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DISSERTATION

TIF-IE, A TRANSCRIPTION FACTOR NECESSARY FOR RIBOSOMAL RNA TEMPLATE COMMITMENT

Submitted by

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Graduate Program in Cell and Molecular Biology

In partial fulfillment of the requirements for

the degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Spring 2001

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March 20, 2001

WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY ANNA MARIA AL-KHOURI ENTITLED *TIF-IE, A TRANSCRIPTION FACTOR NECESSARY FOR RIBOSOMAL RNA TEMPLATE COMMITMENT* BE ACCEPTED AS FULFILLING IN PART THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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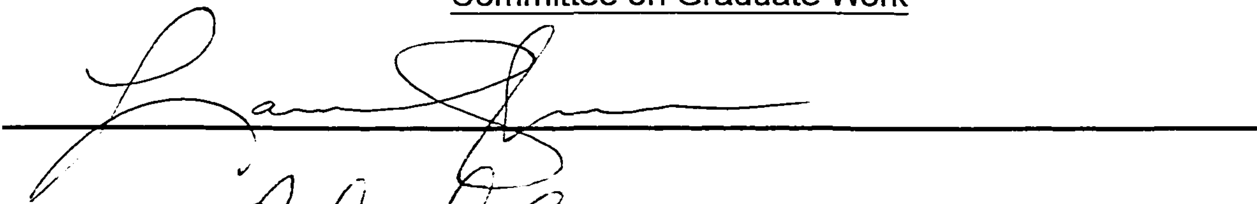

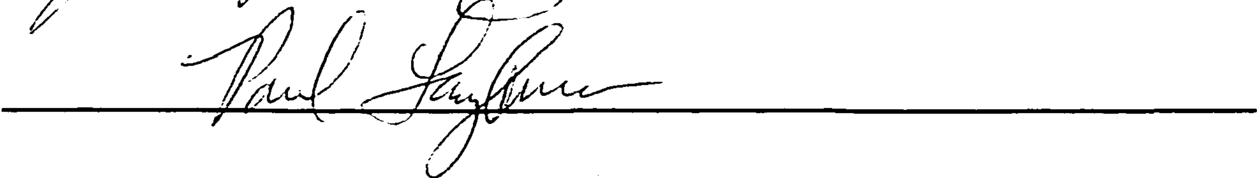

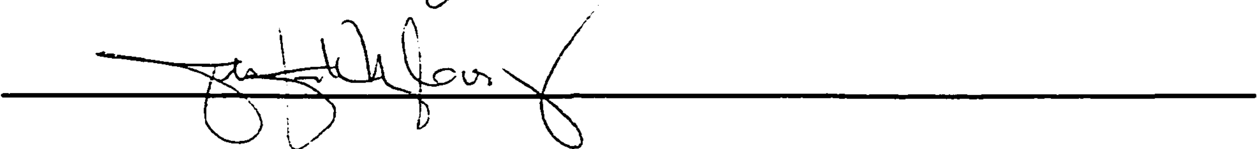

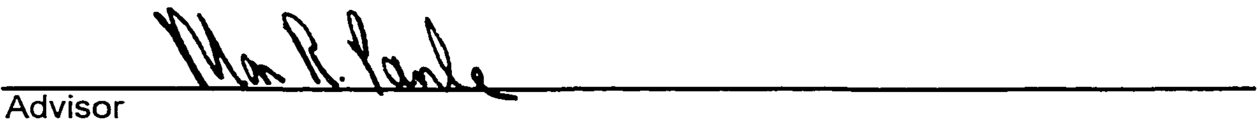

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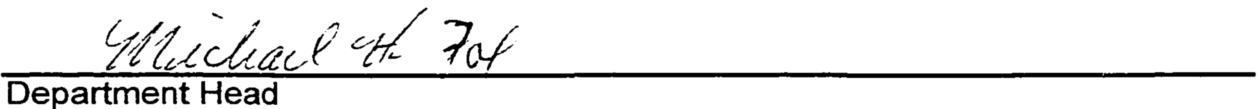

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ABSTRACT OF THE DISSERTATION

TIF-IE, A TRANSCRIPTION FACTOR NECESSARY FOR RIBOSOMAL RNA TEMPLATE COMMITMENT

In the small, free-living amoeba, *Acanthamoeba castellanii*, ribosomal RNA (rRNA) transcription requires in addition to RNA polymerase I (Pol I), a single DNA-binding factor, Transcription Initiation Factor-IB (TIF-IB). TIF-IB is a multimeric protein that contains TATA-binding protein (TBP) and four TBP-associated factors (TAF_Is) that are specific for Pol I transcription with relative molecular weights of 91, 96, 99 and 145. TIF-IB is required for accurate and promoter-specific initiation of rRNA transcription, recruiting and positioning the polymerase on the start site by protein-protein interaction. In *A. castellanii,* partially purified TIF-IB can form a persistent complex with the rDNA promoter while homogeneous TIF-IB cannot. Another factor, TIF-IE, is required along with homogeneous TIF-IB for the formation of a stable complex on the rDNA core promoter. TIF-IE by itself, however, does not bind to the rDNA promoter and thus differs in mechanism from the Upstream Binding Factor (UBF) and Upstream Activating Factor (UAF) that carry out similar complex stabilizing functions in vertebrates and yeast, respectively. In addition to its presence in impure TIF-IB, TIF-IE has been found in highly purified fractions of Pol I, with which it associates, and can be partially separated from the polymerase by rate zonal sedimentation. Renaturation of polypeptides excised from SDS-polyacrylamide

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gels showed that a 141 kDa polypeptide possesses all the known activities of TIF-IE. In other eukaryotic systems, no obviously similar factor to TIF-IE has been reported.

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CHAPTER 1

BACKGROUND AND SIGNIFICANCE

1.1 INTRODUCTION

In nuclei of eukaryotic cells, transcription is carried out by three distinct DNA-dependent RNA polymerases, RNA polymerases I, II, and III. Each is responsible for transcription of a different set of cellular genes. The focus of this chapter is RNA polymerase I (Pol I) transcription. Pol I transcribes the ribosomal RNA (rRNA) gene in the nucleolus to yield a primary transcript. This primary transcript is processed through a series of cleavages to yield the mature rRNA species 18S, 5.8S and 28S that assemble, together with ribosomal proteins and 5S RNA, into ribosomes.

All actively dividing cells synthesize vast amounts of rRNA to ensure that subsequent generations contain the ribosomes necessary to support protein synthesis. In such growing cells, rRNA transcription accounts for about 75% of the total RNA synthesis. This enormous quantity of rRNA can be produced because cells contain multiple copies of the rRNA gene, which are arranged in tandem head-to-tail arrays in most organisms. About half of these copies are transcribed at a very high rate. On average, each active rRNA gene is

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transcribed by approximately 100 Pol I molecules that are spaced about every 100 base pairs (bp) on each gene copy. In contrast, a gene transcribed by RNA polymerase II would have only one to two elongating polymerases, thus producing only 1-2% of the RNA per active gene (Grummt, 1999; Reeder, 1999; Paule, 1998a).

The rate of rRNA synthesis is regulated according to the physiological state of the cell; growth rate without cell division, differentiation into a protein-secreting cell, and proliferation rate all affect transcription activity. rRNA synthesis is down regulated when cells approach stationary phase, are starved of an essential nutrient so they attenuate growth, are exposed to protein synthesis inhibitors, or cease protein secretion. Conversely, rDNA transcription is up regulated on reversal of such conditions. Thus, by responding to changes in the cellular environment, transcription by Pol I ultimately determines ribosome production and the potential for cellular proliferation.

The rRNA transcription rate is regulated at the level of transcription initiation (Reeder, 1999; Grummt, 1999). Therefore, to understand regulation, the components, reactions, and mechanism involved in the initiation process need to be known in detail.

1.2 THE RIBOSOMAL RNA GENE PROMOTER STRUCTURE

The multiple copies of the rRNA gene are separated from one another by a region, which varies greatly in length in different organisms, known as the intergenic spacer (IGS). Within the IGS of various species, a variety of elements

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are located that are involved in regulation of transcription (Fig. 1.1). Despite the lack of sequence conservation, the overall structural organization of these rDNA promoter elements in eukaryotes, extending from human to yeast, is comparable. Starting from the initiation site and moving upstream, these elements (Fig. 1.1) are the following: the core promoter element, the upstream promoter element, the proximal terminator, enhancers, spacer promoters, and transcription terminators.

The core promoter element (CPE) overlaps the site of transcription initiation (+1) by 4-6 bp and extends ~ 50 bp upstream of that site. In most species, this element is necessary and sufficient for initiation of basal rRNA transcription *in vitro*. This suggests that the CPE is the primary agent in recruiting Pol I to the promoter and directing it to the correct initiation site (Paule and White, 2000; Reeder, 1999; Grummt, 1999; Paule, 1998b).

Pol I transcription is noted for being species specific, even between closely related species, and this correlates with the fact that the nucleotide sequence of Pol I promoters have diverged significantly. This species specificity resides solely within the CPE (Grummt, 1999). The CPE also contains the only conserved rRNA promoter sequence element, the ribosomal initiator (rInr). The rInr is an AT-rich, TATA-like sequence surrounding +1. rInr is both the site of DNA strand separation during transcription initiation and weak interaction with a component of the general rRNA transcription factor. Though its sequence resembles the TATA box, it is not an interaction site for TATA-binding protein (TBP) (Paule, 1998b; Radebaugh *et al.*, 1997; Perna *et al.*, 1992).

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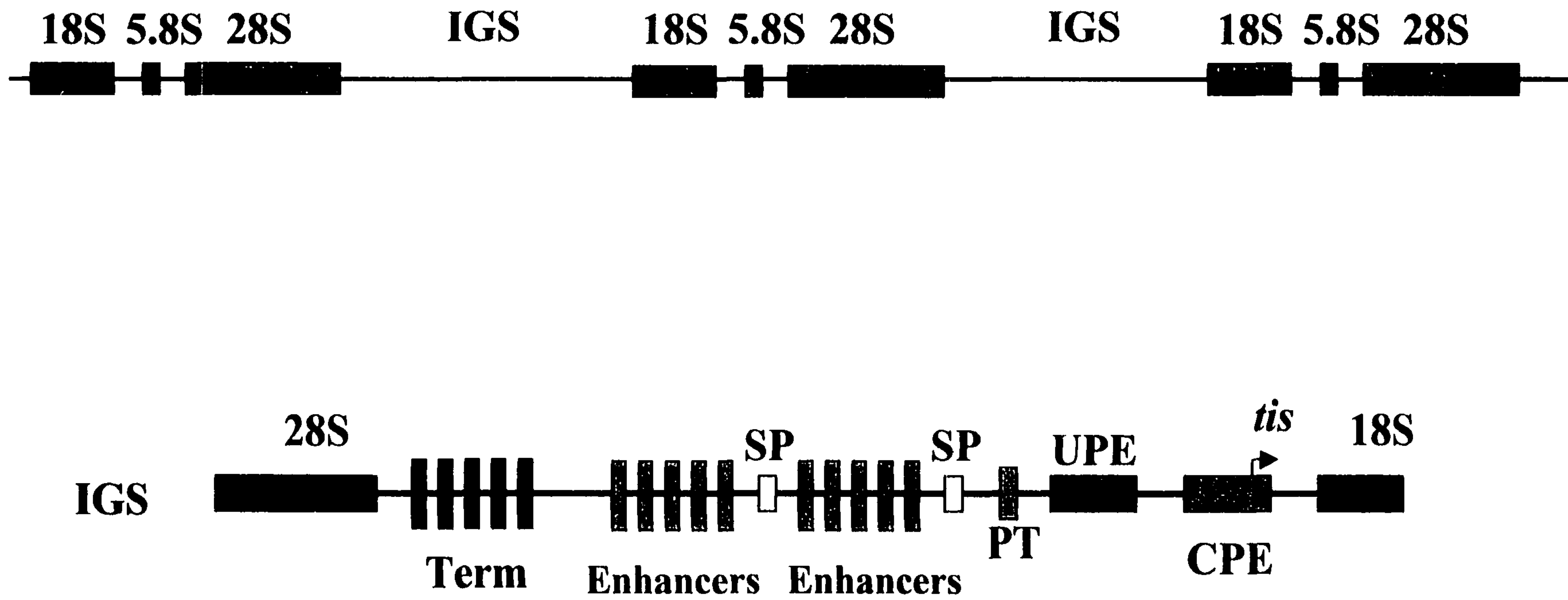

Fig. 1.1. The ribosomal RNA gene is a multi-copy gene and the gene copies are arranged in a Head-to-Tail manner. Each gene is separated from the next by the IGS. A variety of regulatory elements are located within the IGS.

The upstream promoter element (UPE) extends 150-200 bp upstream of the initiation site (+1). It stimulates transcription driven by the CPE, but in most instances is not absolutely required for transcription initiation. Upstream sequences enhance transcription by binding an assembly factor that mediates efficient recruitment of the core factor to the promoter and increases the stability of initiation complexes. Interestingly, in both human and mouse rDNA, the UPE shares a region of significant sequence homology with the CPE. This sequence homology, together with the finding that the correct spacing and helical alignment between the UPE and CPE are crucial for promoter function, suggest that the UPE-binding factor and the core factor interact with both the core and the UPE (Paule and White, 2000; Grummt, 1999; Reeder, 1999; Zomerdijk and Tjian, 1998). The only well studied species in which a UPE has not been identified yet is *A. castellanii*.

Upstream of the UPE, there is a proximal transcription terminator sequence referred to as the proximal terminator (PT). The PT mediates transcription termination by binding a specific transcription termination factor. It also stimulates transcription initiation by several mechanisms. It protects the promoter from wandering polymerases reading through from the upstream rRNA transcription unit or from spacer promoters. Wandering polymerases are capable of displacing trans-acting factors bound to the downstream promoter leading to promoter inactivation (Henderson *et al.*, 1989; Bateman and Paule, 1988). The PT also plays a role in remodelling the repressive chromatin structure over the promoter (Langst *et al.*, 1997). Finally, the transcription termination factor that

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binds to the PT also binds to the terminator sequence at the end of a transcription unit. This factor can oligomerize (Sander and Grummt, 1997), thus bringing together the end of one transcription unit to the beginning of the successive unit. This may allow the faster progression of Pol I from the rRNA gene to the promoter of the next gene.

Ribosomal enhancers are positioned upstream of the PT. These sequence elements enhance transcription from the promoter. They can function in a distance independent manner and in either orientation. The enhancer element is often found in multiple adjacent repeats and is bound by a single transcription factor.

Also within the IGS there is a functional secondary Pol I promoter that is referred to as spacer promoter (SP). This promoter allows the synthesis of relatively short transcripts extending down to the proximal terminator. The SP is only active during limited times during development, and can stimulate transcription from the gene promoter by unknown mechanisms.

Upstream of the SP are the transcription terminators. This repeated sequence element is bound by a transcription termination factor that leads to the end of Pol I progression, production of a 3' end to the transcript and the release of the RNA product and Pol I (Grummt, 1999; Reeder, 1999; Paule, 1998b).

1.3 MECHANISM OF RIBOSOMAL RNA TRANSCRIPTION INITIATION

Pol I is unable to recognize and initiate transcription from the rRNA promoter without the aid of additional proteins, the general transcription factors.

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Initially, a stable complex between transcription factor(s) and the core promoter is assembled. This complex is referred to as the committed complex because it is resistant to challenge by a second template. The committed complex persists through multiple rounds of transcription (Paule and White, 2000). Pol I, possibly associated with another factor, is then recruited to the promoter by protein-protein interaction with the committed complex (Miller *et al.*, 2001; Peyroche *et al.*, 2000; Yamamoto *et al.*, 1996; Schnapp *et al.*, 1993; Kownin *et al.*, 1987). A series of reactions then follow that result in melting of the double-stranded template (Kahl *et al.*, 2000), binding of the first two nucleoside triphosphates, and formation of the first phosphodiester bond. Pol I starts transcribing and clears the promoter (Paule, 1998c).

1.4 RIBOSOMAL RNA TRANSCRIPTION INITIATION FACTORS

Fractionation of cell extracts and testing protein fractions in *in vitro* transcription assays or isolation of mutants specifically defective in rDNA transcription, allowed the identification of the following factors (Table 1) that are minimally required to support rRNA transcription initiation from ribosomal RNA promoter containing templates.

1.4.1 Transcription Initiation Factor-IB (TIF-IB)

A unique aspect of Pol I transcription is the species-specific nature of rRNA promoter recognition (Bell *et al.*, 1989; Grummt *et al.*, 1982; Mishima *et al.*,

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Transcription factor	Organism(s)	Main functions and characteristics
TIF-IB[1]	*Acanthamoeba castellanii*, mouse, human, rat, yeast (*Saccharomyces cerevisiae*), frog (*Xenopus laevis*).	Species-specific, binds to the rRNA core promoter and then recruits and correctly positions Pol I at the transcription start site
TIF-IE	*Acanthamoeba castellanii*	Commits the rDNA template along with TIF-IB, tightly associated with Pol I
UBF	Vertebrates (mouse, human, rat, frog)	Binds to the UPE and recruits TIF-IB to the core promoter, UBF phosphorylation regulates rRNA transcription
UAF	Yeast (*Saccharomyces cerevisiae*),	Binds to the UPE and commits the rDNA promoter on its own, stimulates transcription driven by the CPE
Rrn3p	Yeast (*Saccharomyces cerevisiae*)	Mediates growth-dependent regulation of Pol I transcription, tightly associated with Pol I and involved in Pol I recruitment to the promoter
TIF-IA	Mouse	Mammalian homologue of yeast Rrn3p, tightly associated with Pol I
TIF-IC	Mouse	Tightly associated with Pol I, plays a role in elongation

Table 1. Summary of Pol I transcription initiation factors in different organisms and their characteristics.

