2003

Self-association studies of yeast TATA binding protein using an E. coli two-hybrid system

Michael Sauder

Claire Adams

Michael Fried

Self-association studies of yeast TATA binding protein using an *E. coli* two-hybrid system

Michael Sauder*, Claire Adams, and Michael Fried

Department of Biochemistry and Molecular Biology
Pennsylvania State University College of Medicine
500 University Drive, H171
Hershey, PA 17033

* Corresponding author:

Telephone: 717.285.3495
Telefax: 717.285.5571
Email: ms1217@messiah.edu

Key Words: two-hybrid, TATA binding protein

Abstract

In eukaryotes the TATA binding protein (TBP) binds DNA at a TATA box promoter, recruits RNA polymerase II, and initiates transcription. *In vitro* studies have shown that transcription initiation may be regulated by self-association and oligomerization of TBP. The carboxy-terminal domain of TBP binds the DNA and is the largest part of the molecule, while the small amino terminal domain has an unknown function. We have used an *Escherichia Coli* two-hybrid system to study if the two domains of TBP exhibit any self-association *in vivo*. The pBT bait plasmid encoded the full length bacteriophage λcI protein for fusion with the inserted TBP gene, and the pTRG target plasmid encoded the amino terminal domain of an RNA polymerase II subunit and a linker region for protein fusion with the insert. Cells co-transformed with the two plasmids grew only if there was interaction between the TBP subunits. Such interaction allowed the λcI-TBP fusion protein to recruit the RNA polymerase-TBP fusion protein, initiating transcription of a carbenicillin resistance reporter gene and permitting cell growth. Using this system we found significant interaction between full-length TBP's.

Introduction

The TATA binding protein (TBP) is central to transcription in eukaryotes. In many eukaryotic genes, this protein binds DNA at a TATA box promoter element. Other basal transcription factors then join, helping to initiate transcription, and lastly RNA polymerase II is recruited. When polymerase leaves the promoter, transcription of the gene begins.

A TATA box consists of a specific sequence of nucleotides about 30 base pairs (bp) upstream from the transcription start point of a gene. The TATA box is a consensus sequence; it can vary between different genes, but nevertheless will be recognized by TBP [1]. Often this sequence is TATAAAA or TATATAA, but sometimes it will begin with a C, and the other bases can also vary. Though not quite all eukaryotic genes have a TATA box, TBP is still (almost certainly) involved in their transcription [2, 3]. TBP use is not confined to eukaryotes; organisms in the archaebacterial domain also have a TBP [3]. It is interesting to note that, although eubacteria do not use TBP, many of their genes have a similar consensus sequence (TATAAT) about 10 bp upstream of the transcription start point [2].

The TBP is part of the general transcription factor D involved with class II promoters (TFIID). Also included in TFIID are approximately 14 other TBP-associated factors, also known as transcriptional activating factors (TAF_{II}s) [3]. TBP can bind to DNA by itself and produce a basal level of transcription [4]. However, for protein activators bound to distal DNA enhancer elements to affect the formation of the pre-initiation complex (PIC), the TAF_{II}s must be available [5]. Then specific TAF_{II}s will bind to the gene's initiator site and downstream promoter elements.

TBP binds to the minor groove, which is unusual [6, 7]. This may be so that it can bend the DNA to more easily bring distant activators to bear on the forming PIC [3]. Binding is accompanied by bending of the DNA of approximately 90 degrees [8]. In this case the DNA bends away from the binding protein. This presents a convex surface to TBP, and allows it to gain fuller access to the minor groove.

The binding mechanism involves the insertion of two phenylalanine residues of TBP between the first two T:A bases at the one side of the TATA box, and two more phenylalanine residues between the last 2 bp [6]. This distorts the DNA, forcing it to partially unwind. In fact, judging by the short interphosphate distances and the helical twist angles (18.5°, about half that of B-form DNA), the TATA box DNA approaches the A-form [6]. Beyond the phenylalanine residues, the DNA returns to the regular B-form.

The minor groove, widened from ~5 Å to ~9 Å, is curved along the bottom of TBP (which looks like a saddle) for about 310 nm. The curved, eight-stranded anti-parallel β-sheet on the broad, under part of the protein saddle provides a large hydrophobic surface for DNA binding [9]. This permits direct interaction between the β-sheet and specific amino acid residues on TBP.

The first step in the formation of the PIC is TBP binding to the unique DNA sequence in the TATA box. This results in "template commitment" [2]. Since TBP binds very stably, with an unusually large number of residues (180) required, its dissociation rate is low and it may stay bound through multiple rounds of transcription [2].

The next general transcription factor to join the DNA-TBP complex is TFIIB. This protein helps to stabilize TBP, and also bridges to the polymerase [10]. The three-subunit TFIIA factor either joins next [8, 11]. It works to activate the PIC by disassociating negative cofactors

[3]. A pre-formed TFIIF-RNA polymerase II complex is probably the next to join. Because TFIIF has already bound to polymerase II, TFIIF helps to ensure that polymerase does not join to a non-specific DNA site.

The structure of TBP has been determined with X-ray crystallography [9]. The protein consists of two dissimilar domains. The carboxy terminal domain is the largest part of the molecule (180 residues). It is the DNA-binding part of the protein, and is the most important domain in the action of the protein *in vivo*. Consistent with this evaluation is the fact that its sequence is very similar across species [2].

The C-terminal domain forms a crystal structure that can be readily analyzed with X-ray crystallography [4, 6, 9]. This domain looks somewhat like a saddle sitting astride DNA. It has two 40% identical repeats of 66-67 amino acids. Between these is a more basic area called the basic repeat [9]. This is the concave part of TBP that binds DNA, and it contains many hydrophobic groups. The opposite side of the molecule provides a convex surface, with α-helical regions that provide hydrophilic sites for transcription factor binding [12].

The monomer is the form of TBP active in regulating transcription [2, 13]. But TBP forms dimers *in vitro*, and it has been postulated that TBP forms dimers *in vivo*, since the C-terminal portion of TBP readily forms dimers which are easy to study by X-ray crystallography [9]. Dimerization is seen by many as a method of TBP regulation, and may keep TBP from being degraded. There is evidence that TFIIA disrupts TBP dimers [3]. Others have disputed whether dimerization is a significant event *in vivo* [13]. When two C-terminal domains join, their DNA-binding sites bind to each other, and they may be unlikely to readily dissociate in order to bind a TATA box [12].

The amino terminal domain, which may mediate TBP self-association, is relatively small [13]. It varies in size and sequence depending on species and on its cellular source, but is approximately 61 residues long [6]. Its exact function is not known, but the fact that deletions in the N-terminus increase DNA binding activity suggest it may be important in regulation of TBP [2]. It may function by mediating oligomerization of TBP *in vivo* [13]. It is not essential for DNA binding; mutant TBP missing the N-terminal part can still bind DNA and recruit polymerase successfully, though only at a basal level [8]. There is evidence that when it is present, it undergoes some sort of conformation change during DNA binding [14].

