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DISSERTATION

ANALYSIS OF PROMOTER-TRANSCRIPTION FACTOR INTERACTIONS BY SITE-SPECIFIC PHOTO-CROSS-LINKING

Submitted by

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In partial fulfillment of the requirements for

The degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Summer 2002

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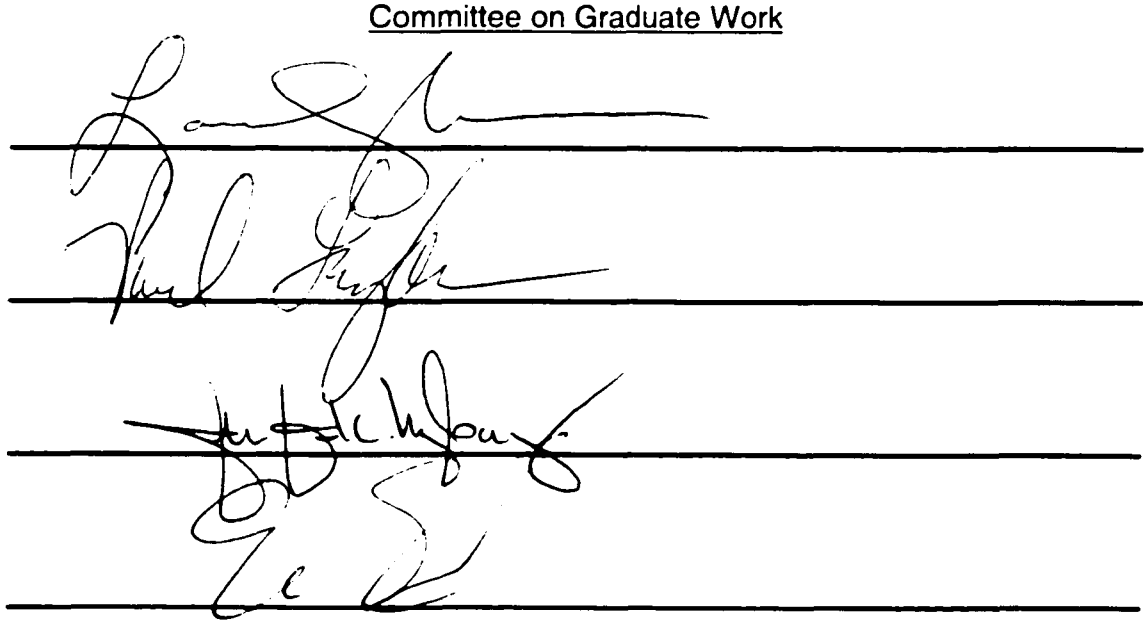

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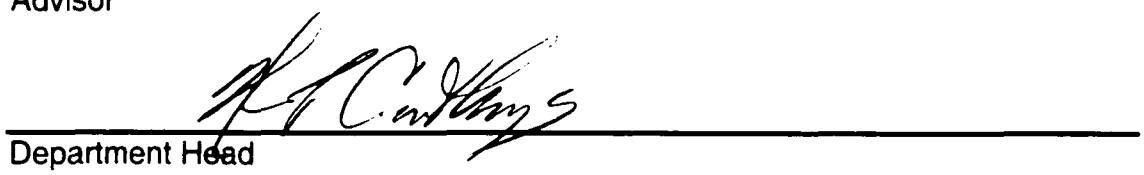

Department Head

ABSTRACT OF THE DISSERTATION

ANALYSIS OF PROMOTER-TRANSCRIPTION FACTOR INTERACTIONS BY SITE-SPECIFIC PHOTO-CROSS-LINKING

Cells depend upon the proper functioning of proteins for cell cycle regulation and controlled cell growth, factors whose malfunction contributes to many human diseases. Transcription of RNA is carried out by DNA-dependent RNA polymerases I, II, and III. Transcription of ribosomal RNA (rRNA) by RNA polymerase (pol) I from DNA is a key point of regulation in the synthesis of ribosomes, the translational machinery for the production of proteins. Ribosomes are composed of rRNA and proteins, the structural and the functional components, respectively. rRNA comprises 75% of the entire RNA in the cell; its transcription is highly active.

Our experimental organism is *Acanthamoeba castellanii*, a free-living amoeba. In *A. castellanii*, Pol I transcribes the genes which form the 35S rRNA preribosomal transcript. Pol I is recruited to the DNA by protein-protein interactions with Transcription Initiation Factor IB (TIF-IB) to form the initiation complex. TIF-IB has been shown by MPE footprinting to protect the DNA from -17 to -67 relative to the transcription initiation site, or *tis* (+1). The *A. castellanii* subunits have been identified as TATA-binding protein (TBP), which is 32kDa in size, and four TBP-associated factors (TAF_Is), having relative molecular masses of 145kDa, 99kDa, 96kDa, 91kDa.

Proteins can be photo-cross-linked to DNA by site-specific derivatization with an aryl-azido group, which is activated to a nitrene upon exposure to UV

light. This results in a covalent cross-link of promoter DNA to the proximal protein subunit. TIF-IB subunits have been partially mapped by photo-cross-linking in the initiation complex before promoter clearance by Pol I by Xiaoliang Gong, a former member of the Paule lab. Using a new, site-specific method, we have completed the cross-linking of TIF-IB in the regions where it had not been done, as well as cross-link Pol I on the promoter in the preinitiation and elongation complexes. The results are similar to cross-linking results with RNA polymerase III, but differ from cross-linking done with RNA polymerase II.

We have also worked in collaboration with Mary Robinson in Laurie Stargell's lab. Mary found enhanced DNA-protein interactions with certain combinations of factors, including TBP, TFIIA and $TAF_{II}40$. We have set out to map these factors, as well as TFIIB and miniTFIIA, on a yeast promoter. TFIIA and TFIIB cross-link specifically on the promoter, whereas TBP and miniTFIIA do not. We have also observed that the addition of the $TAF_{II}40/TAF_{II}19$ heterodimer enhances cross-linking of TFIIB. Also, the $TAF_{II}40/TAF_{II}19$ heterodimer has a strong affinity for DNA, while $TAF_{II}40$ does not cross-link to the DNA.

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I would first like to thank my advisor, Dr. Marvin Paule. Marv has been an amazing mentor. I came into his lab with little experience or knowledge of biochemistry. He taught me to think like a scientist, advised me well but let me work independently. He supported me in times of darkness (literally) and gave me the confidence to work through times of failure. Thanks, Marv!

I would also like to thank Dr. Jenny Nyborg, Dr. Laurie Stargell, Dr. Paul Laybourn, Dr. Norm Curthoys, Dr. Christine Wilcox for serving as my committee members and for taking the time out to guide me. Your advice has been invaluable. I would also like to express my appreciation to Dr. Erica Suchman for stepping in when I needed a new committee member at the last minute.

I am especially thankful to Laurie Stargell and Mary Robinson for a fun collaboration and a different way of thinking. It will serve me well in the future.

I've enjoyed working with everyone in the Paule lab these past years, especially Dr. Cathy Radebaugh, who has been a constant help to me. Thanks to Nick Polakowski, Anna al-Khouri, Joe Gogain, Scott Fields and everyone else for being friends and sometimes roommates.

Most of all, I'd like to thank my family: my husband Dave and my son Rok for being so sweet, my parents for always being so supportive, and my sister Eva for getting me interested in science.

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CHAPTER 1

BACKGROUND AND SIGNIFICANCE

1.1 INTRODUCTION

Eukaryotic transcription is carried out by three distinct DNA-dependent RNA polymerases. This document describes experiments carried out using the *Acanthamoeba castellanii* ribosomal RNA promoter with RNA polymerase (pol) I and its transcription factors, and the yeast *Saccharomyces cerevisiae HIS3* promoter with selected transcription factors. Pol I experiments are discussed in relation to previous results with pol II (Kim et al., 1997) and pol III (Geiduschek and Kassavetis, 2001 and references therein), as well as with respect to pol I transcription systems in other organisms. Therefore, it is beneficial to include a brief background on each of the polymerases and their transcription factors, when relevant, with emphasis on the factors used in the current experiments.

1.2 COMPOSITION OF EUKARYOTIC RNA POLYMERASES I, II AND III

The three eukaryotic DNA-dependent RNA polymerases each have between 10 and 14 subunits (Table 1)(Sentenac, 1985). Since they have

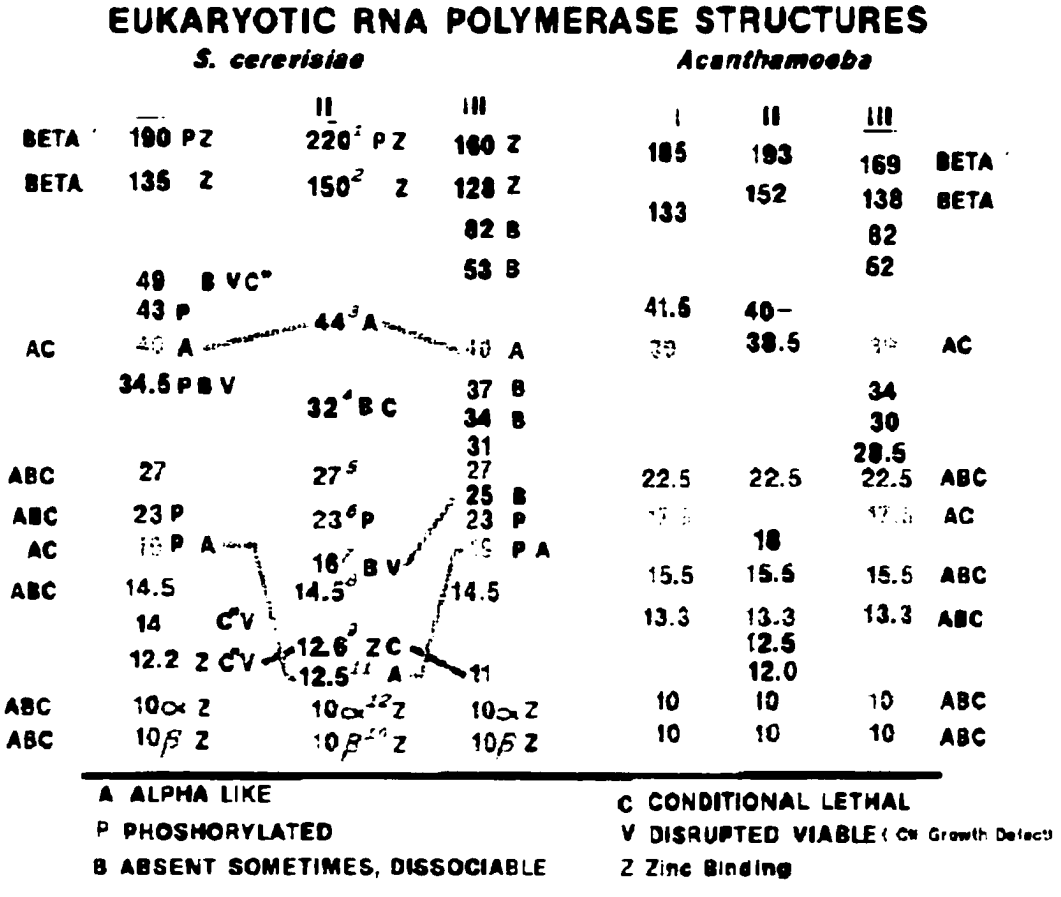


Table 1. Comparison of the eukaryotic polymerase subunits in *Acanthamoeba castellanii* and *Saccharomyces cerevisiae*. The superscripts for yeast pol II indicate an alternate designation. For example, 220 is Rpb1, 150 is Rpb2, etc.

many homologous subunits, we would expect them to be fairly similar in size, shape, and structural organization. Of the three, yeast RNA pol II is the only one whose crystal structure has been solved (Cramer et al., 2000; Cramer et al., 2001; Gnatt et al., 2001). In yeast, all three polymerases have been well characterized. There are five common subunits, ABC27, ABC23, ABC14.5, 10α and 10β. In *Acanthamoeba*, these subunits are ABC 22.5, ABC 17.5, ABC 13.3, ABC 10α and 10β (D'Alessio et al., 1979). There are two additional subunits common to pol I and pol III, AC40 and AC19, which are AC39 and AC15.5 in *Acanthamoeba*. There are five pol I-specific subunits, but only one, A43, is required for viability.

Besides these subunits, there are a number of others that have a high degree of similarity between the three polymerases, shown in table 1. Most notable among these are the two largest subunits, named beta and beta prime in yeast. The pol I subunits are A190 and A135 in yeast and A185 and A133 in *Acanthamoeba*. We will be comparing the *Acanthamoeba* pol I cross-linking to these subunits with yeast pol II subunits B220 and B150 and yeast pol III subunits C160 and C128.

It is also important to note the similarities between the eukaryotic RNA polymerases and bacterial RNA polymerase. Yeast beta and beta prime correspond to the bacterial subunits with the same names, α^{I} corresponds to AC40 and B44, α^{II} is AC19 and B12.5, ω is ABC23.

From their structural similarities, we would expect the DNA to follow the same path through each polymerase during transcription. This is true to some extent. There are, however, differences between the path that DNA takes once it exits pol II versus its path after it exits polymerases I and III. These differences will be discussed at length in Chapter 3, where we compare biochemical data from pol I and pol III with biochemical (Kim et al., 1997) and structural (Cramer et al., 2001) data from pol II.

1.3 TRANSCRIPTION INITIATION IN THE RNA POLYMERASE II SYSTEM

RNA polymerase II transcribes messenger RNA, which is translated by the ribosomes into proteins. The promoter can be divided into core elements and regulatory elements (reviewed in Hampsey, 1998). The core element includes either the TATA box, an Inr (initiator sequence), or both. The TATA box is the binding site for TATA-binding protein (TBP). The TBP-associated factors ($TAF_{II}s$) and other general transcription factors (GTFs) associate directly or indirectly with TBP to form the preinitiation complex (PIC), which assembles on the core element. The Inr serves as a binding site for TATA-less promoters (Lobo et al., 1992).

1.3.1 THE TATA ELEMENT

The TATA box in yeast contains the consensus sequence TATAAA. TBP binds both consensus and nonconsensus elements (Hahn et al., 1989). Many nonconsensus elements allow for transcription, albeit with diminished activity.

This brings up the possibility that factors other than TBP might play a role in recognition of the TATA element (Li and Sherman, 1991).

The GTFs are those factors defined as being required for accurate transcription initiation by pol II *in vitro*. They include TBP, TFIIA, TFIIB, TFIIE, TFIIF, and TFIIH (Green, 2000). We are interested in TBP, TFIIA and TFIIB, as well as the TBP-associated factors, $TAF_{II}40$ and $TAF_{II}19$.

1.3.2 TBP

TBP is required for initiation by all three RNA polymerases (Hernandez, 1993). It was identified as a subunit of the pol I transcription factor SL1 (Comai et al., 1992) and as part of the pol III factor TFIIIB (Huet and Sentenac, 1992; Kassavetis et al., 1992; Lobo et al., 1992). In many pol II systems, it is part of the TFIID complex, which includes the TBP-associated factors (TAF_{II}s) as well.

TBP is composed of two domains. The highly conserved C-terminus encompasses two thirds of the protein and contains two direct repeats. The N-terminal domain is more divergent, but is still conserved among vertebrate forms of TBP (Mittal and Hernandez, 1997). Yeast TBP is 27 kDa in size and is functionally interchangeable with mammalian TBP in *in vitro* transcription systems (Buratowski et al., 1988; Cavallini et al., 1988; Mittal and Hernandez, 1997). The crystal structure of *Arabidopsis* TBP showed that the protein contains a structure resembling a saddle, with which TBP sits on the DNA (reviewed in Nikolov and Burley, 1997). The crystal structures of both the yeast and

Arabidopsis TBP-TATA complexes showed that the TBP saddle induces a kink where it binds the TATA element, bending the DNA by 80° (Kim et al., 1993; Kim et al., 1993). The outside, convex surface of the saddle is available for interaction with TAF_{II}s and other factors. TBP (shown in blue) is pictured in figure 1.1 along with TFIIB (shown in green).

On most pol II promoters, TBP recognizes the TATA element. In the yeast *S. cerevisiae*, the TATA box is located 40 to 120 bp upstream of the initiation site. On TATA-containing promoters, the TBP-TATA interaction begins the assembly of the entire PIC, which is about 4 MDa in size (Green, 2000). TBP also binds nonconsensus TATA elements (Hahn et al., 1989). The stability of such binding is variable and weaker than that for consensus sequences (Stewart and Stargell, 2001). These findings suggest that factors other than TBP might aid in recognizing different TATA boxes (Li and Sherman, 1991; Stewart and Stargell, 2001). It was also found that activation-defective derivatives of TBP were mutated at positions directly contacting the DNA (Lee and Struhl, 1995). Thus, the TATA-TBP interface is also critical for transcriptional activation.

1.3.3 TFIIB

One of the factors that may also be critical for activation is TFIIB, which is necessary for recruitment of pol II (Buratowski et al., 1989). Yeast TFIIB is a 38kDa protein that interacts directly with TBP and pol II, as well

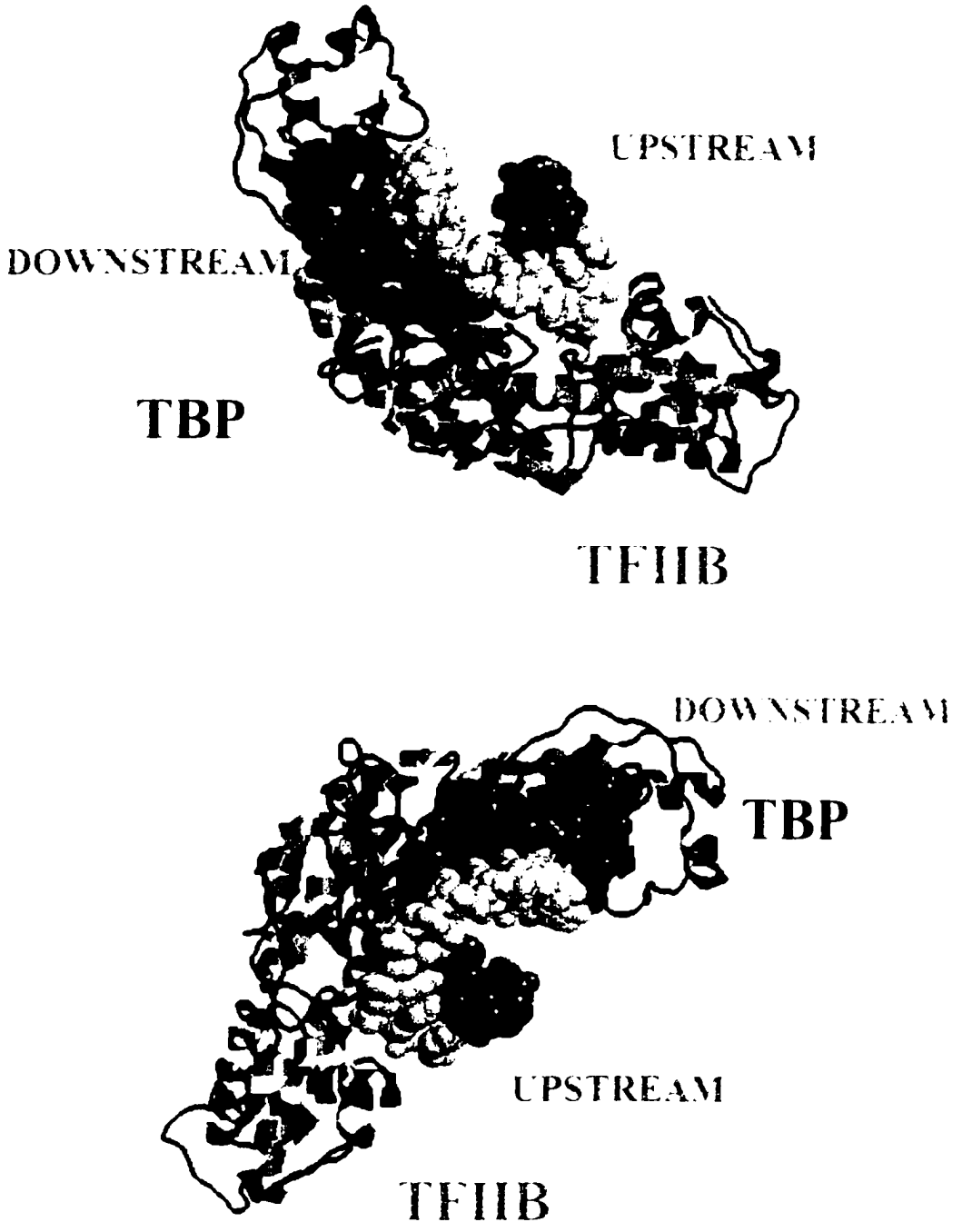


Fig. 1.1 The crystal structure of the TBP-TFIIB-DNA complex. TBP is pictured in green, TFIIB is in cyan, and DNA is in orange and red.

as other factors, including TAF_{II}s of TFIID. TFIIB may also be a direct target of gene-specific transcriptional activators (Lin et al., 1991).

TFIIB contains several structural motifs. The N-terminus contains a zinc-binding domain. This region interacts with the pol II-TFIIF complex. The C-terminus, which contains two imperfect repeats, folds into a protease resistant core that binds TBP (Barberis et al., 1993; Buratowski and Zhou, 1993; Ha et al., 1991). The crystal structure of the TBP-TFIIB-DNA complex (Fig. 1.1) shows that TFIIB contacts both TBP and DNA (Nikolov et al., 1995). The contacts were also demonstrated by footprinting (Lee and Hahn, 1995) and cross-linking experiments (Coulombe et al., 1994; Lagrange et al., 1996).

TFIIB is also important in start site selection. TFIIB is a product of the *SUA7* gene, whose recessive mutations shift transcription start site selection downstream of the normal position (Pinto et al., 1992). The mechanism for start site selection is not well understood, but it appears to involve interaction between TFIIB and the Rpb1 subunit of pol II (Li et al., 1994). Indeed, the downstream start site shift is associated with diminished TFIIB-pol II interaction (Bushnell et al., 1996).

1.3.4 TFIIA

TFIIA is classified as a GTF that is dispensable for basal transcription *in vitro* on a linear template (DeJong et al., 1995), but necessary for transcription *in vivo*, on chromatin templates, or with unpurified pol II. TFIIA interacts with TBP and stabilizes TBP-TATA box

binding (Imbalzano et al., 1994). It also interacts with other transcriptional activators (Ozer et al., 1994; Yokomori et al., 1994) and $TAF_{II}40$ (Kraemer et al., 2001). TFIIA is not required for start site selection (Kang et al., 1995), but it is necessary for the formation of an open promoter complex (Wang et al., 1992).

Yeast TFIIA is composed of two polypeptides of 32 and 13.5 kDa (Wang et al., 1992), designated Toa1 and Toa2, respectively (Wang et al., 1992). Both subunits are required for viability, stressing the importance of TFIIA in the cell.

The crystal structure of the yeast TFIIA-TBP-DNA complex has been solved for two different forms (Geiger et al., 1996; Tan et al., 1996). Tan's structure is pictured in figure 1.2. The structure was determined with the smallest form of TFIIA that remained biologically functional, thus the central region of TOA1 was deleted. TFIIA associates with the side of TBP-DNA opposite that of TFIIB. It is located exclusively upstream of the TATA box.

