vivo and affected protein-DNA interactions in vitro, depending on the position involved. In general, substitutions of the base pairs in the middle of the TATAAA sequences caused the most dramatic effects in TBP binding and higher order complex formation. However, substitutions on the ends of the TATA box, such as TATAAG and CATAAA, had no effect on initial binding of TBP, TFIIA-TBP or TFIIB-TBP. The main defect with these elements was the stability of the TFIIA-TBP-DNA complex, which was compromised on both CATAAA and TATAAG. The removal of the amino-terminus from TBP resulted in stabilization of the cTBP-DNA and TFIIA-cTBP-DNA complexes on both TATAAA (canonical) and CATAAA (off-consensus) and the TFIIA-cTBP complex could no longer distinguish between different core DNA sequences.

These results indicate that in order to achieve high levels of transcriptional output, a productive pre-initiation complex must be assembled at the core promoter. We have found that guanine or cytosine replacements in the consensus TATAAA sequence affects TBP and TFIIA containing complexes, underscoring the critical role these two transcription factors play in transcription initiation at promoters lacking the consensus sequence.

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CHAPTER 2

THE STABILITY OF THE TFIIA-TBP-DNA COMPLEX IS DEPENDENT ON THE SEQUENCE OF THE TATAAA ELEMENT

Chapter two describes the interaction of several components of the RNA polymerase II general transcription machinery with two distinct promoter elements, TATAAA and CATAAA. This work is submitted for publication in the *Journal of Biological Chemistry*. The work appears here exactly as in the submitted manuscript. However, data cited as "data not shown" in the manuscript are shown here as supplemental figures. The citation of the publication appears below.

Stewart, J.J. and L.A. Stargell. 2001. The stability of the TFIIA-TBP-DNA complex is dependent on the sequence of the TATAAA element. J. Biol. Chem. (submitted).

2.1 SUMMARY

To determine the mechanistic differences between canonical and non-canonical TATA elements, we compared the functional activity of two sequences: TATAAA (canonical) and CATAAA (non-canonical). The TATAAA element can support high levels of transcription in vivo while the CATAAA element is severely defective for this function. This dramatic functional difference is not likely due to a difference in TBP binding efficiency since protein/DNA complex studies in vitro indicate little difference between the two DNA sequences in the formation and decay rates of the TBP-DNA complex. In addition, the binding and stability of the TFIIB-TBP-DNA complex is similar for the two elements. In striking contrast, the TFIIA-TBP-DNA complex is significantly less stable on the CATAAA element when compared to the TATAAA element. The importance of the TFIIA-TBP interaction in distinguishing between TATAAA and CATAAA was tested in vivo by fusing a subunit of TFIIA to TBP. We found that fusion of TFIIA to TBP dramatically increases transcription from CATAAA in yeast cells. Taken together, these results indicate that the stability of the TFIIA-TBP complex depends strongly on the sequence of the core promoter element and that the TFIIA-TBP complex plays an important function in recognizing optimal promoters in vivo.

2.2 INTRODUCTION

Initiation of mRNA synthesis by RNA polymerase (Pol) II is the major

step of regulation of eukaryotic gene expression, and occurs at core promoters that typically consist of a TATA box and initiator element (63). The first step in promoter recognition is binding of TATA-binding protein (TBP) to the TATAAA element (reviewed in 4). Recruitment of TBP is a rate-limiting step in transcriptional initiation in vivo at a majority of promoters (6, 27, 67) and promoter occupancy of TBP correlates very well with transcriptional activity (32, 37). The TBP-TATA interaction sets the stage for the nucleation of the remainder of the pre-initiation complex (PIC), which includes TFIIA, -B, -E, -F, -H, -J and Pol II (reviewed in (47). TFIIA and TFIIB have each been shown to make direct contacts with TBP and DNA, and in so doing, stabilize the TBP-DNA complex (reviewed in 15). In addition, TFIIA- and TFIIB-promoter occupancy is coordinated with that of TBP (31), thus interactions between the DNA, TBP, TFIIA and TFIIB are likely to play an important role at a majority of promoters in vivo.

Despite the apparent requirement of the TATAAA sequence in the initiation of Pol II transcription, many natural eukaryotic core promoters lack recognizable TATA elements but still require TBP for transcription initiation (11, 41, 53). This non-canonical or "TATA-less" subclass includes the promoters of several different types of genes, including the ubiquitously expressed "housekeeping" genes, developmentally regulated genes (growth factors, growth factor receptors) and many oncogenes (14, 46, 57). In addition, several instances of multiple, yet functionally distinct elements driving the expression of

a single gene have been described in yeast (36, 55, 62). For example, the yeast *HIS3* gene contains two distinct promoter elements: a canonical TATAAA element (T_R) that initiates from +13; and a second element (T_C) that lacks the canonical TATAAA sequence, extends over approximately 30 base pairs, and initiates from +1 (40). The two tandem yeast *HIS3* promoter elements are utilized differentially depending on the overall levels of *HIS3* production (52, 62). At low levels of overall transcription, both T_C and T_R are utilized with approximately equal efficiencies. However, at high levels of transcription, expression from T_C increases only slightly, while expression from T_R shows a large and dramatic increase. Thus, the non-canonical T_C element saturates at much lower levels of expression than does the canonical TATA element.

The in vivo regulation observed for T_C can be mimicked by substituting T_C with CATAAA, a sequence with a single base pair change of the first thymine in the TATAAA element to a cytosine (22). Since CATAAA can functionally replace T_C, and CATAAA serves as a simpler model than T_C for non-canonical core promoters, we utilized TATAAA and CATAAA to study the mechanistic differences between canonical and non-canonical elements in vitro and in vivo. Electrophoretic mobility shift assays show that the TBP-DNA and the TFIIB-TBP-DNA complexes behave very similarly on TATAAA and CATAAA. In contrast, the TFIIA-TBP complex is much less stable on the CATAAA element compared to the TATAAA element. Transcriptional analysis indicates that the CATAAA element is severely defective for supporting high levels of transcription

in vivo, but a TFIIA-TBP fusion molecule can restore activity from this element. These results suggest that the stability of the TFIIA-TBP-DNA complex is sensitive to the sequence of the core promoter element and that the TFIIA-TBP complex plays an important role in core promoter recognition and the level of transcriptional expression in vivo.

2.3 EXPERIMENTAL PROCEDURES

2.3a Strains and DNA Constructs

The yeast strain yJS156 is a derivative of KY804 (relevant genotype: *MATα ura3-52 trp1-Δ1eu2::PET56 gal2 gcn4-Δ1*) (19), with the chromosomal copy of *SPT15* deleted by a two-step knockout. TBP functions are provided by a *URA3*-marked plasmid containing the TBP promoter, open reading frame and terminator. Plasmid shuffling on 5-fluoroorotic acid was used to create the *TRP1*-marked wild type TBP, Toa2-TBP and TFIIB-TBP fusion strains assayed.

The DNA elements used for the in vivo transcription analysis and the in vitro protein DNA interaction studies were 23 base pair oligonucleotides designed from sequences at the promoter of the yeast *HIS3* gene. The TATAAA oligo contains a core promoter element identical to the *HIS3* T_R with the sequence 5'AATTCC**TATAAA**GTAATGTGGAG 3'. The CATAAA oligo is identical to TATAAA except that the first thymine in the core element is substituted with a cytosine (5'AATTCC**CATAAA**GTAATGTGGAG 3'). To make double stranded probe, a 19 bp complementary oligonucleotide was

synthesized and annealed to the 23 bp oligo described above leaving an adenine and thymine rich overhang on the 5' end of the probe. Klenow enzyme was used to fill in the single stranded overhang with 25 μCi [α-32 P]dATP in the presence of 2.0 μM dTTP.

Plasmids used for the in vivo transcription analysis were derivatives of YCp86 containing the 2 Kb fragment from pUC18 including the *bla* gene and the origin of replication, a 1.9 Kb fragment of yeast DNA containing *cen3* and *ars1*, a 1.1 Kb fragment containing the *URA3* gene and a polylinker. The hybrid *HIS3* promoterYCp86-SC3801 (7, 56) has the wild-type initiation and amino terminal region of the *HIS3* gene fused in frame with a functional *E.coli LacZ* gene and is used to detect levels of transcription in vivo. The promoter region contains a 365-bp *GAL1,10* fragment containing four GAL-4 binding sites fused upstream of the EcoRI-SacI restriction endonuclease sites, between which the TATAAA and CATAAA oligonucleotides (described above) could be inserted. When the two oligos were cloned into this molecule it was renamed pJS3801 (TATAAA) and pJS3803 (CATAAA), respectively.

2.3b Transcriptional analysis

For the experiments measuring activated transcription in vivo, the pJS3801 and pJS3803 constructs (described above) were transformed into yeast strain yJS156. The resulting transformants were used in liquid assays for β-galactosidase activity. The cells were grown in selective medium containing

either 2% raffinose or 2% galactose to mid-exponential growth phase ($OD_{600} \cong 1$-2). Enzyme activities were determined in triplicate and normalized to the OD_{600} of the cultures.

Quantitative RNA analysis was done by S1 nuclease digestion of approximately 50 μg of RNA prepared from cells grown in synthetic complete medium to an OD_{600} of 0.6 (10, 21). Total RNA was prepared by hot-phenol extraction and hybridized with 100-fold excess of radiolabeled single stranded *HIS3*, *DED1* and *tRNA*^{W} probes and subjected to S1 nuclease digestion. The RNA amounts used in each reaction were normalized to the RNA levels obtained from a probe to the intron of the tryptophan tRNA gene (tRNA^{W}).

2.3c Protein Expression and Purification

Untagged, full-length yeast TBP was expressed in *E. coli* strain BL21 (DE3) with a pET11a vector (called pYTBP) as described (5). The soluble fraction was purified over Q, SP, and Heparin HiTrap columns (Pharmacia) equilibrated in buffer A (10 mM sodium phosphate, 100 mM KCl, 5 mM $MgCl_2$, 10% (v/v) glycerol, 1 mM DTT, pH adjusted to 7.5). TBP flowed through the Q column and was eluted from the SP and Heparin columns using linear NaCl gradients in buffer A. Final purification of TBP was by gel filtration with Sephacryl S-100 equilibrated in 100 mM KCl, 40 mM HEPES, 20 mM Tris, 10% glycerol, 1mM dithiothreitol and 0.5 mM phenylmethylsulfonyl fluoride (final pH 7.9).

TFIIB derivatives were cloned into pET-15b for expression and purification of His-tagged proteins from *E.coli* BL21DE3/PlysS (Novagen). Cells were grown in LB medium at 37 °C to an optical density of 0.7. Isopropyl-β-D-thiogalactopyranoside (IPTG, 0.1mM final concentration) was added and the cells were incubated for two hours at 30 °C. Cells were harvested by centrifugation and washed with a 20 mM Tris, 50 mM NaCl buffer. Following sonication, the lysate was incubated with shaking at 4 °C with 1 mL of His-bind resin (Novagen). After two consecutive wash steps with buffers containing 20 mM imidizole and 80 mM imidizole respectively, the his-tagged protein was eluted with buffer containing 200 mM imidizole. The eluate was dialyzed against 100 mM KCl, 40 mM HEPES, 20 mM Tris, 10% glycerol, 1 mM dithiothreitol and 0.5 mM phenylmethylsulfonyl fluoride, final pH 7.9 (EMSA binding buffer without $MgCl_2$). The protein was shown to be 80% pure upon staining with Coomassie blue.

Recombinant yeast TFIIA was purified as described (54). This procedure involves expressing each subunit, Toa1 and Toa2, in separate strains of *E.coli* BL21DE3. IPTG (0.1mM final concentration) was added at an optical density of 0.6 and after the cells were ruptured by sonication, insoluble material was collected by centrifugation. The proteins were then denatured in a buffer containing 8M urea and renatured in the presence of the other subunit. The renatured proteins were dialyzed against 100 mM KCl, 40 mM HEPES, 20 mM Tris, 10% glycerol, 1 mM dithiothreitol and 0.5 mM phenylmethylsulfonyl

fluoride, final pH 7.9 (EMSA binding buffer without $MgCl_2$). The TFIIA was approximately 60 % pure as determined by Coomassie staining.

2.3d Electrophoretic mobility shift assays (EMSA)

Protein-DNA interactions in vitro were studied by incubation of purified proteins with ^{32}P labeled TATAAA and CATAAA probes. Binding reactions contained 10 μM poly[dG-dC] non-specific competitor, 100 mM KCl, 40 mM HEPES, 20 mM Tris, 10% glycerol, 1 mM dithiothreitol and 0.5 mM phenylmethylsulfonyl fluoride, final pH 7.9. ^{32}P labeled TATAAA and CATAAA probes (2.4 picomoles) were incubated with 13.8 nM TBP at 25° C for 30 minutes. The complexes were separated on 5% acrylamide gel containing 50 mM Tris-borate, 1 mM EDTA and 2 mM $MgCl_2$ in both the gel and running buffer. Recombinant yeast TFIIA (5.0 nM) and His-tagged TFIIB (8.5 nM) were isolated and incubated with TBP and probe DNA and treated as described above, except that $MgCl_2$ was omitted from the gel and running buffer. Omission of the divalent cations from the binding reaction allows for the stable formation of only the ternary complex. Gels were transferred to Whatman 3MM paper and dried under vacuum at 80 °C and subjected to phosphorimager screen or autoradiography at -70°C with an intensifying screen.

2.3e Quantitative analysis of DNA binding

The concentration of active protein was determined by DNA titration in

which the reaction mixtures contained known concentrations of radiolabeled probe and a fixed amount of protein (17, 18) and the fraction of DNA bound was determined. By this analysis, the TBP preparation used for most experiments shown in this work was about 6% active, similar to the10% activity reported by others for yeast TBP preparations (18). All TBP concentrations given in the text refer to the concentration of active protein.

TBP and TFIIA-TBP binding kinetics were measured by electrophoretic mobility shift assays (EMSA). Reactions containing 2.4 picomoles of probe DNA and 13.8 nM TBP (or 13.8 nM TBP and 5.0 nM TFIIA) were incubated at 25 °C in a final reaction volume of 360 μL. At the indicated times, the extent of TBP-DNA and TFIIA-TBP-DNA complex formation was analyzed by loading 10 μL of the binding reaction on to the gel. The amount of complex formed was quantitated using the Phosphorimager. The data are presented as the fraction bound at time, t. Fraction bound is calculated from the ratio B/B_{30}, where B represents the amount of complex present at each time point and B_{30} is defined as the amount of complex formed at 30 minutes.

For dissociation kinetic studies, TBP-DNA, TFIIA-TBP-DNA and TFIIB-TBP-DNA complexes were allowed to reach equilibrium and then challenged with 1000 fold molar excess of specific competitor poly[dAdT] for the specified amount of time. The samples were then loaded on to the gel and resolved by EMSA. The data are presented as the fraction bound at time, t. Fraction bound is calculated from the ratio B/B_0, where B represents the amount of complex

present at each time point and B_o is defined as the amount of complex formed in the control lane without added specific competitor.

2.4 RESULTS

2.4a Mechanistic differences between canonical and non-canonical elements may depend on TFIIA activity.

Recent work characterizing a TBP allele defective for interaction with TFIIA (the N2-1 derivative of TBP) has shown that the TFIIA-TBP interaction is important for *HIS3* core element utilization (58). When the amount of RNA initiating from +1 (which is driven by the non-canonical element, T_C) and +13 (driven by TATA) start sites were compared, the N2-1 strain showed a preferential increase in transcription initiating from +13, even when the overall level of transcription was low (58). This is in stark contrast to the utilization of the *HIS3* promoter elements occurring in wild type cells, which shows similar levels of output (22). These results suggest that the loss of the TFIIA-TBP interaction has a more dramatic effect on the non-canonical element (T_C) than on the canonical element (58). To further test the importance of the TFIIA-TBP interaction at the *HIS3* promoter, we examined the effect of introducing a fusion construct of a subunit of TFIIA (Toa2) to TBP (59). The idea is that fusion of TFIIA to TBP increases the effective concentration of TFIIA at the promoter. We compared the amount of RNA initiating from the +1 start site (T_C) and the +13 start site (TATA) in strains containing either Toa2-TBP or wild type TBP.

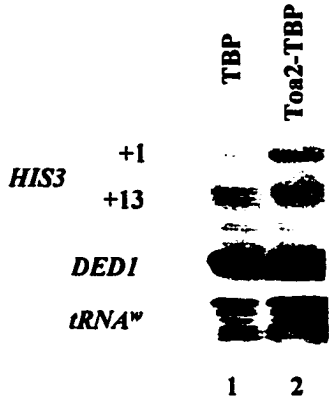

Figure 2.1 *HIS3* gene transcript initiating from +1 is preferentially elevated in the Toa2-TBP fusion strain. Measurements of transcription from yeast strain yJS156 containing either wild type TBP (Lane 1) or the Toa2-TBP fusion (Lane 2). The *HIS3* +1 and +13 initiation sites are indicated. The *DED1* and *tRNA*^{w} transcripts are used as loading controls. The *tRNA*^{w} is transcribed by RNA Polymerase III and should not be affected by the Toa2-TBP fusion molecule.