The N-terminal domain also appears to modulate the self-association of full-length TBP *in vivo* [13]. Using analytical ultracentrifugation one study detected a TBP monomer-tetramer-octamer equilibrium, even over a range of salt concentrations (60 mM to 1 M) and temperatures (4° to 37° C). In this system the tetrameric species was a relatively minor component, and no dimers at all were observed. This study investigated the whole protein, focusing on the difference in behavior between the C-terminal domain and both domains together. Other comparable studies have used only the C-terminal portion of the protein [13]. It appears, then, that full-length TBP does not form dimers, but rather has a monomer-tetramer-octamer equilibrium.

The mechanism of self-association is not entirely clear, but it is known that the C-terminal core domain is a highly rigid structure. Therefore it is likely that the conformation changes necessary to bring about such a self-association are due to the N-terminal domain [13]. Apparently a tryptophan residue at position 26 of the N-terminal portion of the protein (W26) is crucial in mediating the link. The change from monomer to tetramer is accompanied by a "blue shift" in the emission spectrum of the only tryptophan in the N-terminal domain [13]. This may

indicate a change in the indole chromophore of tryptophan from a hydrophilic environment to a less polar one. The change from tetramer to octamer is accompanied by a "red shift," which may signal an increase in the polarity of the environment of the same tryptophan. The exact protein surfaces involved in these reactions are currently unknown, however.

One way to determine whether proteins are interacting, and possibly to see which protein surfaces are involved in such interactions is to use the two-hybrid system. This system involves using a "cut" transcriptional activator protein [15]. Such a protein contains a DNA binding domain which joins to a specific DNA sequence associated with a gene, and a separate transcriptional activation region, which attracts RNA polymerase to begin transcription. If this activating protein is large and the two functions physically separated, the two domains can be isolated.

The two-hybrid system is commonly used in both *E. coli* and the yeast *Saccharomyces cerevisiae*. Use of *E. coli* produces quicker results, because bacteria have a shorter life cycle than yeast [16]. Yeast is a eukaryote, however, and this can be an advantage. The system can also be used in mammals [17].

Each two-hybrid challenge involves co-transforming *E. coli* with two plasmids. The bait plasmid and the target plasmid each have part of the insert gene cloned into them. If the protein products of these two plasmids interact, they will allow a reporter gene to be activated, so the cells will survive. Since the level of expression of the reporter gene is related to the strength of the interaction between the bait and target proteins, and thus how many cell survive, the binding strength between them can be estimated [15,18].

The first step of the two hybrid system is to construct gene fusions. With the BacterioMatch™ two-hybrid vector kit from Stratagene™ this involves cloning the TBP insert

into special plasmids, a bait and a target. The pBT bait plasmid contains the gene for the DNA-binding bacteriophage λcI protein under control of the *lacUV5* promoter next to the insert DNA (Figure 1) [18]. Whatever DNA insert this plasmid contains and expresses (in this case part of TBP) will be a fusion protein with the λcI protein connected to it.

The pTRG target plasmid has a gene for the amino-terminal domain of the RNA polymerase α subunit next to the inserted TBP DNA (Figure 2) [18]. This α subunit, 250 amino acids long, will be part of *E. coli* RNA polymerase; thus whatever insert this plasmid contains and expresses will be bonded to RNA polymerase [19]. The plasmid also contains a tetracycline resistance gene as a selectable marker.

The *E. coli* that Stratagene™ designed for these experiments contain an F' episome with an amp^r gene, and are also engineered to lack restriction repair systems [18]. Thus when they plated on media containing carbenicillin, the only cells that should survive are cells co-transformed with two plasmids containing inserts that associate with each other, allowing RNA polymerase to be recruited to transcribe the amp^r gene. However, because the reporter strain has a background level of ampicillin resistance due to basal transcription of the reporter cassette, plates serving as negative controls can show some growth. This background growth can be distinguished from positive interaction of the inserts by observing if the growth on the challenge plates is significantly higher than the background growth on control plates.

Materials and Methods

Cloning inserts. Full-length yeast TBP was obtained on a pKA9 plasmid from the Frank Pugh lab at the Penn State University Park campus. Using the appropriate primers (Table 1) the N-terminal and C-terminal domains were separately cloned by PCR. Each was then ligated into both the pTRG and pBT vectors [20]. In the pBT plasmid, the *XhoI* and *EcoRI* restriction sites were used, and in the pTRG plasmid *XhoI* and *BamHI* were used. Following the protocol, the ligation was run overnight in a PCR machine, using 1.4 μL of buffer, 6 μL of distilled water, 0.5 μL of plasmid, 2.0 μL of DNA, and 1 μL of T4 DNA polymerase. These vectors were then transformed into two-hybrid bacteria from Stratagene™ which were derived from XL1-Blue MRF' and lack all restriction systems [18].

Confirming Expression. A Western blot was done to confirm that TBP was being expressed in the cells, according to the Perkin Elmer™ protocol for the Western Lightning™ Chemiluminescence Reagent [21]. The gels were 12% acrylamide, and run in a Hoefer miniVE vertical gel system at 25 volts for 1.25 hours. Non-fat milk was used to block the non-specific binding sites on the nitrocellulose membrane. Pierce Clear Blue X-ray CL-XPosure™ film was used to detect the 428 nm chemiluminescence, with an exposure time of 10 minutes for each 5 by 7 inch film.

Minipreps. Plasmid DNA was isolated from the bacteria using the Promega Wizard® *Plus* mini-prep system from bacterial cultures grown overnight in a 30°C air shaker. PCR was done on this DNA to remove the inserts, using primers from Table 1. The PCR results were run on a

0.8% agarose DNA gel, using a Hoefer HE 33 DNA minigel system with 1x TAE buffer [20]. The DNA was then sent to a core facility at the medical center for verification sequencing to confirm that no mutations had occurred. The gels were photographed with a Stratagene™ Eagle Eye II digital camera using Eagle_Sight software (version 3.22).

Two-hybrid challenge. The absorbance of DNA for transformations was measured at 260 nm using an Hitachi U-2000 spectrophotometer, and from this the DNA concentration was calculated, using the equation $[(A_{260}*50*101)/1000]$ to figure the concentration in μg/mL (50 is the dilution factor from the miniprep, and 101 is the dilution factor used in the spectrophotometer). Luria broth (2500 mL) was prepared using 12.5 g yeast extract, 25.0 g bactotryptone, 12.5 g NaCl, and 62.5 g agarose. The media was autoclaved for 10 minutes and four antibiotics added (250 μg/mL carbenicillin, 15 μg/mL tetracycline prepared in 50% EtOH, 34 μg/mL chloramphenicol prepared in 100% EtOH, and 50 μg/mL kanamycin. All antibiotics were filter-sterilized. In addition, half the media had 5 μM IPTG added to increase expression of the *lacUV5* promoter and thus the protein inserts. Then 25 mL of media was poured into each of 82 sterile, polystyrene, 100 x 15 mm petri dishes from VWR.