[1] The nomenclature of this factor varies in different organisms.

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1982; Learned and Tjian, 1982). This species specificity resides within a factor that is essential for the accurate and efficient initiation of transcription *in vitro* (Clos *et al.*, 1986; Learned *et al.*, 1985; Miesfeld and Arnheim, 1984; Mishima *et al.*, 1982). This fundamental transcription initiation factor is variously referred to as Transcription Initiation Factor-IB (TIF-IB) in mouse and *Acanthamoeba castellanii*, SL1 in human and rat, Rib1 in *Xenopus laevis*, Factor D or TFID in mouse, and Core Factor (CF) in the yeast *Saccharomyces cerevisiae* (Table 2). TIF-IB binds to the core domain of the promoter and its ability to form a stable committed complex on the rDNA core promoter in the absence of additional factors varies considerably from species to species as will be discussed later. TIF-IB is responsible for correctly positioning Pol I at the start site. Therefore, it confers promoter selectivity to Pol I.

In all species, TIF-IB is purified as a multisubunit protein complex. It is composed of the TATA-binding protein (TBP) and three or four additional subunits known as Pol I-specific TBP-associated factors (TAF_Is) (Paule and White, 2000). The presence of TBP, which is a component of the basal transcription machinery used by all three nuclear RNA polymerases (Hernandez, 1993), puts TIF-IB into the same class as TFIID and TFIIIB in the Pol II and Pol III systems, respectively (Paule and White, 2000). There are conflicting reports whether TBP is an integral component of yeast CF. While Keys *et al.* (1994) showed that TBP is not a CF subunit, Lin *et al.* (1996) showed that it was associated with purified CF. Steffan *et al.* (1996) reported that TBP is not

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Species	Nomenclature
Acanthamoeba castellanii	TIF-IB (Radebaugh *et al.*, 1994)
Mouse	TIF-IB (Clos *et al.*, 1986)
	Factor D (Tower *et al.*, 1986)
	TFID (Kato *et al.*, 1986)
Human	hSL1 (Learned *et al.*, 1985)
Rat	rSL1 (Smith *et al.*, 1990)
Frog (*Xenopus laevis*)	Rib1 (McStay *et al.*, 1991a)
Yeast (*Saccharomyces cerevisiae*)	CF[2] (Lin *et al.*, 1996)

Table 2. Nomenclature of the core promoter-binding factor in different species.

[2] Despite the homology in function between the yeast CF and the mammalian TIF-IB, there is no obvious amino acid sequence conservation between the Rrn proteins of yeast and the TAFs of mammals.

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required for *in vitro* basal transcription. It is only required to mediate the stimulatory function of the upstream element of the yeast rDNA promoter.

To understand the molecular basis for species-specific promoter recognition of TIF-IB, the individual subunits of human SL1 and murine TIF-IB were isolated and functionally characterized. Human TAF_Is have apparent molecular masses of 48, 63 and 110 kDa (Comai *et al.*, 1994; Zomerdijk *et al.*, 1994; Comai *et al.*, 1992) while murine TAF_Is (mTAF_Is) have apparent molecular masses of 48, 68 and 95 kDa. The mTAF_Is show 77% to 89% amino acid sequence homology to their human counterparts. Despite differences in the primary structure and molecular masses, each mouse or human TAF_I is able to bind to TBP indicating that the interactions between TBP and TAF_Is are conserved (Heix *et al.*, 1997). These findings are in agreement with previous results showing that differences in the N-termini of human and mouse TBP do not contribute to the promoter selectivity of TIF-IB/SL1 (Rudloff *et al.*, 1994). It was also shown that each TAF_I can interact with every other TAF_I subunit in mTIF-IB or hSL1 indicating that the domains mediating the complex network of TAF-TAF interactions are also conserved between human and mouse (Heix *et al.*, 1997). These conserved protein-protein interactions most probably do not play a role in the promoter selectivity of TIF-IB. However, the reconstituted chimeric TIF-IB/SL1 could not support rRNA transcription *in vitro.* Therefore, species specificity is due to a cumulative effect of differences between individual subunits of TIF-IB/SL1 and/or due to differences in the TAF-DNA interactions. Rudloff *et al.* (1994) reported that the human TAF_I48 and TAF_I68 can bind to the

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mouse rDNA promoter and concluded that the largest TAF_I (110) might be responsible for the species-specificity in mouse-human systems.

1.4.2 Vertebrate Upstream Binding Factor (UBF)

UBF was originally isolated as a human protein that binds to the UPE and recruits SL1 to the template (Jantzen *et al.*, 1992; 1990; Bell *et al.*, 1988; Learned *et al.*, 1986). It has been identified in other vertebrates such as mouse (Bell *et al.*, 1990), frog (McStay *et al.*, 1997; Pikaard *et al.*, 1989; Dunaway, 1989), and rat (Smith *et al.*, 1990). In mouse, UBF may also have a role in Pol I recruitment by contacting the polymerase-associated factor PAF53, a murine homologue of the yeast A49 subunit (Muramatsu *et al.*, 1998; Hanada *et al.*, 1996). Efforts to identify UBF homologues in lower eukaryotes including *Acanthamoeba castellanii* and *Saccharomyces cerevisiae* have so far been unsuccessful. In the latter, there is no recognizable UBF gene in its sequenced genome (Reeder, 1999).

UBF purifies as a dimer of 80-100 kDa subunits (Pikaard *et al.*, 1990a,b; Smith *et al.*, 1990; Bell *et al.*, 1990; 1988; Pikaard *et al.*, 1989). It has been cloned from human (Jantzen *et al.*, 1990), rat (O'Mahony and Rothblum, 1991), mouse (Hisatake *et al.*, 1991; Voit *et al.*, 1992), and *X. laevis* (Bachvarov and Moss, 1991; Mcstay *et al.*, 1991a). It has an amino-terminal region that mediates dimerization (O'Mahony *et al.*, 1992; Jantzen *et al.*, 1992; Mcstay *et al.*, 1991), followed by four to six HMG boxes depending upon the species. The latter are DNA-binding domains with high homology to the nonhistone chromosomal high-

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mobility group proteins, HMG 1 and 2. The carboxyl-terminal tail that is required for transcriptional activation is rich in acidic serine and threonine amino acids that are subject to phosphorylation (Jantzen *et al.*, 1992; Voit *et al.*, 1992; Bachvarov and Moss, 1991; Mcstay *et al.*, 1991a; Jantzen *et al.*, 1990). There is strong evidence implicating a role for phosphorylation of UBF in the control of growth-induced increases in rRNA transcription (discussed later).

UBF activity in rDNA transcription probably depends on its ability to bend and wrap DNA, a function that is mediated primarily by the first three HMG boxes (Moss and Stefanovsky, 1995; Putnam *et al.*, 1994). It was proposed that a UBF dimer could wrap 135-160 bp of DNA into a left-handed loop, a structure referred to as the 'enhancesome' (Bazett-Jones *et al.*, 1994; Putnam *et al.*, 1994). The core and the UPE promoter elements could be brought together on the surface of two adjacent enhancesomes. This might lead to TIF-IB binding to the rRNA promoter by its simultaneous interaction with the core and UPE promoter elements (Moss *et al.*, 1998). The requirement for correct helical alignment of the core and UPE supports this notion (Xie and Rothblum, 1992; Windle and Sollner-Webb, 1986). UBF also binds the multicopy ribosomal enhancers, suggesting that a series of enhancesomes could compress the IGS DNA (Paule and White, 2000), bringing closer the end of one rDNA transcription unit and the promoter of the next.

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1.4.3 Yeast Upstream Activating Factor (UAF)

UAF is a yeast transcription factor complex. It consists of three Pol I-specific subunits, Rrn5p, Rrn9p, Rrn10p of apparent molecular masses of 58, 50, 17 kDa, and histones H3 and H4 with relative molecular weights of 18 and 15 kDa respectively, and an uncharacterized 30 kDa protein, p30 (Keener *et al.*, 1997; Keys *et al.*, 1996).

UAF binds specifically and in a stable way to the UPE of the yeast rDNA promoter and commits the rDNA promoter to transcription *in vitro* in the absence of CF. It is unable to direct transcription on its own, but greatly stimulates transcription directed by the core domain and CF, both *in vitro* and *in vivo.* It does so by stabilizing the binding of the entire PIC to the promoter through several protein-protein interactions (Fig. 1.2). The Rrn9p subunit of UAF interacts with TBP and Rrn7p subunit of CF (Keys *et al.*, 1996; Steffan *et al.*, 1996). The interaction of UAF with TBP is important for the activated transcription of rDNA by Pol I *in vivo* (Steffan *et al.*, 1998). TBP also interacts with all three CF subunits with the TBP-Rrn6 interaction being the strongest (Steffan *et al.*, 1996). However, the interaction of TBP with UAF is stronger than with CF. Therefore, in conjunction with TBP, UAF facilitates the recruitment of CF to the rDNA promoter to form a stable pre-initiation complex (Steffan *et al.*, 1996; Keys *et al.*, 1996).

Interestingly, in *S. cerevisiae* mutants defective in UAF, two alternative reversible states exist for rDNA transcription: one favoring transcription by Pol II and the other suitable for Pol I transcription. The presence of UAF in normal cells appears to stabilize the second state, thus achieving a stringent silencing of

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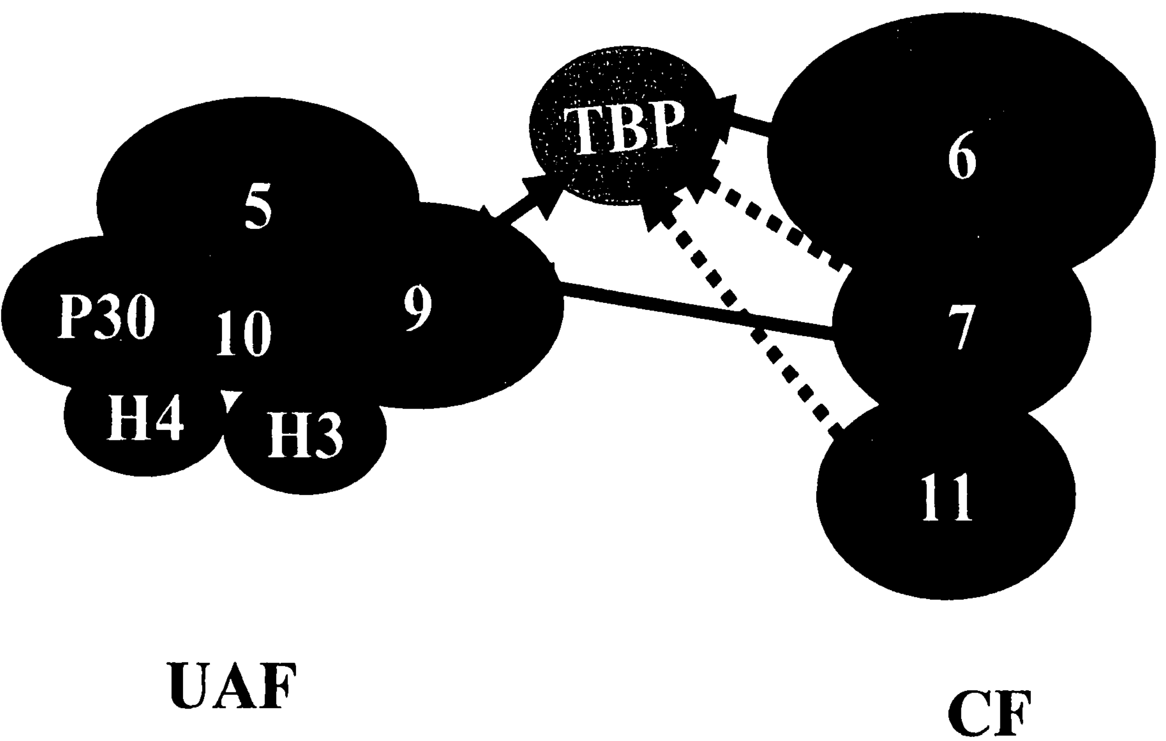

Fig.1.2. The interactions between UAF, TBP and CF.

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rDNA transcription by Pol II (Vu- *et al.*, 1999).

1.4.4 Yeast Rrn3p

Rrn3p is an essential Pol I transcription initiation factor in yeast. It is a monomer of ~ 73 kDa in molecular weight. It interacts with Pol I in the absence of DNA template, leading to an increase in the amount of a transcription-competent pre-initiation complex and as a result transcription is stimulated. However, Rrn3p is not part of the stable pre-initiation complex (Yamamoto *et al.*, 1996)

Rrn3p may be required for the recruitment of Pol I to the core promoter. Peyroche *et al.* (2000) reported that the A43 subunit of Pol I interacts with Rrn3p to form the initiation-competent Pol I-Rrn3p complex. A43 is the only Pol I-specific subunit that is essential for cell viability. They also showed that Rrn3p also interacts with the C-terminus of Rrn6, one of the three subunits of the CF, *in vivo* and *in vitro*. These results suggest that Rrn3p plays a central role in Pol I recruitment to rDNA promoters by bridging the enzyme to the core factor via the A43-Rrn3-Rrn6 interaction.

Rrn3p may be the regulated component of the rRNA transcriptional machinery. Two populations of Rrn3p were found in yeast extracts: a major form of free factor and a minor one associated with Pol I, which is the initiation-competent form of the enzyme *in vitro* (Milkereit *et al.*, 1997). This initiation-competent form (Pol I-Rrn3p) is lacking in transcriptionally inactive extracts from stationary phase yeast cells despite the substantial amounts of Rrn3p and Pol I. Interestingly, Rrn3p dissociates from Pol I during the transcription cycle and

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cannot subsequently reassociate with the enzyme *in vitro* (Milkereit and Tschochner, 1998). Therefore, formation and disruption of the Rrn3p-Pol I complex appears to constitute a molecular switch for regulating rDNA transcription. Bodem *et al.* (2000) reported that the mammalian homologue of yeast Rrn3p is TIF-IA, which is the regulated component in mouse. In addition, Moorefield *et al.* (2000) demonstrated that Rrn3p, the factor mediating growth-dependent regulation of eukaryotic Pol I transcription, is functionally conserved between yeast and mammals. Human TIF-IA can substitute for the yeast gene *in vivo*.

1.4.5 Mouse TIF-IA and TIF-IC

In addition to UBF and SL1, other Pol I transcription factors have been reported in mouse. By the use of an *in vitro* system, Grummt and co-workers showed that two additional factors, TIF-IA and TIF-IC, are required for specific initiation at the mouse rDNA promoter (Schnapp and Grummt, 1991). After the formation of a functional preinitiation complex consisting of promoter-bound TIF-IB and UBF, Pol I, TIF-IA and TIF-IC are recruited to the template to form a transcription-competent initiation complex (Grummt, 1999).

In mouse, growth-dependent regulation of rDNA transcription has been shown to be due to changes in the amount or activity of TIF-IA. This factor is present or active only in extracts derived from rapidly growing cells (Schnapp *et al.*, 1993). TIF-IA is a 75 kDa monomeric globular protein that is tightly associated with Pol I (Clos *et al.*, 1986; Grummt, 1998). Apparently, Pol I and

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TIF-IA associate to form the transcriptionally active enzyme that is capable of initiating transcription from the rDNA promoter. It is required during transcription initiation for the formation of the first phosphodiester bonds of nascent rRNA, but is not required for Pol I recruitment. It is released after initiation and is recruited to preinitiation complexes lacking TIF-IA (Schnapp *et al.*, 1993). Interestingly, yeast Rrn3p and TIF-IA share several properties. They are required for transcription initiation by Pol I (Yamamoto *et al.*, 1996; Schnapp *et al.*, 1993). Both are associated with Pol I and dissociate from the enzyme during transcription (Milkereit and Tschochner, 1998; Brun *et al.*, 1994; Schnapp *et al.*, 1993) and are growth regulated (Grummt, 1998; Milkereit and Tschochner, 1998). Finally, Rrn3p and TIF-IA are monomeric proteins of ~ 75 kDa. Recent data indeed demonstrate that TIF-IA is the mouse homologue of yeast Rrn3p (Bodem *et al.*, 2000). In addition, similar to yeast Rrn3p, Miller *et al.* (2001) recently reported that the human Rrn3p/TIF-IA is essential in the SL1-mediated recruitment of Pol I to the rDNA promoter. This finding is in conflict with an earlier study on the mechanism of TIF-IA that showed that Pol I could be recruited to the promoter without TIF-IA (Schnapp *et al.*, 1993).