Interestingly, the amount of transcription initiating at +1 was 10-fold higher in the Toa2-TBP strain compared to the TBP strain (Figure 2.1). In contrast, the amount of transcription initiating at +13 is essentially the same (within 10 % experimental error) in the two strains. Thus, fusion of TFIIA to TBP causes a preferential increase in transcription from a natural, non-canonical element, indicating the importance of the TFIIA-TBP interaction in transcription initiation at these elements in vivo.

2.4b The CATAAA element is unable to support a high level of transcription in vivo.

Since the CATAAA sequence can functionally replace T_C (22), and CATAAA is a simpler model for non-canonical promoters, we compared the functional activities of CATAAA and TATAAA. The TATAAA and CATAAA elements were cloned individually into the core promoter region of a reporter plasmid containing the *GAL1,10* UAS upstream of the core promoter and the wild type *HIS3* initiation region driving the expression of a *HIS3-LACZ* fusion (7, 56). Thus, the only difference between the two constructs is in the first position of the TATAAA box sequence where TATAAA contains a thymine and CATAAA contains a cytosine. These constructs were used to measure the response to Gal4, a potent acidic activator that stimulates transcription in the presence of galactose.

Under non-inducing conditions (raffinose) both TATAAA and CATAAA

exhibited similar levels of β-galactosidase activity (Table 2.1). In contrast, incubation in galactose medium resulted in a 50 fold increase in activity from the TATAAA driven reporter while the CATAAA driven reporter did not show a significant increase in activity over that of cells grown under non-inducing conditions. Thus, like T_C, the CATAAA element is greatly diminished in its ability to support high levels of transcription in vivo.

2.4c Fusion of TBP and TFIIA results in an increase in expression from CATAAA in vivo.

If CATAAA serves as a good model for T_C (a non-canonical TATA element) then one would predict that the TFIIA-dependence would be similar. To test this hypothesis, we again utilized the Toa2-TBP fusion construct. The response to Gal4 from TATAAA or CATAAA in the strain containing the Toa2-TBP fusion as the sole source of TBP, was measured using the β-galactosidase assay. Incubation in galactose medium results in a 50-fold increase in activity in either the wild type TBP and the Toa2-TBP fusion strain harboring the TATAAA driven reporter (Table 2.1). Whereas activity from the TATAAA element was unaffected by the Toa2-TBP fusion, the CATAAA driven reporter showed a dramatic increase (15-fold) in activity in the fusion strain over the TBP strain containing the CATAAA element. In addition, this increase in activity from CATAAA is specific for TFIIA because no such increase is observed in a TFIIB-TBP fusion strain. Thus, CATAAA and T_C are up-regulated similarly in the

Table 2.1 Gal4 dependent activation of transcription *in vivo*

TBP Derivative[a]	Promoter Element[b]	Raffinose[c,d]	Galactose 18 hrs[c]	Fold Change	Promoter Element[b]	Raffinose[c,d]	Galactose 18 hrs[c]	Fold Change
TBP	TATAAA	7.1 ± 1.7	322.3 ± 20.7	50	CATAAA	5.3 ± 1.5	7.90 ± 1.4	1.5
Toa2-TBP	TATAAA	1.6 ± 0.3	108.7 ± 14.9	50	CATAAA	2.0 ± 1.2	34.3 ± 5.6	15
TFIIB-TBP	TATAAA	3.6 ± 0.38	184.6 ± 30.1	50	CATAAA	2.01±0.29	3.45± 1.24	1.5

[a] The parent yeast strain yJS156 has the chromosomal copy of *Spt15* deleted. TBP functions are provided by a *URA3*-marked plasmid containing the TBP gene. Plasmid shuffling on 5-fluoroorotic acid was used to create the *TRP1*-marked wild type TBPor the Toa2-TBPand TFIIB-TBP fusion strains assayed.

[b] Strains were transformed with a *URA3*-marked *HIS3/Lac Z* fusion plasmid containing either the canonical TATAAA element (pJS3801) or the non-canonical CATAAA element (pJS3803), driven by the *GAL1* UAS.

[c] β-Galactosidase activities were performed with 10^7 cells cultured in a medium containing either 2% raffinose or 2% galactose for the indicated amount of time.

[d] Incubation in the presence of raffinose was the baseline since this carbon source does not stimulate activation in this system but rather de-represses the effect of glucose.

Toa2-TBP background.

2.4d TBP binding affinity and stability are similar on the TATAAA and CATAAA elements.

Since TBP is the sequence specific factor that recognizes the TATA element, it seemed likely that differences in TATAAA and CATAAA element activity might be due to insufficient binding of TBP or instability of the TBP-CATAAA complex. To test this hypothesis, electrophoretic mobility shift assays were used to measure the relative binding and stability of TBP on the TATAAA or the CATAAA element. The two 33 base pair sequences used in this assay differ only in position 11, where TATAAA contains a thymine and CATAAA contains a cytosine.

The rate of formation of the TBP-DNA complex on TATAAA and CATAAA was measured by monitoring complex formation over increasing time (Figure 2.2A and 2.2B). The resulting half-time association ($t_{1/2}$) of 0.9 min was measured for TATAAA and 1.5 min for CATAAA (Figure 2.2C). A comparison over time shows that the formation of the TBP-CATAAA complex is slightly slower at early time points but the absolute amount of complex formed at 30 minutes is similar for both TATAAA and CATAAA.

We next examined the stability of the TBP-DNA complex by determining the dissociation kinetics of the two complexes (Figure 2.3). TBP-DNA complexes were formed, and then challenged with a large excess of an adenine-thymine rich sequence, poly[dAdT]. Poly[dAdT] is used in these

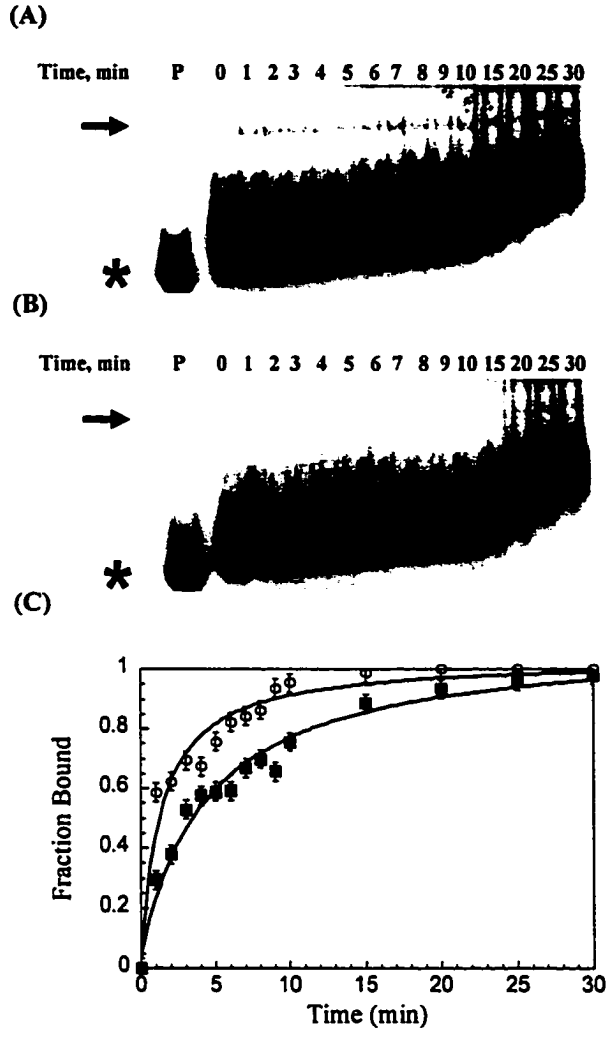

Figure 2.2 Binding of TBP to TATAAA is slightly faster than to CATAAA. A. TBP was added to ^{32}P-labeled TATAAA DNA and at the indicated times, a sample of the reaction was removed and immediately loaded onto a native gel. Free DNA is indicated by an asterisk and the TBP-DNA complex is indicated by a solid arrow. **B.** On rate of TBP binding to CATAAA was measured exactly as described for TATAAA. DNA probe without added TBP is shown in Lane P. **C.** Extent of complex formation at each sampling time was determined as described in "Experimental Procedures" and the results are plotted as fraction bound versus time. The curves fit to the data obtained from the analysis of TATAAA (open circles) and CATAAA (closed squares), respectively. Results from two independent experiments are graphed.

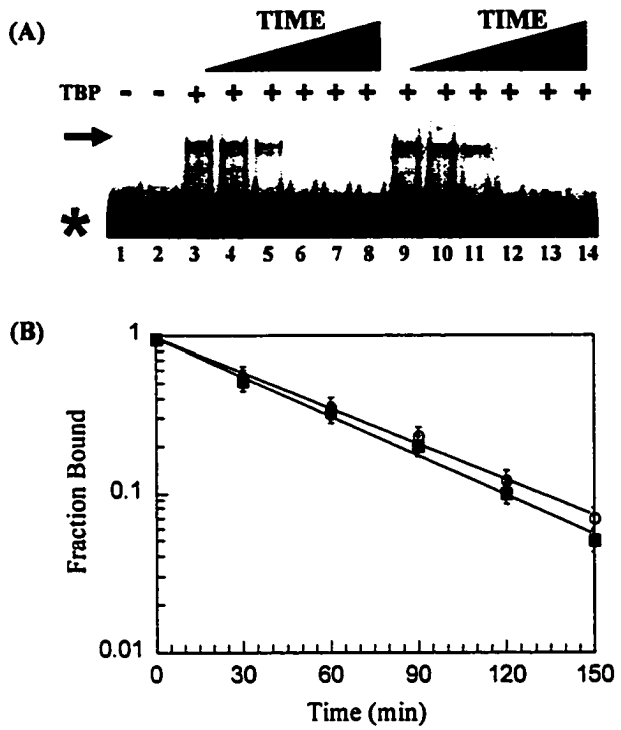

Figure 2.3 Dissociation of TBP from TATAAA and CATAAA DNA is similar. A. Representative gel shift experiment showing probe DNA incubated with TBP for 30 minutes (Lane 3, TATAAA and 9, CATAAA) and then challenged with specific competitor poly[dAdT] at 30 minute intervals for increasing amounts of time (Lanes 4-8, TATAAA and 10-14, CATAAA). DNA alone (no added protein) is shown in Lanes 1 (TATAAA) and 2 (CATAAA). Free DNA is indicated by an asterisk and TBP-DNA complex is indicated by a solid arrow. **B.** Graphical representation of the exponential decay of TBP-TATAAA (open circles) and TBP-CATAAA (closed squares) complexes. Complex remaining at each time point was determined as described under the "Experimental Procedures" and the results are plotted as fraction remaining versus time. Data were means ± SD from three independent experiments.

competition assays as specific competitor because the sequence of alternating adenine and thymine closely resembles a TATAAA element. TBP was incubated with the DNA for 30 minutes, since this results in a similar amount of TBP-DNA complex formed on TATAAA and CATAAA (Figure 2.2 and 2.3), followed by the addition of 1000 fold molar excess of poly[dAdT]. TBP behaves similarly on both elements with a $t_{1/2}$ of 70 min. DNAse I footprinting experiments confirmed that TBP was specifically protecting the TATAAA and CATAAA elements (data not shown). Thus, the off rate of the TBP-DNA complex is similar for both the TATAAA and CATAAA elements. Could the 100-fold difference in activity in vivo be due to the slight difference in early binding kinetics of TBP to TATAAA and CATAAA? Possibly, but we next examined higher order complex formation to determine if more dramatic differences could be observed for the two elements.

2.4e The TFIIA-TBP complex is more stable on the TATAAA element compared to CATAAA.

Since the binding and stability of TBP was not dramatically different on the two elements, we compared the two sequences for their ability to form TFIIA-TBP-DNA complexes. Since the fusion of TFIIA to TBP resulted in elevated levels of transcription from a non-canonical element in vivo (Figure 2.1 and Table 2.1), either the formation or stability of the TFIIA-TBP-DNA complex may be sensitive to the sequence of the TATAAA element.

The rate of formation of the TFIIA-TBP complex was measured on both

elements and found to be the same, within experimental error (Figure 2.4). The resulting half-time association ($t_{1/2}$) of 1.9 min was measured for TATAAA and 1.8 min for CATAAA. This shows that TFIIA can neutralize the subtle kinetic rate difference found on CATAAA versus TATAAA when TBP alone is measured. In addition, the total amount of TFIIA-TBP-DNA complex formed is equivalent for both elements.

In contrast, the stability of the TFIIA-TBP-TATAAA complex differs significantly from the complex formed on the CATAAA element (Figure 2.5). The TFIIA-TBP complex was extremely stable on TATAAA, with a $t_{1/2}$ of 14 hours under the conditions assayed (determined by extrapolation from the exponential decay graph). This half-life is in good accord with previous measurements of the stability of the TFIIA-TBP-TATAAA complex (65). In contrast, the TFIIA-TBP complex on CATAAA shows significant loss of complex over the course of the experiment with a short comparative half-life of only 1.3 hours. Thus, although the TFIIA-TBP complex is rapidly formed to the same extent on both TATAAA and CATAAA, these complexes differ in that the TFIIA-TBP-TATAAA complex is significantly more stable than the complex formed on CATAAA.

2.4f The TFIIB-TBP complex behaves similarly on the two elements indicating the proper formation of certain higher-order complexes.

Since the stability of the TFIIA-TBP-DNA complex is compromised on the

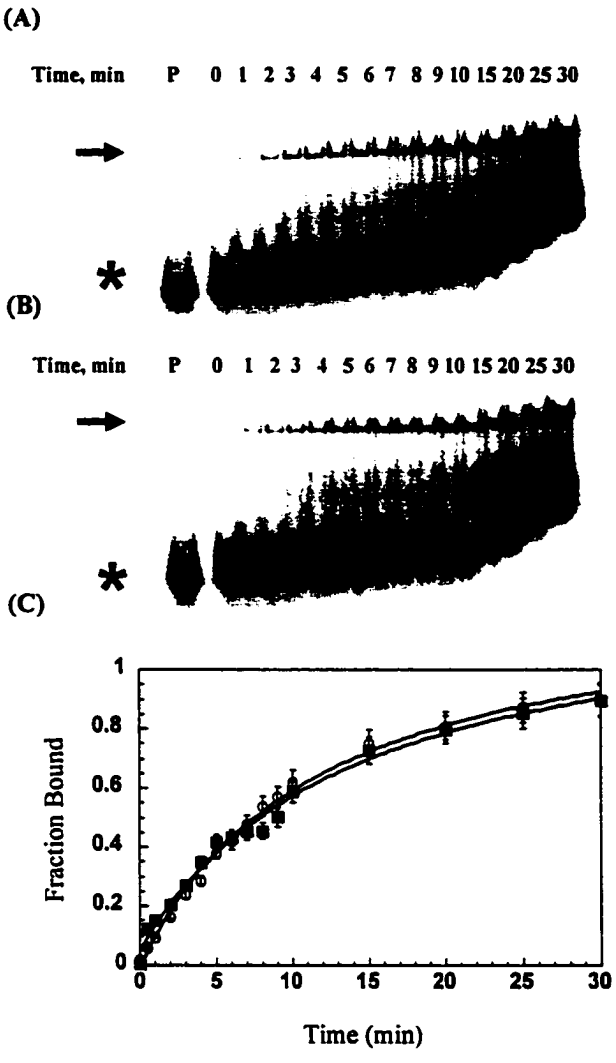

Figure 2.4 Rate of formation of the TFIIA-TBP-DNA complex is the same for TATAAA and CATAAA. A. TBP and TFIIA were added to ^{32}P-labeled TATAAA DNA. Samples of the binding reaction were removed for analysis at the times indicated above each lane. Free DNA is indicated by an asterisk and the TFIIA-TBP-DNA complex is indicated by a solid arrow. Lane P shows a control reaction lacking proteins. **B.** Rate of formation of TFIIA-TBP-DNA complex on CATAAA was measured exactly as described for TATAAA. **C.** The amount of complex formed at each time point was determined and the results are plotted as fraction bound versus time. The curves fit to the data obtained for TATAAA (open circles) and CATAAA (closed squares). Data points from two independent experiments are shown.

