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DISSERTATION

PURIFICATION AND CHARACTERIZATION OF TFIIIA FROM *ACANTHAMOEBA CASTELLANII*

Submitted by

Nicholas Polakowski

Department of Biochemistry and Molecular Biology

In partial fulfillment of the requirements for

the degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Fall 2001

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November 5, 2001

WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY NICHOLAS POLAKOWSKI ENTITLED *PURIFICATION AND CHARACTERIZATION OF TFIIIA FROM ACANTHAMOEBA CASTELLANII* BE ACCEPTED AS FULFILLMENT IN PART THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

Committee on Graduate Work

Advisor

Department Head

ABSTRACT OF DISSERTATION

PURIFICATION AND CHARACTERIZATION OF TFIIIA FROM *ACANTHAMOEBA CASTELLANII*

Our interest has been in studying the mechanisms of RNA polymerase III (Pol III) transcription and the regulation of this process in *A. castellanii*, which was specifically focused on TFIIIA. This factor only activates Pol III-mediated transcription from 5S RNA genes and is the first of three general Pol III-specific transcription factors that is assembled onto the DNA to form the preinitiation complex. Previous work in our laboratory has shown that *A. castellanii* TFIIIA is under direct regulatory control. During the process of *A. castellanii* encystment, both ribosomal RNA (rRNA) and 5S RNA transcription are coordinately down-regulated. The loss of 5S RNA synthesis is associated with the disappearance of TFIIIA activity, and the loss of rRNA synthesis is caused by a modification of polymerase I or a tightly associated factor that is reversible. Based on the mechanism of Pol I regulation, we suspect that TFIIIA is regulated in a similar fashion. Our primary objective was, therefore, to determine if TFIIIA remained present in cysts by using antibodies. Because TFIIIA is not evolutionarily conserved in its primary sequence, antibodies raised against other homologues of this factor have shown little or no cross reactivity with *A. castellanii* TFIIIA. We have, therefore, purified this protein as the preliminary step in elucidating its regulatory control. This factor was found to have a size of 59 kDa, which is significantly larger than all other known TFIIIA homologues. It was also found to bind to and footprint the DNA promoter in a manner more similar to TFIIIA from vertebrates.

To compare the mechanisms that regulate Pol III-mediated transcription in different organisms, we have also examined Pol III-mediated transcriptional regulation in yeast. During stationary phase, Pol III transcription is down-regulated, but the mechanisms involved in the process are not well understood. In studying tRNA synthesis, we have found that *in vivo* observations diverge from what is seen *in vitro*. Specifically, Pol III-mediated transcriptional repression is much more severe *in vivo*. This illustrates that transcriptional regulation cannot be completely recapitulated from yeast whole-cell extracts. However, we did identify an inhibitor that specifically decreases the ability of extracts to support Pol III-mediated transcription. The action of this inhibitor may complement other mechanisms involved in down-regulating Pol III-mediated transcription.

Nicholas Polakowski
Department of Biochemistry
and Molecular Biology
Colorado State University
Fort Collins, CO 80523
Fall 2001

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Chapter 1:

5S RNA Transcription and the Gene-Regulatory Factor, TFIIIA

1.1 The 5S RNA Gene Promoter

The structure of the 5S RNA gene promoter has been exquisitely characterized due to the early development of *in vitro* transcription systems using this gene as a template and the ease of obtaining the gene-specific factor, TFIIIA, from *X. laevis* oocytes (Engelke *et al.*, 1980; Parker and Roeder, 1977). Because the promoter is mainly involved in TFIIIA binding, this has been used as a criterion, in addition to transcriptional activation, in defining the promoter (Sakonju *et al.*, 1981). The boundaries of the 5S RNA gene promoter were first identified by deletion mutagenesis (Bogenhagen *et al.*, 1980; Sakonju *et al.*, 1980). Interestingly, the minimal promoter was found to occupy a region within the transcribed portion of the gene, from +50 to +83 with respect to the transcription initiation site at +1 (Fig. 1.1). Although this stretch of DNA was able to promote transcription, it lacked downstream sequence critical for TFIIIA binding. Methylation and ethylation interference experiments showed that residues +70 to +91 of the 5S RNA gene were found to be involved in tight binding with TFIIIA (Sakonju and Brown, 1982), extending the ICR to +91. Pieler *et al.* (1985) later delineated the 3'-boundary of the ICR even farther downstream, to +97, as C to T transitions in this region of the gene diminished transcription. These effects were not as dramatic as those originating

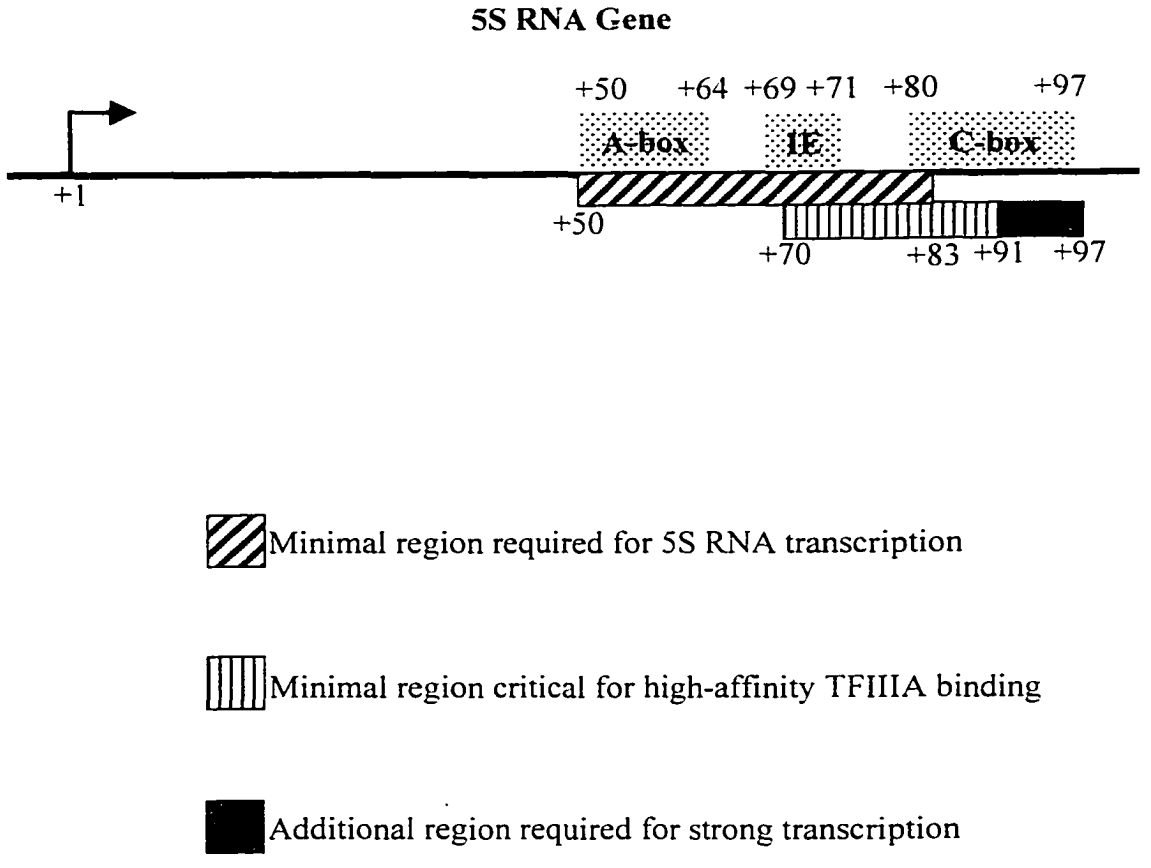

Fig. 1.1. The 5S RNA gene promoter. The promoter is located within the 121 base pair, transcribed portion of the gene and consists of three *cis*-elements: the A-box, the intermediate element (IE), and the C-box. The positions of the *cis*-elements are shown. The minimal promoter boundaries, initially defined by truncating the gene and assaying for transcription, extend from +50 to +83 with +1 denoting the transcription start site as shown. The region denoted as the high-affinity TFIIIA binding site was identified by alkylation interference experiments. Mutations in the promoter that decreased transcription identified the 3'-extension of the promoter that is required for strong transcription. Further site-directed and cassette mutagenesis delineated the three *cis*-elements.

from changes in the DNA slightly upstream, but were, nonetheless, viewed as relevant. With random transition mutants, and methylation interference experiments the important bases were mainly localized at both the 3'- and 5'-boundaries of the promoter (Pieler *et al.*, 1985a; Sakonju and Brown, 1982). Furthermore, linker DNA used to replace sequences within the central region of the promoter produced transcriptionally active templates as long as the overall sequence length was maintained (Bogenhagen, 1985; Pieler *et al.*, 1985b). These results established that two *cis*-elements were located at the upstream and downstream boundaries of the promoter. The two elements have been dubbed the A-box, which is positioned from +50 to +64 on the 5S RNA gene, and the C-box from +80 to +97 (Fig. 1.1). An additional promoter element, extending from +69 to +74, was identified later and termed the intermediate element (Pieler *et al.*, 1987). It had not been detected by linker-scanning mutagenesis as the critical base pairs of this element were consistently maintained in the linker substitutions. The C-box is the only element essential for TFIIIA binding (Pieler *et al.*, 1985b) although the intermediate element also contributes to the stability of the TFIIIA-DNA complex (Pieler *et al.*, 1987). Because TFIIIA makes extensive contacts with the DNA, the intermediate element is additionally thought to position the protein properly over the A-box (Pieler *et al.*, 1987). The A-box is only weakly associated with TFIIIA, but is critical for transcription activation and start site selection (Ciliberto *et al.*, 1983; Pieler *et al.*, 1987). This organization of the 5S RNA gene promoter is conserved among vertebrates and potentially in *A. castellanii* (Geiduschek and Tocchini-Valentini, 1988; Zwick *et al.*, 1991b).

In *Saccharomyces cerevisiae*, the 5S RNA gene promoter structure has been found to vary from that of *X. laevis*. Deleting from the ends of the gene showed that the minimal region require for robust and specific transcription was located from +55 to +99 (Taylor and Segall, 1985). However, cassette mutagenesis and internal

deletions of the gene revealed that, within the minimal promoter, only base pairs +81 to +91 were essential for transcription, which coincides with the C-box found on 5S RNA genes of higher eukaryotes (Challice and Segall, 1989). In comparison to the C-box found on the *Xenopus* gene, five base pair differences are found in the C-box of the *S. cerevisiae* 5S RNA gene. However, methylation interference experiments have shown that, as in higher eukaryotes, the C-box in yeast is critical for the binding of TFIIIA (Challice and Segall, 1989). Interestingly, the consensus A-box sequence (five base pair differences from the A-box of the *Xenopus* somatic gene) is not required for transcription.

In addition to the C-box a *cis*-element encompassing the transcription start site has been identified on the *S. cerevisiae* gene. This promoter element was found to augment transcription (Challice and Segall, 1989) and has been proposed to have a direct role in Pol III recruitment and/or to facilitate DNA melting during initiation of transcription (Geiduschek and Kassavetis, 1992). The spacing between the cis-element at the transcription start site and the C-box is essential for maximal 5S RNA transcription (Challice and Segall, 1989).

1.2 Formation of the Preinitiation Complex on 5S RNA Genes

A preinitiation complex composed of three transcription factors utilized by Pol III is assembled on the 5S RNA gene prior to the recruitment of the polymerase. The three transcription factors in the preinitiation complex are TFIIIA, TFIIIB, and TFIIIC (Segall *et al.*, 1980). Using template commitment assays, the order in which factors are assembled onto the 5S RNA gene promoter has been shown to be TFIIIA, TFIIIC, and TFIIIB (Fig. 1.2) (Bieker *et al.*, 1985; Setzer and Brown, 1985). TFIIIA is the first factor to bind to the DNA (Lassar *et al.*, 1983), and in *Xenopus*, it interacts with the entire promoter region as illustrated by its DNase I footprint on the 5S RNA gene (Engelke *et al.*, 1980). The TFIIIA-DNA complex has been found to be

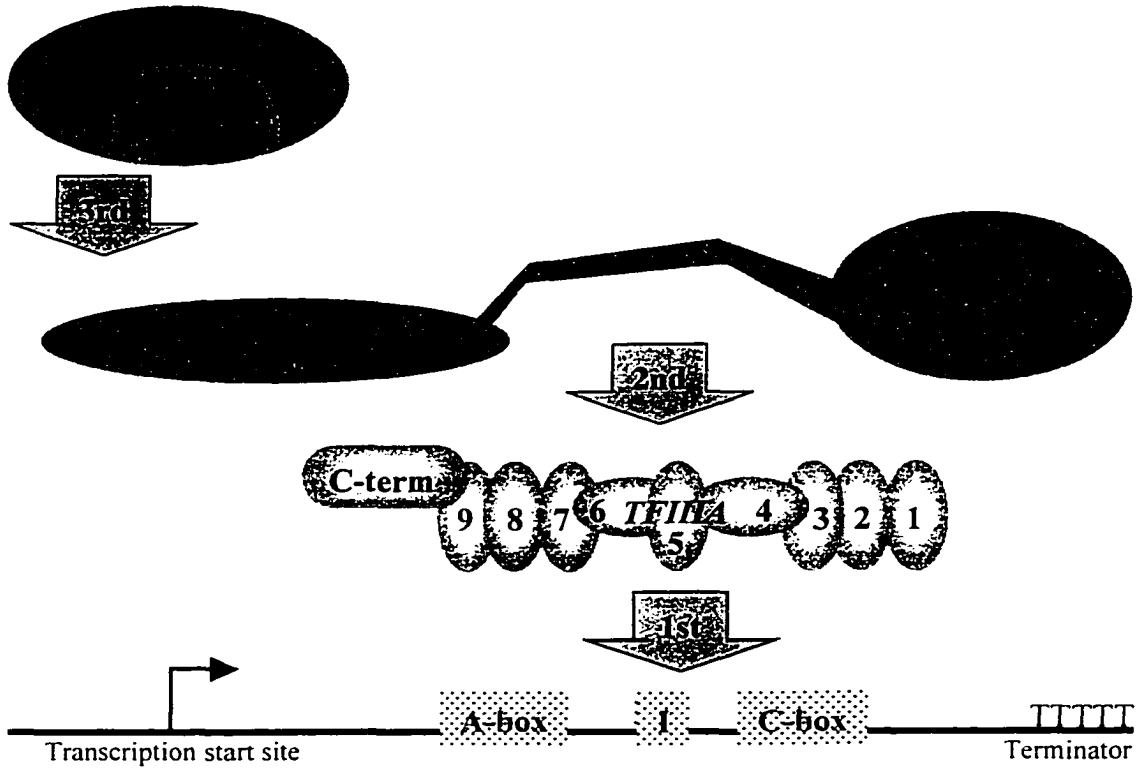

Fig. 1.2. The assembly of the transcription factors on the 5S RNA gene to form the preinitiation complex. TFIIIA, TFIIIC, and TFIIIB bind sequentially to the promoter as shown by the arrows. The series of numbered ovals shown as TFIIIA represent the nine zinc fingers of the protein, with the vertical ovals identifying fingers that contact the DNA and horizontal ovals denoting fingers that link the three DNA binding regions of the protein. In higher vertebrates, the C-terminal region is used for transcription. TFIIIC is shown as two large domains (τA and τB) connected by a hinge in accordance with its structure revealed by electron microscopy (Schultz, *et al.*1989). τA contacts the upstream portion of the gene including the A-box and τB contacts the downstream region of the gene including the polydT terminator sequence (Kassavetis *et al.*, 1994; Chedin *et al.*, 1998). As shown, TFIIIB is the TBP containing Pol III transcription factor and binds nonspecifically to the DNA upstream of the transcription start site (Kumar *et al.*, 1998) following the recruitment by interaction with TFIIIC.

metastable, which is defined by the ability of TFIIIA to disengage from one promoter in solution and bind to another (Lassar *et al.*, 1983). When bound to the 5S RNA gene, TFIIIA recruits TFIIIC, which contacts both TFIIIA (Hayes *et al.*, 1989) and the DNA (Majowski *et al.*, 1987). The addition of TFIIIC stabilizes the complex (Lassar *et al.*, 1983), and TFIIIC functions to recruit and position TFIIIB upstream of the transcription start site (Braun *et al.*, 1992). TFIIIB does not bind specifically to the DNA, but rather, it is recruited by interactions with TFIIIC. In *S. cerevisiae*, the second largest subunit of TFIIIC has been shown to interaction with all three subunits of yeast TFIIIB *in vivo* (Fig 1.3) (Chedin *et al.*, 1998). Binding of the Brf subunit of yeast TFIIIB to the second largest subunit of TFIIIC is the essential interaction mediating the assembly of TFIIIB onto the 5S RNA gene. Site-specific DNA-protein cross-linking and DNase I footprinting experiments have demonstrated that Brf alone can bind to the TFIIIC-TFIIIA-DNA complex (Kassavetis *et al.*, 1992). Once associated with the complex, Brf mediates the recruitment of the additional yeast TFIIIB subunits, TBP and B", to the complex (Kassavetis *et al.*, 1992). Once assembled in the preinitiation complex TFIIIB is able to bind nonspecifically to the DNA (Braun *et al.*, 1989). While the addition of TFIIIA and TFIIIC to the DNA have been found to occur with relative rapidity *in vitro*, the binding of TFIIIB is much slower and, therefore, rate-limiting in the formation of the preinitiation complex (Bieker *et al.*, 1985; Setzer and Brown, 1985). Once integrated into the complex, TFIIIB serves to recruit and position RNA polymerase III for transcription initiation (Kassavetis *et al.*, 1990).

1.3 The Stability of the Preinitiation Complex on 5S RNA Genes

The preinitiation complex formed on the 5S RNA gene has been found to have moderately different properties in the yeast and vertebrate systems. In the vertebrate system, both TFIIIC and TFIIIB increase the overall stability of the

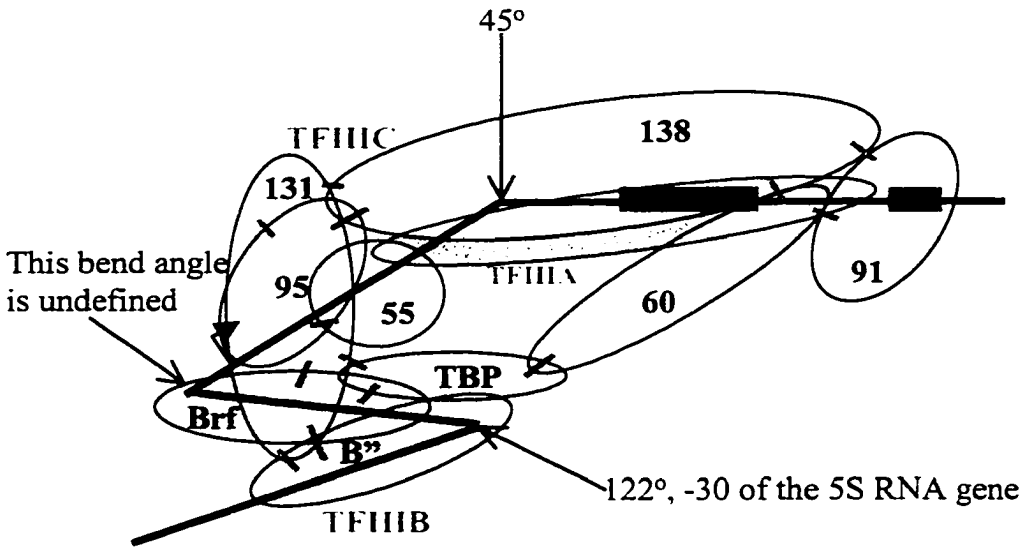

Fig. 1.3. The positioning of the *S. cerevisiae* Pol III transcription factors on the 5S RNA gene. A speculative model of the orientation of the Pol III transcription factors is shown. TFIIIA is shown in red, the TFIIIC subunits are in grey, and the TFIIIB subunits are in blue. The relative positions of subunits have been determined by footprinting and DNA-protein cross-linking (Braun *et al.*, 1989; Braun *et al.*, 1992; Kassavetis *et al.*, 1994). The interactions among the subunits are shown as red lines and have been identified using genetics (Chedin *et al.*, 1998). The DNA is shown as a black line, and the approximated positions of the transcription start site (arrow) and the terminator and C-box elements (green boxes) are indicated. The approximately 45° bend in the DNA induced by TFIIIA and the approximately 122° bend induced by TFIIIB that is centered at -30 (Braun *et al.*, 1992) are shown. The potential bend in the DNA just upstream of the transcription start site (Grove *et al.*, 1999) is also shown. τ60, a component of τ_B that binds to the downstream portion of the gene has been shown to interact with TBP upstream of the transcription start site (Deprez *et al.*, 1999).

complex, which is maintained by retention of all the factors on the DNA, excluding Pol III (Setzer and Brown, 1985). This complex has been shown to resist disassociation by detergent (Maraia, 1996) and high salt concentrations (Carey *et al.*, 1986). Furthermore, TFIIIA, TFIIIB, and TFIIIC remain associated with the DNA in chromatin isolated from *Xenopus* oocytes (Bogenhagen *et al.*, 1982). A different mechanism stabilizes the preinitiation complex in *S. cerevisiae*. TFIIIB alone, once recruited by TFIIIC, is tightly bound to the promoter and remains bound in the presence of high salt concentrations or in the presence of moderate concentrations of the polyanion, heparin (Kassavetis *et al.*, 1989). The stable complex on vertebrate Pol III promoters does not match the strength of the interaction between yeast TFIIIB and the DNA. Although a single stabilizing factor may exist in vertebrate systems (Geiduschek and Kassavetis, 1992), it does not appear to be human TFIIIB. In vertebrate systems, TFIIIB is the least stably associated Pol III transcription factor in the preinitiation complex (Carey *et al.*, 1986; Jahn *et al.*, 1987). However, a component of TFIIIC may exhibit very stable DNA interactions and, therefore, may functionally relate to yeast TFIIIB in complex stabilization (Carey *et al.*, 1986; Geiduschek and Kassavetis, 1992).

1.4 The Preinitiation Complex and the Transcribing Polymerase

Once the preinitiation complex is formed, the gene is programmed for multiple rounds of transcription either through the stable binding of TFIIIB alone, as in yeast, or by the cooperative stability of TFIIIB, TFIIIC, and TFIIIA bound to the DNA as in vertebrates. In vertebrates, these factors remain bound to the DNA during multiple passages of the polymerase (Bogenhagen *et al.*, 1982; Wolffe *et al.*, 1986). Although yeast TFIIIB alone can mediate multiple rounds of *in vitro* transcription, a complex consisting of all the factors is potentially required to mediate multiple rounds of transcription within *S. cerevisiae* cells (Costanzo *et al.*, 2001).

Paradoxically, complexes containing all the Pol III factors cover most of the transcribed portion of the 5S RNA gene (Fig. 1.3), but the polymerase is still able to transgress this region without abolishing the factor complex. Wang and Roeder (1996) have proposed that factors bound within the transcribed portion of the gene are released cyclically from the A-box, the C-box, and the terminator during transcription.

1.5 TFIIIA

TFIIIA has been an intensely studied eukaryotic transcription factor for four major reasons: its abundance and therefore ease of purification from frog oocytes, the simplicity of the *in vitro* 5S RNA transcription system, the nine zinc-finger motifs originally discovered in TFIIIA, and the ability of TFIIIA to specifically bind the 5S RNA transcript in addition to the 5S RNA gene promoter. Because of its extreme abundance in early immature *X. laevis* oocytes (constituting from 5 to 15% of the total soluble protein), TFIIIA was the first eukaryotic transcription factor purified (Engelke *et al.*, 1980) and cloned (Ginsberg *et al.*, 1984). The abundance of TFIIIA in oocytes is due to its additional function in storing a large quantity of 5S RNA to be used later in development. Indeed, TFIIIA was initially purified from the 7S ribonucleoprotein particle (RNP) in association with 5S RNA in *Xenopus* oocytes (Picard and Wegnez, 1979), although its role in 5S RNA transcription was not known at the time. As a transcription factor, TFIIIA was originally isolated from *X. laevis* ovaries and found to activated 5S RNA transcription and bind specifically to the gene (Engelke *et al.*, 1980). The 7S RNP protein and TFIIIA were then shown to be immunologically related, and both proteins produced equivalent polypeptide fragments upon cleavage with cyanogen bromide (Honda and Roeder, 1980). In addition, both proteins activated 5S RNA transcription, and this activity was inhibited by 5S RNA (Honda and Roeder, 1980; Pelham and Brown, 1980). This data showed

that the protein associated with 5S RNA in *Xenopus* oocytes activated 5S RNA transcription. Outside the realm of other oocyte extracts, TFIIIA has proven very difficult to purify mainly because it is frequently much less prevalent in other cell types. A single HeLa cell has been estimated to contain approximately 4000 molecules of TFIIIA (Moorefield and Roeder, 1994), and 3000 to 8000 copies are found in an adult *X. laevis* somatic cell, which is much less than the 10^{12} TFIIIA molecules found in oocytes (Ginsberg *et al.*, 1984; Shastry *et al.*, 1984). Based on the lower number of 5S RNA genes or pseudogenes in the *A. castellanii* genome (at least 4-fold lower) than in the human genome, *A. castellanii* cells are expected to contain less molecules of TFIIIA than a human cells (Sorensen and Frederiksen, 1991; Zwick *et al.*, 1991b). This expectation has been borne out by our purification.

1.5.1 The zinc finger motif

The versatility of TFIIIA in binding specifically to both RNA and DNA is by virtue of its nine zinc fingers, a structural motif first identified in *X. laevis* TFIIIA (Miller *et al.*, 1985). Each zinc finger is composed of a single zinc ion in tetrahedral coordination with two antiparallel β-strands and an α-helix (Fig. 1.4A) (Berg, 1988; Liao *et al.*, 1994). Each zinc ion is specifically bound to a cysteine residue in each β-strand and two histidine residues in the α-helix (Diakun *et al.*, 1986). This structural motif maintains the zinc fingers as independent globular domains when TFIIIA is free in solution (Bruschweiler *et al.*, 1995) and upon its binding to nucleic acids (Wuttke *et al.*, 1997). Although this zinc finger motif is generally conserved, a 3_{10}-helix is found between the β-strands in the amino-terminal finger of *Xenopus* TFIIIA according to its NMR structure and its predicted secondary structure (Wuttke *et al.*, 1997). When TFIIIA is associated with DNA, the α-helix of each interacting finger involved in DNA binding is positioned in the major groove of the DNA. The

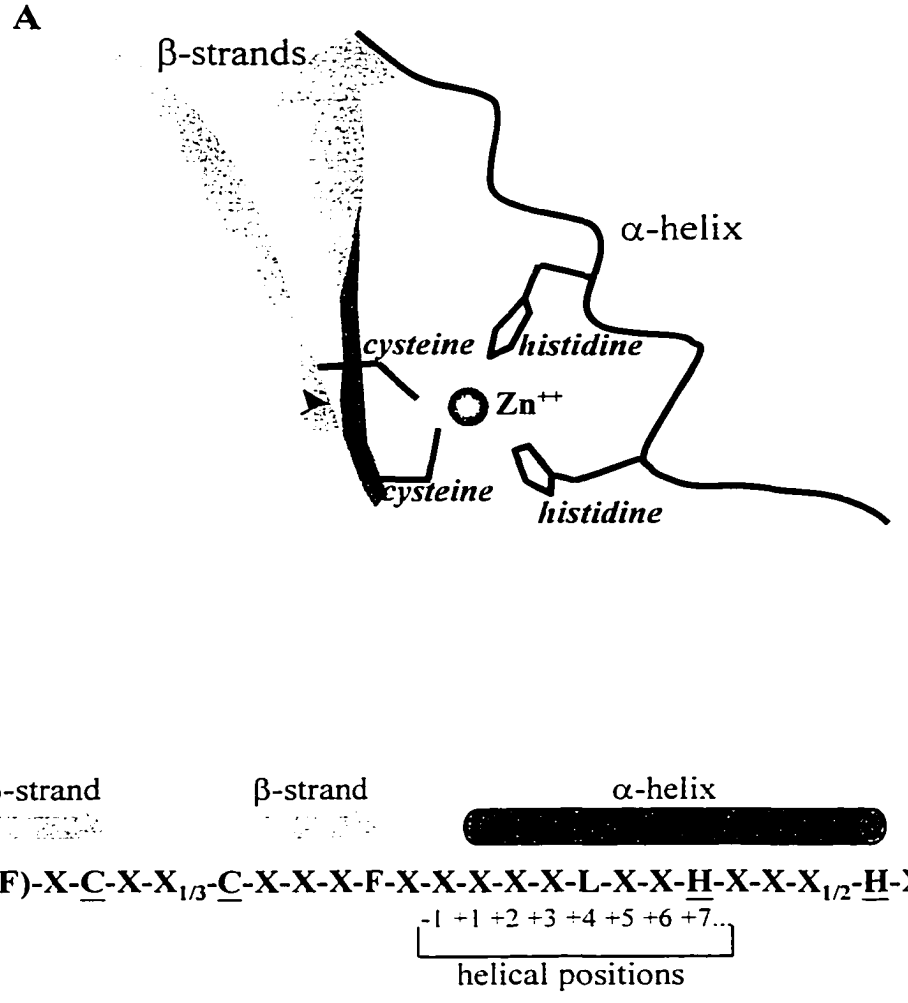

Fig. 1.4. The C2H2 zinc finger motif found in TFIIIA. **A.** the tertiary stucture of the zinc finger is shown with two β-strands (grey, twisted arrows) connected to an α-helix (black spiral). The two cysteine and two histidine residues tetrahedrally coordinated to the zinc ion (sphere) are also shown. **B.** conserved amino acids are shown in the primary sequence of the zinc finger motif. X, any amino acid. Cysteines coordinated to zinc are separated by two to five amino acids, and histidines coordinated to zinc are separated by three or four amino acids. The amino acids composing the β-strands and α-helix are identified by the positions of the secondary structures above the primary sequence. The positions of amino acids with respect to the α-helix are shown below the primary sequence.

use of phage display to select for amino acids in the α-helix that are involved in DNA binding has demonstrated that amino acids positioned in the helix at –1, +3, and +6 are primarily responsible for DNA binding (Jacobs, 1992). In many fingers, the residues positioned at +2 of the α-helix also make critical contacts with the DNA (Choo and Klug, 1994).

The amino acid sequence underlying the zinc finger motif has been found to be extremely variable, although certain amino acids within the motif are considered to be conserved (Fig 1.4B) (Berg, 1988). The conservation of these amino acids is, however, not absolute in all TFIIIA zinc fingers. Tyrosine or phenylalanine is frequently the first amino acid of the zinc finger, which begins the first β-strand of the motif. The two cysteines coordinated to the zinc ion are the next conserved amino acids and are separated from one another frequently by two or four residues. Although the amino acids between the conserved cysteines are variable, acidic residues and, to a lesser extent, proline and glycine more frequently occupy one or more of these positions than the other amino acids (Gaskins *et al.*, 1992). A phenylalanine or, at lower frequency, a tyrosine is found at the last position of the second β-strand. The solution structure of the first three zinc fingers of *Xenopus* TFIIIA has shown that the conserved aromatic residue beginning the first β-strand interacts with the conserved aromatic residue terminating the second β-strand, and this interaction is essential for stabilizing the finger structure (Foster *et al.*, 1997). A conserved leucine is positioned at +4 in the α-helix, and the next conserved residue is the first histidine coordinated to zinc at +7 of the α-helix. Three or four variable amino acids separate the first and second histidines coordinated to the zinc ion. The conserved histidines, cysteines, and aromatic residues (phenylalanines or tyrosines) are critical in maintaining the structural integrity of the zinc finger, but are not involved in nucleic acid binding.

1.5.2 DNA binding by TFIIIA

In all organisms, TFIIIA has the consistent function of activating 5S RNA transcription, which is the only essential function of the yeast homologue (Camier *et al.*, 1995). As discussed in section 1.2, TFIIIA is the first transcription factor to bind to the promoter and nucleates transcription from the 5S RNA gene. Because of the early purification and cloning of *Xenopus* TFIIIA, the interaction of this protein with the 5S RNA gene has been extensively characterized biochemically and structurally. *Xenopus* TFIIIA was initially found to protect the entire 5S RNA gene promoter (+47 to +96) from DNase I cleavage (Engelke *et al.*, 1980). This extensive DNA protection by the 39 kDa protein was shown to be possible because of the extended conformation adopted by TFIIIA (Bieker and Roeder, 1984). Proteolytic cleavage of TFIIIA showed that the DNA binding domain was located within a 30 kDa fragment of the protein (Smith *et al.*, 1984). This fragment was found to contain the nine sequential zinc fingers, suggesting that the zinc fingers mediated DNA binding (Miller *et al.*, 1985). A clearer model of the TFIIIA-DNA complex was obtained by hydroxyl-radical footprinting, which showed that TFIIIA was closely associated with the A-box, the C-box and the intermediate element (Vrana *et al.*, 1988). Footprinted regions were found to be off-set by three nucleotides on each strand of the DNA, indicating that TFIIIA bound in the major groove at the three *cis*-elements. Major groove contacts made by TFIIIA at the C-box and the intermediate element were also supported by methylation interference studies, showing that guanine nucleotides within the C-box and intermediate element were critical for TFIIIA binding (Sakonju and Brown, 1982). Guanine methylation occurs at the N7 position of the base, which affects major groove binding. Changes in the hydroxyl radical footprint with amino- and carboxyl-truncated deletion mutants of TFIIIA showed that the amino-terminal zinc fingers were positioned at the 3'-end of the promoter and the carboxyl-terminal fingers were positioned at the 5'-end of the

promoter (Vrana *et al.*, 1988). Through the extensive characterization of DNA binding by carboxyl-terminal truncated forms of TFIIIA, a model was proposed to define the position of each finger along the DNA (Clemens *et al.*, 1992; Hayes and Clemens, 1992). In this model, the amino-terminal three fingers (1-3) wrap around the DNA in the major groove of the helix at the C-box, the carboxyl-terminal three fingers (7-9) bind in an equivalent manner at the A-box, finger 5 binds in the major groove of the DNA at the intermediate element, and fingers 4 and 6 cross the minor groove, connecting the three DNA-binding domains of TFIIIA (Fig. 1.5A). The x-ray structure of zinc fingers 1-6 bound the 5S RNA gene promoter from +63 to +92 supports this model (Fig. 1.5B) (Nolte *et al.*, 1998).

Interactions between zinc fingers 1-3 and the C-box are necessary and sufficient for DNA-binding by TFIIIA. The 5'-end of the 5S RNA gene can be deleted to +74 without affecting the DNase I footprint of TFIIIA at the C-box (Sakonju *et al.*, 1981). Conversely, deletion of the 3'-end of the gene to +80 eliminates all upstream protection by TFIIIA, showing that TFIIIA cannot bind efficiently to the DNA in the absence of an intact C-box (Sakonju *et al.*, 1981). Alkylation of the promoter at sites in the phosphate backbone or at guanines from +81 to +91 also abolished TFIIIA-DNA interactions, again illustrating the critical nature of the C-box for TFIIIA binding (Sakonju and Brown, 1982). Complementary experiments have shown that a polypeptide with only fingers 1-3 is able to bind the C-box, and this interaction accounts for greater than 90% of the binding energy between TFIIIA and the DNA (Christensen *et al.*, 1991; Liao *et al.*, 1992). The contributions of each of the first three fingers to the total binding energy of the TFIIIA-DNA complex have been determined using broken finger mutants in which the first conserved histidine coordinated to the zinc-ion in a specific finger is replaced by an asparagine to disrupt the zinc finger structure. Disrupting the third finger had the most significant effect on DNA binding relative to disrupting any of the other eight fingers, causing

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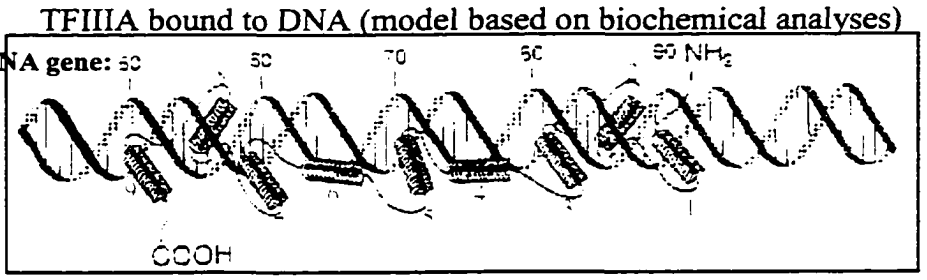

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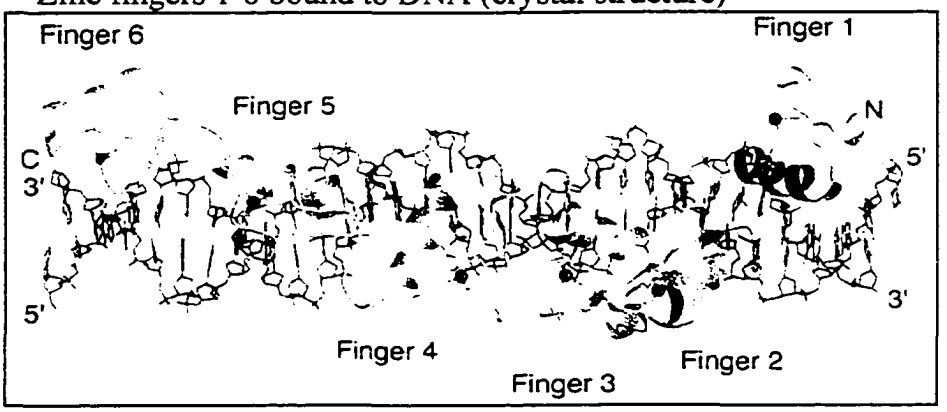

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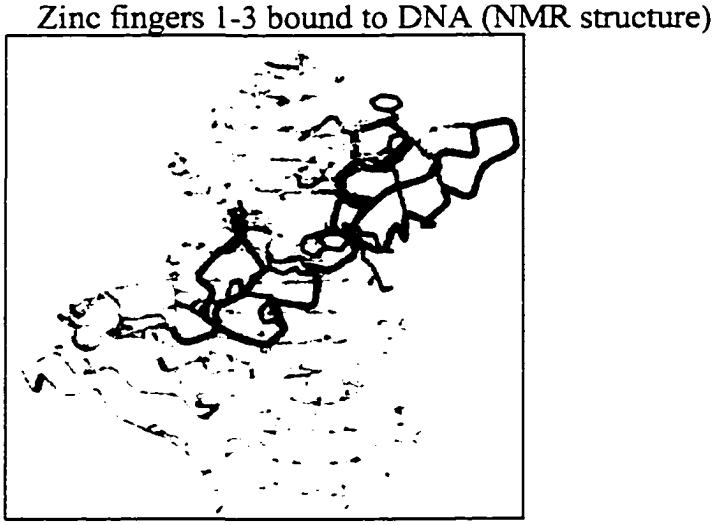


Fig. 1.5. Positioning of the TFIIIA zinc fingers along the 5S RNA gene. A, the model shown was taken from Clemens *et al.* (1992) and shows zinc fingers as tubes that are number 1-9 beginning with the first N-terminal finger. The positions of the three *cis*-elements in the promoter are identified by the red boxes. **B,** this figure was taken from Nolte *et al.* (1998) and shows the crystal structure of fingers 1-6 bound to the DNA. **C,** this figure was taken from Wuttke *et al.*, (1997) and shows fingers 1-3 bound to the DNA.

a 27-fold decrease in binding affinity (Del Rio and Setzer, 1993). Disrupting the second finger caused a modest, 7-fold decrease in DNA-binding, and disrupting finger 1 caused only a 2.6-fold decrease in binding (Del Rio and Setzer, 1993). These data suggest that the first zinc finger is the least critical of the first three fingers for the assembly of TFIIIA onto the DNA. In support of this notion, the DNase I footprint of TFIIIA is still apparent using a deletion mutant lacking the first zinger finger, but the entire footprint is lost when the second finger is additionally deleted (Vrana *et al.*, 1988).