TIF-IC is another essential transcription initiation factor found in mouse (Schnapp *et al.*, 1994; Schnapp *et al.*, 1991). This factor is also found tightly associated with RNA Pol I. It interacts with Pol I in the absence of template (Schnapp *et al.*, 1994) and apparently joins the pre-initiation complex only after prior binding of TIF-IB and UBF (Schnapp and Grummt, 1991). TIF-IC plays a role in elongation by suppressing pausing of Pol I (Schnapp *et al.*, 1994).

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In other systems, a similar transcription factor has not been found. This could be due to differences in experimental systems and methods of analyses. It is also possible that the various factor or Pol I preparations were not sufficiently purified and biochemically defined, leading to apparent discrepancies in the results obtained in different laboratories.

1.4.6 Acanthamoeba castellanii TIF-IE

A novel transcription initiation factor, named TIF-IE, has been found recently in *A. castellanii.* This factor is required along with TIF-IB to form a stable (committed) complex on the rDNA promoter during transcription initiation (Figure 1.3). TIF-IE by itself cannot commit the template, requiring TIF-IB to do so. TIF-IE is present in impure preparations of Pol I and TIF-IB. It can be separated from Pol I by rate zonal sedimentation on a glycerol gradient (Radebaugh *et al.*, 1998).

1.5 ASSEMBLY OF THE COMMITTED COMPLEX

The first step of rDNA transcription initiation *in vitro* is formation of a stable pre-initiation (committed) complex that primes rRNA genes for transcription (Paule and White, 2000). Protein factors bind in a stable way to the rDNA promoter to form the committed complex, which is capable of recruiting Pol I to the promoter region. Once bound to the promoter, these factors remain bound and commit the template to multiple rounds of transcription during which polymerase recruitment, initiation, elongation, termination and subsequent re-initiation occur repetitively.

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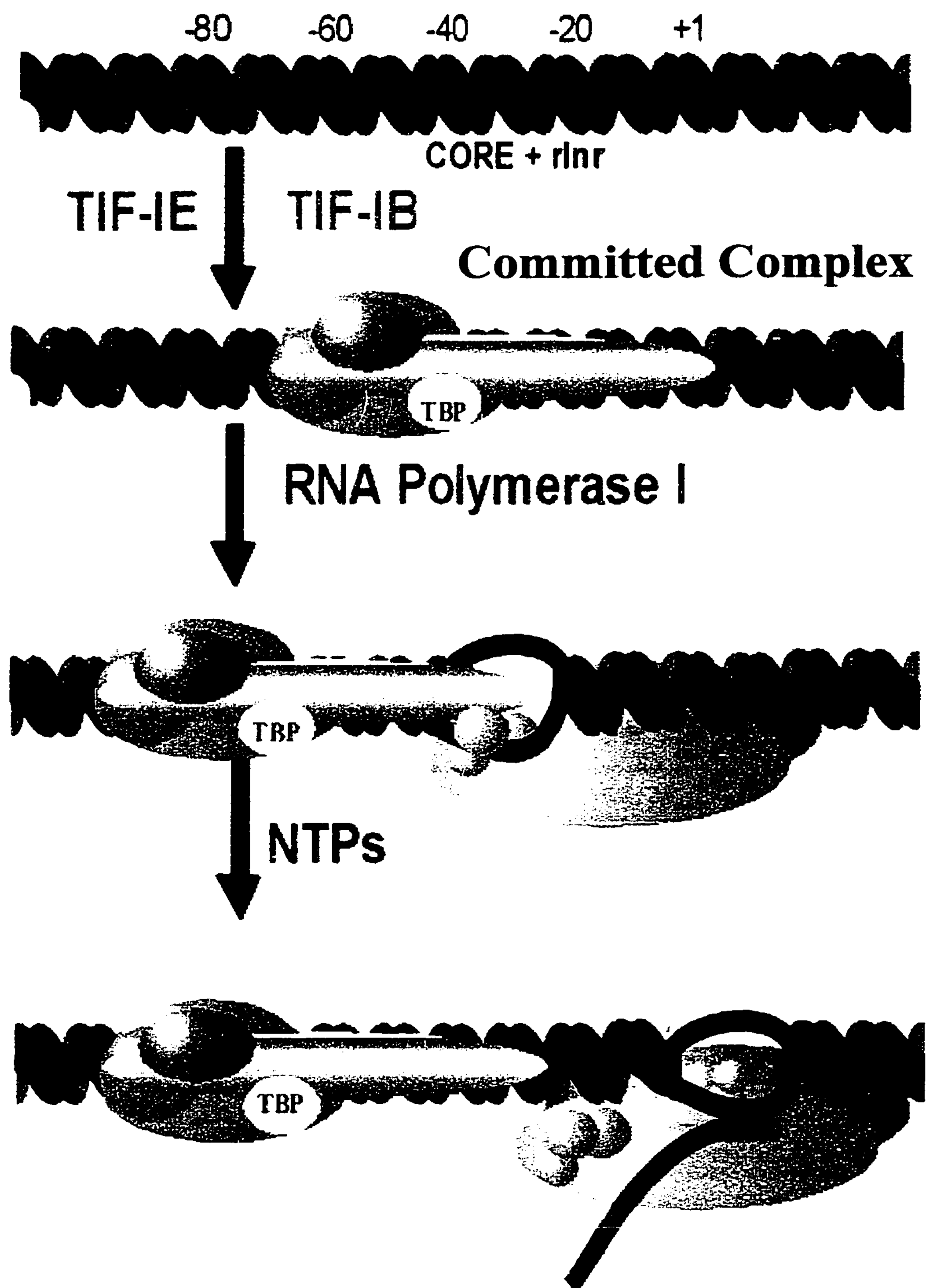

Fig. 1.3 **Mechanism of Transcription Initiation in *Acanthamoeba castellanii***

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The ability of the basal transcription factor, TIF-IB, to form a committed complex on the rDNA core promoter in the absence of additional factors varies considerably from species to species. Human SL1 (hSL1) alone is unable to bind or to protect specific sequences in either the UPE or the CPE. UBF is required along with hSL1 for the formation of a stable complex with the rDNA promoter. UBF binds first to the UPE and apparently aids the binding of SL1 either by interacting with it directly (Bell *et al.*, 1990; Bell *et al.*, 1988; Learned *et al.*, 1986) or by altering the structure of the DNA in the region bound by hSL1 (Bazett-Jones *et al.*, 1994). Recently, Anderson *et al.* (2000) argued that hSL1 is capable of binding to the rDNA promoter by itself and committing the template and that UBF does not alter the affinity of hSL1 for promoter DNA. However, UBF activates Pol I transcription *in vitro* at a step subsequent to the binding of SL1.

To investigate the requirement for the UBF-mediated recruitment of SL1 to the ribosomal DNA promoter, *in vitro* protein-protein interaction assays between intact SL1 complex and a series of UBF deletion mutants were performed. These studies revealed that the carboxy-terminal acidic tail of UBF, which is required for transcriptional activation, is necessary for protein-protein interaction with SL1. UBF lacking the carboxy-terminal domain was shown to be transcriptionally inactive (Tuan, *et al.*, 1999; Jantzen *et al.*, 1992). Moreover, HMG boxes are not essential for this protein-protein interaction. Interestingly, the carboxy-terminal tail of UBF has been shown to be extensively phosphorylated, and the phosphorylation state of UBF appears to be important for its transcriptional activity (Hershey *et al.*, 1995; Voit *et al.*, 1992). *In vitro* protein-protein interaction

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and DNase I footprinting assays showed that the phosphorylation state of UBF plays a crucial role in the recruitment of SL1 to the UPE and CPE of the rRNA promoter. Alkaline phosphatase treatment of UBF completely abolished the ability of UBF to interact with SL1. Moreover, incubation of the dephosphorylated UBF with nuclear extracts from exponentially growing cells was able to restore the UBF-SL1 interaction only in the presence of ATP. In addition, the phosphorylated and alkaline phosphatase-treated UBF could bind with equal affinities to the human rDNA promoter. Therefore, it was determined that the carboxy-terminal activation domain of UBF directly interacts with SL1. UBF phosphorylation-dephosphorylation plays a crucial role in regulating the assembly of a stable and productive initiation complex at the rRNA promoter but does not affect the ability of UBF to recognize and bind to the rRNA promoter (Tuan *et al.*, 1999). SL1 interaction with UBF is mediated by two subunits of the SL1 complex, TBP and TAF_I48 (Hempel *et al.*, 1996; Beckmann *et al.*, 1995; Kwon and Green, 1994). TBP alone is unable to support UBF-dependent activation of transcription by Pol I (Zomerdijk *et al.*, 1994). *In vitro* transcription assays showed that both TBP and TAF_I48 are required to assemble an active SL1 complex able to mediate UBF-dependent transcriptional activation (Beckmann *et al.*, 1995). In conclusion, the formation of the committed complex requires the initial binding of UBF to the rDNA. UBF then communicates with SL1 by means of direct contact of its carboxy-terminal tail with TBP and TAF_I48 (Mcstay *et al.*, 1991a; Bell *et al.*, 1990; Smith *et al.*, 1990; Clos *et al.*, 1986; Tower *et al.*, 1986; Learned *et al.*, 1985; Mishima *et al.*, 1982).

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In *X. laevis*, Rib1 similarly cannot form a stable complex on the *Xenopus* promoter. To form this stable transcription complex, xUBF and Rib1 are both required (Mcstay *et al.*, 1991a). UBF aids the binding of Rib1 by either altering the DNA structure of the promoter region bound by Rib1 (Bazett-Jones *et al.*, 1994) or by preventing the dissociation of TBP from the rather unstable Rib1 in a DNA-independent mechanism (Bodeker *et al.*, 1996).

In Yeast, CF alone or together with TBP cannot form a committed complex on the rDNA promoter. CF does not bind in a stable way to the rDNA template by itself, but its binding is dependent on the initial binding of UAF to the UPE. UAF alone is sufficient and necessary for template commitment (Keys *et al.*, 1996; Keys *et al.*, 1994).

In mouse, it has been previously reported that Factor D (or TIF-IB) was capable of forming the stable or template-committed complex unaided (Schnapp and Grummt, 1991; Tower *et al.*, 1986; Clos *et al.*, 1986; Miesfeld and Arnheim, 1984). Comparison of the amino acid sequence of the murine with the human TAF_Is demonstrated a strong conservation of these proteins. TAF_I48 is the most conserved of the three polypeptides and $TAF_I95/110$ is the least conserved. Human and murine $TAF_I63/68$ reveal pronounced differences in their N- and C-terminal portions. $hTAF_I63$ has a 40-amino acid extension at its N- terminus that the mouse protein lacks. On the other hand, murine TAF_I68 contains an additional 66 amino acids at the C-terminus. This C-terminal extension in mouse has a third zinc finger in mouse that is lacking in human TAF_I63. Two putative zinc finger motifs are conserved in both human TAF_I63 and murine TAF_I68.

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Therefore, a third zinc finger in murine TAF_I68 could explain why mouse TIF-IB can form a stable committed complex in the absence of UBF while SL1 cannot (Heix *et al.*, 1997).

In the rat system, rSL1 alone can recognize the gene promoter. The presence of rUBF however, appears to stimulate transcription and the formation of a template-committed complex (Smith *et al.*, 1990; Smith *et al.*, 1993).

In *A. castellanii*, impure TIF-IB preparations can form stable complexes with the core promoter in a template commitment assay. However, when TIF-IB is purified to apparent homogeneity, its ability to form these complexes is lost. The ability to commit the template can be restored by adding a partially purified factor, which we dub TIF-IE (Radebaugh *et al.*, 1998).

1.6 OBJECTIVES OF THE DISSERTATION

For my dissertation project, I had two main objectives. First, to purify the novel transcription factor, TIF-IE, to homogeneity in order to identify its subunit composition. Second, to investigate its molecular mechanism of action in conferring commitment to homogeneous TIF-IB.

In chapter 2, the subunit composition of this new factor is identified along with its molecular mechanism of action. In chapter 3, partially purified Pol I is shown to copurify with a promoter-DNA binding protein that binds just upstream of the TIF-IB binding site on the promoter. In order to determine if this DNA-binding protein is the same as TIF-IE, it was purified from TIF-IB and Pol I and

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tested for TIF-IE activity in a template commitment assay. Finally in chapter 4, additional observations and future directions are stated.

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CHAPTER 2

CHARACTERIZATION OF A NOVEL RNA POLYMERASE I TRANSCRIPTION INITIATION FACTOR, TIF-IE, REQUIRED FOR TEMPLATE COMMITMENT IN *ACANTHAMOEBA CASTELLANII*

The work presented in this chapter involves the characterization of TIF-IE; determining its subunit composition and its molecular mechanism of action in conferring commitment to TIF-IB. I performed all the experiments and purified most of the necessary proteins. Tara Towers assisted in the purification of RNA Pol I. This work will be submitted to *Molecular and Cellular Biology* for publication.

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CHARACTERIZATION OF A NOVEL RNA POLYMERASE I TRANSCRIPTION INITIATION FACTOR, TIF-IE, REQUIRED FOR TEMPLATE COMMITMENT IN *ACANTHAMOEBA CASTELLANII*

2.1 ABSTRACT

In the small, free-living amoeba, *Acanthamoeba castellanii*, ribosomal RNA (rRNA) transcription requires in addition to RNA polymerase I (Pol I), a single DNA-binding factor, Transcription Initiation Factor-IB (TIF-IB). TIF-IB is a multimeric protein that contains TATA-binding protein (TBP) and TBP-associated factors (TAFs) that are specific for Pol I transcription. This factor is required for accurate and promoter-specific initiation of rRNA transcription. To initiate transcription, TIF-IB binds to the rRNA core promoter and recruits Pol I by protein-protein interactions. In *A. castellanii*, partially purified TIF-IB can form a persistent complex with the rDNA promoter while homogeneous TIF-IB cannot. Another factor, TIF-IE, is required along with homogeneous TIF-IB for the formation of a stable complex on the rDNA core promoter. TIF-IE by itself, however, does not bind to the rDNA promoter and thus differs in mechanism from the Upstream Binding Factor (UBF) and Upstream Activating Factor (UAF) found in vertebrates and yeast, respectively. TIF-IE activity has been found in highly purified fractions of Pol I and can be partially separated from the polymerase by rate zonal sedimentation in a glycerol gradient. Renaturation of polypeptides excised from SDS-polyacrylamide gels showed that TIF-IE activity corresponded

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to a 141 kDa polypeptide. In other eukaryotic systems, a factor similar to TIF-IE has not been reported yet.

2.2 INTRODUCTION

Ribosomal RNA (rRNA) transcription initiation in eukaryotic cells is a multi-step process (Paule, 1998c). Initially, an unusually stable complex of transcription factors forms on the promoter. This complex is resistant to challenge by a second rDNA template, persists through multiple rounds of transcription and is referred to as the committed complex. RNA polymerase I (Pol I), possibly associated with another factor (Rrn3p or TIF-IA), is then recruited to the promoter by protein-protein interactions with the committed complex (Miller *et al.*, 2001; Peyroche *et al.*, 2000; Yamamoto *et al.*, 1996; Schnapp *et al.*, 1993; Kownin *et al.*, 1987). This is followed by melting of the double-stranded DNA (Kahl *et al.*, 2000) and the formation of the first few phosphodiester bonds. This requires TIF-IA/Rrn3p and another factor, TIF-IC, in vertebrates (Schnapp *et al.*, 1994.). Pol I then begins to translocate down the template. Following promoter clearance, the committed complex remains functional and recruits additional RNA polymerases for multiple rounds of transcription (Paule and White, 2000; Paule, 1998c).

A unique aspect of Pol I transcription is the species-specific nature of rRNA promoter recognition (Bell *et al.*, 1989; Grummt *et al.*, 1982; Mishima *et al.*, 1982; Learned and Tjian, 1982). This species specificity resides within a factor that is essential for the accurate and efficient initiation of transcription *in vitro*

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(Clos *et al.*, 1986; Learned *et al.*, 1985; Miesfeld and Arnheim, 1984; Mishima *et al.*, 1982). This fundamental transcription initiation factor is variously referred to as Transcription Initiation Factor-IB (TIF-IB) in mouse and *Acanthamoeba castellanii*, SL1 in human and rat, Rib1 in *Xenopus laevis*, Factor D or TFID in mouse and rat, and Core Factor (CF) in the yeast *Saccharomyces cerevisiae* (Paule and White 2000; Paule, 1998d). TIF-IB binds to the core domain of the promoter. Though all Pol I promoters exhibit a core element, there is relatively little primary sequence conservation in this domain. Once bound to the core, TIF-IB is responsible for correctly positioning Pol I at the transcription start site. Therefore, it confers promoter selectivity to Pol I (Paule and White, 2000; Reeder, 1999).