Co-transformation. Competent cells were prepared and stored at -80° C [20]. Three milliliters of these competent cells were thawed on ice, and 100 μL aliquoted to each of 25 1.5 mL eppendorf tubes. Then 5 μg each of two plasmids were added to each tube according to Table 2. Tubes 1-4 and 6-8 were done in triplicate (eg. 1a, 1b, 1c). The cells with plasmid added were incubated for 30 minutes on ice, heat-shocked for 1 minute and 40 seconds in a 42 °C water bath, and returned to ice for 10 minutes. Then 900 μL of NZY broth [18] was added before the

bacteria were transferred to 25 mL Falcon tubes and incubated in an air shaker (30° C, 200 rpm) for 1.5 hours. 100 μL of each cell suspension was plated. Bacteria from each tube were plated on four plates, two with CTCK media and two with CTCK-IPTG media. Thus each challenge and each control involved twelve plates; six with and six without IPTG (bacteria from the "1a" tube was plated on two CTCK plates and two CTCK-IPTG plates). Such duplication was used to allow averaging of colony counts to minimize the effects of random variations in growth. The plates were incubated for 40-45 hours in a LAB-LINE IMPERIAL II air incubator at 30° C before the colonies were counted.

Results

Verifying ligation procedure. The TBP inserts were inserted into the vectors with restriction endonucleases. Figure 3 is a DNA gel showing results of PCR verifying that full-length TBP was successfully inserted into the pBT plasmid. As mentioned in the legend, Figure 3 also contains five clones of the N-terminal domain of TBP, which demonstrate that the N-terminal domain is much smaller than the full-length TBP. Figure 4 is a similar gel, but of TBP in the pTRG plasmid. This figure also contains five clones of core TBP (the C-terminal domain). Though these proteins were not used in the present experiments, they serve to show that the C-terminal domain is only a little smaller than the full-length TBP.

Western Blotting. Completing Western blots confirmed that TBP was not only inserted into the target vectors but also expressed from them *in vivo*. Figure 5 shows the Western blot result for the pBT plasmid. Both TBP 3 and TBP 5 show expression. Both these inserts are also present in and expressed from the pTRG plasmid, as shown by the Western blot in Figure 6.

Oligomerization of TBP. Only two two-hybrid challenges were successfully completed for this paper. Table 2 reports the results. Here the first successful challenge is shown in red print and the second in blue. In addition, Figures 7 and 8 graph represent the colony counts of the challenge plates and the most significant negative controls. In Figure 7, it is clear that the challenge plates show more growth than most of the control plates. This means that on these plates the Amp^r gene is being more actively transcribed than on the control plates. This increase in transcription must be caused by interaction between the full-length TBP's. Figure 8 is more

equivocal. Here two of the challenge plates (pBT T5: pTRG T5 and pBT T3 : pTRG T5) show hardly any more growth than the controls. The other two challenges (pBT T3 : pTRG T3 and pBT T5 : pTRG T3) show significantly more growth than any of the controls, however.

Discussion

Though this paper reports the results of only two two-hybrid challenges, a total of four challenges with full-length TBP were attempted. The first experiment failed for unknown reasons. The second was successful, and its results are reported here. The third was an attempt to repeat the second but was flawed, probably because the competent cells were not kept consistently chilled. The results of a fourth attempt are ambiguous (Figure 8). What is clear is that further repetition is necessary to perfect and standardize the procedure, and to confirm positive interaction between the TBP's.

In Figure 7 the pBT T3: pTRG control has higher colony counts than some of the challenge plates. It may be that this is not really a negative control at all. There may be some interaction between the T3 clone and the RNAP subunit. However, conclusions can hardly be drawn before more experiments are completed.

As is clear from Figures 7 and 8, the negative controls always exhibit some background growth, even though in this *E. coli* strain the F' episome does contain *lacI*^{q} to repress activity of the *lacUV5* promoter that is controlling expression of the bait and target inserts. Some transcription of these inserts is always occurring, however. In addition, basal transcription of the reporter cassette in the *E. coli* strain provides a background level of ampicillin resistance.

As Figures 7 and 8 show, IPTG appeared to not increase the expression of the TBP inserts, even though the inserts were controlled by the *lacUV5* promoter which should be stimulated by IPTG. In fact, it can be seen from the figures that colony growth was usually a little lower on the IPTG plates. It may be that TBP expression was actually being increased, but

the IPTG plates had fewer colonies simply because the increased levels TBP were toxic to the *E. coli*.

In addition to negative controls, a positive control was run with each challenge (see Table 2). This control involved co-transformation with two plasmids known to interact *in vivo* [18]. One plasmid, called pBT-LGF2, contains the dimerization domain of the Gal4 transcriptional activator protein (Figure 9). The other plasmid, pTRG-Gal11P, contains 80 amino acids from a mutant form of the Gal 11 protein (Figure 10). Cells co-transformed with these plasmids should grow on CTCK media because the interacting proteins can readily activate transcription of the Amp^r gene. In these two-hybrid challenges this positive control provided a way to estimate transformation efficiency and effectiveness of technique.

Repeating the experiment is necessary, but the current results do suggest that TBP oligomerization is significant *in vivo*. This knowledge is noteworthy, since TBP is such a ubiquitous protein, fundamental to transcription in all eukaryotes. Studying the self-association of TBP is valuable because oligomerization may be important in transcription regulation. There are between 30,000 and 50,000 TBP molecules in each yeast cell [5]. It is unlikely that all these molecules are needed all the time. TBP molecules joined to themselves would provide a reserve not currently active but available. Oligomerization may also be involved in minimizing surplus DNA binding, protecting TBP from inactivation and degradation, or limiting TBP transport across the nuclear membrane [13].

One model for TBP self-association shows the N-terminal domain mediating the connection (Figure 11). This model is supported by the ultracentrifugation studies mentioned earlier [13]. The most significant thing about these studies is that no dimers were observed; thus this model contains only monomers, tetramers and octamers. If the equilibrium between these

states occurs as is suggested by this model, this mechanism would provide a simple way to provide a preserve of TBP, shielded from degradation but ready to serve quickly if needed.

Future Work

Dr. Claire Adams will repeat identical two-hybrid challenges to confirm the results presented in this paper. In addition, she may perform a pull-down assay as another way to test for interaction between TBP's. This would involve cloning TBP into a vector containing a multiple histidine tag. The histidines on the resulting fusion protein would be attracted to cobalt ions on the inside surface of a chromatography column. If TBP exhibits self-association, other TBP's put through the column should now stick to these TBP's. All TBP's could then be eluted with an imidazole buffer.

A method to further confirm protein interactions using this Stratagene™ bacterial strain involves the β-galactosidase reporter gene [18]. This method can assist in the identification of false positives resulting from DNA mutations while the plasmids are in the bacteria, and can also help to identify the false positives arising from basal transcription of the promoter that confers carbenicillin resistance in the absence of bait and target protein interaction. In these bacteria the β-galactosidase gene is contained in the F' episome downstream of the Amp^r gene. Specially prepared X-gal indicator plates are used for this test. They are prepared with 80 μg/mL X-gal and 0.2 mM of β-galactosidase inhibitor (phenylethyl-β-D-thio galactoside) in addition to the usual CTCK antibiotics. This inhibitor is included to reduce the background color produced by basal transcription. Cells to be tested are streaked on these plates and incubated at 37° C overnight. Negative colonies will be white to cream in color, while light blue to blue colonies indicate positive interactions between bait and target proteins.