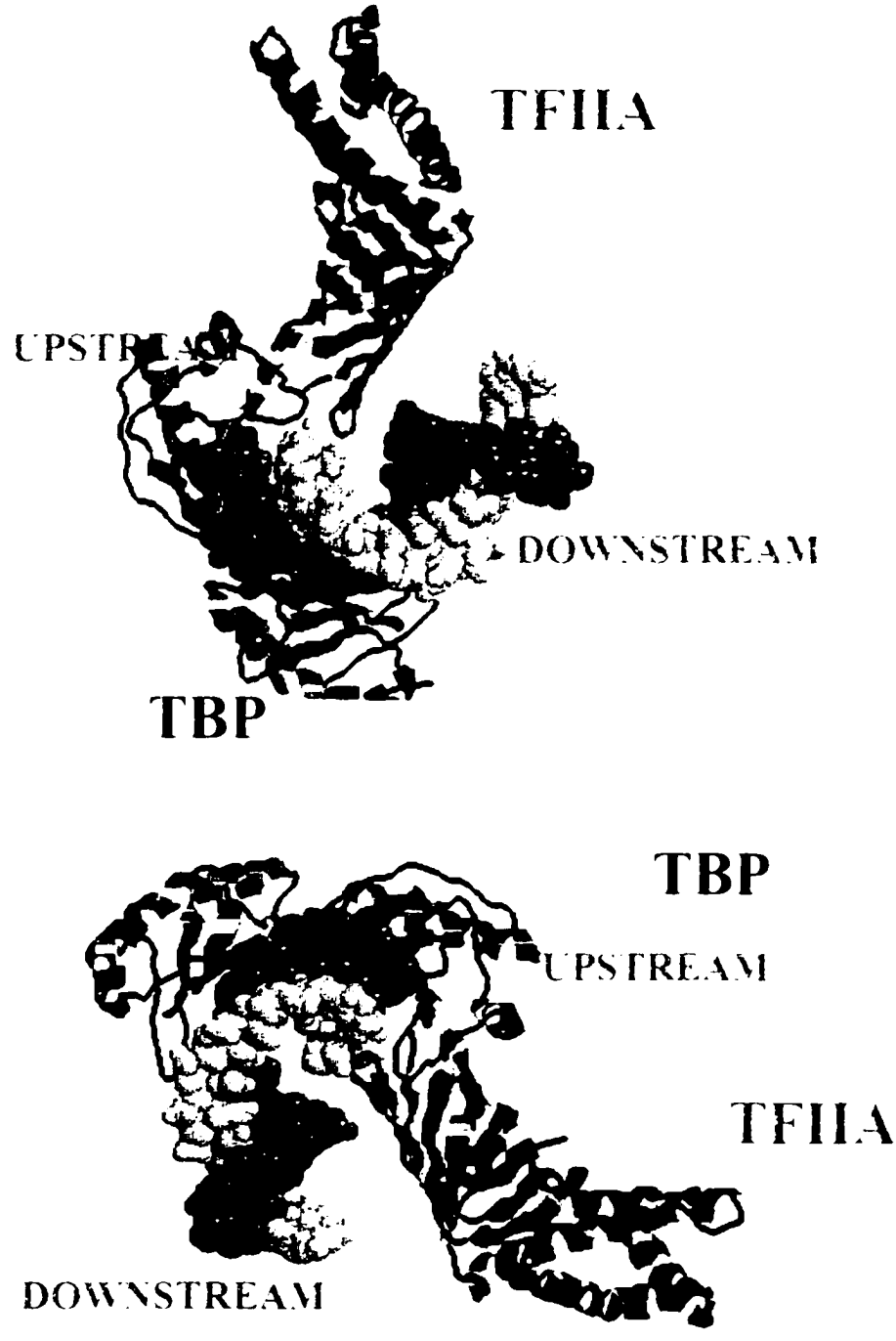


Fig. 1.2 The crystal structure of the TBP-TFIIA-DNA complex. TBP is pictured in green, TFIIA is in blue, and DNA is in orange and red.

1.3.5 TAF_{II} 40 AND TAF_{II} 19

TBP binds a set of TAF_{II}s to form the transcription factor TFIID (Burley and Roeder, 1996), the core factor in pol II transcription. In metazoan systems, TBP alone is sufficient for the nucleation of a functional PIC, but TFIID needs to be part of the complex in order to get activated transcription (Hoffman et al., 1990; Pugh and Tjian, 1990).

Certain TAF_{II}s function by directly contacting activator proteins, whereas others bind GTFs or promoter DNA (Burke and Kadonaga, 1996; Chen et al., 1994; Verrijzer et al., 1995). It has also been noted that TBP may associate with various groups of TAF_{II}s to form complexes distinct from TFIID and whose function is unknown (Timmers et al., 1992).

TAF_{II}40 appears to play an important role in transcription. Mutations in yeast TAF_{II}40 lead to cessation of transcription, implying that TFIID is required at most promoters *in vivo* (Komarnitsky et al., 1999). Yeast TAF_{II}40 has been shown to mediate the interaction between TFIIA and TBP (Kraemer et al., 2001). It was found through mutational analyses of the Toa2 subunit of TFIIA that a disruption of the interaction between TFIIA and TAF_{II}40 results in defects in transcription.

Yeast TAF_{II}19 forms a heterodimer with TAF_{II}40. Their human homologues interact via histone-fold dimerization domains (Birck et al., 1998). TAF_{II}19 by itself is not well characterized, but previous data (Birck

et al., 1998) and data presented in Chapter 4 suggest that it requires its $TAF_{II}40$ partner to be functional.

1.4 TRANSCRIPTION BY RNA POLYMERASE I

Pol I transcribes the majority of the ribosomal RNA, comprising about 75% of the total RNA being actively transcribed in the growing cell. Thus, for energetic reasons, pol I transcription must be tightly regulated. The studies described in this document attempt to illuminate one aspect of Pol I's recruitment to the promoter, the nature of its association with transcription factors and with DNA.

Pol I transcribes almost exclusively class I genes, producing 18S, 5.8S and 28S rRNAs. The RNAs are transcribed as a single polycistronic precursor, which is processed into the mature RNAs (Fig. 1.3). These RNAs, along with the 5S RNA, a pol III product, make up the functional part of the ribosome (reviewed in Paule, 1998). The genes are found in head-to-tail repeats of various numbers, depending upon the organism (reviewed in Long and Dawid, 1980). The *Acanthamoeba* genome has the low copy number of 24 per haploid genome. However, it is a polyploid organism, with N=25, resulting in a total number of 600 rRNA copies per cell (Yang et al., 1994). Animals have copy numbers from 150 to 300, plants have copies in the thousands, and amphibians can have as many as 19,000 copies (reviewed in Paule, 1998).

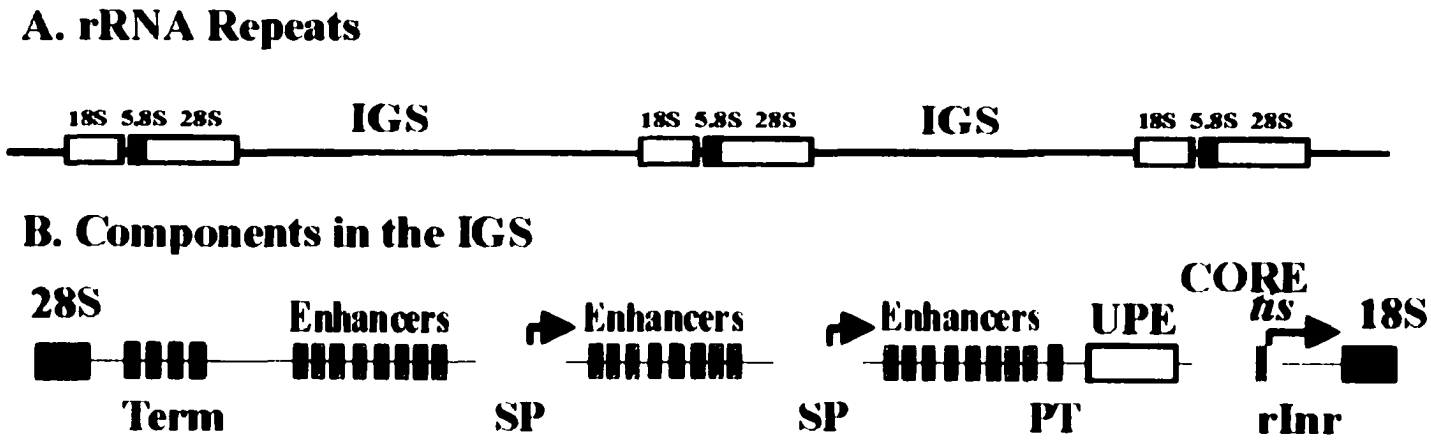


Fig. 1.3 Panel A shows the rRNA gene repeats separated by the intergenic spacer. Panel B shows the transcription elements of a generic eukaryotic promoter.

The main eukaryotic organisms that have been used for studying pol I transcription are *Saccharomyces cerevisiae*, *Acanthamoeba castellanii*, *Arabidopsis*, *Drosophila*, frog, mouse, rat, and human. The transcription factors that have been identified for pol I systems vary from one organism to another.

1.4.1 EUKARYOTIC RIBOSOMAL RNA PROMOTER ELEMENTS

The DNA repeats of the gene clusters mentioned above are separated by intergenic spacers (IGS), which contain all the promoter elements necessary for transcription (Paule, 1998). Although the primary sequences of the promoter are not well conserved among different organisms, there is similarity in how the different promoter elements are arranged. They are located in the same positions relative to the transcription initiation site (*tis*) (Fig. 1.3).

The core promoter is the only element that is required for basal transcription *in vitro* in most organisms. *In vivo*, transcription is extremely weak without other elements. The core promoter encompasses the region from just downstream of the start site up to about –40. It is generally divided into two domains (Iida et al., 1985; Kownin et al., 1985). The only region that is conserved among organisms is the sequence around the *tis*, from about –10 to +10 (Perna et al., 1992). This region is AT-rich, which facilitates melting during initiation. Because it is able to direct accurate transcription on its own in an *in vitro Acanthamoeba* system (Radebaugh et al., 1997), it is similar to the pol II initiator (Inr) element.

The upstream promoter element, or UPE, extends from the core to about –150 relative to *tis*. (Nagamine et al., 1987; Skinner et al., 1984; Windle and Sollner-Webb, 1986; Windle and Sollner-Webb, 1986). The UPE is crucial for activated transcription, and has been identified in all organisms studied except *Acanthamoeba*. In the latter case, basal transcription in so strong *in vitro* that activation may not be detected. Although the distance between the UPE and *tis* varies between organisms, the distance between the UPE and the core promoter in each organism is crucial for activated transcription (Pape et al., 1990; Xie and Rothblum, 1992). These findings suggest that there is functional interaction between the factors that bind the two elements.

The proximal terminator (PT) is a sequence located approximately 200bp upstream of *tis* (Grummt et al., 1986; Henderson and Sollner-Webb, 1986; McStay and Reeder, 1986). It aids in transcriptional termination from upstream transcription units. The PT also stimulates transcription initiation through a number of different mechanisms. One mechanism is dependent on the helical alignment with the UPE and core promoter and requires the trans-acting termination factor (McStay and Reeder, 1990). The PT is also involved in the remodeling of chromatin at the promoter (Langst et al., 1997). Finally, it is believed that the same factor that binds the PT can oligomerize, making it possible to bring together the end of one transcription unit and the beginning of the next,

allowing the polymerase to find its promoter more quickly (Kulkens et al., 1992).

The IGS in some organisms contains functional promoters called spacer promoters (SP) (Cassidy et al., 1987; Moss and Birnstiel, 1979; Tower et al., 1989). The transcription of these elements produces short transcripts terminating at the PT. They may serve to stimulate transcription (De Winter and Moss, 1986; Grimaldi and Di Nocera, 1988), but it is not known it this effect is universal (Labhart and Reeder, 1989; Lucchini and Reeder, 1989).

The IGS also contains ribosomal enhancers, elements that enhance transcription from the core promoter. They can function both upstream and downstream of the promoter and are relatively distance independent (Labhart and Reeder, 1985), but the mechanism by which they stimulate transcription is still poorly understood. They bind one or more of the same factors that bind the promoter proper (Jacob, 1995; Moss and Stefanovsky, 1995).

Termination enhancers, often found in repeats, are located downstream of the coding sequence (Grummt et al., 1985; Labhart and Reeder, 1987; Pfleiderer et al., 1990; Reeder et al., 1987). When bound by a transcription termination factor, these elements halt the polymerase, produce a 3' end to the transcript, and cause the release of the transcript and pol I.

1.4.2 TRANSCRIPTION INITIATION FACTOR IB (TIF-IB)

All organisms have a transcription factor that binds the core element on the pol I promoter and provides minimal basal transcription. This protein is called Transcription Initiation Factor IB (TIF-IB) in *Acanthamoeba* and mouse, Core Factor (CF) in yeast, Rib1 in frog and Selectivity Factor (SL1) in rat and human. TIF-IB in all organisms studied contains TBP and three or four TAF_Is, though in yeast the TBP is weakly bound (Aprikian et al., 2000; Steffan et al., 1996). TIF-IB in Acanthamoeba was shown to recruit polymerase without additional factors *in vitro* (Kownin et al., 1987). TIF-IB in mouse associates with the promoter and, either alone or with TIF-IA and TIF-IC, recruits polymerase to the promoter to form the PIC (Schnapp and Grummt, 1991). TIF-IB in *Acanthamoeba* needs TIF-IE to form a stable complex (Al-Khouri and Paule, 2002) which commits the template and recruits pol I. *Acanthamoeba* TIF-IB is unusual in that it appears to have four TAF_Is rather than the three found in other organisms studied. This finding is, however, coming into question in the present study. SL1 and Core Factor (CF), like TIF-IB, are necessary for transcription initiation. They are, however, much more dependent on activation factors to get efficient levels of transcription. SL1 interacts with Upstream binding Factor (UBF), which binds the DNA just upstream at the UCE. CF interacts with Upstream Activation Factor (UAF), which also binds upstream at a region called the Upstream Promoter Element (UPE), discussed below.

1.4.3 UPSTREAM ACTIVATION FACTOR (UAF)

With the exception of *Acanthamoeba*, pol I systems also require an additional factor necessary for activated transcription. This factor binds a sequence upstream of the core promoter. It is called UAF in yeast and UBF in vertebrates. These two proteins are different in that UBF binds as a homodimer and appears to serve as a DNA architectural factor, looping the DNA, possibly bringing binding sites closer together in order to facilitate transcription. UBF also has activation properties during initiation (Panov et al., 2001) and interacts with both TAF_I48 and the A49/PAF53 subunits of pol I (Hanada et al., 1996). UAF in yeast has a complex subunit makeup consisting of six subunits including histones H3 and H4 (Keener et al., 1997; Keys et al., 1996). The CF-UAF interaction in yeast is different from other systems in that TBP tends to associate more strongly with the activation factor than with CF, apparently bridging between the two on the promoter. It has not yet been tested whether UAF bends the DNA .

Acanthamoeba does have a protein necessary for strong transcription, but it operates in a different manner. TIF-IE stabilizes TIF-IB on the promoter, forming a committed complex that cannot be challenged by another promoter DNA template (Al-Khouri and Paule, 2002; Radebaugh et al., 1998). TIF-IE does not appear to bind the DNA and therefore contacts TIF-IB directly.

1.4.4 TIF-IA/RRN3

Another important factor for all systems is the one that arguably aids in polymerase recruitment to the promoter. This factor is Rrn3p in yeast and human and TIF-IA or C* in mouse. It has yet to be unequivocally identified in *Acanthamoeba*. It is not known exactly how Rrn3p operates. Some studies with human cells indicate that Rrn3p is necessary for polymerase recruitment (Miller et al., 2001), whereas studies with yeast point to Rrn3p being necessary not for recruitment but for a post-recruitment initiation step (Aprikian et al., 2001). Mouse TIF-IA was also not found to be necessary for recruitment, but rather for the formation of the first phosphodiester bonds (Schnapp et al., 1993). Additionally, it is believed that TIF-IA is released after initiation and associates with free pol I to make it an initiation-competent enzyme. Only a small fraction of cellular pol I is found in this complex (Brun et al., 1994; Milkereit and Tschochner, 1998).

1.5 RIBOSOMAL RNA TRANSCRIPTION IN *ACANTHAMOEBA CASTELLANII*

As mentioned, *Acanthamoeba* is unique in that it apparently does not require an activation factor in order to get strong transcription *in vitro*. The basal transcription factor, TIF-IB, recruits pol I to form the PIC. TIF-IB is composed of TBP and four TBP-associated factors, TAF_I145, TAF_I99, TAF_I96, and TAF_I91. The subunits were identified in stained SDS gels (Radebaugh et al., 1998) and confirmed with photo-cross-linking (Gong et al., 1995). However, the validity of

TAF_I99 has come into question in the present study, since it has not been found to cross-link. These data will be discussed in Chapter 3.

TIF-IB binds to the promoter region from –67 to –17 as determined by footprinting assays. Earlier (Gong et al., 1995; Radebaugh et al., 1997) and current photo-cross-linking studies indicate that the DNA region with which TIF-IB comes into contact is even greater, but these sites are not crucial for TIF-IB binding. The promoter can be deleted from the 5' end to -32 and still provide accurate, albeit much weaker, transcription (Iida et al., 1985). Indeed, the promoter can be 5' deleted as far as –6 without complete elimination of specific initiation (Radebaugh et al., 1997). Deletions can also be done from the 3' end up to -19 without compromising the binding of TIF-IB.

It is not known exactly how TIF-IE is recruited to the promoter. TIF-IE is necessary for TIF-IB to be stable on the DNA (Radebaugh et al., 1998), yet it is found in protein purification schemes to be associated with pol I as well as TIF-IB (Al-Khouri and Paule, 2002). TIF-IE does not contact the DNA as determined by footprinting, template order-of-addition experiments (Al-Khouri and Paule, 2002) and photo-cross-linking assays. The TIF-IB/IE complex recruits polymerase to the promoter solely by protein-protein interactions, and remains bound to the DNA after the polymerase has cleared the promoter (Kownin et al., 1987), ready to recruit another pol I molecule (Kahl et al., 2000). We believe that it is TAF_I96 that contacts pol I for several reasons. It is the only subunit whose cross-linking overlaps that of the polymerase (this study). Also, yeast 2-hybrid studies done

with homologous yeast factors (Radebaugh, unpublished) have shown an interaction between Rrn7 of Core Factor and A190 of polymerase. Partial sequencing of TAF_I96 has revealed its homology with Rrn7, underscoring the likelihood of its interaction with pol I. We believe it is this interaction that recruits pol I in yeast when Rrn3p is absent.

The question that remains unanswered is whether an *Acanthamoeba* homologue of Rrn3p is necessary for recruitment of pol I or for a later step in initiation. We have found that there is a protein in purified *Acanthamoeba* pol I which cross-reacts with yeast anti-Rrn3p antibodies, and yeast Rrn3p stimulates transcription in *Acanthamoeba in vitro* systems (Joe Gogain, unpublished results). These data suggest that such a factor does indeed exist, but we do not yet have conclusive evidence.

DNA melting occurs without the addition of ATP, with a 9bp loop forming at the start site (Kahl and Paule, 2001) Once nucleoside triphosphates (NTPs) are added, an RNA transcript is formed, the loop enlarges to 20 bp, with a 9bp DNA-RNA hybrid at steady state (Kahl et al., 2000). Elongation continues as the polymerase clears the promoter, and TIF-IB/IE remain on the DNA, ready to recruit another polymerase molecule. The initiation process is modeled in Fig. 1.4.

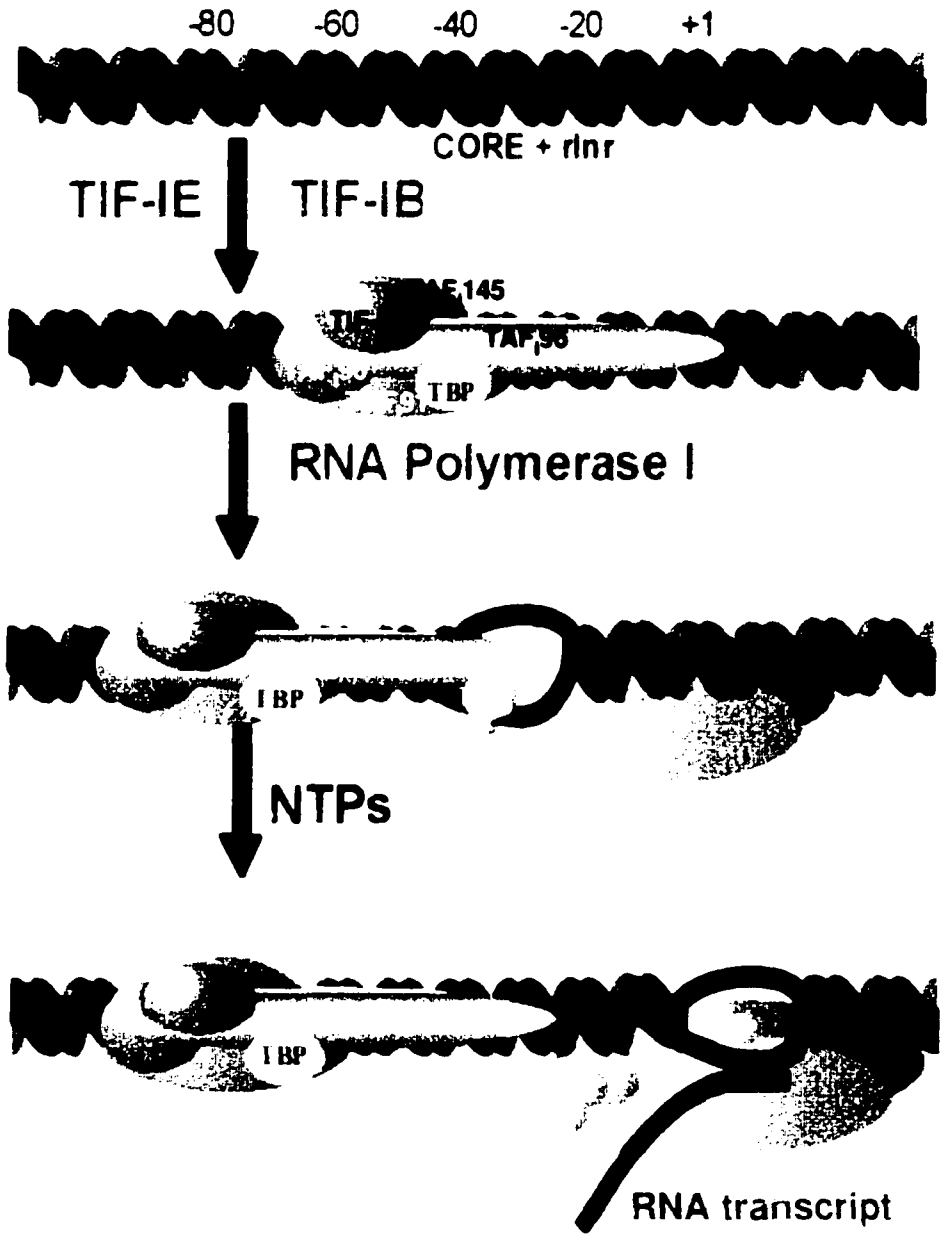


Fig. 1.4 Transcription mechanism in *Acanthamoeba castellanii*

1.6 OBJECTIVES OF DISSERTATION

The *Acanthamoeba* pol I experiments make up the major portion of my dissertation. Interactions between transcription factors are difficult to study in *Acanthamoeba* because of the genes' resistance to cloning. The partial sequencing of TAF_I96, mentioned above, is all we have been able to obtain from the transcription machinery. However, the *Acanthamoeba* system is excellent for doing biochemical experiments. The transcription system is simple, with only two transcription factors plus polymerase necessary for strong transcription *in vitro*. Therefore, we undertook a biochemical approach to finding possible protein interactions by their proximity to one another relative to DNA. We were also particularily interested in pol I-DNA interactions. By utilizing site-specific photo-cross-linking, we have been able to map TIF-IB and RNA polymerase I to the ribosomal RNA promoter region. We have determined that only TAF_I96 cross-links in the same region as the polymerase. We have also compared pol I cross-linking to that done with the other eukaryotic polymerases, and found cross-linking to be more similar to that of pol III than pol II.