In all species, TIF-IB is a multisubunit protein complex. It is composed of TATA-binding protein (TBP) and three or four additional subunits known as Pol I-specific TBP-associated factors (TAF_Is). The presence of TBP, which is a component of the basal transcription machinery used by all three nuclear RNA polymerases (Hernandez, 1993), puts TIF-IB into the same class of transcription factors as TFIID and TFIIIB in the Pol II and Pol III systems, respectively (Paule and White, 2000). The yeast CF might be the only exception to the rule, since there are conflicting reports on whether TBP is an integral component of the yeast CF (Lin *et al.*, 1996; Keys *et al.*, 1994). Though CF does interact with TBP, Pol I specific basal transcription *in vitro* can occur in the absence of TBP (Keener *et al.*, 1998; Steffan *et al.*, 1996). However, *in vivo* overexpression of TBP

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stimulates rRNA transcription (Aprikian *et al.*, 2000), and TBP is needed for transcription activated by upstream activating factor (UAF) (Steffan *et al.*, 1998).

The ability of TIF-IB alone to form a stable complex on the core promoter varies considerably from species to species. Human SL1 is reportedly unable to bind tightly to specific sequences in either the core promoter or the upstream promoter element (UPE) of the rRNA gene. However, in the presence of an accessory transcription factor, Upstream Binding Factor (UBF), SL1 is able to footprint the DNA template. UBF seems to bind first to the UPE and to aid the binding of SL1 either by protein-protein interactions (Bell *et al.*, 1990; Bell *et al.*, 1988; Learned *et al.*, 1986) or by altering the DNA structure of the promoter region bound by SL1 (Bazett-Jones *et al.*, 1994). These interpretations have recently been questioned. J. Zomerdijk has data suggesting that human SL1 can form a committed complex without UBF, and that UBF has a stimulatory role later in initiation (Anderson *et al.*, 2000). Similarly, in *X. laevis*, both UBF and Rib1 are required for the formation of a stable transcription complex on the rRNA promoter (Mcstay *et al.*, 1991a). UBF aids the binding of Rib1 either by altering the DNA structure of the promoter region bound by Rib1 (Bazett-Jones *et al.*, 1994) or by preventing the dissociation of TBP from the rather unstable Rib1 in a DNA-independent mechanism (Bodeker *et al.*, 1996). In *S. cerevisiae*, CF does not bind tightly to the rDNA template. Its stable binding is dependent on the initial binding of an Upstream Activating Factor (UAF) to the UPE. In conjunction with TBP, UAF facilitates the recruitment of CF to the rDNA promoter to form a stable pre-initiation complex (Steffan *et al.*, 1996; Keys *et al.*, 1996; 1994). On the other

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hand, in both mouse and rat, it has been reported that TIF-IB/rSL1 is capable of forming a stable complex unaided. UBF is not required for stable association of TIF-IB with the promoter, but it stimulates it (Smith *et al.*, 1993; 1990; Schnapp and Grummt, 1991; Tower *et al.*, 1986; Clos *et al.*, 1986; Miesfeld and Arnheim, 1984).

In *A. castellanii*, TIF-IB was previously thought to be the only factor necessary for the formation of the committed complex (Paule, 1998d; Bateman *et al.*, 1989; Bateman *et al.*, 1988; Kownin *et al.*, 1987; Bateman *et al.*, 1985; Iida *et al.*, 1985). In fact, partially purified TIF-IB is capable of forming a stable (committed) complex with the rDNA promoter. However, TIF-IB purified to near homogeneity loses this ability to commit to the template. Therefore, it appears that during purification an additional component that helps stabilize the complex of TIF-IB with the promoter is separated from TIF-IB. This component could not be detected during TIF-IB purification presumably because it is too dilute. A novel transcription factor, TIF-IE, is capable of conferring upon homogeneous TIF-IB the ability to commit the ribosomal DNA template. TIF-IE is found associated with Pol I but can be separated partially from the polymerase by rate zonal sedimentation in a glycerol gradient (Radebaugh *et al.*, 1998).

The work presented here establishes that TIF-IE is composed of a single polypeptide and determines its mechanism of action in conferring commitment ability to TIF-IB.

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2.3 MATERIALS AND METHODS

2.3.1 Protein purification from *A. castellanii* extracts

Purification of TIF-IB: TIF-IB was purified to apparent homogeneity starting from a crude nuclear extract as described by Radebaugh *et al.* (1998) with the following modifications. The TIF-IB-containing fraction, obtained by ammonium sulfate precipitation of the nuclear extract, was dialized against 75 mM KCl in HEG_{10} (50 mM HEPES pH 7.9, 0.1 mM EDTA, and 10% glycerol). This fraction was loaded onto a DEAE Sepharose® Fast Flow (Pharmacia Biotech) column that was later developed with a linear gradient from 100 to 750 mM KCl. TIF-IB was further purified by BioRex® 70 (Bio-Rad) chromatography, two rounds of promoter-DNA SEPHAROSE CL-4B affinity chromatography, followed by rate zonal sedimentation in a glycerol gradient (Fig. 2.1).

Purification of RNA polymerase I and TIF-IE: Pol I was purified from a whole cell extract (Fig. 2.2) and its activity was analyzed in a non-specific transcription assay (Spindler *et al.*, 1978). TIF-IE was separated from RNA polymerase I at the last step of purification: rate zonal sedimentation in a glycerol gradient. At this stage of purification, TIF-IE is not homogeneous (Radebaugh *et al.*, 1998).

2.3.2 Plasmids and templates

Plasmids pGG4C and/or pEBH10 were used for the preparation of the DNAs used in electrophoretic mobility shift assays (EMSAs), Methidiumpropyl-EDTA·Fe (II) [MPE·Fe(II)] footprinting and template binding order-of-addition assays. Plasmid pGG4C contains a 114-base pair fragment of the *A. castellanii*

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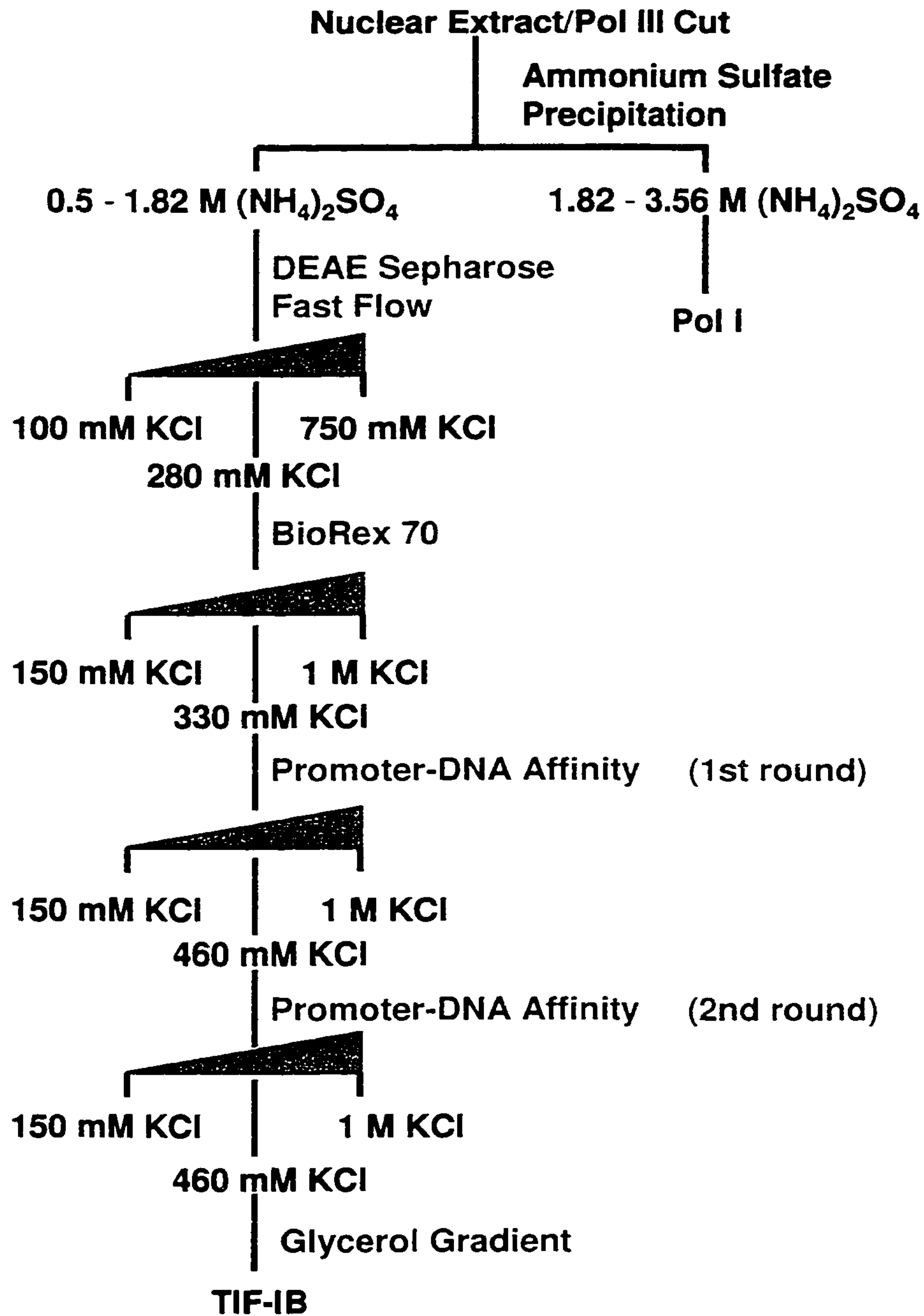

Fig. 2.1. Flow diagram for the purification of *A. castellanii* TIF-IB. Solid triangles indicate linear gradients, with the starting salt concentrations listed on the left and the final salt concentrations on the right

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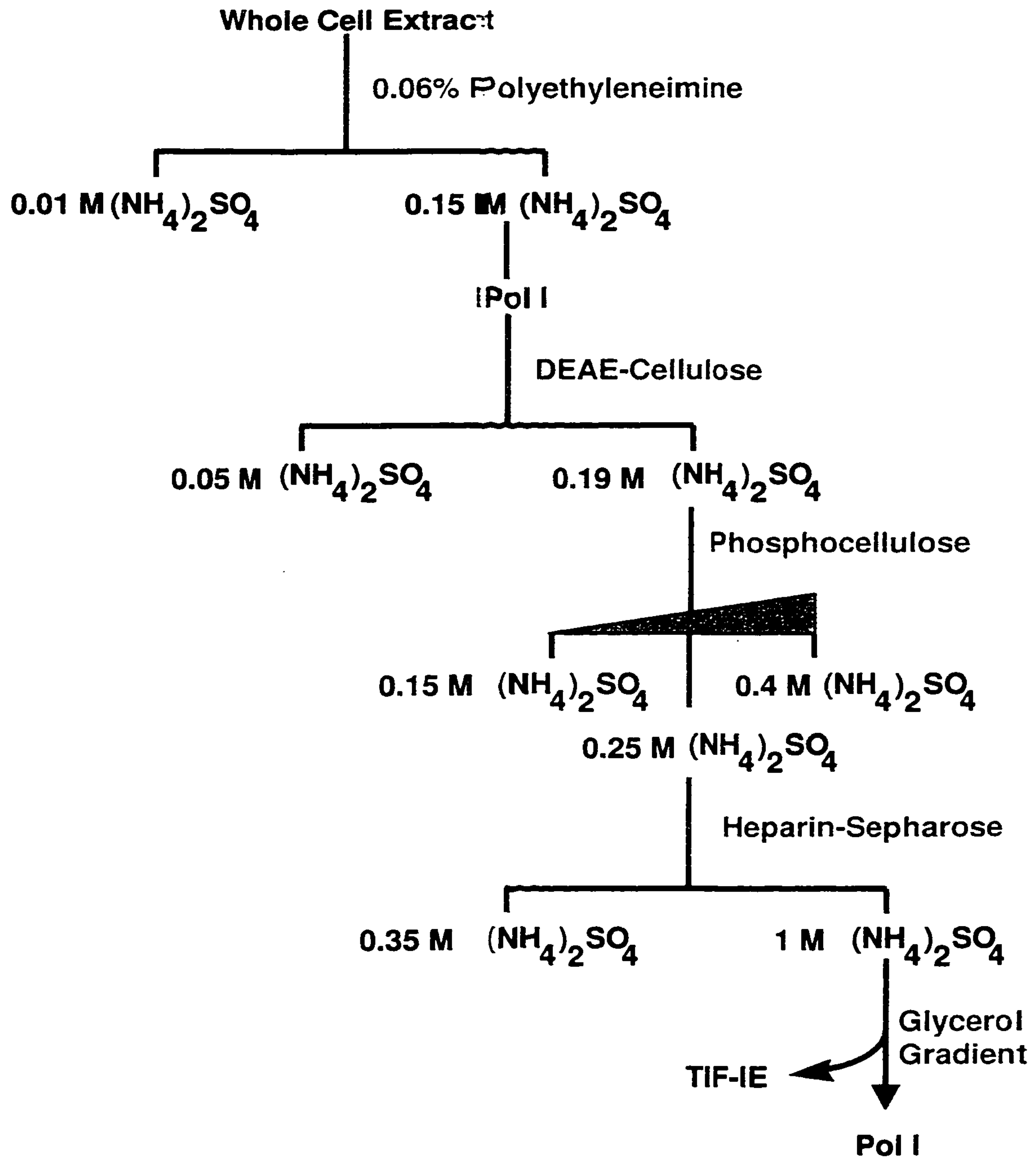

Fig. 2.2 Flow diagram for the purification of *A. castellanii* RNA polymerase I and TIF-IE. TIF-IE is separated from Pol I at the last step of purification, rate zonal sedimentation in a glycerol gradient.

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core promoter, from -96 to +18, relative to the transcription initiation site (tis) (+1), subcloned into the *Not*I site of pBluescript II SK$^-$ (Geiss *et al.*, 1997). Plasmid pEBH10 contains the *A. castellanii* core promoter from -120 to +80 cloned into *Hinc*II site of pUC8 (Bateman *et al.*, 1985). For the electrophoretic mobility shift and MPE·Fe(II) footprinting assays, DNA fragments were prepared from the plasmids by initial digestion with *Bam*HI that cleaves pGG4C downstream of +18 and pEBH10 upstream of -120. For the template binding order-of-addition assays, pGG4C was initially digested upstream of -96 with *Sac*II. In each case, this was followed by treatment with shrimp alkaline phosphatase to dephosphorylate the 5′ ends and then with phenol/$CHCl_3$. DNA concentrations were determined by absorbance at 260 nm. The linear plasmids (2 μg) were then 5′-end-labeled with T4 polynucleotide kinase. pGG4C/*Bam*HI and pGG4C/*Sac*II were then digested with *Sac*I and *Xba*I, respectively. *Sac*I cleaves pGG4C/*Bam*HI upstream of -96 to generate a fragment of 150 bp while the *Xba*I cleaves pGG4C/*Sac*II downstream of +18 to generate a fragment of 127 bp. pEBH10/*Bam*HI was digested downstream of +80 with *Pst*I to generate a 217 bp fragment. Each labeled fragment was separated from linear vector on a 1.75-2% agarose gel, visualized by autoradiography, excised, eluted and purified with QIAEX II Gel Extraction Kit (Qiagen) using the manufacturer's protocol. After purification, the specific activity of each labeled DNA fragment was estimated by liquid scintillation counting.

Plasmids pAr6 and pEBH10 were used for the preparation of the templates used in the template commitment assays. pAr6 contains the *A.*

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castellanii rRNA promoter from –683 to +219 cloned into the *Sma*I site of pUC8 (Radebaugh *et al.*, 1998). The restriction digests of pAr6 with *Hind*III and pEBH10 with *Nde*I produce 240 and 309 nucleotide runoff RNAs respectively.

2.3.3 Electrophoretic mobility shift assay (EMSA)

The 5′-end-labeled 150-bp *Bam*HI/*Sac*I fragment of pGG4C and the 217-bp *Bam*HI/*Pst*I fragment of pEBH10 were used in EMSAs. The binding conditions were the same as described by Geiss *et al.* (1997) except that 0.5 mg/ml bovine serum albumin was used instead of 0.05 mg/ml. DNA was incubated with proteins for 20 min at 25°C. Reactions were stopped on ice and loaded immediately on a low-cross-linking nondenaturing polyacrylamide gel (5% acrylamide; 80:1 [wt/wt] ratio of acrylamide to N,N'-methylene bisacrylamide) as described by Gong *et al.* (1995). Gels were run at 200 V for 1.5 h, dried, and exposed to phosphor storage screens. Data was analyzed by phosphorImaging on a Storm phosphorimager (Molecular Dynamics, Sunnyvale, CA) and quantified by using ImageQuant software (version 5.1).

2.3.4 Template commitment assay

Template commitment assay conditions were the same as described by Radebaugh *et al.* (1998). The minimum amount of template required for binding all the available TIF-IB in the reaction was determined for pEBH10/*Nde*I (DNA A) and pAr6/*Hind*III (DNA B) and used in the following protocol. The first template(s) (DNA A and/or DNA B) was preincubated with TIF-IB alone or with TIF-IE for 10

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min. at 25°C. The second template (DNA B) or buffer was then added, and preincubation continued for another 10 min. RNA synthesis was initiated by the addition of Pol I and proceeded for another 30 minutes. Run-off RNAs were analyzed as described by Radebaugh *et al.* (1998).