Besides testing for interaction between known proteins, the two-hybrid system can be used to determine what protein domains mediate the interaction. It can even be used to identify

specific amino acid residues that produce self-association. This "reverse two-hybrid" system would be a useful tool to confirm the theory that the N-terminal domains of TBP mediate its self-association. The procedure involves first mutating the gene encoding for the protein. PCR under sub-optimal conditions is one way to do this. Then the mutated DNA is re-cloned into a hybrid vector. The mutant vector is co-transformed into a cell along with the wild-type partner hybrid plasmid. If the β-galactosidase reporter gene is being used, screening on X-gal and noting the percentage of white and light blue colonies provides an estimate of the effectiveness of the mutagenesis.

Acknowledgements

I wish to thank Dr. Michael Fried for allowing me to work in his lab, Dr. Claire Adams for patiently explaining procedures and answering questions and Dr. Lawrence Mylin for encouraging me to get involved with research at Hershey Medical Center.

Table Legends

Table 1. Primer sequences along with their functions. All sequences are written 5' to 3'. The forward primers cover the multiple cloning site and extend over the TBP insert.

Table 2. Aliquoting plasmids for co-transformation. Five micrograms each of two different plasmids were aliquoted to three identical tubes containing competent cells (1a, 1b, 1c). The four challenge plates are simply different combinations of the two TBP clones. One positive control is used, and the negative controls serve to monitor background growth. Control # 11 has no plasmid added to the competent cells, and should provide no colony growth. This table also shows the colony counts for the two successful two-hybrid experiments. The first is in red print, and is also shown in graphical form in Figure 7. The second is in blue print, and is the basis of Figure 8.

NAME	FUNCTION	SEQUENCE
pBT 034	pBT Forward Sequencing Primer	TCC GTT GTG GGG AAA GTT ATC
pTRG 029	pTRG Forward Sequencing Primer	CAG CCT GAA GTG AAA GAA
pBT 035	Forward PCR primer	TGA CTG AAT TCG GGT GGT GGT GGT AGC GGT GGT GGT GGT AGC GGT GGT GGT GGT AGC ATG GCC GAT GAG GAA CGT TTA AAG GA
	Reverse PCR primer for both pBT and pTRG	GAA GAC TCG AGC ATT TTT CTA AAT TCA CTT AG

Table 1
M. Sauder

	Plasmid Combination	CTC K	CTCK-IPTG	CTC K	CTCK-IPTG
Challenges					
1	pBT T5 : pTRG T5	256	219	145	123
2	pBT T3 : pTRG T3	304	236	309	281
3	pBT T5 : pTRG T3	186	188	405	392
4	pBT T3 : pTRG T5	156	159	85	79
+ Control					
5	pBT LGF2 : pTRG Gal11	400	427	570	590
- Controls					
6	pBT : pTRG	78	50	75	66
7	pBT T3 : pTRG	202	111	151	130
8	pBT : pTRG T5	66	35	111	102
9	pBT LGF2 : pTRG	125	44	38	45
10	pBT : pTRG GAL11	170	54	196	177
11	No Plasmid	0	0	0	0

Table 2
M. Sauder

Figure Legends

Figure 1. This map of the Stratagene™ pBT bait plasmid shows the chloramphenicol resistance selectable marker, the p15A origin, and the *lacUV5* promoter next to the lambda-cI DNA-binding protein and the multiple cloning site where the TBP domains were inserted.

Figure 2. Map of the Stratagene™ pTRG target plasmid showing the gene for tetracycline resistance which acts as a selectable marker, the ColE1 origin, and the *lac* promoter near the RNAP-alpha subunit.

Figure 3. An agarose gel showing TBP inserts removed from the pBT plasmid by PCR. (a) The GeneRuler™ 100 bp DNA Ladder Plus, with fragment sizes shown in bp. (b) Five clones of the N-terminal domain of TBP. (c) Three clones of full-length TBP. (d) Primer dimers show up on all the lanes.

Figure 4. An agarose gel showing TBP inserts removed from the pTRG plasmid by PCR. (a) The GeneRuler™ 100 bp DNA Ladder Plus. (b-f) Five clones of full-length TBP, 1 through 5 respectively. (g) Five clones of core TBP.

Figure 5. Western blot of full-length TBP expressed from the pBT plasmid. The indicated band on lanes d and f show expression of the 54 kD protein. These lanes contain the TBP clones numbered 3 & 5, which clones can also be seen on the DNA gel in Figure 3. The top band, common to all lanes, and the other unknown bands are background protein due to the fact that we

used a non-monoclonal antibody for the blot. The marker is a prestained, broad range, premixed protein marker from New England Biolabs, and its fragments sizes are indicated in kilo-Daltons on the left of the figure.

Figure 6. Western blot of full-length TBP expressed from the pTRG plasmid. All the lanes show TBP expression (54 kD). Lanes a & b both contain TBP 3 and lane c has TBP 5. The faints bands and the streaks are unknown background proteins present because we did not use a monoclonal antibody. The marker is the same as in Figure 5.

Figure 7. This chart of colony growth on both CTCK and CTCK-IPTG media graphs the results of the challenges and the most significant negative controls of one of the two-hybrid experiments (see Table 2). Each column represents six identical plates, with their colony counts averaged.

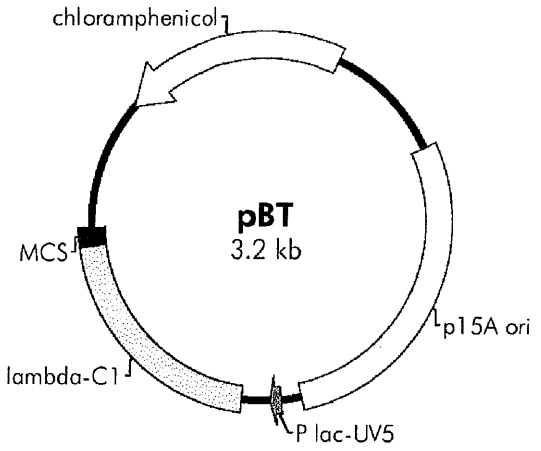
Figure 8. This figure reports the results of the second successful two-hybrid challenge. As in Figure 7, each column represents six identical plates.

Figure 9. This map of the Stratagene™ pBT-LGF2 plasmid shows the chloramphenicol resistance selectable marker, the p15A origin, and the LGF2 protein next to the lambda-cI DNA-binding protein.

Figure 10. This map of the Stratagene™ pTRG-Gal11P plasmid shows the tetracycline resistance gene, the ColE1 origin, and the Gal11 protein next to the RNAP-alpha subunit.