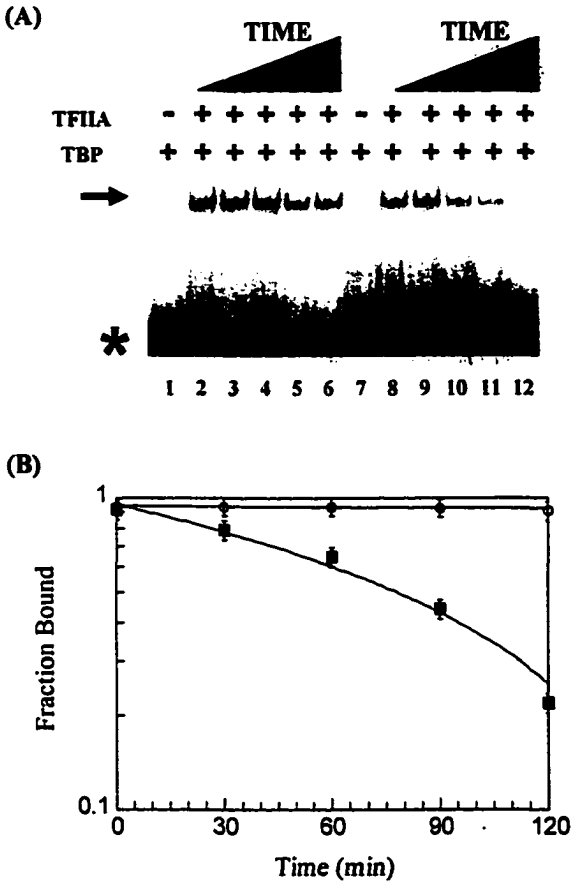

Figure 2.5 The TFIIA-TBP complex is less stable on CATAAA compared to TATAAA. A. Gel shift assay showing the binding and stability of the TFIIA-TBP complex on TATAAA and CATAAA. TBP and TFIIA were pre-incubated with the DNA elements (Lane 2, TATAAA and 8, CATAAA) for 30 minutes before the dissociation-time courses were initiated by the addition of poly[dAdT] specific competitor (Lanes 3-6, TATAAA and 9-12, CATAAA). Each subsequent lane in the series represents 30 additional minutes of incubation with competitor. The TBP only lanes show that the TBP fails to form a stable shifted band in the absence of Mg^{2+} ions (Lane 1, TATAAA and 7, CATAAA). Free DNA is indicated by an asterisk and the TFIIA-TBP-DNA complex is indicated by a solid arrow. **B.** Graphical representation of the exponential decay of TFIIA-TBP-TATAAA complex (open circles) and TFIIA-TBP-CATAAA complex (closed squares) calculated exactly as in Figure 3. Data were means ± SD from three independent experiments.

CATAAA element, we wished to determine if this was specific to the TFIIA-TBP complex, or whether a loss of stability is also observed for other higher order complexes. Thus, we compared the binding and stability of the TFIIB-TBP-DNA complex on TATAAA and CATAAA. As is the case for TBP and TFIIA, the relative amount of initial binding of the TFIIB-TBP complex is very similar on both elements (Figure 2.6). In contrast to the results obtained with TFIIA, there is no observable difference in the stability of the TFIIB-TBP-DNA complex on TATAAA versus CATAAA. Both TFIIB-TBP-TATAAA and TFIIB-TBP-CATAAA complexes have a $t_{1/2}$ of 125 min under the conditions assayed. We conclude that the formation of the TFIIB-TBP higher-order complex behaves similarly on the two elements, indicating that the difference in stability of the TFIIA-TBP-DNA complex on CATAAA is specific to the complex containing TFIIA.

2.5 DISCUSSION

The closer a core promoter element is to the consensus sequence TATAAA, the greater the output from the promoter (7, 44, 56, 66, 69). In addition, binding of TBP to the TATA box is a rate-limiting step in transcription (6, 27, 28, 67), and TBP occupancy at the promoter correlates very well with transcriptional initiation at a majority of promoters (32, 37). One would therefore hypothesize that the recognition of the TATA element by TBP is a critical and sequence-specific event in transcription initiation. And yet, a comparison of the crystal structures of TBP bound to a number of TATA

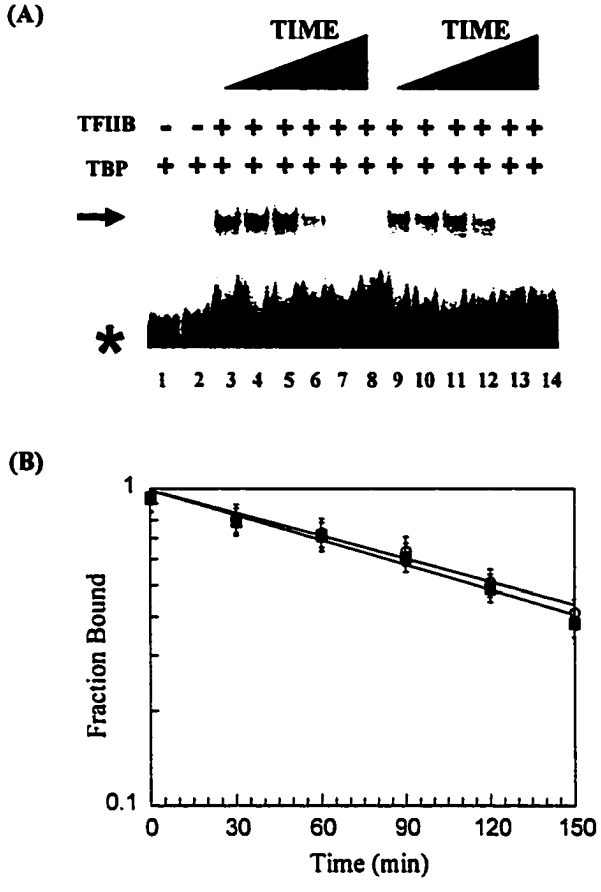

Figure 2.6 Dissociation of the TFIIB-TBP complex from both TATAAA and CATAAA follows similar kinetics. A. Representative gel shift experiment showing the binding and stability of the TFIIB-TBP complex on TATAAA and CATAAA. The TBP and TFIIB proteins were pre-incubated with the elements for 30 minutes before the addition of specific competitor (Lane 3, TATAAA and 9, CATAAA). Dissociation-time course at 30 minute intervals were initiated with poly[dAdT] specific competitor (Lanes 4-8, TATAAA and 10-14, CATAAA). TATAAA and CATAAA probes with TBP only are shown in Lanes 1 and 2, respectively. TBP alone fails to form a stable shifted band in the absence of Mg^{2+} ions. **B.** Exponential decay of TFIIB-TBP-TATAAA complex (open circles) and TFIIB-TBP-CATAAA complex (closed squares) was calculated as described in Figure 3. Free DNA is indicated by an asterisk and the higher-order complexes are indicated by a solid arrow. Data were means ± SD from three independent experiments.

variants revealed no differences in TBP conformation or the trajectory of the DNA (51). Likewise, we find that TBP recognizes and binds to TATAAA or CATAAA with similar affinities and the decay rate of the TBP-DNA complex on the two the elements is identical. On a structural basis, the substitution of a cytosine for the thymine in position one of the TATAAA element should allow for TBP binding because no steric clashes exist in the minor groove between the exocyclic NH_2 group from the guanine and the adjacent side chains of TBP (50, 51). Since we observe a 50-fold difference in expression level from these two elements in vivo, we examined higher-ordered interactions to determine which factors play a critical role in modulating the output of gene expression on non-canonical elements.

The crystal structures of both the TFIIA-TBP-TATAAA and the TFIIB-TBP-TATAAA complexes, and a large body of biochemical and genetic studies, show that these transcription factors play a role in binding and stabilizing the TBP-DNA interaction (3, 13, 20, 24, 33, 45, 64). We found that the TFIIA-TBP-DNA complex formed on CATAAA was significantly less stable than the complex formed on TATAAA in vitro. Moreover, a fusion of TFIIA and TBP resulted in a 15-fold increase in expression from a promoter containing the CATAAA element in vivo. Thus, increasing the effective concentration of TFIIA at the promoter via a TBP fusion can increase the transcriptional output from CATAAA in vivo. This effect is specific to the TFIIA-TBP complex, since the stability of the TFIIB-TBP complex was identical on the two elements in vitro,

and fusion of TFIIB to TBP had no effect in vivo.

How can a loss of stability of the TFIIA-TBP complex account for such dramatic differences in expression in vivo? A high level of gene output not only depends on initial induction of the gene but also on the number of times the gene is continuously transcribed or reinitiated (16, 23, 72). Analysis of the fate of several general transcription factors during the transition from initiation to elongation has shown that many factors are released from the DNA template whereas TBP, TFIIA and some activators remain associated (71, 72). The sequence of the TATA element has also been shown to be critically important to the rate at which reinitiation occurs, and TATA-less promoters fail to show rapid reinitiation (69, 70). Since TBP and TFIIA remain associated with the core promoter after polymerase escape, and these factors have been implicated in directing rapid reinitiation, it seems likely that a difference in stability of this complex could result in significant changes in the rate of reinitiation. The hypothesis that CATAAA is defective for reinitiation can also explain the overall low amounts of transcription measured for this element in vivo.

TBP binds relatively promiscuously to both TATA and non-TATA containing DNA (14, 17, 18, 61, 65). This apparent lack of specificity of TBP for its cognate site is at least partially due to the fact that TBP binds to the minor groove of DNA (25, 26, 34, 60), which provides few functional groups to direct specificity of binding (50). Genetic and biochemical studies suggest that the cell has devised several means of increasing the specificity of TBP for TATA-

containing promoters. Certain TBP-Associated Factors (TAFs) (42), Mot1 (2, 43), NC2 (35), and the Not complex (10), each may play a role in regulating transcription from non-canonical TATA elements. Interestingly, TFIIA can counteract the negative effects on TBP-DNA complex formation of the N-terminus of TAF145 (30), of Mot1 (1), and there are genetic interactions between TFIIA and NC2 (68). Thus, loss of a stable TFIIA-TBP-DNA interaction on non-canonical elements could tip the balance to favor the effects of these negative factors. In addition, transcriptional output could be effected on a number of other levels since TFIIA has also been implicated in core promoter functions (39, 48, 58) and activated transcription (8, 9, 12, 29, 38, 49). Taken together with the results presented here, this suggests that the stability of the TFIIA-TBP-DNA complex plays a critical functional role in promoter selectivity by increasing the specificity for consensus TATA elements in vivo.

2.6 ACKNOWLEDGEMENTS

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SUPPLEMENTAL FIGURES

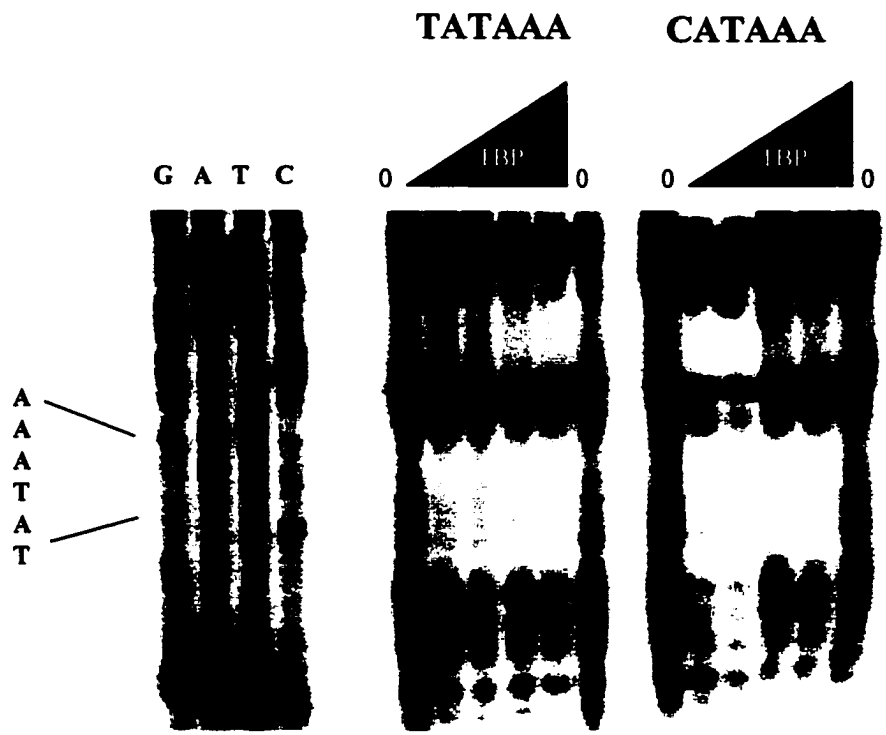

Figure 2.7 DNase I footprinting analysis shows protection from TBP bound to the TATAAA and CATAAA sequences. The cleavage products resulting from DNase I digestion were resolved on a denaturing polyacryamide gel and the chemical identities of the products were assigned by reference to the sequencing markers (G,A,T,C), taking into account the differences in mobility of the fragments due to the presence or absence of a 3'-phosphate group. Each set of lanes corresponds to digestion of the DNA in the absence (flanking lanes) and presence of increasing amounts of TBP (middle lanes).

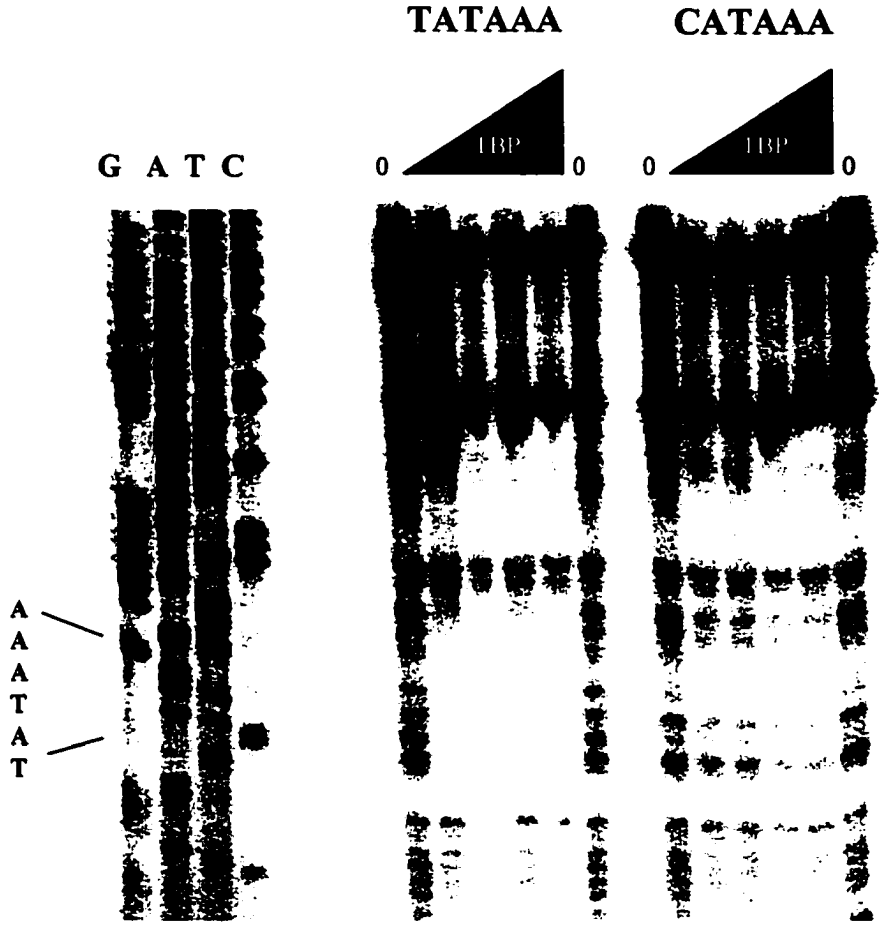

Figure 2.8 DNase I footprinting analysis of TFIIA-TBP bound to TATAAA or CATAAA shows no protection is observed in the CATAAA lanes, indicating that this sequence does not support stable TFIIA-TBP-CATAAA complex. Each set of lanes corresponds to digestion of the DNA in the absence (flanking lanes) and presence of the TBP/TFIIA complex. In this case, the concentration of TBP protein is increasing across the middle lanes while the concentration of TFIIA remains the same.

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CHAPTER 3

FUNCTIONAL ANALYSIS OF TATAAA VARIANTS REVEALS DIVERSE MECHANISTIC DEFECTS

Chapter three describes a mutational analysis of the consensus sequence of the TATAAA element. This work came about from a collaboration between Julie A. Fischbeck and myself and has been submitted for publication in *Biochemistry*. Julie A. Fischbeck is co-author because she conducted the in vivo transcriptional analysis of the TATAAA variants using the β-galactosidase assay (Figure 3.1 and 3.7). I have contributed all of the data shown in figures 3.2, 3.3, 3.4, 3.5 and 3.6. The literature citation for this chapter upon acceptance to a professional journal will be:

Stewart, J.J., J.A. Fischbeck and L.A. Stargell. 2001. Functional analysis of TATAAA variants reveals diverse mechanistic defects. Biochemistry, to be submitted January 2001.

3.1 ABSTRACT

To determine mechanistic differences in transcription initiation between TATAAA and off-consensus sequences, we compared the functional activity of thirteen variant elements, each having a replacement of either a cytosine or guanine base for one of the six positions in the TATAAA element. All of the substituted elements were severely compromised for transcription in yeast cells. In vitro, many of these TATA box derivatives were defective for complex formation with TBP, TFIIA-TBP and/or TFIIB-TBP. Thus, as predicted, off-consensus TATA elements form poor binding sites for TBP and this compromised interaction can affect higher order complex formation. In contrast, two elements (CATAAA and TATAAG) are not affected in the initial formation of the TBP, TFIIA-TBP or TFIIB-TBP complexes. However, these two elements are unable to form a stable TFIIA-TBP-DNA complex in vitro and fusion of TFIIA to TBP specifically restores activity from these two elements in vivo. Taken together, our results indicate that the interplay between the sequence of the TATA element and the components of the general transcription machinery can lead to variations in the kinetics of pre-initiation complex formation, or stability of these complexes, and these differences may account for the observed effects of the diverse sequences on transcription.

3.2 INTRODUCTION

Consensus binding sites have been defined for most of the known DNA

binding transcription factors and it is usually these sites that yield maximal amounts of gene transcription (1, 9, 17-19, 30, 32). Mutational analysis and random selection for functional TATA elements has defined TATAAA as the consensus sequence for the TATA binding protein (TBP) in yeast (5, 25, 31). However, many derivatives of this sequence also confer TATA function in vivo, albeit with diminished activity. Transcription from non-canonical promoter elements remains TBP dependent (6) and functional analysis of mutated TATA elements reveals that yeast and human TBP have nearly identical TATA sequence preferences (22), underscoring the evolutionary conservation of the TBP-TATA interaction.