2.3.5 Methidiumpropyl-EDTA·Fe (II) [MPE·Fe(II)] footprinting

The 5'-end-labeled 150 bp *Bam*HI/*Sac*I fragment of pGG4C was used as the template strand of the rRNA promoter. The binding conditions for footprinting were the same as in EMSAs. DNA and proteins, in a final reaction volume of 20 μl, were incubated for 20 min at 25°C. 1 μl of 70 μM MPE and 50 μM $(NH_4)_2Fe(SO_4)_2$ solution and 1 μl of 0.5 M dithiothreitol were added to the reactions and incubation continued for another 15 min at 25°C. MPE·Fe(II) reactions were stopped and processed as described by Geiss *et al.* (1997). DNA was analyzed on denaturing (7M urea) 10% sequencing gels, 1X Tris-Borate-EDTA. Gels were run at 20 milliamps for 2 to 3 hours. The gel was then dried and exposed to a phosphor storage screen overnight. Data was analyzed by phosphorimaging and ImageQuant software.

2.3.6 SDS-polyacrylamide gel electrophoresis and staining of proteins

TIF-IE fractions were precipitated with chloroform-methanol as described by Wessel and Flugge (1984). The protein pellets were resuspended in 1x SDS loading buffer and electrophoresed through an SDS polyacrylamide gel by standard methods (Garfin, 1990). Gels were stained using Coomassie Brilliant

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Blue R-250 as described (Sambrook *et al.*, 1989) or with silver stain (Blum *et al.*, 1987).

2.3.7 Renaturation of proteins from SDS-polyacrylamide gels

Renaturation was performed essentially as described by Hager and Burgess (1980), but with some of the modifications reported by Kretzchmar *et al.* (1992) and Briggs *et al.* (1986).

Acetone precipitation of TIF-IE sample: Five volumes of cold acetone (-20°C) were added to the TIF-IE sample. The sample was allowed to precipitate for 30 min. in a dry ice-ethanol bath and then centrifuged for 30 min. at 10,000 x g at 4°C. The acetone supernatants were poured off and protein pellets were dried under vacuum for 2 min.

SDS polyacrylamide gel electrophoresis and negative staining: The TIF-IE pellet was resuspended in 20-30 μl 1x SDS loading buffer, heated for 5 min. at 95°C and electrophoresed on a 6-10% SDS-PAGE. To visualize the protein bands and excise them, a Zinc Stain & Destain Kit for Electrophoresis (Bio-Rad; Hercules, CA) was used and staining and destaining were carried out using the manufacturer's protocol. Individual gel slices were then excised, put into siliconized microcentrifuge tubes, and soaked in two changes of 1 ml of 1 mM dithiothreitol for 15 min.

Elution: The 1 mM dithiothreitol solution was decanted and discarded. 0.5-1 ml (depending on the size of gel slice) of elution buffer was added to each gel slice, and the gel was crushed with several strokes of a small Teflon pestle

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that fit tightly inside the 1.7 ml microcentrifuge tube. The elution buffer contained 50 mM Tris pH 7.9, 0.1 mM EDTA, 0.1% SDS, 5 mM dithiothreitol, and 150 mM NaCl. The proteins were allowed to elute for 21 h at room temperature with continuous agitation.

SDS removal and acetone precipitation: The residual polyacrylamide was removed by centrifugation, and the eluates were transferred to 15-ml siliconized Corex tubes. The eluates were then precipitated with five volumes of cold acetone as described above and centrifuged at 10,000 x g for 30 min. at 4°C. The acetone supernatants were poured off and the precipitates were rinsed once gently with 1 ml of ice cold 80% acetone and 20% dialysis buffer (100 mM KCl, 0.2% Nonidet P-40, 50 mM HEPES pH 7.9, 20% glycerol, 0.1 mM EDTA, 1 mM dithiothreitol) to remove the last traces of residual SDS. This was followed by centrifugation at 10,000 x g for 10 min. at 4°C. Supernatants were poured off and protein pellets were allowed to dry at room temperature.

Guanidine hydrochloride treatment and renaturation: The resulting protein pellets were resuspended in 50 μl of buffer containing 6 M guanidine hydrochloride, 100 mM KCl, 0.2% Nonidet P-40, 50 mM HEPES pH 7.9, 20% glycerol, 0.1 mM EDTA, and 1 mM dithiothreitol. The pellets were dissolved thoroughly and allowed to stand at room temperature for 30-40 min. The solution was then dialyzed against 500 ml of dialysis buffer (composition described above) with 0.1 mM phenylmethanesulfonyl fluoride and 1 mM benzamidine for 21 h at 4°C to remove the guanidine hydrochloride and permit the proteins to renature.

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2.3.8 Template binding order-of-addition assay

The 5′-end-labeled 127-bp *Sac*II/*Xba*I fragment of pGG4C (DNA A) and 217-bp *Bam*HI/*Pst*I fragment of pEBH10 (DNA B) were used in this assay. The binding conditions were the same as in EMSAs. The labeled fragment(s), DNA A and /or DNA B, was preincubated with either TIF-IB alone, TIF-IB plus TIF-IE, or TIF-IE alone for 15 min. at 25°C. For the formation of a complex with TIF-IB on each template, TIF-IE amounts were limiting. The second template, DNA A or DNA B, and either TIF-IB or TIF-IE were then added in the secondary incubation period, which continued for another 15 min. Reactions were stopped on ice and loaded on 5% nondenaturing polyacrylamide gels as described by Gong *et al.* (1995). Gels were run at 200 V for 3.5 h, dried, exposed to phosphor storage screens. Data was analyzed by phosphorImaging and quantified by using ImageQuant software.

2.4 RESULTS

2.4.1 TIF-IE is required along with glycerol gradient-purified TIF-IB to form a committed complex on the rRNA promoter.

Radebaugh *et al.* (1998) reported that while TIF-IB purified through the first round of promoter-DNA affinity chromatography (Fig. 2.1) could form a committed complex with the rRNA core promoter in a template commitment assay, glycerol gradient-purified TIF-IB could not. A novel transcription factor, TIF-IE, was required along with the latter fraction for the formation of this stable

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complex. The source of TIF-IE is described below, following demonstration of the assays used.

In this work, we confirmed that glycerol gradient-purified TIF-IB cannot form a stable complex with the rDNA promoter in a template commitment assay (Fig. 2.3). In this assay, two promoter-containing DNA templates, pEBH10/*Nde*I (DNA A) and pAr6/*Hind*III (DNA B), were used. In the initial incubation period, sufficient DNA A and/or DNA B was added to bind all the available promoter-binding factors. Glycerol gradient-purified TIF-IB was incubated either alone or with added TIF-IE and was given the opportunity to form a complex with the DNA. This complex was then challenged in a subsequent incubation period with a second template (DNA B) whose transcription product was distinguishable from the first by length (in a runoff assay). Pol I was added last to start the RNA synthesis phase, which proceeded for 30 min. DNA A and DNA B produced 309 and 240 nucleotide runoff RNAs respectively (lanes 1 and 2). A pause product at about 270 nucleotides was also produced from DNA A (lane 2). DNA A and DNA B were transcribed equally when they were both present during the initial incubation period (lane 3). The difference in the intensity of the bands observed in lane 3 is due to the difference in number of labeled nucleotides incorporated into the two RNA products (67 and 49 labeled nucleotides in the RNA products of DNA A and DNA B respectively). When only DNA A was incubated with TIF-IB in the initial period and DNA B was added during the secondary period (lane 4), transcription levels from both templates were nearly identical to when both templates were present in the initial incubation period (compare lanes 3 and 4).

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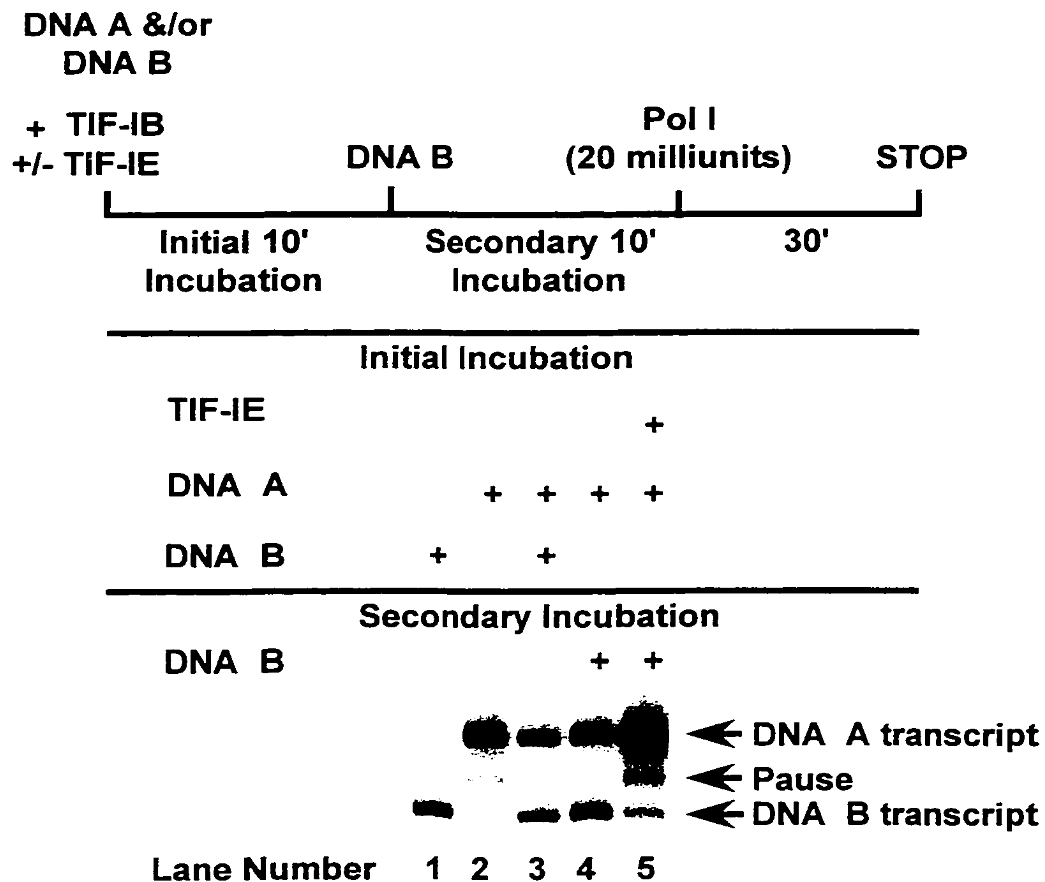

Fig. 2.3. TIF-IE is required along with glycerol gradient-purified TIF-IB to form a committed complex on the rDNA promoter. DNA A and DNA B produce 309 and 240 nucleotide runoff RNAs respectively (lanes 1, 2, and 3). Template commitment assays of glycerol gradient-purified TIF-IB (1 μl) (lane 4), with the addition of TIF-IE (0.5 μl) (lane 5) are shown. DNA A and DNA B represent pEBH10/*Nde*I and pAr6/*Hind*III templates respectively. The RNA transcripts synthesized from DNA A and DNA B in lanes 3, 4, and 5 were quantified and their ratios (A/B) in each lane are 1.1, 1.4, and 20.4 respectively.

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Therefore, glycerol gradient-purified TIF-IB was unable to form a stable complex on the first DNA template (DNA A) during the initial incubation period. However, upon the addition of TIF-IE along with TIF-IB to DNA A in the initial incubation period, the second template (DNA B) failed to be transcribed efficiently (lane 5). TIF-IE also stimulated transcription 2.3 fold (compare lanes 2 and 5). Stimulation occurs even in the absence of a second template and only when TIF-IE is initially incubated with TIF-IB and the DNA (data not shown). We conclude that TIF-IE is required along with glycerol gradient-purified TIF-IB for the formation of a stable (committed) complex on the rDNA core promoter.

2.4.2 Direct measurement of TIF-IB binding activity.

To more directly test the hypothesis that TIF-IE stabilizes the binding of TIF-IB to the rRNA core promoter and therefore allows the formation of the committed complex, EMSA was used to determine the binding activity of TIF-IB alone, TIF-IB plus TIF-IE, and TIF-IE alone to the rDNA promoter. TIF-IB purified through one round of promoter-DNA affinity chromatography is capable of binding stably to the promoter (Fig. 2.4, lane 2). However, further purification of TIF-IB through a second round of promoter-DNA affinity chromatography followed by rate zonal sedimentation in a glycerol gradient caused TIF-IB to lose its ability to bind stably to the rDNA core promoter (lane 3). Consistent with the template commitment assays, this suggests that during purification an additional component that helps in the formation of the TIF-IB-promoter complex is separated from TIF-IB.

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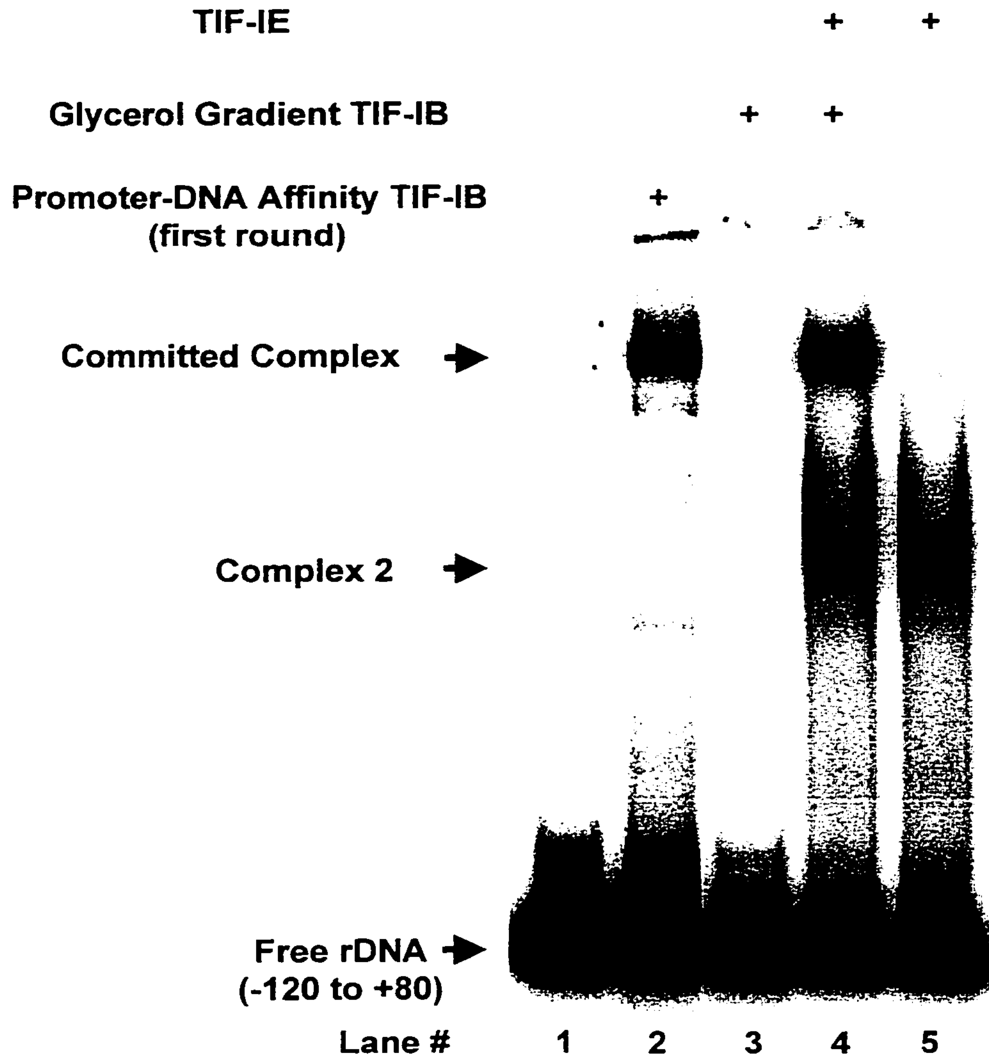

Fig. 2.4. TIF-IE is required along with glycerol gradient-purified TIF-IB for the formation of a stable complex on the rDNA promoter. EMSAs of 1 μl of TIF-IB purified through one round of the promoter DNA affinity column (lane 2), of glycerol gradient-purified TIF-IB alone (2 μl) (lane 3) or with TIF-IE (0.5 μl) (lane 4) and of the TIF-IE sample alone (0.5 μl) on the rRNA promoter (lane 5).

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The addition of TIF-IE to glycerol gradient-purified TIF-IB allowed the formation of a complex with the core promoter (lane 4) with an electrophoretic mobility identical to the complex formed with promoter-DNA affinity-purified TIF-IB (lane 2). The identical electrophoretic mobilities of the two complexes suggest they contain identical protein components, i.e. the TIF-IE purified from Pol I is likely the component lost during rate zonal sedimentation of TIF-IB. TIF-IB and TIF-IE are both required for the formation of a stable complex on the rDNA core promoter and for template commitment. The TIF-IE sample alone produced a different complex on the same template (Fig. 2.4, lanes 4 and 5, Complex 2). Since, at this stage of purification, TIF-IE was not homogeneous it was not clear whether this DNA binding activity observed was due to TIF-IE or a contaminant.