The yeast project was done in collaboration with Mary Robinson, in Laurie Stargell's group. Mary was interested in the interactions of TFIIA and TFIIB with the *HIS3* promoter in *S. cerevisiae*. She had also seen effects of TAF40 on TFIIA in an EMSA, and wanted to further characterize this interaction. The cross-linking we undertook included TBP, TFIIA, TFIIB and the heterodimer of $TAF_{II}40$ and

$TAF_{II}19$. Our experiments produced many interesting results involving each of these factors. They will be described in detail in Chapter 4.

CHAPTER 2

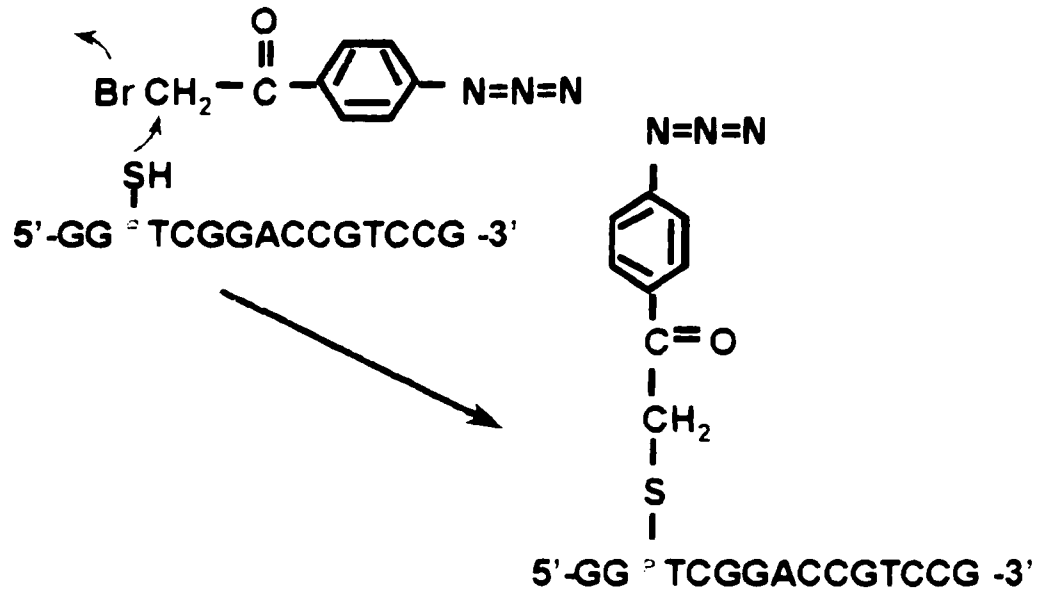
METHODS

2.1 SITE-SPECIFIC PHOTO-CROSS-LINKING

The photo-cross-linking reagent that was used to create a reactive site on the DNA is *para*-azidophenacyl bromide (*p*-apb). *P*-apb can be attached to a known site of a macromolecule, in this case DNA, by nucleophilic displacement at the α-bromo ketone moiety (Figure 2.1 panel A) (Hixson and Hixson, 1975). Subsequent irradiation of the reagent gives a nitrene, which may insert into a protein forming a cross-link (Figure 2.1 panel B).

The advantage of using this reagent is that it can be placed between any two desired bases on the DNA regardless of sequence. Also, the derivative can be found in either DNA groove, allowing for more thorough cross-linking (Fig. 2.2). The disadvantage is that the phenacyl ring can undergo rearrangemant (Tate et al., 1998) (Figure 2.3). This causes the reagent to preferentially react with certain groups, in particular amino groups. The cross-linking may then be stronger at certain sites because of the local composition of the protein rather

Panel A



Panel B

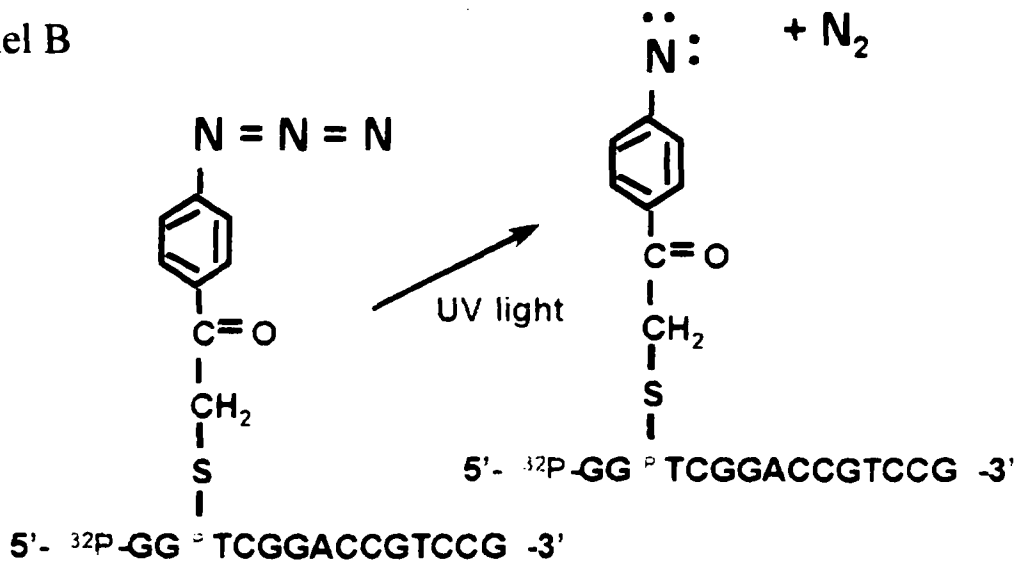


Fig.2.1 Derivatization of the DNA. Panel A shows the reaction between p-apb and a sulfhydryl group on the DNA that results in DNA derivatization. Panel B shows the reaction of the azide group with UV light. A reactive nitrene, which can form a covalent bond with a proximal protein, is formed.

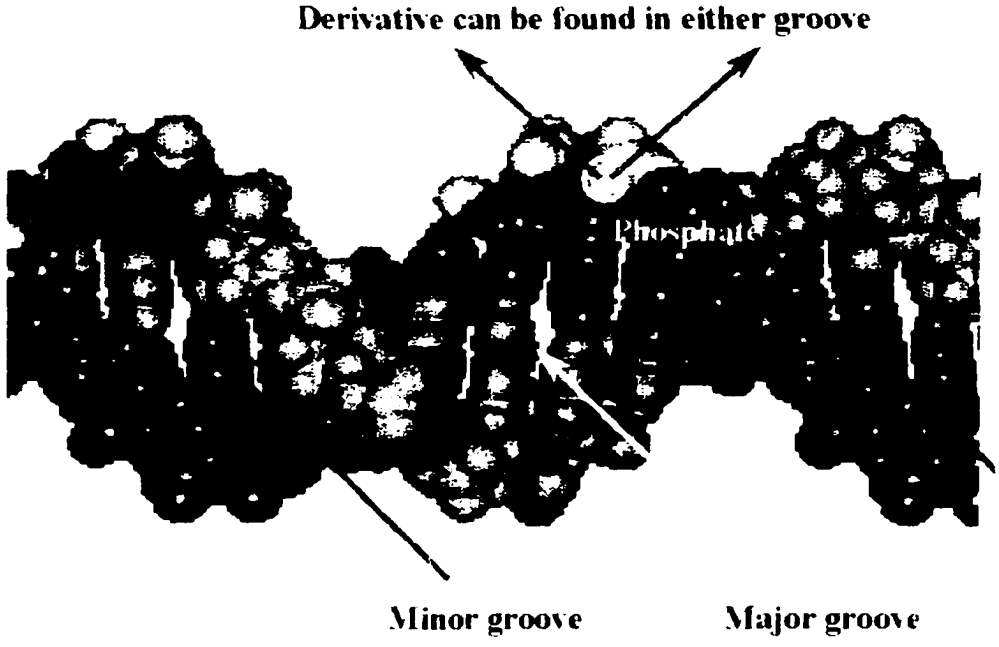


Fig. 2.2 Derivative location on the DNA. The derivative can be found in both the major and the minor groove. Derivatization results in a 50:50 ratio of the diastereomers.

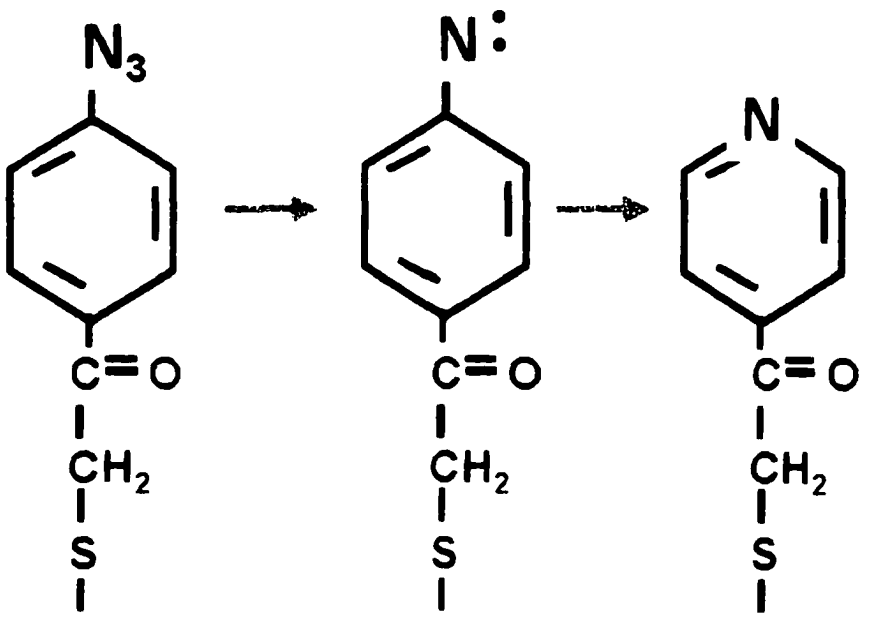


Fig. 2.3 Rearrangement of the phenacyl ring. The azidobenzoyl moiety can rearrange to form a group that is preferentially reactive with amino groups.

than the number of proximal protein molecules or their relative distance to the DNA. Thus, we can not compare strength of cross-linking between different sites, but we can compare changes in cross-linking intensity at the same site with the addition of other proteins. This is demonstrated in the yeast pol II experiments described in Chapter 4.

The following method for making derivatized templates describes the procedure for *Acanthamoeba* rRNA. The method used for yeast pol II templates is simpler, and described in the manuscript mentioned above.

To derivatize DNA, we order primers that contain a sulfhydryl instead of a hydroxyl group on a backbone phosphate of the DNA. Once the DNA is derivatized, all subsequent work must be performed under reduced lighting conditions. Daylight and fluorescent light must be excluded. A single 60W tungsten bulb is permissible (Naryshkin et al., 2001). After attachment of the phenacyl azide, we radiolabel the primer at the 5' end using [γ-^{32}P]-ATP in a reaction with polynucleotide kinase in a 20 minute reaction. The kinase is inactivated by a 5 minute incubation at 65°C. The derivative must not be heated to temperatures higher than 70°C (Naryshkin et al., 2001). Excess radiolabel is purified away on Chroma-spin+TE 10 columns (Clontech), leaving the primer in TE buffer. The primer can be tested for derivatization by running it next to an nonderivatized primer on an 11% polyacrylamide 7M urea gel (Figure 2.4). The gel is run for at least 2 hours at 25 milliamps to achieve satisfactory distinction

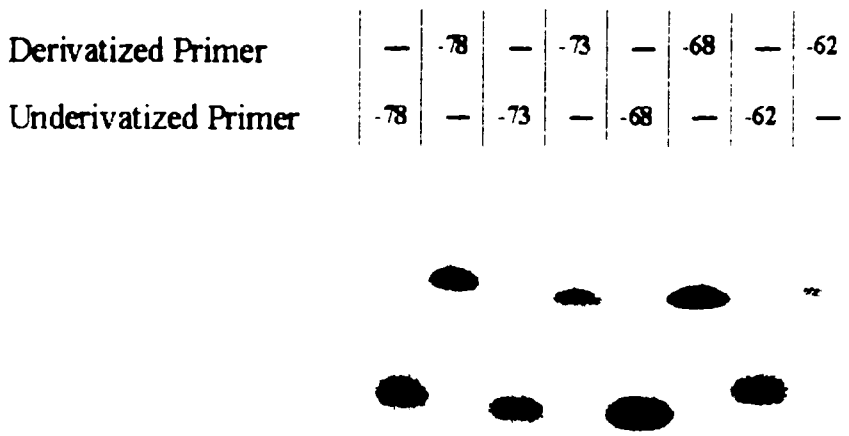


Fig. 2.4 Nonderivatized and derivatized primers electrophoresed on a 7M urea, 11% polyacrylamide gel. Lanes 1, 3, 5 and 7 show nonderivatized primers. Lanes 2, 4, 6 and 8 show decreased mobility of derivatized primers. Close to 100% of each primer is derivatized.

between the two primers. In all our experiments derivatization was close to 100%. The primer is then annealed to single-stranded plasmid DNA (pbs-x (+)) which contains the *Acanthamoeba* rRNA promoter from −120 to +80. In our case, we used only the template strand of DNA and RNA-like primers. The annealing reaction is done by incubating 10pmol each of a labeled, derivatized primer and an upstream primer with 1 pmol single-stranded DNA in a buffer containing 15mM Tris-Cl pH 8.0, 25mM KCl, and 3.5mM $MgCl_2$ for 5 minutes in 150ml water at 65°C and then allowing it to cool to room temperature (about 90 minutes). Primer extension and ligation are done by addition of 3U T4-DNA polymerase and 7.5U T4-DNA ligase. The reaction is incubated for 15 minutes at 25°C and then 35 minutes at 37°C. The reactions are stopped by addition of 10% SDS to a final concentration of 0.25%. The SDS is removed by spin column. The fragment of interest is cut out by restriction digest with BamHI and Hind III. The entire reaction is loaded onto a 5% non-denaturing polyacrylamide gel to separate the fragment of interest (Fig. 2.5). The gel is exposed to a phosphorimage screen for 5 minutes. The posphorimage is aligned with the gel, and the DNA template is excised. Passive elution in buffer containing 10mM Tris-Cl pH 8.0, 200 mM NaCl, and 1mM EDTA takes place overnight at 30°C. The templates are then ethanol precipitated and resuspended in TE. 1μl of each template is counted. For the cross-linking, we use about 5000cpm per reaction. Some of the primers label consistently better than others, and some also appear to anneal better than others. We must assume that each cross-linking reaction contains approximately the same amount (30fmol) of derivatized, labeled template.

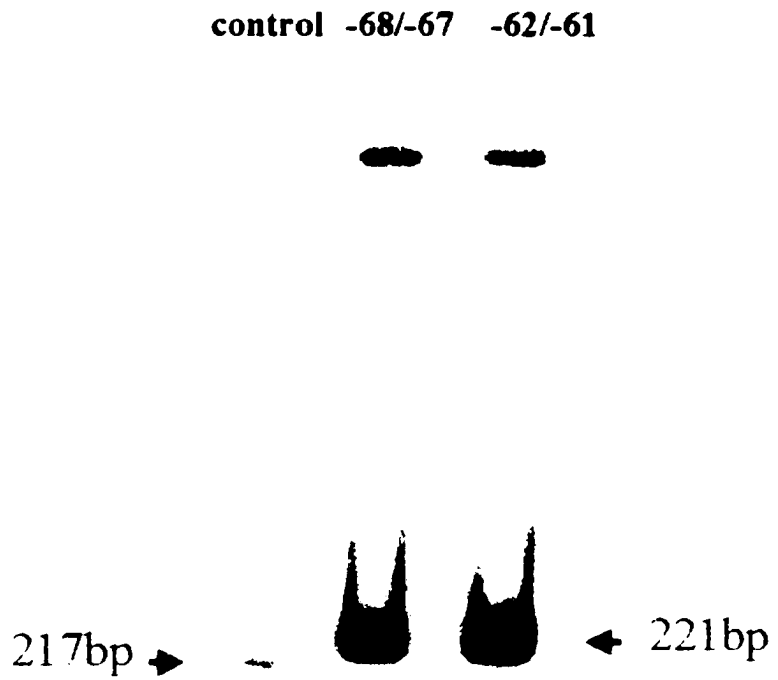


Fig. 2.5 Restriction digests of the filled-in plasmid DNA are electrophoresed on a native 5% polyacrylamide gel in order to separate out the derivatized templates.

The cross-linking for both *Acanthamoeba* and yeast is performed by incubating DNA and proteins under the same conditions as for an electrophoretic mobility shift assay (EMSA). With *Acanthamoeba*, we did EMSAs with about one out of four templates to make sure that the derivative did not interfere with protein binding. EMSAs for yeast were not done with the templates.

Reactions for both systems were assembled and cross-linking was performed as described in the manuscripts. Once the protein and DNA are cross-linked, the DNA is digested and proteins denatured the same way in both *Acanthamoeba* and yeast, also described in the manuscripts. *Acanthamoeba* reactions are run on 7.5% SDS polyacrylamide gels to best separate the TAF_Is and large polymerase I subunits, from 185 to 91kDa, and on 12% gels to see TBP (29kDa) and smaller polymerase subunits. Yeast reactions are run on 16% gels to separate proteins between 27 and 50kDa. Prestained markers have been loaded on the gels. The gels are then dried and exposed to phosphorimage screens for 2 to 3 days. The dried gels are aligned with the phosphorimages of the gels, so that the bands seen can be assigned molecular weights based on the prestained marker electrophoretic mobilities (Gong et al., 1995).

2.2 PROTEIN PURIFICATION

Proteins for the yeast cross-linking were provided by Mary Robinson in the Stargell lab using His-tagged proteins expressed in *E.coli* cells. The exception is the $TAF_{II}40/TAF_{II}19$ heterodimer, which was graciously provided by Song Tan.

Some of the proteins for *Acanthamoeba* cross-linking were kindly provided by members of the Paule lab. Anna Maria al-Khouri provided TIF-IB and TIF-IE, Cathy Radebaugh provided TIF-IB, and Tara Towers provided pol I. Much of the TIF-IB used was also purified by Scott Fields and me as described below.

2.2.1 PURIFICATION OF TIF-IB

TIF-IB was purified from a nuclear extract by the method of Radebaugh *et al.* (1998). The TIF-IB containing fraction was obtained by ammonium sulfate precipitation of the nuclear extract. This fraction was dialyzed to 100mM KCl in HEG10 and loaded onto a DEAE Sepharose Fast Flow (Pharmacia) column and eluted with a gradient of 100mM to 750mM KCl. The TIF-IB containing fractions, as assayed by specific transcription assays, were pooled and further purified on a BioRex 70 (BioRad) column and eluted with a 150mM to 1M KCl linear gradient. The TIF-IB containing fractions were purified on a promoter-DNA sepharose affinity column.

2.3 ALTERNATIVE METHODS FOR MAKING DERIVATIZED TEMPLATES

2.3.1 IMMOBILIZING DNA ON PARAMAGNETIC BEADS

Following is a short description of the methods we initially attempted to use for making derivatized DNA templates. In order to incorporate *p*-apb into DNA to make derivatized templates, it is necessary to anneal a derivatized

primer to single-stranded DNA, and proceed with elongation and ligation reactions as described in Chapter 2. We first attempted to make templates for protein-DNA photo-cross-linking by immobilizing the DNA on Streptavidin paramagnetic beads (Promega). A DNA fragment containing the promoter region is obtained by restriction digest of the pEBH10 plasmid with PvuII, which leaves blunt ends. The fragment is first biotinylated on both ends. One of the biotinylated ends is subsequently cut off by restriction digest with PstI, leaving a 360bp fragment. The DNA is then incubated with the beads in 0.5X SSC buffer to allow adhesion, then washed with 0.1X SSC buffer. To obtain single-stranded DNA, we denature the DNA by adding NaOH to 0.1M at RT, then washing with 0.1X SSC. Primers are annealed and elongated, and DNA is ligated as described.

The advantage of using double stranded DNA immobilized on beads is that the two DNA strands are easy to separate, and each subsequent reaction can be done in a new buffer simply by washing the beads between reactions. We know that we had successful DNA denaturation, primer annealing and elongation. In our control, double stranded DNA was annealed to beads and denatured. We then annealed a cold primer and extended with NTPs that included [^{32}P]-dATP. After washing the DNA on the beads, the majority of the radiolabel was in the bead reaction and not in the wash.

The problem we could not overcome was in cutting the DNA off the beads to do the cross-linking reactions. Although the restriction enzymes we were using for this purpose have sites on the DNA that were about 185bp away from the bead, it is apparently not far enough to allow the enzymes to work. Unfortunately, we could not find other restriction sites for obtaining a longer DNA fragment that would suit our purposes.

2.3.2 SEPARATING DNA STRANDS USING POLYACRYLAMIDE GEL ELECTROPHORESIS

The next method we tried for making DNA templates involved cutting out a shorter, 221bp, promoter fragment from the pEBH10 plasmid. The fragment must have one 3' overhang and one 5' overhang, so that when the strands are separated by gel electrophoresis, one is longer than the other, and therefore has a different mobility. We used BamHI and PstI, so that the template strand is 209 nucleotides and the RNA-like strand is 217 nucleotides in length. A fraction of the fragment is radiolabeled on both ends, so that the two strands are visible on the gel (Fig. 2.6). The fragment is electrophoresed on an 8M urea, 5% polyacrylamide gel, which is exposed to film for 15 minutes. The developed film is then aligned with the gel and the strand of interest, in our case, the template strand, is excised and passively eluted overnight in buffer containing 0.1% SDS, 0.5M ammonium acetate, and 10mM magnesium acetate. The supernatant is

Fig. 2.6 Double stranded DNA fragments electrophoresed under denaturing conditions on an 8M urea, 5% polyacrylamide gel. The template strand (209 bp) is excised from the gel and the single stranded DNA is eluted.

ethanol precipitated and the DNA strand is ready to be used for derivatized templates.

Although this method worked and was used for some initial cross-linking, the yield was very low because of the numerous gel purifications involved. The PEBH10 plasmid has a low copy number, so we tried to increase our yield by starting with more double stranded fragment, as described below.

2.3.3 PCR OF DOUBLE STRANDED DNA PROMOTER FRAGMENT

In order to increase the amount of DNA fragment we were using to obtain single stranded DNA, we used PCR to amplify the fragment. We tested the amplified fragment in an EMSA and were surprised to find that it was not shifted by TIF-IB. After attempting EMSAs with different conditions (Fig. 2.7), including different buffers and polymerases, Honglue Shen in our lab sequenced the PCR products and found they were riddled with sequence errors. At this point, we changed to the method described in Chapter 3.