Figure 11. One model of TBP oligomerization. On the left is a TBP monomer with the N-terminal domain shown as a black circle. This monomer is in equilibrium with a tetramer form, which is in equilibrium with an octamer form. The self-association is mediated by the N-terminal domains.

chloramphenicol resistance ORF 2770–219
p15A origin of replication 581–1493
***lac-UV5* promoter** 1556–1586
λC1 ORF 1671–2341
multiple cloning site 2342–2394



pBT Multiple Cloning Site Region
(sequence shown 2327–2394)

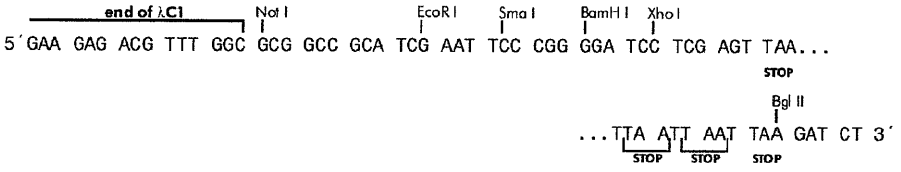


Figure 1
M. Sauder

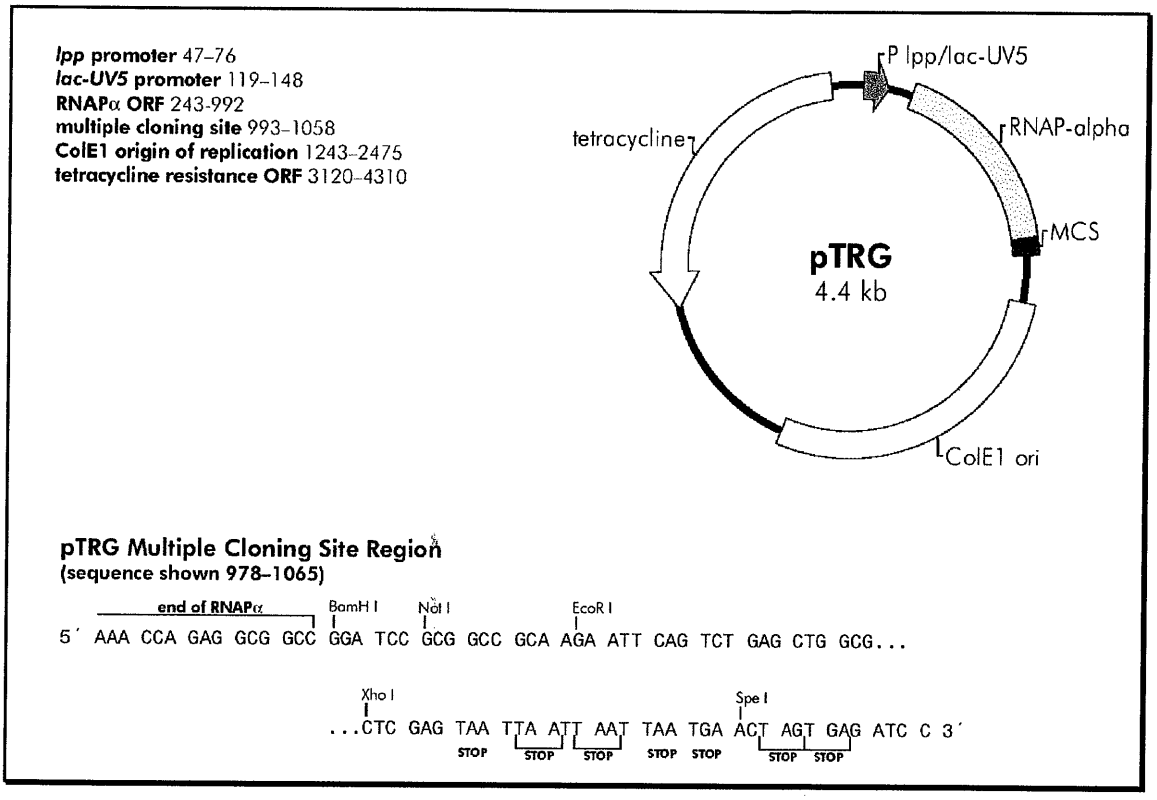


Figure 2
M. Sauder

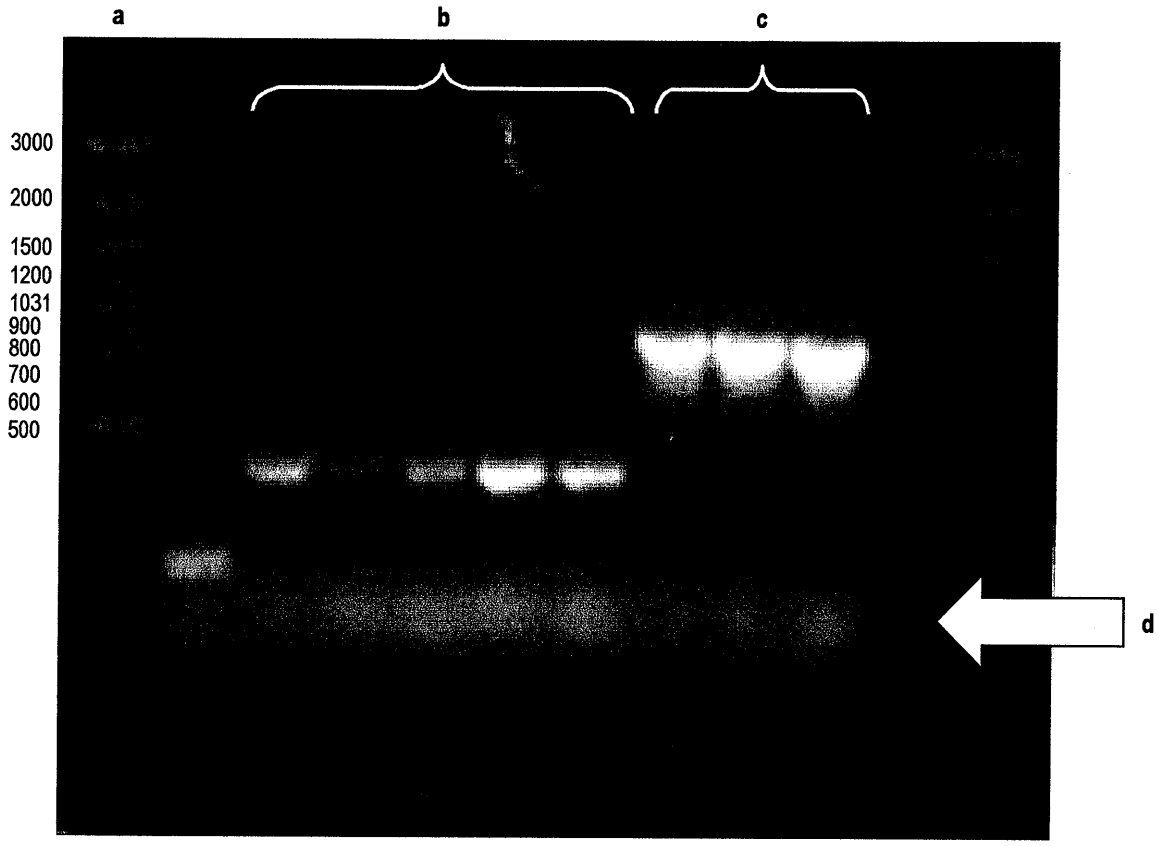


Figure 3
M. Sauder

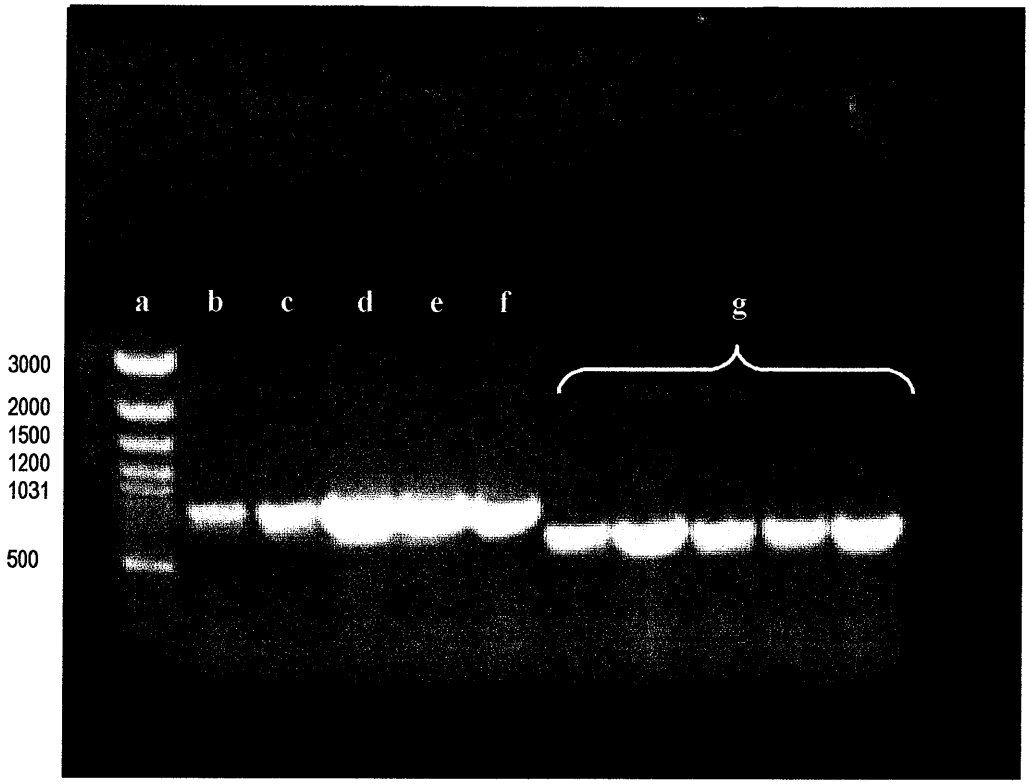


Figure 4
M. Sauder

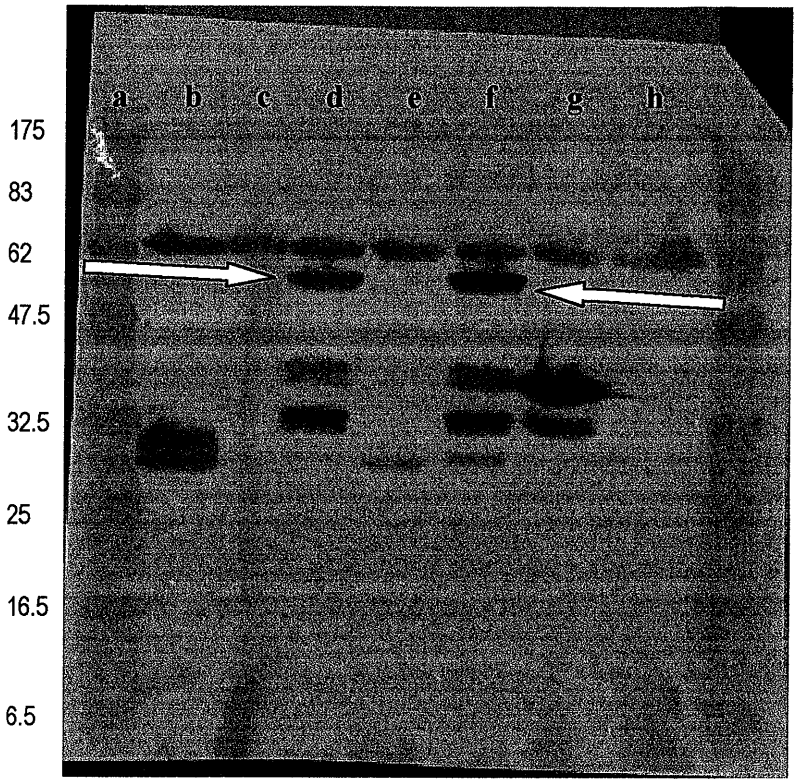


Figure 5
M. Sauder

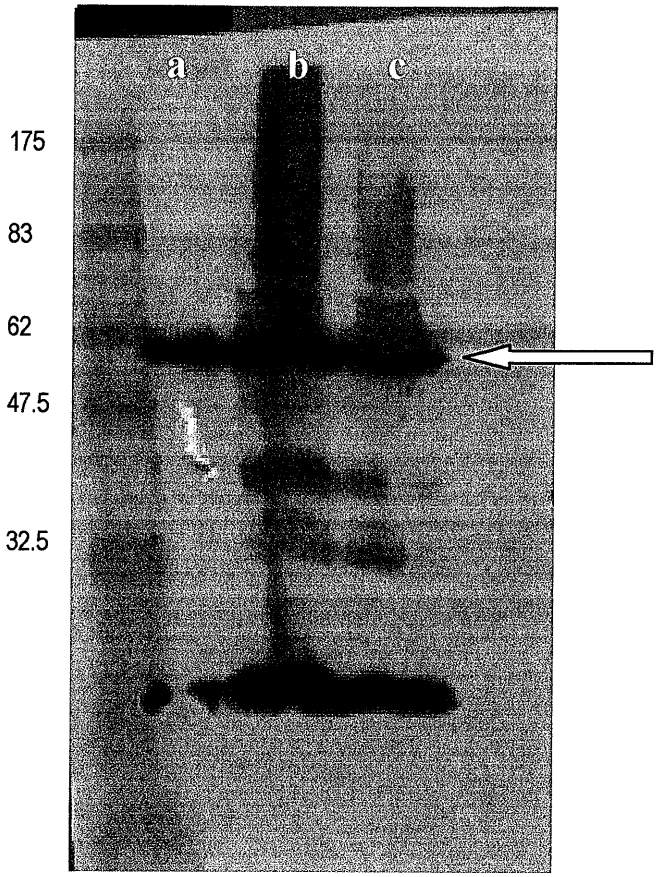


Figure 6
M. Sauder

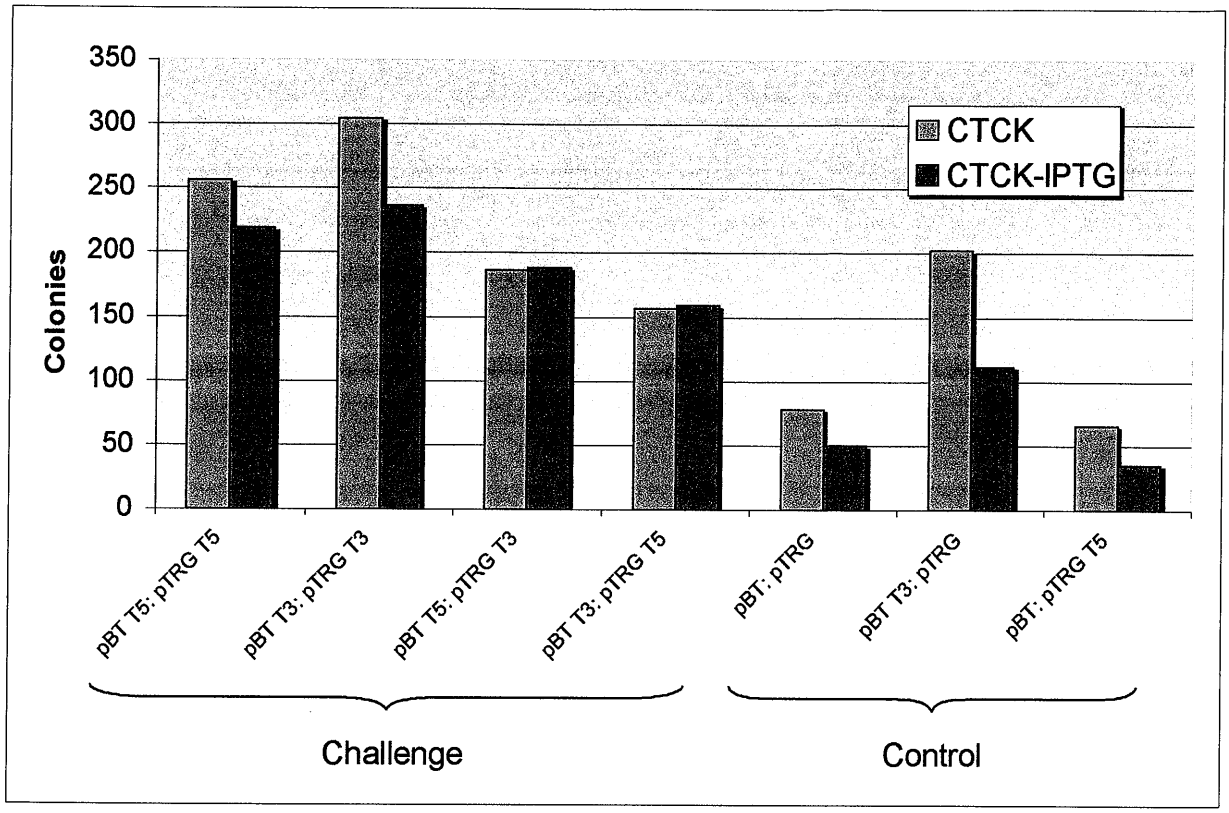


Figure 7
M. Sauder

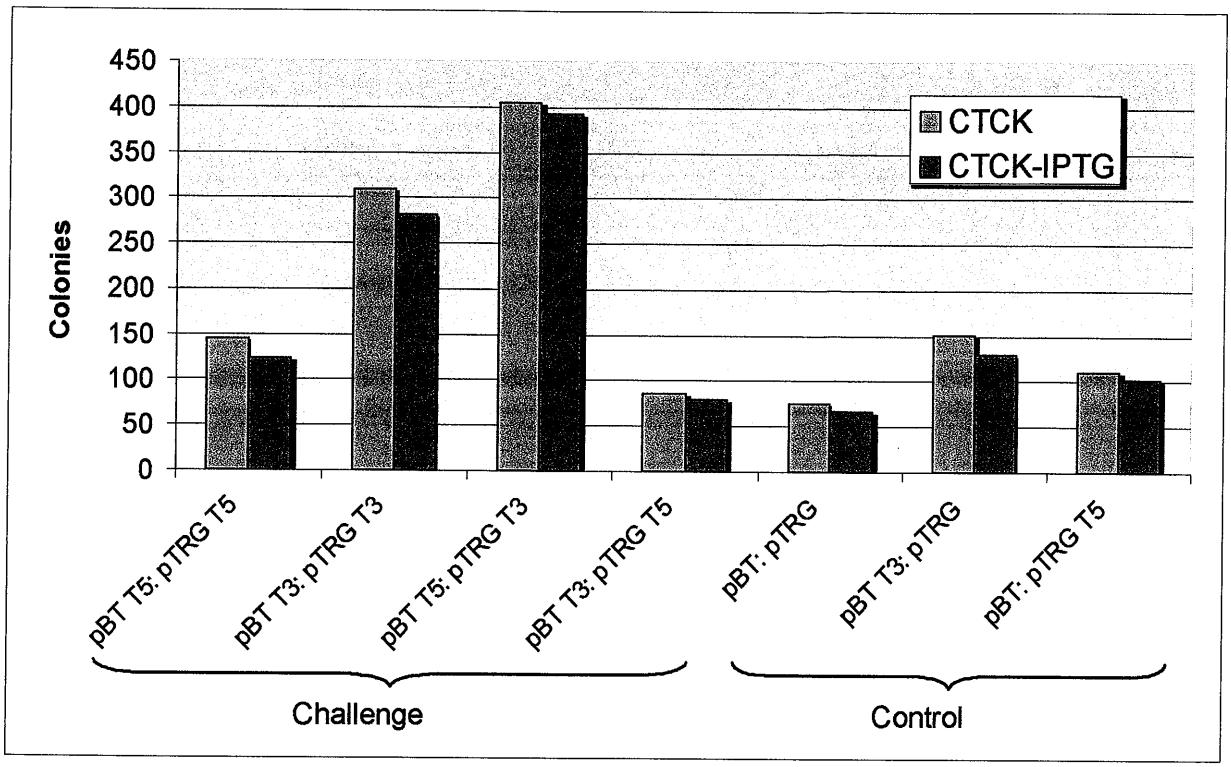


Figure 8
M. Sauder

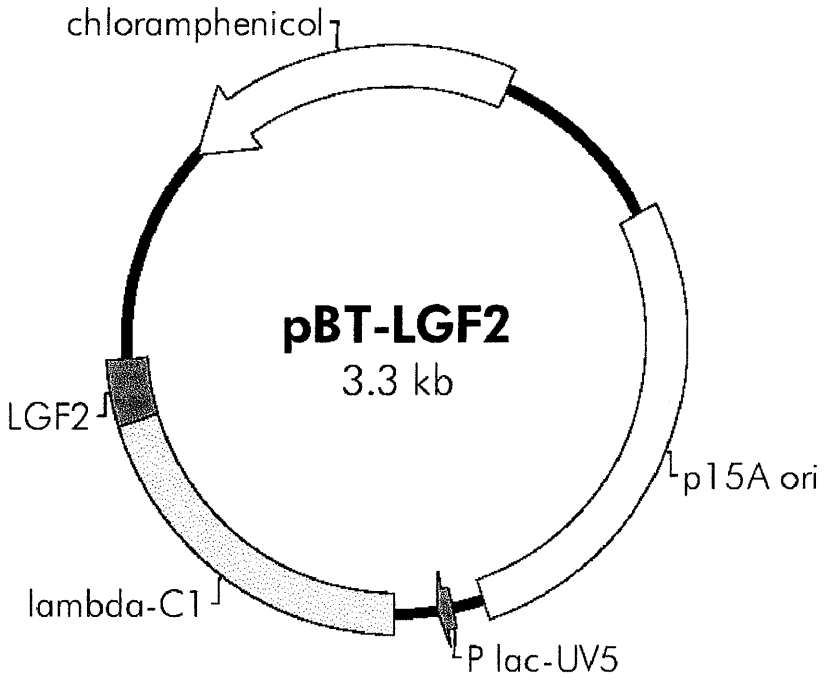


Figure 9
M. Sauder

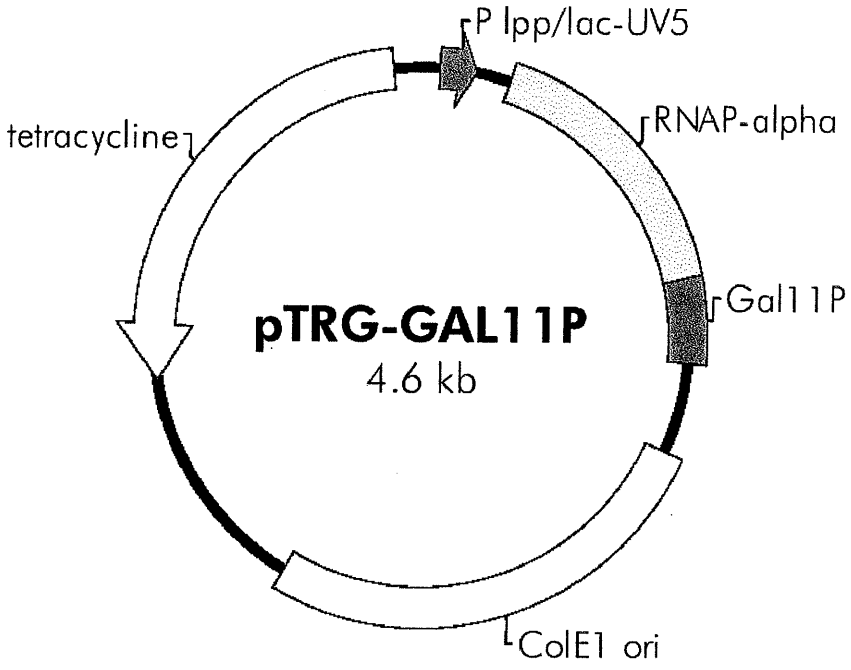