2.4.3 TIF-IE is partially separated from RNA polymerase I by rate zonal sedimentation in a glycerol gradient.

We have not been able to recover significant amounts of TIF-IE from either the DNA-affinity column washes or from the glycerol gradient fractions of TIF-IB. We can barely detect TIF-IE activity in the glycerol gradient fractions, presumably because TIF-IE is highly diluted. However, incubation of homogeneous TIF-IB plus Pol I in the initial incubation of a template commitment assay led to stable complex formation (data not shown). Radebaugh *et al.* (1998) found that TIF-IE is present in significant amounts in the Pol I heparin-Sepharose fraction and could be partially separated from Pol I during the last step of purification, rate zonal sedimentation in a glycerol gradient (Fig. 2.2). Glycerol gradient fractions were assayed for TIF-IE activity in an EMSA-stimulation assay.

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As shown above, glycerol gradient-purified TIF-IB cannot form a stable complex on the ribosomal promoter (Fig. 2.5, compare lanes 2 and 3). Adding fractions from rate zonal sedimentation of Pol I (2-19) to glycerol gradient-purified TIF-IB (Fig. 2.5, lanes 4-21), showed that TIF-IE is present in the fractions, with the peak activity sedimenting in fractions 6 and 7 (Fig. 2.5, lanes 8 and 9). Pol I activity was detected by a non-specific transcription assay (Fig. 2.5, bar graph above lanes 14-21) in fractions 12-19, with the peak activity present in fractions 14 and 15. This explains the huge complexes formed in lanes 16 and 17.

Even after one round of rate zonal sedimentation, Pol I was still associated with TIF-IE. Small amounts of TIF-IE could be resolved from glycerol gradient-purified Pol I by a second round of rate zonal sedimentation, but the TIF-IE activity still overlapped with the non-specific polymerase activity (data not shown). This strongly suggests an interaction between Pol I and TIF-IE so that some, but not all TIF-IE is released from the enzyme by rate zonal sedimentation (see Discussion).

2.4.4 Stimulation of TIF-IB binding to the rDNA core promoter by TIF-IE is dose dependent.

Titration of glycerol gradient-purified TIF-IB (1-3 μl) to the promoter DNA did not result in a detectable complex in EMSA (Fig. 2.6A, lanes 3, 5, and 7). However, the addition of TIF-IE along with glycerol gradient-purified TIF-IB to the promoter rDNA allowed the formation of a complex (Fig. 2.6A, lanes 4, 6, and 8) at a level that is dependent on the dose of TIF-IB. Provided that

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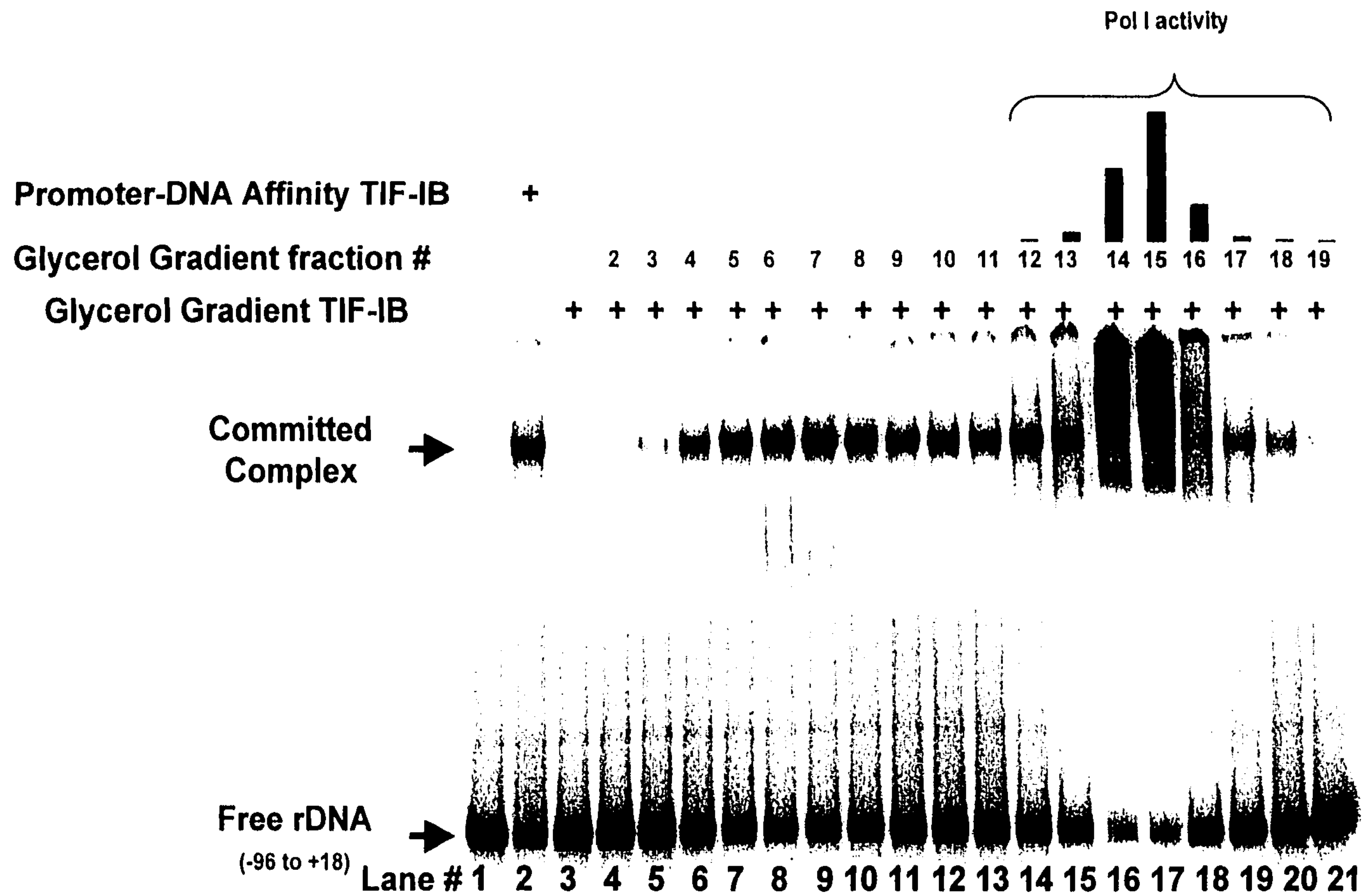

Fig. 2.5. Rate zonal sedimentation of TIF-IE in a glycerol gradient. EMSAs of promoter-DNA affinity-purified TIF-IB (2 μl) (lane 2), of glycerol gradient-purified TIF-IB alone (2 μl) (lane 3) or with (0.5 μl) of glycerol gradient fractions of Pol I 2 to 19 (lanes 4-21) on the rRNA promoter.

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Fig. 2.6. Stimulation of glycerol gradient-purified TIF-IB EMSA by TIF-IE is dose dependent. (A) EMSAs of promoter DNA affinity-purified TIF-IB (1 μl) (lane 2), of increasing amounts of glycerol gradient-purified TIF-IB alone (lanes 3, 5 and 7) or with 0.5 μl of TIF-IE (lanes 4, 6 and 8) on the rRNA promoter. (B) EMSAs of glycerol gradient-purified TIF-IB alone (lanes 2 and 5) or with increasing amounts of TIF-IE (lanes 3, 4, 6 and 7), and of TIF-IE alone (lanes 8 and 9).

TIF-IB amounts were not limiting, the amount of complex formed was also dependent on the dose of TIF-IE (Fig. 2.6B, compare lanes 3 and 4, 6 and 7).

The dose-dependency on TIF-IE for complex formation was confirmed by MPE·Fe(II) footprinting. Promoter-DNA affinity-purified TIF-IB alone protected the rRNA promoter from MPE cleavage in a region extending from –67 to –17 with respect to the tis (+1) (Fig. 2.7, lane 3), while glycerol gradient-purified TIF-IB did not (lane 4). However, the addition of increasing amounts of TIF-IE to glycerol gradient-purified TIF-IB (lanes 5 and 6) stimulated the formation of a complex on the rRNA promoter that produced the same pattern of protection from MPE cleavage as promoter-DNA affinity-purified TIF-IB (lane 3). The extent of protection from MPE cleavage was also dependent on the dose of TIF-IE (Fig. 2.7, compare lanes 5 and 6). TIF-IE alone did not protect the promoter from MPE cleavage in the promoter region (lanes 7 and 8). Therefore, the region of rDNA promoter extending from –67 to –17 was protected from MPE cleavage only by the cooperative binding of both TIF-IB and TIF-IE. The dose dependency suggests TIF-IE enters into the complex stoichiometrically rather than exerting some catalytic modification of TIF-IB.

2.4.5 Subunit composition of TIF-IE.

TIF-IE sediments near the top of the glycerol gradient of Pol I. Therefore, glycerol gradient fractions 2-10 were analyzed on 10% SDS–PAGE to reveal polypeptide(s) consistently found associated with TIF-IE activity as detected by an EMSA. The EMSA (Fig. 2.8A) showed that TIF-IE activity was found in glycerol gradient fractions 3-10, with the peak activity in fraction 6. SDS-PAGE of

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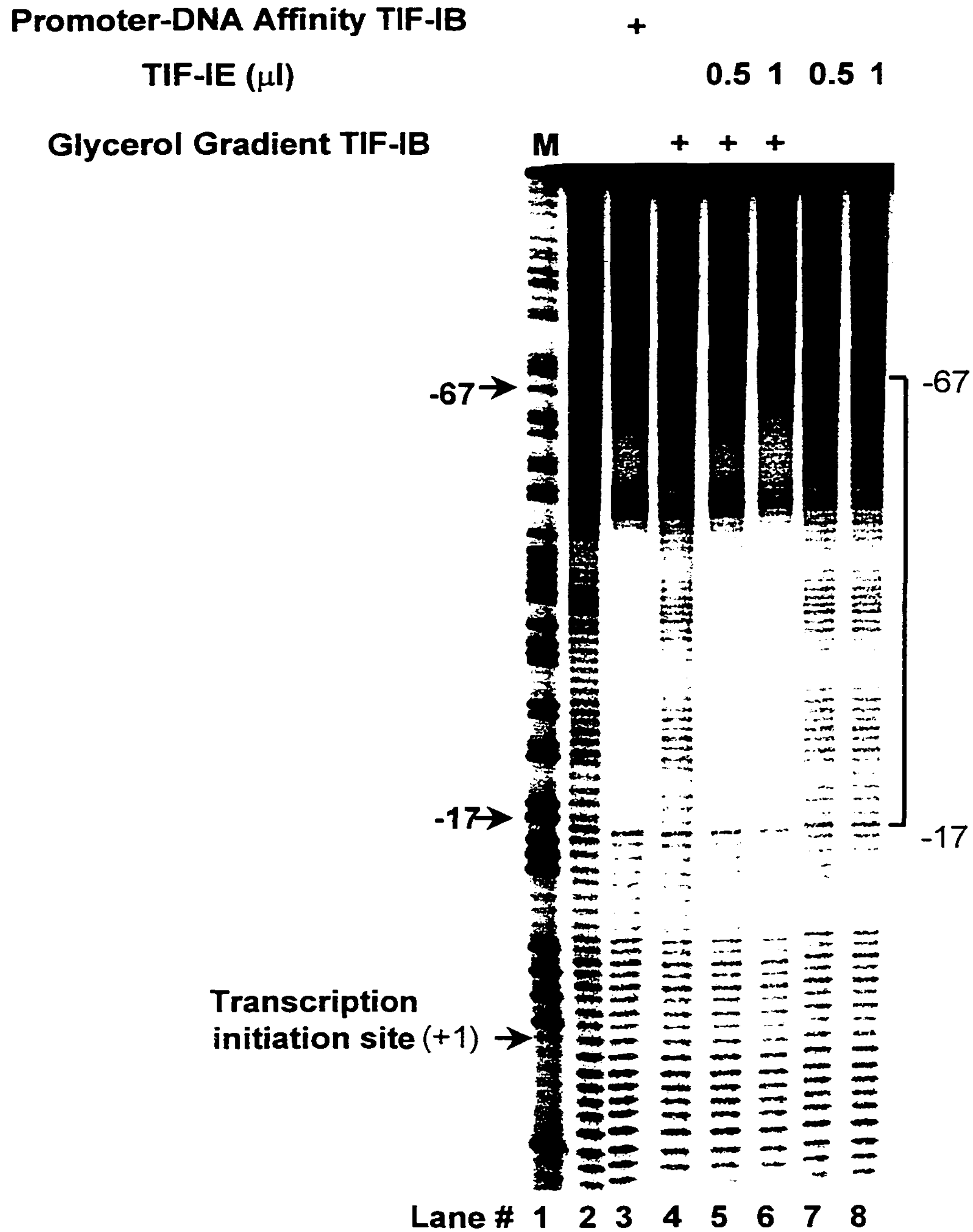

Fig. 2.7. TIF-IE is required along with glycerol gradient-purified TIF-IB for the formation of a stable complex on the rDNA and its effect is dose dependent. MPE·Fe(II) footprints of promoter-DNA affinity-purified TIF-IB (2 μl) (lane 3), of glycerol gradient-purified TIF-IB alone (3 μl) (lane 4) or with increasing amounts of TIF-IE (lanes 5 and 6), and of TIF-IE sample alone (lanes 7 and 8) on the template strand of the rRNA promoter.

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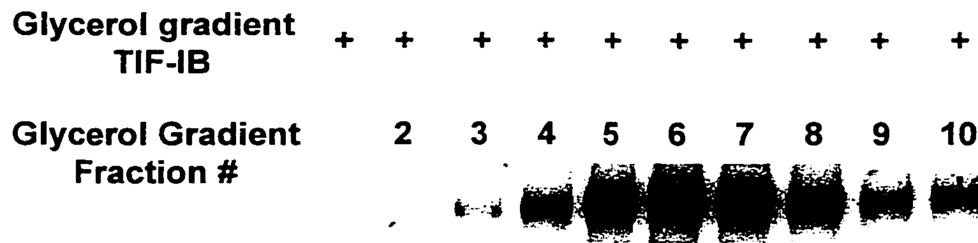
(A)



(B)

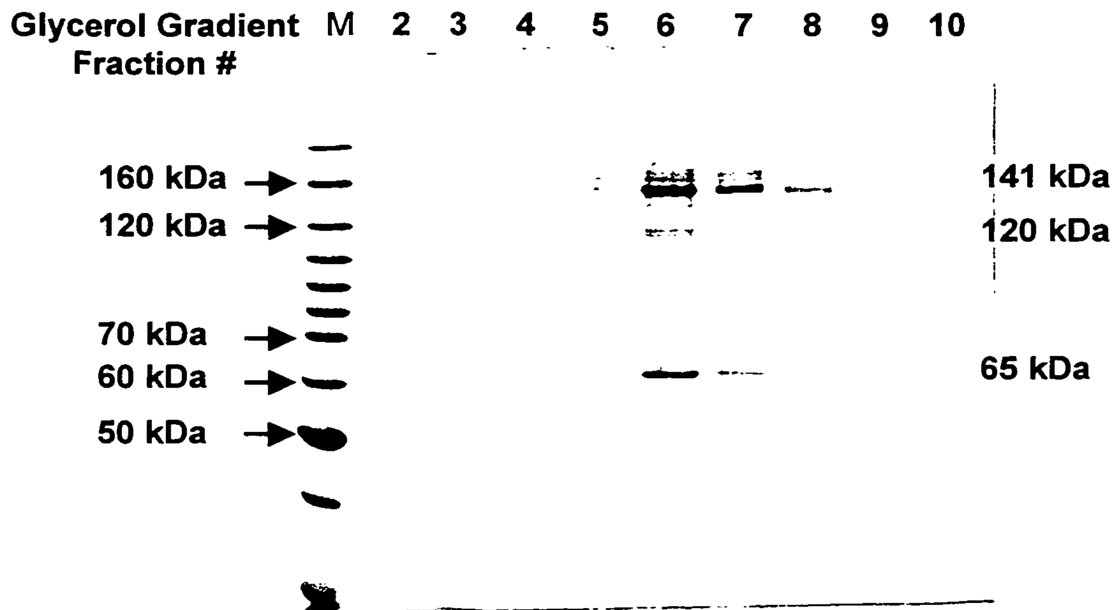


Fig. 2.8. Three major polypeptides of relative molecular weights of 65, 120, and 141 kDa were consistently found associated with TIF-IE activity. A, EMSA showing complexes formed between glycerol gradient-purified TIF-IB (2 μl) and 0.5 μl of glycerol gradient fractions of Pol I (2-10) on the rDNA promoter region from -120 to +80. **B**, a 10% SDS-polyacrylamide gel of 40 μl of the glycerol gradient fractions of Pol I assayed in **(A)** stained with Coomassie Blue. Molecular weights of the size markers are shown on the left.