In our initial cross-linking experiments, we found that polymerase cross-linked at every site tested. In order to make certain we had only specific cross-linking, we did experiments in which we titrated polymerase and competitor in different combinations (Fig. 2.8, 2.9 and 2.10). We found that at a certain concentration of polymerase and competitor, described above, we could get

Template DNA	C	C	A	A	A	A	H	H
dNTPs	-	-	M	M	S	S	M	M
TIF-IB	-	+	-	+	-	+	-	+

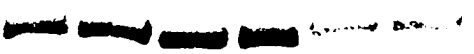


Fig. 2.7 EMSA of rRNA promoter DNA PCR reactions. TIF-IB shifts the control DNA (pEBH10 promoter fragment), but not the DNA from PCR reactions made from the same fragment.
C = Control, A = Anka's PCR reactions, H = Honglue's PCR reactions, M = Masteramp reagents (Epicentre), S = Standard reagents

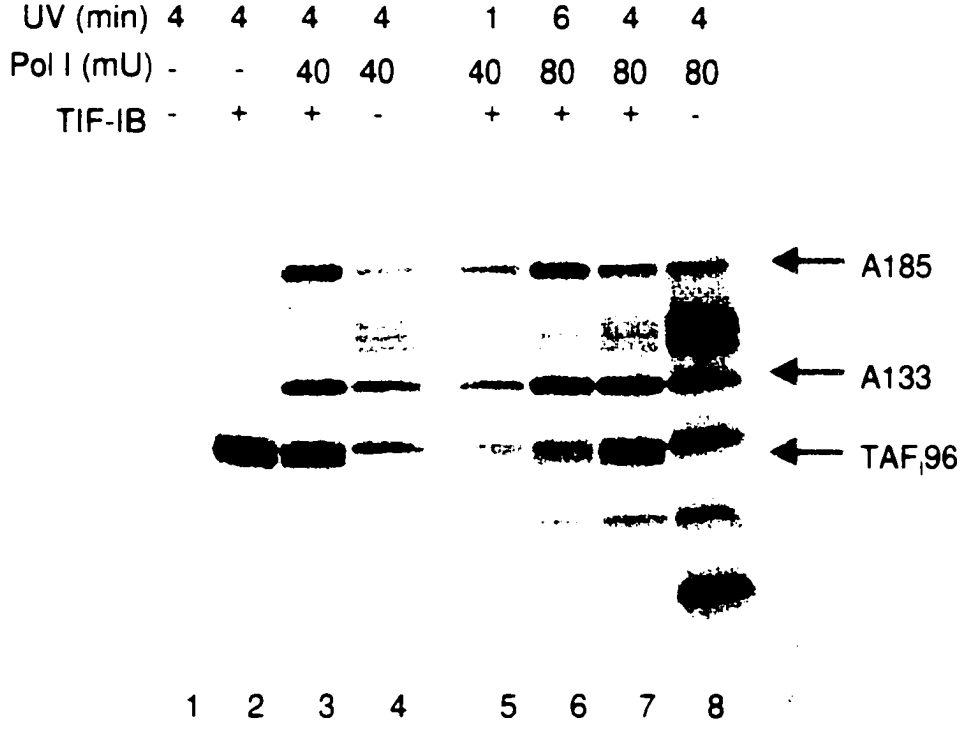


Fig. 2.8 Examining specificity of pol I cross-linking. The arrows point to subunits of TIF-IB (TAF$_I$96) and RNA pol I (A133 and A185) cross-linking at these levels of polymerase. Pol I should only be recruited in the presence of TIF-IB. However, lanes 4 and 8 do not contain TIF-IB, but A133 and A185 still appear. There are also many nonspecific bands in the polymerase cross-linking.

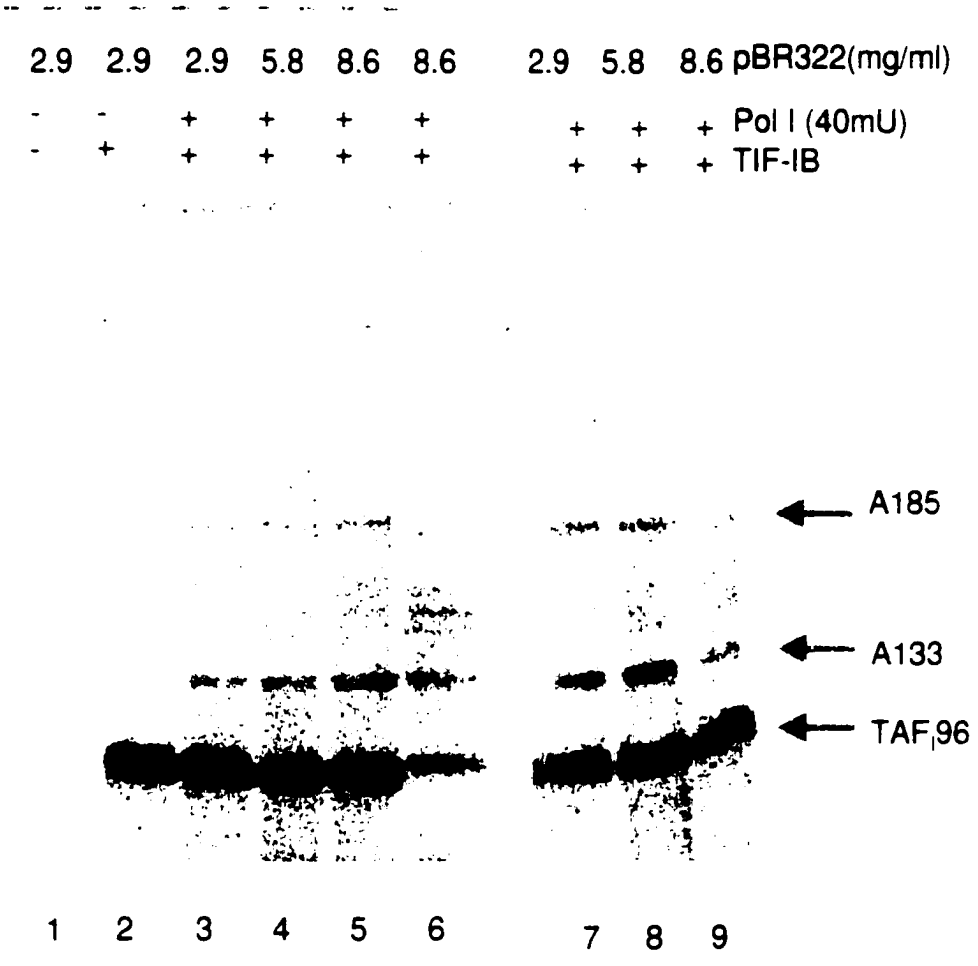


Fig. 2.9 Titration of circular and linear nonspecific competitor plasmid pBR322to eliminate nonspecific cross-linking of pol I. The amounts of competitor used is not enough to eliminate all pol I cross-linking in the absence of TIF-IB.

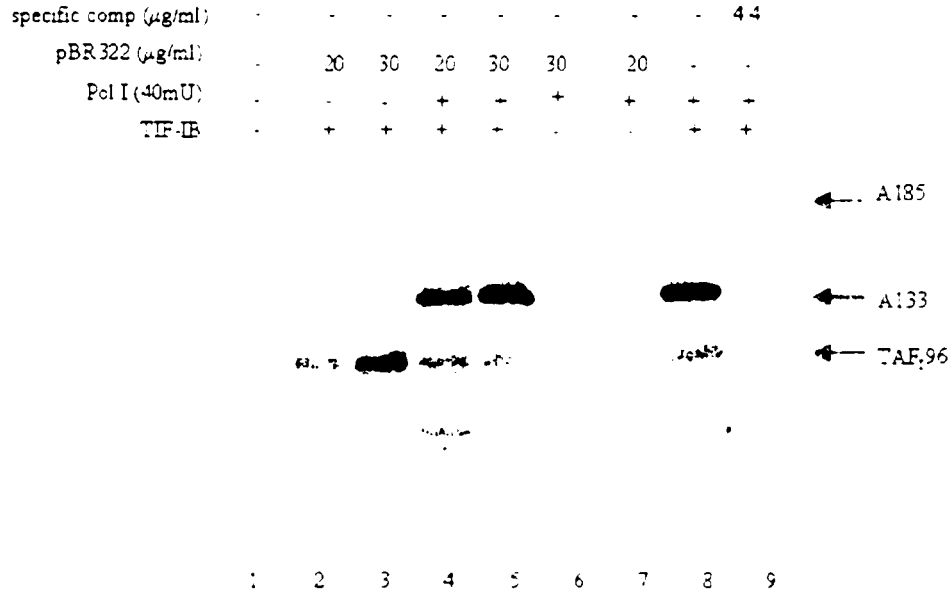


Fig. 2.10 Titrations of pBR322 nonspecific competitor in pol I cross-linking at the start site. The ideal amount of competitor to be used with 40mU polymerase is 20mg/ml, shown in lane 8. Pol I does not cross-link at these amounts in the absence of TIF-IB, but does cross-link in its presence (lane 4).

specific and often intense cross-linking of polymerase at certain sites and not at others.

CHAPTER 3

SITE-SPECIFIC PHOTO-CROSS-LINKING OF FUNCTIONAL RNA POLYMERASE I TRANSCRIPTION INITIATION AND ELONGATION COMPLEXES

The work presented here appears in the same form as it was submitted to Molecular and Cellular Biology, with the following exceptions. The manuscript is presently under review. The references are in a format to fit with the remainder of the dissertation. They have been omitted from the end of the manuscript, and are included instead with the remaining references at the end of this document to avoid redundancy. Also, some figures were not included in the manuscript.

SITE-SPECIFIC PHOTO-CROSS-LINKING OF FUNCTIONAL RNA POLYMERASE I TRANSCRIPTION INITIATION AND ELONGATION COMPLEXES

3.1 ABSTRACT

The TIF-IB/TIF-IE complex plus RNA polymerase I (pol I) form the initiation complex on the ribosomal RNA (rRNA) promoter of *A. castellanii*. TIF-IB photo-cross-links to the promoter from –78 to +10 relative to the *tis* (+1). TIF-IE does not bind the DNA and does not appear in the photo-cross-linking. Though TBP, a subunit of TIF-IB, does not bind DNA using its TATA-binding saddle, it does photo-cross-link to a 22bp sequence that does not resemble a TATA box. Pol I is recruited to the DNA by protein-protein interactions with TIF-IB. The photo-cross-linking results suggest that pol I does not extend significantly upstream of the promoter-proximal border of the factor complex. This contrasts significantly with similar analyses of pol II, but is in agreement with pol III data.

3.2 INTRODUCTION

RNA polymerase I (pol I) transcribes the precursor that is processed into the 28S, 18S and 5.8S RNAs. These RNAs, along with proteins and 5S RNA, an RNA polymerase III product, make up the ribosome. Since ribosomal RNA comprises about 75% of the total RNA being transcribed in the growing cell, it is energetically necessary to tightly regulate its transcription.

Acanthamoeba castellanii, a simple eukaryote, provides a good model for studying pol I transcription. The preinitiation complex (PIC) includes only three proteins. Transcription initiation factor IB (TIF-IB) is the factor that recognizes the promoter (Iida et al., 1985). TIF-IB is composed of TATA-binding protein (TBP) and four TBP-associated factors (TAF_Is), named for their sizes of 145, 99, 96 and 91 kDa. Transcription initiation factor IE (TIF-IE) is an accessory factor necessary for stable binding of TIF-IB to the core promoter (Radebaugh et al., 1998) but does not appear to bind DNA itself (Al-Khouri and Paule, 2002). Pol I is recruited to the promoter by TIF-IB through protein-protein interactions. Pol I makes no sequence-specific contacts with DNA (Kownin et al., 1987). In *Acanthamoeba*, recruitment is the regulated step of transcription (Bateman et al., 1985).

We had been interested in mapping TIF-IB to the promoter in order to determine which subunits may be interacting with pol I and therefore may be involved in regulation. These experiments were initially carried out

using photo-cross-linking with 5-[*N*-(*p*-azidobenzoyl)-3-aminoallyl]-dUMP, a modified nucleotide that is incorporated into the DNA in place of thymine and extends its photo-activable derivative into the major groove (Gong et al., 1995). Subsequently, footprinting and minor groove-binding drug-inhibition studies (Geiss et al., 1997) indicated that TIF-IB binds largely in the minor groove. TBP is also known to make DNA contacts in the minor groove. Thus, it seemed logical to test photo-cross-linking reagents that either targeted the minor groove, or, better, were unbiased as to groove.

We were also intrigued by the fact that RNA polymerase II (pol II) has been shown to cross-link far upstream of the transcription initiation site (*tis*) on the DNA (Kim et al., 1997). We were curious about whether pol I also extended upstream and what implications that would have in regard to its contact with TIF-IB. In order to further define TIF-IB binding and to determine where pol I contacts the DNA without regard to which groove is contacted, we have performed experiments using a derivative that is linked to the DNA sugar-phosphate backbone, thus extending into both the major and minor grooves.

We have found that TBP in TIF-IB cross-links over a 22 base pair stretch of DNA, about twice that of yeast pol II, and excluding much of the TAF binding. TIF-IB TAF_Is cross-link both upstream and downstream of TBP, from –78 to +10 relative to the *tis*. Pol I was found to cross-link downstream to +16, in agreement with where it had been previously footprinted (Bateman et al., 1985) and upstream no farther than –13. We

believe that because TIF-IB is so large (463 kDa) and it binds so tightly (K_D = 59 pM) (Radebaugh et al., 1998), it excludes pol I binding upstream on the promoter. The unusually large TBP footprint is discussed in terms of the different role of TBP in rRNA transcription.

3.3 MATERIALS AND METHODS

3.3.1 DERIVATIZED PROMOTER DNA FRAGMENTS

Oligodeoxyribonucleotides were derivatized by the method of Bartholomew (personal communication). All reactions before U.V. irradiation were performed under reduced lighting. Oligodeoxyribonucleotides 15 to 17 nucleotides in length with a phosphorothioate between the second and third residue (Macromolecular Resources and IDT) were derivatized in a 150μL reaction by mixing a 75μL solution of 7.5nmoles oligodeoxyribonucleotide in 100mM triethylammonium bicarbonate buffer pH 7.93 with 75μL 100mM *p*-azidophenacyl bromide (Sigma) in acetonitrile. Reactions were incubated 4 hours at 25°C then lyophilized in the Speedvac at medium heat setting. Pellets were resuspended in 100μL deionized water and extracted 3 times with water-saturated isobutanol and 4 times with water-saturated ethyl ether. Oligodeoxyribonucleotides were diluted to 10pmol/μL with 10mM TrisCl-1mM EDTA pH 8.0. 10pmol oligodeoxyribonucleotides were

phosphorylated with [γ^{32}P]-ATP and annealed to pBSx(+) single stranded plasmid (pUC 18 containing the RNA polymerase I promoter from –120 to +80) (Gong et al., 1995), followed by primer extension and ligation (Travers and Buckle, 2000). Dithiothreitol was omitted from all reactions, as it reduces phenyl azides to photo-inert phenyl amines (Radebaugh et al., 1998). The plasmid was digested with BamHI and HindIII to produce a 221bp fragment, which was purified on a 5 % native polyacrylamide gel and passively eluted.

3.3.2 PHOTO-CROSS-LINKING

20μL binding reactions contained 30 fmol derivatized DNA fragment, 0 or 40 mU *Acanthamoeba* RNA pol I (Spindler et al., 1978), and no TIF-IB/TIF-IE or the amount needed to shift all template DNA in an electrophoretic mobility shift assay, 20mM HEPES pH 7.9, 10mM $MgCl_2$, 1% NP-40, 2.5mg/ml BSA, 10mM 2-mercaptoethanol, 0.3 mg/ml pBR322, 0.1mM EDTA, and 10% glycerol. Reactions were incubated 20min at 25°C, then irradiated for 4 min at 365nm (11mJ/mm2) with ultraviolet light (Spectroline model SB-100p). Complexes were digested as described (Gong et al., 1995) and electrophoresed on 7.5% and 12% SDS polyacrylamide gels, which were dried, exposed to phosphor storage screens (Kodak) for 2-4 days, and visualized on a Storm phosphorimager (Molecular Dynamics).

3.3.3 PURIFICATION OF PROTEINS

RNA pol I was purified as described (Iida and Paule, 1992) through the heparin-sepharose stage. TIF-IB/TIF-IE was purified through one round of the DNA promoter affinity column (Radebaugh et al., 1998). 1X oligo TIF-IB contains sufficient TIF-IE for the formation of stable complexes with the promoter DNA. In one test experiment, additional TIF-IE was added, but was not found to cross-link to the DNA (Fig. 3.1).

3.3.4 MODELING

DeepView (ver. 3.7) was used to create a single pdb file from 1I50 (Cramer et al., 2001) plus a DNA PDB file, then Rasmol (ver. 2.6) created an α-carbon backbone representation of the polymerase.

3.2 RESULTS

3.2.1 POSITIONS OF DNA DERIVATIZATION AND FACTOR BINDING

Modified DNA templates were made by incorporating single derivatization sites located on primers into the *Acanthamoeba* rRNA promoter from –78 to +16 relative to *tis*. This part of the promoter was chosen because it encompasses TIF-IB and pol I binding as shown by DNase I and methidiumpropyl-EDTA·Fe(II) footprinting assays (Bateman

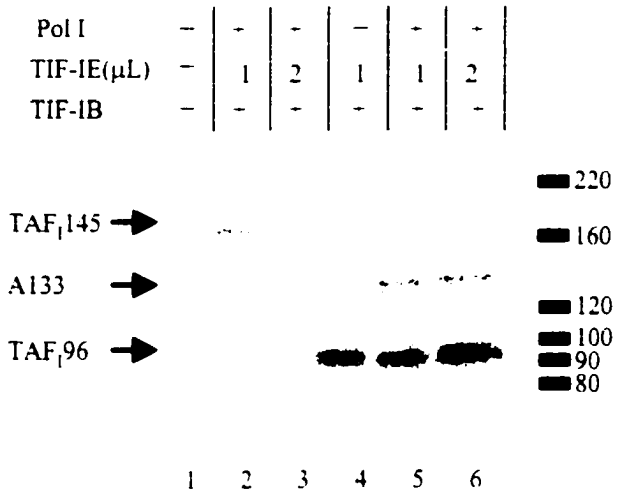


Fig. 3.1 Cross-linking of TIF-IE. This is a sample gel with TIF-IB, pol I and TIF-IE. TIF-IE was not found to cross-link to the DNA.

et al., 1985; Paule et al., 1991). The modified derivatization sites were 2-6 base pairs apart, resulting in 27 DNA fragments each derivatized at a single, distinct site. Each primer used for promoter synthesis was tested for derivatization by comparing its slower mobility with nonderivatized primers on a denaturing polyacrylamide gel (Fig. 2.4 in Chapter 2). Derivatization efficiency approached 100%. Templates were tested for TIF-IB binding using electrophoretic mobility shift assays (EMSA). Sample assays are shown for two derivatized DNAs (Fig. 3.2). TIF-IB was titrated until 100% of the template forms complexes with slower mobility (lanes 2 and 4) than the free DNA template (lanes 1 and 3). Pol I binding was not tested by EMSA, as the DNA-TIF-IB-Pol I complex is so large that it stays in the well in this assay. We know, however, that pol I is active and that the full initiation complex is formed because we can induce transcription elongation by adding nucleoside triphosphates (NTPs) to the reaction mixture. We can also footprint the bound pol I both before elongation and at several stalled sites downstream of the *tis*. In these assays, template usage approaches 100% and nearly all preinitiation complexes (PICs) formed are transcriptionally active (Kahl et al., 2000; Kim et al., 1997).

3.2.2 SPECIFICITY OF PHOTO-CROSS-LINKING

In order to verify that TIF-IB and pol I were cross-linking specifically, we tested cross-linking under a variety of conditions. One example of these data is shown in Fig. 3.3. No cross-linking bands are

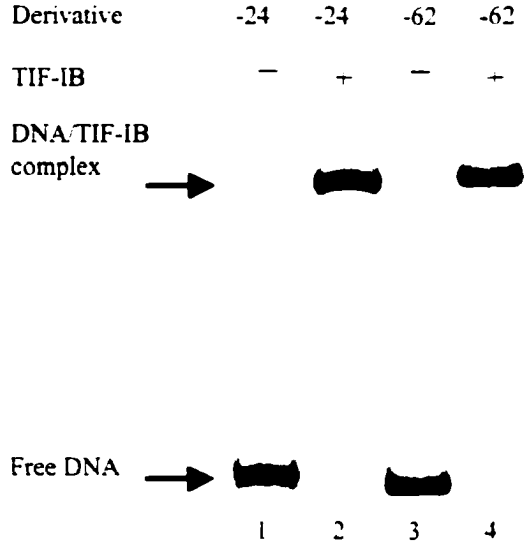


Fig. 3.2 EMSA of derivatized templates complexed withTIF-IB. Lanes 1 and 3, DNA templates alone; lanes 2 and 4, the complex that forms with the addition of TIF-IB.

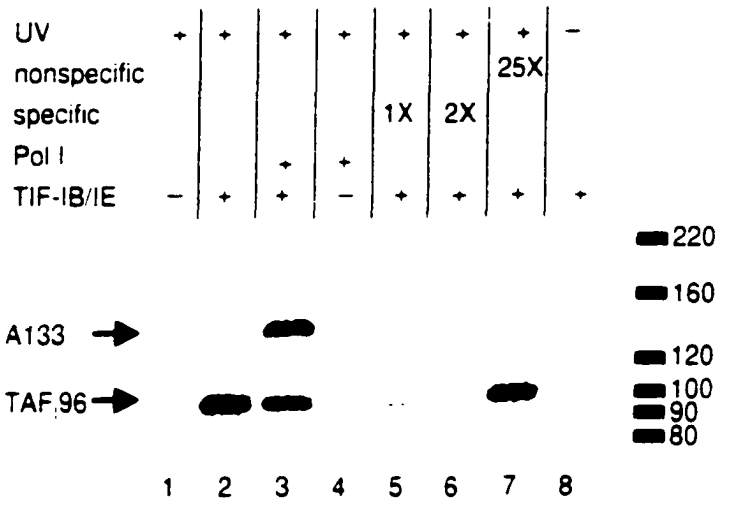


Fig. 3.3 Control cross-linking at the start site. Lane 1, DNA alone; lane 2, the addition of TIF-IB/IE; lane 3, TIF-IB/IE plus pol I; lane 4, pol I alone with DNA; lane 5, TIF-IB/IE with the addition of 1X specific competitor; lane 6, TIF-IB/IE with the addition of 2X specific competitor; lane 7, TIF-IB/IEwith the addition of 25X nonspecific competitor; lane 8, TIF-IB/IE and DNA without UV irradiation.

found in the absence of TIF-IB and pol I (lane 1), indicating that the DNA is fully digested and that BSA, a stabilization protein, is not cross-linking. In contrast, when TIF-IB/IE is bound to the DNA derivatized at –1, strong cross-linking to the 96 kDa subunit occurs (lane 2). After addition of pol I to the committed complex, a new strongly cross-linked band corresponding to the A133 subunit (the β subunit homolog, (D'Alessio et al., 1979) is obtained (lane 3). Pol I does not cross-link in the absence of TIF-IB under the conditions used (lane 4), in agreement with its inability to recognize promoters in the absence of TIF-IB. However, it is critical to note that if too much polymerase is added, or not enough non-specific competitor is used, pol I cross-links over the entire template, demonstrating its strong nonspecific affinity for DNA (Fig. 2.8, 2.9 and 2.10 in Chapter 2). Therefore, in these experiments the controlled titration of pol I and competitor DNA was carried out to eliminate this spurious background. TIF-IB cross-linking is abolished by the addition of specific competitor (lanes 5 and 6), but remains strong in the presence of excess non-specific competitor (lane 7), demonstrating the sequence specific interaction of TIF-IB/IE with the promoter DNA. As expected, photo-cross-linking does not occur in the absence of exposure to UV light (lane 8). This sample control experiment demonstrates the effectiveness of the method in mapping the positions of the transcriptional components in the PIC.