The solution structure of zinc fingers 1-3 of *Xenopus* TFIIIA bound to a segment of the 5S RNA gene from +79 to +93 has provided further detail into the binding of TFIIIA at the C-box (Wuttke *et al.*, 1997). The structure confirmed that the zinc fingers wrap around the DNA in the major groove of the helix (Fig. 1.5C). As expected the majority of the interactions between the polypeptide and the DNA were shown to be in the region of the 5S RNA gene from +80 to +91 (Fig. 1.6). Interestingly, the zinc finger interactions with the DNA were found to overlap along the DNA. For example, tryptophan 28 at the + 2 position in the α-helix of finger 1 was found to interact with guanine +87, histidine 58 at the same position in finger 2 contacted thymine +88, and lysine 87 within finger 3 was found to contact the phosphate following cytosine +87 (Fig. 1.6). These overlapping interactions suggest that zinc fingers 1-3 are closely clustered on the C-box, which is supported by the continuous TFIIIA footprint over this region of the DNA (Vrana *et al.*, 1988). In support of the biochemical data suggestive of a weaker interaction between finger 1 and the DNA, this finger was found to have a different orientation in the major groove, resulting in a less intimate interaction between finger 1 and the DNA (Wuttke *et al.*, 1997).

Two conserved linkers of the motif TGEKP connect the first three zinc fingers, and these linkers have been found to be critical for DNA binding. NMR

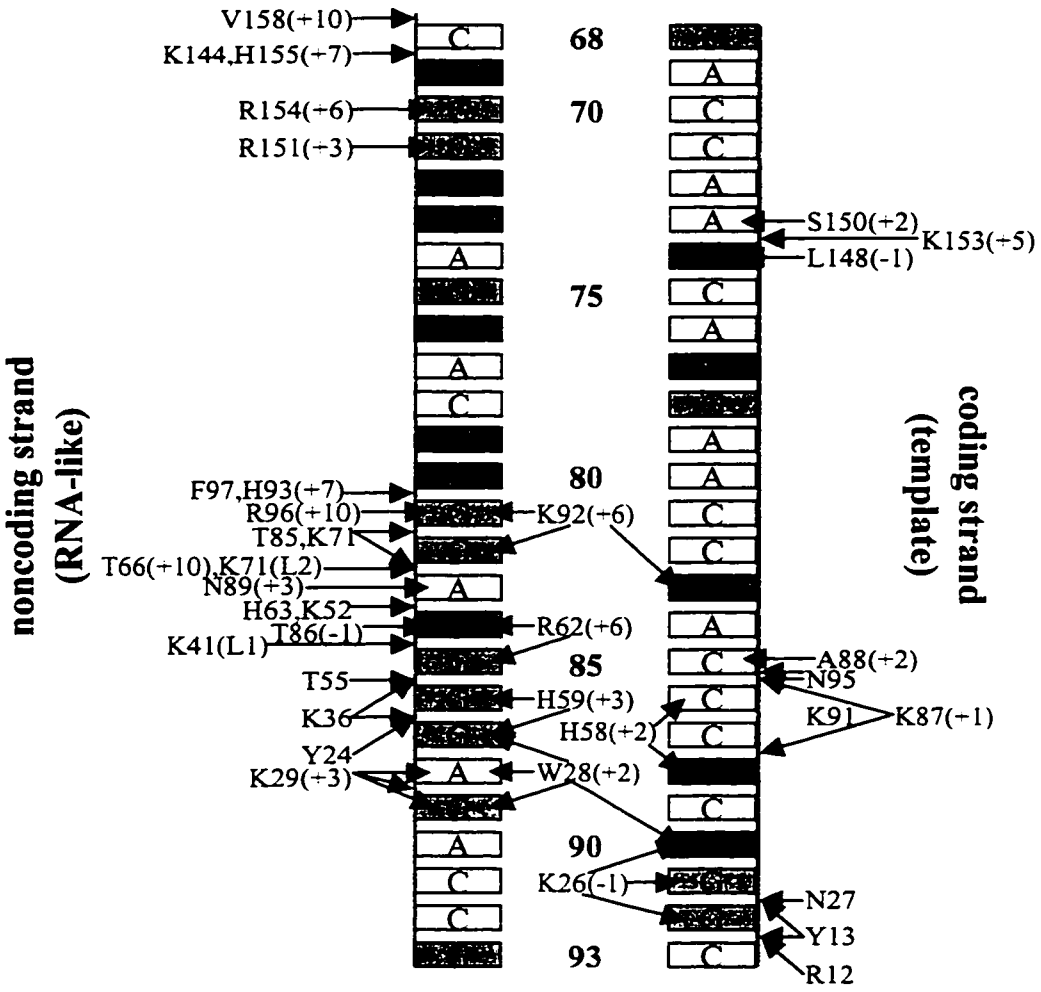


Fig. 1.6. The amino acid-DNA contacts derived from the NMR and crystal structures. The amino acid-DNA contacts indicated by the arrows were derived from the finger 1-3/DNA complex solved by NMR (Wuttke *et al.*, 1997) and/or from the finger 1-6/DNA complex solved by x-ray crystallography (Nolte *et al.*, 1998). DNA bases are shown as colored rectangles: guanine, red; cytosine, yellow; adenine, lavender; thymine, green. The sugar-phosphate backbone of the DNA is shown as two blue lines. The nucleotide positions within the 5S RNA gene are identified between the complimentary strands. Arrows extending onto the rectangles depict amino acid-base contacts. Arrows to the blue lines between the rectangles denote amino acid-DNA backbone contacts. Numbers in parenthesis indicate the position of the amino acid with respect to the zinc finger α-helix. "L1" or "L2" in parenthesis indicates that the amino acid is within the first or second linker, respectively.

studies have shown that these linkers limit the flexibility between zinc fingers 1-3, which may contribute to properly positioning these fingers on the DNA (Bruschweiler *et al.*, 1995). Also by restricting the movement of the first three fingers in solution, the linkers may help to reduce the loss in entropy during the formation of the TFIIIA-DNA complex, thereby making DNA binding more energetically favorable. The solution structure of fingers 1-3 bound to the C-box has supported the role of the TGEKP linker motif in properly positioning these zinc fingers on the DNA (Wuttke *et al.*, 1997). The threonine at the first position of the linker was found to form hydrogen bond contacts within the linker and with the adjacent finger, which was found to stabilize the polypeptide-DNA complex. Substituting this threonine with serine has been shown to cause an 11-fold decrease in TFIIIA binding, demonstrating the importance of this residue for DNA binding (Clemens *et al.*, 1994). The glycine at the second position of the linker is necessary to cap the α-helix of the adjacent amino-terminal finger, allowing the carboxyl-terminal amino acids of the linker to form an extended configuration (Wuttke *et al.*, 1997). This extended structure of the linker is proposed to allow adjacent fingers to maintain continuous major groove contacts with the DNA (Berg, 1990). A mutation of the glycine in the second linker to serine was shown to eliminate the DNase I protection pattern by TFIIIA, illustrating the importance of this residue in DNA binding (Smith *et al.*, 1991). In the solution structure the lysines in the first and second linkers are positioned to form hydrogen bond contacts with the phosphate backbone of the DNA (Fig. 1.6). These interactions may be critical in stabilizing the complex, because mutating the lysine in the second linker to a glutamate was found to abolish the TFIIIA footprint (Smith *et al.*, 1991).

Other mutational analyses of the first two linkers have indicated that the entire linker and not specific amino acids within this motif is important for DNA binding. Less than a 3-fold decrease in DNA binding has been observed using

mutant proteins in which one or two amino acids in the first or second linker was replaced by corresponding amino acids from either of two other *Xenopus* zinc finger proteins, p43 or Xfin (Zang *et al.*, 1995). In contrast, DNA binding was shown to be abolished or severely compromised by replacing the entire first or second linker in TFIIIA with a p43 linker, exemplifying the importance of the entire TGEKP motif (Choo and Klug, 1993).

The central zinc fingers (4-6) are essential for connecting the zinc finger clusters 1-3 and 7-9 that bind at opposite ends of the DNA promoter. Contacts between finger 5 and the intermediate element are necessary to anchor the central fingers on the DNA. Mutagenesis (You *et al.*, 1991), missing nucleoside analyses (Hayes and Tullius, 1992), and methylation interference experiments (Sakonju and Brown, 1982) have shown that contacts made at the intermediate element with finger five contribute to the overall DNA binding affinity of TFIIIA. These contacts are only possible when finger 4 is present, which serves to link the amino-terminal three high-affinity DNA binding fingers to finger 5. Although finger 4 does not make strong DNA contacts (Nolte *et al.*, 1998), disrupting this finger has been shown to eliminate the upstream TFIIIA footprint (Smith *et al.*, 1991). This loss of strong TFIIIA contacts in the upstream region of the promoter suggests that finger 4 functions to properly position finger 5 on the DNA. This notion is supported by other experiments in which the disruption of finger 4 was found to significantly lower the DNA binding affinity of TFIIIA (Del Rio *et al.*, 1993). Finger 6 connects fingers 5 to the carboxyl-terminal three fingers, allowing the carboxyl-terminal fingers to interact with the A-box. This role of finger 6 is illustrated by the loss of DNase I protection in the 3'-portion of the A-box when it is disrupted (Del Rio *et al.*, 1993). In other experiments, disrupting finger 6 was found to result in the same loss in the upstream TFIIIA footprint that has been observed by disrupting finger 4 (Smith *et al.*, 1991). This finding suggests that finger 6 influences the role of fingers four and five in DNA binding.

In the context of the full-length protein, zinc fingers 7-9 have been shown to contribute poorly to the DNA binding affinity of TFIIIA. The weak binding of the carboxyl-terminal three fingers is exemplified by DNase I protection patterns using either deletion mutants of the DNA promoter or truncated forms of TFIIIA. The 3'-portion of the footprint was retained when the A-box of the promoter was deleted (Sakonju *et al.*, 1981) or when the carboxyl-terminal fingers were deleted (Vrana *et al.*, 1988). In contrast, deleting the amino-terminal fingers (Vrana *et al.*, 1988) or a portion of the C-box (Sakonju *et al.*, 1981) was found to abolish the entire TFIIIA footprint. These early experiments showed that, in the absence of contacts between the amino-terminal zinc fingers and the DNA, binding of fingers 7-9 to the A-box is not detected.

The weak binding of zinc fingers 7-9 to the A-box may be caused by strain in the TFIIIA-DNA complex. Because TFIIIA binds to a relatively long stretch of DNA, DNA-protein contacts in one region of the complex are likely to influence contacts in another region. Structural data of the amino-terminal fingers bound to DNA have shown that the intimate contacts made by fingers 1 and 2 with the DNA cause a local widening and deepening in the major groove (Nolte *et al.*, 1998; Wuttke *et al.*, 1997). This DNA distortion could lead to compaction of the major groove in a region encompassing the A-box, thereby hindering DNA binding by fingers 7-9. In support of this notion, a truncated form of TFIIIA, lacking the amino-terminal three fingers, has been shown to bind specifically to the 5S RNA gene with a binding energy of 83 % of the binding energy of full-length TFIIIA (Kehres *et al.*, 1997). As discussed in this section, fingers 1-3 have been shown to account for 95 % of the DNA binding energy of TFIIIA (Christensen *et al.*, 1991; Liao *et al.*, 1992). This discrepancy in the binding affinities of fingers at opposite ends of TFIIIA can be explained by zinc fingers 1-3 inhibiting DNA binding by the carboxyl-terminal fingers. Fingers 5 and 6 may also inhibit DNA binding by the three carboxyl-terminal fingers, as polypeptides

containing fingers 5-9 and 6-9 have not been shown to bind the 5S RNA gene, while a polypeptide containing only fingers 7-9 was found to bind weakly to the DNA (Theunissen *et al.*, 1992). The carboxyl-terminal three fingers may have similar adverse effects on DNA binding by the amino-terminal fingers, as polypeptides containing fingers 1-5 and 1-7 have a higher binding affinity than full-length TFIIIA (Clemens *et al.*, 1994). Experiments in which different combinations of two zinc fingers were disrupted have shown that the outermost fingers of TFIIIA produce the strongest negative influences on DNA binding of the fingers at opposite ends of the protein (Kehres *et al.*, 1997).

Fingers 7-9 appear to bind cooperatively to the A-box rather than as three separate binding units. Protection of the entire A-box from DNase I cleavage is observed only with the complete set of amino-terminal fingers. Removal of finger 9 has been found to substantially reduce the protection over the entire A-box, and with the additional removal of finger 8, the footprint over the A-box is completely lost (Clemens *et al.*, 1994). Moreover, disruption of finger 8 alone in the context of full-length TFIIIA has been found to completely eliminate A-box protection (Del Rio *et al.*, 1993). Therefore, finger 7 is unable to bind efficiently to the DNA in the absence of fingers 8 and 9. This dependency of finger 7 on fingers 8 and 9 is further supported by the sensitivity of finger 7 in the TFIIIA-DNA complex to proteolysis observed when finger 9 or fingers 8 and 9 have been deleted from the protein (Clemens *et al.*, 1994). Together, these results demonstrate fingers 7-9 depend on one another for A-box binding.

1.5.3 The transcription activating domain of TFIIIA

The regions of *Xenopus* TFIIIA required to activate transcription are located in the carboxyl-terminal portion of the protein. Disrupting fingers 7, 8, or 9 has been shown to eliminate transcriptional activation by TFIIIA (Del Rio and Setzer, 1993),

which complements results that have shown the A-box to be essential for transcription (section 1.1). Additionally, removal of a 10 kDa carboxyl-terminal segment of TFIIIA by proteolysis also eliminates transcriptional activation (Smith *et al.*, 1984). This domain was found to lie outside of the region containing the zinc fingers, and the use of truncated forms of TFIIIA confirmed that the carboxyl-terminal domain was required for transcription. TFIIIA with 31 amino acids deleted from the carboxyl-terminus was found to activate transcription, but deleting an additional 19 amino acids abolished transcription (Vrana *et al.*, 1988). Using cassette and random point mutagenesis, the minimal domain required for transcription was found to encompass amino acids 291-304 of *Xenopus* TFIIIA (Fig. 1.8), having the sequence RSLASRLTGYIPPK (Mao and Darby, 1993). Moreover, the spacing between this motif and finger 9 was also shown to be essential for optimal transcriptional activation (Mao and Darby, 1993). Based on these results and the results from disrupting fingers 7-9, binding of fingers 7-9 to the A-box has been proposed to position the carboxyl-terminal domain to recruit and properly position another Pol III-mediated transcription factor on the promoter. The subsequently recruited factor may be TFIIIC, based on experiments in which a 34 kDa fragment of TFIIIA lacking the carboxyl-terminal domain is not stabilized on the DNA by the TFIIIC-containing fraction (Hayes *et al.*, 1989).

1.5.4 RNA binding by TFIIIA

TFIIIA has also been found to bind specifically to 5S RNA, and this function has been found to be essential during early *Xenopus* development (Pelham and Brown, 1980). To accommodate the high level of protein synthesis required during *Xenopus* embryonic development, a large quantity of 5S RNA is produced earlier in oogenesis, and this RNA is stored until it is needed (Wolffe and Brown, 1988). The nascent 5S RNA that is rapidly produced in *Xenopus* oocytes is assembled into

a complex with TFIIIA in the nucleus (Guddat *et al.*, 1990). This complex, termed the 7S ribonucleoprotein particle (7S RNP), is transported to and sequestered in the cytoplasm (Picard and Wegnez, 1979). Therefore, TFIIIA serves as part of a storage particle for 5S RNA, and this function accounts for the massive quantity of TFIIIA in *Xenopus* oocytes (Honda and Roeder, 1980).

Interactions between TFIIIA and 5S RNA have been found to be complex because of the higher order structures formed by the RNA and the effects that TFIIIA is speculated to have on these structures, which may affect the stability of theTFIIIA-5S RNA complex either positively of negatively (Searles *et al.*, 2000). The secondary structure of the 5S RNA consists of five stems or helices, numbered I to V, and five loop domains, denoted A to E (Fig. 1.7). TFIIIA nuclease protection has shown contact over almost the entire 5S RNA molecule (Sands and Bogenhagen, 1991). However, only certain domains of the RNA are critical for TFIIIA binding. Comprehensive cassette mutagenesis of the 5S RNA has demonstrated that disrupting loop A produces the most detrimental effect on TFIIIA binding (You *et al.*, 1991). A separate study testing TFIIIA binding to a comprehensive set of 5S RNA point mutants indicated that loop E was the most critical domain for TFIIIA binding (Rawlings *et al.*, 1996). The differences in the results of these studies is unclear, but may be due to the different assays used to measure the dissociation constants (a nitrocellulose filter binding assay versus an electrophoretic mobility shift assay [EMSA]) or to differences in the base substitutions at the same sites in the 5S RNA. Regardless of the contrasting results, both loop A and E are the most important regions of the 5S RNA molecule for TFIIIA binding. Both studies also showed that disrupting helices II and V cause a loss in binding affinity. However, the specific sequences comprising these helices were not found to be important for TFIIIA binding. These mutagenesis results have been corroborated by the ability of TFIIIA to bind efficiently to a truncated 5S RNA molecule that contains only loop A, helix

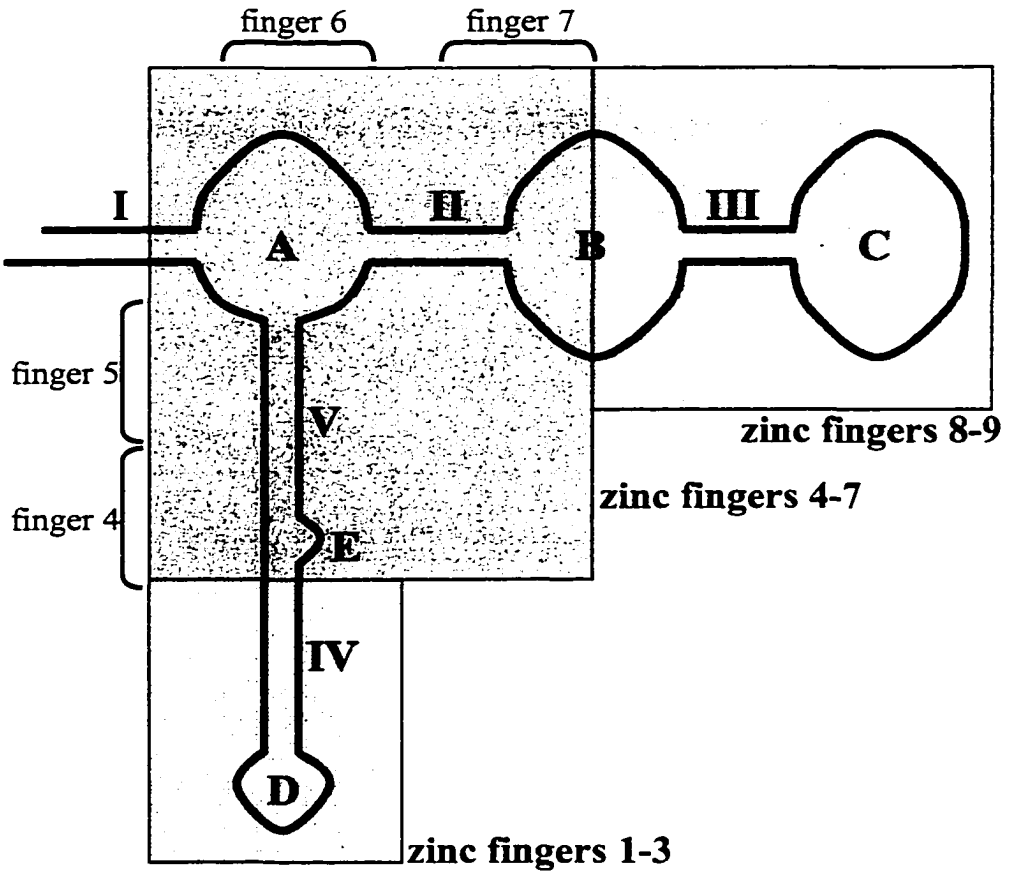


Fig. 1.7. The secondary structure of 5S RNA and the contacts made by TFIIIA. Groups of zinc fingers that contact the boxed regions of the 5S RNA are indicated in bold. Bracketed regions of the 5S RNA show contacts made by the indicated individual fingers. The red box covers the region of the 5S RNA that is essential for high affinity and specific binding by TFIIIA. The blue boxes cover regions of the 5S RNA that are expendable for TFIIIA binding.

II, helix V, loop E, and part of helix IV (McBryant *et al.*, 1995; Neely *et al.*, 1999; Searles *et al.*, 2000). In contrast to high affinity DNA binding, which occurs predominantly through TFIIIA-C-box interactions, the 5S RNA domains all produce relatively weak contributions toward TFIIIA binding, but the sum of these domain interactions is essential for high affinity binding by TFIIIA.

Truncated versions of TFIIIA have been used to define the regions of the protein that are essential for 5S RNA binding. Limited proteolysis of TFIIIA initially showed that RNA binding was mediated by the region of the protein containing the nine zinc fingers (Sands and Bogenhagen, 1991). Further sequential deletions of the fingers from both ends of the protein revealed that a polypeptide consisting of only fingers 4-7 retained approximately 95 % of the RNA binding energy of full-length TFIIIA, as determined using an EMSA (Clemens *et al.*, 1993). This finding was supported by experiments in which individual zinc fingers were disrupted in the context of the full-length protein; disrupting fingers 4-6 were found to cause the most significant decrease in RNA binding affinity (Setzer *et al.*, 1996). Similarly, mutations in fingers 4 or 6 that did not disrupt the zinc finger structure were also found to cause a significant loss in binding affinity (Friesen and Darby, 1997). Together these results suggest that fingers 4 and 6 are mainly responsible for the high affinity binding of TFIIIA to 5S RNA. Finger 6 also has been found to help mediate the specificity of the TFIIIA-5S RNA interaction. Disruption of this finger or its cognate RNA binding site increases the nonspecific association of TFIIIA with 5S RNA (Setzer *et al.*, 1996). In addition, finger 5 plays a role in RNA binding specificity. A truncated polypeptide lacking this finger produces multiple shifted complexes in an EMSA (Clemens *et al.*, 1993). Furthermore, disrupting finger 5 in the context of the full-length protein reduces RNA binding affinity by only 2.6-fold, but this mutant does not produce the same nuclease protection of the 5S RNA molecule as wild type TFIIIA (Setzer *et al.*, 1996). Among the central zinc fingers involved in RNA binding,

finger 7 contributes least to the thermodynamic binding energy (Searles *et al.*, 2000). Indeed, a polypeptide containing only fingers 4-6 was found to be sufficient for 5S RNA binding (Theunissen *et al.*, 1992). However, finger 7 may contribute to the specificity of 5S RNA binding (Neely *et al.*, 1999). The discovery that the central zinc fingers were utilized for RNA binding has led to the proposition that different sets of zinc fingers confer high affinity binding to the different nucleic acid types (Clemens *et al.*, 1993).

Although zinc fingers 4-7 have been shown to be sufficient for 5S RNA binding, additional regions of TFIIIA may augment RNA binding. Several mutations in the 5S RNA that are known to decrease the binding affinity of full-length TFIIIA were found to have more severe effects on the binding affinity of a polypeptide containing only fingers 4-7 (McBryant *et al.*, 1995). These differences in RNA binding were proposed to arise from fingers 1-3, 8, and/or 9 energetically compensating for loss of RNA contacts made by fingers 4-7. Disrupting finger 1 in the context of full-length TFIIIA has been found to cause a significant decrease in the RNA binding affinity (Setzer *et al.*, 1996). The role of finger 1 in RNA binding is unknown. Perhaps this finger counters negative influences on RNA binding contributed by other fingers. For example, mutations in the region of the 5S RNA that is contacted by fingers 8 and 9 have been found to increase the binding affinity with TFIIIA. Therefore, finger 1 may buffer negative affects of fingers 8 and 9 on RNA binding. Hitherto, the dissociation constant of a polypeptide containing only fingers 2-9 has not been determined, so the role for finger 1 is completely speculative.

A model predicting the positions of the nine zinc fingers on the 5S RNA has been proposed in which the fingers are extended along the secondary structure of the RNA (Fig. 1.7). Deletion of fingers 1-3 has been found to result in the loss of nuclease protection in the region of helix IV, suggesting that the amino-terminal

fingers contact this region of the 5S RNA (Theunissen *et al.*, 1992). Finger 4 has been shown to interact with loop E and the adjacent portions of helices IV and V based on the loss of nuclease protection in this region in complexes containing full-length TFIIIA with finger 4 disrupted (Setzer *et al.*, 1996). Furthermore, mutations in loop E of the RNA or in finger 4 of TFIIIA have been found to cause similar decreases in the stability of the protein-RNA complex (McBryant *et al.*, 1995). Fingers 5 and 7 were originally proposed to contact helices V and II, respectively, based on the identity of the sequences of these helices with sequences in the intermediate element and the A-box of the DNA promoter (Clemens *et al.*, 1993). Mutations in helix V that did not disrupt the helical structure, but altered the sequence equivalent to the intermediate element, were found to decrease TFIIIA binding, suggesting that finger 5 makes sequence specific contacts within the major groove of helix V (Rawlings *et al.*, 1996). The finger 7-helix II interaction was confirmed by experiments in which the presence of helix II in the RNA secondary structure was found to confer a higher binding affinity to a polypeptide containing fingers 4-7 than to a polypeptide containing fingers 4-6 (Searles *et al.*, 2000). Moreover, the presence of finger 7 in the truncated polypeptide extends the footprint to include helix II of full-length 5S RNA (Searles *et al.*, 2000). Finger 6 is known to contact loop A based on similar changes that occur in the footprint, the dissociation constant, and in the dissociation kinetics of the RNA-protein complex by disrupting either loop A or by disrupting finger 6 (Setzer *et al.*, 1996). Fingers 8 and 9 have been shown to interact with helix V based on the loss of nuclease protection over this region of the RNA with a polypeptide lacking these fingers (Theunissen *et al.*, 1992).

A limited number of experiments have shown that amino acids at the same positions in the zinc finger α-helix that are used for DNA binding are also used for RNA binding (Fig. 1.4). Alanine substitutions at position -1, +2, +3, and +6 in the α-

helix of finger 4 or 6 were shown to result in a large reduction in the RNA binding affinity of the finger 4-7 polypeptide (Friesen and Darby, 1997). The lysine at -1 and the serine at +2 of finger 4 were found to be especially significant for RNA binding, as these residues were selected for RNA binding by phage display (Friesen and Darby, 1997). Mutating the TWT motif in finger 6 (positions -1, +1, and +2 of the α-helix) caused a severe decrease in RNA binding affinity, with a mutation at threonine -1 showing the most severe defect (Clemens *et al.*, 1993). Structural and biochemical evidence support the role of amino acids at this position in fingers 1,3, and 5 playing a crucial role in DNA binding (Nolte *et al.*, 1998; Wuttke *et al.*, 1997). The TWT motif was specifically chosen for mutagenesis based on the critical role of zinc finger 6 in RNA binding and the presence of this motif at the same position in zinc finger 6 of p43 (Joho *et al.*, 1990). p43 is another *Xenopus* protein with nine zinc fingers that also binds specifically to 5S RNA, but does not bind to the gene. Interestingly, finger 6 of TFIIIA contacts loop A and not a helical domain in the RNA. Therefore, this association is expected to contrast dramatically with complexes formed between zinc fingers that bind in the major groove of the DNA regardless of the involvement of an equivalently positioned amino acid used in nucleic acid binding.

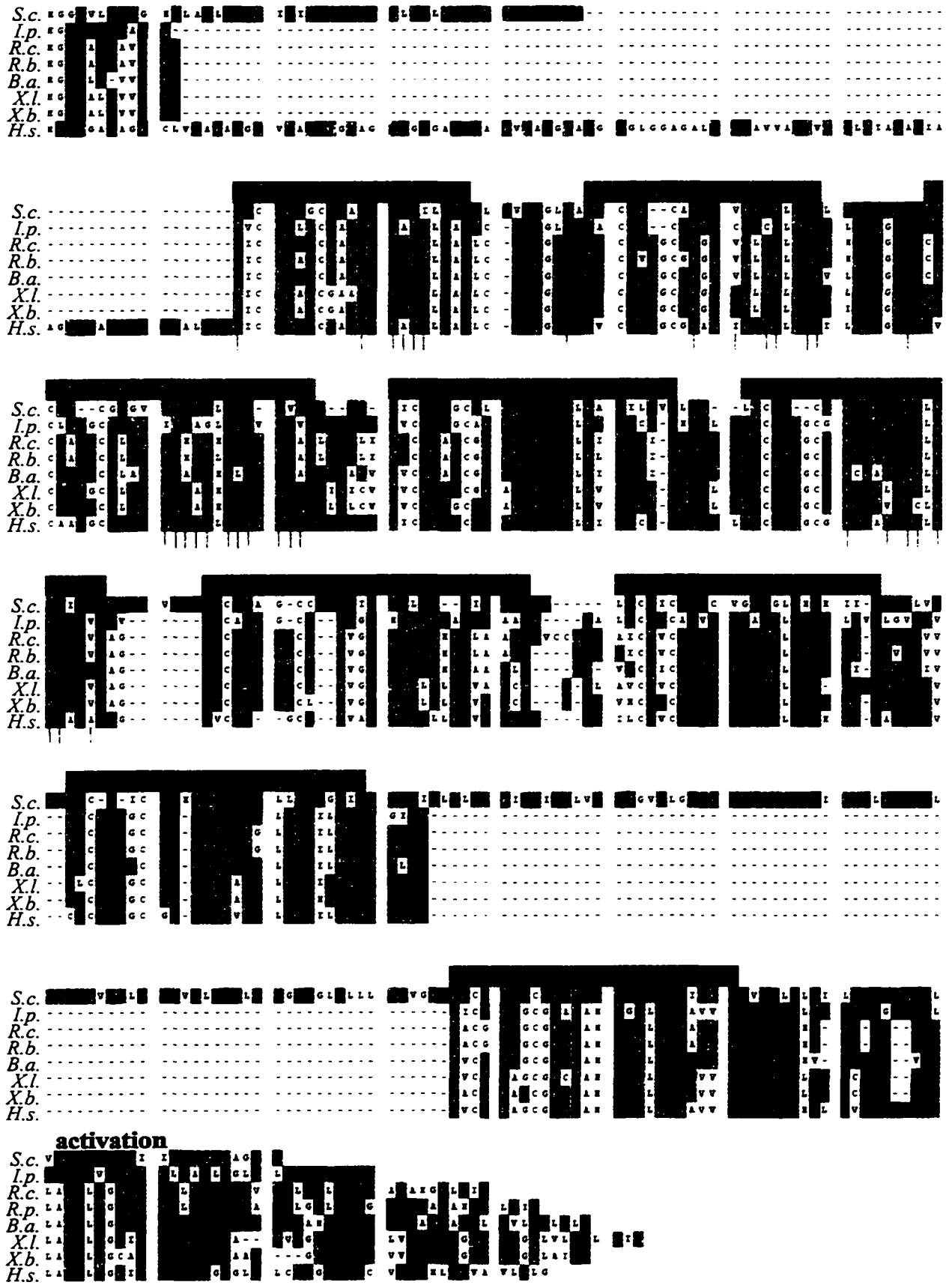
1.5.4 A comparison of the known TFIIIA homologues

Homologues of *X. laevis* TFIIIA have only been cloned from four other amphibians, a species of catfish, human, and yeast (Table 1.1). TFIIIA has not been cloned from other organisms for two main reasons. First, the amino acid sequence of TFIIIA is not well conserved among species as shown by the amino acid alignment of the known TFIIIA homologues (Fig. 1.8). Therefore, a common sequence among the known TFIIIA clones does not exist for identifying novel TFIIIA homologues in many other organisms. Amino acid sequences potentially

Table 1.1. The known TFIIIA homologues. Data for the five amphibian TFIIIA homologues was obtained from Gaskins and Hanas (1990) and Gaskins *et al.*, (1992). Data for TFIIIA from *S. cerevisiae* was obtained from Archambault *et al.* (1992). Data for TFIIIA from catfish was obtained from Ogilvie and Hanas (1997). Data for TFIIIA from human was obtained from information deposited in the Genbank database. The length of each protein is shown as the number of amino acids. The percent identity among the different proteins is presented as a relative comparison to TFIIIA from *X. laevis*. Only an estimation of the percent identity for TFIIIA from *S. cerevisiae* is presented, because some domains present in this protein are absent from TFIIIA from *X. laevis* and vice versa. Transcription domain identifies proteins possessing the conserved C-terminal RSLASRLTGYIPPK motif. Although TFIIIA from catfish lacks this domain, the homologues still weakly activates transcription in a nuclear extract from mouse.

organism	common name	length	size (kDa)	identity (%)	transcription domain
S. cerevisiae	Yeast	429	51	~23	no
I. punctatus	Catfish	322	37	43	no
R. catesbeina	American bullfrog	335	38	63	yes
R. pipiens	Northern leopard frog	335	38	62	yes
B. americanus	American toad	339	40	61	yes
X. laevis	African clawed frog	344	40	100	yes
X. borealis	Kenyan clawed frog	339	40	84	yes
H. sapiens	Human	423	42	58	yes

Fig. 1.8. The amino acid sequence alignment of the known TFIIIA homologues. Sequences were aligned with respect to the amino-terminal domains, the nine zinc fingers, and the transcriptional activation domain found in higher eukaryotes. Included are the amino acid sequences from *Saccharomyces cerevisiae* (*S.c.*), *Ictalurus punctatus* (*I.p.*), *Rana catesbeiana* (*R.c.*), *R. pipiens* (*R.p*), *Bufo americanus* (*B.a.*), *Xenopus laevis* (*X.l.*), *X. borealis* (*X.b.*) and *Homo sapiens* (*H.s.*). The zinc fingers are numbered 1 to 9 and are indicated above the alignment by green rectangles. The transcriptional activation domain in *X. laevis* TFIIIA is indicated by an orange rectangle above the alignment. Orange lines below the alignment identify the amino acids of *X. laevis* TFIIIA within zinc fingers 1-6 that potentially contact the DNA based on structural data (Wuttke *et al.*, 1997; Nolte *et al.*, 1998). Amino acids are identified in colored boxes by their one letter codes: methionine and serine, yellow; amino acids with aromatic side-chains, olive green; proline, green; all other amino acids with nonpolar side chains, grey; asparagine and glutamine, light blue; basic amino acids, dark blue; acidic amino acids, red. The alignment was done using Omiga version 2.0 (Oxford Molecular).



corresponding to TFIIIA homologues were not identified in the *Arabidopsis thaliani*, *Caenorhabditis elegans*, *Zea mays*, *Musculus musculus*, or *Drosophila melanogaster* available genome sequences by testing the known TFIIIA sequences against these genomes using the BLASTp program (Altschul *et al.*, 1997). However, because of some sequence conservation among vertebrate TFIIIA homologues, the sequences of the four amphibian and the human TFIIIA homologues were identified by screening cDNA libraries with a DNA probe from *X. laevis* TFIIIA (Drew *et al.*, 1995; Gaskins and Hanas, 1990; Gaskins *et al.*, 1992). The second reason TFIIIA has not been cloned from a variety of organisms is because it is extremely rare in most cell types (section 1.5). Only limited success has been achieved purifying TFIIIA from cells in which this protein is present at extremely low quantities (Moorefield and Roeder, 1994; Wang and Weil, 1989). In contrast, the purification and cloning of TFIIIA from *X. laevis* and catfish were facilitated by the relative abundance of this protein in immature oocytes from these organisms, greater than 5% of the total protein (Engelke *et al.*, 1980; Ogilvie and Hanas, 1997).

The variation in the amino acid sequences among the TFIIIA homologues causes differences in their biochemical properties. Subtle differences have been found in the DNase I footprints of the amphibian proteins on the *Xenopus* somatic 5S RNA gene. *X. laevis* and *X. borealis* TFIIIA have the same footprint boundaries on both strands of the DNA (section 1.5.2), but each protein produces some unique hypersensitive cleavage sites. On the coding and noncoding strands of the DNA, *X. borealis* TFIIIA produces hypersensitive cleavage at +63, while a hypersensitive cleavage site in this segment of the DNA bound by *X. laevis* TFIIIA is observed only on the noncoding strand and is displaced one position downstream to +62 (Gaskins *et al.*, 1989). Hydroxyl radical footprinting and structural data do not show *Xenopus* TFIIIA contacts within this segment of the 5S RNA gene. Therefore, variations in the

amino acid sequence between the *X. borealis* and *X. laevis* proteins that cause the alterations in the footprint are unclear. More extensive differences in the TFIIIA footprint on the *X. borealis* 5S RNA gene are observed between the two amphibian genera, *Xenopus* and *Rana* (Gaskins *et al.*, 1992). *Rana* TFIIIA exhibits a much weaker DNase I footprint over the 3'-end of the promoter that extends over the intermediate element and the A-box. Two base-pair differences in the A-box between *Xenopus* and *Rana* may be related to the weaker 3'-portion of the footprint (Fig. 1.9). Mutagenesis and footprinting studies have shown that zinc fingers 7-9 contact the A-box (section 1.5.2), and amino acids in fingers 7 and 8 that are implicated in DNA binding (positions -1, +3, and +6 of the α-helix) are found to vary between *Rana* and *Xenopus* (Fig. 1.8). Interestingly, a similar weakening in the 3'-region of the footprint was found by disrupting finger 8 of *X. laevis* TFIIIA, suggesting that variations specifically in finger 8 between *Rana* and *Xenopus* TFIIIA are responsible for differences in the 3'-portion of the footprint. The DNase I footprint of *B. americanus* on the coding strand of the Xenopus somatic gene has also been reported (Gaskins *et al.*, 1992). The pattern of protection was found to be more similar to the *Xenopus* TFIIIA footprints than to the *Rana* TFIIIA footprints.

The DNase I protection pattern of human TFIIIA on the *X. laevis* somatic 5S RNA gene has been found to be similar to the *X. laevis* TFIIIA footprint (Seifart *et al.*, 1989). The similar binding properties of the two proteins is not surprising considering that their respective 5S RNA gene promoters differ at only one position, which is found within the A-box (Fig. 1.9). Both proteins protect the same extent of DNA on the coding strand, and both proteins yield a hypersensitive cleavage site at +62 on the noncoding strand of the DNA. However, the DNase I footprint of human TFIIIA on the noncoding strand extends downstream beyond the 3'-edge of the *Xenopus* TFIIIA footprint (+96) to +99. Because the amino acids in finger 1 that contact the DNA are identical in both proteins except at +1 of the α-helix (Fig. 1.8),

Fig. 1.9. The alignment of the 5S RNA gene promoter from organisms in which TFIIIA has been cloned and from *Acanthamoeba castellanii*. The nucleotide sequences from *A. castellanii* (*A.c.*), *Saccharomyces cerevisiae* (*S.c.*), *Ictalurus punctatus* (*I.p.*), *Rana catesbeiana* (*R.c.*), *R. pipiens* (*R.p*), *Bufo americanus* (*B.a.*), *Xenopus laevis* (*X.l.*), *X. borealis* (*X.b.*) and *Homo sapiens* (*H.s.*). The colors shown the conservation of the sequence: dark blue, most conserved; green, moderately conserved; red, least conserved. The positions of the A-box, the intermediate element (IE), and the C-box are indicated by green rectangles below the alignment. The alignment was done using Omiga version 2.0 (Oxford Molecular).

the extended footprint of human TFIIIA may arise from the divergent and extended amino-terminal domain of this protein. In support of this hypothesis, removal of the amino-terminal region of *Xenopus* TFIIIA may effect a slight loss in the 3'-border of its footprint (Clemens *et al.*, 1994), suggesting that the amino-terminal region of TFIIIA may have a minor influence on DNase I protection. Another difference in the footprints of the two proteins is the overall weaker protection of the *Xenopus* gene by human TFIIIA (Seifart *et al.*, 1989). Because the *cis*-element that is essential for TFIIIA binding, the C-box, is identical in the *Xenopus* and human 5S RNA genes (Fig. 1.9), the weaker binding of human TFIIIA may be due to differences in its amino-acid sequence. Out of 24 amino acids in *Xenopus* TFIIIA that potentially contact the DNA, 7 are not conserved in human TFIIIA (Fig. 1.8). The human 5S RNA gene promoter differs from the *Xenopus* somatic gene promoter by a single base pair that is located within the A-box (Fig. 1.9). Because human TFIIIA has been observed to bind more strongly to the human promoter than to the *Xenopus* promoter, it is possible that human TFIIIA relies more strongly on interactions with the A-box or other sequence elements within the human 5S RNA gene.