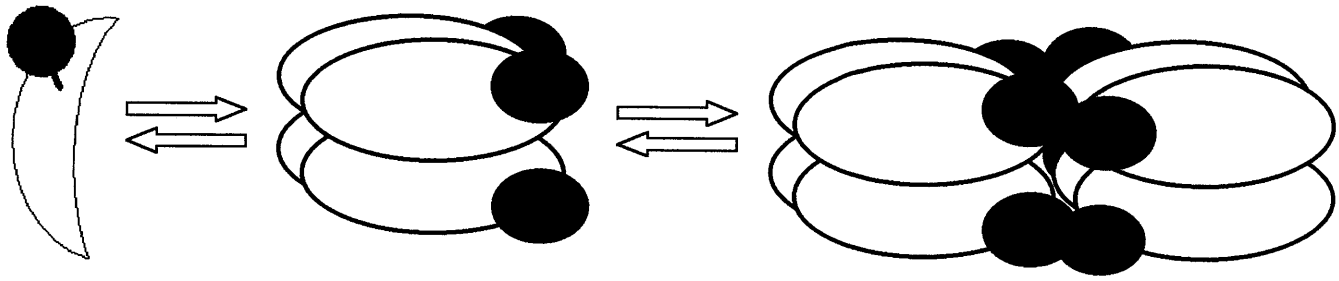


Figure 10
M. Sauder



8 TBP Monomers 2 TBP Tetramers 1 TBP Octamer

Figure 11
M. Sauder

References

[1] **Hahn, S., S. Buratowski, P. A. Sharp, and L. Guarante.** 1989. Yeast TATA-binding protein TFIID binds to TATA elements with both consensus and nonconsensus DNA sequences. Proc. Natl. Acad. Sci. USA. **86:**5718-5722.

[2] **Horikoshi, M., T. Yamamoto, Y. Ohkuma, P. A. Weil, and R. G. Roeder.** 1990. Analysis of structure-function relationships of yeast TATA box binding factor TFIID. Cell. **61:**1171-1178.

[3] **Pugh, F. B.** 2000. Control of gene expression through regulation of the TATA-binding protein. Gene. **255:**1-14.

[4] **Kim, J. L., D. B. Nikolov, and S. K. Burley.** 1993. Co-crystal structure of TBP recognizing the minor groove of a TATA element. Nature. **365:**520-527.

[5] **Lee, T. I., and R. A. Young.** 1998. Regulation of gene expression by TBP-associated proteins. Genes & Dev. **12:**1398-1408.

[6] **Kim, Y., J. H. Geiger, S. Hahn, and P. B. Sigler.** 1993. Crystal Structure of a yeast TBP/TATA-box complex. Nature. **365:**512-520.