3.2.3 ACANTHAMOEBA rRNA TRANSCRIPTION COMPLEXES

Photo-cross-linking was carried out for three different complexes. TIF-IB/TIF-IE was incubated with derivatized promoter DNA fragments to map TBP and the individual TAF_Is. In separate reactions, Pol I was also added to form the PIC. Pol I and TIF-IB were cross-linked at numerous sites along the promoter region to see how far each protein extends, both upstream and downstream, and to see how much overlap there is between TIF-IB and pol I. Cross-linking was also performed with the same set of proteins in the elongation complex, after pol I was moved to +7 by the addition of 2 NTPs. Our ability to move the polymerase in this way demonstrates that we have a complete, active *Acanthamoeba* rRNA transcription complex. These same proteins produce strong transcripts in an *in vitro* transcription assay. Footprinting demonstrates that a very high fraction of these complexes are transcriptionally active, with few dead-end inactive complexes (Kahl et al., 2000).

3.2.4 TAF_I CROSS-LINKING

The TIF-IB TAF_Is cross-link from –78 to +10 on the rRNA promoter (Fig. 3.4, 3.5 and 3.6). TIF-IE does not cross-link to the DNA, consistent with its not binding DNA (Al-Khouri and Paule, 2002). TAF photo-cross-linking was identical whether or not RNA polymerase I is present. Only the data for the quaternary complex is shown. The largest TIF-IB subunit,

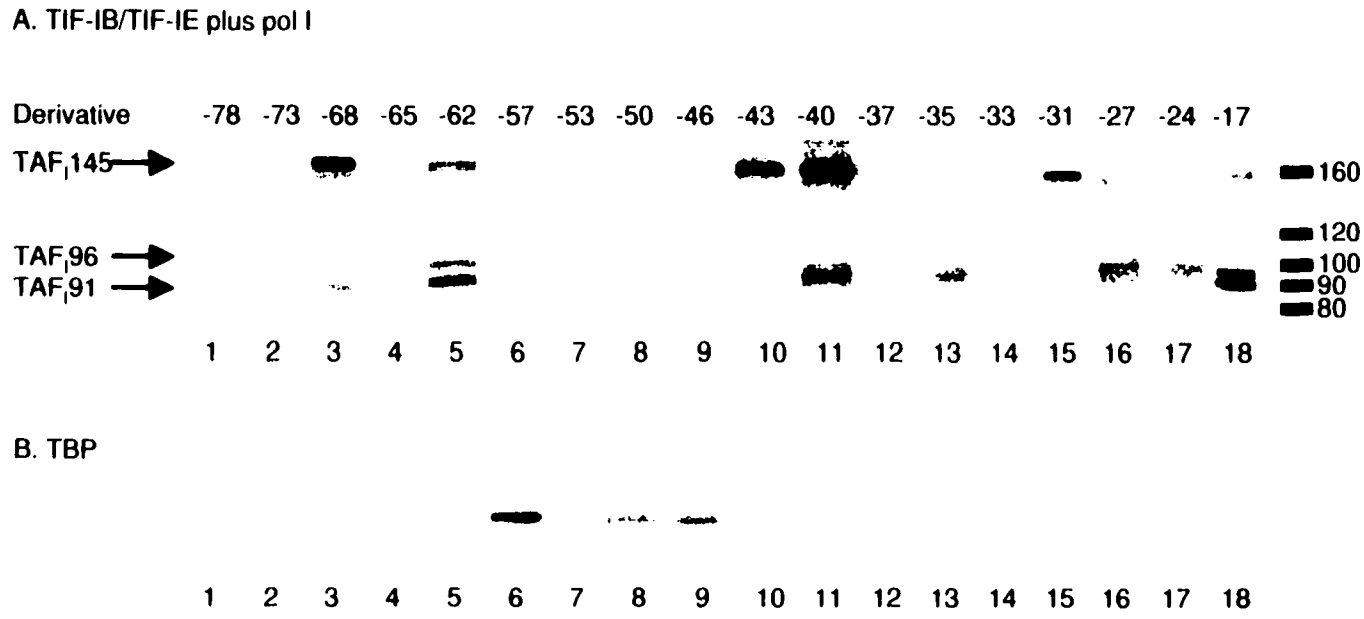


Figure 3.4 Photo-cross-linking with TIF-IB/IE and pol I in the initiation complex at sites from –78 to –17 relative to *tis*
Panel A: TAF$_I$145 cross-links along this entire region, as does TAF$_I$96, with few cross-linked sites in the upstream part of TBP cross-linking (see panel B). TAF$_I$91 cross-links mostly upstream (lanes 2-5) with a single cross-linking site farther downstream at –17 (lane 18).
Panel B: TBP cross-links from –57 to –35 (lanes 6-13), covering a region of about 22bp.

Figure 3.5 Photo-cross-linking with TIF-IB/IE and pol I just upstream of the start site. The A133 subunit of polymerase cross-links upstream as far as –11 (lane 4) in the initiation complex. TAF$_I$96 cross-linking overlaps that of pol I.

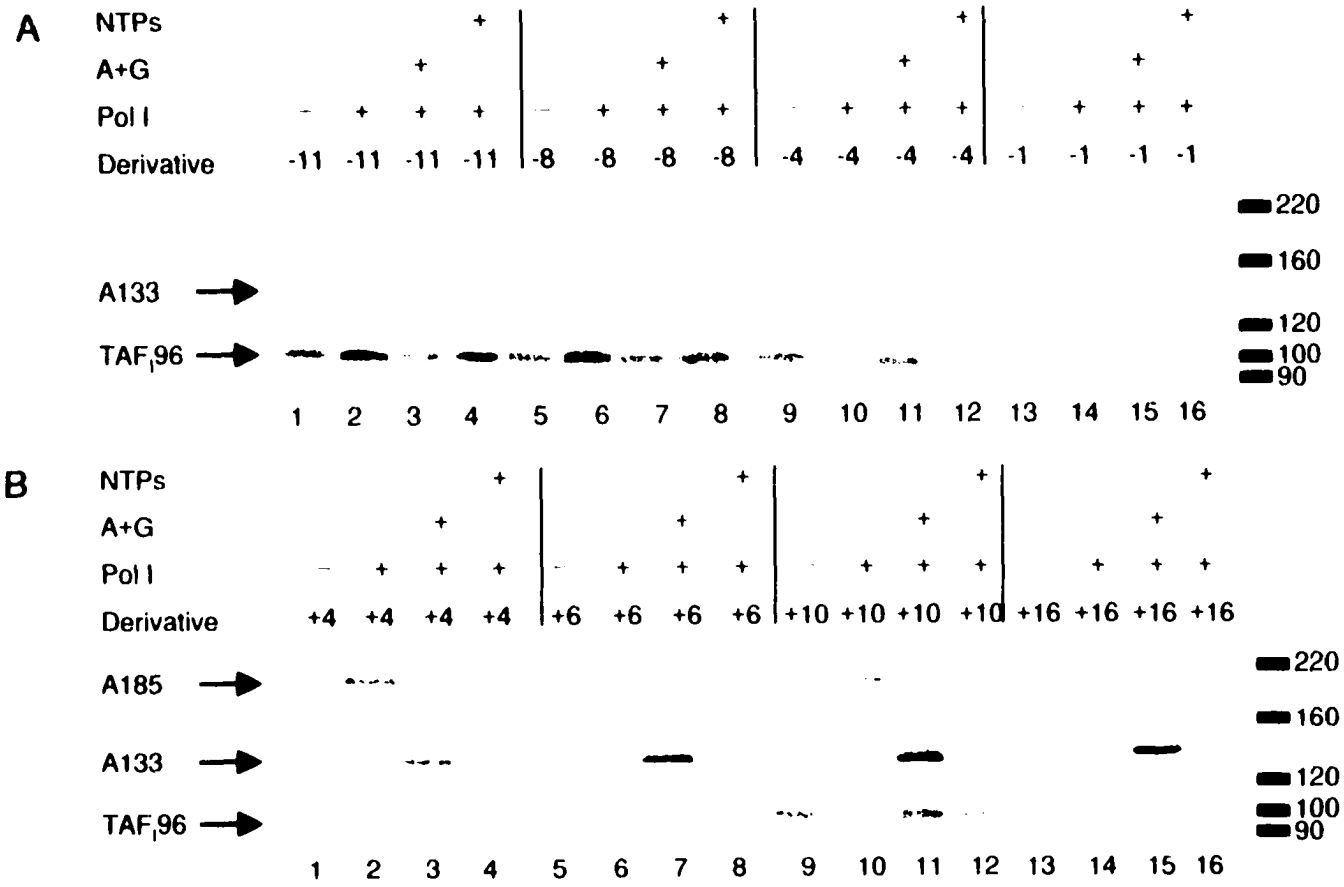


Fig. 3.6 Cross-linking of TIF-IB/IE and pol I in the initiation complex, elongation complex, and after promoter clearance by pol I. Panel A: Cross-lininking upstream of the start site. Panel B: Cross-linking downstream of the start site.

TAF_I145, is found from –78 to –17 (Fig. 3.4A), cross-linking mainly on one side of the helical axis (Fig. 3.9). It does not cross-link to sites more proximal to the *tis* (Fig. 3.5 and 3.6).

TAF_I96 cross-links similarly to TAF_I145 in the far upstream regions at –78, -73, -68, and –62 (Fig. 3.4A, lanes 1-5). It does not cross-link in most of the region where TBP is in proximity to the DNA (Fig. 3.4A and 3.4B, lanes 6-9), with the exception of -35 (lane 13). Farther downstream, there is pronounced cross-linking of TAF_I96. The cross-linking is almost continuous from –40 all the way to the start site at –1 (Fig. 3.4A, lanes 11-18, Fig. 3.6A). TAF_I96 also shows up one helical turn downstream of the start site, at +10 (Fig. 3.6B, lanes 9-12). TAF_I91 cross-links only far upstream, at –78, -73, -68 and –62, and then once downstream at –17 (Fig. 3.4A), perhaps suggesting that it is located on the outside of TIF-IB relative to the DNA. Curiously, in the present study TAF_I99 was not found to cross-link at any site, though in an earlier study using 5-[*N*-(*p*-azidobenzoyl)-3-aminoallyl]-dUMP-derivatized template (Gong et al., 1995), this subunit was localized between -38 and -66 (see Discussion). Otherwise, the present study is in substantial agreement with the earlier one.

3.2.5 TBP

TBP cross-links in one area of the promoter, from –57 to –35 (Fig. 3.4B). Especially in the upstream region it appears to exclude the cross-linking of the TAF_Is, suggesting that it is in close proximity to the DNA. This region does not include a TATA element, and TBP is not believed to use its TATA-binding surface to interact with the DNA in the Pol I factor (Radebaugh et al., 1998). Interestingly, it cross-links over a much larger region, 22 bp, than yeast TBP in pol II initiation complexes, 10 bp (see Discussion).

3.2.6 RNA POLYMERASE I

Pol I was cross-linked in both the initiation and in the elongation complexes. In the initiation complex, the two largest subunits, A185 and A133, were found to cross-link at various sites from –11 to +16 (Fig. 3.5, lanes 4,6,8 and Fig. 3.6A and B, lanes 2,6,10,14). A133 cross-links at every site tested in that region with the exception of +6. A185 cross-links at +4, +6, +10 and +16, every site tested downstream of +4. No pol I subunits were cross-linked at any of the sites from –14 to –78 (Fig. 3.4A and 3.5), so that the farthest upstream site at which we saw polymerase in the initiation complex was –11 (Fig. 3.5).

Pol I can be initiated and stalled at specific sites along the template by supplying a limited number of the four NTPs (Kahl et al.,

2000). For example, addition of ATP and GTP allows the formation of a heptamer RNA, but not an octamer RNA (5'AAAGGGAC). In the elongation complex, with the polymerase stalled at +7, the farthest upstream we see A133 cross-linking is –4 (Fig. 3.6A, lane 11); cross-linking at -8 and -11 is lost (Fig. 3.6A, lanes 3 and 7). This is between 4 and 7 nucleotides downstream from the upstream limit where A133 was cross-linked in the initiation complex (Fig. 3.5, lane 4), approximately the same distance the polymerase was moved. A185 cross-linking, whose most upstream site before initiation was +4 (Fig. 3.6B, lane 2), disappears altogether (Fig. 3.6B, lanes 3, 7, 11, 15). These data suggest that either this region of polymerase has moved even farther downstream than +16, or the DNA is situated such that the reactive derivative is not juxtaposed with a reactive portion of the polymerase (see Discussion).

3.3 DISCUSSION

We have used site-specific photo-cross-linking to map the subunits of TIF-IB/IE and RNA polymerase I on the *Acanthamoeba* rRNA promoter, pol I in the elongation complex, and TIF-IB/IE after promoter clearance.

3.3.1 TIF-IB

The different subunits of TIF-IB cross-link continuously along the promoter region from -78 to the start site, with only two tested sites without any cross-linking (derivatives at -37 and -14). This agrees with uranyl nitrate, hydroxyl radical, and dimethyl sulfate footprinting experiments, which have shown that TIF-IB wraps around and protects DNA along the entire region from –67 to -17 (Geiss et al., 1997). TAF_I96 also cross-links at a site 10 bp downstream of the start site. However, this interaction does not result in strong footprinting with either DNase I, uranyl, methidiumpropylEDTA-Fe(II) or EDTA-Fe(II) in solution (Bateman et al., 1985; Geiss et al., 1997). Photo-cross-linking is, therefore, more sensitive than footprinting at both ends of the interaction region in detecting protein in proximity to the DNA.

Our cross-linking indicates that TAF_I145, TAF_I91 and TBP interact with the region from –78 down to –17, but not farther downstream. Only TAF_I96 extends farther downstream. It makes sense that the region near the start site should be more accessible for pol I to readily make contact with the DNA. Indeed, this idea agrees with the cross-linking results for pol I (see below). Experiments with deletion mutations of the DNA promoter have shown that the region between –17 and –31 is required for stable TIF-IB/IE binding to the DNA, and the region from –32 to –47 only stabilizes the complex (Paule et al., 1991). Footprinting has shown TIF-IB is wrapped nearly completely around the DNA in the –32 to –20 section of

the promoter. Regions downstream of –17 and upstream of –47 are dispensable for stable binding. This would suggest that the region from –17 to –47 is most important for TIF-IB binding, whereas the farthest upstream interaction sites may serve only to slightly stabilize binding, or have little effect on the energetics of the interaction. In the region upstream of –47, TIF-IB protects two minor grooves from uranyl and EDTA-Fe(II) cleavage, the upstream of these quite weakly.

Contrasting with the importance of the –17 to –47 region, 5' deletion mutations can extend all the way through most of the promoter, down to –6, without eliminating correct polymerase I positioning and initiation. This faithful transcription is barely detectable, and TIF-IB/IE binding to this deletion mutant is not detectable by footprinting or electrophoretic mobility shift (Radebaugh et al., 1997). Thus, TIF-IB is making some sequence-specific contacts in the downstream region of the promoter, even though these contacts do not result in significant binding energy. TAF_I96 is the subunit implicated in this contact with the promoter by the photo-cross-linking.

We have found that TAF_I96 photo-cross-links throughout the entire region, starting at –78 and extending to –1, and also cross-links at a single, isolated site one helical turn downstream of the *tis*. This downstream cross-linking of TAF_I96 implies that it is contacting or in close proximity to the RNA-like strand of the melted bubble (Kahl et al., 2000) within the "jaws" of the polymerase (see below and Fig. 3.6). From these

results, we hypothesize that this is the TIF-IB subunit that is most important for recruiting and making contact with pol I. It is the only TAF whose cross-linking overlaps with the polymerase. The gene for this *A. castellanii* subunit has been partially cloned. Its sequence shows similarity to the yeast, *S. cerevisiae*, TAF_I encoded by RRN7 (C. Radebaugh, unpublished). We have found that Rrn7p interacts with the A190 subunit of yeast pol I in yeast two hybrid experiments, in accordance with the above hypothesis (C. Radebaugh, personal communication). In yeast, pol I can be recruited to the promoter in complex with the yeast homologue of TIF-IB by promoter-bound UAF, and in the absence of any other known factor (Aprikian et al., 2001). This is in substantial agreement with the notion that interaction between TIF-IB/Rrn7p and pol I serves a critical role in polymerase recruitment.

In contrast to earlier work from this laboratory, we did not detect any cross-linking to TAF_I99. It is interesting that in the earlier study, TAF_I99 only cross-linked at positions where TAF_I145 also was found, opening the possibility that TAF_I99 is a degradation product of TAF_I145. If so, this would bring *A. castellanii* TIF-IB into line with other species in having only three TAF_Is instead of four.

3.3.2 RNA POLYMERASE I

We have determined that the polymerase cross-links to about 27 bp of DNA, from −11 to +16 relative to *tis*. This is in close agreement with methidiumpropylEDTA-Fe(II) footprinting, which estimated 26 bp at a number of stalled sites close to the *tis*. It appears that the two largest subunits of polymerase, which have been shown in the pol II crystal structure to form the "jaws" of the polymerase (Cramer et al., 2001), are the only subunits to make substantial contact with the DNA. We have occasionally observed photo-cross-linking of the ABC22.5, the *Acanthamoeba* homologue of yeast ABC27 (RPB5), at +6 (data not shown). This subunit lies at the "lips" of the jaw in the X-ray structure of pol II (Cramer et al., 2000). It was reported to cross-link to the DNA in pol II between +5 and +15 (Kim et al., 1997) and between +11 and +16 in pol III (Bartholomew et al., 1993). In pol I, however, the intermittent nature of the cross-linking suggests the DNA might take a slightly different path past this region of the enzyme. Even more likely, RNA polymerase I itself has a slightly different conformation so that this subunit is not as accessible to the DNA as in pol II or pol III.

The cross-linking done with human pol II showed that the two largest subunits cross-linked along the length of the promoter region, making substantially more contact than in pol I or pol III. In the yeast pol III system, the two largest subunits also cross-link along the entire stretch of the polymerase footprint, as is the case with *Acanthamoeba* pol I.

However, unlike in the pol I and pol II systems, as many as seven other subunits of pol III cross-link to the DNA, implying that there is some protein proximity to the DNA that is specific to the different polymerases (Bartholomew et al., 1993).

In distinct contrast to RNA polymerase II cross-linking, which showed cross-linking to upstream DNA all the way to – 53 (Kim et al., 1997), we do not find any pol I subunits cross-linking to the promoter upstream of -11. There are several reasons why we believe that our results are an accurate reflection of the *in vivo* situation. From a technical viewpoint, we have carefully titrated competitor DNA in order to eliminate the nonspecific binding and cross-linking to DNA of polymerase alone (Fig. 3.3). Second, where cross-linking occurs, reproducible and clearly visible bands appear in the gels (Fig. 3.4, 3.5 and 3.6), and even after extreme computer enhancement of the digitized data, we do not see any cross-linking at the upstream sites (data not shown). In the upstream region, other subunits of TIF-IB clearly cross-link at nearly every site, so there is no systematic problem with application of the method. More critically, however, our reconstituted system closely mimics the active *in vivo* complex: a completely homologous *Acanthamoeba* rRNA transcription system, with all components from a single species, was used. We can elongate the transcript to the end of the DNA template or we can stall the polymerase by depletion of two NTPs (Fig. 3.6A and B). This reconstituted system demonstrates extremely efficient template usage;

nearly 100% of the templates form active PICs capable of promoter clearance (Kahl et al., 2000), in contrast to the low efficiency by pol II reconstituted systems. When this is done, cross-linking to pol I is strong before translocation, and is lost completely or nearly completely following promoter clearance, or shows the expected movement downstream in the stalled complex (Fig. 3.6). We produce high levels of transcripts with the same proteins in an *in vitro* transcription assay under identical conditions, with the exception of dithiothreitol replacing 2-mercaptoethanol. We have also performed cross-linking experiments with yeast pol II transcription factors TBP, TFIIA and TFIIB on the yeast HIS3 promoter. Our results are in substantial agreement with those of Kim et al., who cross-linked human TFIIA and TFIIB on a viral promoter, demonstrating the reproducibility of the method in our hands (Bric, Robinson, Paule & Stargell, in preparation). Also, since our downstream cross-linking of pol I is similar to that of pol II, there is no reason we should not detect upstream cross-linking were it actually present.

There are several reasons why, in our system, polymerase might not be expected to cross-link upstream. The transcription factor TIF-IB is so large it may preclude any other protein binding to the promoter. We have found that in footprinting assays, neither DNase nor MPE-Fe(II) have access to the region of DNA bound by TIF-IB (Kownin et al., 1987), and it is therefore unlikely that a protein as large as polymerase would have that access. The cross-linking done

with pol II included transcription factors TBP, TFIIB and TFIIF. These together are only about half the size of TIF-IB and cross-link a span of only 33bp (Kim et al., 1997), as opposed to 88 bp for TIF-IB. Perhaps significantly, this is not the minimal functional pol II transcription initiation complex, which would include TFIIE, TFIIH, and on many promoters, the TAFs as well. The effect these might have on cross-linking of the upstream DNA was not evaluated (Kim et al., 1997). In addition, different TBP interactions in pol I and pol II PICs may have a significant effect on the trajectory of the upstream DNA as it exits the enzyme (see the model below). Finally, when we move pol I downstream by seven nucleotides, the upstream border of cross-linking to polymerase also moves downstream by the same distance. This is not surprising, since previous MPE-Fe(II) footprinting assays of TIF-IB and pol I have shown that there is very little overlap of the two factors on the promoter in the vicinity of the DNA (Kahl et al., 2000; Paule et al., 1991). Our results are in substantial agreement with the similarly functional RNA polymerase III complex. The upstream border of pol III cross-linking is at –21 in the PIC, and moves the predicted distance upon stalling the polymerase at +17. Thus, our results with the pol I complex (Fig. 3.6) mirror closely the pol III data, lending universality to our results.

3.3.3 TBP

TBP cross-links over a 22bp segment of the promoter, from –57 to –35. Over the upstream part of that region, it is the only factor to contact the DNA (Fig.

3.4B). Although TBP must be in close proximity to the DNA, we do not believe that it is actually binding any particular sequence. This upstream region of the promoter is not critical for binding of TIF-IB (Kownin et al., 1988). TBP in the context of TIF-IB is not located over a consensus TATA-box sequence, although there is a perfect pair of nested TATA-boxes farther downstream on the promoter, surrounding the *tis* (Radebaugh et al., 1994). TBP alone, not in the context of TIF-IB, strongly footprints and cross-links to these downstream sites (X.Gong, unpublished data). Indeed, TATA-box oligos do not inhibit transcription by pol I, though they do so for pol II and pol III in parallel experiments, and the rRNA promoter is not bent where TBP binds as it is in normal TBP-TATA box complexes (Radebaugh et al., 1994). This may be critical to the interaction of upstream DNA with the polymerase (see below).