The DNase I footprint of catfish TFIIIA has not been reported, but based on the divergence of its amino acid sequence (Fig. 1.8) and in the sequence of the catfish 5S RNA gene (Fig. 1.9), this protein is not expected to bind to the *Xenopus* 5S RNA gene. As expected, yeast TFIIIA has not been found to bind to the *Xenopus* promoter (Archambault *et al.*, 1992). The footprint of yeast TFIIIA on its gene is quite different from the those of the vertebrate homologues (see below).

TFIIIA homologues in which the transcriptional activation domain identified in *X. laevis* has been conserved are able to activate 5S RNA transcription from a *X. laevis* egg extract. The carboxyl-terminal motif is conserved in the *Xenopus* and *Rana* genera and in *H. sapiens* and *B. americanus* (Fig. 1.8). *X. laevis* and *X. borealis* have been found to produce similar levels of transcription from a *X. laevis*

egg extract with the *X. borealis* 5S RNA gene, while transcription is much weaker with *R. pipiens* and *R. catesbeina* TFIIIA (Gaskins *et al.*, 1989). Weak transcription with the *Rana* TFIIIAs may be related to the weaker 5'-footprint produced by these homologues on the *X. borealis* 5S RNA gene, considering that A-box binding is essential for transcription. Transcription using *B. americanus* TFIIIA has not been reported. However, based on its strong protection of the *Xenopus* somatic 5S RNA gene promoter, including over the A-box (Gaskins *et al.*, 1992), its transcriptional activation properties are expected to be more similar to *Xenopus* TFIIIA than to *Rana* TFIIIA. Human and *Xenopus* TFIIIA have been found to produce similar levels of transcription with their respective genes in a HeLa cell extract (Waldschmidt *et al.*, 1990). Interestingly, catfish TFIIIA was able to produce weak transcription from a rodent nuclear extract, although this homologue lacks the transcriptional activation domain found in higher vertebrates (Ogilvie and Hanas, 1997). In comparison, 5S RNA transcription with *Xenopus* TFIIIA was 20-fold greater in the same rodent nuclear extract.

Characterization of yeast TFIIIA has revealed that its properties are significantly different from the properties of *Xenopus* TFIIIA. The deduced amino acid sequence of yeast TFIIIA shows that it is only approximately 23 % similar to the sequence of *X. laevis* TFIIIA (Archambault *et al.*, 1992). This divergence between the two proteins may be partly related to the high degree of variation in sequences of the yeast and *Xenopus* 5S RNA genes (Fig. 1.9). Yeast TFIIIA has been found to produce a shorter footprint than *Xenopus* TFIIIA, protecting only +65 to +95 of the gene from DNase I cleavage (Braun *et al.*, 1989). The shorter footprint may be related to an insertion of 81 amino acid between fingers 8 and 9 in yeast TFIIIA that is not found in other homologues (Fig. 1.8). This insert could potentially disrupt DNA binding by the carboxyl-terminal fingers, but regardless of this potential effect, the 81 amino acid domain has been found to be required for transcription (Milne and

Segall, 1993). Therefore, significantly different domains in yeast and *Xenopus* TFIIIA elicit transcriptional activation. In addition to the 81 amino acid insert, zinc finger 1 of yeast TFIIIA has been found to be essential for transcriptional activation. Yeast TFIIIA mutants that lack finger 1 or in which finger 1 is disrupted are unable to recruit TFIIIC as observed by EMSA, and these mutants cannot activate 5S RNA transcription *in vitro* or *in vivo* (Milne and Segall, 1993; Rothfels *et al.*, 1998). Together, these results have illustrated the significant differences between yeast and *Xenopus* TFIIIA.

A comparison of the known TFIIIA homologues indicates that the well-defined properties of *X. laevis* TFIIIA may not apply to other homologues. To address this possibility, TFIIIA has been characterized from *A. castellanii* (Chapter 2). Additional interest in *A. castellanii* TFIIIA relates to its regulatory control of 5S RNA transcription during *A. castellanii* encystment. Nuclear extract prepared from cysts has been shown to be unable to support 5S RNA transcription, which is due to the loss of TFIIIA activity (Matthews *et al.*, 1995). A similar loss of TFIIIA activity has been observed in *Xenopus* eggs following meiosis (White, 1998). In the *Xenopus* system, loss of TFIIIA activity has been proposed to occur by feedback inhibition in which TFIIIA becomes sequestered in the cytoplasm in the 7S RNP (Pelham and Brown, 1980). However, proof of this mechanism has not been reported. It is therefore of interest to elucidate the mechanism of TFIIIA regulation during *A. castellanii* encystment to determine if loss of TFIIIA activity is based upon interactions with 5S RNA or a completely novel mechanism.

Chapter 2:

Purification and Characterization of TFIIIA from *Acanthamoeba castellanii*

The work described in this chapter has not been published, but is written in a format to facilitate in writing a manuscript for publication. I have done all of the work presented in this chapter.

2.1 Abstract

TFIIIA is exclusively required to activate RNA polymerase III (Pol III) transcription from 5S RNA genes. Although all known TFIIIA homologues harbor nine zinc fingers that mediate DNA binding, very limited sequence homology is found among these proteins, which reflects unique properties of some TFIIIA homologues. For example, the *Acanthamoeba castellanii* homologue directly regulates 5S RNA transcription. We have purified and characterized *A. castellanii* TFIIIA as a step toward obtaining a clearer understanding of these differences and of the regulatory process. This protein was found to be 59 kDa, significantly larger than all other TFIIIA homologues isolated to date. Nevertheless, it exhibited a 50 base pair DNase I footprint, which is similar to that produced by the smaller TFIIIAs from vertebrates, but distinct from the smaller footprint of the 51 kDa TFIIIA of yeast. Similar footprinting is not reflected in greater sequence similarity between the *A. castellanii* and vertebrate promoters.

2.2 Introduction

TFIIIA activates transcription of 5S RNA genes by RNA polymerase III. Early studies in *Xenopus* demonstrated that TFIIIA is the first transcription factor to contact the DNA. It binds along the entire length of the approximately 50 bp type I promoter located within the transcribed portion of the gene (Engelke *et al.*, 1980). Mutational analysis has shown that the promoter consists of three *cis*-elements: a 5'-proximal A-box, a 3'-proximal C-box, and an intermediate element in the middle of the promoter (Pieler *et al.*, 1987). Hydroxyl radical footprinting and missing nucleoside analysis has shown that TFIIIA contacts all three promoter elements (Hayes and Tullius, 1992; Vrana *et al.*, 1988). Formation of the TFIIIA-DNA complex allows the subsequent sequential integration of TFIIIC and TFIIIB into the complex (Bieker *et al.*, 1985; Setzer and Brown, 1985). Following the addition of TFIIIB, Pol

III can be recruited to activate transcription (Bogenhagen *et al.*, 1982). In yeast and human, the same sequence of events is needed for gene activation (Geiduschek and Kassavetis, 2001; Paule and White, 2000).

TFIIIA from *X. laevis* has been well characterized. In *Xenopus* oocytes, TFIIIA also functions to store 5S RNA in the cytoplasm during development (Honda and Roeder, 1980; Pelham *et al.*, 1981). Because of this role, TFIIIA can constitute up to 15% of the soluble protein in oocytes, which led to the early purification and cloning of this transcription factor (Engelke *et al.*, 1980; Ginsberg *et al.*, 1984). From the amino acid sequence, *X. laevis* TFIIIA was found to have nine sequential C2H2-type zinc fingers (Miller *et al.*, 1985). An amino-terminal proteolytic fragment of TFIIIA containing all the zinc fingers was found to retain the same ability to protect the 5S RNA gene from DNase I cleavage as full length TFIIIA, but lost transcription activity (Smith *et al.*, 1984), illustrating the role of the zinc fingers in DNA binding. Changes in the TFIIIA footprint effected by truncating the protein showed that the amino-terminal fingers were positioned at the 3'-end of the promoter and the carboxyl-terminal fingers were position at the 5'-end (Vrana *et al.*, 1988). Interactions between the amino-terminal three fingers and the promoter were found to be most critical for DNA binding, which is exemplified by the ability of a polypeptide containing only these three fingers to bind DNA with high affinity (Christensen *et al.*, 1991; Liao *et al.*, 1992). In contrast the carboxyl-terminal three fingers were shown to be essential for transcriptional activation, as disrupting any one of these fingers by mutating a histidine coordinated to the zinc ion inactivates transcription (Del Rio *et al.*, 1993).

The DNA binding and transcriptional activating properties of other TFIIIA homologues vary from those that have been defined for *X. laevis* TFIIIA. TFIIIA has been cloned from four other frog species (Gaskins and Hanas, 1990; Gaskins *et al.*, 1992) using the *X. laevis* clone as a probe, from catfish [Ogilvie and Hanas, 1997]

using peptide sequence from the purified factor, from human (Drew *et al.*, 1995) using the conserved TGEKP linker sequence connecting the amino-terminal three zinc fingers, and from *S. cerevisiae* by serendipity (Archambault *et al.*, 1992). Interestingly, the amphibian TFIIIA homologues have been found to produce slightly different footprints on the *X. borealis* somatic 5S RNA gene, and the ability to activate transcription in a *X. laevis* egg extract was also found to differ with the use of TFIIIA homologues from two different amphibian genera (Gaskins *et al.*, 1989), illustrating that even among closely related species the properties of TFIIIA can differ.

Functional differences are further supported by the lack of strong amino acid sequence conservation among known TFIIIA homologues. This lack of conservation has also hindered the identification and cloning of additional homologues. *S.cerevisiae* TFIIIA was identified as a 9 zinc finger-containing gene adjacent to a cloned RNA polymerase subunit (Archambault *et al.*, 1992). Although *S. cerevisiae* TFIIIA, like all of the other homologues, has nine zinc fingers, it is only approximately 23% homologous to the *Xenopus* factor at the amino acid level. One significant difference between *Xenopus* and *S. cerevisiae* TFIIIA is their size; yeast TFIIIA is approximately 12 kDa larger than the 39 kDa *Xenopus* protein. The larger size of *S. cerevisiae* TFIIIA is primarily due to an 81 amino acid domain inserted between zinc fingers 8 and 9 (Archambault *et al.*, 1992) that is not found in other TFIIIA homologues. This domain is required for transcription (Milne and Segall, 1993). In contrast, TFIIIA from *Xenopus* and other higher vertebrates possess a 15 amino acid sequence within their carboxyl-terminal domains that is essential for transcription (Mao and Darby, 1993). This domain is not found in TFIIIA from yeast or catfish. Despite its larger size, TFIIIA from *S. cerevisiae* protects an approximately 16 base pair smaller segment of the 5S RNA gene from DNase I cleavage than *Xenopus* TFIIIA (Braun *et al.*, 1989), suggesting that the similarly

positioned zinc fingers may function differently in the two proteins. Indeed, the first zinc finger in yeast and *Xenopus* function differently; this finger is primarily used for DNA binding in *Xenopus* (Vrana *et al.*, 1988), but is also required for the recruitment of TFIIIC and transcriptional activation in yeast (Rothfels *et al.*, 1998).

Because TFIIIA has only been characterized from a small number of vertebrates and *S. cerevisiae*, a complete understanding of the possible variations in this protein has not yet been achieved. Indeed, database searches reveal the sequence of only eight TFIIIA genes. As a prelude to cloning, we report here the purification and characterization of TFIIIA from *Acanthameoba castellanii* (*Ac*TFIIIA), an organism in which it has key regulation functions (Matthews *et al.*, 1995). *Ac*TFIIIA was specifically photo-cross-linked to the 5S RNA gene at two separate sites on the promoter and was additionally identified by southwestern blotting. *Ac*TFIIIA produced a DNase I pattern of protection similar to that produced by TFIIIA homologues in higher vertebrates. These experiments correlated TFIIIA activity with a 59 kDa polypeptide, which is significantly larger than any of the known TFIIIA homologues.

2.3 Materials and Methods

2.3.1 Purification of TFIIIA.

With the exception of collecting the cells at 1,840 X *g* rather than 3,270 X *g*, *A. castellanii* trophozoite cells were grown and harvested as described (Radebaugh *et al.*, 1994). Nuclear extract was prepared from individual batches of 50 to 55 L of cells with 260 L of cells processed in total, as described (Radebaugh *et al.*, 1994; Zwick *et al.*, 1991a), but with three modifications. First, cells were homogenized with three passages through a LSC Homogenizer LH-21 (Yamato Scientific, Japan) at 1000 rpm, resulting in breakage of >90 % of cells. Second, the cell homogenate was

centrifuged 15 min. at 8,700 X *g*. Third, the precipitated proteins were collected by centrifugation for 30 min at 4,420 X *g*.

Basic chromatography buffer (HEG) consisted of 50 mM HEPES, pH 7.9, 20% glycerol, 0.2 mM phenylmethane sulfonyl fluoride, 0.2 mM EDTA and 1 mM dithiothreitol (DTT). Additional components supplemented this buffer depending on the chromatography step. All columns were equilibrated and washed with at least 5 column volumes of starting buffer prior to loading. Whatman P11 (phosphocellulose) was prepared per manufacturer's instructions and was additionally treated overnight with two changes, 13 bed volumes each, of 1.5 M KCl, 250 mM HEPES, pH 7.9, and 0.2 mM EDTA prior to equilibration. All chromatography procedures were performed at 4 °C, and all KCl concentrations were verified by conductivity measurements versus a standard curve of KCl in water. The first P11 column (2.5 cm diameter, 390 mL bed volume) was equilibrated using HEG buffer with 100 mM KCl. The total nuclear extract (~3.8 g of protein, 213 mL) was applied to the column at a linear flow rate of 13.4 cm/hr. Protein eluting from the column was detected by absorbance at 280 nm. The flow-through fraction (570 mL, 1,041 mg of total protein) containing TFIIIA was collected and the apparent concentration of KCl was increased to 450 mM by adding HEG with 1.7 M KCl; benzamidine was also added to a final concentration of 1 mM. This fraction was then gently mixed on ice for 1 hr to allow dissociation of TFIIIA and 5S RNA.

The second P11 column (2.5 cm diameter, 104 mL bed volume) was equilibrated using HEG with 450 mM KCl and 1 mM benzamidine. The first P11 flow-through fraction, adjusted to 450 mM KCl, was applied to the column at a linear flow rate of 13.4 cm/hr. The column was then washed with the buffer used to equilibrate the column and developed with a 500 mL linear salt gradient from 450 mM KCl to 1.2 M KCl in HEG, 1 mM benzamidine. TFIIIA eluted predominantly between 510 and 590 mM KCl. Thirteen fractions constituting the peak of DNA-

binding activity were pooled (~81 mL and 4.3 mg total protein), transferred to dialysis tubing (MWCO 12,000 to 14,000; Spectrum Laboratories, Rancho Dominguez), and dialyzed 6 hr against 2 L HEG with 110 mM KCl, 1 mM benzamidine, and 5 mM DTT in place of 1 mM, which reduced the concentration of KCl to 140 -150 mM. The higher DTT concentration stimulated DNA binding of TFIIIA in the next step.

A total of 21.6 mg calf thymus DNA (Sigma, St. Louis) in 16 mL of 50 mM HEPES, pH 7.9, was added to the dialyzed pool from the second P11 column, reducing the concentration of KCl to about 120 mM. $MgCl_2$ and $ZnCl_2$ were then added to this fraction to final concentrations of 7.5 mM and 2.5 μM, respectively. This solution was then incubated at room temperature for 20 min with gentle agitation. Prior to loading the promoter-DNA affinity column, any precipitates that had formed in the solution were removed by centrifugation for 10 min at 7,000 rpm in a JA10 rotor (Beckman, Fullerton) at 4 °C. The supernatant is the Affinity Load.

A promoter-DNA affinity column (1.5 cm diameter, 7 mL bed volume) was equilibrated using HEG without EDTA and with DTT elevated to 5 mM containing 110 mM KCl, 7.5 mM $MgCl_2$, 2.5 μM $ZnCl_2$, and 1 mM benzamidine. The Affinity Load was then applied to the affinity column at a linear flow rate of 5.66 cm/hr. The column was subsequently washed with equilibration buffer and developed with a 35 mL linear salt gradient from 110 mM KCl to 1 M KCl. TFIIIA activity eluted between 340 and 470 mM KCl.

2.3.2 Determination of protein concentrations

Different procedures, depending on the stage of purification, were required to determine the protein concentration. The protein concentration of each batch of nuclear extract was determined using the Enhanced Protocol of a BCA protein assay (Pierce, Rockford) as described by the manufacturer using the supplied

bovine serum albumin (BSA) protein standard. Protein concentrations of the flow-through fraction and the pooled fractions from the second P11 column were determined by a modified Bradford microassay procedure (Bio-Rad, Hercules) as described by the manufacturer, also using the same BSA as the protein standard. The total protein in the pool of purified TFIIIA activity was determined from a Coomassie Brilliant Blue G-250-stained 11% SDS-polyacrylamide gel used to resolve a substantial portion of this protein. Prior to being loaded on the gel, proteins were precipitated using a chloroform-methanol procedure (Wessel and Flugge, 1984) with 100 ng of cofilin added as a carrier. The relative intensities of Coomassie-stained protein bands were determined using the Photometrix 1D Gel Analysis Program (version 2.51; Non Linear Dynamics, England). β-galactosidase, carbonic anhydrase, and myoglobin were resolved on the same gel and utilized as protein standards.

2.3.3 Preparation of the DNA-promoter-affinity resin

The DNA coupled to the resin consisted of 5 head-to-tail repeats of the *A. castellanii* 5S RNA gene from +15 to +96, which was originally cloned into the BamHI site of pT7T3α18 (Life Technologies, Rockville) and was a generous gift from Mike Zwick. A fragment containing the multimer was excised using *Eco*RI and *Hind*III, purified as described (Radebaugh *et al.*, 1998), and subsequently coupled to cyanogen bromide-activated Sepharose CL-4B (Pharmacia, Piscataway) as described (Kadonaga and Tjian, 1986).

2.3.4 Fractionation of TFIIIB, TFIIIC, and Pol III for DNA-Protein Photo-cross-linking

Nuclear extract applied to a P11 column was fractionated as described previously (Matthews *et al.*, 1995). TFIIIB, Pol III, and TFIIIC were found in the 0.3, 0.45, and 0.65 M KCl step elutions, respectively.

2.3.5 Fractionation of TFIIIB, TFIIIC, and Pol III for *in vitro* transcription assays

Protein retained on the first P11 column (described under "Purification of TFIIIA") was eluted using HEG with 700 mM KCl and detected by absorbance at 280 nm. This fraction was diluted to 450 mM KCl using HEG and batch-loaded for 1 hr onto 90 mL of P11 resin equilibrated using HEG with 450 mM KCl. The slurry was then filtered through a 417 qualitative filter disc (VWR, San Francisco), and all three components found in the filtrate were precipitated by making the solution 70% saturated in ammonium sulfate, stirring overnight, and centrifuging at 12,100 x *g* for 10 min in a JA17 (Beckman, Fullerton) rotor (Matthews *et al.*, 1995). The precipitate was resuspended in dialysis buffer (50 mM HEPES, pH 7.9, 120 mM KCl, 10 % glycerol, 0.2 mM phenylmethane sulfonyl fluoride, 0.2 mM EDTA, 1mM benzamidine and 1 mM DTT) at a volume of 0.25 X the load volume. The solution was then transferred to dialysis tubing (MWCO 12,000 to 14,000; Spectrum Laboratories, Rancho Dominguez) and dialyzed against 2 L of dialysis buffer at 4 °C for 6 hr. This fraction was relatively free of contaminating TFIIIA and had a low background of 5S RNA transcription.

2.3.6 Electrophoretic mobility shift assays

Preparation of the DNA probe and the electrophoretic mobility shift assays (EMSAs) were done as described (Matthews *et al.*, 1995), but with the following

modifications. A 260-bp fragment of pAc5S.3dI5'-34 (Zwick *et al.*, 1991a) was obtained by EcoRI-HindIII digestion and labeled by a fill-in reaction. The standard EMSA reaction contained 100 mM KCl, 50 mM HEPES (pH 7.9), 7.5 mM $MgCl_2$, 2.5 μM $ZnCl_2$, 5 mM DTT, 10% glycerol, 4 μg acetylated BSA, and 20,000 cpm of probe in a total volume of 20 μL. Reactions were incubated at 25 °C for 20 min and then loaded directly on a 5% nondenaturing polyacrylamide gel as described (Matthews *et al.*, 1995) that had been prerun for 20 min at 200 V. Reactions were electrophoresed for 1.3 hr at 200 V at room temperature.

A variation of the standard EMSA reaction was used to quantify DNA binding activity for the purification table. A PCR amplified DNA fragment from –2 to +136 of the 5S RNA gene was end-labeled. Each reaction contained 1 μL of the protein fraction and 1.9 fmol of DNA probe of known specific activity. The reaction with the first P11 flow-through fraction and the second P11 pool were supplemented with 300 ng and 60 ng of calf thymus DNA, respectively, as nonspecific competitor DNAs. These two reactions were then incubated at 25 °C for 10 min prior to adding probe. One unit of TFIIIA DNA-binding activity is defined as that amount needed to shift 1 fmol of probe, in probe excess, under the standard conditions.

2.3.7 Southwestern blotting

The probe used for hybridization was a 5S RNA gene fragment from -2 to +136 that was internally radiolabeled in a PCR reaction. Protein fractions were resolved on an 11% SDS-polyacrylamide gel and electrotransferred to a PVDF membrane. The membrane was incubated with 50 mL of renaturation buffer (50 mM HEPES [pH 7.9], 100 mM KCl, 7.5 mM $MgCl_2$, 50 μM $ZnCl_2$ 0.1 %, Nonidet P40, 10 % glycerol, 5 mM DTT, 1 mM benzamidine, and 0.2 mM PMSF) at room temperature for a total of 4.5 hr with two changes of the buffer. The second change of renaturation buffer contained 20 μM $ZnCl_2$, and the third change contained 8 μM

$ZnCl_2$. The membrane was then blocked with 50 mL of renaturation buffer containing 3 μM $ZnCl_2$ and 5% non-fat milk proteins for 1 hr and subsequently hybridized with probe in 3 mL of binding buffer (50 mM HEPES [pH 7.9], 100 mM KCl, 7.5 mM $MgCl_2$, 2.5 μM $ZnCl_2$, 10 % glycerol, 0.1 mg/mL BSA, and 5 mM DTT) at room temperature for 2 hr. After hybridization, the membrane was washed twice with 25 mL of 50 mM HEPES (pH 7.9), 100 mM KCl, 7.5 mM $MgCl_2$, and 5 mM DTT, and twice with 25 mL of 50 mM HEPES (pH 7.9), 50 mM KCl, 7.5 mM $MgCl_2$, and 5 mM DTT. Washes were done at room temperature for 15 min on a rocking platform. Dried membranes were exposed to phosphorstorage screens for 48 hours.

2.3.8 *In vitro* transcription assays

Each transcription reaction contained 50 mM HEPES (pH 7.9), 100 mM KCl, 7.5 mM $MgCl_2$, 10 % glycerol, 2 mM DTT, 2 μg of α-amanitin per mL, 600 μM each of ATP, CTP, and GTP, 50 μM UTP, 5 μCi (α-^{32}P)UTP (3,000 Ci/mmol), and 42 fmol pBS+5S.3 (Matthews *et al.*, 1995), 7.2 μL of the TFIIIB/TFIIIC/Pol III fraction, and 2 μL of an affinity purified TFIIIA fraction in a final volume of 30 μL. Reactions were started by adding the NTP mixture (3.5 μL) and were then incubated at 25 °C for 1.5 hr. They were stopped by adding 11 μL of a freshly prepared mix of 5 μL proteinase K (10 mg/mL) plus 6 μL stop mix (250 mM NaCl, 1 % SDS, 20 mM Tris-HCl [pH 7.6], 5 mM EDTA), and incubated at 50 °C for 30 min. The RNA was precipitated by adding 5 μL of linear polyacrylamide (5 mg/mL), 154 μL 10 mM Tris-HCl (pH 7.5), 1 mM EDTA (pH 8.0), 100 μL of 7.5 M ammonium acetate, 1000 cpm of the standard EMSA probe (as a recovery standard) and 900 μL of 95% ethanol. Samples were chilled at –20 °C for 10 min, and then the RNA was collected by centrifugation for 20 min at maximum speed in an Eppendorf centrifuge. The samples were then processed as describe (Matthews *et al.*, 1995).

2.3.9 SDS-PAGE and silver-staining of proteins

SDS-PAGE was performed as described previously (Laemmli, 1970). To each sample, 6X SDS loading buffer (Ausubel *et al.*, 1994) was added to a final concentration of 1X in the sample. Samples were resolved on an 11% separating gel with a 4% stacking gel. The gel was stained with silver as described (Blum *et al.*, 1987).

2.3.10 DNase I footprinting

The plasmid pAc5S.3dI5'-34 (Zwick *et al.*, 1991a) was digested with either *Eco*RI or *Hind*III, the 5'-ends of the linearized DNA were dephosphorylated with shrimp alkaline phosphatase and were heated at 65 °C for 15 min to inactivate the enzymes. The linearized plasmids were then end-labeled with T4 polynucleotide kinase. The labeled DNA was precipitated with 7.5 M ammonium acetate and 95 % ethanol, and each linearized plasmid was subsequently digested with the alternate restriction enzyme. The fragments were then purified on a 1.2 % agarose gel and extracted using Qiaex II beads (Qiagen, Valencia).

For footprinting, protein-DNA interaction conditions were essentially the same as those used for the EMSA, except the reaction volume was 50 μL and 10,000 cpm of probe was added to each reaction. Following protein-DNA association, 0.1 units of DNase I (2 μL) was added to each sample and reactions were incubated at 25 °C for 2 min. Reactions were then transferred to a dry ice/ethanol bath to inactivate the DNase I enzyme and were treated with proteinase K and precipitated as described under "*In vitro* transcription assays". Digestion products were suspended in 7 μL of loading solution (90% formamide, 10 mM EDTA, 0.1 % bromphenol blue, 0.1 % xylene cyanol) and resolved on an 8 % polyacrylamide, 7 M urea, 1X TBE gel as described previously (Geiss *et al.*, 1997). Labeled digestion fragments were

visualized as described previously (Matthews *et al.*, 1995) and compared to a G + A chemical sequencing ladder.

2.3.11 Site-specific DNA-photo-cross-linking

Two oligodeoxyribonucleotides were synthesized with a phosphorothioate in the place of the normal phosphodiester linkage between the second and third nucleotides at the 5'-end of the molecules. One oligodeoxyribonucleotide corresponded to the RNA-like strand of the 5S RNA gene from +65 to +80. The other corresponded to the RNA-like strand from +85 to +100. Oligodeoxyribonucleotides were suspended to a concentration of 100 pmol/μL in 100 mM triethylammonium bicarbonate, pH 8.0, and 75 μL of this solution was combined with 75 μL of 100 mM azidophenacyl bromide in acetonitrile to link the azidophenacyl group to the phosphorothioate. This and all subsequent steps were performed under reduced light. The reaction was incubated at 25 °C for 4 hr. The solvent was then evaporated under vacuum, and the oligodeoxyribonucleotide pellets were suspended in 100 μL of sterile, deionized water. The solutions were subsequently extracted three times with 100 μL of water-saturated butanol and four times with 200 μL of water-saturated diethyl ether. To remove residual diethyl ether, samples were incubated at 37 °C for 15 min. The oligodeoxyribonucleotides were then diluted with 10 mM Tris, 1 mM EDTA, pH 8.0 to a final volume of 600 μL. Aliquots of 2 μL (approximately 20 pmol) were dried under vacuum and stored at –20 °C.

The preparation of the probes has been described (Kim *et al.*, 1999). The derivatized primers were annealed to pBS+5S.3 (Matthews *et al.*, 1995) or pBS+5SΔ86 single-stranded plasmid DNA of the RNA-ike strand. The latter plasmid contained an insertion of the 5S RNA gene from –129 to +85. Probes from pBS+5S.3 were prepared by digesting the DNA with either *Eco*RI and *Hind*III or

*Eco*RI and *Sma*I to produce fragments of pBS+5S.3 that contained –126 to +219 or –126 to +96 of the 5S RNA gene, respectively. Probes from pBS+5SΔ86 were prepared by digesting the DNA with *Eco*RI and *Hind*III to produce a fragment that contained the 5S RNA gene from –129 to +85.

The conditions used for DNA-protein binding were the same as those used for the EMSA. Following binding, reactions were u.v. irradiated with 11 mJ/mm^2 at 365 nm for 4 min. The cross-linked protein-DNA in the samples was analyzed as described previously (Gong *et al.*, 1995).

2.4 Results

2.4.1 Purification of TFIIIA

A. castellanii TFIIIA was purified to near homogeneity from a nuclear extract by three chromatography steps (Fig. 2.1A). Enrichment of the 59 kDa TFIIIA was observed on a silver-stained SDS-polyacrylamide gel (Fig. 2.1B) of each fraction. Overall, *A. castellanii* TFIIIA was purified 5037-fold from the first PC flow-through fraction to a specific activity of 196,000 units/mg protein (Table 2.1).

Nuclear extract was prepared from cells harvested in early log phase to ensure the full activity of the protein, which becomes inactivated later in the growth cycle (Matthews *et al.*, 1995). Passage of this material through a phosphocellulose (PC) column resulted in TFIIIA eluting mainly in the 0.1M KCl flow-through fraction as described (Matthews *et al.*, 1995). Human and *Xenopus* TFIIIA in crude extracts have also been found to elute in the PC flow-through fraction (Segall *et al.*, 1980; Shastry *et al.*, 1982), a property attributed to the formation of a complex with 5S RNA (Seifart *et al.*, 1989). In both the vertebrate and *Acanthamoeba* systems, a minor portion of the TFIIIA is retained on the PC column, presumably because it is free of 5S RNA. Attempts to purify this TFIIIA was unsuccessful (data not shown), resulting in highly contaminated final preparations of TFIIIA (data not shown). The

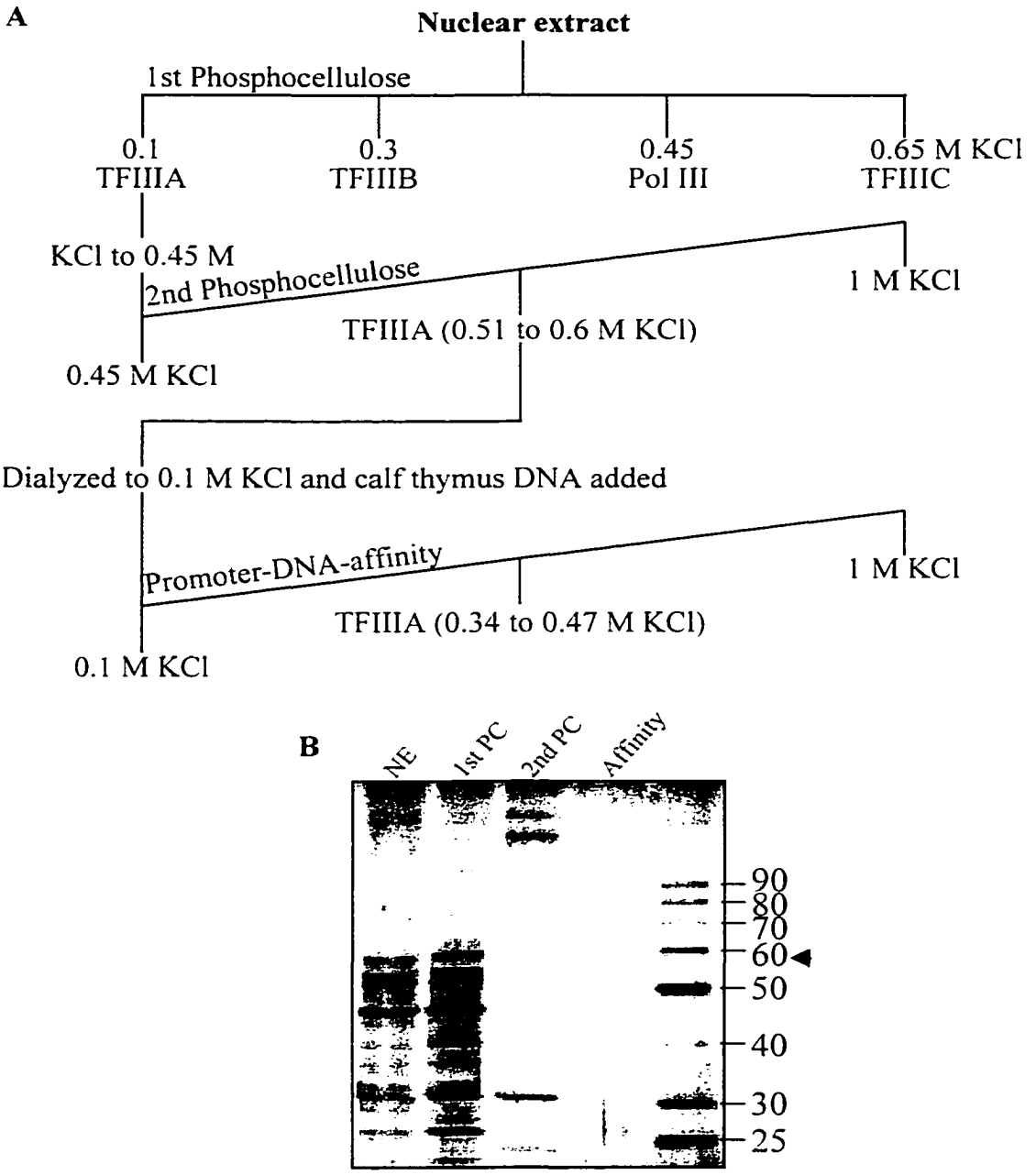


Fig. 2.1. *A. castellanii* TFIIIA was purified from nuclear extract using three chromatographic steps. **A**, the schematic representation depicts the general purification strategy. The separation of the Pol III factors with the first phosphocellulose column is indicated. **B**, the 11 % SDS-polyacrylamide gel stained with silver shows the relative purity of TFIIIA at each stage of the purification. NE, 0.5 μg nuclear extract; 1st PC, 0.5 μg of the 0.1 M KCl flow-through fraction from the first phosphocellulose column; 2nd PC, 0.5 μg of the TFIIIA-containing fractions pooled from the second PC column; affinity, 19 μL of fraction 21 from the promoter-DNA-affinity column (Fig. 2.3C). The arrow shows the protein band correlated with TFIIIA activity.

Table 2.1 Purification of TFIIIA from *A. castellanii* nuclear extract. A Bradford assay (Bradford, 1976) was used to determine the protein concentrations of the 1st PC flow through fraction and the fractions pooled from the 2nd PC column. Because the Bradford assay produced an inaccurate measurement of the protein concentration of the nuclear extract, a BCA protein assay (Pierce) was used for this fraction. The protein concentration of the pooled fractions from the promoter-DNA-affinity column was determined by comparison of proteins in this fraction to protein standards on a Coomassie-stained SDS-polyacrylamide gel. TFIIIA activity was determined by EMSA, with 1 unit of activity defined as the amount of protein required to shift 1 fmol of a radiolabeled DNA fragment containing the 5S RNA gene from -2 to +136. A comparison of the protein bands in the Coomassie-stained gel and the total units in the purified fraction indicates that 0.1% of the TFIIIA is active in this fraction. Based on the estimation that TFIIIA was purified from 5.2 X 10^{11} *A. castellanii* cells, each cell has 171 molecules of TFIIIA.

Fraction	Volume (mL)	Total protein (mg)	Units	specific activity (units/mg)	Fold purification	%
Nuclear extract	213	3797	-	-	-	-
1st PC flow-through	470	1041	147,828	142	3.65	100
2nd PC pool	81.3	4.3	28,268	6574	169	19
Affinity pool	6.13	0.003	588	196,000	5037	0.4

*Ac*TFIIIA that binds to the PC column elutes at 0.65 M KCl along with TFIIIC (Matthews *et al.*, 1995). In contrast, human TFIIIA binds more tightly, eluting at 1 M KCl (Seifart *et al.*, 1989). Moorefield (Moorefield and Roeder, 1994) was able to take advantage of this tighter binding to the cation exchanger BioRex 70 to obtain highly enriched human TFIIIA, skipping PC chromatography altogether, but *Ac*TFIIIA elutes from BioRex 70 at too low a salt (330 mM KCl) for this to be effective. Alternatively, nucleic acids have been removed from human TFIIIA using DEAE chromatography (Moorefield and Roeder, 1994), but this procedure was found to cause proteolytic degradation of *Ac*TFIIIA (data not shown).

Therefore, the use of two, consecutive PC chromatography steps was found to be optimal for significant purification of *Ac*TFIIIA in high yield. This approach was previously shown to produce a fraction highly enriched in human TFIIIA activity (Seifart *et al.*, 1989). The first PC flow-through fraction, which contained most of the TFIIIA, was adjusted to 0.45 M KCl to disrupt TFIIIA-nucleic acid interactions, which allowed TFIIIA to bind to a second PC column equilibrated to 0.45 M KCl. This concentration of KCl was close to the upper limit at which *Ac*TFIIIA can bind to the PC column, and at this concentration of KCl, 83% of the contaminating protein did not bind. The column was then developed with a linear KCl gradient, and the peak of TFIIIA eluted between 0.5 and 0.6 M KCl. An electrophoretic mobility shift assay (EMSA) was used to identify the column fractions that contained TFIIIA DNA-binding activity (Fig. 2.2A). Two shifted complexes are formed in the EMSA; the lower complex is presumably similar to the *Xenopus* TFIIIA proteolytic fragment missing its C-terminus that retains DNA-binding capability (J. Gottesfeld, pers. comm.). The pool of TFIIIA (fractions 17 to 29) exhibited a 46-fold increase in specific DNA-binding activity over the preceding PC flow-through fraction (Table 2.1). Many protein bands that correlated with the DNA binding activity of TFIIIA were present in a silver-stained SDS-polyacrylamide gel of the second PC fractions (Fig. 2.2B).