[7] **Burley, S. K., and R. Roedor.** 1996. Biochemistry and structural biology of transcription factor IID (TFIID). Annu. Rev. Biochem. **65:**769-799.

[8] **Roeder, R. 1996.** The role of general initiation factors in transcription by RNA polymerase II. TIBS. **21:**327-335.

[9] **Nikolov, D. B., S. Hu, J. Lin, A. Gasch, et al.** 1992. Crystal structure of TFIID TATA-box binding protein. Nature. **360:**40-46.

[10] **Kornberg, R.** 1996. RNA polymerase II transcription control. TIBS. **21:**325-326.

[11] **Tan, S., Y. Hunziker, D. F. Sargent, and T. J. Richmond.** 1996. Crystal structure of a yeast TFIIA/TBP/DNA complex. Nature. **381:**127-134.

[12] **Daugherty, M. A., M Brenowitz, and M. G. Fried.** 1999. The TATA-binding protein from *Saccharomyces cerrevisiae* oligomerizes in solution at micromolar concentrations to form tetramers and octamers. J. Mol. Biol. **285:**1389-1399.

[13] **Daugherty, M. A., M Brenowitz, and M. G. Fried.** 2000. Participation of the Amino-Terminal Domain in the Self-Association of the Full-Length Yeast TATA Binding Protein. Biochemistry. **39:**4869-4880.