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these glycerol gradient fractions (2-10) revealed that three major polypeptides were consistently found associated with TIF-IE activity. These had relative molecular weights of 65, 120, and 141 kDa (Fig. 2.8B). To identify which of the three polypeptides is associated with TIF-IE activity, polypeptide bands were excised from SDS-polyacrylamide gels and renatured. A TIF-IE sample was initially fractionated on a 10% SDS-PAGE and the proteins were subsequently eluted and renatured from seven different gel slices. After excision of the gel slices, part of the gel including the lanes for the size markers and another TIF-IE sample was silver stained to show the polypeptides present in each slice (Fig. 2.9A). Due to the high and differential sensitivity of the silver staining technique, several minor polypeptides in addition to the three shown in Fig. 2.8B were seen. The renatured eluates were assayed for TIF-IE activity in an EMSA-stimulation assay (Fig. 2.9B). The renatured eluate of gel slice # 2 exhibited activity (Fig. 2.9B, lane 5). Gel slice # 2 contained several polypeptides with relative molecular weights between 100 and 160 kDa (Fig. 2.9A). These results exclude the possibility of the 65 kDa polypeptide being a required TIF-IE subunit. To pin down which 100–160 kDa polypeptide(s) has TIF-IE activity, we subjected another TIF-IE sample to 6% SDS-PAGE to better resolve these polypeptides. A negative (zinc) staining kit (Bio-Rad; Hercules, CA) was used to visualize the polypeptide bands before their excision from the gel. This negative staining method produced an opaque white background in the gel, while the proteins were not stained and remained as a clear band in the gel. This staining allowed

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Fig. 2.9. The molecular weight of TIF-IE is between 100 and 160 kDa. A, a section of a 10% SDS-polyacrylamide gel that was silver stained after gel slicing showing the probable polypeptides in each slice of TIF-IE lane. **B**, EMSAs of glycerol gradient-purified TIF-IB alone (0.5 μl) (lane 2) or with TIF-IE (0.5 μl) (lane 3), or with the renatured eluates of gel slices 1-7 (9.5 μl) (lanes 4-10) on the rRNA promoter.

the excision of nine gel slices containing only one or two polypeptides each. The proteins contained in each gel slice were eluted, renatured, and assayed for TIF-IE activity in an EMSA (Fig. 2.10A). TIF-IE activity was detected in renatured eluates of gel slices # 2-4 (Fig. 2.10A, lanes 3-5) with a strong peak in slice # 3 (Fig. 2.10A, lane 4). Each renatured eluate was resolved by SDS-PAGE (Fig. 2.10B) and silver stained. The eluate of gel slice # 3 contained only a 141 kDa polypeptide. TIF-IE activity detected in the gel slices flanking # 3 (Fig. 2.10A, lanes 3 and 5) is due to the presence of small amounts of the 141 kDa polypeptide in these slices, probably because of diffusion during processing (Fig. 2.10C). Therefore, we conclude that TIF-IE is a single subunit protein with a relative molecular weight of 141 kDa. The sedimentation rate of TIF-IE is consistent with it being mostly a monomer (data not shown). To provide conclusive evidence that the 141 kDa renatured protein possessed all the activities attributed to TIF-IE, we examined it in a template commitment assay. Indeed, the 141 kDa renatured protein exhibited TIF-IE activity comparable to the starting material (Fig. 2.11, compare lanes 5 and 6).

2.4.6 Mechanism of action of TIF-IE.

In some vertebrates, the formation of the committed complex requires an accessory transcription factor, UBF, in addition to TIF-IB. UBF binds stably to the UPE and was reported to help recruit TIF-IB to the template (Mcstay *et al.*, 1991a; Bell *et al.*, 1988; Learned *et al.*, 1986). Similarly in yeast, an additional transcription factor, UAF, binds first to the UPE and, in concert with TBP, helps

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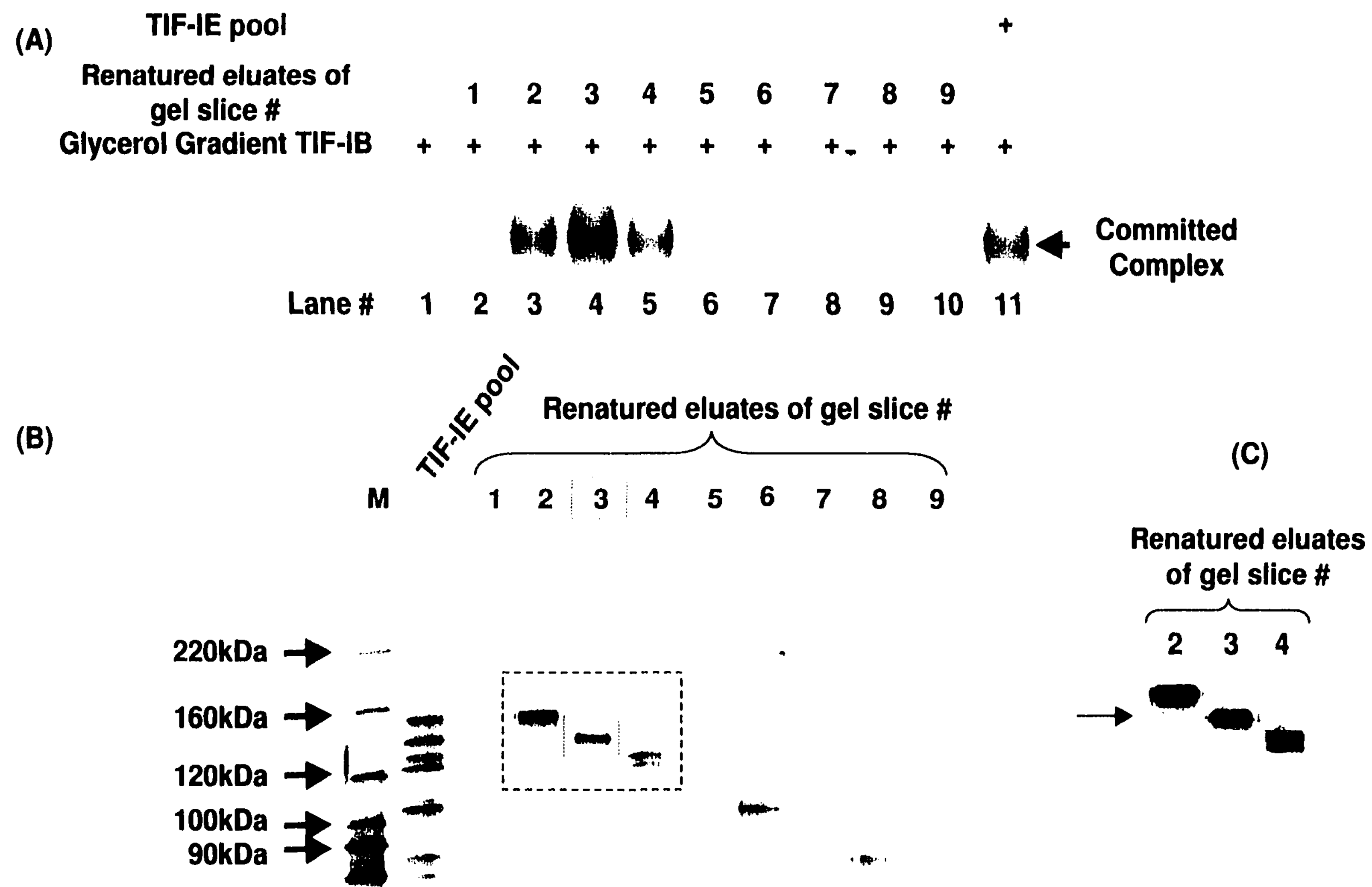

Fig. 2.10. TIF-IE has an apparent molecular weight of 141 kDa. A, EMSAs showing complexes formed between the rRNA promoter and glycerol gradient-purified TIF-IB alone (lane 1), or with the renatured eluates of gel slices (1-9) (lanes 2-10), or with TIF-IE (lane 11). **B**, a 6% SDS-polyacylamide gel of the renatured eluates of gel slices (1-9) assayed in (A) stained with silver. **C,** inset of SDS-polyacrylamide gel showing polypeptides of eluates of gel slices # 2-4.

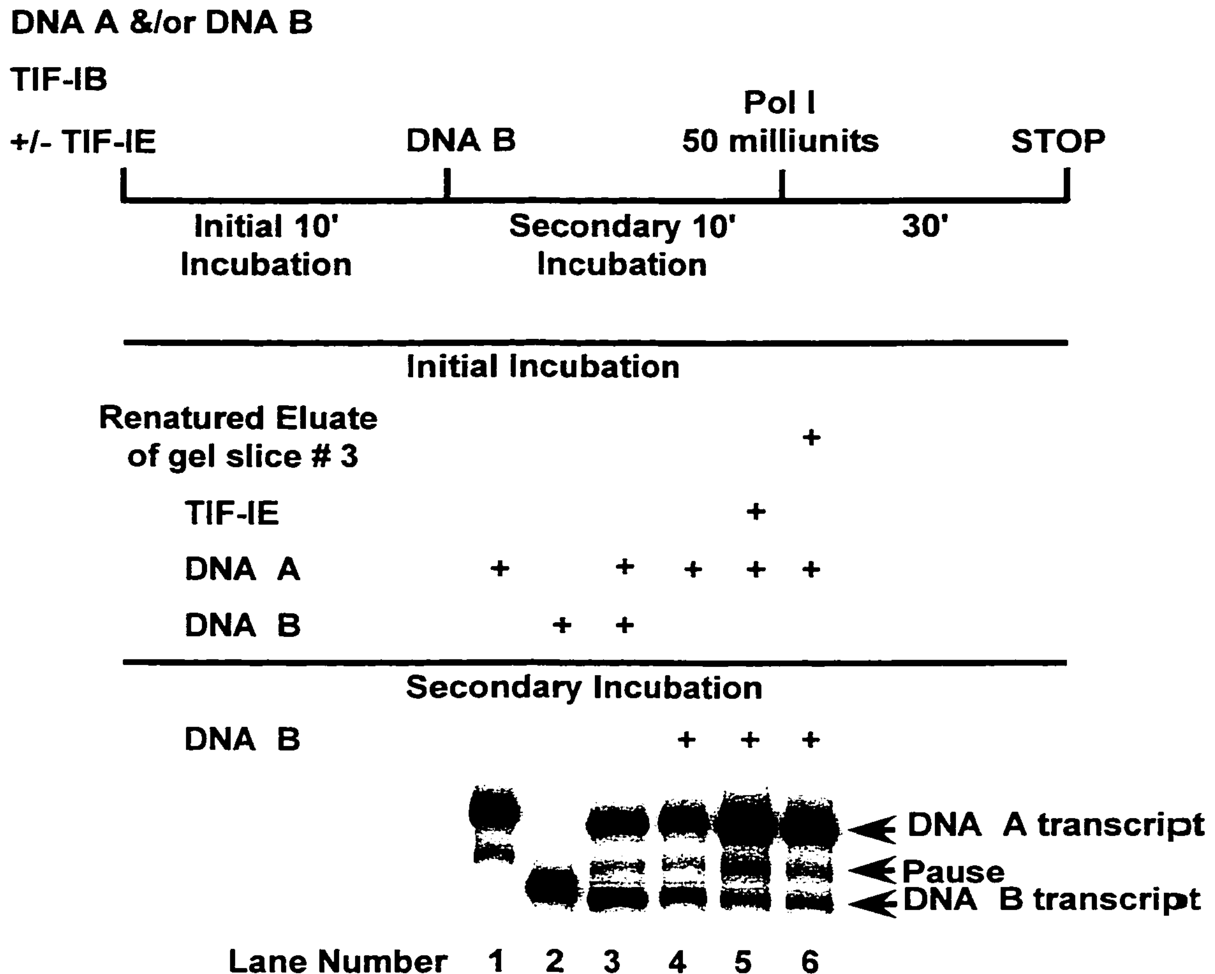

Fig. 2.11. The 141 kDa renatured polypeptide is able to confer commitment to glycerol gradient-purified TIF-IB. DNA A and DNA B produce 309 and 240 nucleotide runoff RNAs respectively (lanes 1, 2, and 3). Template commitment assays of glycerol gradient-purified TIF-IB alone (2 μl) (lane 4), or with 1 μl of TIF-IE (lane 5), or with 5 μl of the 141 kDa renatured eluate of gel slice # 3 (lane 6) are shown. DNA A and DNA B represent pEBH10/*Nde*I and pAr6/*Hind*III templates respectively. The RNA transcripts synthesized from DNA A and DNA B in lanes 3, 4, 5 and 6 were quantified and their ratios (A/B) in each lane are 1.7, 2.3, 11.96, 8.3 respectively.

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recruit CF to the promoter (Keys *et al.*, 1996; 1994). TIF-IE is similar to these two factors in that it is required for the formation of the committed complex in *A. castellanii.* Therefore, it is important to elucidate whether or not initial binding of TIF-IE to the rRNA promoter is required for recruiting glycerol gradient-purified TIF-IB and committing the rDNA template. A template binding order-of-addition assay was developed for that purpose in which two 5′-end-labeled rRNA promoter templates of different lengths were used. The differences in template length allowed separation of the different sized committed complexes in an EMSA (Fig. 2.12, lanes 4 and 5). When both DNA templates were present with TIF-IE and TIF-IB in the initial incubation period, equal amounts of complexes were formed on the two templates (lane 6). To determine if TIF-IE binds stably to the rRNA promoter before the recruitment of TIF-IB, TIF-IE was incubated with only one of the templates in an initial incubation period, followed by the addition of the second template along with TIF-IB in a secondary incubation period (Fig. 2.12, lanes 7 and 8). The same ratio of complexes formed as when both templates were present simultaneously in the initial incubation period (Fig. 2.12, compare lanes 6-8). Therefore, in the initial incubation, TIF-IE did not form a stable complex with the template. In contrast, TIF-IB forms a very weak and unstable complex that is later stabilized by the addition of TIF-IE in the secondary incubation period (Fig. 2.12, lanes 1, 2, 9 and 10). The addition of TIF-IE alone to the DNA templates did not result in formation of any stable complex in this portion of the gel (Fig. 2.12, lanes 11 and 12). We conclude that the initial binding of TIF-IE to the promoter is not required for the recruitment of TIF-IB, and

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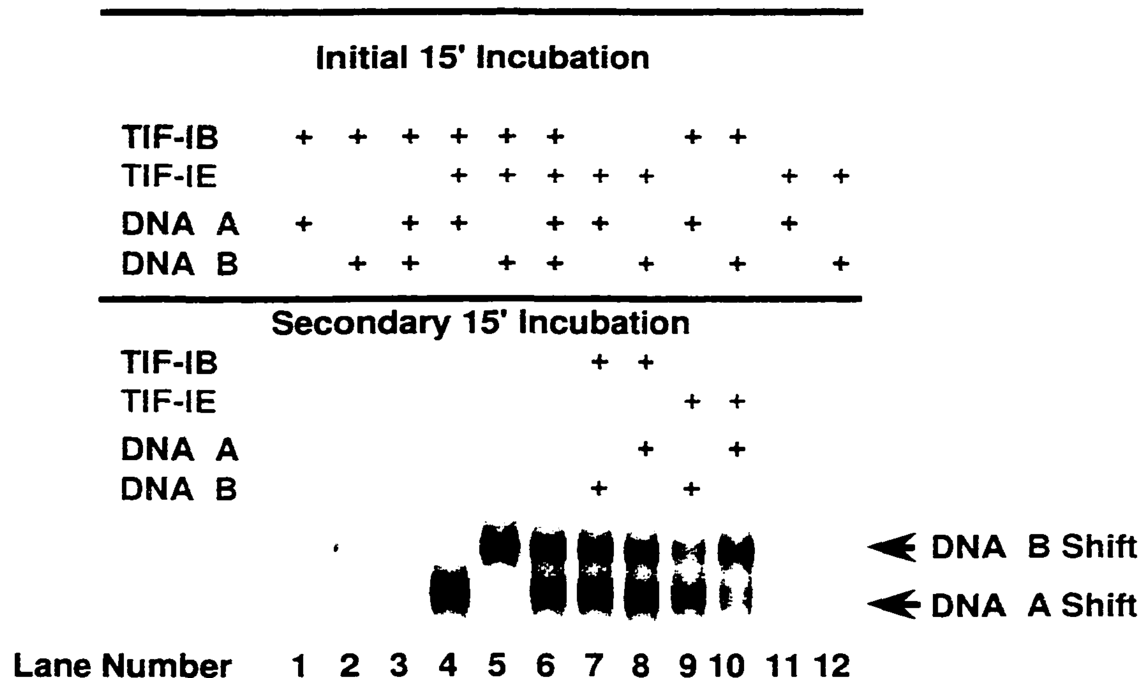

Fig. 2.12. The initial binding of TIF-IE to the rRNA promoter is not required for the recruitment of homogeneous TIF-IB. EMSAs showing complexes formed during the initial incubation period between DNA A and TIF-IB alone (1 μl) (lane 1) or plus TIF-IE (0.25 μl) (lane 4); DNA B and TIF-IB alone (lane 2) or with TIF-IE (lane 5); both DNA templates and TIF-IB alone (lane 3) or with TIF-IE (lane 6). EMSAs in lanes 7-10 show the effect of order of addition of the different factors and DNA templates on complex formation. EMSAs of TIF-IE sample alone with DNA A and DNA B are shown in lanes 11 and 12 respectively. The complexes formed in lanes 6, 7, 8, 9 and 10 on both templates were quantified and their ratios (B/A) in each lane are 0.89, 0.84, 0.76, 0.5, 1.9 respectively.