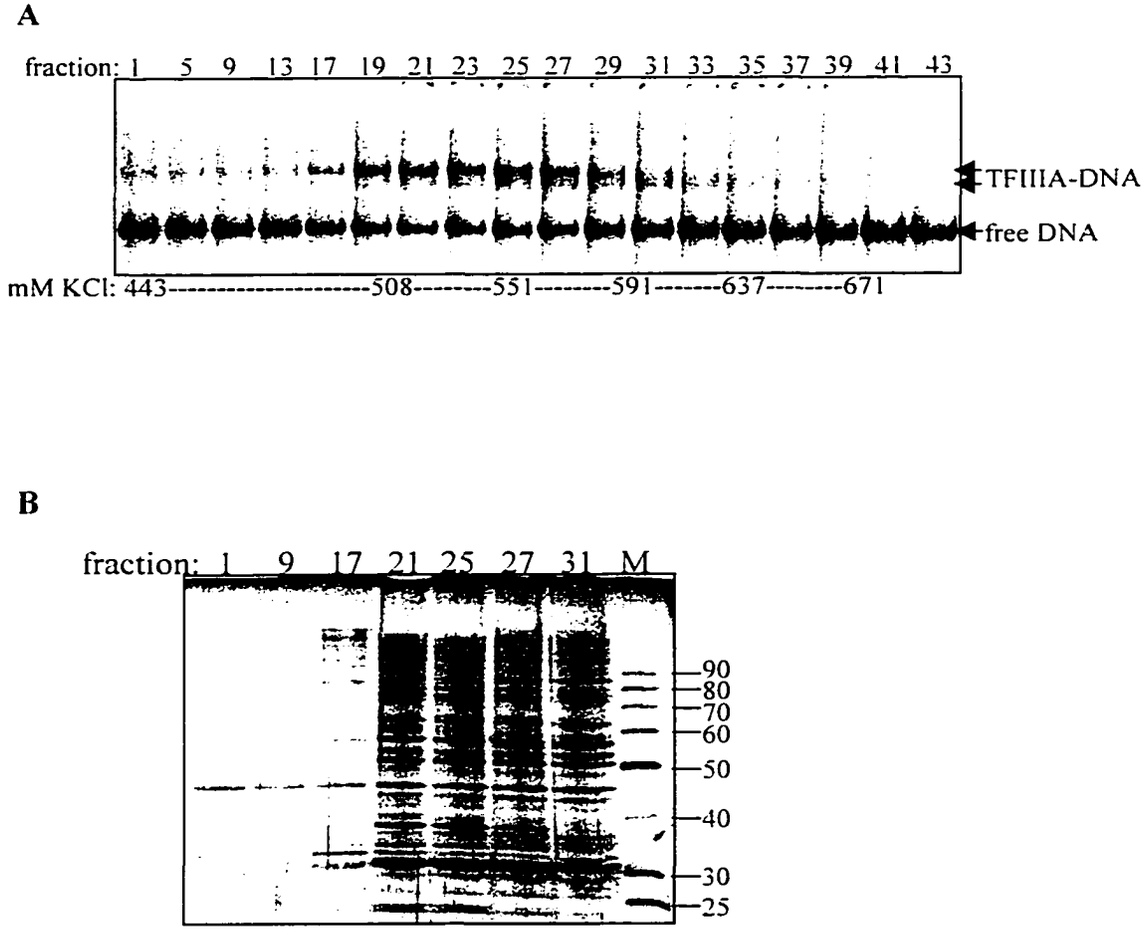


Fig. 2.2. The DNA-binding activity detected in fractions eluted from the second PC column. A, the DNA binding activity of TFIIIA was tested by EMSA using 3 μL of the indicated column fractions and 20.000 cpm of end-filled-in DNA containing the 5S RNA gene (*EcoRI/ HindIII* digested pAc-PS1) in a 20 μL reaction. The shifted complexes and the free DNA are identified by arrows at the right of the gel. The concentration of KCl across the gradient is shown below the gel. **B**, a subset of the fractions used in the EMSA was resolved on an 11 % SDS-polyacrylamide gel (21 μL of the indicated fraction/lane) that was then stained with silver. The lane labeled "M" was loaded with 0.25 μL of the Benchmark Protein Ladder (GIBCO) with protein molecular weights shown at the right.

The second PC column pool was adjusted to 120 mM KCl (see Methods) and applied to a promoter-DNA-affinity column. The DNA attached to Sepharose 4B resin consisted of five head-to-tail repeats of the *A. castellanii* 5S RNA gene (Zwick *et al.*, 1991b) from +17 to +96 (+1 denotes the transcription start site). A head-to-tail repeat from +47 to +100 of the *A. castellanii* 5S RNA gene, which encompassed the minimal proposed ICR (Pieler *et al.*, 1985a; Sakonju *et al.*, 1980), exhibited weaker interactions with *Ac*TFIIIA (data not shown) and was not used. Calf-thymus DNA was added to the TFIIIA-containing fraction 20 min prior to loading the promoter-DNA-affinity column as a nonspecific competitor. The quantity of competitor DNA used was the amount required to produce a distinct TFIIIA-DNA complex in an EMSA. Other competitor DNAs tested (poly(dI-dC)), salmon sperm, poly(dA-dT), pBS plasmid) did not result in the same high purification, but sometimes led to higher yields.

A linear KCl gradient eluted TFIIIA between approximately 350 and 500 mM KCl. The DNA-binding activity of TFIIIA was localized to fractions 19 to 23, with peak activity in fraction 21 (Fig. 2.3A). When combined with a protein fraction containing TFIIIB, TFIIIC, and Pol III, the same fractions with DNA-binding activity also activated 5S RNA transcription *in vitro* (Fig. 2.3B). Transcription activity was observed in additional fractions outside those with DNA-binding activity because of the increased sensitivity of the transcription assay. The DNA-binding and transcription activities correlated exactly with a protein band of 59 kDa, which was the only major protein observed in the column fractions (Fig. 2.3C).

2.4.2 Southwestern blotting

Southwestern blotting was used to test whether the 59 kDa protein was able to bind to the 5S RNA gene. Proteins in the TFIIIA-containing fractions purified through the second PC column and the promoter-DNA-affinity column were resolved

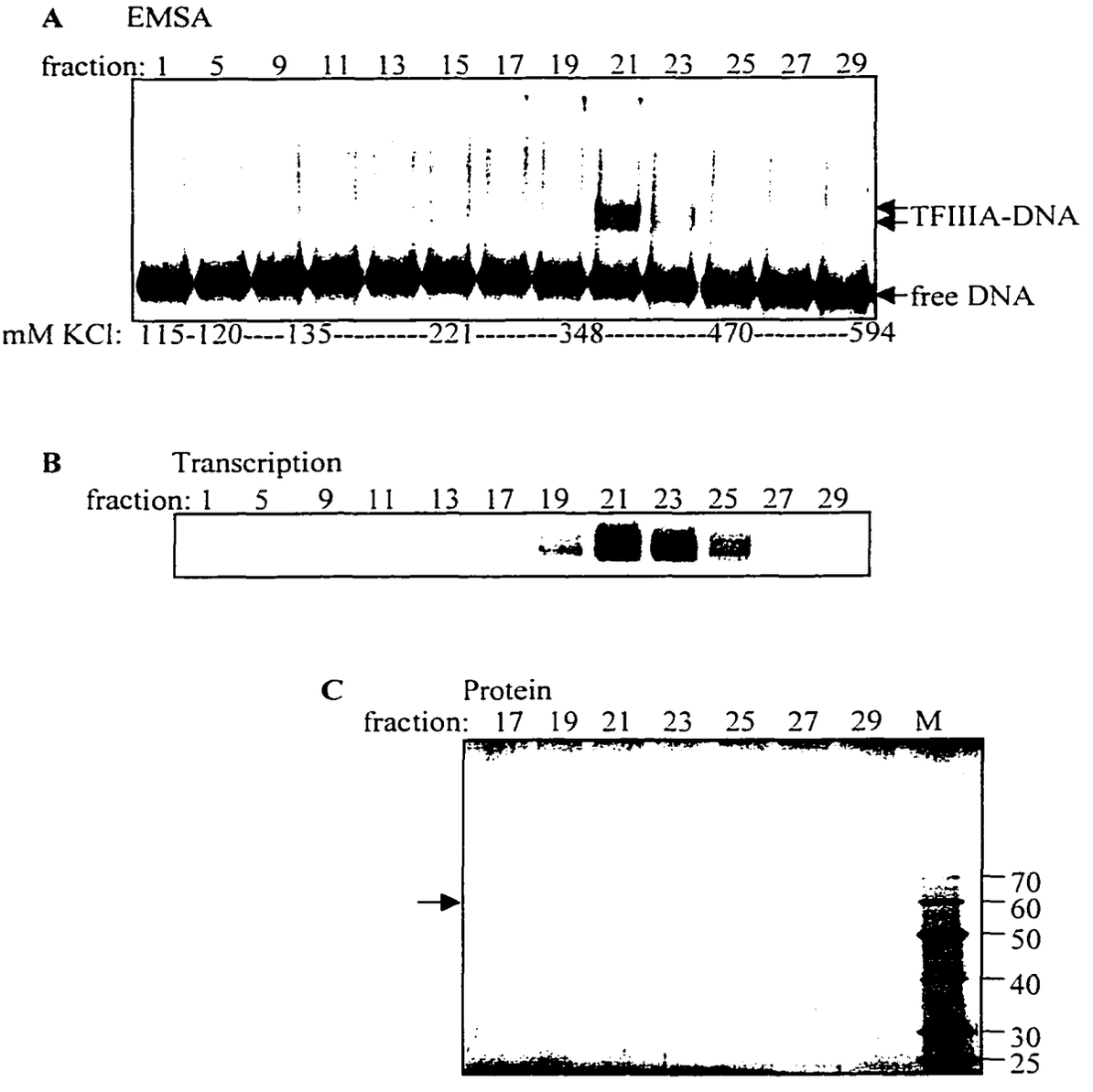


Fig. 2.3. Transcription activation and DNA-binding activities of TFIIIA correlate with a 59 kDa protein band in promoter-DNA-affinity column fractions. **A.** the DNA binding activity of TFIIIA of the indicated column fractions was tested exactly as described in the legend of Fig. 2.2. **B.** activation of 5S RNA transcription by TFIIIA was tested using 2 μL of the indicated column fractions and a protein fraction containing TFIIIB, TFIIIC, and Pol III in a 30 μL *in vitro* transcription reaction. **C.** a subset of the fractions used in the TFIIIA activity assays was resolved on an 11 % SDS-polyacrylamide gel (19 μL of the indicated fraction/lane) that was then stained with silver. The lane labeled "M" was loaded with 0.25 μL of the Benchmark Protein Ladder (GIBCO) with protein molecular weights shown at the right. The arrow identifies the protein that correlated with TFIIIA activity.

through an 11% SDS-polyacrylamide gel and electroblotted to a PVDF membrane. Proteins were renatured on the membrane and tested for the ability to bind to the 5S RNA gene. A single unique protein of approximately 59 kDa was found to bind to the 5S RNA gene (Fig. 2.4). This was even the case in the impure Second PC pool fraction (cf. Fig 2.2B with 2.4). We conclude the protein that specifically binds 5S DNA is TFIIIA, and has the same molecular weight as the protein correlated with TFIIIA activity in the promoter-DNA-affinity fractions.

2.4.3 DNase I footprinting

DNase I footprinting was performed to identify the region of the 5S RNA gene to which *Ac*TFIIIA bound. Using fractions eluted from a promoter-DNA-affinity column, the expected TFIIIA footprint centered over the 5S RNA gene promoter was observed only in fractions corresponding to the peak DNA binding activity of TFIIIA as determined by EMSA (Fig. 2.5). The *Ac*TFIIIA footprint was found to extend from approximately +44 to +97 of the 5S RNA gene. *Ac*TFIIIA was additionally found to cause hypersensitive cleavage at +64 on the RNA-like strand. This protection pattern is nearly identical to those observed using TFIIIA from higher eukaryotes. For example, TFIIIA from *X. laevis* protects +47 to +96 of the 5S RNA gene from DNase I cleavage and produces hypersensitive cleavage at +62 on the RNA-like strand (Engelke *et al.*, 1980). The similarities in the *Ac*TFIIIA and *X. laevis* TFIIIA footprints indicated that these proteins form similar complexes with the DNA. The known DNA binding properties of *X. laevis* TFIIIA were, therefore, used to further characterize *Ac*TFIIIA.

2.4.4 Protein-DNA photo-cross-linking using purified TFIIIA fractions

Site-specific photo-cross-linking was used to test whether the 59 kDa protein present in the purified TFIIIA preparation interacted with regions of the 5S RNA

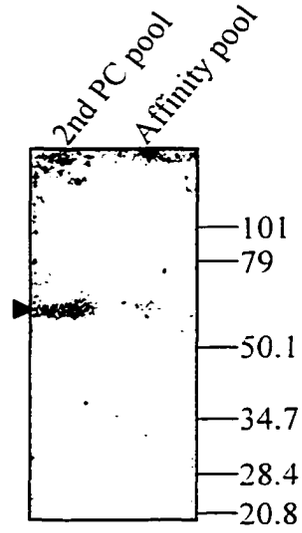


Fig. 2.4. Southwestern blotting of *A. castellanii* TFIIIA. TFIIIA purified through a second PC column and TFIIIA purified through a promoter-DNA-affinity column (32 μL of each fraction) were resolved on an 11 % SDS-polyacrylamide gel. Proteins were then transferred to a PVDF membrane and renatured on the membrane. The membrane was then hybridized with an internally ^{32}P-labeled 5S RNA gene fragment from –2 to +136 of the gene that was prepared by PCR. The arrow shows a protein that bound to the 5S RNA gene probe in both fractions, which was estimated to be 62 kDa based on the positions of the markers shown at the right of the memebrane.

gene known from footprinting to be contacted by *Ac*TFIIIA. This technique involves linking a photo-active azidophenacyl group to a synthetically added phosphorothioate group in the radiolabeled DNA backbone (Lagrange *et al.*, 1996); the conjugate was separated by two nucleotides from the radiolabeled phosphate. Upon u.v. irradiation, the azide is converted to a nitrene that covalently cross-links to proteins within 11 Å of the phosphate backbone (Mayer and Barany, 1995). Following digestion of the DNA, proteins interacting with the DNA near the modified site are identified as radiolabeled bands on an SDS-polyacrylamide gel. Protein-DNA photo-cross-linking has been used to detail the transcription complexes on yeast tRNA (Bartholomew *et al.*, 1990; Bartholomew *et al.*, 1991) and 5S RNA genes (Braun *et al.*, 1992) as well as on the *A. castellanii* rRNA gene promoter (Gong *et al.*, 1995).

Assuming *Ac*TFIIIA binds the 5S RNA promoter similarly to the *Xenopus* factor, as suggested by the strong similarity of their footprints, two different probes were constructed. One was modified at nucleotide +86 on the RNA-like strand and the other modified at +66 on the same strand (Fig. 2.6A). The +86 site lies within the C-box, which is the promoter element indispensable for TFIIIA binding in *Xenopus* (Pieler *et al.*, 1987). The solution structure of N-terminal three zinc fingers of *X. laevis* TFIIIA bound to the DNA shows that zinc fingers 1 and 2 are in close proximity to the phosphate backbone of the DNA at +86 (Wuttke *et al.*, 1997). Therefore, this probe should be highly effective in capturing *A. castellanii* TFIIIA on the DNA. The photo-active site at +66 is expected to be less efficient in contacting TFIIIA, because it is not within the boundaries of any of the three promoter elements, although it is within the footprinted region of the DNA (Fig. 2.6A). The crystal structure of the first six zinc fingers of *Xenopus* TFIIIA bound to 5S DNA show this site is in proximity to zinc finger 6 (Nolte *et al.*, 1998).

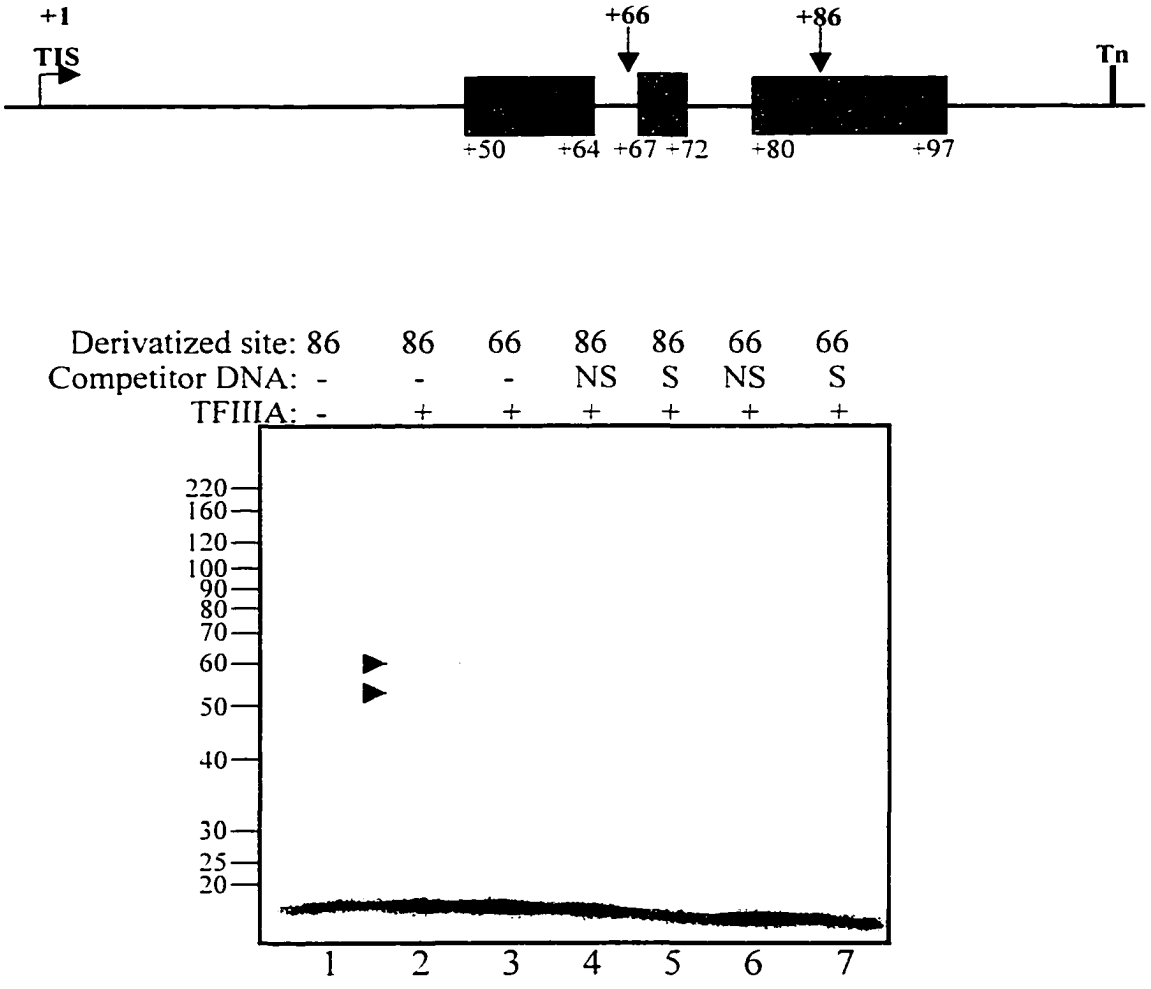


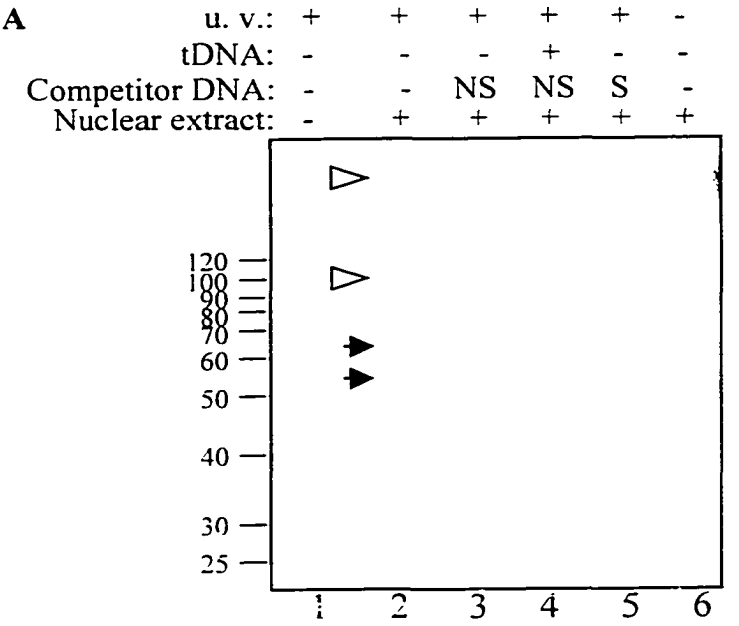
Fig. 2.6. The 59 kDa protein is specifically u. v. photo-cross-linked to the *A. castellanii* 5S RNA gene promoter. **A.** a schematic representation of the 5S RNA gene shows the relative positions of each photoderivative with respect to the transcription start site at +1. The vertical arrows at +66 and +86 identify each of the sites that were modified in the coding strand of the DNA. The promoter elements are shown as shaded boxes with their boundaries labeled below each box. IE, intermediate element; TIS, transcription initiation site; Tn, termination site. Photoaffinity probes consisted of an *Eco*RI/*Hind*III pBS+5S.3 DNA. **B.** six μL of affinity-purified TFIIIA (+) was cross-linked to 7500 cpm of the indicated photoaffinity probe in a 30 μL reaction. Protein markers are indicated at the left of the panel. NS, reactions preincubated with 8 ng of pBS(-) nonspecific competitor DNA; S, reactions preincubated with 8 ng pBS+5S.3 specific competitor DNA; -, reaction lacking TFIIIA. Labeled peptide are indicated with arrows.

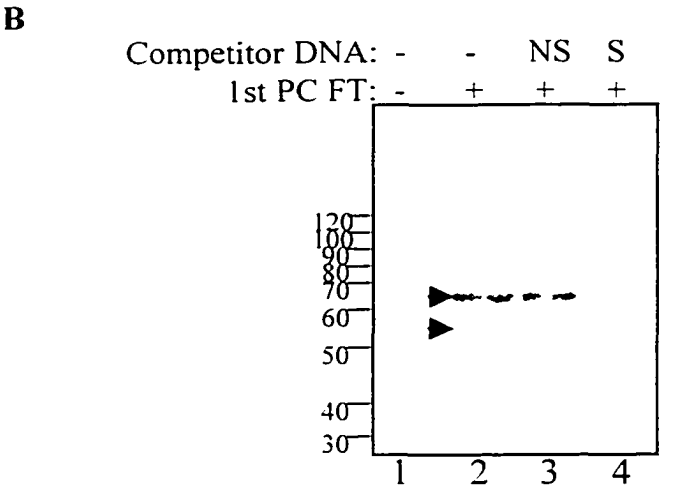
Both probes were tested with purified *A. castellanii* TFIIIA, and with each probe, the 59 kDa protein was detected (Fig. 2.6B, lanes 2-4 and 6). Another protein migrating at 54 kDa was also observed, which was probably the degradation product related to the lower complex observed in the EMSA (Fig. 2.4A). As expected, cross-linking of the 59 kDa protein to the +86 photoaffinity probe was more efficient than cross-linking to the +66 probe (Fig. 2.6B, compare lane 2 to 3 and lane 4 to 6). This difference could also be caused by variability in cross-linking efficiency because of different amino acids of the protein in proximity to each derivative (Tate *et al.*, 1998). Photo-cross-linking of the 59 kDa protein was sequence specific. The addition of plasmid DNA containing the 5S RNA gene as competitor abolished reaction with the 59 kDa protein (Fig 2.6, lanes 5 and 7) while an equal amount of plasmid DNA lacking this insert only slightly reduced the signal (Fig. 2.4B, lanes 4 and 6). These results show that the 59 kDa protein identified in purified TFIIIA fractions is able to interact specifically with the 5S RNA gene in regions known to be contacted by TFIIIA, and that no other protein in the preparation photo-cross-links.

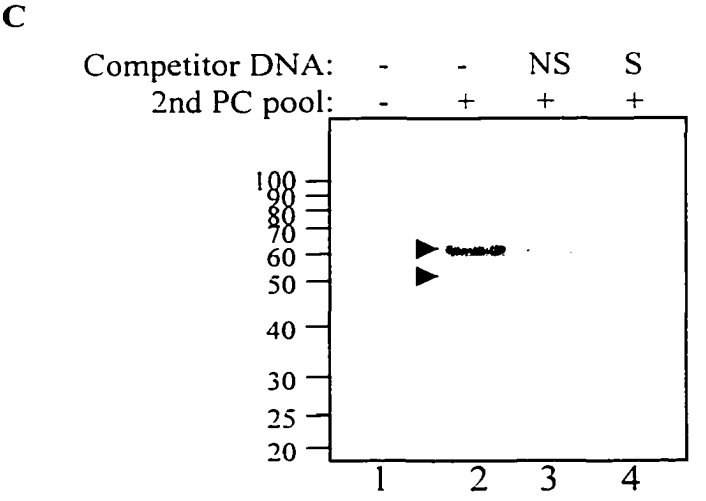
2.4.5 Protein-DNA photo-cross-linking using crude preparations of TFIIIA

Because the 59 kDa protein is the predominant component in the highly purified TFIIIA, the results obtained above could be misleading. Cross-linking in fractions from earlier stages in the purification was used to further test the specificity for cross-linking the 59 kDa protein. The fractions tested included nuclear extract, the flow-through fraction from the first PC column, and TFIIIA pooled from the second PC column. In all these fractions, the 59 kDa protein is only a small fraction of the total protein (Fig. 2.1B). Nevertheless, in the photo-cross-linking experiments, a pair of proteins near 60 kDa specifically cross-linked to the 5S RNA gene (Fig. 2.7). Depending on which fraction was tested, the size of the labeled protein

Fig. 2.7. The 59 KDa protein is detected in all TFIIIA-containing fractions. In each panel, proteins of the indicated fraction (+) were tested for the ability to be specifically cross-linked to the 5S RNA gene. -, reactions without the indicated fraction; NS, reactions preincubated with pBS(-) nonspecific competitor DNA; S, reactions preincubated with pBS+5S.3 specific competitor DNA; black arrows, polypeptides correlated with TFIIIA. **A**, nuclear extract (73 μg) with 12 μg of calf thymus DNA was cross-linked with 4600 cpm of the +66 photoaffinity probe in a 30 μL reaction. Lane 6 shows a reaction that was not u.v.-irradiated. The indicated reactions were treated with 150 ng of specific or nonspecific competitor DNA. tDNA, reaction preincubated with 15 ng of a $tRNA^{met}$ gene (*Eco*RI/*Hind*III digested pArabMet, #4 [Matthews *et al.*, 1995]); grey arrows, labeled polypeptides other than those correlated with TFIIIA. **B**, The first PC flow-through fraction (15 μg) with 4 μg of calf thymus DNA was cross-linked with 4600 cpm of the +66 photoaffinity probe in a 20 μL reaction. The indicated reactions were treated with 160 ng of specific or nonspecific competitor DNA. **C**, TFIIIA activity from the second PC column (6 μL) with 240 ng of polydIdC DNA, 120 ng of polydAdT DNA, and 3 μg of BSA was cross-linked with the +86 photoaffinity probe in a 30 μL reaction. The indicated reactions were treated with 350 ng of specific or nonspecific competitor DNA.







appeared to fluctuate from approximately 65 kDa in nuclear extract and the PC flow-through fraction to 62 kDa in the pooled activity from the second PC column. These fluctuations in the apparent size of the of the labeled protein are caused by variations in the size of the DNA tag covalently linked to the protein. Differential digestion of the DNA cross-linked to the polypeptides has indeed been shown to alter the electrophoretic mobility of labeled polypeptides (Gong *et al.*, 1995). Because cruder fractions were used in these experiments, elevated amounts of general nonspecific competitor DNA had to be added to the reactions to reduce interactions between nonspecific proteins and the photoaffinity probes. These large amounts of nonspecific competitor DNA partially quench the DNA digestion reactions following u.v. irradiation. It is also possible that a component(s) in the cruder fractions inhibits the digestion reaction. Although there were small differences in the electrophoretic mobilities of the radiolabeled bands, the characteristic "doublet" detected using the purified TFIIIA fraction (Fig. 2.6 B) were consistently observed, supporting the notion that the same proteins were labeled in each experiment.

The 59 kDa protein was specifically cross-linked in the crude nuclear extract using the +66 probe (Fig. 2.7A, lanes 3 and 5). The efficiency with which the 59 kDa subunit cross-linked to the probe in nuclear extract was unexpected. In an EMSA, nuclear extract has been found to induce the formation of higher-order complexes on the DNA that obscure detection of TFIIIA-DNA complexes. However, the specific higher-order complexes would contain TFIIIA as well as some or all of the other Pol III general transcription factors. It was, therefore, expected that the probe would react with multiple polypeptides, particularly in this region, with subunits of TFIIIC (Paule and White, 2000). Two other polypeptides were indeed detected in addition to the 59 kDa protein: one in the range of 100 kDa and another above the 220 kDa marker (lanes 2 to 5). Detection of these polypeptides was reduced by the addition

of either a DNA fragment containing the *Arabidopsis thalianii* $tRNA^{met}$ gene, which should bind TFIIIC but not TFIIIA, or plasmid DNA containing the 5S RNA gene (lanes 4 and 5). The intensity of the >220 kDa polypeptide signal decreased by 33% and 44% with the addition of tRNA and 5S RNA gene competitor DNA, respectively. The two larger polypeptides detected in nuclear extract were not detected in the PC flow-through fraction that is free of TFIIIC (Fig. 2.7B).

2.4.6 Photo-cross-linking using crude preparations of individual Pol III factors

Hitherto the 59 kDa protein has been detected in all fractions possessing TFIIIA activity. We, therefore, wanted to demonstrate that this protein is not cross-linked in fractions that lack TFIIIA activity. Segall *et al.* (1980) initially reported the separation of the Pol III general transcription factors from human whole-cell extracts using PC chromatography. This method has similarly proven effective for the separation of the *A. castellanii* factors: TFIIIA has been shown to elute with 0.1 M KCl, TFIIIB with 0.3 M KCl, Pol III with 0.45 M KCl, and TFIIIC with 0.65 M KCl (Matthews *et al.*, 1995). As mentioned above, some TFIIIA is retained on the column and elutes with TFIIIC. These fractions were tested for the presence of the 59 kDa protein in u.v. photo-cross-linking experiments (Fig. 2.8).

As shown above, the 59 kDa protein was cross-linked to the +66 probe using either nuclear extract or the first PC flow-through fraction (Fig 2.8A, lanes 2 and 3), and it was additionally detected in the 0.65 M KCl, TFIIIC-containing fraction (Fig. 2.8A, lane 6). However, this protein was not observed in either the TFIIIB or Pol III-containing fraction (Fig 2.8A, lanes 4 and 5). Identical cross-linking of the 59 kDa was obtained using the +86 probe (Fig. 2.8B). These results show that the 59 kDa protein is only found in fractions possessing TFIIIA activity.

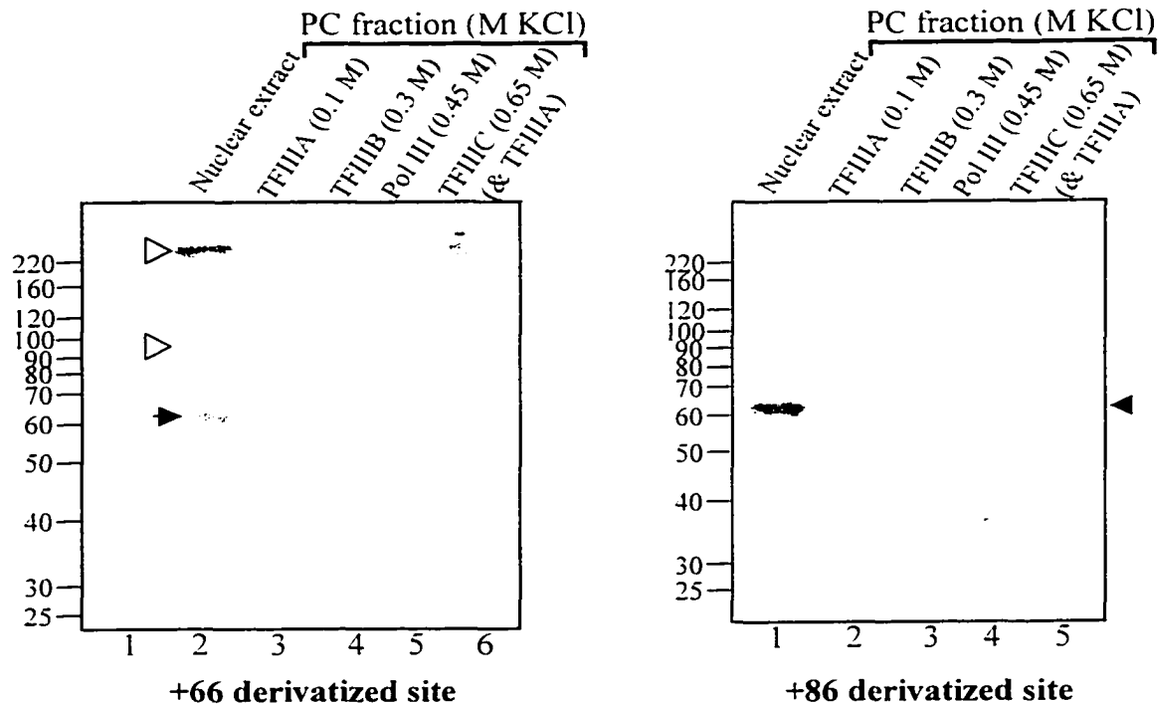


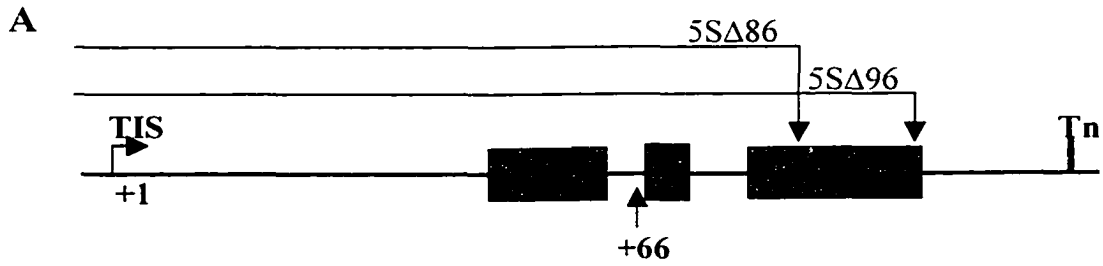
Fig. 2.8. The 59 KDa protein is absent in fractions lacking TFIIIA activity. PC column fractions containing the separated Pol III factors were tested with the photoaffinity probes to determine the fractions that possessed the protein correlated with TFIIIA activity. Above each lane, the concentration of KCl applied to the column and the Pol III factor eluted in the fraction are indicated. Each PC fraction (15 μg of protein) with 4 μg of calf-thymus DNA was cross-linked with 2900 cpm of the +66 photoaffinity probe (**A**) or with 1800 cpm of the +86 photoaffinity probe (**B**). Nuclear extract (37 μg) with 8 μg of calf-thymus DNA was cross-linked with each photoaffinity probe. Black arrows, polypeptides correlated with TFIIIA; transparent arrows, labeled polypeptides other than those correlated with TFIIIA.

Using the +66 photoaffinity probe, the ~100 kDa and >220 kDa polypeptides were again detected in nuclear extract and were also detected in the 0.65 M KCl, TFIIIC-containing fraction (Fig 2.8A, lanes 2 and 6), possibly supporting the hypothesis that these are TFIIIC subunits alluded to above.

Other than the 59 kDa protein and a 35 kDa polypeptide detected in the 0.45 M PC fraction, polypeptides detected with the +86 probe in the nuclear extract and the PC fractions were only weakly labeled (Fig 2.8B). The 35 kDa polypeptide was not detected in nuclear extract. This polypeptide may be related to a complex previously detected by EMSA that was determined to be caused by nonspecific protein binding based on DNA competition assays (Matthews *et al.*, 1995).

2.4.7 Photo-cross-linking using a 5S RNA gene fragment lacking part of the C-box

Although TFIIIA has been localized predominantly to the flow-through fraction of the first PC column (Matthews *et al.*, 1995), we were concerned that the intensity in labeling of the 59 kDa protein was approximately equivalent in this and in the PC 0.65M KCl fractions (lanes 2 and 5). Strong labeling of the 59 kDa protein in the PC 0.65M KCl fraction could be explained by the stabilizing effects TFIIIC induces on the TFIIIA-DNA complex (Lassar *et al.*, 1983). Alternatively, the 59 kDa labeled band could correspond to another protein that also binds specifically to the 5S RNA gene. This notion is unlikely considering the DNA-binding properties of the 59 kDa protein have, thus far, paralleled those of TFIIIA. Nevertheless, to definitively relate the DNA-binding properties of TFIIIA with those of the 59 kDa protein, this protein was tested for the ability to bind either of two 5S RNA gene fragments, each truncated to a different position at the 3'-end (Fig. 2.9A). The 5SΔ86 fragment lacked 11 bp of the C-box (Pieler *et al.*, 1985a), including four guanines critical for DNA interactions with *Xenopus* TFIIIA (McConkey and Bogenhagen, 1987; Sakonju and



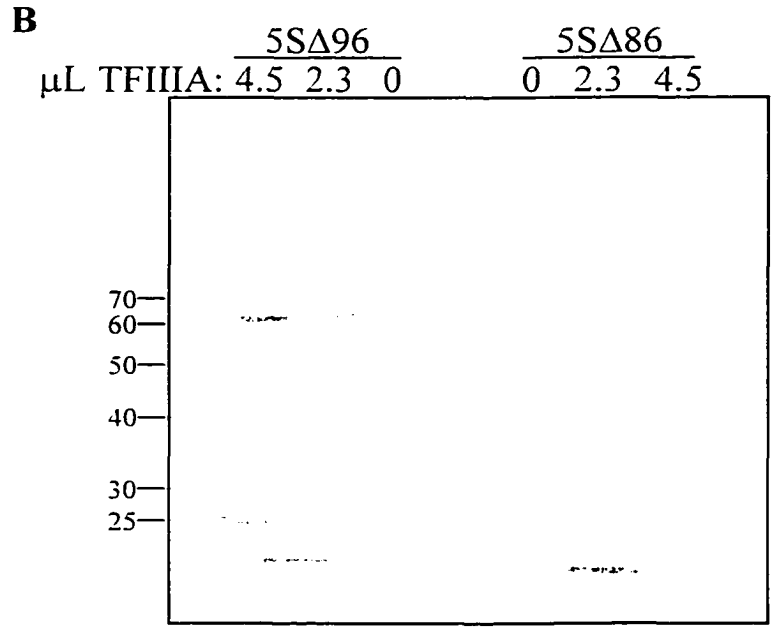


Fig. 2.9. Partial deletion of the C-box abolishes interactions between the 59 kDa protein and the 5S RNA gene. Two photoaffinity probes trucated at different positions at the 3'-end of the 5S RNA gene were tested for the ability to cross-link the 59 kDa protein from promoter-DNA-affinity purified TFIIIA. **A**, the positions at which the gene was truncated are shown: 5SΔ86 extends to +85 and 5SΔ97 extends to +96 in the 5S RNA gene. Both probes contained the photoderivative positioned at +66 on the coding strand. **B**, the specified quantities of affinity purified TFIIIA were cross-linked with 2600 cpm of each indicated photoaffinity probe in a 20 μL reaction.

Brown, 1982). This DNA fragment was found to be incapable of forming a complex with *A. castellanii* TFIIIA as determined by EMSA (data not shown). In contrast, the 5SΔ96 fragment, which included the critical base pairs required for *Xenopus* TFIIIA binding, was able to form a complex with *A. castellanii* TFIIIA (data not shown).