[14] **Lieberman, P. M., M. C. Schmidt, C. C. Kao, and A. J. Berk.** 1991. Two distinct domains in the yeast transcription factor IID and evidence for a TATA box-induced conformation change. Mol. Cell. Biol. **11:**63-74.

[15] **Fields, S.** 1993. The two-hybrid sytem to detect protein-protein interactions. METHODS: A companion to methods in enzymology. **5:**116-124.

[16] **Hopper, J.** 2001. The yeast two-hybrid toolbox. Class Handout.

[17] **Vidal, M., P Braun, E Chen, J. D. Boeke, and E. Harlow.** 1996. Genetic characterization of a mammalian protein-protein interaction domain by using a yeast reverse two-hybrid system. Proc. Natl. Acad. Sci. USA. **93:**10321-10326.

[18] Stratagene. 2001. BacterioMatch Two-Hybrid System Vector Kit Instruction Manual.

[19] **Dove, S. L., J. K. Joung, and A. Hochschild.** 1997. Conversion of the subunit of *Escherichia coli* RNA polymerase into a transcriptional activator or an activation target. Genes Dev. **12:**745-754.

[20] **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Labororatory Press. 2nd ed. Cold Spring Harbor, NY.

[21] Perkin Elmer™ Western Lightning™ Chemiluminescence Protocol.