The fact that TBP cross-links over twice as long a region in our system as yeast TBP does in a pol II system also suggests that its interaction with DNA is different in the two systems. One intriguing possibility that is in accord with the available data is a TBP dimer rather than a monomer in the pol I factor. TBP is known to readily dimerize, but the biological significance of this is unknown (Campbell et al., 2000). The *Acanthamoeba* TIF-IB complex can be supershifted in an EMSA, and forms not one, but two supershifted bands we interpreted as the binding of one or two immunoglobulins (Radebaugh et al., 1994) There is a single MPE accessible site at the putative dyad (–49/–50) of such a dimer (Bateman and Paule, 1988). A TBP dimer would be immune to inhibition

by TATA oligonucleotides, as is found in the pol I system, because the dimer forms by mutual interaction between the TATA-binding surfaces of the two TBP molecules (Campbell et al, 2000 and references therein). Alternatively, two molecules of TBP could bind in succession, the second reversing the bend introduced by the first. However, this does not explain the lack of inhibition by oligonucleotides. Finally, the non-conserved N-terminal domain might be in close enough proximity to the DNA in TIF-IB to cross link, resulting in the extended reactivity. This domain is rather small in *Acanthamoeba*, however, making this explanation less likely (Wong et al., 1992).

3.3.4 MODEL OF THE PATH OF DNA THROUGH RNA POLYMERASE I

Figures 3.7 and 3.8 show a model for the path of DNA through pol I derived from the x-ray crystallographic structure of yeast pol II (Cramer et al., 2001) and the data described above. The three eukaryotic RNA polymerases share five subunits in common, and in addition, the two largest subunits are largely conserved between enzymes and across species. Thus, though there are bound to be subtle differences between the enzymes, because there is no crystal structure for polymerase I, as a first approximation we used the crystal structure for yeast pol II on which to base a low resolution mating of the DNA and the enzyme. In the model, the DNA is seen to lie in the open groove formed by the two largest

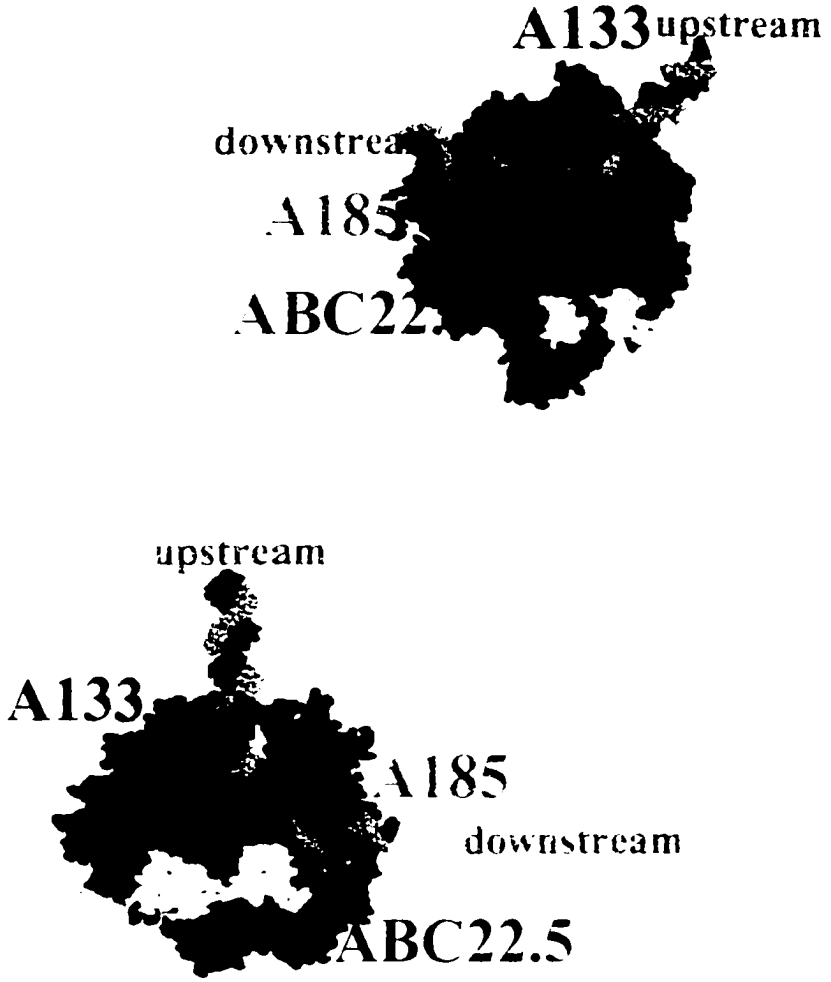


Fig. 3.7 Model of the path of DNA through RNA pol I. This model is a composition of the structural data known for pol II and the cross-linking described. The three polymerase subunits with which DNA cross-links are *Acanthamoeba* A185, A133 and ABC22.5. Their Counterparts in yeast pol II are A190, A135 and ABC27, Pictured in the model. In our model, the DNA enters Into the groove formed between A185 and A133, the two largest subunits of pol I. It bends approximately 90° at the active site, and exits without wrapping around pol I. It does not make close contact with ABC22.5.

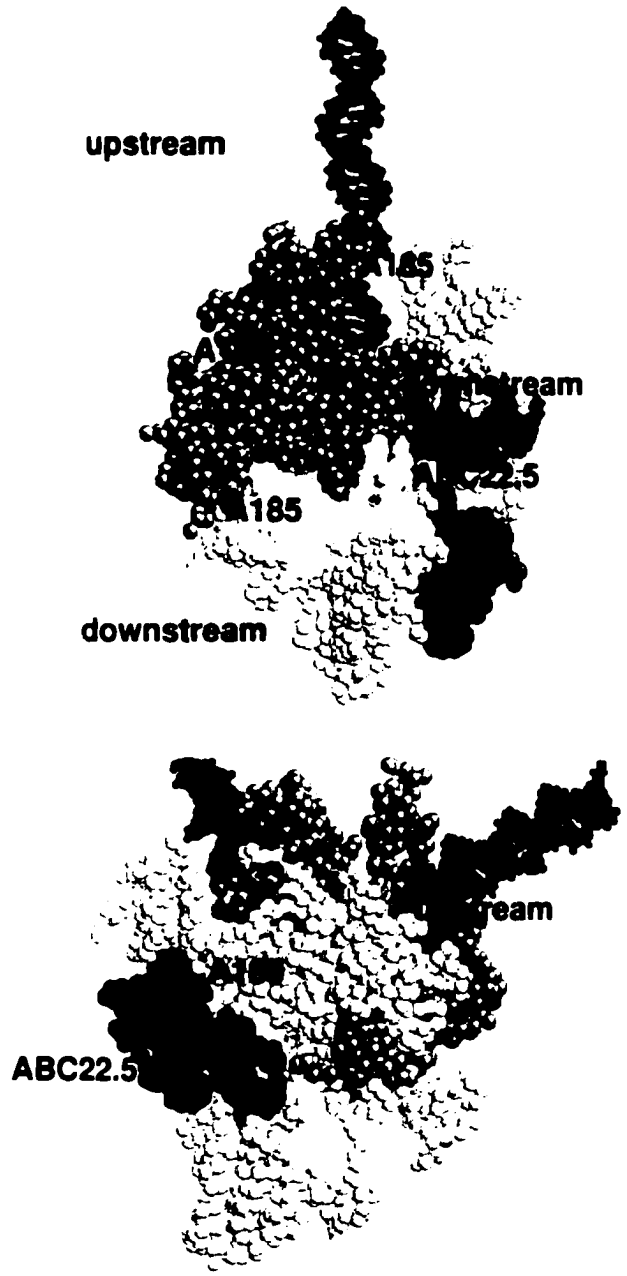


Fig. 3.8 DNA model in manuscript.

subunits of the enzyme. The downstream DNA enters from the left and proceeds until it encounters the "wall" at the active site, whereupon it makes a nearly right angle bend and then exits the polymerase. This general scheme is based upon the models for the Taq polymerase (Zhang et al., 1999) and the co-crystal structure of elongating yeast RNA polymerase II (Gnatt et al., 2001). This model is entirely consistent with the photo-cross-linking data from this study, which is summarized in figures 3.9 and 3.10. However, because the DNA largely contacts only the two largest subunits, there is little detail revealed about the exact path of the DNA. A steady state melted bubble of 19 bp is present in the pol I elongation complex, with 9 bp of DNA-RNA hybrid. The size of this bubble and hybrid was revealed by reaction with $KMnO_4$ and diethylpyrocarbonate (Kahl et al., 2000). In the model, these structures are not modeled because not enough is known about their locations or paths through the polymerase.

The biggest difference between this model and that determined earlier for pol II (Kim et al., 1997) is in the extent of contact between the upstream DNA and the enzyme. There are several reasons that may account for this difference. First, TIF-IB largely covers all of the upstream DNA, out to –78, precluding interaction with polymerase I (see Discussion above). Second, for the DNA to bend around pol II, TBP had to induce a structural bend that turned the DNA back toward the polymerase (Kim et al., 1997). The weight of evidence, including direct measurement of DNA

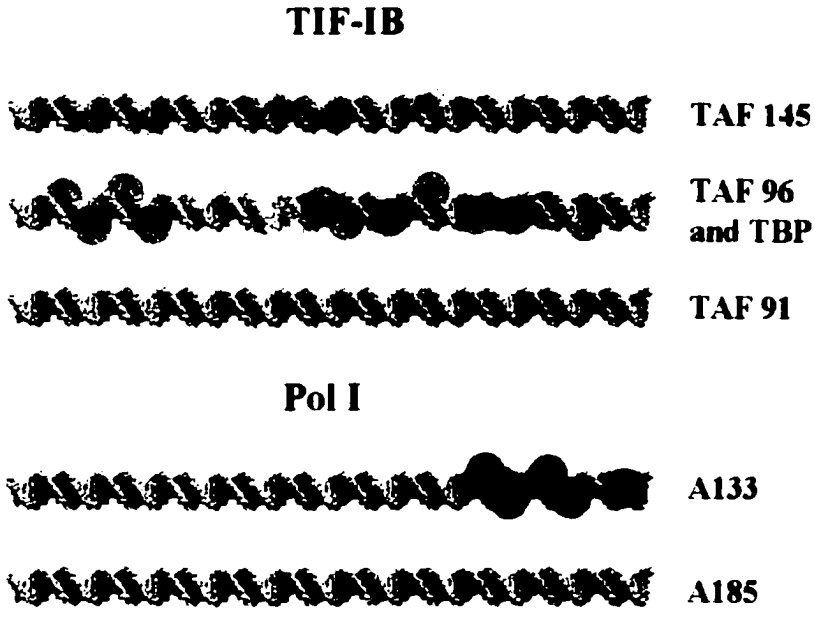


Fig. 3.9 Cross-linkings of TIF-IB and pol I mapped onto the rRNA promoter. Colored circles represent 11 angstrom clouds to simulate the reach of the derivative.

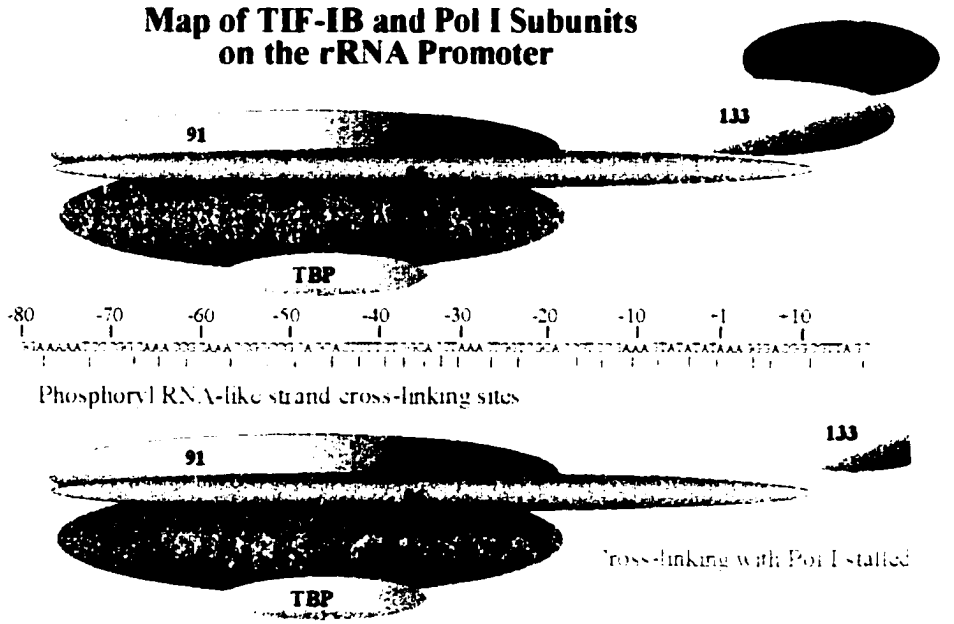


Fig. 3.10 Model of TIF-IB and pol I subunits on the rRNA promoter.

bending (Gnatt et al., 2001; Gong et al., 1995), suggests that in the pol I system, TBP does not similarly bend the DNA. These two effects would be lost as soon as the polymerase leaves the PIC during elongation. Nevertheless, we do not observe extended photo-cross-linking of the upstream DNA in an elongation complex stalled at +7 (Fig. 6), suggesting the DNA simply does not interact in the upstream region. This is perhaps not surprising in light of the highly acidic character of the "outer" surface of the polymerase.

3.3.5 ACKNOWLEDGEMENTS

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CHAPTER 4

PHOTO-CROSS-LINKING RNA POLYMERASE II TRANSCRIPTION FACTORS ON THE YEAST *HIS3* PROMOTER

4.1 INTRODUCTION

There are several reasons why we have undertaken the cross-linking experiments in the yeast pol II system. Mary Robinson in the Stargell group has laid the foundation for these experiments by doing EMSAs and footprinting assays with the proteins TBP, $TAF_{II}40$ and $TAF_{II}19$, and TFIIA. She has found enhanced DNA-protein interactions with certain combinations of factors. We have set out to map these factors, as well as TFIIB and miniTFIIA, on a yeast promoter, which had previously not been done. We observed that TFIIA and TFIIB cross-link specifically on the promoter, whereas TBP and miniTFIIA do not. We have also noted that the addition of the $TAF_{II}40/TAF_{II}19$ heterodimer enhances cross-linking of TFIIB, whereas $TAF_{II}40$ alone does not. Also, the $TAF_{II}40/TAF_{II}19$ heterodimer has a great affinity for DNA, while $TAF_{II}40$ does not cross-link to the DNA. These results, along with Mary's experiments, will be included in a manuscript that will be submitted for publication.

4.2 MATERIALS AND METHODS

4.2.1 DERIVATIZED PROMOTER DNA FRAGMENTS

Oligodeoxyribonucleotides were derivatized by the method described in Chapter 3 with the following modifications. Oligodeoxyribonucleotides phosphorylated with [γ^{32}P]-ATP were annealed to a 76bp single stranded fragment containing the *HIS3* promoter. Both RNA-like and template strand DNAs were used, in conjunction with primers of the opposite strand. Primer extension and ligation was carried out as described previously, without the need for subsequent restriction enzyme digestion.

4.2.2 PHOTO-CROSS-LINKING

18μL binding reactions contained 30 fmol derivatized DNA fragment plus various combinations of the following factors: 12.5ng TBP, 50ng TFIIA, 50ng miniTFIIA, 6.25 TFIIB, 62.5 $TAF_{II}40/TAF_{II}19$ and 250 $TAF_{II}40$ with the following buffer conditions. Reactions were incubated 30min at 25°C, then irradiated for 4 min at 365nm (11mJ/mm2) with ultraviolet light (Spectroline model SB-100p). Complexes were digested as described (Gong et al., 1995) and electrophoresed on 16% SDS polyacrylamide gels, which were dried, exposed to phosphor storage screens (Kodak) for 2-4 days, and visualized on a Storm phosphorimager (Molecular Dynamics).

4.2.3 PROTEINS

Proteins for the yeast cross-linking were provided by Mary Robinson in the Stargell lab using His-tagged proteins expressed in *E.coli* cells. The exception is the $TAF_{II}40/TAF_{II}19$ heterodimer, which was provided by Song Tan.

4.3 RESULTS

4.3.1 POSITIONS OF DNA DERIVATIZATION

Modified DNA templates were made by incorporating derivatized primers into the *Saccharomyces cerevisiae HIS3* promoter. The derivatized sites extended from –12 to +10 relative to TATA box, with +1 between the first T and A of that promoter element. This part of the promoter was chosen because it encompasses binding of TBP, TFIIA and TFIIB as shown by cross-linking experiments using yeast TBP, yeast and human TFIIA and human TFIIB on a viral promoter (Lagrange et al., 1996). This region of the promoter contains the possible binding sites predicted from the crystal structures of TFIIA-TBP-DNA (Tan et al., 1996) and TFIIB-TBP-DNA (Nikolov et al., 1995). The modified derivatization sites were four base pairs apart, with six derivatization sites on each strand, resulting in 12 DNA fragments each derivatized at a single, distinct site. The RNA-like strand is designated with an R and the template strand with a T. The derivatized sites are pictured in Fig. 4.1.

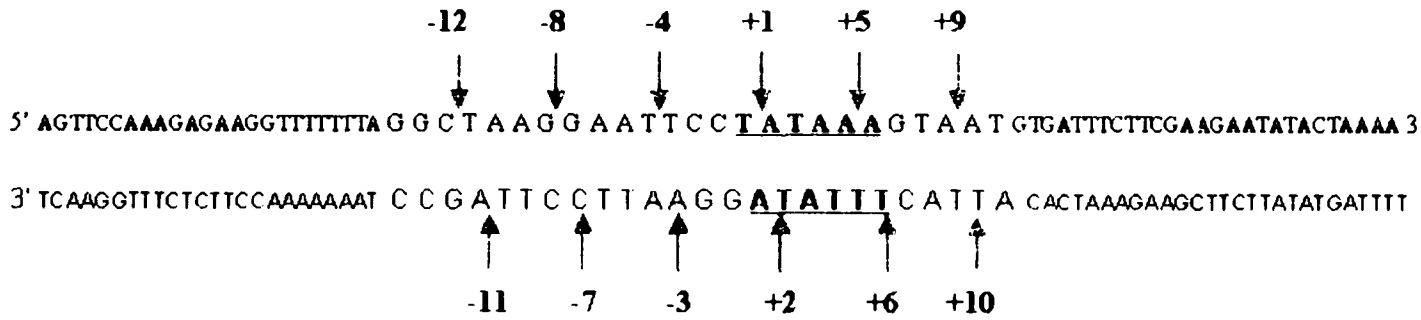

Fig **4.1** Derivatized sites on the HIS3 promoter Red arrows indicate sites examined for protein binding The TATA element is underlined

4.3.2 YEAST PARTIAL INITIATION COMPLEXES

Photo-cross-linking was carried out to examine a number of protein-DNA complexes. A titration was performed with each factor that was used in the cross-linking. The criterion for the amount of protein to be used was that no factor should cross-link to the DNA in the absence of TBP. This is because TFIIA and TFIIB bind to the promoter region specifically only in the presence of TBP. We found a wide range of DNA affinity among the different factors, which will be discussed below. TBP was cross-linked alone to the promoter, then in complexes with TFIIA, miniTFIIA, and TFIIB. We examined cross-linking with each of these complexes in the presence and absence of $TAF_{II}40$ and, separately, the $TAF_{II}40/TAF_{II}19$ heterodimer.

4.3.3 TBP CROSS-LINKING

We cross-linked TBP at +1, where we expected it to be present, and at the same time at −12R or at +9R, under a variety of conditions. When a titration of poly(dGdC) was used as nonspecific competitor, there was no decrease in the cross-linking at any of the sites tested. When poly(dAdT) was used as a competitor at the −12R site, TBP cross-linking was reduced, indicating competition (Fig. 4.2). However, when poly(dAdT) was titrated simultaneously at −12R and +1R, TBP cross-linking

DNA template	-12	-12	-12
Poly(dAdT) ng	10	100	1000
TBP (670 fmol)	+	+	+
	1	2	3

Figure 4.2 Cross-linking of TBP with a titration of poly(dAdT) at the -12 site on the RNA-like strand of DNA. TBP is competed out high concentrations of poly(dAdT) (lane 3).

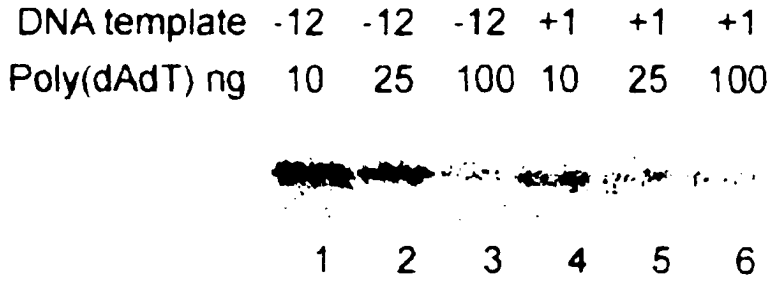

Figure 4.3 Cross-linking of TBP with a titration of poly(dAdT) at the -12 and +1 sites on theRNA-like strand of DNA. TBP is competed at both sites. but the competition from 10 to 100 ng of poly(dAdT) is 4.3-fold at the -12 site and only 2.6-fold at the +1 site.

diminished to the same level at both sites (Fig. 4.3, lanes 3 and 6). However, since the cross-linking at –12R was stronger to begin with (Fig. 4.3, lanes 1 and 4), TBP cross-linking is competed out 4.3 fold at that site and only 2.6 fold at +1R. When we titrated down TBP itself, we found loss of cross-linking to be equal at all sites tested (Fig. 4.4).

Since it is possible that the N-terminal region was causing the cross-linking upstream and downstream of TATA, we repeated the same experiments with coreTBP. CoreTBP is missing the N-terminal domain, but it is known to bind DNA more strongly than full-length TBP. Again, there was no difference in intensity of cross-linking at non-TATA sites than it was at TATA. Indeed, when we used lesser amounts of cTBP in the presence of poly(dAdT), cross-linking was abolished at all sites (Fig. 4.5, lanes 2-4). We included TFIIA in the reactions to see if it would induce stronger TBP cross-linking at TATA, but again found no difference between –12R and +1R (Fig. 4.6). TFIIA, however, cross-links only at –4R on the RNA-like strand (Fig.4.6, lane 4), despite its dependence on TBP for cross-linking.

In order to determine if TBP is recognizing an AT-rich sequence, we compared TBP cross-linking on a yeast template to TBP on a different DNA. We tested the ribosomal RNA promoter from *Acanthamoeba castellanii*, which is GC-rich (Fig. 4.7). The derivatized site on this promoter is at –50R. There is no TATA box here, but TBP cross-links here in the context of TIF-IB, the endogenous pol I

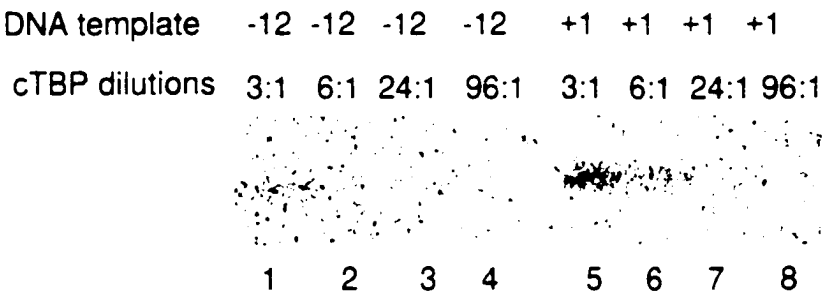

Fig. 4.4 Titration of cTBP. Dilutions of cTBP resulted in loss of cross-linking at all sites. This gel shows weaker cross-linking at -12 than +1, but the results vary from one experiment to another. We could not consistently cross-link cTBP at +1 without also cross-linking it at other sites.