Each DNA fragment was prepared as a photoaffinity probe modified at nucleotide +66, and was then tested for the ability to cross-link proteins in a purified TFIIIA fraction (Fig 2.9B). The 59 kDa protein was cross-linked to the 5SΔ96 probe, and the intensity of the signal was dependent on the quantity of protein added: a 2-fold increase in the purified fraction used yielded a 1.6-fold increase in the band intensity. No polypeptides were labeled using 5SΔ86 with either amount of purified TFIIIA added. These results showed that a DNA fragment deficient in binding *Xenopus* TFIIIA is also deficient in interaction with the 59 kDa protein, demonstrating that the 59 kDa protein possesses equivalent DNA-binding properties to those of *Xenopus* and *A. castellanii* TFIIIA.

2.5 Discussion

TFIIIA homologues have been characterized from only seven vertebrates and yeast, and these were found to diverge significantly. This key factor for 5S RNA transcription is the regulatory target for this gene in *A. castellanii* (Matthews *et al.*, 1995), so to initiate a study of regulatory mechanism, it was purified, characterized and compared with TFIIIAs from the other species. TFIIIA is a 59 kDa protein in this organism, the largest known example among those that have been characterized. Although its large size is most similar to another lower eukaryote, *S. cerevisiae*, promoter interaction and susceptibility to proteolysis of *Ac*TFIIIA is more similar to the vertebrate, *Xenopus*. As in most cell types other than oocytes, *Ac*TFIIIA was found to be present at very low quantities within the cell. Based on the DNA binding activity of TFIIIA in the first PC flow-through fraction, only 170 molecules of TFIIIA

were estimated to be present in each *A. castellanii* cell. However, because a small portion of TFIIIA was retained on the first PC column, this estimate is likely to be slightly low. Nevertheless, such low abundance complicated effective purification of large amounts of the protein.

*Ac*TFIIIA was purified using chromatography steps similar to those that were used to enrich for human TFIIIA activity by Seifart (Seifart *et al.*, 1989). As with human TFIIIA, *Ac*TFIIIA from a crude extract does not bind to a PC column and is mainly in the 0.1 M KCl flow-through fraction. Two observations suggest the lack of binding is because of the association of TFIIIA with nucleic acids: A small portion of the TFIIIA from the crude extract that is potentially free of nucleic acids is retained on the column. Dissociation of the factor and nucleic acids by high salt concentration in the flow through prior to application to a second PC column resulted in binding of TFIIIA to the matrix. TFIIIA is known to specifically bind 5S RNA (Honda and Roeder, 1980; Pelham and Brown, 1980), so this is the probable nucleic acid with which it is associated in the crude extract.

In cofractionating with TFIIIC from the first PC column developed with a linear salt gradient, TFIIIA eluted at a slightly higher concentration of KCl than the concentration at which TFIIIA eluted from the second PC column (data not shown). This variation in the elution profile suggests that *Ac*TFIIIA retained on the first PC column was associated with TFIIIC. Indeed, potential interactions between TFIIIA and TFIIIC or TFIIIC subunits have been found. Photo-cross-linking experiments using purified yeast TFIIIA have resulted in labeling of the τ91 subunit of TFIIIC in addition to TFIIIA, indicating the presence of this subunit in purified TFIIIA preparations (Braun *et al.*, 1992). In the human system, reactivation of 5S RNA transcription in crude extracts required the addition of TFIIIA as well as TFIIIC following immunodepletion with an antibody against TFIIICα (Lagna *et al.*, 1994).

The possible interactions between TFIIIA and TFIIIC on the first PC column may have contributed to the inability to further purify this fraction of TFIIIA.

Although *Ac*TFIIIA binds less efficiently to the PC column than does human TFIIIA, applying the first PC flow-through fraction to a second PC column under conditions of high ionic strength was still found to be an efficient step in the purification. Within a narrow range of elevated salt concentrations (400-450 mM KCl), *Ac*TFIIIA was found to dissociate from nucleic acids while retaining the ability to bind to the PC column. At lower salt concentrations within the range, more contaminating proteins were retained along with *Ac*TFIIIA on the PC column. At higher salt concentrations, TFIIIA did not bind avidly to the column, and leakage of TFIIIA from the column was found to occur during loading. The first PC flow-through fraction was adjusted to 0.45M KCl, which approximated the upper limit allowable for *Ac*TFIIIA to bind to the column and was close to the salt concentration at which TFIIIA is first detected eluting from the column in a linear gradient (approximately 500 mM KCl). Human TFIIIA does not elute until much higher KCl concentration, so this strategy is not as efficacious for *Acanthamoeba* as for the human factor. Nevertheless, there is a large increase in specific activity of 46.3-fold over that of the first PC flow-through fraction, though only a 19% recovery of TFIIIA activity is obtained (Table 2.1).

Promoter-DNA-affinity chromatography was the final step in the purification. Three rounds of affinity chromatography had been used successfully to purify human TFIIIA (Moorefield and Roeder, 1994). However, exposing *Ac*TFIIIA to three rounds of affinity chromatography resulted in a large reduction in DNA binding activity (data not shown). Therefore, a single affinity chromatography step was optimized by using calf thymus DNA to sequester proteins that could bind nonspecifically to the column.

*Ac*TFIIIA purified through the affinity column was purified more than 5,000-fold over the first PC flow-through fraction. The overall increase in *Ac*TFIIIA purity from the nuclear extract is significantly greater, but DNA binding activity in the nuclear extract could not be determined by EMSA, so this cannot be estimated. The final yield of TFIIIA was extremely low because the purification scheme was developed mainly to identify the protein band corresponding to *Ac*TFIIIA. It has been reported that a significant amount of human TFIIIA activity is lost using chromatography buffers at pH 7.9, but not with buffers at pH 7.0. However, no difference in yield was detected when *Ac*TFIIIA was purified using chromatography buffers at either pH. The greatest reduction in activity occurred during affinity chromatography. The low yield appears to be because of binding to calf thymus DNA during the loading step. However, though the use of other competitor DNAs led to higher yields, they also led to significantly lower fold purification.

Although the use of calf thymus DNA during affinity chromatography was required to obtain homogenous *Ac*TFIIIA, this nonspecific competitor DNA may have also sequestered a large portion of the TFIIIA and led to the large loss of activity. The use of well defined competitor DNAs, such as linearized plasmid DNA or poly(dI-dC), in place of calf thymus DNA has been found to significantly increase the yield of TFIIIA from the affinity column, but reduce the purity. Two alterations of the second PC chromatography step have also been found to increase the yield of *Ac*TFIIIA. First, the first PC flow-through fraction can be applied to the column at 400 mM KCl rather than 450 mM KCl, which eliminates leakage of TFIIIA from the column during chromatography. Second, replacing the linear salt gradient with a single, high-salt step elution has also been found to reduce the loss of TFIIIA activity. These changes in the purification procedure have been found to result in a TFIIIA fraction that is contaminated with additional proteins. However, because *Ac*TFIIIA is now known to be 59 kDa, it is possible to distinguish this protein from

contaminants on a SDS-polyacrylamide gel. With the changes in the purification scheme that increase the yield of *Ac*TFIIIA and the knowledge of the size of *Ac*TFIIIA, it may be possible to obtain enough material for polypeptide sequencing.

*Ac*TFIIIA was identified in purified fractions by EMSA and was coincident with a 59 kDa protein. Several approaches were used to verify that the 59 kDa protein is *Ac*TFIIIA: First, southwestern blotting showed that the 59 kDa polypeptide renatured in situ on the blot was able to bind radiolabelled 5S DNA (Fig. Z.4). Second, extensive site-specific DNA-protein photo-cross-linking was used (Figs. 2.6-2.9). Not only did the 59 kDa protein photo-cross-link from the highly purified fraction, but also from less concentrated and purified fractions even including the crude nuclear extract. Only fractions exhibiting TFIIIA activity cross-linked. Cross-linking was sequence specific; 5S RNA gene mutants not able to bind TFIIIA in an EMSA did not cross link. These techniques were valuable in that they required only small quantities of protein. Attempts to detect TFIIIA-activity in a purified fraction that was eluted and renatured from an SDS-polyacrylamide gel slice were unsuccessful, presumably because the quantity of TFIIIA in the purified fraction was too low for detection with this assay. However, DNA-binding activity of TFIIIA was recovered from a gel slice using TFIIIA pooled from the second PC column, with activity localized in a gel slice containing proteins ranging from 49 kDa to 60 kDa (data not shown).

The results show that *Ac*TFIIIA is the largest TFIIIA homologue that has been identified to date (Moorefield and Roeder, 1994; Wyszko *et al.*, 1997) (Archambault *et al.*, 1992; Ginsberg *et al.*, 1984). The second largest known TFIIIA homologue (51 kDa) is found in *S. cerevisiae*, and its large size is primarily due to an 81 amino acid insert between its carboxyl-terminal two zinc fingers. *Ac*TFIIIA is not likely to harbor this same domain, as this expansion segment is specifically required for 5S RNA transcription in *S. cerevisiae* (Archambault *et al.*, 1992; Milne and Segall, 1993;

Rowland and Segall, 1998). Furthermore, the 81 amino acid insert appears to be correlated with the truncated footprint produced by TFIIIA from *S. cerevisiae*; *Ac*TFIIIA produced a DNase I protection pattern more similar to those of by *Xenopus* and human TFIIIA, suggesting the additional molecular weight in *Ac*TFIIIA does not interrupt the nine zinc fingers.

In addition to *Ac*TFIIIA, two polypeptides of ~100 kDa and >220 kDa were crosslinked to the 5S RNA gene in nuclear extract and the 0.65 M KCl, TFIIIC-containing fraction. Cross-linking of these polypeptides was found to be reduced by the addition of the 5S RNA gene or a $tRNA^{met}$ gene competitor DNA. These results suggest that the two polypeptides correspond to subunits of TFIIIC. Further purification of TFIIIC in conjunction with a comprehensive set of photoaffinity probes along the 5S RNA gene might confirm these initial findings.

Supplementary Figures for Chapter 2

The following figures (10-20) were addressed in Chapter 2 as "data not shown" or additional support the results presented in Chapter 2.

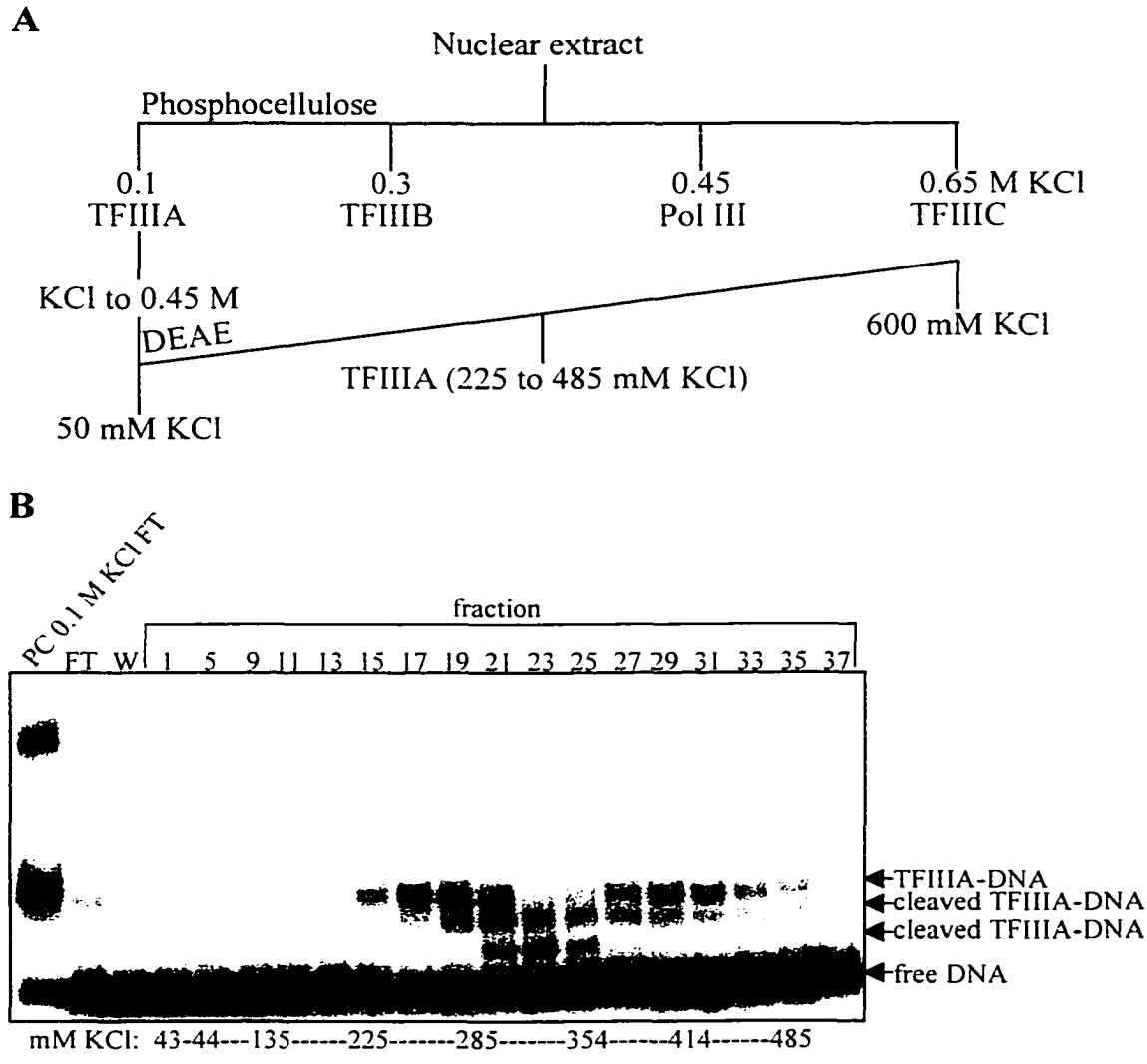


Fig. 2.10. ***A. castellanii*** **undergoes proteolytic degradation during DEAE chromatography.** **A.** DEAE chromatography was used as the second step in the purification scheme. The phosphocellulose (PC) 0.1 M KCl flow-through fraction was applied to a DEAE Sepharose Fast Flow column (Pharmacia; 10 mg of protein/mL of column resin) at a flow rate of 0.5 X column volume (mL)/hr. **B**, the DNA binding activity of TFIIIA was tested by EMSA using 3 μL of the indicated fraction and 20,000 cpm of end-filled-in DNA containing the 5S RNA gene (*Eco*RI/*Hind*III digested pAc-PS1) in a 20 μL reaction. The concentration of KCl in every fifth fraction across the gradient is shown below the gel. FT, DEAE column flow-through fraction; W, DEAE column wash fraction. Degradation is apparent from the complexes below the TFIIIA-DNA complex that are potentially formed by TFIIIA cleavage products that retain the ability to bind DNA. This degradation was only observed after the column was developed and did not occur during the extensive period (up to 30 hr) in which the column was loaded.

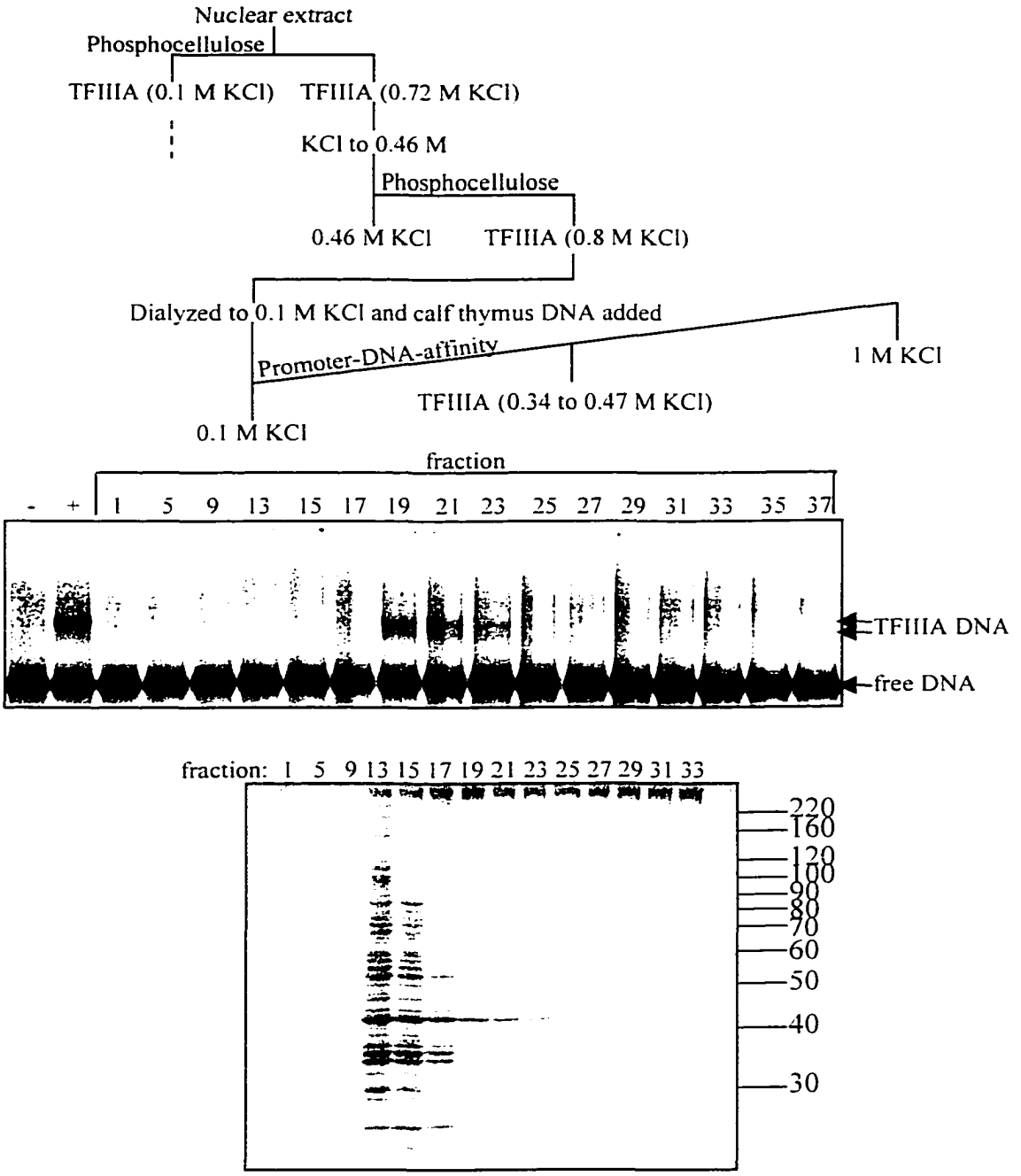


Fig. 2.11. The fraction of TFIIIA bound to the first PC column could not be purified to homogeneity. A, the fraction of TFIIIA bound to the first PC column was purified as shown. Utilizing a second PC column equilibrated at high salt was done to remove separate TFIIIC from TFIIIA: the kinetics of TFIIIA and TFIIIC binding to the PC column may differ, and at high salt concentrations this difference may result in the selective retention of TFIIIA on the PC column (Beth Moorefield, personal communication).

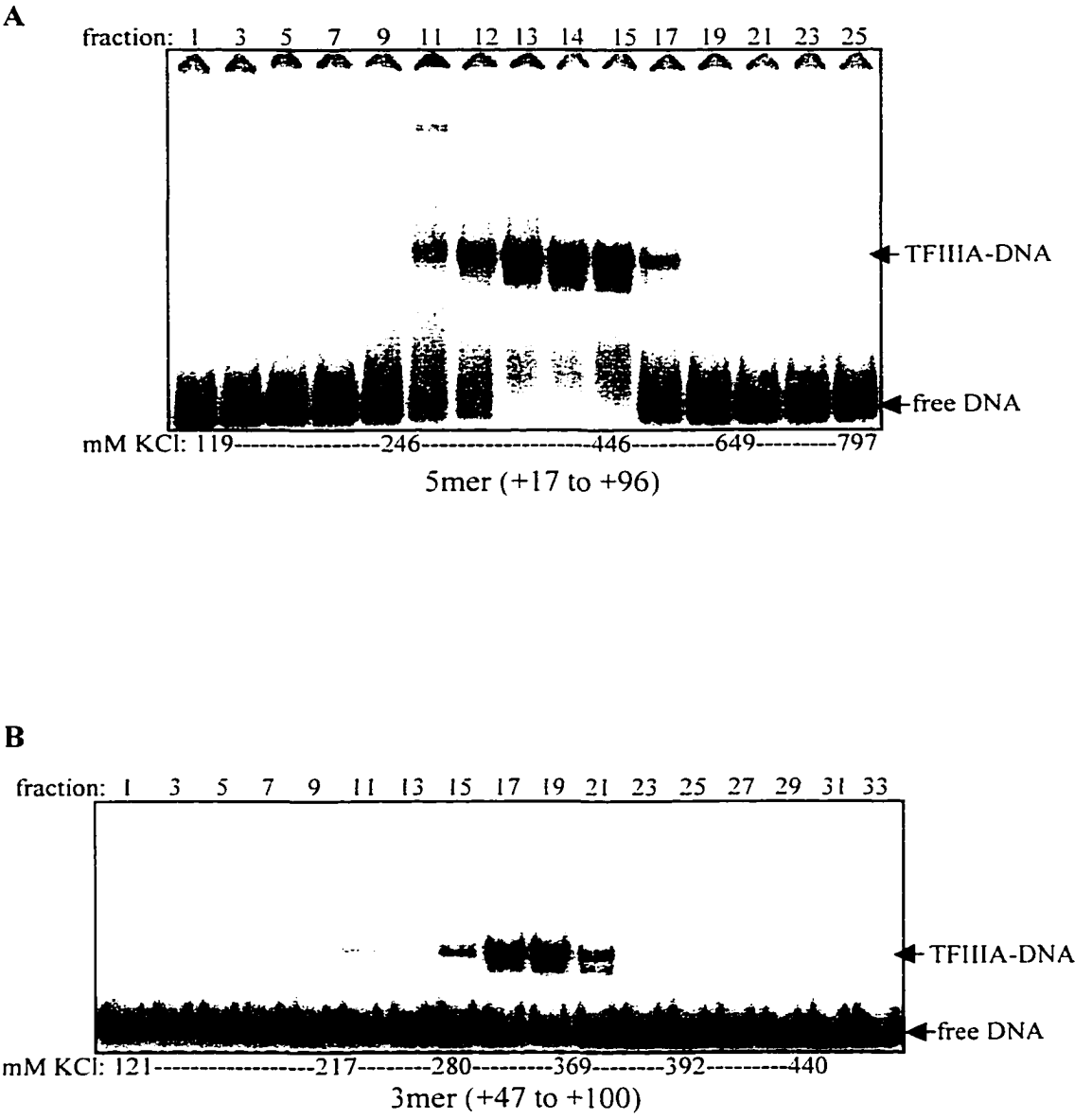


Fig. 2.12. A higher concentration of KCl is required to eluted *A. castellanii* TFIIIA from the 5mer DNA column (+17 to +96 of the 5S RNA gene) than from the 3mer column (+47 to +100 of the gene). **A.** TFIIIA eluted from the 5mer column was predominantly in fractions from 345 to 446 mM KCl, as determined by EMSA. **B.** TFIIIA eluted from the 3mer column was found predominantly in fractions from 310 to 340 mM KCl.

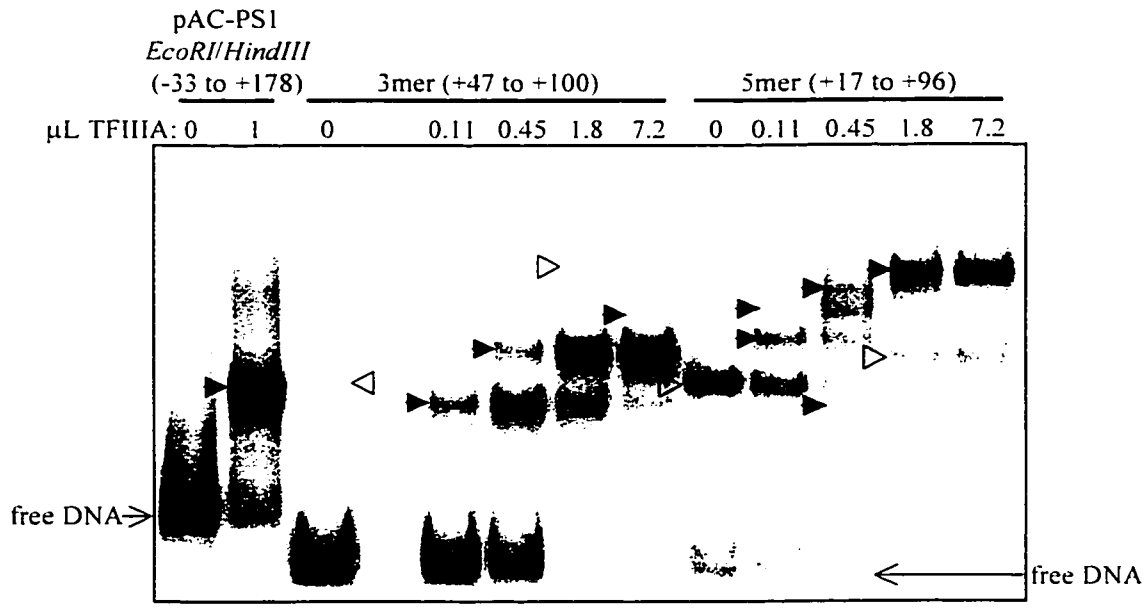


Fig. 2.13. *A. castellanii* TFIIIA binds with higher affinity to the +17 to +96 5mer of the 5S RNA gene than to the +47 to +100 3mer of the gene. An EMSA was used to test the TFIIIA binding affinity for the 3mer and 5mer DNAs. *Eco*RI/*Hind*III digestion fragments that contained the 3mer or 5mer were radiolabeling by an end-fill-in reaction. Increasing amounts of promoter-DNA-affinity column purified TFIIIA, as indicated, were added to either 0.35 fmol of the 3mer probe or 0.29 fmol of the 5mer probe in a 20 μL reaction. pAc-PS1 digested with *Eco*RI and *Hind*III was used with and without TFIIIA in the EMSA control reactions. Black arrows, potential TFIIIA-DNA complexes; transparent arrows, unknown or nonspecific complexes. Loss of the free DNA shows that TFIIIA sequestered the 5mer probe more efficiently than the 3mer probe. Only two TFIIIA molecules appear to bind efficiently to the 3mer probe, while five molecules of TFIIIA appear to bind to the 5mer probe.

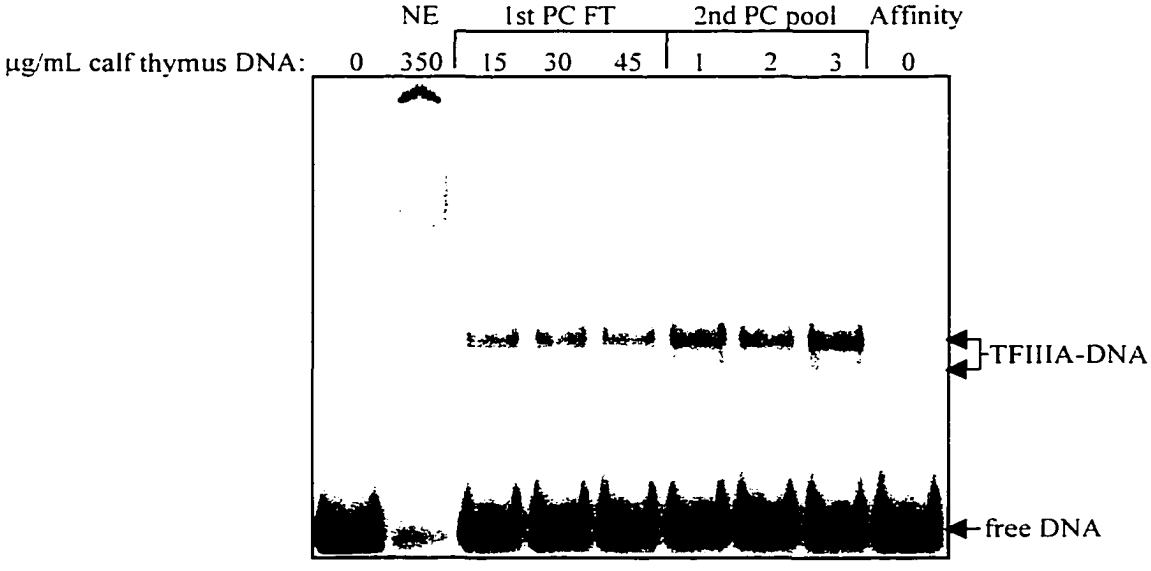


Fig. 2.14. EMSA to quantify DNA binding activity of TFIIIA at each stage of the purification. The indicated fractions (1 μL of each) were tested in 20 μL EMSA reactions with the indicated amounts of calf thymus DNA and 1.9 fmol of a ^{32}P end-labeled probe consisting of the 5S RNA gene from -2 to +136. NE, nuclear extract; 1st PC FT, the 0.1 M KCl flow-through fraction from the first phosphocellulose (PC) column; 2nd PC pool; pooled TFIIIA activity from the second PC column; affinity, pooled TFIIIA activity from the promoter-DNA-affinity column.

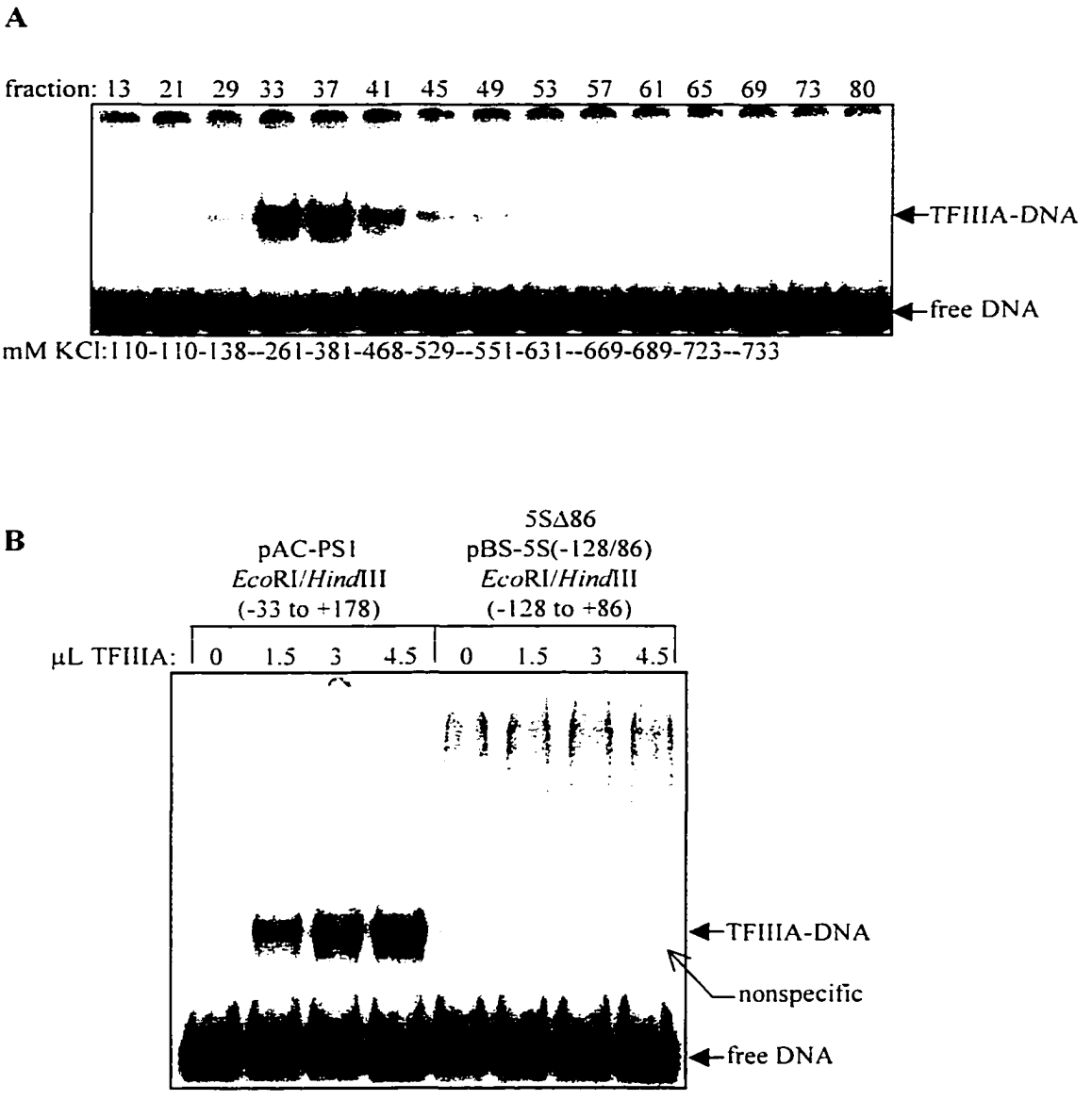


Fig. 2.15. *A. castellanii* TFIIIA is able to bind the 5S RNA gene when it is truncated to +96 from the 3'-end, but is unable to bind to the gene when it is truncated to +85 from the 3'-end. A. TFIIIA is able to bind with high affinity to a promoter-DNA-affinity column with matrix-attached DNA consisting of a single copy of the 5S RNA gene truncated from the 3'-end to +96 (digested with *Sma*I). B. the ability of TFIIIA to bind to a DNA fragment containing the 5S RNA gene from -128 to +86 was tested by EMSA. The indicated quantities of promoter-DNA-affinity purified TFIIIA were combined with 0.5 fmol of the standard EMSA probe or a DNA fragment containing the 3'-truncated 5S RNA gene. Both DNA fragments were labeled by a ^{32}P end-fill-in reaction.

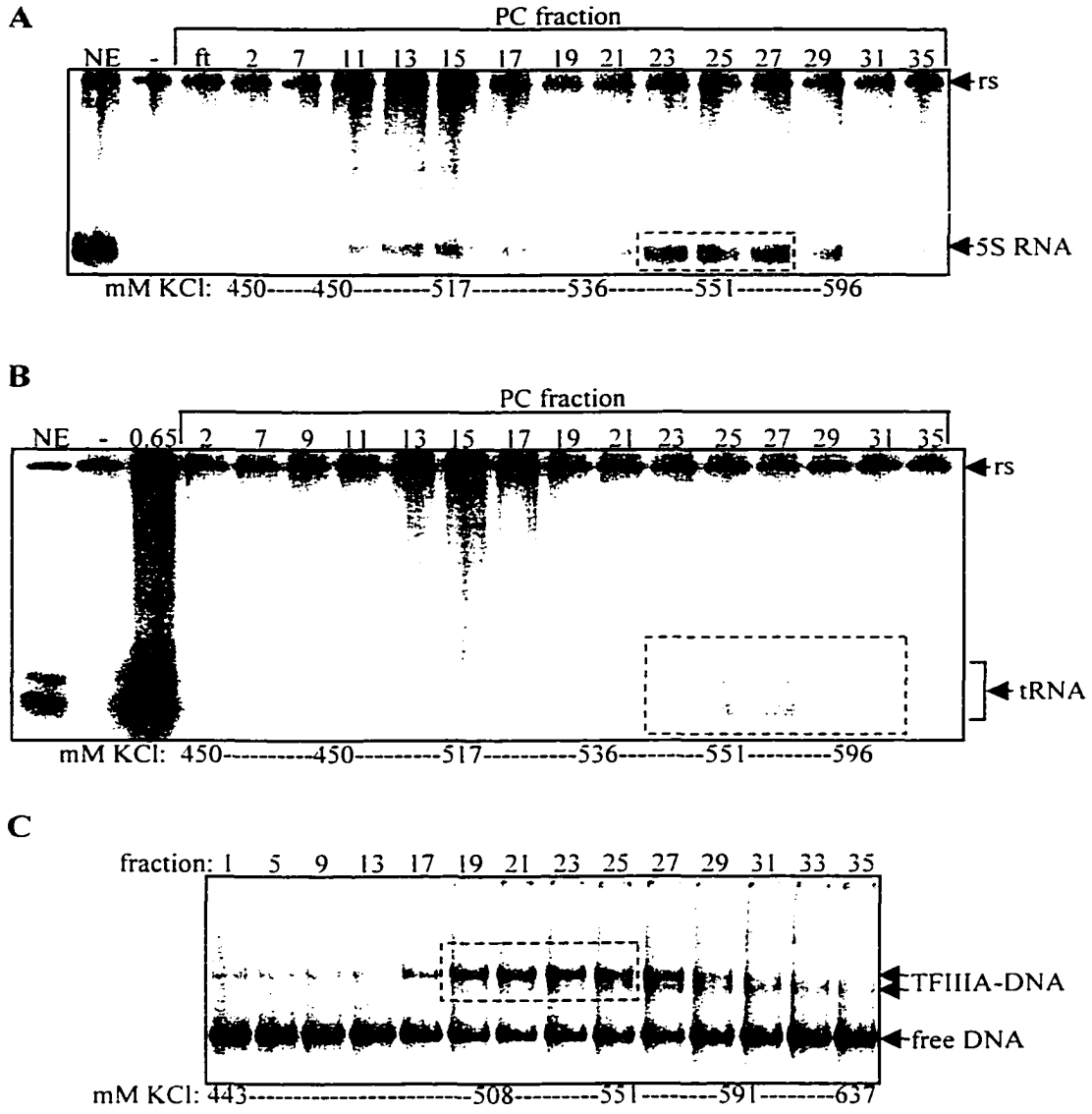
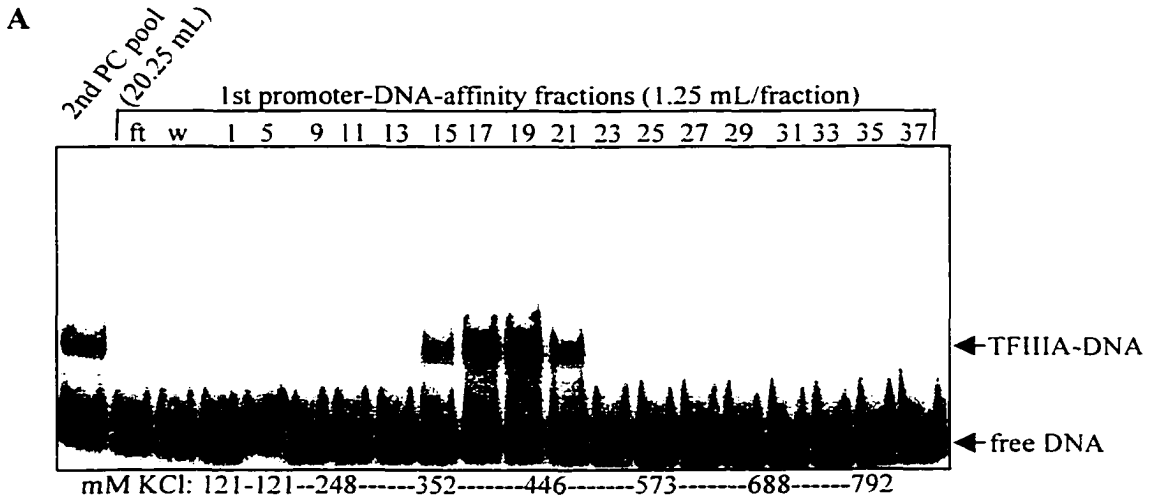
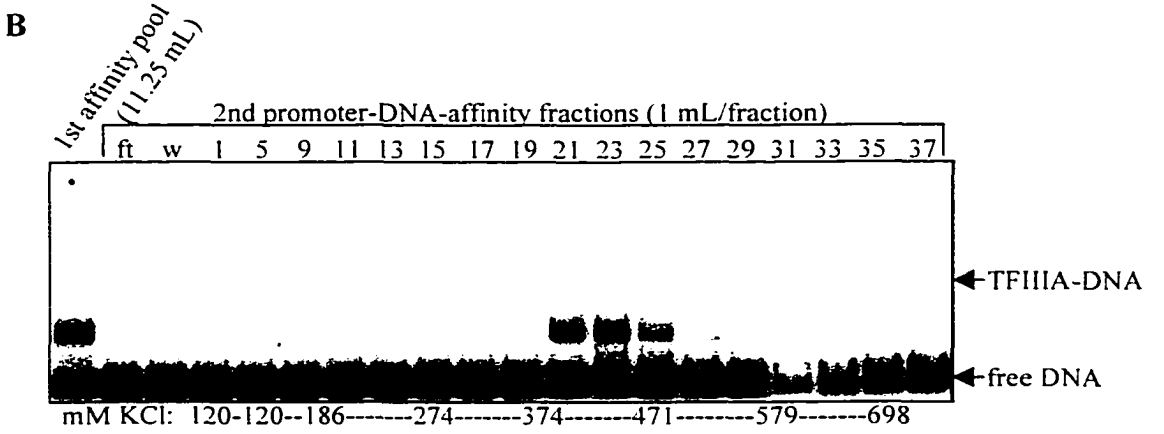
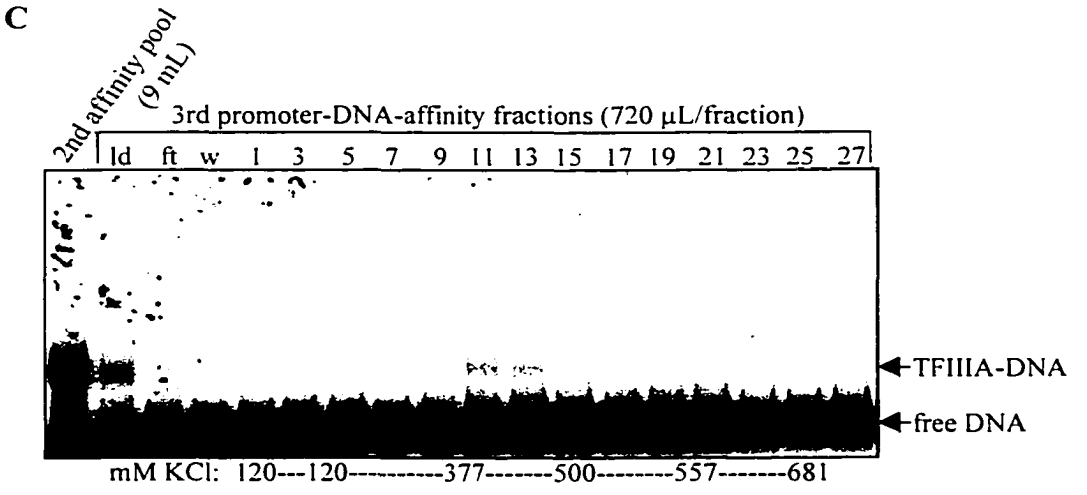


Fig. 2.16. TFIIIA coelutes with TFIIIC from the first PC column at a higher KCl concentration than that required to elute TFIIIA from the second PC column. For the *in vitro* transcription reactions, except those supplemented with 3 μL of nuclear extract (NE), 3 μL of the 0.3 M KCl PC fraction (TFIIIB) and the 3 μL of the 0.45 M KCl PC fraction (Pol III) were used. **A.** 5S RNA transcription using fractions eluted with a linear salt gradient from the first PC column are shown. The KCl concentration of every fifth fraction is shown below the gel. The box shows fractions potentially containing both TFIIIA and TFIIIC, which eluted approximately between 545 and 570 mM KCl. rs, recovery standard (standard EMSA probe); -, 5S RNA transcription with the 0.3 and 0.45 M KCl PC fractions alone. **B.** tRNA transcription is shown. The box shows fractions containing TFIIIC, which eluted between approximately 545 and 600 mM KCl. 0.65, positive control for tRNA transcription (TFIIIC activity). **C**, the DNA binding activity of TFIIIA eluted from the second PC column (Fig. 2.2) shows the peak of TFIIIA activity eluted between 500 and 555 mM KCl.