In previous work we compared the functional activity of two sequences: TATAAA and CATAAA (29). TATAAA represents the canonical TATA box and CATAAA served as a model for off-consensus elements (13). In vitro studies showed little difference between TATAAA and CATAAA in the formation and stability of the TBP-DNA complex, although CATAAA was unable to support high levels of transcription in vivo (29). Moreover, the binding and stability of the TFIIB-TBP-DNA complex is similar for the two elements. In contrast, although the TFIIA-TBP-DNA complex can form initially to the same extent on TATAAA and CATAAA, this complex is significantly less stable on the CATAAA element. The decrease in functional activity at CATAAA could be suppressed in vivo by expression of a fusion construct of TFIIA and TBP (29). Thus, the stability of the TFIIA-TBP complex depends strongly on the sequence of the

core promoter element and the TFIIA-TBP complex plays an important function in distinguishing optimal promoters in vivo.

In order to determine whether a decrease in stability of the TFIIA-TBP-DNA complex is a general mechanistic property of off-consensus elements, we compared different non-canonical sequences for their ability to direct transcription in vivo, and to form complexes with TBP, TFIIA, and TFIIB in vitro. We systematically replaced each position of the six base pair consensus sequence with either a cytosine or guanine base. Transcription analysis indicates that each of the substituted elements is severely defective for high levels of transcriptional output in vivo. Electrophoretic mobility shift assays with TBP, TFIIA and TFIIB, demonstrate that many of these TATA box derivatives are defective for formation of certain complexes, indicating sequence-specific defects in complex formation. Only one element displayed properties similar to CATAAA (29): the TATAAG element was unaffected for the formation of TBP-DNA, TFIIA-TBP-DNA and TFIIB-TBP-DNA complexes, but the TFIIA-TBP complex was found to be much less stable on this element. Overall, the data suggest that different positions of the TATA box can influence the kinetics of factor binding leading to multiple mechanisms for transcription regulation.

3.3 EXPERIMENTAL PROCEDURES

3.3a Transcriptional analysis

Plasmids used for measuring activated transcription in vivo were

derivatives of YCp86 containing the hybrid *HIS3* promoter and the wild-type initiation and amino terminal region of the *HIS3* gene fused in frame with functional *Escherichia coli LacZ* (5, 25). The promoter region contains a 365-bp *GAL1, 10* fragment containing four *GAL4* binding sites fused upstream of the EcoRI-SacI restriction endonuclease sites, between which the substituted element oligonucleotides could be inserted. When the synthesized oligos were cloned into this molecule they were renamed pJS3801- pJS3814 (Table 3.1).

For the experiments measuring activated transcription *in vivo*, the pJS3801-pJS3814 constructs (described above) were transformed into yeast strain yJS156, a derivative of KY804 (relevant genotype: *MATα ura3-52 trp1-Δ1 leu2::PET56 gal2 gcn4-Δ1*) (12) with the chromosomal copy of *Spt15* deleted by a two-step knockout. TBP functions are provided by a *URA3*-marked plasmid containing the TBP promoter, open reading frame and terminator. Plasmid shuffling on 5-fluoroorotic acid was used to create the *TRP1*-marked wild type TBP, TFIIB-TBP or the Toa2-TBP fusion strains assayed. The resulting transformants were used in liquid assays for β-galactosidase. The cells were grown in selective medium containing either 2% raffinose or 2% galactose to mid-exponential growth phase ($OD_{600} \cong 1$-2). Enzyme activities were determined in duplicate and normalized to the OD_{600} of the cultures.

3.3b Protein expression and purification.

Full-length yeast TBP was expressed in *Escherichia coli* strain BL21 DE3 (4). The protein was purified from the soluble fraction with Q, SP, and Heparin

HiTrap columns (Pharmacia). The resulting fractions were equilibrated in 100 mM KCl, 40 mM HEPES, 20 mM Tris, 10% glycerol, 1mM dithiothreitol and 0.5mM phenylmethylsulfonyl fluoride (final pH 7.9). The protein was shown to be 90% pure upon staining with Coomassie blue.

GST-TFIIB was produced by fusing the open reading frame to glutathione S-transferase (GST) and transformed into *E.coli* BL21 DE3 (Novagen). Cells were grown in LB medium at 37°C to an optical density of 0.7. Isopropyl-β-D-thiogalactopyranoside (IPTG, 0.1mM final concentration) was added and the cells were incubated for two hours at 30 °C. Cells were harvested by centrifugation and washed with a 20 mM Tris, 50 mM NaCl buffer. Following sonication, the lysate was incubated with shaking at 4°C with glutathione resin. After two consecutive wash steps, the protein was eluted using 5 mM reduced glutathione in 50mM Tris buffer. The eluate was equilibrated in 100 mM KCl, 40 mM HEPES, 20 mM Tris, 10% glycerol, 1mM dithiothreitol and 0.5mM phenylmethylsulfonyl fluoride (final pH 7.9). The protein was shown to be 75% homogeneous on SDS-PAGE upon staining with Coomassie blue.

Recombinant yeast TFIIA was purified as described (23). This procedure involves expressing each subunit, *TOA1* and *TOA2*, in separate strains of *E.coli* BL21DE3. Cells were ruptured by sonication, insoluble material was collected by centrifugation. Each insoluble pellet was resolublized in 8M urea. Each subunit was renatured in the presence of the other subunit and

dialyzed against the buffer described in the TBP and GST-TFIIB purification. The TFIIA was approximately 60 % pure as determined by Coomassie staining.

3.3c Electrophoretic mobility shift assays (EMSA)

The DNA elements used in vitro protein DNA interaction studies were 23 base pair oligonucleotides (29). The TATAAA oligo contains the sequence 5'AATTCC**TATAAA**GTAATGTGGAG 3'. The other elements were synthesized with the appropriate base substitution and prepared in the exact same way.

Protein-DNA interactions *in vitro* were studied by incubation of purified proteins with ^{32}P internally labeled probes. Binding reactions contained 10 μM poly[dG-dC] non-specific competitor, 100 mM KCl, 40 mM HEPES, 20 mM Tris, 10% glycerol, 1mM dithiothreitol and 0.5mM phenylmethylsulfonyl fluoride (final pH 7.9). TBP (13.8 nM) and the TATA element probe (2.4 picomoles) was incubated for 30 minutes at room temperature and the complex was separated on 5% acrylamide gel containing 50 mM Tris-borate, 1 mM EDTA and 2 mM $MgCl_2$ in both the gel and running buffer. Recombinant yeast TFIIA (5.0 nM) and GST-tagged TFIIB (8.5 nM) were incubated with TBP and probe DNA as described above except $MgCl_2$ was omitted from the gel and running buffer. Omission of the divalent cations from the binding reaction allows for the stable formation of only the ternary complex. For dissociation kinetic studies, TBP-DNA, TFIIA-TBP-DNA and TFIIB-TBP-DNA complexes were allowed to reach equilibrium and then challenged with 1000 fold molar excess of specific

competitor poly[dAdT] for the specified amount of time. The samples were then loaded on to the gel and resolved by EMSA.

3.4 RESULTS

3.4a Single cytosine or guanine replacements in the TATAAA element severely impair transcription in vivo.

A set of elements based on the yeast consensus TBP-binding site (TATAAA) were designed (Table 3.1). Variants of the TATA element with cytosine bases substituted at every position of the consensus TATAAA sequence were termed the C series. Since we were concerned about the effect of substituting a purine (adenine) for a pyrimidine (cytosine), we also constructed a series of guanine base substitutions at every position of the TATAAA sequence (G series). In each of the TATA box variants, the single base change was restricted to the six base pairs of the yeast consensus sequence while the flanking sequences remained identical. To test for transcriptional capability in vivo, each element was cloned individually into the core promoter region of a reporter plasmid containing the *GAL1,10* UAS and the *HIS3* initiation region driving the expression of a *HIS3-LacZ* fusion (5, 25). These constructs were used to measure the response to Gal4, a potent acidic activator that stimulates transcription in the presence of galactose.

Incubation in galactose medium for 18 hours caused an induction in β-

Table 3.1 DNA oligos used in this study

Name	*Sequence*[a]
pJS3801	GAATTCC**TATAAA**GTAATGTGGAGCTC
pJS3803	GAATTCC**C**ATAAAGTAATGTGGAGCTC
pJS3804	GAATTCC**T**C**T**AAAGTAATGTGGAGCTC
pJS3805	GAATTCC**TA**C**A**AAGTAATGTGGAGCTC
pJS3806	GAATTCC**TAT**C**A**AGTAATGTGGAGCTC
pJS3807	GAATTCC**TATA**C**A**GTAATGTGGAGCTC
pJS3808	GAATTCC**TATAA**C**A**GTAATGTGGAGCTC
pJS3809	GAATTCC**G**ATAAAGTAATGTGGAGCTC
pJS3810	GAATTCC**T**G**T**AAAGTAATGTGGAGCTC
pJS3811	GAATTCC**TA**G**A**AAGTAATGTGGAGCTC
pJS3812	GAATTCC**TAT**G**A**AGTAATGTGGAGCTC
pJS3813	GAATTCC**TATA**G**A**GTAATGTGGAGCTC
pJS3814	GAATTCC**TATAA**G**G**TAATGTGGAGCTC

[a] The 23 base oligos are shown with the six base TATA boxes in bold letters. Bases altered relative to the TATA sequence are underlined.

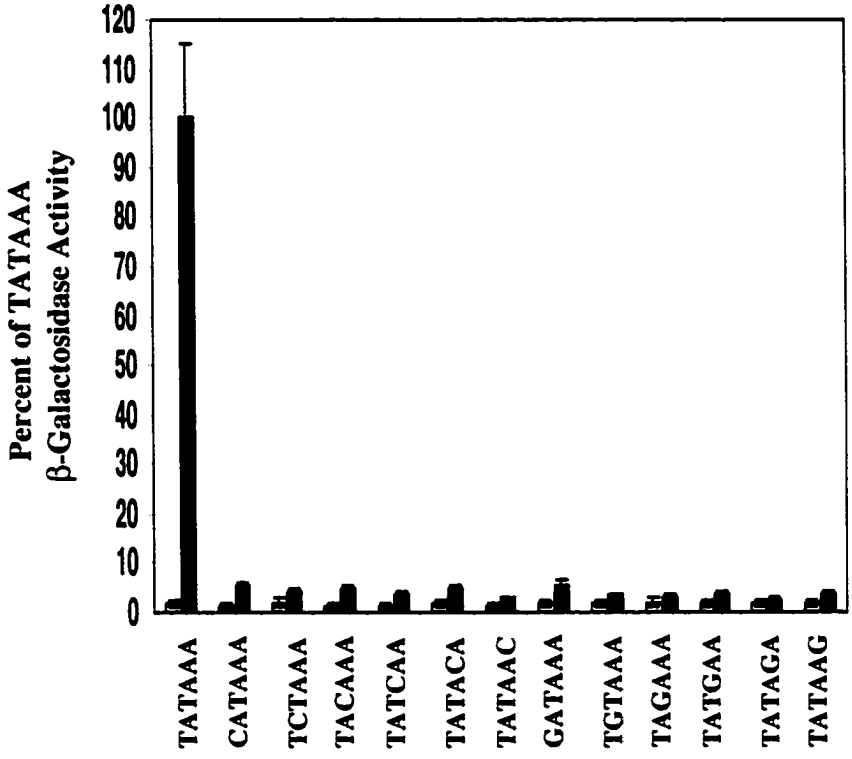


Figure 3.1 All of the TATAAA variants are severely impaired for activated transcription in vivo. The yeast strain yJS156 was transformed with a *HIS3/Lac Z* fusion plasmid containing either the canonical TATAAA element or the indicated non-canonical element, driven by the *GAL1,10* UAS. β-galactosidase activities were performed with cells cultured in a medium containing either 2% raffinose (gray columns) or 2% galactose (black columns) for 18 hrs. Incubation in the presence of raffinose was the baseline since this carbon source does not stimulate activation in this system but rather de-represses the effect of glucose.

galactosidase activity in cells containing the TATAAA driven reporter (Figure 3.1). In contrast, the reporters driven by the non-canonical elements did not show a significant increase in β-galactosidase activity over that of cells grown in raffinose medium alone (no galactose). Thus, the non-canonical elements were unable support high levels of transcription induced by the activator Gal4. In order to explore the mechanistic defects exhibited by each of these TATAAA variants, the extent of protein-DNA complexes formed on each element was determined in vitro.

3.4b Internal base substitutions in the TATAAA sequence disrupts TBP binding.

Each of the C substituted elements were tested for the ability to form the TBP-DNA complex (Figure 3.2A). As reported in previous work (29), substitution of the first T position with a C (CATAAA), did not alter the amount of TBP-DNA complex formed at equilibrium compared to TATAAA. Two other elements (TACAAA and TATAAC) exhibited fairly robust TBP binding (60-70% of TATAAA activity). Substitutions at the second, fourth and fifth positions A with C (TCTAAA, TATCAA and TATACA) were less well tolerated, with less than 50% of the TBP-DNA complex formed on TATAAA or CATAAA (Figure 3.2A and 3.2C).

TBP-DNA complex formation was also measured on the G substituted elements (Figure 3.3A). Interestingly, a substitution of the last A with G

Figure 3.2. The formation of the transcription factor complexes on the C series of non-canonical elements is sequence dependent. A. Representative EMSA experiment showing recombinant yeast TBP incubated with each of the fourteen ^{32}P labeled probes. Free DNA is denoted by an asterisk and the TBP-DNA complex is indicated by arrows. **B.** Representative EMSA experiment showing recombinant yeast TBP, recombinant TFIIA and GST-TFIIB incubated with the ^{32}P labeled probes. Free DNA is denoted by an asterisk, while the IIA-TBP-DNA and IIB-TBP-DNA complex are indicated by the appropriate arrows. **C.** Graphical representation of the amount of protein complexes formed on the C series of non-canonical elements. The x-axis shows the position of the substitution in each element graphed as a function of the total amount of binding to the canonical TATAAA element.

(A)

(B)

(C)

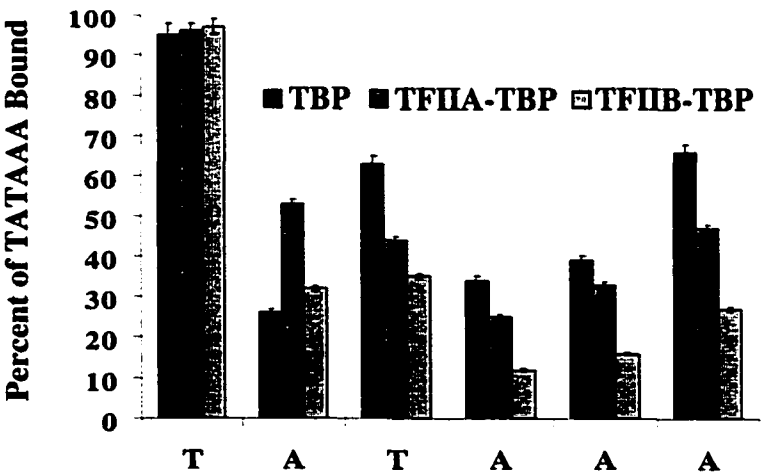
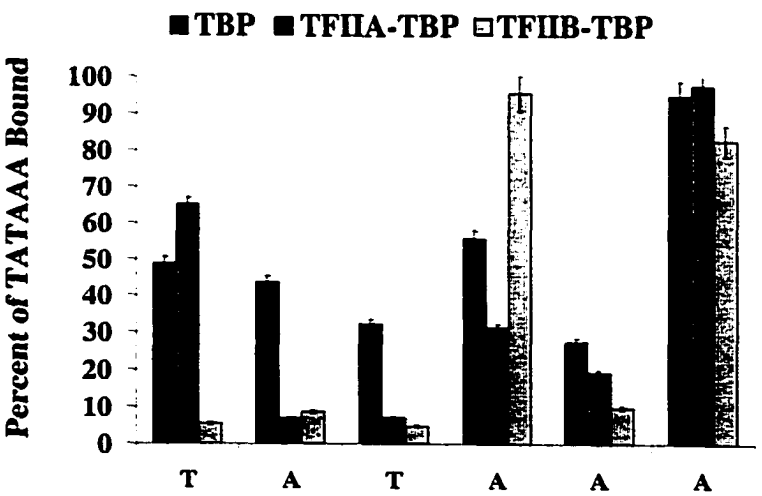


Figure 3.3. The formation of the TBP-DNA complex on the G series of non-canonical elements also shows sequence dependent binding defects. A. Representative EMSA experiment showing recombinant yeast TBP incubated with the element indicated. Free DNA is denoted by an asterisk and the TBP-DNA complex is indicated by arrows. **B.** Representative EMSA experiment showing recombinant yeast TBP, recombinant TFIIA and GST-TFIIB incubated with the ^{32}P labeled probes. Free DNA is denoted by an asterisk, while the TFIIA-TBP-DNA and TFIIB-TBP-DNA complex are indicated by the appropriate arrows. **C.** Graphical representation of the amount of protein complexes formed on the G series of non-canonical elements. The x-axis shows the position of the substitution in each element graphed as a function of the total amount of binding to the canonical TATAAA element.