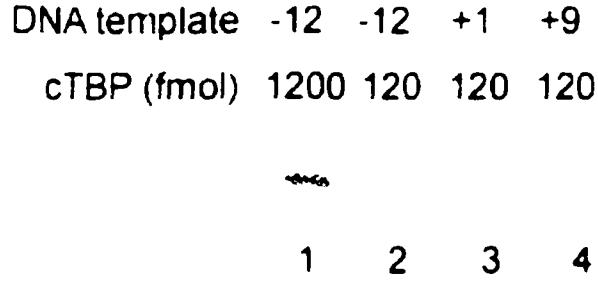

Figure 4.5 Cross-linking of cTBP at -12. +1 and +9 on the RNA-like strand of DNA. cTBP does not cross-link at low concentrations when poly(dAdT) is present (lanes 2.3.and 4).

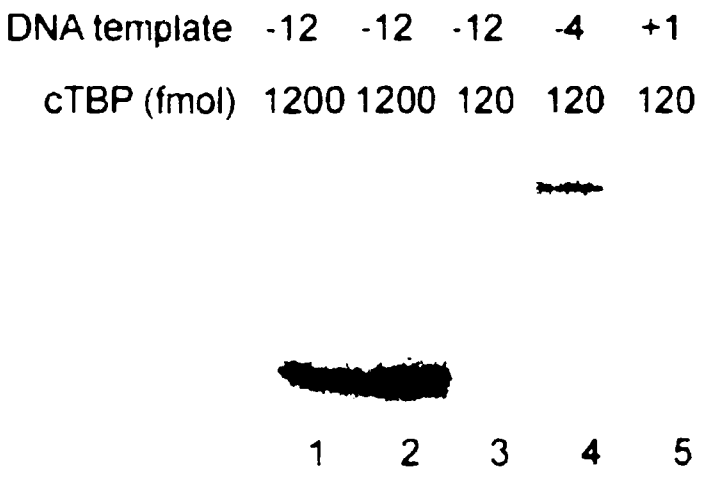

Figure 4.6 Cross-linking of cTBP in the presence of TFIIA with poly(dGdC) as competitor. cTBP cross-links equally well at -12 as it does at +1 (lanes 3 and 5).

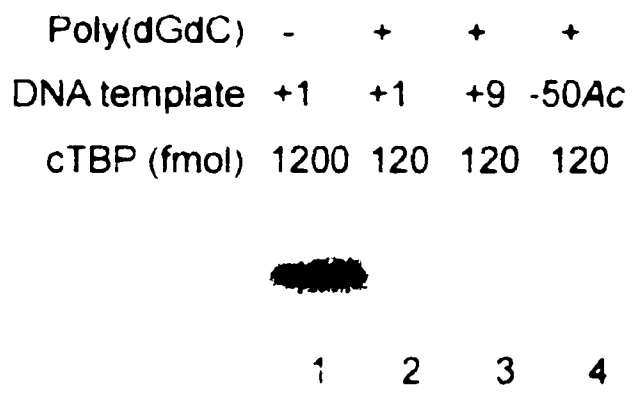

Figure 4.7 Cross-linking with cTBP at +1 and +9 on the RNA-like strand of the yeast template, and -50 on the RNA-like strand of the *Acanthamoeba castellaneii* template. cTBP cross-links equally well at +1 and +9 (lanes 2 and 3), but does not cross-link at -50 of *Acanthamoeba*.

transcription factor. We used poly(dGdC) as a nonspecific competitor, since poly(dAdT) has too strong an affinity for TBP, and abolishes TBP cross-linking at all sites. We found that on the yeast template there was cross-linking at +9R, a TATA-less site (Fig.4.7, lane 3). The cross-linking at +1R was no stronger (Fig.4.7, lane 2). However, under the same conditions, there was no cross-linking of TBP at –50R on the template (Fig. 4.7, lane 4). We believe that the nonspecific cross-linking is a result of the sequence of the DNA rather than an intrinsic property of TBP itself. TBP prefers AT-rich sequences, and it appears that the yeast *HIS3* promoter is so AT-rich that TBP cross-links everywhere. This does not mean that TBP is binding stably at all these sites, but rather that it is at least temporarily in the vicinity of DNA. The fact that TFIIA cross-links at only one site on the RNA-like strand, despite promiscuous TBP cross-linking, gives us reason to believe that the TBP-TATA complex at this position is different from the interaction that may take place with TBP and DNA at a non-TATA site.

Thus, TBP was found to cross-link at every site tested, from –12 to +9 on the RNA-like strand (-12R and +9R), and from –11 to +10 on the template strand (-11T and +10T) (Fig. 4.8-4.13, lanes 1-8). Since we expected cross-linking only in the vicinity of the TATA-box (+1 to +6), as was found with yeast TBP on a viral promoter, we performed control experiments to rule out nonspecific cross-linking.

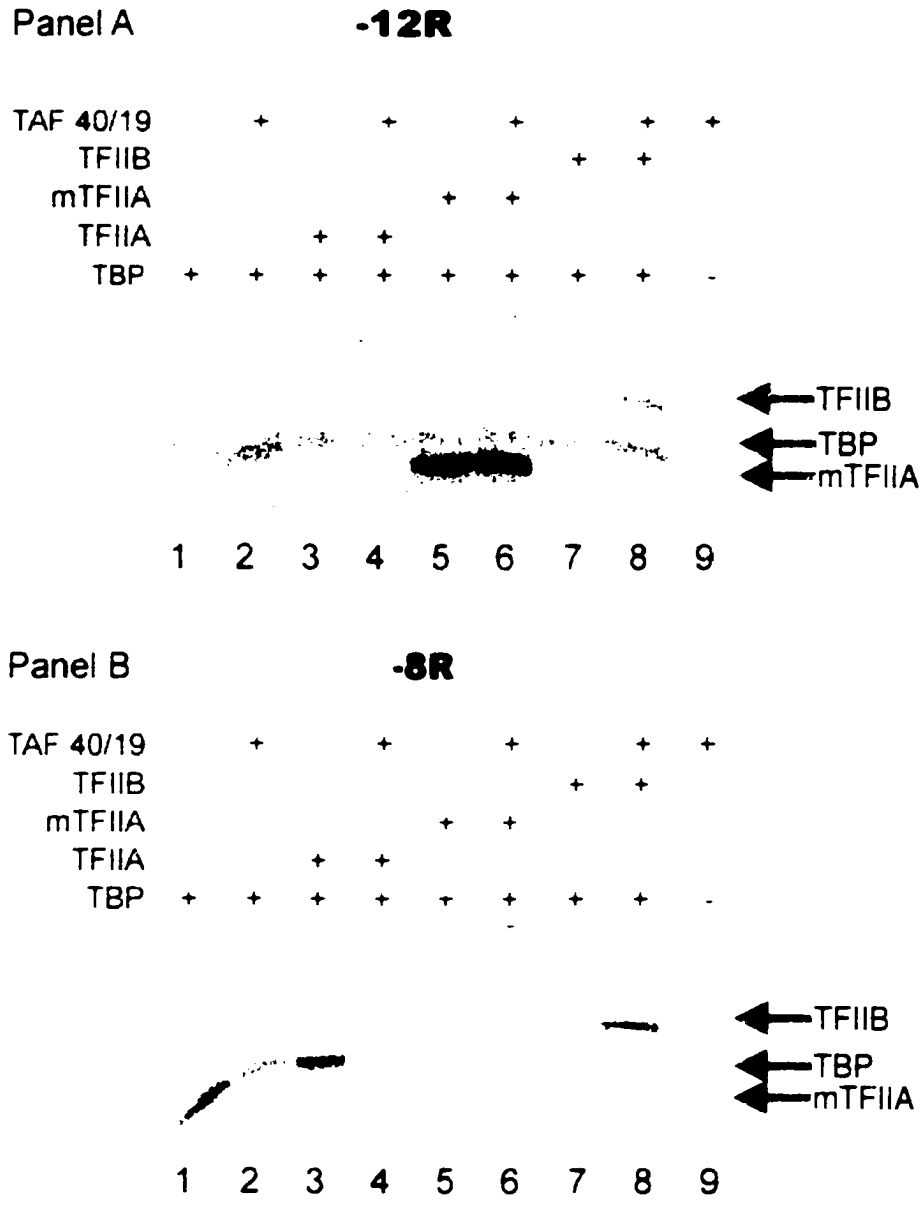

Fig. 4.8 Cross-linking of derivatives -12 and -8 on the RNA-like strand of DNA with the proteins indicated. In both panels TBP cross-links at every tested site. MiniTFIIA cross-links in lanes 5 and 6. TFIIB cross-linkswith the addition of TAF40/TAF19 at both sites (panels A and B, lane 8).

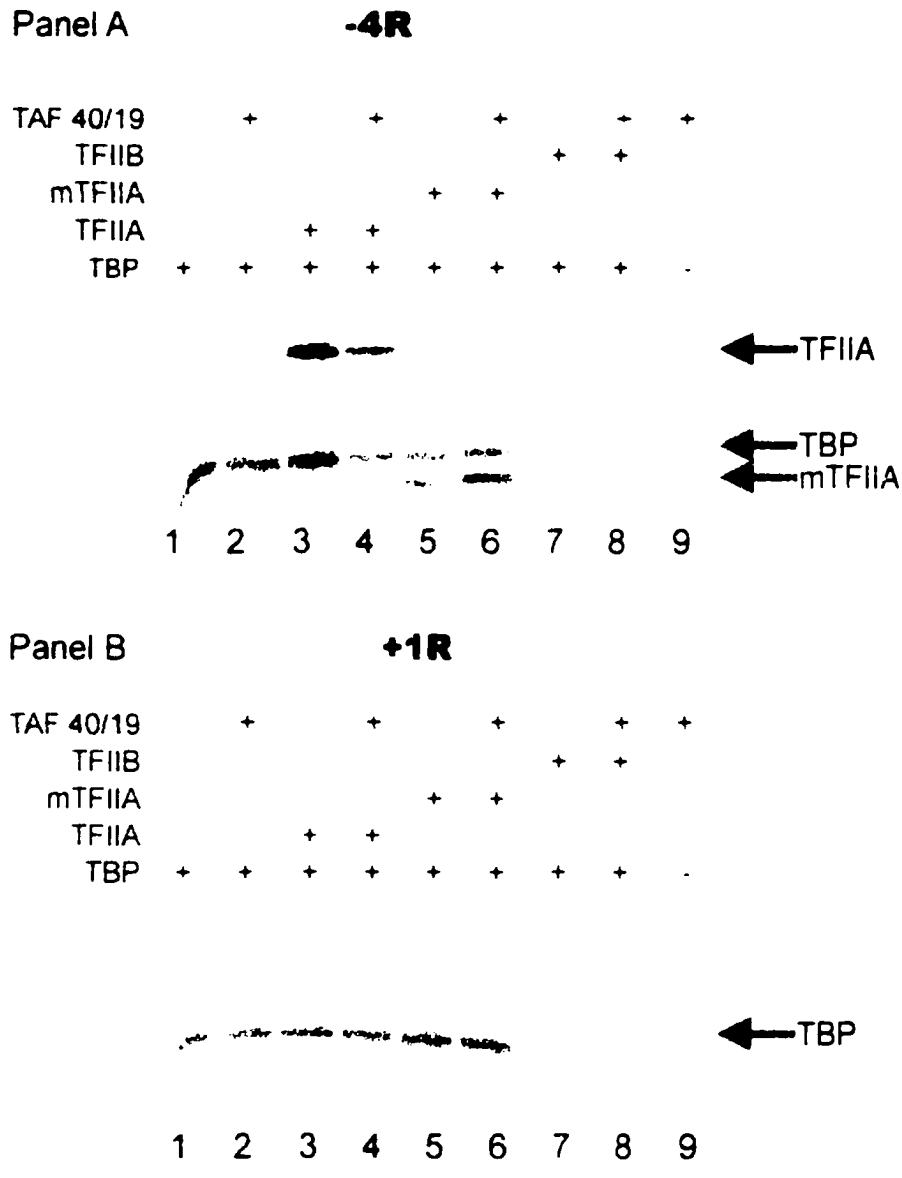

Fig. 4.9 Cross-linking of derivatives -4 and +1 on the RNA-like strand of DNA with the proteins indicated. In both panels TBP cross-links at every tested site. TFIIA cross-links at -4R in panel A, lanes 3 and 4. MiniTFIIA cross-links in lanes 5 and 6. TFIIB does not cross-link at either site (panels A and B, lanes 7 and 8)

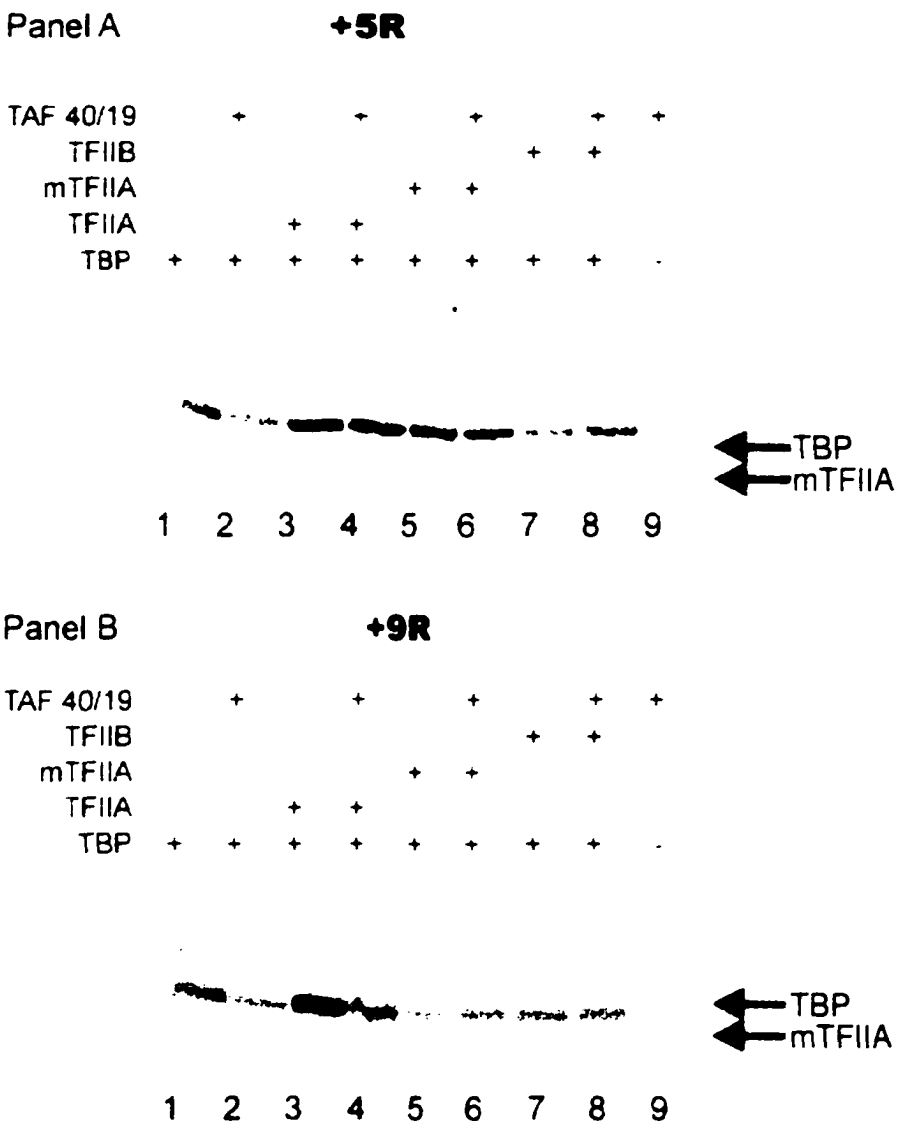

Fig 4.10 Cross-linking of derivatives +5 and +9 on the RNA-like strand of DNA with the proteins indicated. In both panels TBP cross-links at every tested site. MiniTFIIA cross-links weakly in both panels, lanes 5 and 6. TFIIB does not cross-link at either site (panels A and B, lanes 7 and 8).

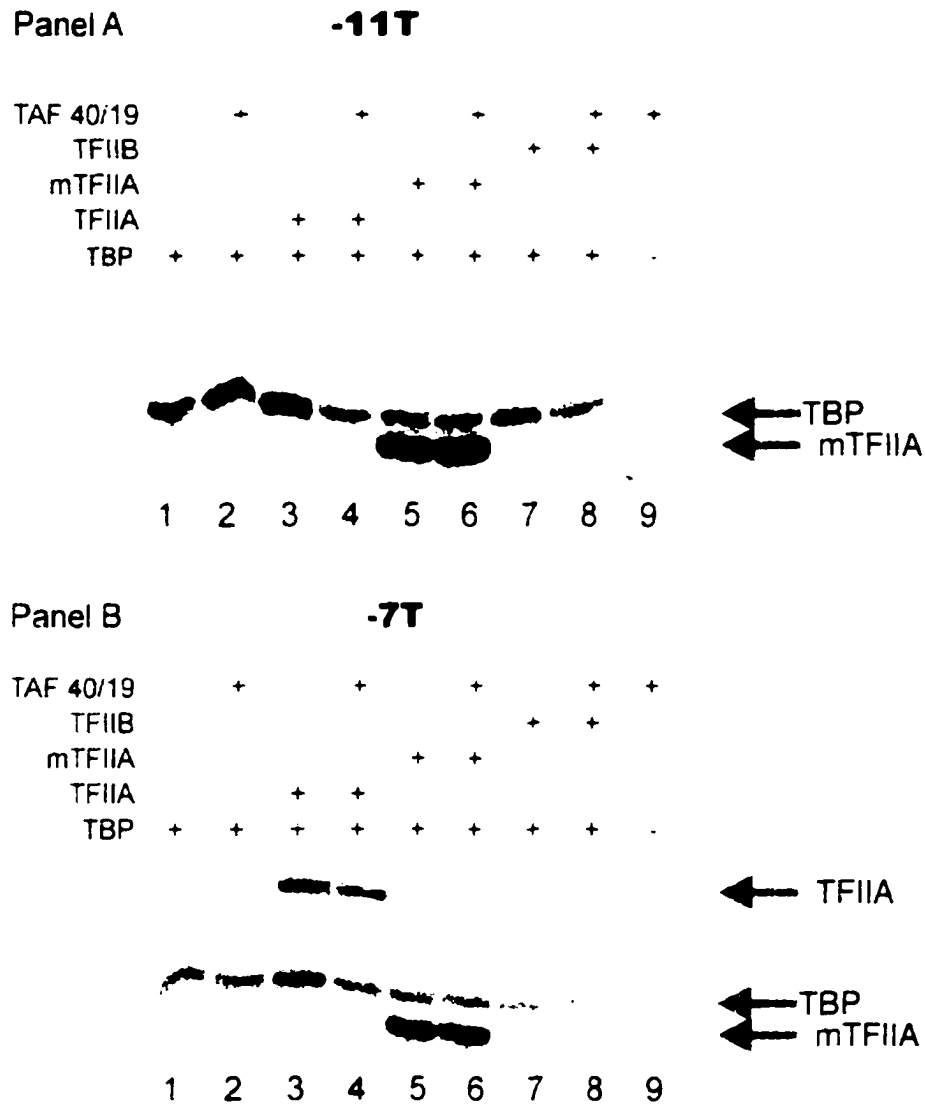

Fig. 4.11 Cross-linking of derivatives -11 and -7 on the template strand of DNA with the proteins indicated. In both panels TBP cross-links at every tested site. TFIIA cross-links strongly at -7T (panel B, lanes 3 and 4) and very weakly at -11T. MiniTFIIA cross-links strongly in both panels, lanes 5 and 6. TFIIB cross-links very weakly with the addition of TAF40/TAF19 at both sites (panels A and B, lane 8).

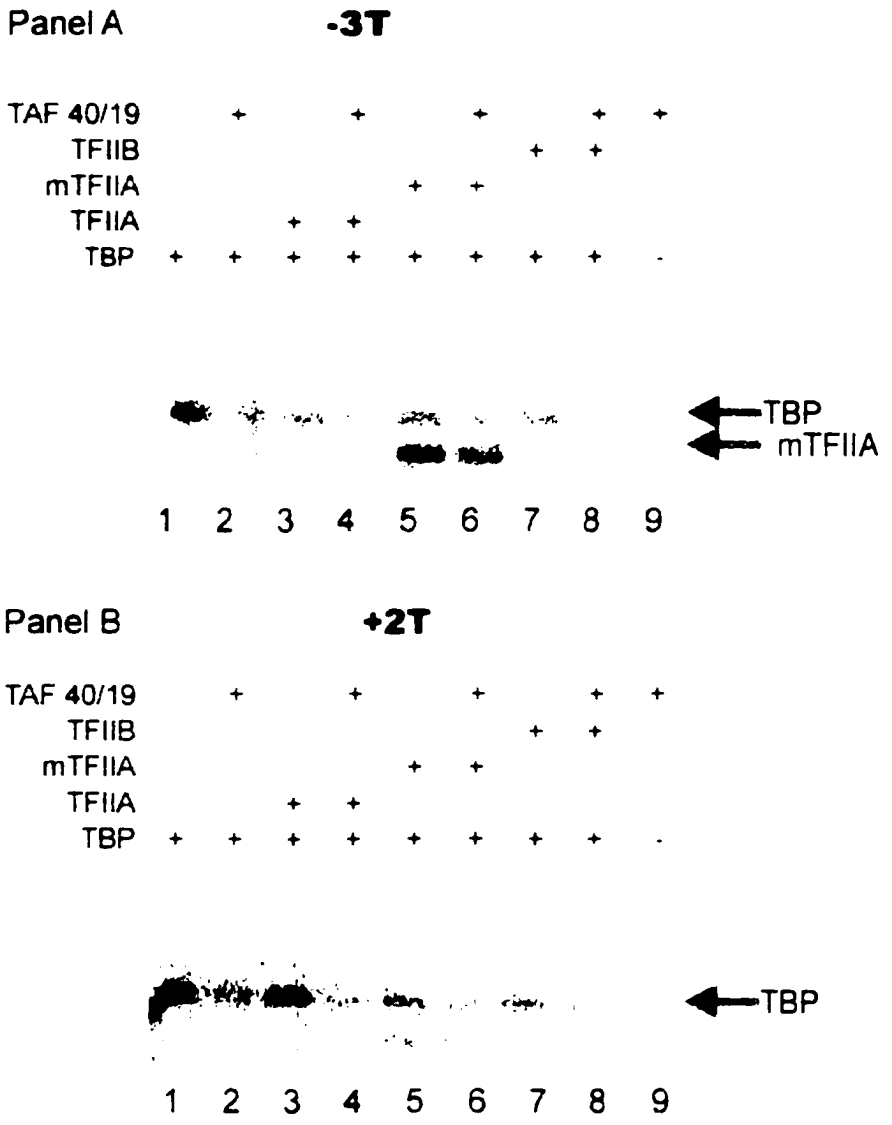

Fig. 4.12 Cross-linking of derivatives -3 and +2 on the template strand of DNA with the proteins indicated. In both panels TBP cross-links at every tested site. MiniTFIIA cross-links strongly at -3T and weakly at +2T (panels A and B, lanes 5 and 6). No other proteins cross-link at these sites.