Fig. 2.17. TFIIIA activity was significantly reduced after three rounds of promoter-DNA-affinity chromatography. The DNA binding activity of the fractions eluted from the three affinity columns was tested by EMSA. The KCl concentration of every fifth fraction eluted from each column is shown below each EMSA gel. ft. flow-through fraction; w. column wash fraction. ld. loaded fraction. **A.** BSA (2.6 mg) was added to TFIIIA pooled from the second PC column. and this fraction was dialyzed to 166 mM KCl and additionally diluted to 120 mM KCl. Prior to loading the first affinity column (1.5 cm diameter. 5 mL bed volume). 0.2 mg polydI·dC DNA and 0.2 mg polydA·dT nonspecific competitor DNA were added to the fraction. Approximately 50 % of the TFIIIA activity pooled from the second PC column was recovered from the first affinity column using the defined competitor DNAs as opposed to calf thymus DNA and by adding BSA to stabilize TFIIIA. **B.** BSA (4.2 mg). but not nonspecific competitor DNA. was added to the fraction applied to the second affinity column (1.5 cm diameter. 4 mL bed volume). This fraction was diluted to 120 mM KCl prior to loading. **C.** the fraction applied to the third affinity column was dialyzed to 120 mM KCl. No nonspecific competitor DNA or BSA was added to this fraction prior to loading.







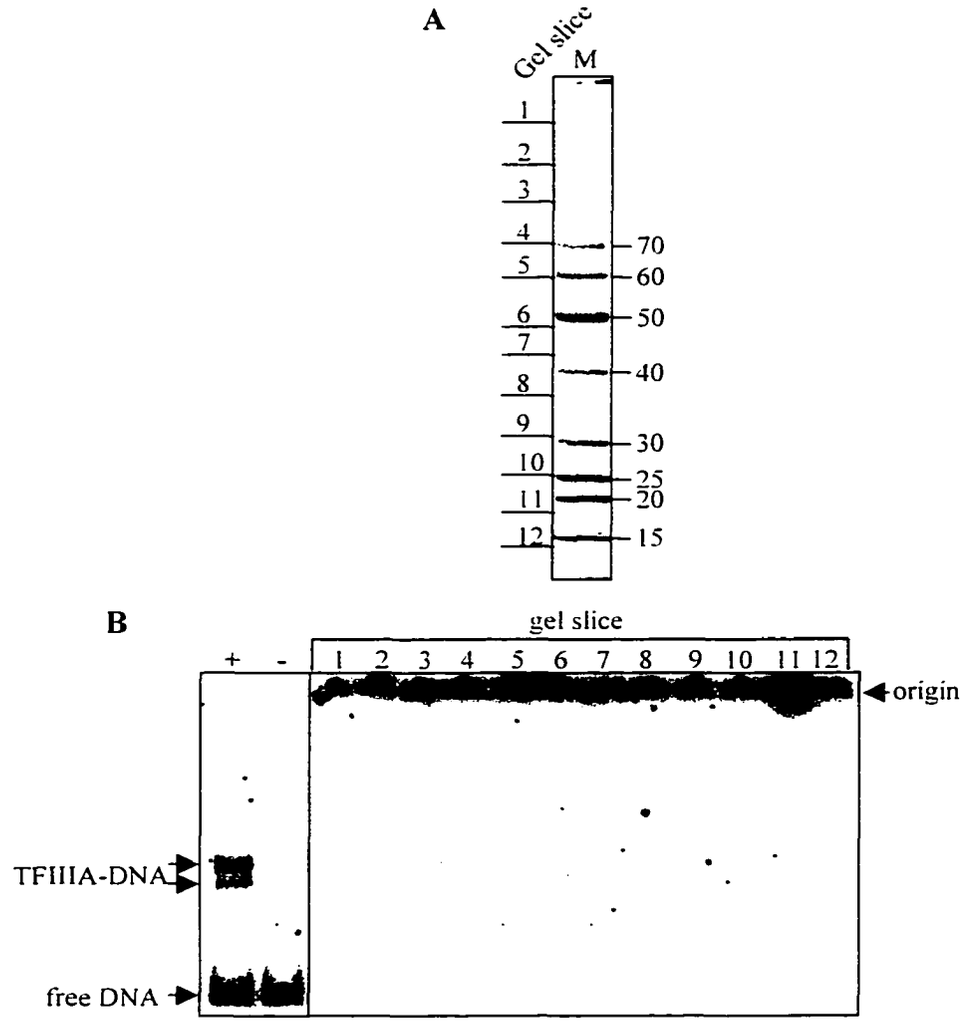


Fig. 2.18. DNA-binding activity of TFIIIA was recovered from an SDS-polyacrylamide gel slice containing proteins ranging in size from 48 kDa to 60 kDa. **A**, the 11% SDS-polyacrylamide gel stained with Coomassie shows the size range of the proteins from each gel slice. Slices were taken from a lane loaded with 40 μL of TFIIIA pooled from the second PC column. Proteins were extracted and renatured as described (Ossipow *et al.*, 1993), except the elution-renaturation buffer (ERB) lacked 0.1 % Trasylol and contained 50 mM DTT, 0.2 mM PMSF, and 1 mM benzamidine. In addition, crushed polyacrylamide slices were suspended with 1 vol (mg of slice/μL) of ERB and then passed through a 0.2 μm filter to remove the polyacrylamide. The supernatant was dialyzed against 50 mM HEPES (pH 7.9), 85 mM KCl, 5 μM $ZnCl_2$, 10% glycerol, 5 mM DTT, 1 mM benzamidine, and 0.2 mM PMSF. **B**, proteins extracted from each indicated gel slices (20 μL of each sample) were tested for TFIIIA activity by EMSA using 40,000 cpm of a double end-labeled DNA fragment containing the 5S RNA gene in a 22 μL reaction. The dried gel was cut into two pieces, and the control lanes were exposed to a phosphorimager screen for one day, while the experimental lanes were exposed for two days. +, 2 μL of purified TFIIIA; -, reaction solution alone.

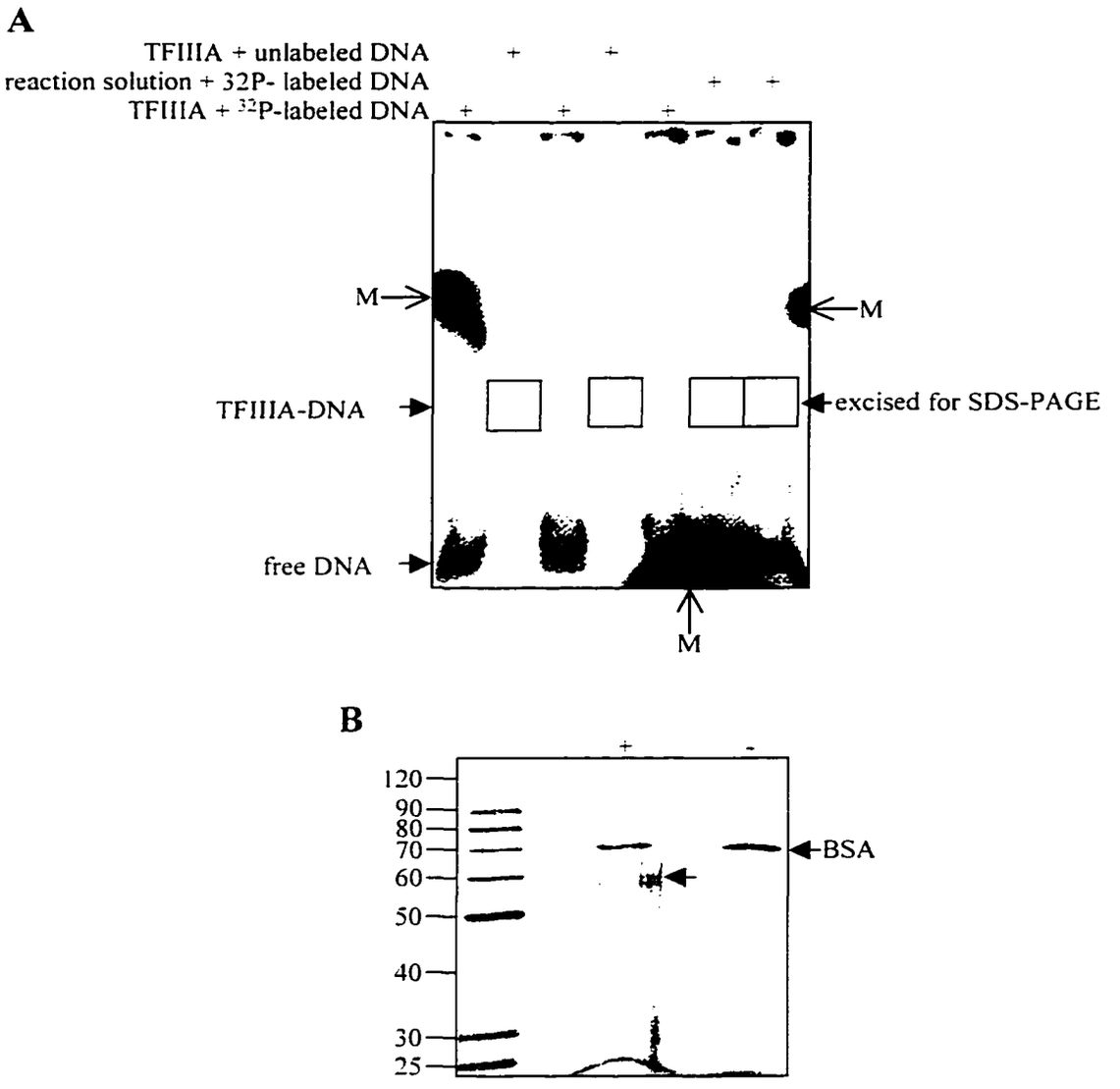


Fig. 2.19. A protein migrating with the TFIIIA-DNA complex in a native polyacrylamide gel is the same size as *A. castellanii* TFIIIA. **A**, TFIIIA-DNA complexes were resolved in an EMSA gel. Complexes were formed with either 3 μL of affinity-purified TFIIIA and 20,000 cpm of *Eco*RI/*Hind*III digested pAC-PS1 or 31.9 μL of dialyzed, affinity purified TFIIIA containing 0.5 mg/mL of BSA and approximately 150 ng of unlabeled *Eco*RI/*Hind*III digested pAC-PS1. All EMSA reactions were 35 μL. M, α-^{32}P DNA spotted onto the gel for orienting the gel. **B**, gel slices were resolved in an 11% SDS-polyacrylamide gel that was subsequently stained with silver. Slices had been removed from the lanes of the EMSA gel containing the potential unlabeled DNA-TFIIIA complexes and the lanes containing the reaction solution with 0.5 mg/mL BSA. Slices were excised based on the positions of the radiolabeled DNA-TFIIIA complexes. A protein similar in size to *A. castellanii* TFIIIA (arrow) was observed in the lane that contained the TFIIIA-DNA complex (+), but not the reaction solution with BSA alone (-).

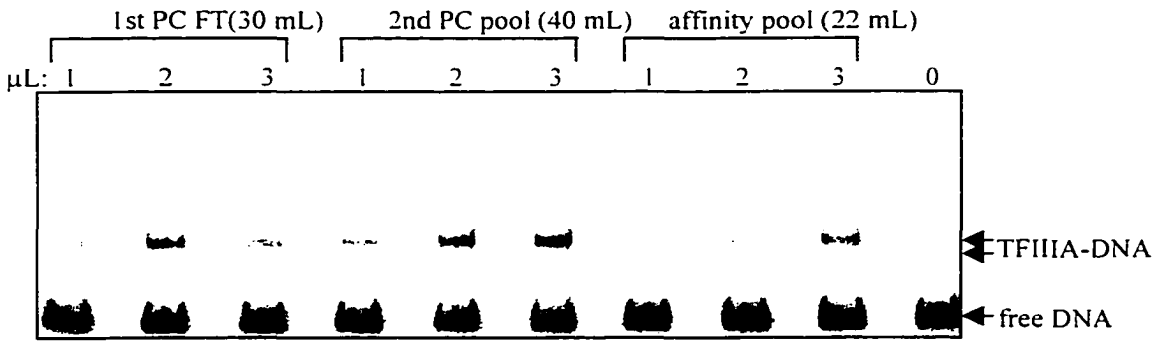


Fig. 2.20. The yield of TFIIIA is greater when the first PC flow through fraction is applied to the second PC column at 0.4 M KCl. The DNA binding activity of TFIIIA was tested at three stages of a previous purification procedure by EMSA. Indicated amounts of each fraction were tested with 10.000 cpm *Eco*RI/*Hind*III digested pAc-PS1 in a 20 μL reaction. The previous procedure was similar to the present procedure used to purify TFIIIA except for three general differences. (1) The KCl concentration of the first PC flow-through fraction was raised to 0.4 M instead of 0.45 M. (2) The previous promoter-DNA-affinity column was used (3 repeats from +47 to +100 of the 5S RNA gene attached to the column matrix). (3) PolydI·dC (184 μg) and of polydA·dT (92 μg) nonspecific competitor DNAs were used instead of calf thymus DNA. Approximately 160% of the TFIIIA activity in the first PC flow-through fraction was recovered from the second PC column. This increase in activity may be due to the separation of TFIIIA from inhibitors of its activity that are present in the first PC flow-through fraction. Approximately 30% of the TFIIIA activity from the first PC flow-through fraction remained after affinity chromatography. These estimations in the yield are speculative because the EMSA reactions were not performed more than once.

Chapter 3: TFIIIA Must Be Cloned to Further Study its Regulation

The work described in this chapter describes preliminary results that may be used to determine the direction of a project to elucidate the mechanism regulating TFIIIA in *A. castellanii*.

3.2 Abstract

The loss of TFIIIA activity effects the down-regulation of 5S RNA transcription during *A. castellanii* encystment. This process is coordinated with the down-regulation in rRNA transcription by Pol I. Experimental evidence has shown that Pol I-mediated transcription is regulated by a posttranslational modification of the polymerase, suggesting that a similar modification of TFIIIA may be involved in coordinating the reduction in transcription from the 5S and rRNA genes. Preliminary results suggest that TFIIIA cannot be detected in trophozoite cell extracts by immunoblotting because of the small quantity of this protein found in the cells. Therefore, it is unlikely that potentially inactive TFIIIA in cyst cell extracts could be detected using antibodies against TFIIIA. Any further investigation into TFIIIA regulation should therefore initially involve cloning the protein and determining if it possesses any motifs that could potentially be modified.

3.1 Regulation of 5S rRNA transcription during *A. castellanii* encystment.

The primary objective of this research has been to purify and characterize TFIIIA from *A. castellanii* in order to initiate future studies into the regulation of this factor. *A. castellanii* cells adopt one of two growth states in response to surrounding conditions. In a nutrient-rich medium, close to neutral pH, cells undertake rapid growth and division and are observed as having numerous lamellapodia and filopodia. These cells, termed trophozoites, accommodate this rapid growth rate by synthesizing large quantities of ribosomal RNA (rRNA) that are required for high levels of protein synthesis. Both Pol I and III are utilized to produce the complete set of rRNA molecules: Pol III transcribes 5S RNA, while Pol I transcribes a precursor to the three largest rRNA components. A change in the growth state of these cells occurs when they encounter adverse conditions, such as when they are transferred to a basic medium, lacking nutrients. Under adverse growth conditions, the cells adopt a dormant and protective state, morphologically characterized by their rounded appearance and the formation of two outer protective layers surrounding each cell: one composed of polysaccharide and the other of protein. These cells, termed cysts, exhibit a coordinate down-regulation in 5S and rRNA transcription (Matthews *et al.*, 1995). The decrease in Pol I transcription is caused by the loss in the ability of the polymerase to initiate specific transcription, which is proposed to be evoked by a modification in one of the enzyme subunits or a tightly associated factor (Paule *et al.*, 1984). In contrast, the disappearance of TFIIIA and not polymerase III activity leads to the loss of 5S RNA synthesis (Matthews *et al.*, 1995). Unlike what has been reported in *S. cerevisiae* (Clarke *et al.*, 1996), an inhibitor does not specifically target 5S RNA synthesis in *A. castellanii*. So far, the mechanism controlling TFIIIA activity during encystment remains unknown.

It has been hypothesized that TFIIIA undergoes a posttranslational modification or becomes sequestered away from the promoter during encystment.

This notion is based on changes in the Pol I enzyme as *A. castellanii* cells change from trophozoites to cysts and *vice versa*. Loss of specific transcription activity by cyst Pol I has been correlated with a modification in its AC39 subunit, although the nature of this modification remains unclear. Interestingly, in encysted cells that are stimulated to reenter the growth/division cycle, Pol I activity is rapidly restored in conjunction with a reversal of the AC39 modification. Therefore, Pol I seems able to cycle between active and inactive forms, indicating that TFIIIA may be similarly manipulated in order to sustain coordinate regulation between 5S RNA and rRNA transcription. It is possible that TFIIIA is regulated by phosphorylation, in a similar manner as the yeast transcription factor ADR1. Like TFIIIA, ADR1 has two C2H2-type zinc fingers that are essential for its function (Blumberg *et al.*, 1987; Hartshorne *et al.*, 1986). Phosphorylation of ADR1 at a site carboxyl-terminal to its zinc fingers blocks its ability to activate transcription (Denis *et al.*, 1992). Because TFIIIA *from A. castellanii* has not been cloned, the existence of possible phosphorylation sites on the protein is not known. Further investigation of *Ac*TFIIIA should involve cloning this protein.

3.2 Cloning TFIIIA.

The success of an initial attempt to clone *Ac*TFIIIA is currently unclear. The pool of TFIIIA from the promoter-DNA-affinity column (Chapter 2) was concentrated using repeated rounds of methanol and chloroform precipitation (Wessel and Flugge, 1984). In the first round of precipitation, 300 μL of the purified TFIIIA fraction was processed with 100 ng of cofilin that was added as a carrier protein. In each subsequent round, the protein pellets from the previous round were resuspended with an additional 300 μL of the purified TFIIIA fraction, and the proteins in this solution were reprecipitated. This procedure was repeated until the entire purified TFIIIA fraction had been processed. The concentrated proteins were

then resolved on an 11% SDS-polyacrylamide gel, which was then stained with Coomassie (Fig. 3.1A), and the band corresponding to TFIIIA was excised and sent to the HHMI Biopolymer Facility at Yale University for internal peptide sequencing. In using this procedure, only 1 to 5 pmol of TFIIIA was recovered from approximately 5.2 X 10^{11} *A. castellanii* cells. This low yield of TFIIIA limited the amount of polypeptide sequence obtained and additionally limited the certainty of these sequences. Modifying the purification scheme as described (Section 2.5) could possibly increase the yield of TFIIIA. Additional, TFIIIA may not precipitate efficiently with methanol and chloroform, as more bands were observed on the Coomassie-stained gel than on a silver-stained gel containing 19 μL of purified TFIIIA that had not been precipitated (Fig. 2.1B). Therefore, an alternative protein precipitation or concentration methods may be necessary to increase the yield of TFIIIA.

Only three polypeptide sequences were obtained:

1 = N,G,(T,S)-F-L-H,(E,N)-P,(E,N)-S-Q-L-(R)

2 = E,L-F,L-A,Q-R-N-T

3 = Q,T,A,D,S,V-V-H-D,(Q)-(G),(L)-L-(R).

Amino acids are shown according to their single letter abbreviations. Amino acid positions are separated by hyphens; commas separate multiple amino acids detected at a single position; weakly detected amino acids are shown in parenthesis. Of these three sequences, only sequence 1 contained a sufficient number of consecutive amino acids with limited codon degeneracy to designing a PCR primer for cloning TFIIIA (Fig. 3.1B). The degeneracy of this primer was further limited by using the known codon bias of *A. castellanii* cloned proteins. Prior to PCR, cDNA was synthesized from RNA extracted from *A. castellanii* by reverse transcription using a polydT primer that contained an extended adapter sequence at its 3'-end. The cDNA was then amplified by PCR using the degenerate TFIIIA

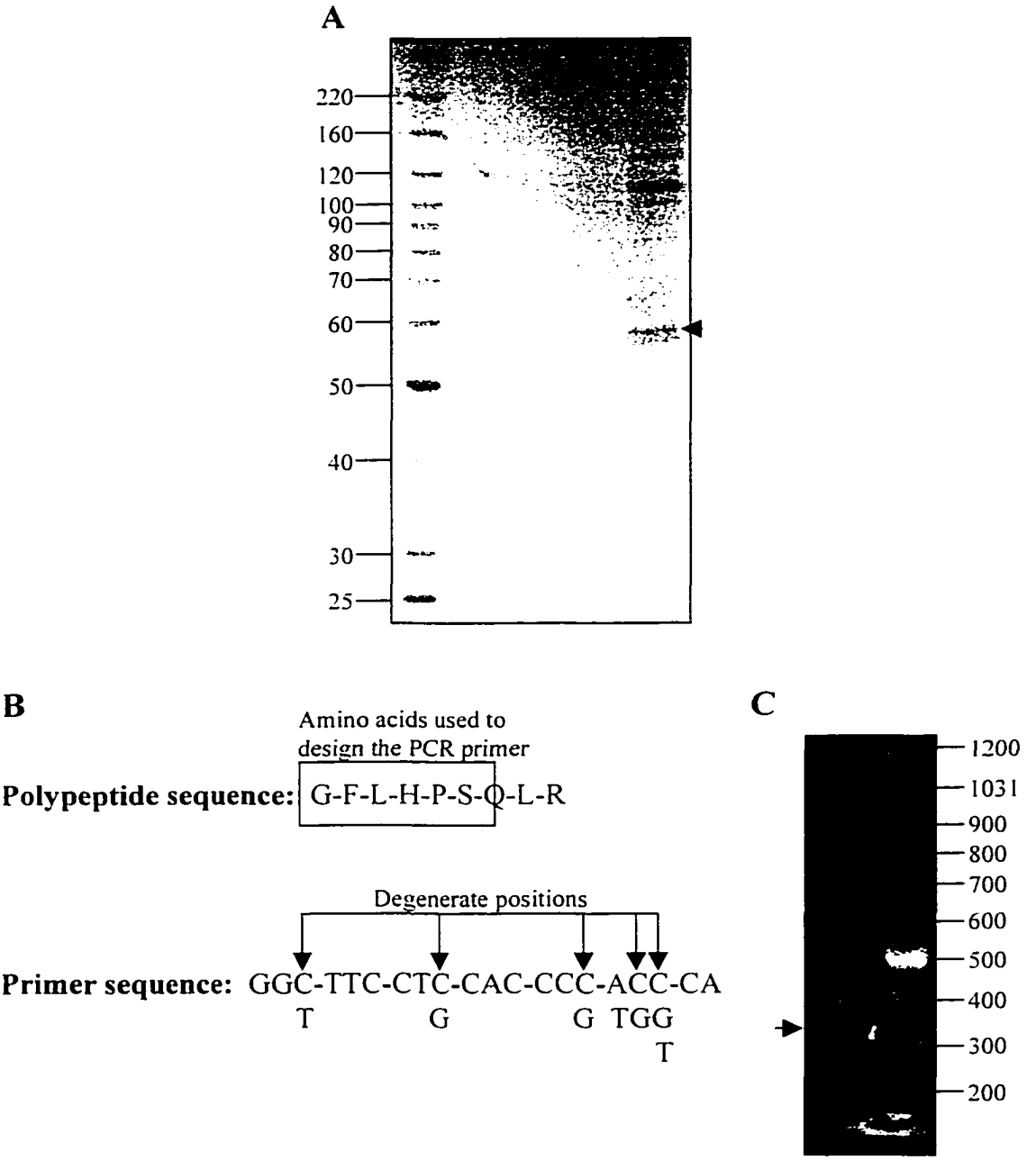


Fig. 3.1. A preliminary attempt to clone TFIIIA. **A.** shown are the proteins from the purified TFIIIA fraction (Chapter 2) that were concentrated an resolved on an 11% polyacrylamide gel that was subsequently stained with Coomassie. The arrow shows the band correlated with TFIIIA that was excised and sent to HHMI Biopolymer Facility at Yale University for internal peptide sequencing. **B.** of the three polypeptide sequences obtained, only this one was used to design a degenerate PCR primer. The boxed amino acids were used to generate the PCR primer. Q is partially boxed because only the first two nucleotides of the codon were incorporated into the PCR primer. The degeneracy of the primer was limited by the known codon biases in *A. castellanii* **C.** a 350 bp product was detected after two rounds PCR amplification starting with *A. castellanii* cDNA.

primer and a primer that was equivalent to the adapter sequence of the polydT primer. Two rounds of PCR were performed: in the first round, a touch-down strategy was used in which the annealing temperature was above the Tm of the primers at the beginning of the PCR reaction, but was decrease by one degree after every two cycles, so that the annealing temperature was below the Tm of the primers by the end of the reaction (Roux and Hecker, 1997). In the second round of PCR, a single, high annealing temperature was used that approximated the Tm of the primers. A low quantity of a 350 bp product was observed following the second round of amplification (Fig. 3.1C). This PCR product has not yet been cloned and sequenced. Additional PCR primers to the *Ac*TFIIIA sequence may be needed if cloning is to be successful. Therefore, methods to elucidate the mechanism of TFIIIA regulation in *A. castellanii* may be limited to cloning the protein and determining if it contains any domains or motifs that may be involved in regulation.

3.3 *A. castellanii* TFIIIA cross-reactivity with antibodies raised against other TFIIIA homologues.

The primary objective was to determine if TFIIIA remained present in cysts, which could be determined simply by immunoblotting. Unfortunately, antibodies have not yet been raised against this protein. However, with the recent determination of the size of *A. castellanii* TFIIIA, it became possible to test if antibodies raised against TFIIIA from other organisms could cross-reacted with this protein. Antibodies against either *S. cerevisiae* or *X. laevis* TFIIIA were tested for cross-reactivity with proteins in the first PC flow-through fraction, but were not found to bind specifically to *A. castellanii* TFIIIA in this fraction (Fig 3.2A and B). This negative result may have been due to the extremely low quantities of TFIIIA in cruder protein fractions because, in the second PC pool, a protein close to the size of *A. castellanii* TFIIIA was found to cross-react weakly with the *X. laevis* antibody

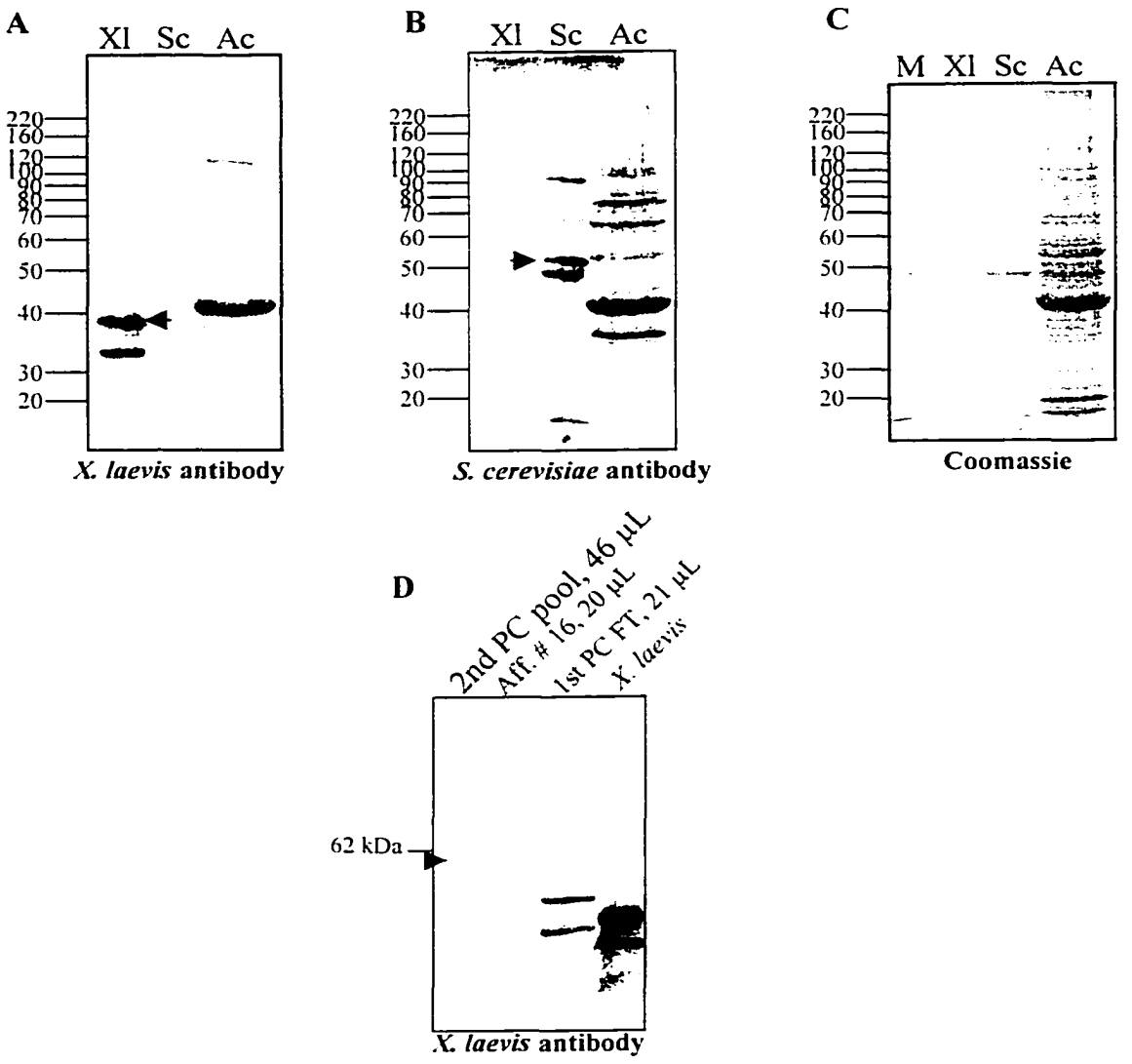


Fig. 3.2. Antibodies raised against *X. laevis* and *S. cerevisiae* TFIIIA do not cross-react with *A. castellanii* TFIIIA in the first PC flow-through fraction. For all immunoblotting experiments, proteins were resolved on 11% polyacrylamide gels and transferred to PVDF membranes (Gelman Laboratory). Membranes were blocked with fraction V BSA (Sigma) and then incubated with the primary antibody 12-16 hrs. The *S. cerevisiae* polyclonal antibody was kindly provided by Jacqueline Segall. The *X. laevis* monoclonal antibody and heparin agarose purified *X. laevis* TFIIIA were generous gifts from Joel Gottesfeld. Xl, heparin agarose purified TFIIIA from *X. laevis*; Sc, *S. cerevisiae* TFIIIA-containing fraction from a BioRex 70 575 mM KCl step elution of a whole-cell extract (Taylor *et al.*, 1985); Ac, *A. castellanii* TFIIIA from the first PC flow-through fraction. **A**, immunoblot using the *X. laevis* antibody. The arrow identifies TFIIIA from *X. laevis*. **B**, immunoblot using the *S. cerevisiae* antibody. The arrow identifies TFIIIA from *S. cerevisiae*. **C**, coomassie stained proteins in each fraction. **D**, immunoblot testing the *A. castellanii* second PC pool and affinity fraction 16 (Chapter 2 supplementary Fig. 2.11).

(Fig. 3.2D). Based on the specific activity in the second PC pool, approximately 1 ng of TFIIIA was used in comparison to 0.5 μg of the purified *Xenopus* TFIIIA fraction. Therefore, the cross-reactivity of *A. castellanii* TFIIIA with the *Xenopus* antibody may be greater than the cross-reactivity apparent from the immunoblot. These results suggest that immunobloting may not be sensitive enough to monitor *Ac*TFIIIA in nuclear extract as cells encyst, because TFIIIA is likely to be in extremely low abundance in this fraction and likely masked from detection by other cross-reacting proteins (Fig. 3.2A).

3.4 An unidentified factor possibly enhances TFIIIA binding to the 5S RNA gene.

An unidentified factor may facilitate TFIIIA binding to the 5S RNA gene. TFIIIA activity has been repeatedly found to be lost during attempts to multiple rounds of affinity chromatography as final steps in the purification. In one purification procedure, TFIIIA activity was significantly reduced after the second round of promoter-DNA-affinity chromatography (Fig. 3.3A). TFIIIA activity lost in the column flow-through fraction was only a small fraction of the total TFIIIA from the fraction loaded onto the column, suggesting that most of the TFIIIA was inactivated during chromatography or was separated from a component essential for activity. Fraction 19 contained the residual peak of DNA-binding activity. To determine if a component that stimulated DNA binding activity had been separated from TFIIIA during chromatography, fraction 19 was combined with other chromatography fractions in an EMSA (Fig. 3.3B). Upon combining fraction 19 with the flow-through fraction from the column, stimulation of DNA binding-activity was observed. The DNA-binding activity of the flow-through fraction and fraction 19 in combination was more than 4-fold above the summation of their DNA-binding activities alone. The increased DNA binding was not produced when BSA was added to fraction 19,

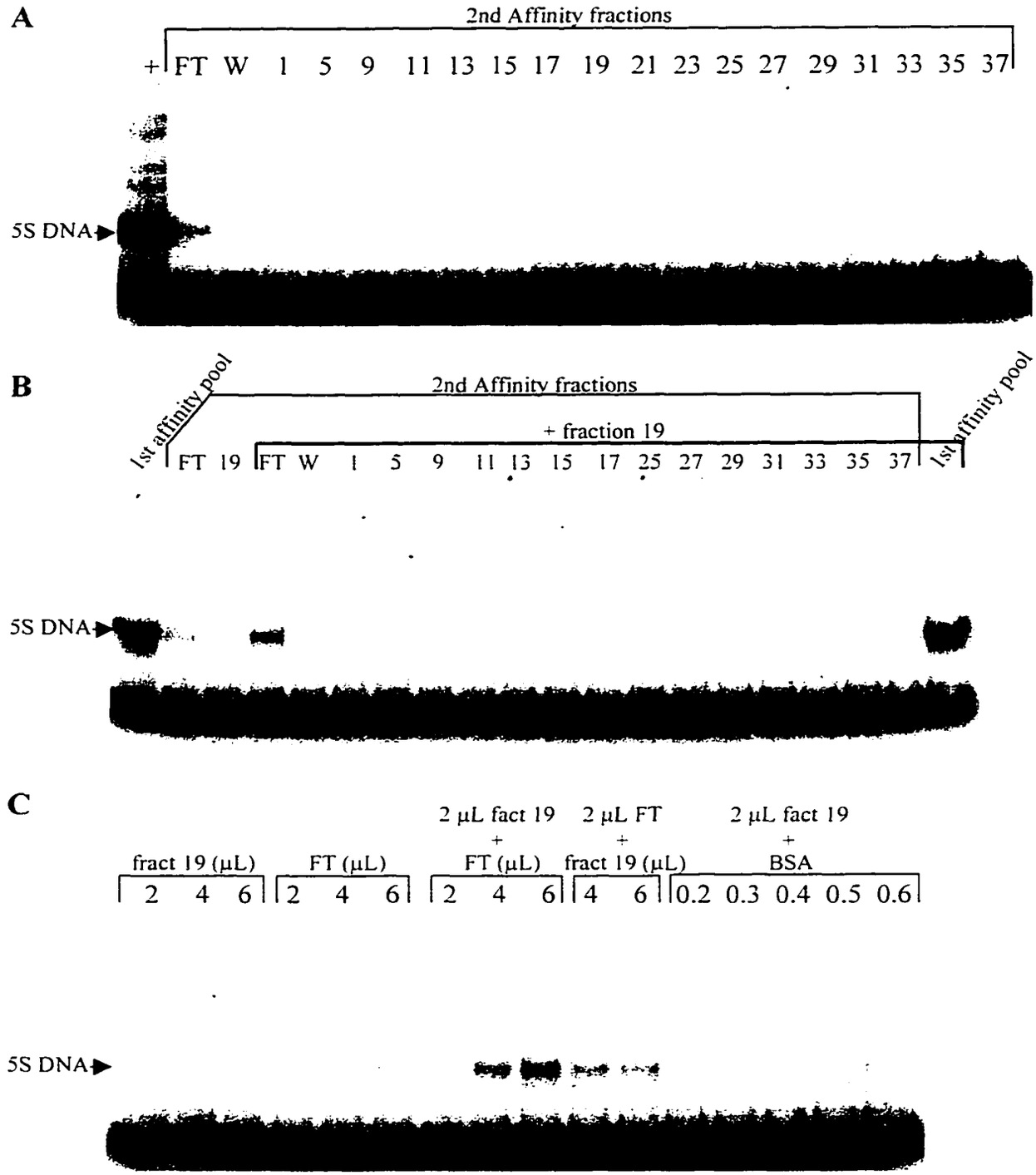


Fig. 3.3. Weak DNA-binding by TFIIIA is stimulated by an unidentified factor. A. the EMSA shows that TFIIIA activity was significantly reduced after a second round of affinity chromatography. W, column wash; FT, column flow-through. **B.** in the EMSA, fraction 19 from the second affinity column (the peak of TFIIIA activity) was combined with other fractions from the column. A more than additive increase in DNA-binding activity occurred when fraction 19 was combined with the FT fraction. **C**, the EMSA shows that DNA-binding activity in fraction 19 was stimulated specifically by the FT fraction, but not by BSA

indicating that this was not a nonspecific protein effect that stabilized TFIIIA (Fig. 3.3C). Therefore, an unknown factor may stimulate low levels of TFIIIA binding-activity. Although not determined, it is also possible that the combination of the flow-through fraction and fraction 19 would have also stimulated 5S RNA transcription. If this were so, it could be similar to a situation observed in the vertebrate Pol III system in which a protein identified as TFIIIA-intP has been found to interact with TFIIIA and, thereby, stimulate transcription (Moreland *et al.*, 2000). TFIIIA-intP has also been found to mediate effects in the tumor necrosis factor pathway (Li *et al.*, 1999), but how this relates to Pol III-mediated transcription remains unclear. Nevertheless, a similar factor is not likely to be present in a single-celled organism such as *A. castellanii*, although a newly discover yeast Pol III subunit, C17, has been found to be similar in sequence to a hormone receptor (Ferri *et al.*, 2000). These similarities between factors used for Pol III-mediated transcription and proteins involved in signal transduction may reveal novel mechanism by which Pol III is regulated.