(A)

(B)

(C)



(TATAAG) could form the TBP-DNA complex to the same degree as TATAAA and CATAAA, indicating that a purine substitution is slightly better tolerated at this position than a pyrmidine. This is more dramatically shown when comparing the activity of GATAAA (50%) to CATAAA (nearly 100%) and TAGAAA (45%) to TACAAA (65%) indicating that pyrimidine for a purine substitution is more detrimental. As is the case of the C series, internal substitutions in the TATAAA element significantly disrupt TBP binding. The TATGAA and GATAAA substitutions were the most functional showing 50% of TATAAA and CATAAA binding. In contrast, the TATAGA element showed the most compromised ability to form the TBP-DNA complex, only about 27% of the amount of complex formed on the control elements (Figures 3.3A and 3.3C).

3.4c Higher-order complex formation also depends on the sequence of the core element.

Higher-order complex formation was measured for the C series of elements using TATAAA and CATAAA as controls (Figures 3.2B and 3.2C). TFIIA-TBP-DNA complex formation on the elements ranged from TCTAAA with 53% of TATAAA and CATAAA binding, to TATCAA showing the most dramatic decrease in TFIIA-TBP-DNA binding (25%). A similar trend is observed with the formation of the TFIIB-TBP-DNA complex on the C series of elements, although TFIIB complexes seem to be more significantly affected by the cytosine base substitutions with activities ranging from 12-35% of control

binding (Figures 3.2B and 3.2C).

TFIIA-TBP and TFIIB-TBP complex formation on the G substituted elements was also determined (Figure 3.3B). In the case of the TFIIA-TBP-DNA complex formation, the TATAAG element could form this complex to the same extent as TATAAA and CATAAA. In contrast, the remainder of the elements showed varying amounts of the TFIIA-TBP-DNA complex formed with TGTAAA and TAGAAA (both ≅ 7.0%) showing the most dramatic decreases (Figures 3.3B and 3.3C). In the case of the TFIIB-TBP-DNA complex formation, TATAAG and TATGAA formed this complex to a similar extent as TATAAA (Figure 3.3B). The TATGAA element is an interesting case because this element is compromised for both TBP and TFIIA-TBP complex formation but not TFIIB-TBP-DNA complex formation, suggesting a TFIIB-dependent conformational change is stabilizing the TBP-DNA interaction. All of the remaining elements tested do not show significant TFIIB-TBP-DNA complex formation, with amounts of complex formed at ≤10% of TATAAA and CATAAA (Figure 3.3B and 3.3C).

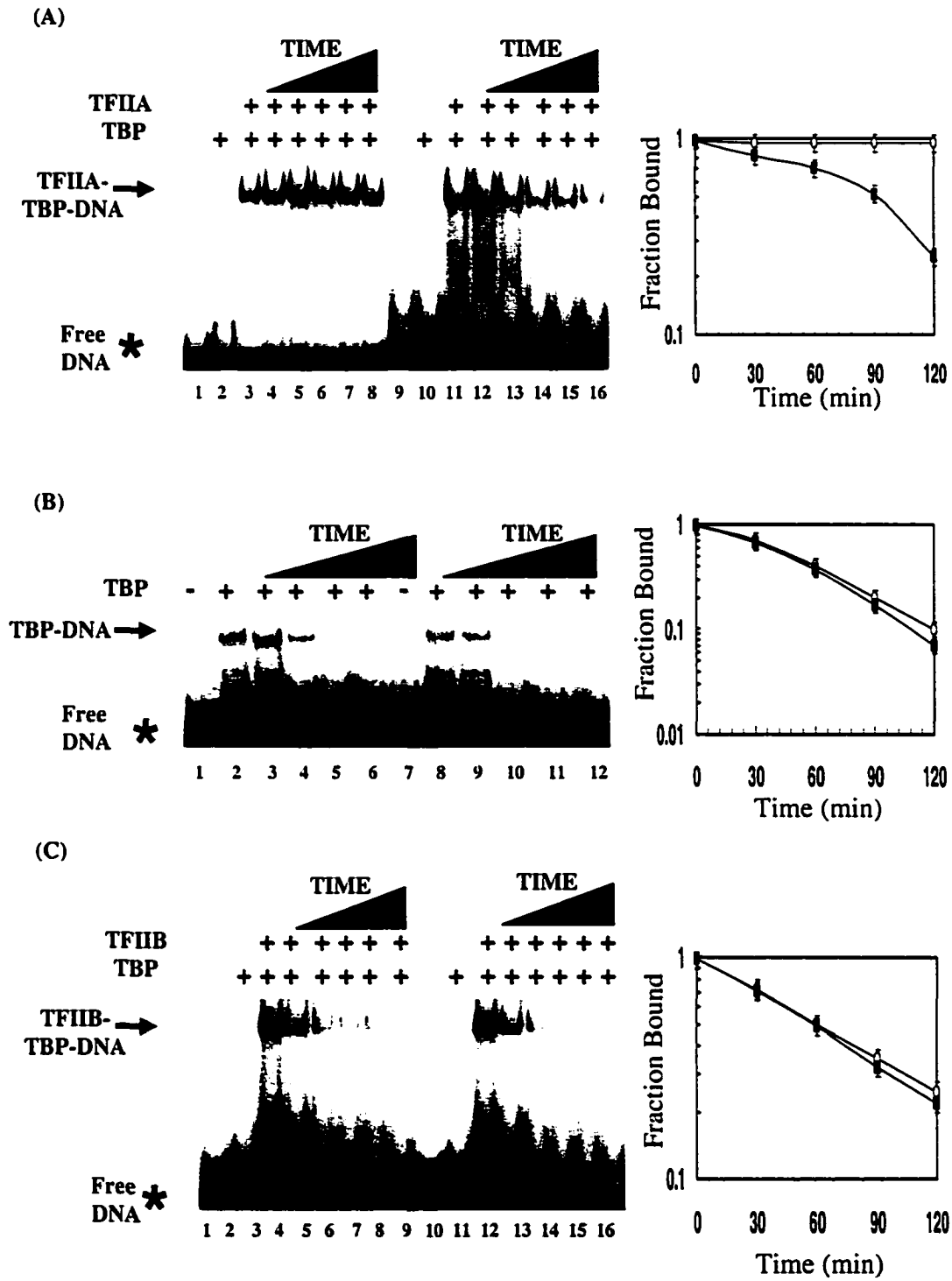
3.4d The TFIIA-TBP-DNA complex is destabilized on TATAAG.

In the studies presented above, many of the elements have specific defects in protein-DNA complex formation (Figure 3.2 and Figure 3.3). However certain substitutions of the first (with a C) and last (with a G) positions of the TATAAA box do not appear to affect initial complex formation of TBP-

DNA, TFIIA-TBP-DNA or TFIIB-TBP-DNA complexes. We have demonstrated that the CATAAA element is unable to form a stable TFIIA-TBP-DNA complex (29). To test the hypothesis that TATAAG may have a similar defect in TFIIA-TBP-DNA complex stability, electrophoretic mobility shift assays were used to measure the relative binding and stability of the TFIIA-TBP complex on both TATAAA and TATAAG. A comparison of the absolute amount of binding of TFIIA-TBP to each of the elements shows very similar amounts of complex formation on both elements (Figure 3.4A). The stability of the TFIIA-TBP-DNA complex was determined by examining the dissociation kinetics for the two complexes. The stability of the TFIIA-TBP-TATA complex on TATAAA differs significantly from the complex formed on the TATAAG element. The TFIIA-TBP complex was extremely stable on TATAAA, with little or no loss of complex over the course of the experiment. The TFIIA-TBP complex on TATAAG showed significant loss of complex, with almost complete loss of complex over the two-hour time-course.

Since the stability of the TFIIA-TBP-DNA complex is compromised on the TATAAG element, we wished to determine if this was specific to the TFIIA-TBP complex, or whether a loss of stability is also observed for other transcription factor-DNA complexes. The TBP-DNA complexes form and decay with a similar kinetic rate on both TATAAA and TATAAG (Figure 3.4B). The TFIIB-TBP-DNA complex behaved very similarly on both TATAAA and TATAAG as well (Figure 3.4C). In conclusion, TBP is binding to TATAAA and TATAAG in

Figure 3.4. The TFIIA-TBP-TATAAG complex is less stable than the TFIIA-TBP-TATAAA complex. A. Representative EMSA showing the binding and stability of the TFIIA-TBP complex on TATAAA and TATAAG. TBP and TFIIA were pre-incubated with the DNA elements for 30 minutes (Lanes 3 TATAAA and 11 TATAAG) before the dissociation-time courses were initiated with poly[dAdT] specific competitor (lanes 4-8 TATAAA and 11-16 TATAAG) with each subsequent lane in the series representing 30 more minutes incubation with specific competitor. The TBP only lanes show that the TBP alone fails to form a stable shifted band in the absence of Mg^{2+} (Lanes 2 TATAAA and 10 TATAAG). Free DNA is indicated by an asterisk and the TFIIA-TBP-DNA complex is indicated by a solid arrow. Far right, graphical representation of the exponential decay of TBP-TATAAA (open circles) and TBP-TATAAG (closed squares) complexes. Complex remaining at each time point was determined as described under the "Experimental Procedures" and the results are plotted as fraction remaining versus time. Data were averages ± SD from two independent experiments. **B.** Representative EMSA showing the binding and stability of the TBP complex on TATAAA and TATAAG. TBP was incubated with the DNA elements for 30 minutes (Lanes 2 TATAAA and 8 TATAAG) before the dissociation-time courses were initiated with poly[dAdT] specific competitor (lanes 3-6 TATAAA and 9-12 TATAAG) with the next lane in the series representing 30 more minutes incubation with specific competitor. Free DNA is indicated by an asterisk and the TBP-DNA complex is indicated by a solid arrow. Far right, graphical representation of the exponential decay of TBP-TATAAA (open circles) and TBP-TATAAG (closed squares) complexes. Data were averages ± SD from two independent experiments. **C.** Representative EMSA experiment showing the binding and stability of the TFIIB-TBP complex on TATAAA and TATAAG. The TBP and TFIIB proteins were pre-incubated with the elements for 30 minutes before the addition of specific competitor (lanes 3 TATAAA and 11 TATAAG). Dissociation-time course at 30 minute intervals were initiated with poly[dAdT] specific competitor (lanes 4-9 TATAAA and 12-16 TATAAG). TATAAA and TATAAG probes with TBP only are shown in lanes 2 and 11 respectively. TBP alone fails to form a stable shifted band in the absence of Mg^{2+} ions. Free DNA is indicated by an asterisk and the TFIIB-TBP-DNA complex is indicated by a solid arrow. Far right, graphical representation of the exponential decay of TBP-TATAAA (open circles) and TBP-TATAAG (closed squares) complexes. Data were averages ± SD from two independent experiments.



such a manner that allows for the formation of the TFIIB-TBP higher-order complex and the difference in stability of the TFIIA-TBP-DNA complex on TATAAG, like CATAAA (29), is specific to the complex containing TFIIA.

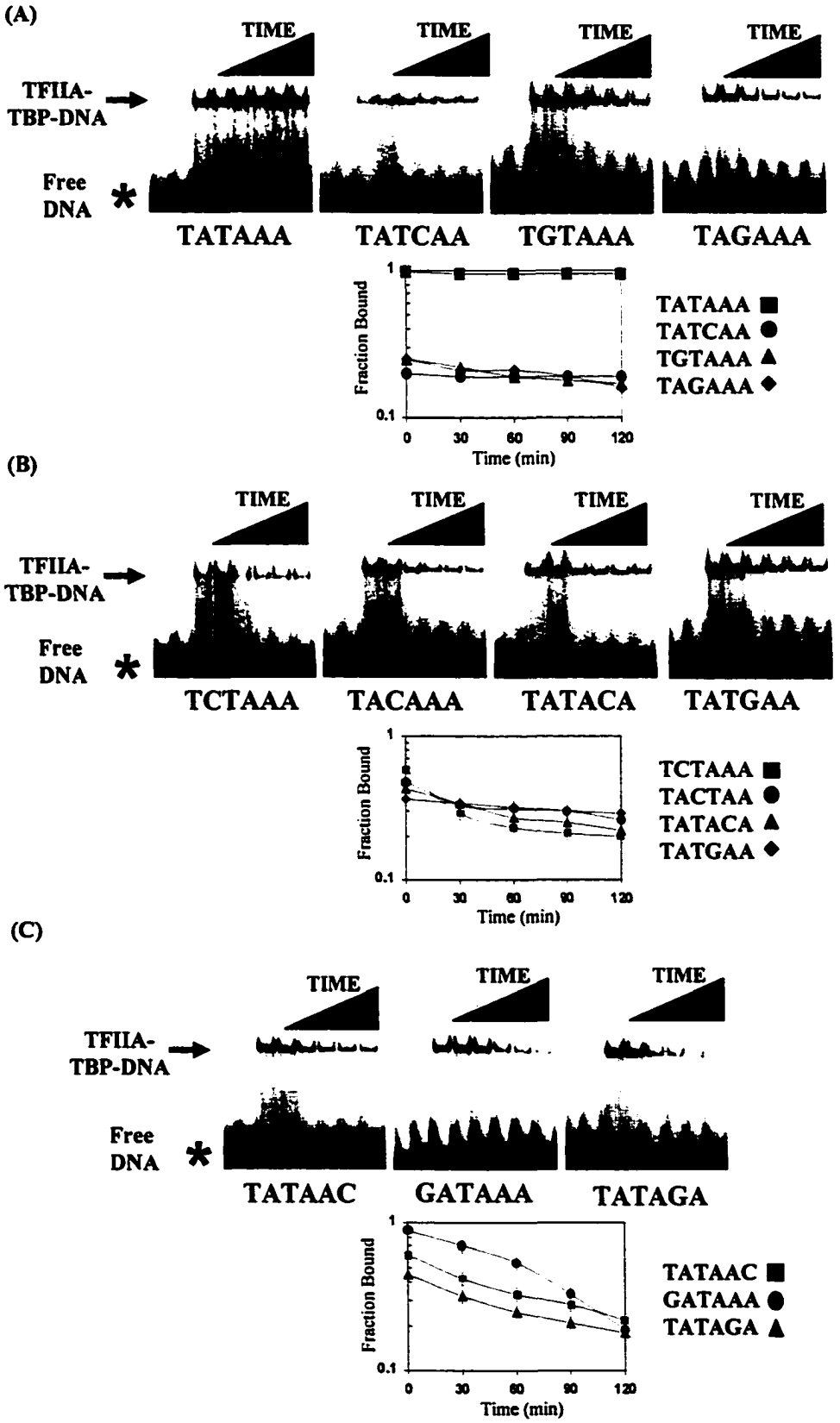
3.4e Lack of stability of the TFIIA-TBP-DNA complex is also observed for other elements.

EMSA were conducted to measure the stability of the TFIIA-TBP-DNA complex on the remaining TATAAA variants. Although elements TATCAA, TGTAAA and TAGAAA do not form TFIIA-TBP-DNA complex to the same extent as TATAAA, the amount of complex that does form is stable over the course of the experiment (Figure 3.5A). Elements TCTAAA, TACAAA, TATACA and TATGAA also do not form the TFIIA-TBP-DNA complex to the same extent as TATAAA but are moderately stable with approximately a 20% loss of complex during the time-course of the competition (Figure 3.5B). Interestingly, TATAAC, GATAAA and TATAGA are very unstable showing a significant loss of complex over the course of the experiment similar to TATAAG (Figure 3.5C). Also, TATAAC, GATAAA and TATAGA (along with CATAAA and TATAAG) all have substitutions near the ends of the TATAAA sequence, indicating a role for the end positions in destabilizing TFIIA-TBP-DNA.

3.4f The stability of the TFIIB-TBP-DNA complex depends strongly on the sequence of the TATA element.

EMSA competition experiments were conducted to measure the stability

Figure 3.5. The stability of the TFIIA-TBP-DNA complex is dependent on the sequence of the TATAAA element. A. Representative EMSA showing the binding and stability of the TFIIA-TBP complex on core promoter elements that, like TATAAA, exhibit infinite stability. TBP and TFIIA were incubated with the DNA elements for 30 minutes before the dissociation-time courses were initiated by adding the specific competitor, poly[dAdT]. Controls of DNA probe alone (Lane 1 of each experiment) and TBP without the addition of TFIIA in the absence of Mg^{2+} (Lane 2 of each experiment) are shown. Each subsequent lane represents 30 more minutes incubation with specific competitor (Lanes 3-8 of each experiment). Free DNA is indicated by an asterisk and the TFIIA-TBP-DNA complex is indicated by a solid arrow. Below, graphical representation of the exponential decay of the TFIIA-TBP-DNA complexes. Complex remaining at each time point was determined as described under the "Experimental Procedures" and the results are plotted as fraction remaining versus time. Data were means ± SD from two independent experiments. **B.** Representative EMSA showing the binding and stability of the TFIIA-TBP complex on elements that exhibit slight loss of complex over time. The experimental design is exactly as described for panel A. Below, graphical representation of the exponential decay of the TFIIA-TBP-DNA complexes. Data were means ± SD from two independent experiments. **C.** Representative EMSA showing the binding and stability of the TFIIA-TBP complex on elements that show formation of very unstable TFIIA-TBP-DNA complexes. The experimental design is exactly as described for panel A. Below, graphical representation of the exponential decay of the TFIIA-TBP-DNA complexes. Data were means ± SD from two independent experiments.