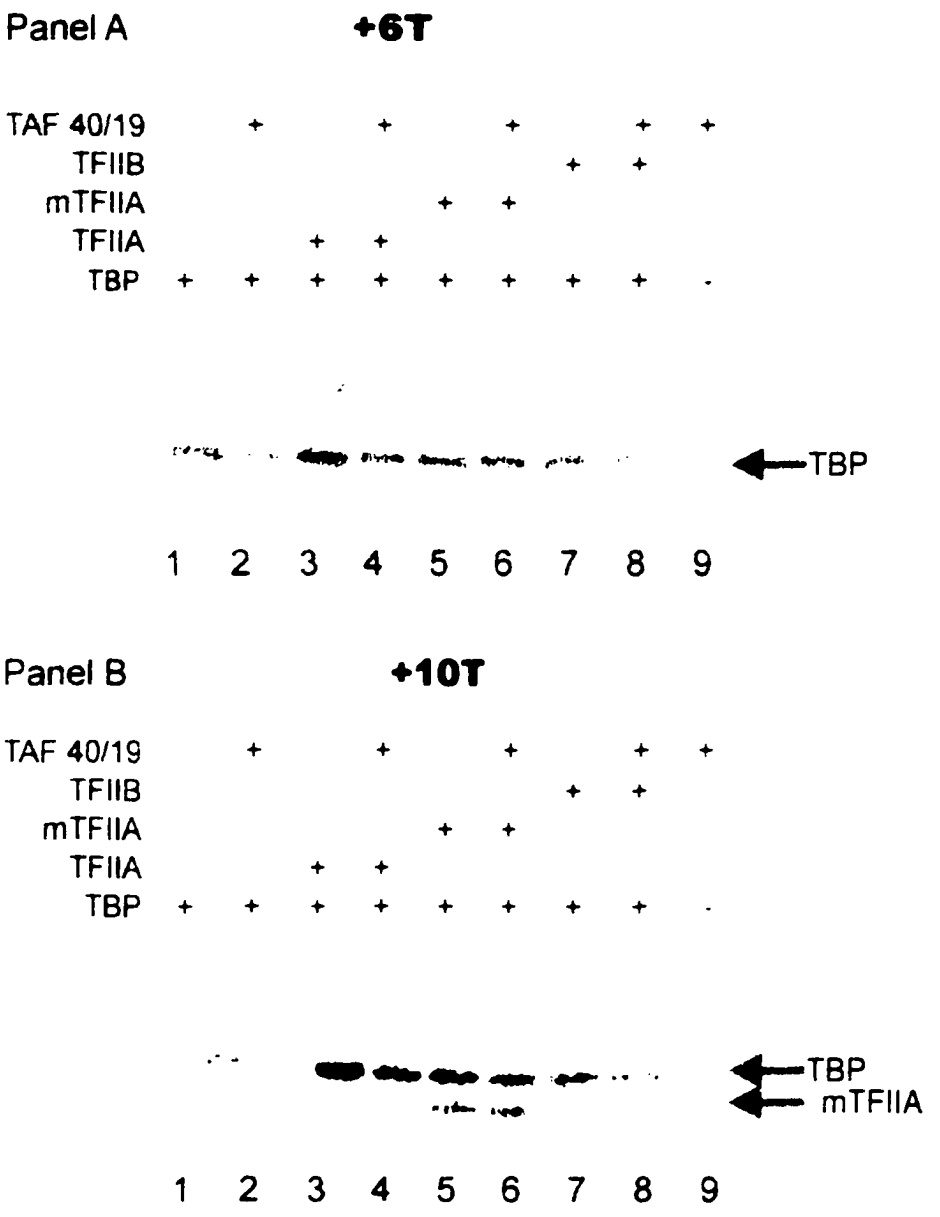

Fig. 4.13 Cross-linking of derivatives +6 and +10 on the template strand of DNA with the proteins indicated. In both panels TBP cross-links at every tested site. MiniTFIIA cross-links strongly at +10T (panel B, lanes 5 and 6).
No other proteins cross-link at these sites.

4.3.4 TFIIA, MINITFIIA AND PHOSPHORYLATED TFIIA CROSS-LINKING

The cross-linking experiments with TFIIA are shown in lanes 3 and 4 in figures 4.8 through 4.13. Lane 3 shows TFIIA cross-linking with TBP alone, while lane 4 reactions also contain the $TAF_{II}40/TAF_{II}19$ heterodimer. Toa1 of TFIIA was found to cross-link strongly at –4R and –7T on the DNA (Fig. 4.9 panel A and Fig. 4.11 panel B). Toa2 cross-linking could not be clearly identified on any of the gels. Toa1 does not cross-link in the absence of TBP, even at high concentrations (Fig. 4.14). Since TBP does cross-link everywhere, TFIIA must either recognize a particular sequence of DNA, or a certain conformation of TBP that exists only on the TATA box.

MiniTFIIA, a truncated version of TFIIA missing a nonconserved region of Toa1 (Geiger et al., 1996), was cross-linked at every site as well. These results are shown in lanes 5 and 6 in figures 4.8 through 4.13. MiniTFIIA experiments were done for 2 reasons. To determine if the missing region, which contains phosphorylation sites (Solow et al., 1999), was involved in TFIIA's recognition of TBP or the DNA. We were also interested in examining $TAF_{II}40$ cross-linking, and since Toa1 cross-linked to DNA electrophoreses at the same molecular weight as $TAF_{II}40$, we used the truncated form of Toa1 as well as full length Toa1 in the $TAF_{II}40$ and $TAF_{II}40/TAF_{II}19$ experiments. We found that miniTFIIA does not have the specificity of full length TFIIA. MiniTFIIA, seen in lanes 5 and 6 in all

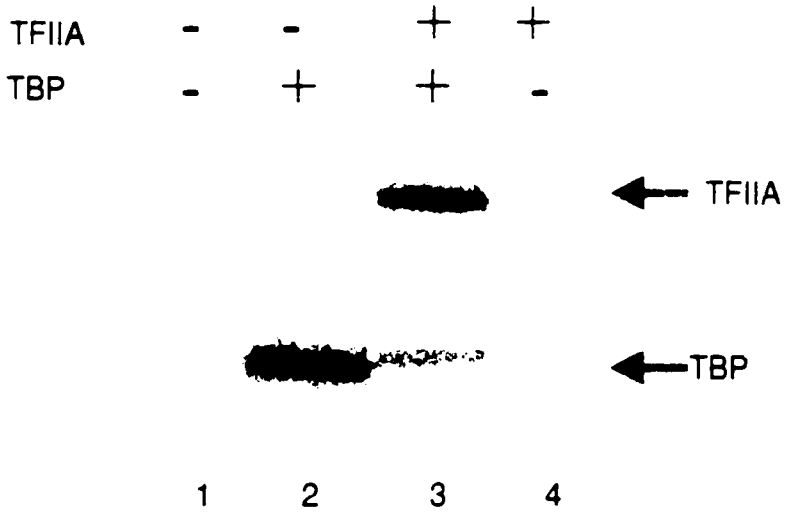

Fig. 4.14 TFIIA cross-linking at -4R in the presence and absence of TBP. TFIIA does not cross-link in the absence of TBP.

the gels, cross-linked strongly at –12R (Fig.4.8 panel A), -11T, –7T (Fig. 4.10, panels A and B) and –3T (Fig. 4.11 panel A). MiniTFIIA cross-linked with about the same intensity as TBP at –8R (Fig. 4.9 panel A), -4R (Fig.4.9 panel A) and +10R (Fig. 4.12 panel B). At all other sites, cross-linking was weak, but visible with phosphorimage enhancement. Since miniTFIIA did not cross-link in the absence of TBP, it appears that miniTFIIA binds TBP whether or not TBP is on the TATA box.

Phosphorylation has been shown to enhance TBP-TFIIA-DNA interactions (Solow et al., 1999). Thus, we were curious about whether the pattern of TFIIA cross-linking would change if TFIIA were phosphorylated. These experiments showed the exact same TFIIA cross-linking as those with full-length unphosphorylated TFIIA (4.15).

4.3.5 TFIIB CROSS-LINKING

TFIIB appears to have high affinity for DNA. It cross-linked strongly with and without TBP in preliminary experiments (4.16). However, when diluted appreciably, TFIIB cross-linked reproducibly only in the presence of TBP and only at specific sites. These results are presented in lanes 7 and 8 in all gels. Lane 7 shows TFIIB cross-linking with TBP and lane 8 reactions contain the $TAF_{II}40/TAF_{II}19$ heterodimer as well. TFIIB cross-linked at –12R and –8R (Fig. 4.8 panels A and B) and at –11T (Fig. 4.11 panel A). There appears to be no TFIIB cross-linking downstream of the TATA box.

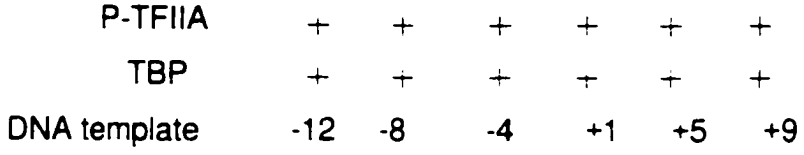

Fig. 4.15 Cross-linking with phosphorylated TFIIA on the template strand. P-TFIIA cross-linking is identical to that of unphosphorylated TFIIA.

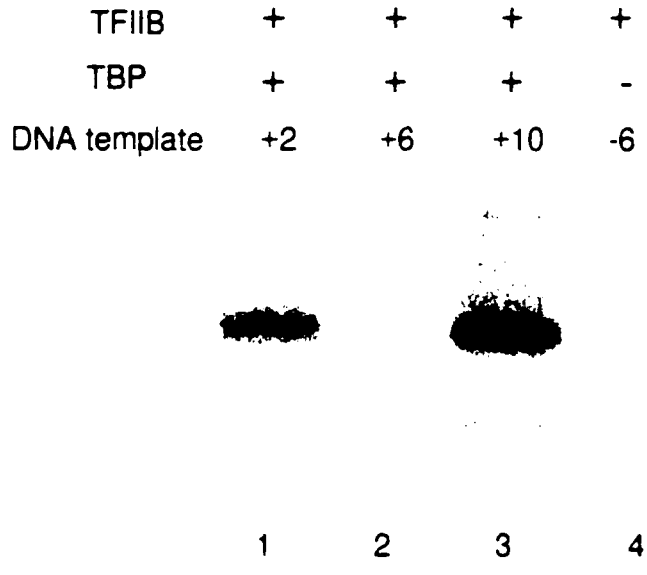

Fig. 4.16 TFIIB cross-linking. TFIIB cross-links even in the absence of TBP. When diluted, its cross-linking becomes specific.

4.3.6 $TAF_{II}40$ AND $TAF_{II}40/TAF_{II}19$ CROSS-LINKING

$TAF_{II}40$ was cross-linked with DNA alone, TBP, TBP and TFIIA, TBP and miniTFIIA, and TBP and TFIIB. $TAF_{II}40$ did not cross-link at any site, even at high concentrations relative to the other factors (Fig.4.17). The cross-linking with $TAF_{II}40/TAF_{II}19$ was very different. Both proteins of the heterodimer cross-link with DNA alone as well as the other factors do. When $TAF_{II}40/TAF_{II}19$ is titrated down so that it no longer cross-links with DNA alone, it ceases to cross-link in the presence of any other factors as well.

$TAF_{II}40/TAF_{II}19$ does not change TBP cross-linking, but when it is added to reactions containing TBP and TFIIB, it increases TFIIB cross-linking up to 40-fold (lane 8 in Fig. 4.8 panels A and B and Fig.4.11 panel A). When $TAF_{II}40/TAF_{II}19$ is added to TBP plus TFIIA or miniTFIIA, their cross-linking does not intensify. TFIIA cross-linking actually appears diminished, but it actually does not change when it is quantified relative to TBP cross-linking.

4.4 DISCUSSION

We have used site-specific photo-cross-linking to map partial RNA pol II transcription preinitiation complexes on the yeast *HIS3* promoter (Fig. 4.18). Our studies include TBP, TFIIA, miniTFIIA, phosphorylated TFIIA, TFIIB, $TAF_{II}40$

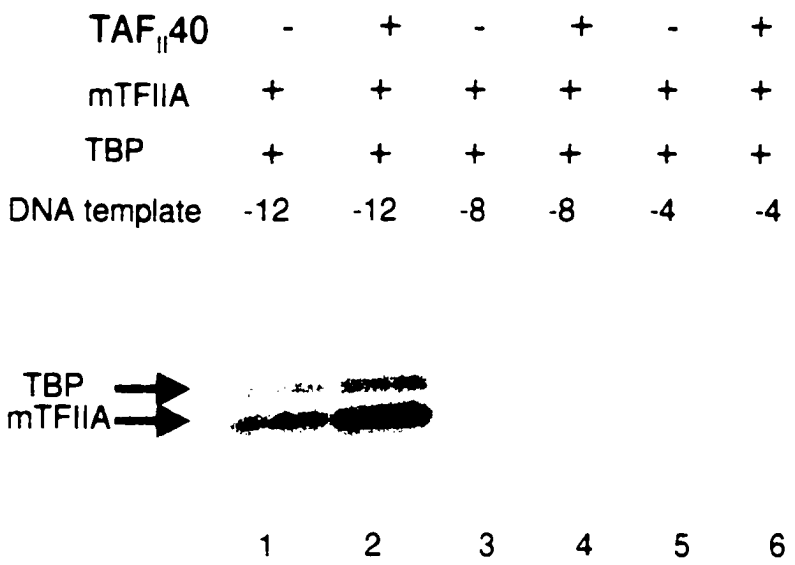

Fig. 4.17 TAF$_{II}$40 cross-linking. TAF$_{II}$40 does not cross-link at any site, with any combination of factors.

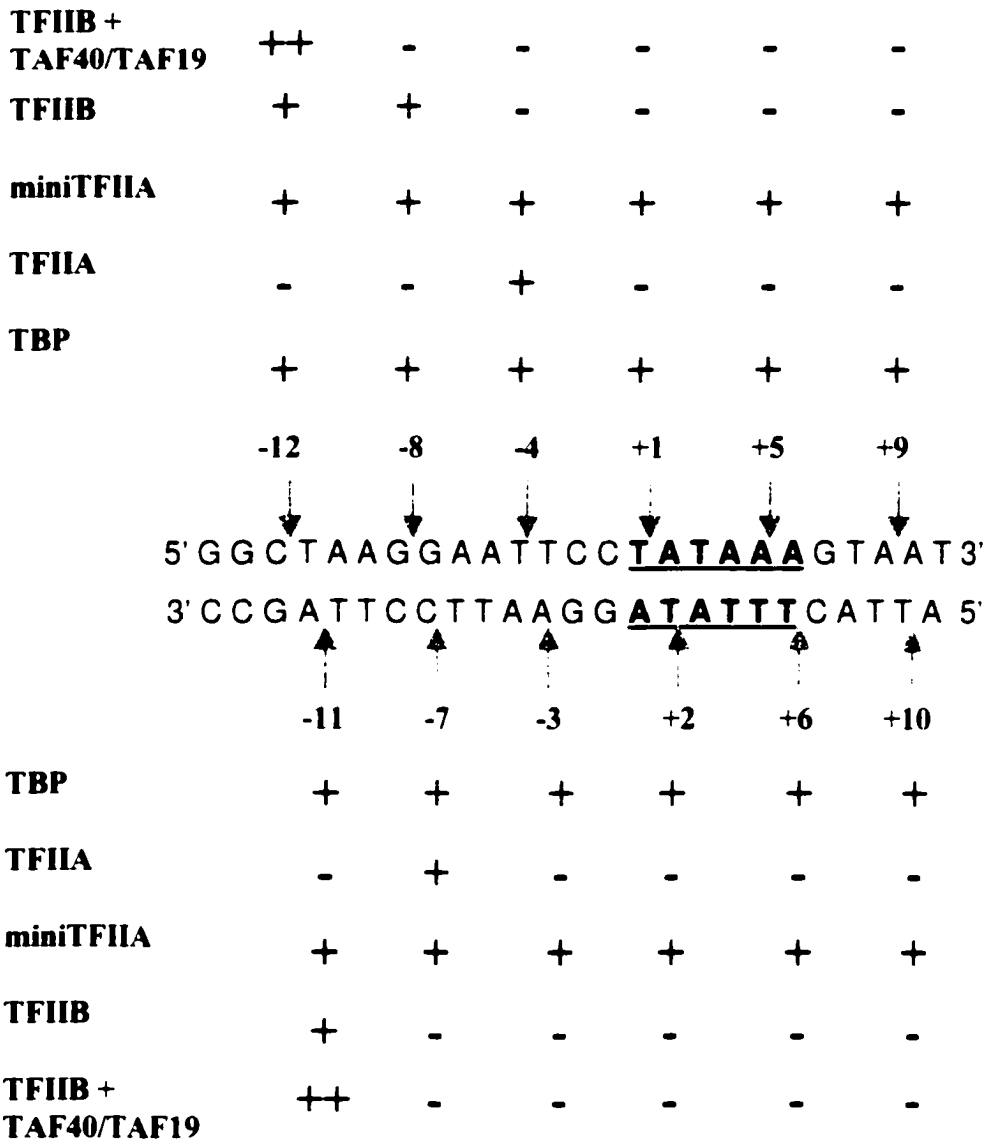

Fig. 4.18 Compilation of cross-linking data from yeast transcription factors on the *HIS3* promoter.

and the $TAF_{II}40/TAF_{II}19$ heterodimer. The cross-linkings not only indicate which proteins are in the vicinity of the DNA, but also how the addition of some proteins affects the cross-linking of others.

4.4.1 TBP

TBP cross-linking appeared at every site tested on both DNA strands, from –12 to +11 relative to the TATA box. When yeast TBP was cross-linked on a viral promoter, TBP crossed linked only at the TATA box (Lagrange et al., 1996), leading us to expect the same result. We tested TBP cross-linking with titrations of different competitors and titrations of TBP itself at sites near TATA and farther upstream and downstream of that element. In the end, we could not lose TBP cross-linking away from TATA without also losing cross-linking at TATA. The only indication that TBP binds more strongly on the TATA box than elsewhere is that cross-linking at –12, where we do not expect to find TBP, is competed out relatively more than at +1. The competitor used was poly(dAdT), which behaved as a specific competitor, completely eliminating cross-linking at high concentrations.

The sequence of the *HIS3* promoter is extremely AT-rich, whereas the viral promoter used by Lagrange *et al.* is not. We believe that TBP binds at least fleetingly to AT-rich sequences, which is in agreement with results noted by others. This binding is probably not stable enough for nucleation of transcription complexes, as a mutated TATA box can not support high levels of transcription *in vivo* (Stewart and Stargell, 2001).

TBP appears to bind long enough, however, to be captured in photo-cross-linking, in which a covalent bond is formed instantaneously between DNA and protein. Thus we believe that the TBP cross-linking we see at sites other than the TATA box is the result of nonspecific interaction with an AT-containing DNA sequence. We believe that the TBP-TATA cross-linking is specific because of the pattern of cross-linking of the other factors, discussed below.

4.4.2 TFIIA, MINI TFIIA AND PHOSPHORYLATED TFIIA

TFIIA requires TBP in order to bind specifically. TFIIA cross-linking, at –4R and –6T, agrees perfectly with yeast TBP cross-linking done previously (Lagrange et al., 1996), where TBP cross-linked only at TATA. There are two possibilities for why TFIIA cross-links so specifically while TBP cross-links everywhere. One is that TFIIA is recognizing a sequence of the promoter and thus being recruited to the DNA. This, however, is not likely, since TFIIA does not cross-link in the absence of TBP. Another possibility is that TBP changes conformation when it binds DNA, but only at the TATA box. TFIIA is recognizing this different conformation of TBP, while it does not recognize TBP when it is located elsewhere. *In vivo*, it is most likely that TBP does not bind DNA stably enough at nonconsensus TATA sites for TFIIA to be recruited. Finally, it is possible that TFIIA is recruited by the stable TBP-TATA complex and then also recognizes the DNA sequence in that region of the promoter, stabilizing its own binding.

We do not have a way of distinguishing this possibility using the current experiments.

We cross-linked MiniTFIIA, which is missing a nonconserved region of Toa1. We were surprised to find that miniTFIIA cross-links at almost every site, along with TBP, while full-length TFIIA cross-linked at only two sites. It is likely that the missing region contains elements that are necessary for specific recognition of TBP on the TATA box. Since there are several phosphorylation sites on that part of TFIIA, we performed cross-linking with phosphorylated TFIIA. We were interested in whether phosphorylation may be a point of regulation for TFIIA recruitment. Our results, however, do not support this hypothesis, since the cross-linking with phosphorylated TFIIA are identical to those with full length TFIIA.

4.4.3 TFIIB

TFIIB was found to cross-link at –12R, -8R and –11T. These sites are just upstream of where TFIIA cross-linked. Previous cross-linkings, which had been done with human TFIIB, showed that it cross-linked at numerous sites both upstream and downstream of TATA (Lagrange et al., 1996). However, human TFIIA also cross-linked at numerous sites, whereas yeast TFIIA did not. Therefore, we believe our results to be compatible with what should be expected for the yeast factors.

TFIIB also cross-linked the DNA alone quite strongly unless it was diluted to about one eighth the concentration of TFIIA. At that point, weak

TFIIB cross-linking was seen only in the presence of TBP, which is our criterion for specific cross-linking. However, with the addition of $TAF_{II}40/TAF_{II}19$, cross-linking increased dramatically, underscoring the importance of the TFIID complex.

4.4.4 $TAF_{II}40$ AND $TAF_{II}40/TAF_{II}19$

In Mary's experiments, the addition of $TAF_{II}40$ to TFIIA resulted in an increased TFIIA-DNA band in an EMSA. It did not, however, change the mobility of the complex. We decided to add $TAF_{II}40$ to our cross-linkings to see if it either cross-links to the DNA or changes the TFIIA and TFIIB cross-linkings. We were surprised to find that it did neither. When we used the $TAF_{II}40/TAF_{II}19$ heterodimer in the same reactions, the results with TFIIB were dramatic. TFIIB cross-linking increased up to 40-fold.

CHAPTER 5

FUTURE DIRECTIONS

The *Acanthamoeba* RNA polymerase I transcription system has been very well characterized by biochemical assays. The current photo-cross-linking experiments are in agreement with what has been found about TIF-IB and TIF-IE by footprinting. Pol I cross-linking is in agreement with what can be discerned from previous footprinting experiments. One more experiment that could add to our knowledge of *Acanthamoeba* pol I is uranyl nitrate footprinting. Since uranyl nitrate is a small molecule, it may be able to give us higher resolution for pol I footprinting at the overlap with TIF-IB. We would expect such data to agree with our cross-linking results.

Photo-cross-linking experiments would also be useful in examining the yeast pol I transcription system. The yeast pol I factors have all been cloned. Cathy Radebaugh in our lab, as well as many other groups, are examining the interactions of those factors using yeast genetics. If we could cross-link those factors to the DNA, not only would we learn more about yeast factor interactions with the DNA, but we would also be able to compare the yeast system with the *Acanthamoeba* system.

The future experiments that could be done with the yeast pol II system are endless. Cross-linking could be done with any combination of factors. Ideally, an entire transcription complex on a yeast promoter could be analyzed. However, such an enormous project is beyond the interest of most labs, since the pol II

system contains so many factors. The present project could be expanded to include cross-linking with TFIIA, TFIIB and $TAF_{II}40/TAF_{II}19$ together on the promoter. Footprinting experiments will be done to look for enhancement of TFIIA and TFIIB binding by $TAF_{II}40/TAF_{II}19$, which can then be compared to the enhancement we saw in the cross-linking.

Although the information that can be obtained from cross-linking experiments is limited, as it is from any other single assay, it is a valuable biochemical tool that, in conjunction with other assays, can tell us a great deal about specific protein-DNA interactions and the structure of the transcriptional complexes.

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