Chapter 4:

Regulation of RNA Polymerase III Transcription in *Saccharomyces cerevisiae*

The work described in this chapter is very preliminary and not publishable. However, additional experiments could culminate in a publication. This chapter has been written in a format to facilitate in writing a manuscript. The work was done with the aid of two undergraduates, Jill Giggy and Jacob Boysen. The *in vitro* transcription assay presented in Fig. 4.7B was done by Jacob Boysen.

4.1 Abstract

The mechanisms involved in RNA polymerase III (Pol III) transcriptional regulation in *Saccharomyces cerevisiae* are not clear, partly as a consequence of the difficulties in interpreting results from *in vitro* experiments. Previous work done using whole-cell extracts has shown that a decrease in the limiting TFIIIB subunit, Brf, is partly responsible for the suppression of Pol III-mediated transcription that occurs during the arrest of cell growth in stationary phase. Here we have shown that an inhibitor of Pol III-mediated transcription is also present in extracts prepared from stationary phase cells. This inhibitor did not decrease Pol I transcription *in vitro*, suggesting that the effects on Pol III-mediated transcription are specific. These results are discussed with respect to previous findings concerning the down-regulation of Pol III-mediated transcription in growth-arrested *S. cerevisiae* cells.

4.2 Introduction

RNA polymerase III transcribes a diverse array of small RNA molecules (White, 1998), including tRNAs and 5S RNA, which are both required for translation. Although tRNAs and 5S RNA are transcribed by RNA polymerase III, these two gene types have different promoter structures. The promoter on tRNA genes consists of an A-box positioned at +10 with respect to the transcription start site (+1) and a B-box positioned from 30 to 60 base-pairs downstream of the A-box (Geiduschek and Kassavetis, 1992). The A-box dictates the transcription start site, and the B-box is essential for TFIIIC binding and therefore the assembly of preinitiation complexes on tRNA genes. In *Saccharomyces cerevisiae*, only a single *cis*-element from +81 to +94 is required for 5S RNA transcription (Challice and Segall, 1989). This element is analogous to the C-box of higher eukaryotic 5S RNA gene promoters that is essential for TFIIIA binding (Pieler *et al.*, 1987).

Formation of the preinitiation complex on a tRNA gene requires the ordered binding of the Pol III general transcription factors, TFIIIC and TFIIIB (Kassavetis *et al.*, 1989). TFIIIC binds first to the DNA, contacting the A-box and the B-box. The TFIIIC-DNA complex is stable, with most of the binding energy localized to interactions at the B-box (Marzouki *et al.*, 1986). TFIIIB is then recruited to the complex strictly through protein-protein interactions with TFIIIC.

The assembly of the preinitiation complex on the 5S RNA gene is slightly more complex, binding to the DNA first and then recruiting TFIIIC. *S. cerevisiae* TFIIIA has been found to protect +65 to +95 of the gene from DNase I cleavage (Braun *et al.*, 1989). TFIIIA promotes the binding of TFIIIC to the complex by protein-protein contacts; the additional DNA-TFIIIC contacts extends the DNase I protection pattern over most of the transcribed portion of the gene (Braun *et al.*, 1989).

On tRNA and 5S RNA genes, TFIIIB from *S. cerevisiae* does not bind DNA in the absence of TFIIIC. However, once TFIIIB is integrated into the complex, it binds tightly to the DNA and resists dissociation from the DNA by the polyanion heparin or high concentrations of NaCl (Kassavetis *et al.*, 1989). However, TFIIIC is effectively stripped from the DNA by heparin or under conditions of high ionic strength, and on 5S RNA genes, TFIIIA is also stripped from the DNA by heparin treatment (Kassavetis *et al.*, 1990). The heparin-treated complexes that retain only TFIIIB are able to recruit polymerase for multiple rounds of transcription, demonstrating the critical role of TFIIIB in polymerase recruitment (Kassavetis *et al.*, 1990).

The rates of tRNA and 5S RNA transcription are regulated according to the need for protein synthesis, which frequently correlates to the rate of cell growth and proliferation. In an actively growing *S. cerevisiae* cell, ribosomal RNA transcription, including 5S RNA transcription, and tRNA transcription represents approximately 70% of the total RNA synthesis in the cell (Zaragoza *et al.*, 1998). In contrast, ribosomal and tRNA transcription is frequently reduced in slower growing cells in order for the cells to maintain metabolic efficiency. These changes in tRNA and 5S RNA transcription in response to the cellular growth rate have been observed in *S. cerevisiae* cells grown under a variety of defined conditions. A reduction in Pol III-mediated transcription has been found to occur in *S. cerevisiae* cells that cease to grow upon progression into stationary phase. Using stationary phase cell extracts from wild type cells, an approximately 3-fold decrease in Pol III-mediated transcription has been found (Sethy *et al.*, 1995). Because of this modest reduction in transcription, cells harboring a mutation in the τ131 subunit of TFIIIC have been used to study the down regulation of Pol III-mediated transcription in stationary phase. Extracts prepared from these cells support higher levels of Pol III-mediated transcription and exhibit a 20-fold decrease in transcription evoked by stationary

phase growth arrest (Sethy *et al.*, 1995). Using this mutant yeast strain, a decrease in the limiting subunit of TFIIIB, Brf, was found to be primarily responsible for the decrease in Pol III-mediated transcription, as the addition of Brf to inactive cell extracts stimulated tRNA synthesis. The addition of TFIIIC was also found to stimulate transcription, but to a lesser extent.

The antibiotic rapamycin has been shown to induce a stationary phase-like state in *S. cerevisiae* cells and has also been used to study Pol III-mediated transcriptional down regulation in *S. cerevisiae* (Zaragoza *et al.*, 1998). Extracts prepared from rapamycin-treated cells were found to support less Pol III-mediated transcription that untreated cells, and this reduction in transcription was attributed to a decrease in polymerase activity and, to a lesser extent, in TFIIIB activity. These results demonstrate that the reduction in transcription in rapamycin-treated cell extracts may be caused by a different mechanism than that affecting Pol III-mediated transcription in stationary phase cell extracts prepared from the τ131 mutant yeast cells. Moreover, the addition of active Pol III factors to rapamycin-treated cell extracts and stationary phase cell extracts has identified potential regulatory targets , but has not defined the mechanism regulating Pol III-mediated transcription in *S. cerevisiae* stationary phase cells.

In the following experiments, the effect of stationary phase growth arrest on Pol III-mediated transcription was examined using a wild type yeast strain. Changes in transcription supported by cell extracts at different times in stationary phase were compared with changes in tRNA synthesis within cells in log phase and at different times in stationary phase. Extracts were found to exhibit only a transient reduction in Pol III-mediated transcription during stationary phase in contrast to a permanent inactivation of tRNA synthesis within the cells, suggesting that the properties of the extracts do not fully recapitulate *in vivo* regulation. The transient inactivation 5S and tRNA transcription was observed in three separate experiments, correlating this

event with a true regulatory mechanism. Analysis of an inactive stationary phase cell extract indicated that Pol III-mediated transcription was abrogated by an inhibitor in the extract that did not affect Pol I transcription.

4.3 Materials and Methods

4.3.1 Growth of cells and preparation of extracts

The cell extraction procedure was adaptation of two procedures that have been described (Klekamp and Weil, 1982; Schultz *et al.*, 1991). Extracts were derived from a single yeast colony (strain YS18) that was transferred to 100 mL of YPD medium and incubated at 30 °C with shaking for 14-20 hr; after 20 hr of growth, cells were in early log phase. Aliquots (50-100 mL) were then transferred to 1 L volumes of YPD, and these cultures were incubated at 30 °C with shaking for 22-26 hr. After this time the cell densities of the cultures were measured by A_{600} and/or direct cell counting and cells were harvested periodically. Cells were collected by centrifugation at 5,000 rpm and 4 °C for 10 min in a Beckman JA-10 rotor, and the cell pellets were suspended in 300-400 mL of solubilization buffer (0.4 M $(NH_4)_2SO_4$, 0.2 M Tris [pH 8.1], 10 % glycerol, 10 mM $MgCl_2$). Following a second, equivalent centrifugation step, the cell pellets were transferred to a 30 mL syringe and pressed into a liquid-nitrogen bath. The resultant cell strands were ground under liquid nitrogen for 30 min using a mortar and pestle. The cell homogenate was then suspended in solubilization buffer containing 10 mM 2-mercaptoethanol, 1 mM PMSF, and 1 mM benzamidine at 2 mL buffer/g of cells, and this slurry was chilled at 0 °C for 20 min. A subsequent centrifugation step at 33,000 rpm and 4 °C in a Beckman Ti50.2 rotor for 1 hr was used to pellet cell debris. The clear portion of the supernatant was collected, and proteins in this fraction were precipitated with $(NH_4)_2SO_4$ at 70 % of saturation (0 °C). Protein precipitates were then collected by centrifugation at 10,000 rpm and 4 °C in a JA-10 rotor, and protein pellets were

suspended in dialysis buffer (20 mM HEPES [pH 7.9], 100 mM KCl, 0.2 mM EDTA, 20% glycerol, 10 mM β-mecaptoethanol, 0.2 mM PMSF, and 1mM benzamidine) at 0.25 mL of buffer C/mL of clear supernatant (above). Samples were transferred to dialysis tubing (Spectra/Por) with a molecular weight cut off of 12,000-14,000 and dialyzed against 2 L of dialysis buffer. The final protein samples were frozen in liquid nitrogen and stored at –80 °C.

4.3.2 Q-column chromatography

Fractionation of yeast extracts using Macro-Prep high Q anion exchange resin (Bio Rad) as been described previously (Clarke *et al.*, 1996; Riggs *et al.*, 1995).

4.3.3 DEAE chromatography

Fractionation of yeast extracts was done essentially as described (Riggs and Nomura, 1990), except DEAE Sepharose Fast Flow resin (Pharamacia) was used in place of DEAE Sephadex A-25 (Sigma).

4.3.4 *In vitro* transcription assays

Plasmids used for the *in vitro* transcription assays were pBS-y5S, harboring the yeast 5S RNA gene from 57-bp upstream to 95-bp downstream of the gene, pBS-TY(GUA)J2, harboring a tRNA tyrosine gene from 64-bp upstream to 25-bp downstream of the gene, pArabMet#4 (Matthews *et al.*, 1995), and pYr11-316 (Schultz *et al.*, 1991). The standard Pol III-mediated transcription reaction used for yeast extracts contained 20 mM HEPES (pH 7.9), 90 mM KCl, 12 mM $MgCl_2$, 12 % glycerol, 0.3 mM EDTA, 0.3 mM DTT, 7 u RNasein (Promega), 600 μM each ATP, CTP, and GTP, 50 μM UTP, 5 μCi of (α-^{32}P)UTP (3,000 Ci/mmol), 200 ng of the plasmid pBS- (Stratagene), and 40 fmol pBS-y5S or pBS-TY(GUA)J2 in a volume

of 50 μL. Reactions were incubated at 25 °C for 15 min prior to the addition of the nucleotides (5.5 μL) to start transcription, which then proceeded at 25 °C for an additional 30 min. Reactions were terminated and samples were processed as described under "Materials and Methods" in Chapter 2.

The standard Pol III-mediated transcription reaction used for *A. castellanii* nuclear extract is described under "Materials and Methods" in Chapter 2.

The standard Pol I transcription reaction used for the D-300 fraction contained 20 mM Tris (pH 7.9 [25 °C]), 0.1 M KCl, 10 mM $MgCl_2$, 10 % glycerol, 1 mM DTT, 8 u RNasein, 500 μM each ATP, CTP, GTP, 15 μM UTP, 5 μCi of (α-^{32}P)UTP (3,000 Ci/mmol), and 10 ng pYr11-316 digested with *Eco*RV in a volume of 40 μL. Samples were incubated at 25 °C for 10 min prior to the addition of the nucleotides (2.5 μL) to start the reaction, which then proceeded at 25 °C for an additional 30 min. The yeast Pol I transcription reactions were then processed similarly to the yeast Pol III reactions.

4.3.5 S1 nuclease assays

The S1 nuclease assays have been described previously (Iyer and Struhl, 1996), as has the oligodeoxynucleotide used to detect tRNA synthesis (Cormack and Struhl, 1992). Oligodeoxynucleotides were end-labeled with (γ-^{32}P)ATP and T4 polynucleotide kinase (MBI Fermentas) as described by the manufacturer. Probes were subsequently purified using Micro Bio-Spin 6 Chromatography Columns (Bio Rad) as described by the manufacturer. The annealing reactions contained 20 μg of RNA and 0.2 pmol of probe.

4.4 Results

4.4.1 Synthesis of tRNA is significantly and permanently reduced within *S. cerevisiae* stationary phase cells.

An S1 nuclease assay was used to measure the relative levels of newly synthesized tRNA transcripts from cells harvested at different stages during log phase growth and at different times of stationary phase growth arrest. Because tRNA and 5S RNA are stable, measuring their overall abundance in the cell by northern blotting does not represent the true rate of transcription from their genes. The S1 nuclease assay can be used to measure the rate of synthesis of a stable RNA provided that the RNA has a region that is rapidly processed following transcription. Such regions exist as introns within nascent tRNA transcripts, but not within nascent 5S RNA transcripts. Therefore, it was only possible to measure cellular levels of tRNA transcription and not 5S RNA transcription. Oligodeoxynucleotides that annealed to an intron found within a family of tryptophan tRNA genes and within an isoleucine tRNA gene intron were initially used to measure the changes in the rate of Pol III-mediated transcription. These introns are rapidly processed following tRNA synthesis (Cormack and Struhl, 1992). For comparison, the level of Pol II transcription from the *HTA2* gene that produces histone H2A was measured (Choe *et al.*, 1982).

The relative levels of synthesis of the three RNA types were examined. A single yeast colony of the YS18 strain was inoculated into of YPD medium and grown overnight. Cells from aliquots of the culture were harvested the following day at various intervals (Fig. 4.1A). The relative levels of the two nascent tRNA transcripts and of the HTA2 transcript were measured using the S1 nuclease assay at each time point (Fig. 4.1.B). These S1 nuclease assays were only performed once. These initial S1 nuclease assays showed that a continuous decrease in $tRNA^{W}$ synthesis occurred from log phase to stationary phase. Phosphorimager

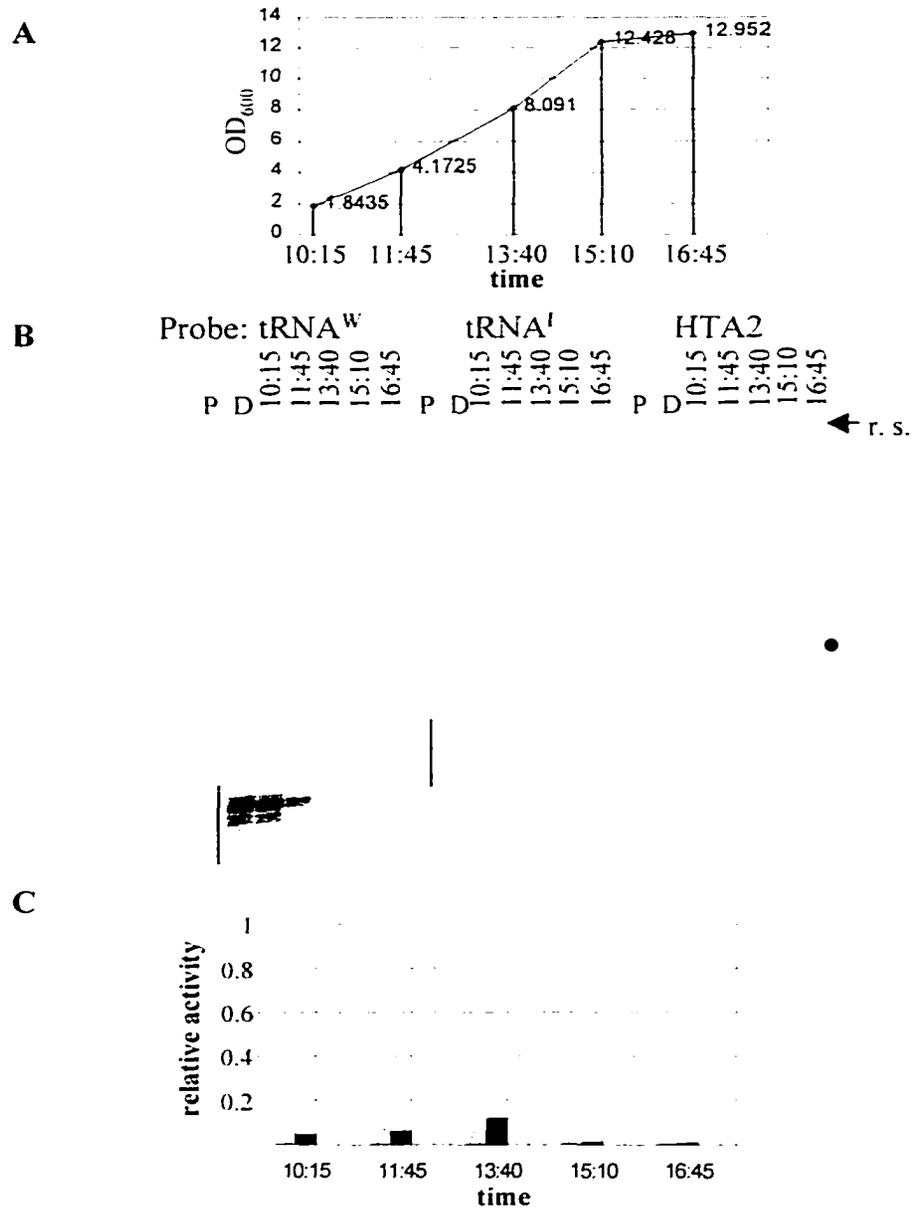


Fig. 4.1. A reduction in tRNA and HTA2 synthesis occurs as *S. cerevisiae* cells enter stationary phase growth arrest. **A**, the culture densities at the times when 15 mL of cells were harvested after a 500 mL culture of YS18 cells was started are shown. **B**, levels of and nascent $tRNA^W$, nascent $tRNA^I$, and HTA2 mRNA in the RNA extracted from the cells at each time point were determined using an S1 nuclease assay in which $tRNA^W$, $tRNA^I$, and HTA2 oligodeoxynucleotide probes were hybridized with 25 μg of RNA overnight. P, undigested probes; D, digested probes without RNA; rs, a radiolabeled DNA internal recovery standard added after S1 digestion. The dot indicates bands corresponding to HTA2 mRNA, and the bars identify nascent tRNA transcripts. **C**, the bar graph shows the quantification of the S1 nuclease assay results. Lightly shaded bars show the relative levels of HTA2 mRNA. Black bars show the relative levels of nascent $tRNA^I$ transcripts, and white bars show the relative levels of $tRNA^W$ transcripts.

quantification of the results using Image Quant 5.1 (Molecular Dynamics) showed a 29.8-fold reduction in $tRNA^W$ synthesis between the first and last time points (Fig. 4.1C). A similar continuous decrease in $tRNA^I$ synthesis was not observed, although a clear reduction in transcription occurred in stationary phase (Fig. 4.1C). These calculations have not been confirmed by S1 nuclease analysis on the same RNA set. The nascent $tRNA^I$ transcripts produced a much weaker signal than the nascent $tRNA^W$ transcripts, which may stem from less transcriptional activity from the $tRNA^I$ gene compared with the family of $tRNA^W$ genes or a lower efficiency in the ability of the oligodeoxynucleotide to bind to the nascent $tRNA^I$ transcripts. HTA2 synthesis appeared to increase slightly during log phase and then decrease in stationary phase. A similar pattern of HTA2 expression was observed in a microarray of transcription before, during, and after the postdiauxic shift in *S. cerevisiae* (DeRisi *et al.*, 1997).

The large decrease in $tRNA^W$ synthesis was confirmed in another S1 nuclease experiment. The culture was processed as described above. In addition to measuring the OD_{600} of the cell culture, the cells were counted (Fig. 4.2A). Variability was evident between the cell counts and light-scattering measurement, and this variability probably arises from cell clumping and cells rapidly settling out of suspension. EDTA was found to be ineffective in separating aggregates of cells in stationary phase. Because of the previously observed variation in the levels of $tRNA^I$ synthesis and the lower signal from these nascent transcripts, levels of $tRNA^I$ synthesis were not measured in this experiment. A drastic decrease was again observed in $tRNA^W$ synthesis as cells approached stationary phase. Quantification of the results showed a 3.8-fold reduction in $tRNA^W$ synthesis at the 11:45 time point (Fig. 4.2B). At 11:45 hr, the cells appeared to have entered stationary phase (Fig. 4.2A). This experiment was only performed once, so this measurement is potentially inaccurate. The level of $tRNA^W$ synthesis continued to decline at subsequent time

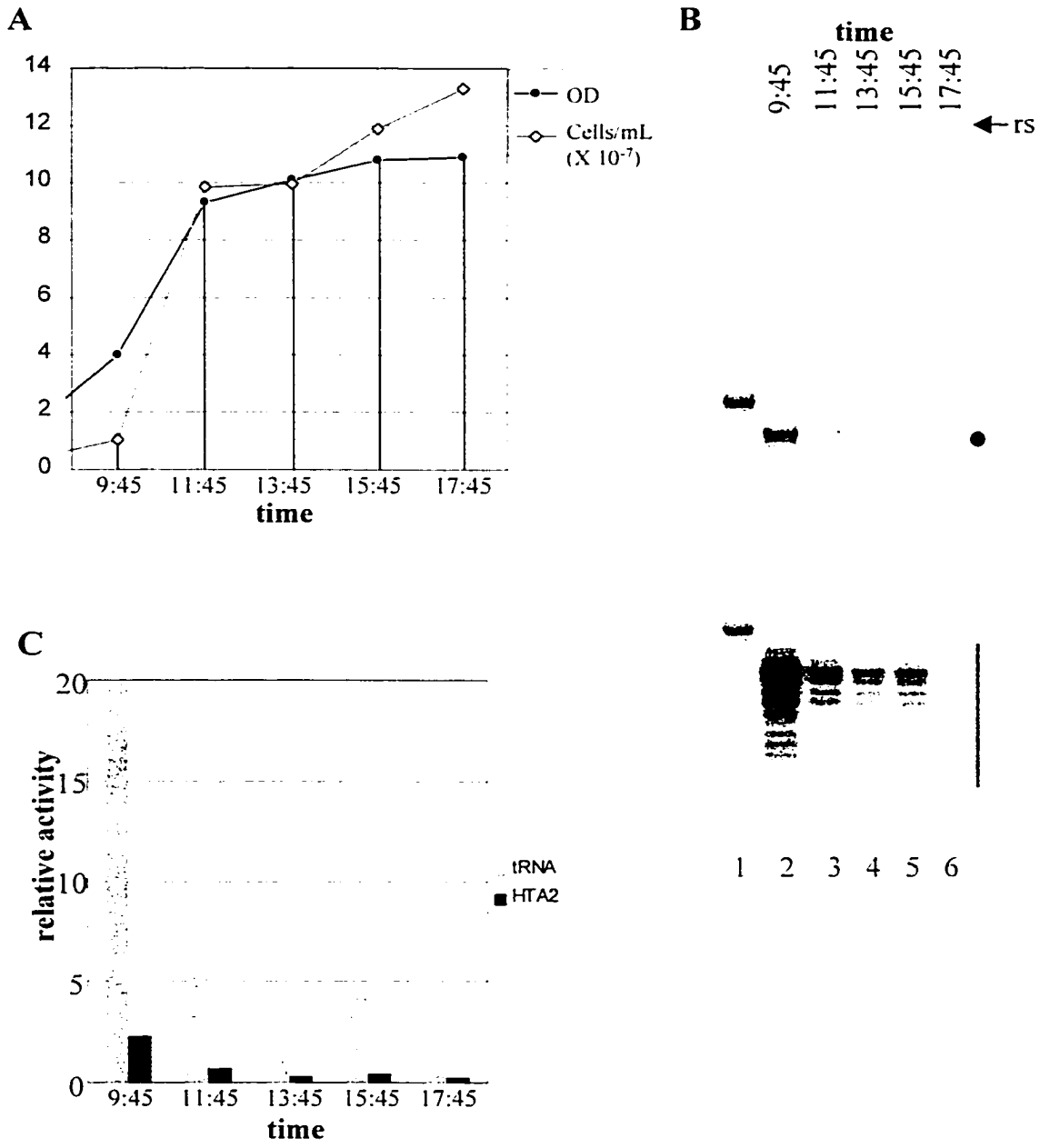


Fig. 4.2. A reduction in tRNA^W and HTA2 mRNA synthesis occurs within *S. cerevisiae* cells during stationary phase growth arrest. **A**, the culture densities at the times when the cells were harvested after the 100 mL culture of YS18 cells was started are shown. Cells were counted and the light scattering of the culture was compared. **B.** levels of nascent tRNA^W and HTA2 mRNA at the indicated time points were determined using an S1 nuclease assay as described (legend of Fig. 3.1). Lane 1 shows the undigested probes. The dot shows bands corresponding to HTA mRNA, and the bars show nascent tRNA transcripts. **C.** the bar graph shows the quantification of the S1 nuclease assay results. Lightly shaded bars show the relative levels of nascent tRNA^W transcripts, and black bars show the relative levels of HTA2 mRNA.

points in stationary phase, although not as dramatically. A 3.9-fold reduction in HTA2 synthesis was observed at the second time interval, similar to the decrease in tRNA synthesis. However, a 54.7-fold reduction in $tRNA^W$ synthesis was observed between the first and last time point, but only a 7.9-fold decrease was observed in HTA2 synthesis, illustrating a dramatic down regulation in $tRNA^W$ synthesis. A comparison of the culture densities and the pattern in the reduction of $tRNA^W$ synthesis in this and the previous experiment suggest that this experiment encompassed slightly later times in stationary phase.

The S1 nuclease assay was then used to determine if any changes in tRNA synthesis occur at later times in stationary phase. YS18 cells were inoculated into YPD medium and grown overnight. The culture was processed as described for the previous two experiments. The concentration of cells determined at 24 hr (1×10^8 cells/mL) indicated that the cells had progressed into stationary phase (Fig. 4.3A). Therefore, cells harvested at all of the time points were in stationary phase. In comparison to log phase cells in which $tRNA^W$ synthesis was observed (Fig. 4.1B, time point 10:15), $tRNA^W$ was not detected in stationary phase cells at each of the time points in the current experiment (Fig. 4.3B). The difference in the pattern of radiolabeled bands corresponding to nascent $tRNA^W$ transcripts has been observed when older probes are used (Cathy Radebaugh, personal communication); in this experiment, probes were used that had been prepared 8 days earlier.

4.4.2 5S and tRNA transcription are transiently down-regulated in extracts prepared from stationary phase cells.

Extracts were prepared from cells at different times in stationary phase to compared the down regulation of *in vitro* Pol III-mediated transcription with the decrease in $tRNA^W$ synthesis that occurs within the cells. A single yeast colony (strain YS18) was transferred to 100 mL of YPD medium and grown overnight.

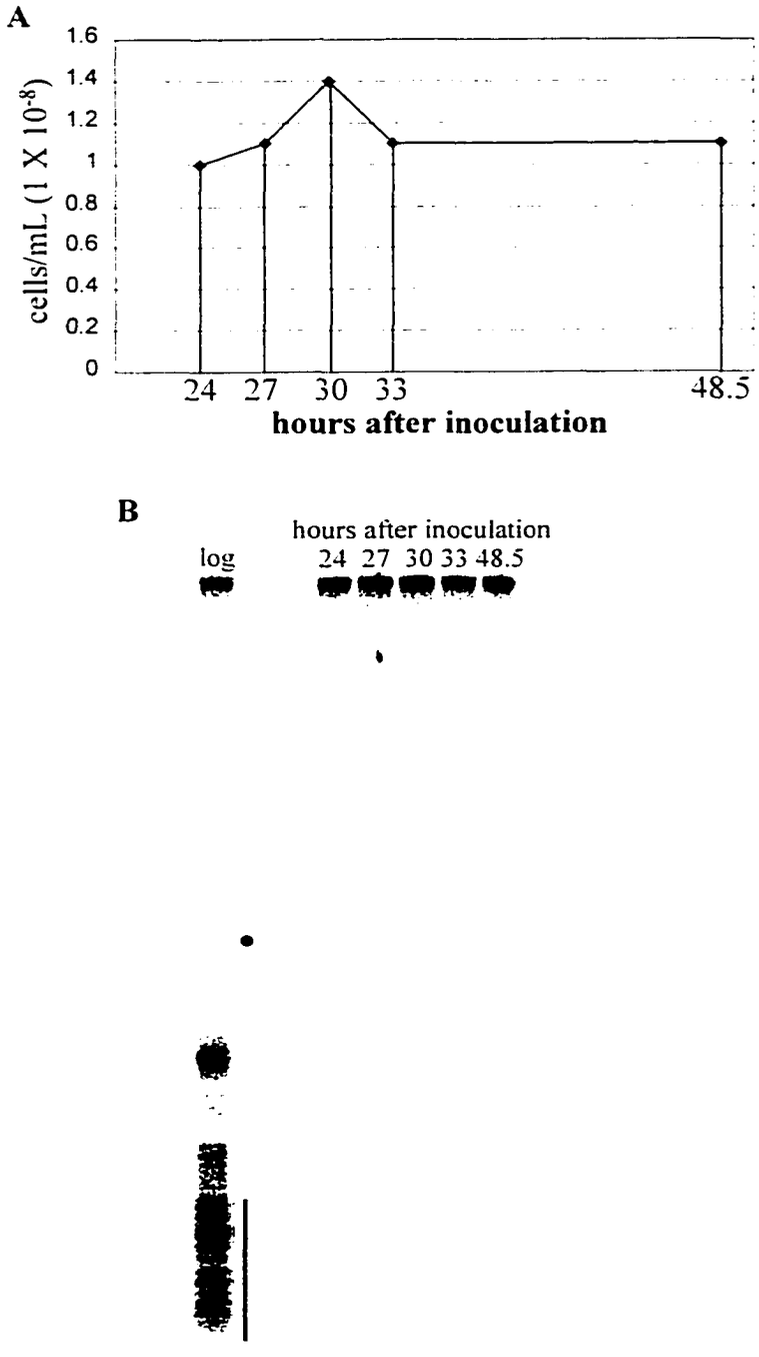


Fig. 4.3. During at least the first 24 hr in stationary phase, $tRNA^W$ synthesis is not detected within *S. cerevisiae* cells. **A**, shown are the concentrations of cells at times after 100 μL of YS18 cells from a culture (≥1 X 10^8 cells/mL) were inoculated into 1 L of YPD. The graph shows that, at each time point, cells were in stationary phase. **B**. the relative quantities of HTA2 mRNA and nascent $tRNA^W$ transcripts from the cells at each time point were determine as described (legend of Fig. 3.1) and compared with the level of nascent $tRNA^W$ transcripts from 10:15 hr (Fig. 3.1). The dot shows bands originating from HTA2 mRNA, and the bar shows bands from nascent $tRNA^W$ transcripts.

Based on the growth rate of the YS18 cells used in this experiment, the cell density following growth overnight was estimated to be 1.59 X 10^7 cells/mL, indicating that the cells were in log phase. Five aliquots of 100 μL from this culture were transferred to 5 flasks, each containing 900 mL of YPD medium, and these cultures were grown overnight. Cells were harvested at intervals of 4 hrs or greater, and at these times, the culture density was measured (Fig. 4.4A). The OD_{600} measurements in this experiment are approximately 5-fold lower than in previous experiments because a different spectrophotometer was used. Different spectrophotometers have been found to produce different readings from the same culture. For example, a DU 640 spectrophotometer (Beckman) shows a $\geq$ 10-fold higher reading than a Spectronic 21D spectrophotometer (Milton Roy) of the same cell culture. Although the different spectrophotometers produced different readings, they all have been found to measure the relative changes in cell density similarly. Therefore, an approximation of the time at which cells reach stationary phase can be determined using different spectrophotometers.

Whole-cell extracts were prepared to test *in vitro* 5S RNA transcription. Each extract and time point is identified according to the number of hours after the 900 mL cultures were prepared. A sufficient quantity of cells for extract preparation was not obtained from the 900 mL culture at the first time point (21.2 hr), so the level of Pol III-mediated transcription was not assessed in a log phase cell extract. Extracts were prepared from cells harvested at the latter four time points (27.8, 31.7, 36.7, and 46.2 hr), and a comparison was made of the level of 5S RNA transcription supported by these extracts. The earliest stationary phase extract (27.8 hr) supported the highest level of transcription (Fig. 4.4B). The 31.7 hr extract exhibited a 4.3-fold reduction in its ability to support 5S RNA transcription (Fig. 4.4C). This calculation is limited to a single experiment in which these extracts were analyzed. In the two later stationary phase cell extracts (36.7 and 46.2 hr), the ability to

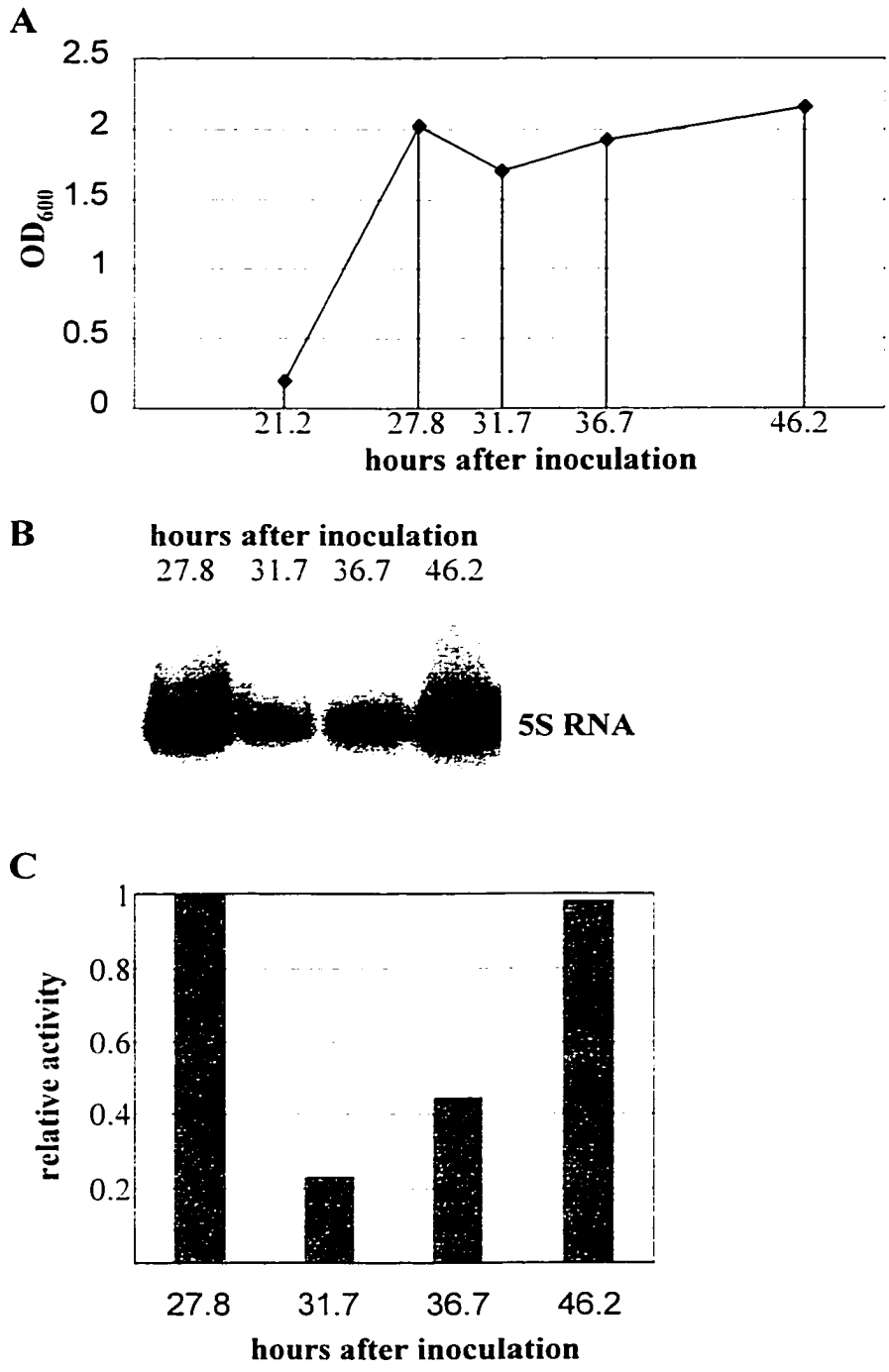


Fig. 4.4. Extracts prepared from cells in early stationary phase are transiently suppressed in their ability to support 5S RNA synthesis. **A**, cells were allowed to progress into stationary phase, as show by the growth curve, and were harvested at the four latter time points, which indicate the times after the 900 mL cultures were started. **B**, whole-cell extracts (88 μg), prepared from cells at each time point, were tested for the ability to support 5S RNA synthesis in a *in vitro* transcription reaction. **C**, the bar graph shows the quantification of the *in vitro* transcription results.

support 5S RNA transcription was partially restored, indicating the largest decrease in 5S RNA transcription occurred transiently in the stationary phase cell extracts (Fig. 4.4B). A transient decrease in transcription was obtained using *S. cerevisiae* YS18 cells and using the protease deficient yeast strain, BJ926.