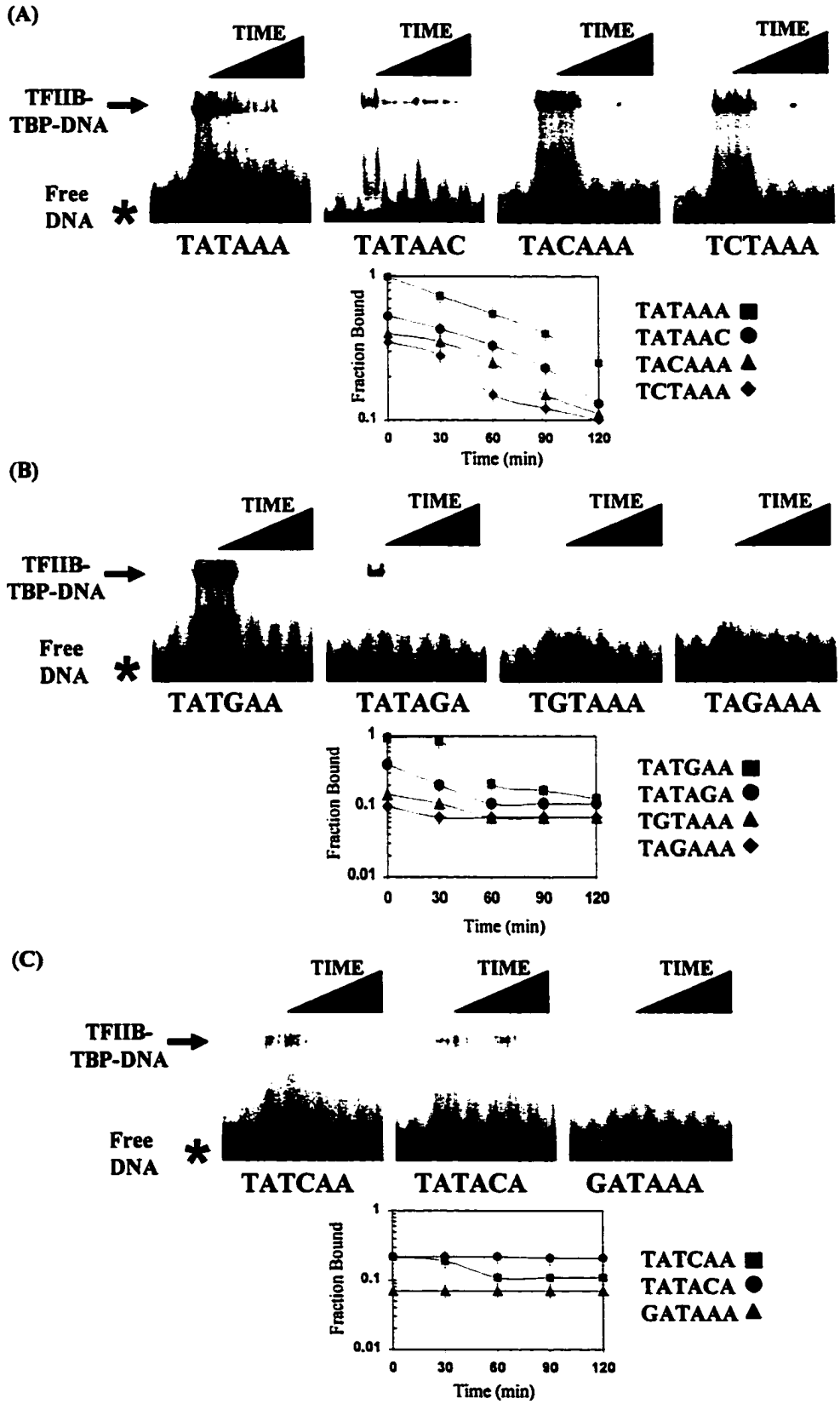


of the TFIIB-TBP-DNA on the TATA variants. Although the elements TATAAC, TACAAA and TCTAAA do not form the TFIIB-TBP-DNA complex to the same extent as TATAAA, the kinetics of the loss of complex from these elements is very similar to that of TATAAA (Figure 3.6A). The TGTAAA, TAGAAA, TATGAA and TATAGA form different initial amounts of the TFIIB-TBP-DNA complex but these elements share the trend that there is nearly complete loss of the complex very quickly during the competition, usually in less than 30 minutes (Figure 3.6B). The final group contains elements TATCAA, TATACA and GATAAA that form very low initial amounts of the TFIIB-TBP-DNA complex. The small amount of TFIIB-TBP-DNA complex formed on these elements is more stable than the complex formed on the previously described class of elements, but no more stable than the TFIIB-TBP-TATAAA complex.

3.4g Fusion of TBP and TFIIA results in an increase in expression from CATAAA and TATAAG in vivo.

Our in vitro results suggest that the lack of formation of stable protein-DNA complexes could contribute to the extremely low levels of transcription observed from several of the non-canonical elements in vivo. One would predict that if these complexes could be stabilized in vivo, this would result in an increase in transcriptional activity. To test this hypothesis, we utilized the Toa2-TBP and TFIIB-TBP fusion molecules. Presumably, fusing TBP directly to a subunit of TFIIA and TFIIB will artificially increase the effective concentration of

Figure 3.6. The stability of the TFIIB-TBP-DNA complex is influenced by the sequence of the TATAAA element. A. Representative EMSA showing the binding and stability of the TFIIB-TBP complex on core promoter elements that exhibit similar kinetics of TFIIB-TBP-DNA complex dissociation to TATAAA. TBP and TFIIB were incubated with the DNA elements for 30 minutes before the dissociation-time courses were initiated with poly[dAdT] specific competitor. Controls of DNA probe alone (Lane 1 of each experiment) and TBP without the addition of TFIIB in the absence of Mg^{2+}(Lane 2 of each experiment) are shown. Each subsequent lane represents 30 more minutes incubation with specific competitor (Lanes 3-8 of each experiment). Free DNA is indicated by an asterisk and the TFIIB-TBP-DNA complex is indicated by a solid arrow. Below, graphical representation of the exponential decay of the TFIIB-TBP-DNA complexes. Complex remaining at each time point was determined as described under the "Experimental Procedures" and the results are plotted as fraction remaining versus time. Data were means ± SD from two independent experiments. **B.** Representative EMSA showing the binding and stability of the TFIIB-TBP complex on elements that exhibit extremely rapid loss of complex over time. The experimental design is exactly as described for panel A. Below, the graphical representation of the exponential decay of the TFIIB-TBP-DNA complexes. Data were means ± SD from two independent experiments. **C.** Representative EMSA showing the binding and stability of the TFIIB-TBP complex on elements that do not form much initial TFIIB-TBP-DNA complex but that complex appears to be stable. The experimental design is exactly as described for panel A. Below, graphical representation of the exponential decay of the TFIIB-TBP-DNA complexes. Data were means ± SD from two independent experiments.



these proteins at the promoter element being tested.

The response to Gal4 in the strain containing the Toa2-TBP or the TFIIB-TBP fusion as the sole source of TBP was measured for all 13 elements using the β-galactosidase assay. Incubation in galactose medium for 18 hours results in significant induction in activity in both the Toa2-TBP and TFIIB-TBP fusion strain harboring the TATAAA driven reporter (Figure 3.7A and 3.7B), as expected for a canonical element. Strikingly, the Toa2-TBP fusion strains containing the CATAAA and TATAAG driven reporters showed a dramatic increase in activity to approximately 30% of TATAAA activity (Figure 3.7A). This increase in CATAAA and TATAAG activity is specific for TFIIA because no such increase is observed in the TFIIB-TBP fusion strain (Figure 3.7B). The increase is also specific to these elements since none of the other elements showed any increase in activity in the Toa2-TBP fusion strains (Figure 3.7). Thus, transcriptional defects of unstable TFIIA-TBP-DNA complex can be specifically suppressed by fusing TFIIA and TBP.

3.5 DISCUSSION

TBP is required for transcription initiation at all promoters transcribed by RNA polymerase II. The importance of the TBP-TATA interaction has been illustrated by studies showing that the binding of TBP to the TATA box is rate limiting at a majority of TATA-containing promoters and therefore a likely target of transcription regulation (14, 16). And yet, TBP has been shown to bind

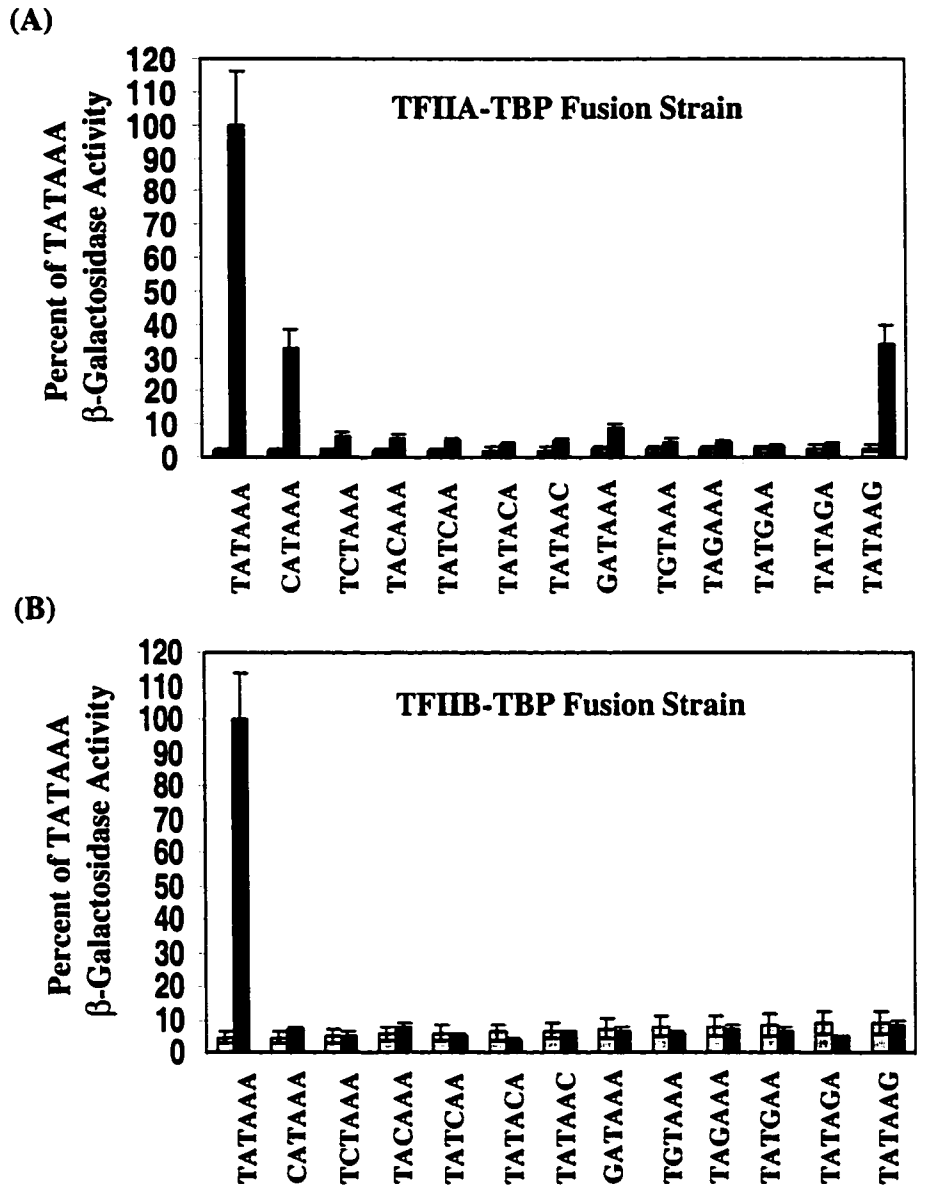


Figure 3.7 Fusion of TFIIA-TBP allows for some activated transcription from TATAAG in vivo. Yeast strain yJS156 was transformed with the β-galactosidase assay probe plasmid containing either the canonical TATAAA element or the indicated non-canonical element. β-galactosidase activities were performed with yeast cells cultured in a medium containing either 2% raffinose (gray columns) or 2% galactose (black columns) for 18 hrs.

promiscuously to both TATAAA and CATAAA, a non-canonical element (29). To determine if this holds true for other non-canonical sequences, eleven other variations of the TATAAA sequence were tested. We have found that cytosine and guanine substitutions at the middle positions of the TATAAA sequence result in decreased TBP binding. Although TBP binds to the minor groove of DNA, which provides few functional groups to direct binding specificity (15, 24, 27), minor groove differences do arise from an exocyclic NH_2 protruding from the guanine. This protruding group has been predicted to disrupt the TBP-DNA interaction at many positions by sterically clashing with hydrophobic residues on the concave undersurface of TBP (Table 3.2) (22). Replacement of A for C or G at postion 2 clashes with TBP residue Leu-163. Substitutions at position 3 from T to C or G clashes with Val-119. At position 4, the A to C or G replacement clashes with Val-119, and at position 5 the A to C and A to G substitution is precluded by Val-29 (22). Substitutions on the ends of the TATA box (position 1, CATAAA and position 6, TATAAG) are sterically allowed (22) and we find that these elements can form the TBP-DNA complex to the same extent as TATAAA.

In addition, the TBP-TATA interaction may be dependent on the unique flexibility that this sequence exhibits (8, 10). This has been shown using gel analysis (2, 28), fluorescence resonance energy transfer (FRET) (21) and cyclization analysis of TBP complexed to different TATA sequences (7). The DNA flexibility is believed to contribute to the stability of the TBP-DNA

Position 1	Position 2	Position 3	Position 4	Position 5	Position 6
T 79.5%	A 83.5%	T 91.4%	A 89.2%	A 71.0%	A 84.8%
C 11%	T 13.9%	A 4.4% A Tract	T 8.4%	T 27.7%	G 9.5%
A 5.0%	C 1.3% Steric Clash Leu163	C 3.3%	C 1.7% Steric Clash Val119	C <1% Steric Clash Val119	C 2.9%
G 4.5%	G 1.3% Steric Clash Leu163	G <1% Steric Clash Val119	G <1% Steric Clash Val119	G <1% Steric Clash Val119	T 2.8%

Table 3.2 Representation of the frequency of each base at all six positions of the TATA box, as determined by weighted matrices analysis of >500 eukaryotic RNA pol II promoter regions (3). Modeled thermodynamic considerations of the TBP-TATA interaction at each position are overlaid (22).

interaction because TBP forms the most stable conformation on severely bent or distorted DNA. This phenomenon could explain the inability of certain TATAAA variants to form a stable TBP-DNA complex. Perhaps the base substitutions are affecting the TBP-DNA interaction due to the thermodynamic differences in the rigidity of the triple bond between C-G or G-C base pairing and the double bond of the A-T or T-A base pairing.

In the cell, TBP recognition of the TATA box sets the stage for the nucleation of the remainder of the pre-initiation complex (PIC), which includes TFIIA, -B, -E, -F, -H, -J and Pol II (reviewed in 20). TFIIA and TFIIB have each been shown to make direct contacts with TBP and DNA, and in so doing, stabilize the TBP-DNA complex (reviewed in 11). We found that elements which exhibited a decrease in the TBP-DNA interaction were typically also compromised in forming the higher-order complexes with TFIIA and TFIIB. Generally speaking, neither TFIIA nor TFIIB could overcome defects in TBP binding to the TATAAA variants, with one notable exception. The presence of TFIIB, but not TFIIA, restores TBP binding to the TATGAA element to the level of complex formed on TATAAA. This result suggests that TFIIB specifically introduces a conformational change in the TBP-DNA complex that can overcome the steric clash between the exocyclic NH_2 group on the guanine and Val-119 of TBP.

Although TATAAG was able to form the initial TBP-DNA, TFIIA-TBP-DNA and TFIIB-TBP-DNA complexes to the same extent as the control

elements TATAAA and CATAAA, measurements of the stability of the TFIIA-TBP-DNA showed that, like CATAAA (29), TATAAG was defective for the formation of a stable TFIIA-TBP-DNA complex. Experiments measuring the stability of the TBP-DNA complex and TFIIB-TBP-DNA complex on TATAAG revealed that these complexes behaved indistinguishably from TATAAA and CATAAA. Therefore, the difference in stability of protein complexes formed on CATAAA and TATAAG is specific to the higher order complex containing TFIIA and a TFIIA-TBP fusion molecule can restore activity form these two elements in vivo. Significantly, the other TATAAA variants did not show an increase in transcriptional activity in the TFIIA-TBP fusion strain even though some of these elements showed measurable loss of TFIIA-TBP-DNA complex during the competition experiments. Thus, stabilizing the TFIIA-TBP interaction could only restore activity from the elements CATAAA and TATAAG, where the stability of the TFIIA-TBP-DNA complex appears to be the primary mechanistic defect.

Overall, the data presented indicate that single base substitutions in the consensus TATA sequence leads to transcriptional reduction, due to diverse mechanistic defects, which are dependent on the position involved (Table 3.3). Internal base substitutions in the TATAAA element are not commonly found in core promoter elements driving the expression of eukaryotic genes (3). This is consistent with our findings that internal substitutions disrupt TBP binding and higher-order complex formation. In contrast, substitutions at the first and last position are biologically relevant in that CATAAA and TATAAG elements are

Position 1	Position 2	Position 3	Position 4	Position 5	Position 6
T	A	T	A	A	A
C	C	C	C	C	C
TBP binding 96%	TBP binding defective **25%**	TBP binding 65%	TBP binding defective **35%**	TBP binding defective **40%**	TBP binding 70%
TFIIA-TBP-DNA complex 97% **very unstable**	TFIIA-TBP-DNA complex 55%↑ moderately stable	TFIIA-TBP-DNA complex 45% moderately stable	TFIIA-TBP-DNA complex **25%** very stable	TFIIA-TBP-DNA complex **35%** moderately stable	TFIIA-TBP-DNA complex 50% **very unstable**
TFIIB-TBP-DNA complex 99% moderately stable	TFIIB-TBP-DNA complex **30%**↑ moderately stable	TFIIB-TBP-DNA complex **35%** moderately stable	TFIIB-TBP-DNA complex **10%** very stable	TFIIB-TBP-DNA complex **15%** very stable	TFIIB-TBP-DNA complex **25%** moderately stable
G	G	G	G	G	G
TBP binding 48%	TBP binding defective **41%**	TBP binding defective **35%**	TBP binding 50%	TBP binding defective **25%**	TBP binding 98%
TFIIA-TBP-DNA complex 65%↑ **very unstable**	TFIIA-TBP-DNA complex **7%** very stable	TFIIA-TBP-DNA complex **7%** very stable	TFIIA-TBP-DNA complex **30%** moderately stable	TFIIA-TBP-DNA complex **20%** **very unstable**	TFIIA-TBP-DNA complex 99% **very unstable**
TFIIB-TBP-DNA complex **5%** very stable	TFIIB-TBP-DNA complex **9%** very unstable	TFIIB-TBP-DNA complex **4%** very unstable	TFIIB-TBP-DNA complex 98%↑ very unstable	TFIIB-TBP-DNA complex **10%** very unstable	TFIIB-TBP-DNA complex 90% moderately stable

Table 3.3 Summary of the in vitro protein-DNA interactions with the thirteen TATAAA derivatives. The main defects are indicated in bold lettering. The arrows indicate situations where the higher-order complex can increase overall binding over TBP-DNA complex formation.

utilized in approximately ten percent of all eukaryotic protein coding genes (Table 3.2) (3). We have demonstrated that CATAAA and TATAAG share a common mechanism of transcription regulation, namely the altered stability of the TFIIA-TBP-DNA complex. This suggests that the altered stability of the TFIIA-TBP-DNA complex is a useful and widespread mechanism for regulation of gene transcription from non-optimal core promoter elements in vivo.

3.6 ACKNOWLEDGEMENTS

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CHAPTER 4

THE N-TERMINAL DOMAIN OF TBP IS INVOLVED IN PROMOTER SELECTIVITY

Chapter four includes a number of functional experiments demonstrating the possible role of the N-terminus of TBP in the regulation of RNA polymerase II transcription initiation. The work presented in this chapter came about through a collaboration with a rotation student Neelu Yadav and myself. I have contributed all the data shown in figures 4.1, 4.2 and 4.3 while Neelu contributed all of the yeast two-hybrid data, which are mostly not shown. Future directions, when accomplished, will include additional collaborators.

4.1 ABSTRACT

To determine the possible functional role of the N-terminal domain (NTD) of TBP in RNA pol II transcription, we studied the effect of deletion of the first 63 amino acids of TBP both in vivo and in vitro. TBP lacking the NTD could not support cell viability at high temperatures in vivo. This dramatic in vivo effect is not likely to be due to a loss of interaction between the carboxy-terminal domain of TBP (cTBP) and other components of the general transcription machinery since no protein-protein interactions were detected with the NTD using the two hybrid assay. The NTD has been shown to be involved in the regulation of cTBP binding and stability on the DNA. Removal of the NTD from TBP results in stabilization of the TBP-DNA and TFIIA-TBP-DNA complex on both canonical TATAAA and off-consensus CATAAA elements. Moreover, the removal of the NTD from TBP also neutralizes the ability of the TFIIA-TBP complex to distinguish between different DNA sequences. These results indicate that the N-terminus of TBP plays a role in regulating cTBP-DNA interactions and works in concert with TFIIA to distinguish weaker core promoter elements.

4.2 INTRODUCTION

Genomic and cDNA clones encoding TATA binding protein (TBP) have been isolated from a number of species including fungi, insects, plants and mammals (2, 6-8, 10-13, 15, 17). *Saccharomyces cerevisiae* TBP consists of a single peptide of 240 amino acids which can be divided into the N-terminal

domain (NTD) residues 1-63 and a highly conserved C-terminal domain (cTBP) made up of residues 64-240. Biochemical and genetic experiments have shown that only the highly conserved carboxy terminal core domain of TBP (~80 % sequence identity between all species) is required for the DNA binding function (16). The N-terminal domain varies both in length and sequence between different species and the role of this domain in RNA pol II transcription initiation remains unclear. In addition, structural information regarding this domain is not available, since all of the available high resolution structures used TBP lacking this domain (9, 18, 19).

Removal of the NTD from human or *Drosophila* TBP results in a loss of responsiveness to the transcriptional activator Sp1 (23, 24). This suggests the NTD in these species is playing a role in the protein-protein interactions that regulate the activity of the TFIID complex. However, when compared to yeast, the amino-terminal domains in flies and humans are quite different in both length and sequence composition. Other experiments imply a role for the *Saccharomyces cerevisiae* NTD in regulating the DNA binding activity of TBP. Removal of the yeast NTD results in faster binding to DNA and a much more stable TBP/DNA complex (20, 21), suggesting an inhibitory role for this domain. Studies measuring the ability of full length TBP to associate into dimers and other higher oligomers demonstrate that TBP dimerization is unfavored by the presence of the NTD (1). Since the dimerization interface of cTBP coincides with the DNA-binding surface and the NTD interferes with TBP dimerization, it is

possible that the NTD may interact with the DNA binding surface of TBP and influence the kinetics of TBP binding to the DNA.

To address the role played by the N-terminus of TBP in transcription in vivo, we created a yeast strain in which cTBP was the sole source of TBP for the cell. We found that expression of TBP lacking the NTD results in a temperature sensitive phenotype in vivo, suggesting a mechanistic penalty for using TBP lacking that NTD to drive transcription in the cell. Full-length yeast TBP binding in vitro has been well characterized on TATAAA and CATAAA and the TFIIA-TBP-DNA complex is significantly less stable on the CATAAA element when compared to the TATAAA element (26). To measure cTBP competency for DNA binding, cTBP-TATAAA and cTBP-CATAAA complex formation and stability was measured in vitro. We find that cTBP forms a very stable complex with both TATAAA and CATAAA and the kinetics of dissociation is similar for both sequences. Thus, without its N-terminus, TBP could no longer distinguish between the two sequences, suggesting an important role for the NTD of TBP in core promoter recognition.

4.3 EXPERIMENTAL PROCEDURES

4.3a Yeast strains and plasmids

The yeast strain yJS156 is a derivative of KY804 (relevant genotype: *MATα ura3-52 trp1-Δleu2::PET56 gal2 gcn4-Δ1*) (14) with the chromosomal copy of *SPT15* deleted by a two-step knockout. TBP functions are provided by

a *URA3*-marked plasmid containing the TBP promoter, open reading frame and terminator. Plasmid shuffling on 5-fluoroorotic acid was used to create the *TRP1*-marked wild type TBP and cTBP strains used to determine growth phenotypes. The cTBP construct contains a deletion of amino acids 5-63 of the TBP ORF, encompassing the majority of the NTD (3).

All yeast strains used in the yeast two-hybrid assay were transformants of MaV103 (28). MaV103 contains the *GAL1* promoter (with four Gal4 binding sites) fused to the *HIS3* promoter and structural gene; *GAL4* and *GAL80* are both deleted in the strain.

To generate the AD-NTD clone, amino acids 1-63 of the TBP open reading frame were amplified using PCR and cloned into the 2μm *LEU2* activation domain (pACT2.2) vector (AD) (5) which contains the *ADH1* promoter, a nuclear localization sequence, an HA epitope and, the Gal4 activation domain (residues 768-881). PCR primers were designed to introduce a Nde1 site at the start of the TBP ORF and a BamH1 site after amino acid 63 and the engineered stop codon. The DNA-binding domain construct (DB-NTD) was created by subcloning the Bgl II fragment of AD-NTD into the DNA-binding domain (pPC97) (28) vector (DB) (CEN-*TRP3*) which contains the *ADH1* promoter, a nuclear localization sequence, HA epitope and the Gal4 DB.

4.3b Yeast two-hybrid assays and phenotypic studies.

Both Gal4 DB and Gal4 AD plasmids were transformed into the yeast

strain MaV103 using a standard lithium acetate transformation. The resulting strains were grown in the appropriate selection medium. Cells were streaked on to the appropriate plates that either contained or lacked 3-aminotriazole (AT). Cells were grown at 30°C for 4-7 days. For phenotypic studies, strains were streaked to plates with rich medium containing either glucose (YPD) or alternate carbon sources (YP galactose, glycerol or ethanol) and incubated at the following temperatures: 16, 30, 37 or 39°C.

4.3c Protein Expression and purification.

Full-length yeast TBP was expressed in *Escherichia coli* strain BL21 DE3 and was purified from the soluble fraction with Q, SP, and Heparin columns (1). The TBP was equilibrated and stored in EMSA binding buffer without Mg^{2+} (26, 27). The protein was shown to be 90% homogeneous by SDS-PAGE.

N-terminally deleted TBP (cTBP) was prepared by trypsin cleavage of full length TBP (1). The two products were separated by ion exchange chromotography and the identity of cTBP was confirmed by mass spectroscopy.

Recombinant yeast TFIIA was purified as described (25). This procedure involves expressing each subunit, *TOA1* and *TOA2*, in separate strains of *E.coli* BL21DE3. Cells were ruptured by sonication, insoluble material was collected by centrifugation. Each insoluble pellet was resolublized in 8M urea. Each subunit was renatured in the presence of the other subunit and equilibrated in EMSA binding buffer without Mg^{2+} (26, 27). The TFIIA was

approximately 60 % pure as determined by Coomassie staining.

4.3d Electrophoretic mobility shift assays (EMSA))

The DNA elements used in vitro protein DNA interaction studies were 23 base pair ^{32}P internally labeled TATAAA and CATAAA oligonucleotides (26). Protein-DNA interactions *in vitro* were studied by incubation of TBP (13.8 nM) and TFIIA (5.0 nM) with the DNA probes (2.4 picomoles) and separation of the resulting complexes on a 5% acrylamide gel (26, 27). For dissociation kinetic studies, TBP-DNA, TFIIA-TBP-DNA complexes were allowed to reach equilibrium and then challenged with 1000 fold molar excess of specific competitor poly[dAdT] for the specified amount of time and resolved by EMSA. The data are presented as the fraction bound at time, t. Fraction bound is calculated from the ratio B/B_o, where B represents the amount of complex present at each time point and B_o is defined as the amount of complex formed in the control lane without added specific competitor (26, 27).

4.4 RESULTS

4.4a Deletion of the N-terminus of TBP results in temperature sensitivity.

To examine the physiological relevance of NTD in transcription initiation in vivo, full length TBP and cTBP (TBP minus the NTD) were expressed in yeast strain yJS156. cTBP supported cell viability at 30 °C, 16 °C and on YP galactose, YP ethanol and YP glycerol but exhibited a temperature sensitive

phenotype at 37 °C and 39 °C compared to the full length TBP (Figure 4.1). These results suggest deletion of the N-terminus of TBP may be affecting overall transcription initiation in the cell. Since TBP is a universal transcription factor required by all three RNA polymerases, is the loss of the NTD affecting transcription from RNA pol II, II and III?

4.4b The N-terminus of TBP does not interact with other components of the general transcription machinery via the yeast two-hybrid assay.

Yeast cells using cTBP as their sole source of TBP exhibit a temperature sensitive phenotype at high temperatures. This could be due to the loss of interaction with one or more components of the general transcription machinery. To test this hypothesis, we cloned the portion of TBP encoding the first 63 amino acids (NTD) into both Gal4 DNA Binding (DB) and Gal4 Activation Domain (AD) plasmids. The design of this clone placed the AD or DB on the N-terminus of the NTD protein. The DB-NTD construct was used to show that the NTD could not artificially recruit the components of the general transcription machinery and did not allow the MAV103 cells to grow on selective medium containing as low as 5mM AT (data not shown). This construct was then used to detect interactions with a large panel of AD constructs, including several types of activators, both subunits of TFIIA, TFIIB, and all of the TBP associated factors (TAFs). No interactions between DB-NTD and the AD constructs were measured by the ability of the MAV103 strain to grow on selective medium.

TBP Derivative	YPD 30°C	YPD 37°C	YPD 39°C	YPD 16°C
Wt TBP	+++	+++	++	+++
cTBP	+++	+	-	+++

TBP Derivative	YP Galactose	YP Ethanol	YP Glycerol
Wt TBP	+++	+++	+++
cTBP	+++	+++	+++

Figure 4.1. Growth phenotypes of the different TBP derivatives. Yeast strain yJS156 was transformed with *TRP1*-marked wild type TBP or cTBP as the sole source for TBP in the cell. The resulting strains were grown on rich medium (YPD) and incubated at the temperatures indicated or grown at 30°C on the indicated carbon sources.

containing AT (data not shown). The negative results were not the result of a destablization of the NTD protein, since both AD-NTD and DB-NTD strains produced detectable amounts of the N-terminus of TBP as assayed by immunoblot analyses of whole cell yeast extracts (data not shown). These results suggest that the role of NTD of TBP may not be interaction with the other components of the general transcription machinery but rather regulating cTBP function.

4.4c The N-terminal domain of TBP inhibits DNA binding.

To test whether or not deletion of the N-terminus of TBP could affect recognition and binding of cTBP to the TATA box, EMSA competition assays were carried out to measure the differences in binding and stability of cTBP and on TATAAA and CATAAA (Figure 4.2). The initial formation of the TBP-DNA complex was similar for TATAAA and CATAAA. The stability of the cTBP-DNA complex was determined by challenging the formed complex at two hour intervals with a 1000 fold molar excess of Poly[dAdT] competitor (Figure 4.2 A and B). As with TBP-TATAAA and TBP-CATAAA, the kinetics of cTBP-DNA complex dissociation is similar on both elements. However, removal of the NTD of TBP causes a significant stabilization of the cTBP-DNA complex with a $T_{1/2}$ of 5 hours compared to the TBP-DNA complex with a $T_{1/2}$ of 70 min (26).

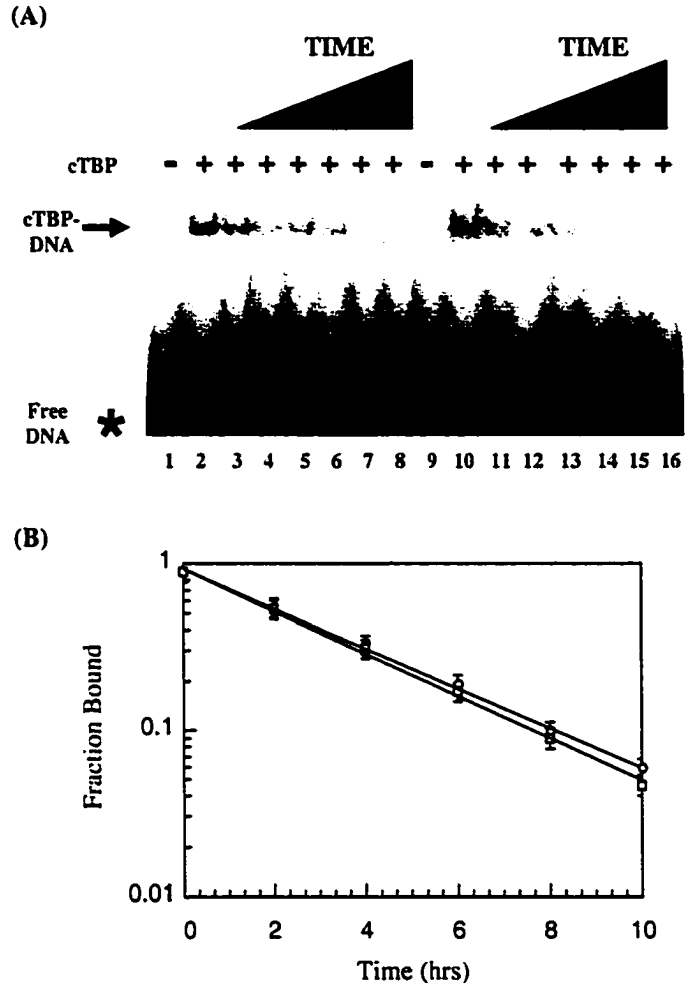

Figure 4.2 Dissociation of cTBP from TATAAA and CATAAA DNA is similar. A. Representative gel shift experiment showing probe DNA incubated with TBP (Lane 2, TATAAA and 10, CATAAA) and then challenged with specific competitor poly[dAdT] at 30 minute intervals (Lanes 3-8, TATAAA and 11-16, CATAAA). DNA alone (no added protein) is shown in Lane 1 (TATAAA) and 9 (CATAAA). Free DNA is indicated by an asterisk and TBP-DNA complex is indicated by a solid arrow. **B.** Graphical representation of the exponential decay of TBP-TATAAA (open circles) and TBP-CATAAA (closed squares) complexes. Results are plotted as fraction remaining versus time. Data are means ± SD from three independent experiments.