A transient decrease in *in vitro* tRNA transcription was also found to occur during stationary phase. *S. cerevisiae* BJ926 cells were grown to a density of 3.5 X 10^7 cells/mL (log phase) in YPD medium, and 20 μL aliquots of this culture were transferred to four flasks, each containing 1L of YPD medium, and cultures were then grown overnight. Cells were harvest and the culture densities determine at 18.2, 20.2 and 36 hours after starting the 1L cultures (Fig. 4.5A). The culture density was measured at 11 hr, but the quantity of cells at this time was too low for preparing an extract. The culture density did not change significantly between 18.2 and 20.2 hr, indicating that cells had entered stationary phase by 18.2 hr. Extracts were prepared from cells harvested at the 18.2, 20.2, and 36 hr time points, and these extracts were tested for their ability to support 5S and tRNA transcription.

At each of the three time points, different quantities of extract were tested in the *in vitro* transcription reactions to determine the amount of each extract that supported the highest level of Pol III-mediated transcription. From initial experiments in which the *in vitro* Pol III-mediated transcription system was optimized, *S. cerevisiae* whole-cell extracts were found to contain intrinsic inhibitors of transcription. In addition, standardizing transcription reactions according to the quantity of extract protein added to the reactions may produce inaccurate comparisons of Pol III-mediated transcription supported by different extracts (Laurie Stargell, personal communication). Pol III-mediated transcription was maximal when approximately 80 μg of protein from the control, active extract or the reactivated stationary phase cell extract (36 hr) was used. Transcription did not change significantly when different amounts of the inactive extracts were used in

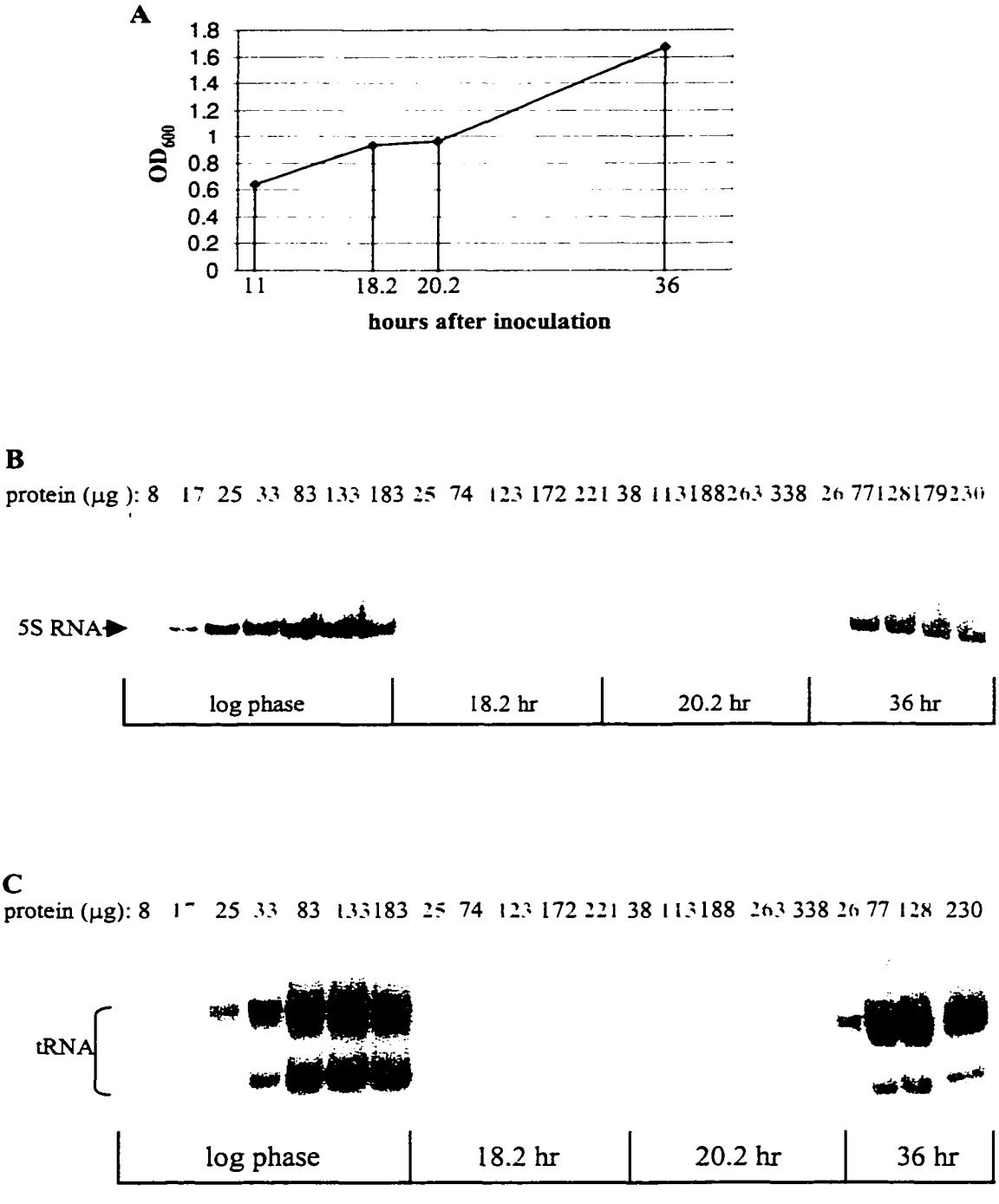


Fig. 4.5. tRNA synthesis is transiently reduced in addition to 5S RNA synthesis in stationary phase cell extracts. A, the growth curve shows the culture densities at four times after the 1L cultures of BJ926 cells were started. Cells were harvested at the latter three time points. **B**, the indicated quantities of whole-cell extracts from the indicated time points were tested for the ability to support 5S RNA transcription. **C**, extracts were tested for the ability to support tRNA transcription. The indicated amounts of extract are show in alternating blue and black font for clarity.

transcription reactions. Because of the diffusion of radiolabeled bands corresponding to 5S and tRNA transcripts from the inactive extracts, the amount of these extracts that produced the highest level of transcription could not be determined. However, greater than 80 μg of each inactive extract (18.2 and 20.2 hr) was required to produce optimal Pol III-mediated transcription.

The levels of 5S RNA transcription were low in the 18.2 and 20.2 hr extracts compared to a log phase extract, while in the 36 hr extract, 5S RNA transcription was found to increase (Fig. 4.5B). Likewise, tRNA transcription was reduced in the 18.2 and 20.2 hr extracts, but increased in the 36 hr extract (Fig. 4.5C). The similar transient reduction of 5S and tRNA transcription in early stationary phase cell extracts, suggested that a general component of the Pol III-mediated transcription machinery (TFIIIB, TFIIIC, or polymerase) was affected.

The S1 nuclease assay results could not be compared directly with the *in vitro* transcription results, because these experiments were performed separately using different cell cultures. However, speculative comparisons between the results of each experiment were made based on the relative changes in the cell growth curve shown for each experiment and based on the times cultures were incubated prior to harvesting the cells. *In vitro* transcription by Pol III was reactivate in stationary phase approximately 10-15 hr after cells had entered stationary phase (Fig. 4.4A, compare 21.2 or 27.8 hr to 36.7 hr). In contrast, nascent tRNA transcripts were not detected in stationary phase cells at any time point for a duration of at least 24 hr in stationary phase. Therefore, tRNA synthesis was not reactivated within stationary phase cells over the duration in which Pol III-mediated transcription was reactivated in stationary phase cell extracts. Although the transient decrease in Pol III-mediated transcription was only observed in vitro, this result was consistently observed, suggesting it was related to a actual mechanism of transcriptional down regulation occurring in the cells.

4.4.3 The reduction in Pol III-mediated transcription *in vitro* is due to an inhibitor

Although the transient decrease in Pol III-mediated transcription from whole-cell extracts was not observed within cells (S1 nuclease assay results), it was consistently observed *in vitro*, utilizing different sets of stationary phase cell extracts. Therefore, this transient loss of Pol III-mediated transcription is possibly related to an actual mechanism used to down-regulate transcription within cells. A transient change in transcription could be caused by a posttranslational modification in one or more components of the transcription machinery or the effect of an inhibitor. Along these lines, TFIIIB, TFIIIC, and Pol III each undergo phosphorylation within *S. cerevisiae* (Chedin *et al.*, 1998; Ghavidel and Schultz, 1997), although the effects of these modifications are not completely clear. An inhibitor has also been identified that specifically inactivates 5S RNA transcription (Clarke *et al.*, 1996), though this has not been corroborated by other reports (Sethy *et al.*, 1995; Zaragoza *et al.*, 1998). The 31.7 hr extract (Fig. 4.4) was tested for the presence of an inhibitor of Pol III-mediated transcription in extract mixing experiments (Fig. 4.6). Increasing amounts of either active or inactive extract alone or combined was added to transcription reactions (Fig. 4.6A). A linear increase in the amount of active extract per reaction resulted in an approximately linear increase in 5S RNA synthesis (lanes 1-3). Using the same quantities of inactive extract 5S RNA transcription appeared to plateau (lanes 4-6). Interestingly, upon mixing 20 μg of inactive extract with 40 μg of active extract, 5S RNA synthesis increased almost to the level obtained using 60 μg of active extract alone (Fig. 4.6B). Because this mixing experiment was performed only once this result may be erroneous, but if it is accurate, it implies that the limiting factor required for Pol III-mediated transcription (namely TFIIIB) is active in each extract. Therefore, the inactive extract may contain an inhibitor of transcription that is functionally suppressed by components in the active extract, or

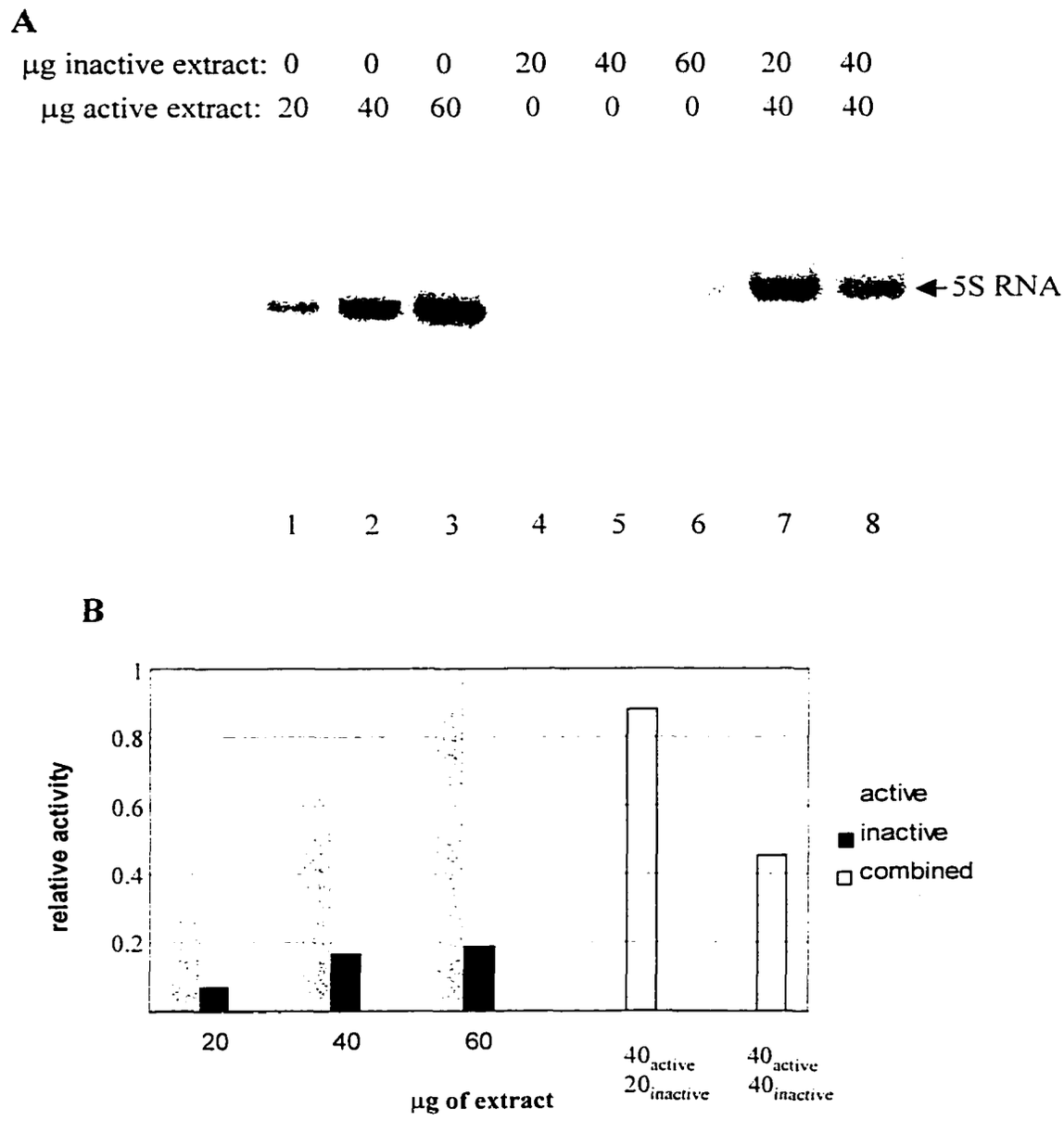


Fig. 4.6. The reduction in Pol III-mediated transcription in stationary phase extracts is due to an inhibitor. **A.** indicated amounts of active extract and inactive (Fig. 3.4, 31.7 hr) extract were tested alone or combination for the ability to support 5S RNA synthesis in an *in vitro* transcription assay. **B.** the bar graph shows the quantification of the transcription results. Lightly shaded bars show the relative levels of 5S RNA synthesis from the indicated quantities of the active extract. The black bars show the relative levels of 5S RNA synthesis from the indicated quantities of the inactive extract. The darkly shaded bars show the relative levels of 5S RNA synthesis from the indicated quantities of both extracts combined in one reaction.

that is sequestered by an excess of its target when 40 μg of active extract is combined with 20 μg of inactive extract. When 40 μg of both extracts were combined (lane 8), the level of 5S RNA synthesis was lower than that produced by 40 μg of active extract alone, further suggesting that a transcriptional inhibitor was present in the inactive extract.

4.4.4 The inhibitor of Pol III-mediated transcription is separated from the Pol III transcription machinery by Q-column chromatography.

In order to separate the inhibitor from the Pol III transcription machinery, the inactive stationary phase cell extract was applied to a Q-Sepharose column (Pharmacia). This column was used previously to resolve an inhibitor of 5S RNA synthesis in extracts from histidine-starved cells (Clarke *et al.*, 1996). TFIIIA was reported to elute from the column at 140 mM KCl, TFIIIB and TFIIIC at 250 mM KCl, Pol III between 300 and 500 mM KCl, and the inhibitor at 550 mM KCl (Fig. 4.7A). Q-column fractions eluting between 210 mM KCl and 330 mM KCl were able to stimulate 5S RNA transcription when combined with limiting amounts of active extract (Fig. 4.7B). The greatest stimulation occurred using fraction 17, corresponding to approximately 310 mM KCl, suggesting that Pol III effected the most significant increase in 5S RNA synthesis. In contrast, fraction 11, which should contain TFIIIA, did not augment 5S RNA synthesis, corroborating previous results in which TFIIIA was found not to be limiting in yeast whole-cell extracts (Braun *et al.*, 1989). TFIIIB and TFIIIC were probably predominantly found in fractions 13 and 15, which also stimulated transcription. These factors also were possibly present in fraction 17, considering that a relatively sharp linear salt gradient was applied to the column. So far, we have not tested each fraction with respect to the components required for Pol III-mediated transcription.

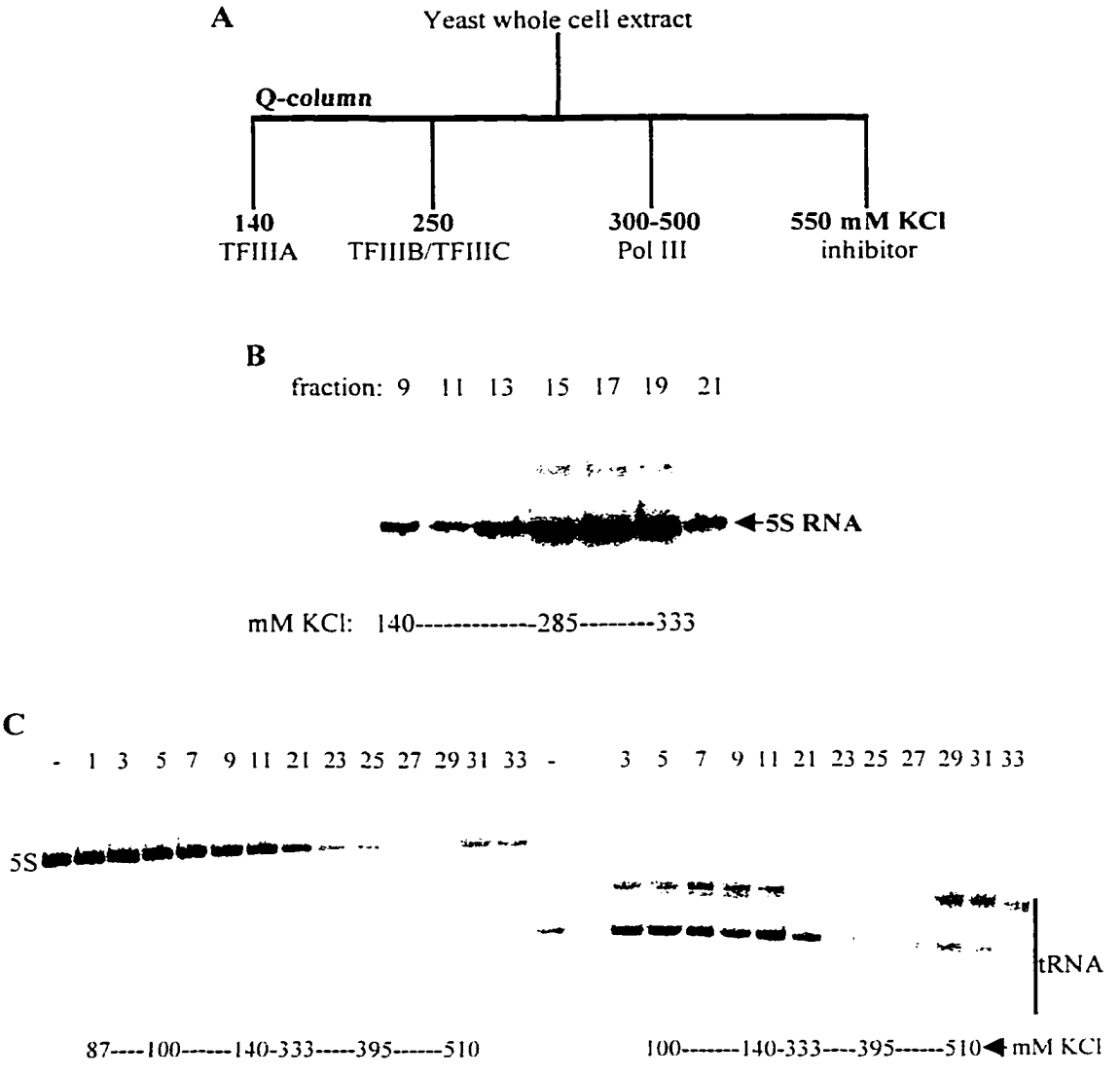


Fig. 4.7. Q-column separation of the inhibitor from Pol III and its transcription factors. The inactive extract (Fig. 3.4, 31.7 hr) was applied to a Q-column, which was then developed with a linear KCl gradient. **A**, the diagram shows the KCl concentrations at which Pol III and each of its factors elutes. Also shown is the point of elution of a previously identified inhibitor of 5S RNA synthesis (Clarke *et al.*, 1996). **B**, Q-column fractions (7 μL) that possibly contained Pol III factors and/or polymerase were tested for their ability to augment 5S RNA transcription when combined with 20 μg of an active whole-cell extract. The concentration of KCl in every fifth fraction is indicated below the gel. **C**, the indicated Q-column fractions (7 μL) were combined with 50 μg of active extract in *in vitro* transcription reaction to test if any of these fractions suppressed 5S RNA synthesis (left half of the gel) or tRNA synthesis (right half of the gel). For clarity, fractions that increased transcription were omitted from the experiment.

The peak of inhibitor activity eluting from the Q-column was found in fractions 23 and 25, corresponding to 363 mM KCl and 395 mM KCl, respectively (Fig. 4.7C). Both fractions repressed 5S RNA synthesis as well as tRNA synthesis. In contrast, the inhibitor identified by Clarke *et al.* (1996) eluted from the Q-column at 550 mM KCl and did not affect tRNA transcription, suggesting that the inhibitor identified in the histidine-starved cell extracts is different from the inhibitor identified in the stationary phase cell extracts.

4.4.5 The inhibitor does not affect Pol I transcription.

Yeast whole-cell extracts intrinsically contain general inhibitors of transcription. To eliminate the possibility that the inhibitor of transcription in the stationary phase cell extract was nonspecific, the Q-column-purified inhibitor was tested for the ability to reduce *in vitro* Pol I-mediated transcription. Yeast whole-cell extracts lose the ability to support Pol I-mediated transcription in mid log phase, corresponding to a stage at which Pol III-mediated transcription is still active (Clarke *et al.*, 1996). Therefore, the events controlling Pol I and Pol III-mediated transcription are probably uncoupled, suggesting that the inhibitor will not affect Pol I-mediated transcription if it specifically targets the Pol III transcription machinery. To obtain a protein fraction that supported Pol I-mediated transcription, yeast whole cell extracts were fractionated through a DEAE column, and the active Pol I transcription machinery was eluted in a 350 mM KCl fraction as described. This fraction supported active Pol I-mediated transcription, and the addition of Q-column fraction 25, which contained the inhibitor (cf Fig. 4.7C), to DEAE 350 mM KCl fraction did not reduce transcription (Fig. 4.8A). However, the quantity of the DEAE 350 mM KCl fraction added to the reactions supported a high level of Pol I-mediated transcription, which could have eclipsed any effects by the inhibitor. Using a smaller quantity of the DEAE fraction in combination with more of the inhibitor (Q-column

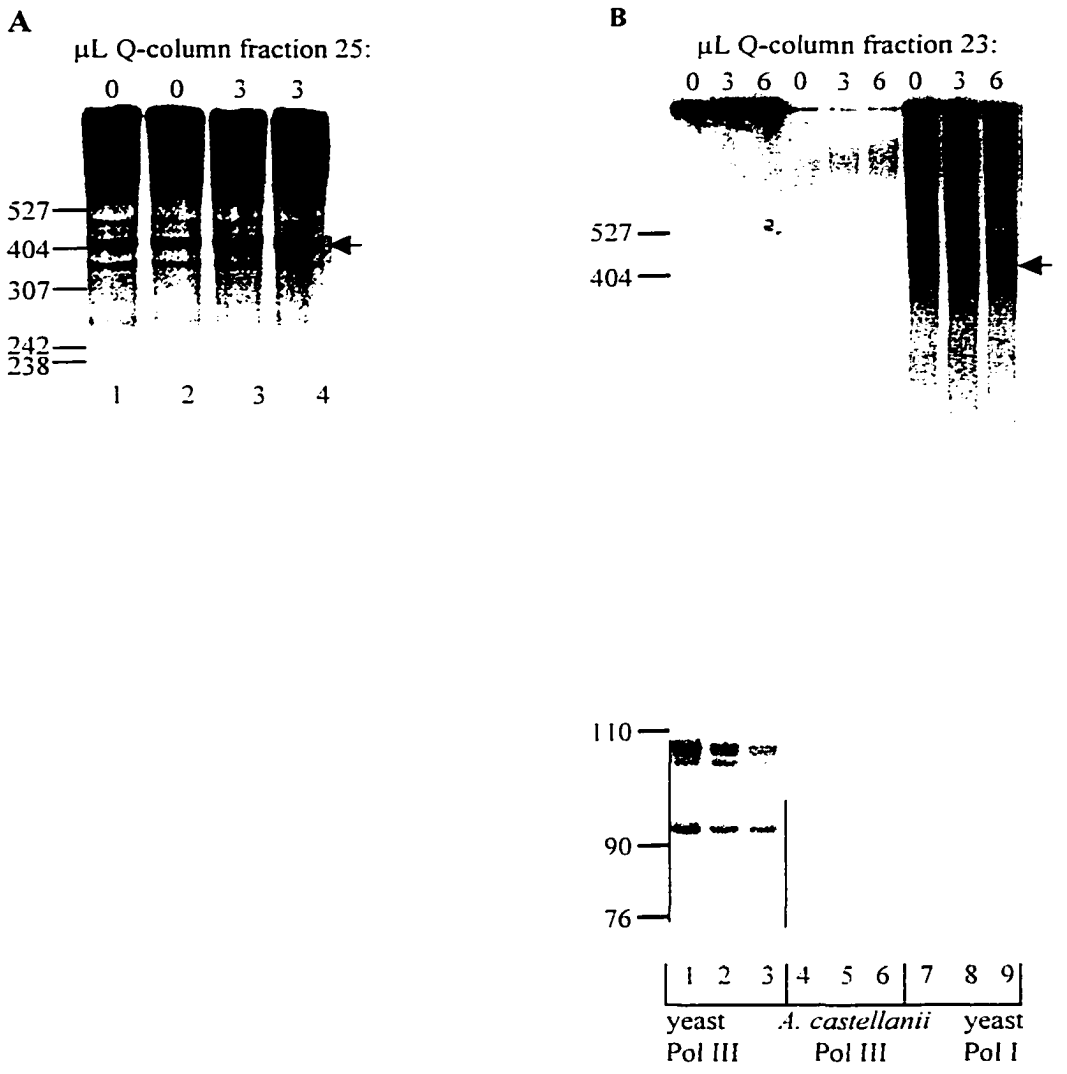


Fig. 4.8. The inhibitor separated by the Q-column does not affect Pol I transcription. A yeast whole-cell extract was applied to a DEAE column, and a 350 mM KCl step elution yielded a protein fraction that supported Pol I transcription. **A.** this DEAE fraction (7.5 μL) was assayed alone or in combination with the inhibitor (as indicated) to test if the inhibitor suppressed Pol I transcription. Positions of labeled DNA markers are denoted at the left. The 416 nt run-off transcript synthesized by Pol I is marked by the arrow. **B.** increasing amounts of the inhibitor (as indicated) were combined with either 88 μg of an active yeast whole-cell extract that supported Pol III-mediated transcription, with 21 μg of an *A. castellanii* nuclear extract that supported Pol III-mediated transcription, or with 3.3 μL of the DEAE fraction that supported Pol I-mediated transcription. tRNA synthesis was tested using the extracts that supported Pol III-mediated transcription. The vertical bars indicate the positions of the tRNA transcripts, and the transcript synthesized by Pol I is shown with the arrow.

fraction 23) still did not reduce Pol I-mediated transcription (Fig. 4.8B, lanes 7-9), suggesting that the inhibitor affecting Pol III-mediated transcription did not affect Pol I-mediated transcription.

The inhibitor was also tested with *Acanthamoeba castellanii* nuclear extract to determine if the inhibitor could also decrease Pol III-mediated transcription in this system. A linear decrease in tRNA synthesis was indeed evoked by a linear increase in the amount of Q-column fraction 23 added to the nuclear extract (Fig. 4.8B, lanes 4-6). Therefore, the *S. cerevisiae* and *A. castellanii* Pol III systems respond similarly to the inhibitor, indicating that the inhibitor targets an evolutionarily conserved component of the Pol III-mediated transcription machinery.

4.5 Discussion

Down regulation of Pol III-mediated transcription parallels the decrease in protein synthesis that takes place as yeast cells enter stationary phase. The loss of Pol III-mediated transcription relates to the role of Pol III in synthesizing 5S RNA and tRNA, which are directly involved in translation. From experiments in which whole-cell extracts were analyzed, TFIIIB, TFIIIC, and the polymerase were each found to be potential targets affecting Pol III-mediated transcriptional inactivation in *S. cerevisiae*. The addition of TFIIIC and the Brf subunit of TFIIIB were found to revitalize Pol III-mediated transcription in stationary phase cell extracts (Sethy *et al.*, 1995), but in extracts from rapamycin-treated cells, the addition of TFIIIB and, to a greater extent, Pol III revitalized transcription (Zaragoza *et al.*, 1998). Direct inhibition of protein synthesis with cycloheximide also reduces the activity of TFIIIB, specifically of B" and Brf (Dieci *et al.*, 1995). In each of these reports, TFIIIB has consistently been found to be targeted, which should globally repress all Pol III-mediated transcription, as TFIIIB is required on all class III genes in yeast. However, in inducing growth arrest by starving cell for histidine, a reduction in 5S RNA

synthesis and not tRNA synthesis was found (Clarke *et al.*, 1996). Interestingly, all of the Pol III-mediated transcription factors in an extract prepared from the starved cells were shown to be active, and the loss of 5S RNA synthesis was found to be caused by a inhibitor protein (Clarke *et al.*, 1996). All of these reports demonstrate that different effects on the Pol III-mediated transcription machinery in whole-cell extracts can occur by varying the conditions used to elicit growth arrest in *S. cerevisiae*. Experiments presented in this chapter were done to compare the reduction in Pol III-mediated transcription from whole-cell extracts (*in vitro*) with the reduction of Pol III-mediated transcription within cells (*in vivo*) during stationary phase. As opposed to the mutant cell strain used by Sethy *et al.* (1995), wild type *S. cerevisiae* cells were used in this study. A significant difference was found between the down regulation of Pol III-mediated transcription *in vivo* and changes in factor activity *in vitro*. This disparity could reflect multiple tiers of Pol III-mediated transcriptional regulation undertaken within the cell that are not fully reconstituted in a cell extract.

Extracts prepared from cells in early stationary phase were shown to have a reduced capacity to support 5S and tRNA synthesis, which was found previously (Sethy *et al.*, 1995). In the earlier report by Sethy *et al.* (1995), Pol III-mediated transcription in a wild type cell extract was decreased only approximately 3-fold from a log phase cell extract, and therefore, cells harboring a mutation in the τ131 subunit of TFIIIC were utilized. These cells exhibited increased Pol III-mediated transcription, but remained susceptible to regulation, and extracts prepared from the mutant cells in stationary phase were found to exhibit a 20-fold decrease in transcription. In an experiment presented in this chapter, an approximately 4.5-fold decrease in 5S RNA transcription was observed between extracts prepared at the first and second time points in stationary phase (Fig. 4.4), and in another experiment a similar decrease in tRNA transcription was observed (Fig. 4.5). These results

suggests that the decrease in Pol III-mediated transcription in stationary phase cell extracts from wild type cells is much greater than the 3-fold reduction reported previously considering that log phase cell extracts were not examined, and the 4.5-fold reduction in transcription occurred during early stationary phase.

Extracts from later time points in stationary phase recovered the ability to support 5S and tRNA transcription (Fig. 4.4 and 4.5), demonstrating that the time in stationary when cells are harvested is critical to obtaining a maximal reduction in Pol III-mediated transcription from whole-cell extracts. It is possible that Sethy *et al.* (1995) did not observe a significant decrease in *in vitro* transcription using a wild type cell extracts because the extracts had partially restored their ability to support Pol III-mediated transcription.

In contrast to the stationary phase cell extracts, a permanent reduction in the amount of nascent tRNA transcripts was found *in vivo*, as determined in the S1 nuclease assays. These results demonstrate that whole-cell extracts do not recapitulate the regulation of Pol III-mediated transcription within cells. The loss or alteration in components required for transcription or transcriptional regulation may influence results obtained using the cell extracts. During the extraction procedure, factors involved in regulating Pol III-mediated transcription may be lost because of their stable association with class III genes, which could result in their removal from the extract with the chromatin pellet. For example, TFIIIB exhibits a strong interaction with DNA, which has led to a disproportionate loss of this factor in extracts relative to the other Pol III factors (George Kassavetis, personal communication). Pol III-mediated transcription may also be affected nonspecifically by components of the extract that are not related to transcription within an intact cell. For instance, Pol I transcription is inactive in certain whole-cell extract preparations and only becomes reactivated following a DEAE chromatography step. These

examples illustrate that caution should be taken in drawing conclusions from the properties of a whole-cell extract.

Even with the differences between the *in vivo* and *in vitro* results, the decrease in Pol III-mediated transcriptional activity of stationary phase cell extracts may have still been a specific effect of regulation. This temporal loss of transcription was reproduced in separate experiments and in using two yeast strains: YS18 and BJ926. It should be noted that an earlier report has shown that intrinsic inhibitors of Pol III-mediated transcription are present in yeast whole-cell extracts (Taylor and Segall, 1985). However, the effects of these inhibitors in our active extracts were found to be minimal, which may relate to modifications in our procedure used to prepare extracts. Moreover, extracts prepared from log phase cells were never found to be inactive. Therefore, the reduced level of Pol III-mediated transcription from the early stationary phase extract was, in all likelihood, not an artifact from the preparation of the extract.

To determine the mechanism decreasing Pol III-mediated transcription in the inactive extract, we compared the levels of *in vitro* transcription in reactions containing different proportions of to inactive extract. Upon combining 40 μg of each extract in a reaction, a clear decrease in transcription was observed relative to the level of transcription from 40 μg of active extract alone. This indicated that an inhibitor was acting on Pol III-mediated transcription in the inactive extract, which disagreed with earlier reports. Using extracts prepared from the mutant cells described above, Sethy *et al.* (1995) did not observe a change in the level of transcription when 100 μg of active extract was supplemented with an equivalent amount of inactive extract. This disparity in the results may have arisen because the inhibitor is transiently activated during stationary phase, which has a temporary effect on Pol III-mediated transcription. As discussed above, Sethy *et al.*, (1995) possibly did not use stationary phase cell extracts that were maximally decreased

for Pol III-mediated transcriptional activity, in which case they would not detect the inhibitor. Alternatively, combining 100 μg of active and inactive extract in an *in vitro* transcription reaction (Sethy *et al.*, 1995) may have been an quantity of extract greater than that required to saturate transcription in the reaction, which could have caused the inhibitor to be squelched by an excess of its target. An extract mixing experiment has also been done with a rapamycin-treated cell extract and an untreated cell extract, using only 10 μg of each extract, and again, an inhibitor was not detected (Zaragoza *et al.*, 1998). Interestingly, combining the rapamycin-treated and untreated cell extracts appeared to elevate transcription above the level that would be expected if each extract had an additive effect in the transcription reaction. A similar result was observed in an experiment presented in Fig. 4.6 when 20 μg of inactive extract was added to 40 μg of active extract; the level of 5S RNA transcription was near that obtained using 60 μg of active extract alone. The more that additive increase in pol III-mediated transcription that has been observed could be explained by a component of the active extract that suppresses the effects of the inhibitor. Because inhibition of 5S RNA transcription was observed when 40 μg, but not 20 μg, of inactive extract were combined with the active extract suggests that an inhibitor in the extract binds to one of the components required for Pol III-mediated transcription. Specifically, by increasing the amount of the inhibitor in the reaction, the inhibitor and its target would be driven into an association with one another.

The inhibitor was separated from the Pol III-mediated transcription machinery by Q-column chromatography to show clearly the effects of the inhibitor on Pol III-mediated transcription. This step was used previously to resolve an inhibitor that differentially suppressed 5S RNA transcription in extracts prepared using cells that had been starved for histidine in order to arrested growth (Clarke *et al.*, 1996). The inhibitor that we have identified affects both 5S and tRNA synthesis, suggesting that it is not the same inhibitor that had been described. Regardless of the different

properties of the two inhibitors, Q-column chromatography was still used because it did not entail preliminary step elutions to remove nonspecific inhibitors that may be present in the whole-cell extract. Other chromatographic procedures used to purify the yeast Pol III-mediated transcription factors do utilized such steps (Taylor and Segall, 1985) and may have resulted in the loss of a potentially specific inhibitor present in the inactive stationary phase cell extract. The Q-column fractions that reportedly contained TFIIIB, TFIIIC, and Pol III (Clarke *et al.*, 1996) were able to augment transcription in a reaction containing a low amount of active extract, while fractions eluted from the column at higher salt concentrations inhibited Pol III-mediated transcription. The fractions that caused the maximum decrease in 5S RNA transcription had a similar affect on tRNA transcription, suggesting that the same inhibitor targets 5S and tRNA transcription.

The results from Q-column chromatography have only shown that an inhibitor was separated from the Pol III-mediated transcription machinery and that certain components required for Pol III-mediated transcription exhibit activity in the early stationary phase extract. Currently, the Q-column fractions from the inactive extract have not been compared to Q-column fractions from an active extract compare the relative activities of the general Pol III factors from each extract. Furthermore, experiments have not been done to definitively relate this inhibitory activity to the loss of Pol III-mediated transcription in the inactive extract. However, we have shown that the inhibitor does not suppress Pol I transcription, suggesting that it is specific for Pol III. As discussed above, an inhibitor of Pol III-mediated transcription was not previously identified in extracts prepared from stationary phase cells. Instead, a decrease in the already limiting amounts of Brf was found to reduce the level of Pol III-mediated transcription in extracts prepared from stationary phase cells (Sethy *et al.*, 1995). The limiting quantity of Brf observed in whole-cell extracts is partially related to the procedure used to prepare the extract. Due to its tight

association with DNA (Kassavetis *et al.*, 1989), any TFIIIB sequestered on Pol III-mediated genes is probably lost during the extraction procedure. In relation to this, a significant portion of B" can be recover by extracting the DNA pellet with a high concentration of urea (Kassavetis *et al.*, 1992). Therefore, Brf may not be as limiting within the cell as it is in the extract. In supporting this notion, overexpressing Brf within cells was not found to augment increase Pol III-mediated transcriptional activity *in vivo* (Sethy-Coraci *et al.*, 1998).

Because our results are preliminary, and do not point toward an effect on a specific component required for Pol III-mediated transcription, we can only speculate as to the target of the inhibitor. Based on the ability of the inhibitor to decrease Pol III-mediated transcription from *A. castellanii* nuclear extract, the inhibitor probably targets an evolutionarily conserved component of the Pol III-mediated transcription machinery. As discussed above, our inactive extract behaved similarly to the extract prepared from rapamycin-treated cells in that it augmented transcription from active extracts under certain conditions (Fig. 4.6). The addition of polymerase and, to a lesser extent, the addition of TFIIIB was found to restore Pol III-mediated transcription in the extract prepared from rapamycin-treated cells (Zaragoza *et al.*, 1998). Protein phosphatase 2A (PP2A) was implicated in down-regulating Pol III transcription by rapamycin because PP2A is a target in the rapamycin pathway and was previously shown to decrease tRNA synthesis *in vivo* and *in vitro* (van Zyl *et al.*, 1992). Van Zyl et al. (1992) showed that the addition of polymerase or TFIIIB restored Pol III-mediated transcription to the extracts containing deregulated PP2A (van Zyl *et al.*, 1992), which parallels the rapamycin results. PP2A was proposed to activate an inhibitor of Pol III-mediated transcription (van Zyl *et al.*, 1992). Perhaps the inhibitor that we have identified is the same as the proposed inhibitor activated by protein PP2A. If the inhibitor targets TFIIIB and/or the polymerase, as suggested by the properties of the rapamycin-treated cell extract and the extracts prepared

from cells containing upregulated PP2A, then the inhibitor could decrease Pol III-mediated transcription in *A. castellanii*, because Pol III and at least two subunits of TFIIIB exhibit evolutionary conservation.

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