4.4d The N-terminus of TBP plays a role in destabilizing the TBP-TFIIA complex on the CATAAA element.

EMSA competition assays were carried out to measure the differences in binding and stability of TFIIA-cTBP and on TATAAA and CATAAA (Figure 4.3). The initial formation of the TFIIA-cTBP complex was measured on both elements and found to be the equivalent. The stability of the TFIIA-cTBP-DNA complexes was determined by challenging the formed complex at timed intervals with a 1000 fold molar excess of Poly[dAdT] specific competitor (Figure 4.3 A, B and C). The TFIIA-cTBP-TATAAA and TFIIA-cTBP-CATAAA complexes both exhibit similar stabilty during the first two hours of the experiments (Figure 4.3 B) and similar decay kinetics at longer time points with a $T_{1/2}$ of 36 hours (Figure 4.3C). In contrast, the same complex with full length TBP was shown to be extremely unstable on CATAAA, with a short comparative half-life of only 1.9 hours compared to the TFIIA-TBP-TATAAA complex half-life of 14 hrs. (26). These data indicate that without the N-terminus of TBP, the TFIIA-cTBP complex could no longer distinguish between consensus and off-consensus sequences, suggesting a role for the NTD in allowing TBP to distinguish between strong and weak core promoters.

4.5 DISCUSSION

Our results indicate that the N-terminal domain of TBP affects the structure and the stability of the TBP-DNA complex, especially in the context of

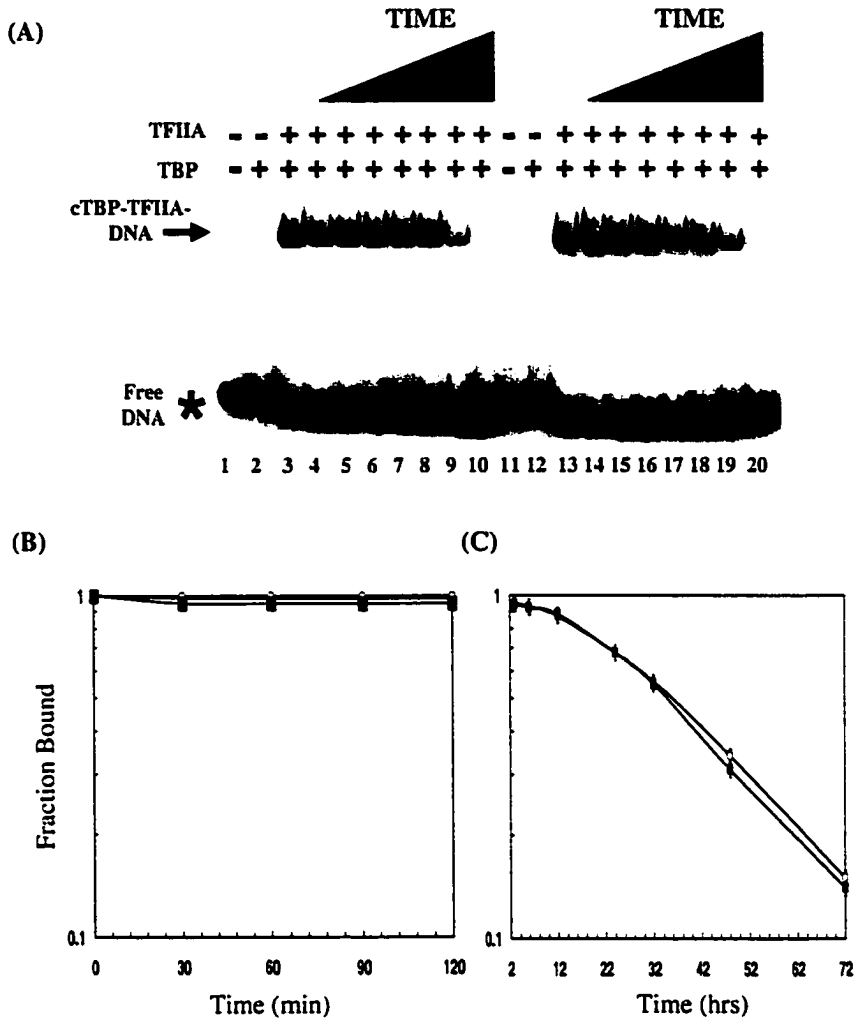

Figure 4.3 Dissociation of TFIIA-cTBP from TATAAA and CATAAA DNA is similar. A. Representative gel shift experiment showing probe DNA incubated with TFIIA-TBP (Lane 2, TATAAA and 12, CATAAA) and then challenged with specific competitor poly[dAdT] at 30 minute intervals (Lanes 3-10, TATAAA and 13-20, CATAAA). Controls TBP alone is shown in Lane 2 (TATAAA) and 12 (CATAAA) and DNA alone (no added protein) is shown in Lane 1 (TATAAA) and 11 (CATAAA). Free DNA is indicated by an asterisk and TBP-DNA complex is indicated by a solid arrow. **B.** Graphical representation of the exponential decay of TBP-TATAAA (open circles) and TBP-CATAAA (closed squares) for the first two hours. **C.** Graphical representation of the exponential decay of the complexes from 2 -72 hours. Data are means ± SD from three independent experiments.

TFIIA. The major effect that the NTD has on the stability of the TBP-DNA complex is detected by native gel electrophoresis where the NTD increases the dissociation rate of the TBP-DNA complex by a factor of 4 (26). Our data are consistent with the hypothesis that a conformational change involving the NTD is one of the isomerizaiton steps in the formation of a stable TBP-DNA complex (16). Based on previous results (1), we hypothesize that when TBP is free in solution, the NTD is present in a conformation that interferes with formation of a stable TBP-DNA complex, possibly by occluding the DNA binding surface on TBP. An isomerization of the complex is required to change the conformation of TBP to form the stable TBP-DNA complex. The same isomerization step that moves the NTD out of the way of the DNA binding domain also might expose another interaction surface on TBP. This mechanism would ensure that only TBP molecules that are bound to DNA in a productive manner are able to compete for the binding of additional factors, such as TFIIA.

4.6 FUTURE DIRECTIONS

4.6a Transcriptional and genetic analysis

Since TBP is a universal transcription factor required by all three RNA polymerases, deletion of the N-terminus of TBP may be affecting overall transcription in the cell. We would like to know if this is the case or if the cTBP defect at high temperatures is specific for RNA pol II. This information could be elucidated by transcriptional analysis and yeast genetics. Total amounts of

transcription can be measured using polyA RNA or direct measurements of rRNA by RNA Pol I and tRNA by RNA Pol III.

An intragenic complementation assay could be used to determine if cTBP is functional for RNA Pol III transcription at the restrictive temperature. Complementation depends on the ability of two temperature sensitve TBP mutants, each conferring a different functional defect, to support cell viability when they are present in the cell at the same time (4). If cTBP is able to complement the growth defect of a TBP mutant, F155S, then we can conclude that the defect does not involve RNA pol III transciption. To determine if a loss of Pol I transcription was the cause of the cTBP phenotype we could utilize a plasmid-based system (pNOY103) in which the rRNAs are synthesized from a Pol II promoter (22). If cTBP is strictly defective for Pol I transcription, then this Pol II-driven rRNA transcript should rescue the temperature sensitive phenotype when the cells are cultured in galactose-containing medium.

4.6b Continuation of the Yeast two-hybrid assay

As mentioned previously, the design of the DB-NTD and AD-NTD constructs used thus far placed these DB and AD on the N-terminus of the NTD. In the normal context of TBP, the c-terminal domain of TBP is attached to amino acid 63, leaving the N-terminal end of the NTD free. Thus, the design of these constructs may be inhibiting potential interactions by the blockage of the N-terminus of the NTD. To address this concern, new constructs in the

opposite orientation have been designed by me and created by Dr. Sue Kraemer to position the DB and AD on the C-terminal side of the NTD. These constructs are currently being tested in the two-hybrid assay for interactions with other proteins.

4.6c What is the structure of the NTD of TBP?

Little is known about the conformation of the NTD of TBP because all the high resolution crystal structures of TBP available at this time were solved lacking this domain (9, 18, 19). In order to gain a better understanding of the role of the NTD in the cell, structural studies of this domain are currently underway by a Masters student, Greg Smith, in collaboration with Dr. Kevin Lumb's laboratory. These experiments will include circular dichroism and NMR to determine what secondary structure, if any, the NTD adopts. These experiments, along with the other future directions proposed throughout this chapter will allow us to answer more questions about how this important domain of TBP functions in transcription.

4.7 ACKNOWLEDGEMENTS

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CHAPTER 5

PERSPECTIVES AND FUTURE DIRECTIONS

The work presented in this dissertation has been focused on understanding transcription regulation of promoters transcribed by RNA polymerase II lacking canonical TATA boxes. When CATAAA (our model for non-canonical) was compared to canonical TATAAA, this element was found to be severely defective for in vivo transcription driven by the Gal4 activator. In vitro studies showed a loss of stability of the TFIIA-TBP-CATAAA complex, identifying a novel mechanism in transcriptional regulation involving destabilization of the TFIIA-TBP complex on this sequence.

In order to determine how universal is the TFIIA dependent regulatory mechanism, a survey of eleven additional elements was conducted. Single base substitutions of each position of the consensus TATAAA sequence led to abolishment of transcriptional response in vivo and diverse protein-DNA complex defects (depending on the position involved). However, the TATAAG element was found to behave like CATAAA in that it could support the initial formation of TBP-DNA, TFIIB-DNA and TFIIA-DNA complexes, but the TFIIA-TBP complex was not stable. The instability of the TFIIA-TBP-DNA complex on

CATAAA and TATAAG is believed to cause the transcriptional defects exhibited by these elements in vivo, thus, the loss of TFIIA from the pre-initiation complex might be exploited by the cell to decrease transcription from suboptimal core promoter elements.

Taken together, these results suggest that not only is TBP binding the core promoter important for transcription regulation but also other aspects of TBP function, including interacting productively with TFIIA. We have shown that artificially stabilizing the TFIIA-TBP-DNA complex on both CATAAA and TATAAG, using a fusion molecule, results in increased transcription in vivo. One important question that arises from these results is what are the implications of the loss of the TFIIA-TBP-DNA interaction in the context of all of the other cellular components? For example, interactions with other components of the general transcription machinery, such as the N-terminus of TBP, TBP associated factors (TAFs), and RNA pol II itself, as well as the influence of other regulatory complexes, and phosphorylation of transcription factors are possible ways for the cell to regulate transcription initiation from non-canonical elements.

5.1 THE N-TERMINAL DOMAIN OF TBP

Work investigating the possible involvement of the amino-terminus of TBP (NTD) in core promoter recognition has shown that when the NTD was lacking from TBP (cTBP) cells could not support viability at high temperatures in

vivo. The NTD has been shown to be involved in the regulation of cTBP binding and stability on the DNA. We found that removal of the NTD from TBP results in stabilization of the cTBP-DNA and TFIIA-cTBP-DNA complexes on both TATAAA and CATAAA. The removal of the NTD from TBP also neutralizes the ability of TFIIA to distinguish between different DNA sequences. This suggests that the N-terminus of TBP plays an inhibitory role in the cell by destabilizing TBP-DNA interactions and to distinguish weaker core promoter elements.

Structural studies of the NTD of TBP are currently underway by Greg Smith in collaboration with Dr. Kevin Lumb's laboratory. Circular dichroism and NMR will be used to determine what secondary structure, if any, the NTD adopts. These experiments will give us a better understanding of this domain to TBP, since none of the available high resolution structures of TBP contain the NTD, and allow us to answer more questions about how this important domain of TBP functions in transcription.

5.2 THE POSSIBLE ROLE OF TBP-ASSOCIATED FACTORS.

TBP-associated factors (TAFs) are believed to play a role in core promoter recognition, especially in the absence of a canonical TATA box (reviewed in 3, 7,13). Recent work has shown that one of the TBP associated factors, TAF40, interacts directly with both TBP and TFIIA and enhances TFIIA-TBP-DNA complex formation in vitro (8). Consequentially, we hypothesize that

this factor may be able to enhance the TFIIA-TBP-DNA interaction on the C series and G series of non-canonical elements (Figure 5.1). Mary Robinson has developed the EMSA assay to test this hypothesis and is currently investigating the ability of TAF40 to affect the TFIIA-TBP-DNA complex on these other elements.

5.3 INTERACTION WITH THE NOT COMPLEX AND SPT3

TBP is also associated with transcriptional repressors *in vivo* that generally affect TBP function by inhibiting TBP-DNA interactions. Transcriptional repressors in yeast include certain TAFs (10), Mot1(ADI) (1, 11), and the NOT complex (5). Interestingly, mutations in the NOT (Negative On Transcription) proteins cause an increase in transcription from the +1 start site of *HIS3*, indicating the ability of the NOT proteins to differentiate between strong and weak promoters. Genetic and biochemical evidence suggests that the products of the NOT genes, along with Mot1 and Spt3p, are associated in a complex that serves to regulate TBP function (4).

Although the ability of the NOT complex to repress transcription at TATA-less promoters is well established, the mechanism by which it does this is unknown. Based on our findings, we hypothesize that in the absence of TFIIA to stabilize the TBP-DNA interaction on CATAAA and TATAAG, Mot1p removes TBP from the DNA in an ATP dependent manner (1). Once free, the TBP can then either bind to another, possibly more canonical, TATA element or interact

Figure 5.1 Possible mechanisms of transcription regulation at non-canonical elements due to the loss of stability of the TFIIA-TBP complex.

with Spt3p, which has been shown to enhance TBP binding to DNA (6). Spt3p is a target for regulation by the NOT complex (4) and this NOT dependent regulation is believed to further disfavor TBP binding to non-canonical elements (Figure 5.1). To examine the possible involvement of the NOT complexes in our system, we have obtained mutant NOT background strains (gifts from the Collart laboratory). These strains have been shown to preferentially increase transcription from the *HIS3* non-canonical promter (T_C), presumably because the mutations inhibit NOT regulation of the Spt3-TBP complex, thus allowing Spt3 stabilization of the TBP binding to non-canonical elements. The NOT mutant strains could be utilized to investigate if the NOT complex can preferentially increase transcription from the substituted core elements that I designed.

5.4 RAPID REINITIATION

A high level of gene output not only depends on initial induction of the gene but also on the number of times the gene is continuously transcribed or reinitiated (15,16, 17). Analysis of the fate of several general transcription factors during the transition from initiation to elongation has shown that many factors are released from the DNA template whereas TBP, TFIIA and some activators remain associated (16,17). The sequence of the TATA element has also been shown to be critically important to the rate at which reinitiation occurs, and TATA-less promoters fail to show rapid reinitiation (14,15). Since TBP and

TFIIA remain associated with the core promoter after polymerase escape, and these factors have been implicated in directing rapid reinitiation, it seems likely that a difference in stability of this complex could result in significant changes in the rate of reinitiation. We hypothesize that CATAAA and TATAAG are defective for reinitiation due to loss of a stable TFIIA-TBP-DNA complex and this explains the overall low amounts of transcription measured for these elements in vivo (Figure 5.1).

5.5 TRANSCRIPTION FACTOR PHOSPHORYLATION

Casein kinase II (CK2) is a ubiquitous Ser/Thr kinase which phosphorylates a large number of proteins including several transcription factors. Sequence analysis of TBP reveals two putative phosphorylation sites for CK2; one on an upper helix of the carboxy-terminal domain, and the other overlapping the division between the amino-terminal domain and the carboxy-terminal domain. The phosphorylation of *Saccharomyces cerevisiae* TBP by CK2 has been shown to reduce its TATA box binding activity (9). It would be reasonable to speculate that the cell uses phosphorylation of TBP to induce conformational changes in the protein that affect the interaction of TBP with the DNA. The phosphorylation site located at the C-terminal end to the NTD is particularly interesting since phosphorylation of this site may induce structural changes in the NTD, allowing this domain to occlude the DNA binding surface of cTBP and inhibit binding. Dephosphorylation might also induce a

comformational change forcing the NTD away from the concave undersurface of TBP and allow for TBP binding. In vitro EMSA assays developed by Greg Smith could be utilized to test the effects of phosphorylation on TBP and cTBP on DNA binding to TATAAA as well as the C series and G series of non-canonical elements.

Toa1, the large subunit of yeast TFIIA, has also been shown to be phosphorylated by CK2 in vivo. This phosphorylation stabilizes the TFIIA-TBP-DNA complex and is required for high-level transcription (12). So unlike the phosphorylation of TBP, phosphorylation of TFIIA enhances the binding and stability of the TFIIA-TBP complex on DNA, leading to a possible mechanism for the cell to stabilize this interaction on non-canonical core elements in vivo. In vitro EMSA assays could be utilized to determine if phosphorylation of TFIIA enhances binding of the TFIIA-TBP complex to TATAAA or stabilizes the TFIIA-TBP complex on elements defective for this interaction, such as CATAAA and TATAAG.

5.6 SUMMARY

The experiments proposed above, combined with the foundation of work presented in this dissertation, will certainly advance our understanding of transcription initiation by RNA pol II.

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