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it appears that TIF-IB may bind weakly to the promoter before TIF-IE joins the complex.

Glycerol gradient-purified TIF-IE contains a component that binds DNA in an EMSA, forming a complex with greater electrophoretic mobility than the committed complex (Fig. 2.4, Complex 2). However, consistent with our conclusion above, renatured TIF-IE does not form this complex in an EMSA (Fig. 2.13, lanes 5 and 6), suggesting Complex 2 was due to a contaminating protein in the glycerol gradient fraction.

We also noted that the DNA binding activity that produced Complex 2 sedimented similarly, but not exactly as TIF-IE in the glycerol gradient of Pol I (Fig. 2.14). To test this further, two fractions from the glycerol gradient, one that exhibited Complex 2 formation (fraction 6) and one that did not (fraction 10), were used to stimulate TIF-IB binding in an EMSA. The TIF-IE activities of the two were normalized and tested side by side (Fig 2.15). Clearly, both fractions could stimulate TIF-IB binding equally (Fig. 2.15, compare lanes 3 and 5, 8 and 10), but only fraction 6 formed Complex 2 (lanes 4 and 9). In these experiments, TIF-IB was not limiting because the same amounts of committed complex were formed when an additional increment of TIF-IB was added (lanes 3 and 8, 5 and 10). Therefore, the two TIF-IE activities were equalized as argued. We conclude from all of the above that TIF-IE does not form a complex with the DNA template in the absence of TIF-IB.

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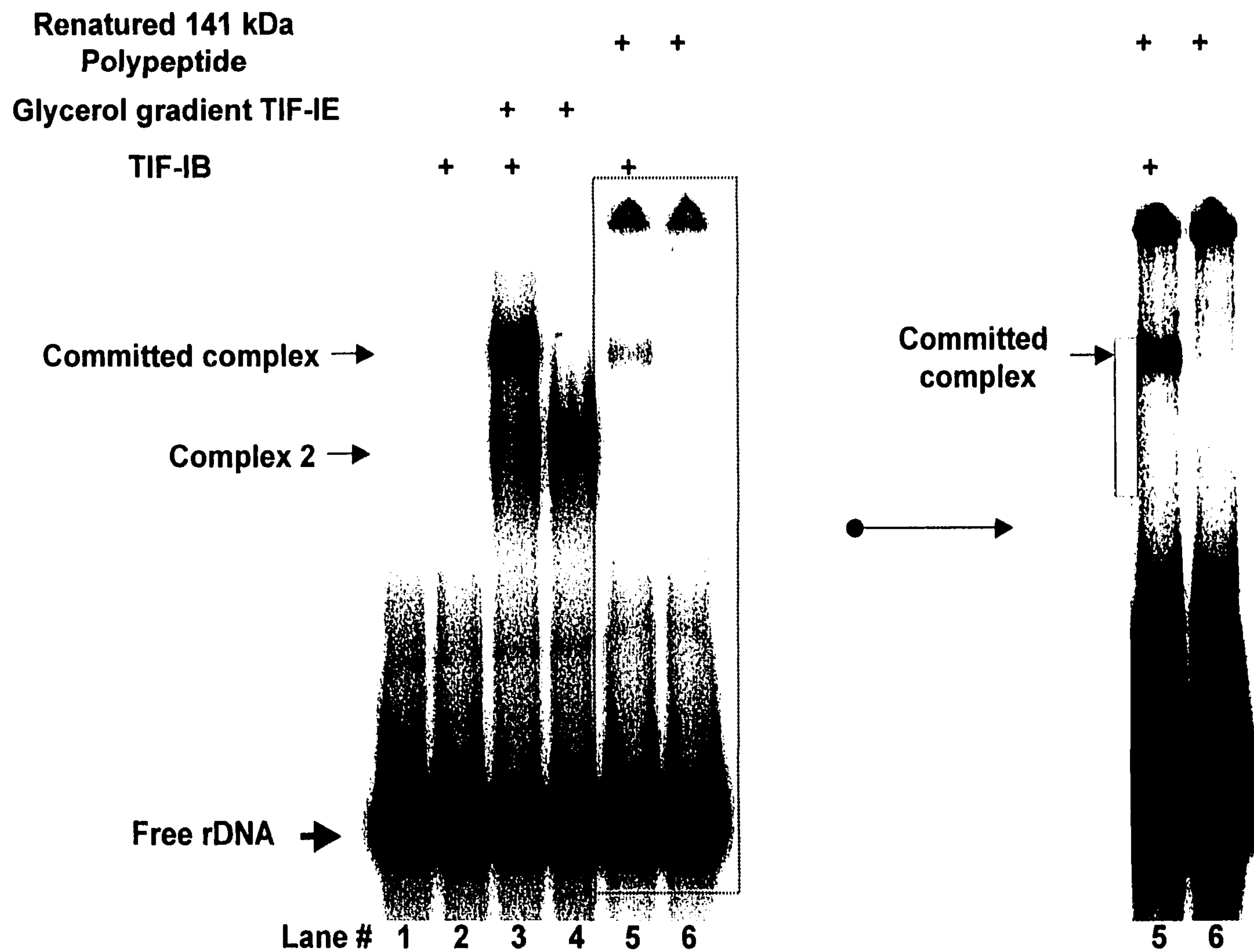

Fig. 2.13. The renatured 141 kDa polypeptide of TIF-IE does not form Complex 2 on the rRNA promoter seen in impure TIF-IE. EMSAs of 0.5 µl of glycerol gradient-purified TIF-IB alone (lane 2) or with 0.5 µl of impure TIF-IE (lane 3) or with 9.5 µl of the renatured 141 kDa TIF-IE (lane5). Lanes 4 and 6 show EMSAs of impure TIF-IE and the renatured TIF-IE respectively.

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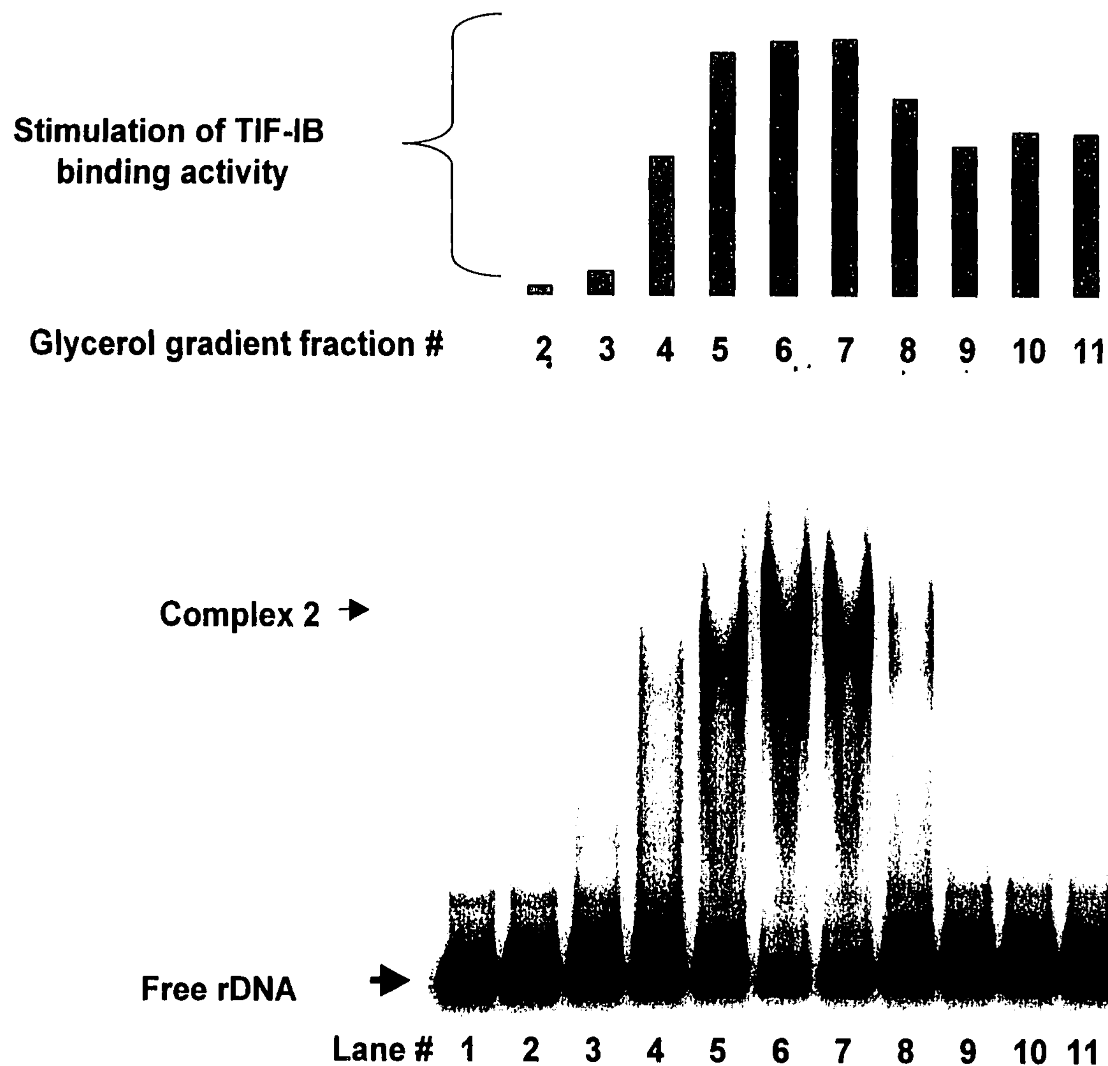

Fig. 2.14 The DNA-binding activity that produces Complex 2 sediments similarly as TIF-IE in the glycerol gradient of Pol I. EMSAs of 0.5 μl of glycerol gradient fractions of Pol I # 2-11 (lanes2-11). The bar graph above indicates TIF-IE activity in each fraction.

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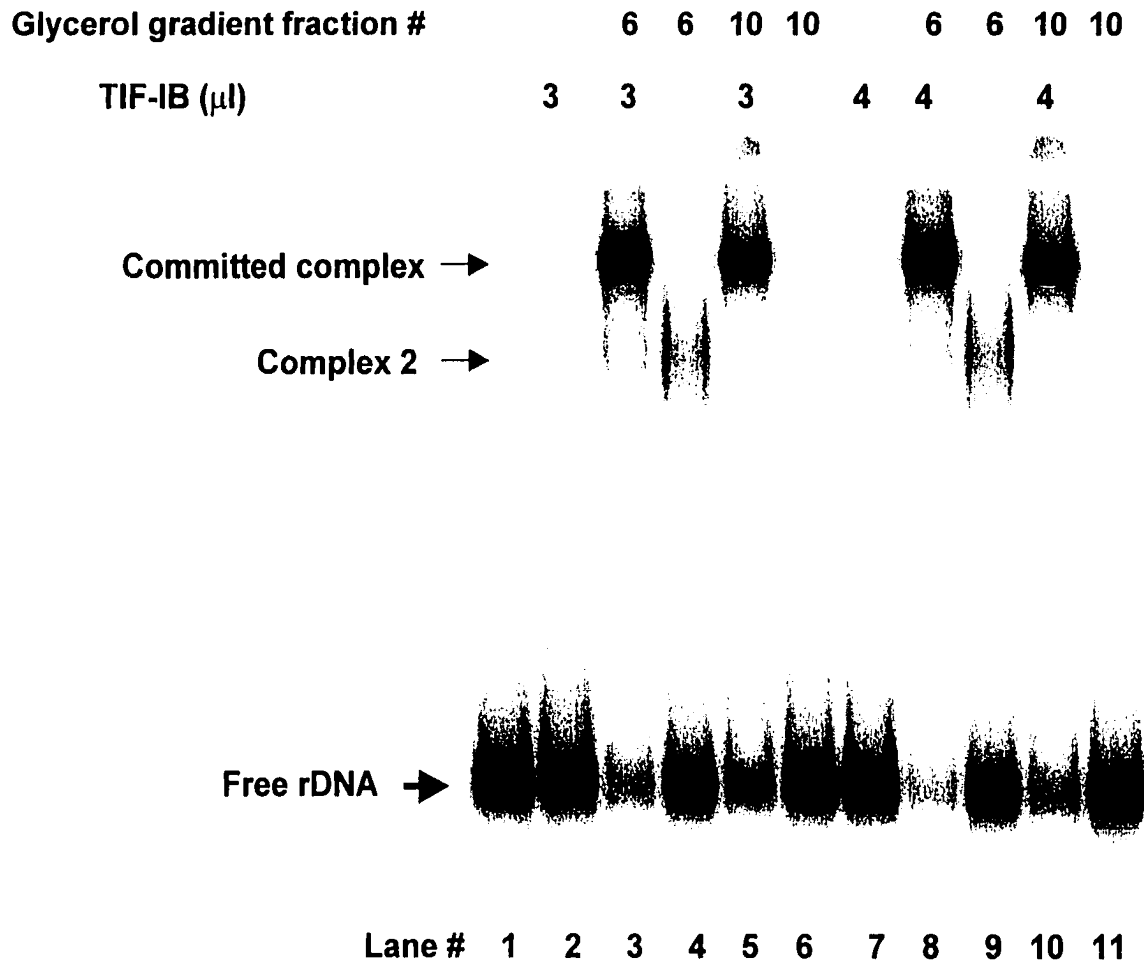

Fig. 2.15 TIF-IE activity does not correlate with the DNA-binding activity (Complex 2). EMSAs of glycerol gradient-purified TIF-IB alone (lane 2 and 7) or with 0.5 μl or 0.7 μl of glycerol gradient fractions of Pol I # 6 and # 10 respectively (lanes 3, 5, 8 and 10). EMSAs of 0.5 μl and 0.7 μl of glycerol gradient fractions of Pol I # 6 and # 10 are shown in lanes 4, 6, 9 and 11).

2.5 DISCUSSION

The fundamental transcription initiation factor, TIF-IB, was purified to apparent homogeneity as described by Radebaugh *et al.* (1998) with the addition of another round of promoter-DNA affinity chromatography, followed by rate zonal sedimentation in a glycerol gradient. While TIF-IB purified through one round of the promoter-DNA affinity column was capable of forming a persistent (committed) complex on the core promoter in a template commitment assay and in an EMSA, the glycerol gradient-purified TIF-IB was not. Apparently the modified purification procedure separated an additional component from TIF-IB. This component is essential for the formation of a stable complex on the rDNA promoter.

The component that is separated from TIF-IB appears also to be associated with RNA polymerase I. A novel transcription factor, TIF-IE, was separated from impure preparations of Pol I. TIF-IE is required for conferring commitment to glycerol gradient-purified TIF-IB in a template commitment assay. TIF-IE is speculated to be the same factor that is separated from TIF-IB during purification. We also show in an EMSA that a stable complex forms between homogeneous TIF-IB and the rDNA only in the presence of TIF-IE. Complex stabilization by TIF-IE is dose dependent; both EMSA and MPE·Fe(II) footprinting assays showed this.

TIF-IE can only be partially separated from Pol I by rate zonal sedimentation. Pol I purified through two rounds of glycerol gradient sedimentation is still associated with TIF-IE even though these gradients contain the nonionic detergent NP-40

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(data not shown). This explains why glycerol gradient-purified TIF-IB can still mediate specific transcription in the presence of Pol I even though it does not bind tightly to the rDNA promoter on its own.

Subunit composition of TIF-IE and its mechanism of action. Polypeptides associated with TIF-IE activity were individually excised from an SDS-polyacrylamide gel and renatured. EMSAs showed that a 141 kDa polypeptide stabilized TIF-IB binding to the promoter and template commitment assays confirmed this polypeptide was necessary for formation of the committed complex in combination with TIF-IB. The peak activity of TIF-IE sediments between the positions of bovine serum albumin (66 kDa) and aldolase (158 kDa), which is most consistent with a monomer of the 141 kDa polypeptide making up TIF-IE (data not shown).

In other eukaryotic systems, a factor similar to TIF-IE has not been reported yet. However, in other species, the core promoter-binding factor cannot commit the template on its own. In some vertebrates, the formation of the committed complex requires an accessory transcription factor, UBF, in addition to TIF-IB. UBF interacts with the UPE and the core promoter and helps recruit TIF-IB to the template (Jantzen *et al.*, 1992; 1990; Mcstay *et al.*, 1991a; Bell *et al.*, 1988; Learned *et al.*, 1986). Similarly in yeast, an additional transcription factor, UAF, is necessary for the formation of the committed complex. Like UBF, UAF binds to the UPE and, in conjunction with TBP, it facilitates the recruitment of CF to the rDNA promoter and enables the factors to form a stable committed complex (Keys *et al.*, 1996; 1994). *A. castellanii* TIF-IE is functionally similar to

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these two factors in that it is required for the formation of the committed complex. TIF-IE is also similar to UBF and UAF in its stimulatory effect on rRNA transcription. UBF and UAF stimulate but are not absolutely required for basal transcription, and their respective core promoter-binding factors form weak complexes with the promoter. However, the extent of stimulation by TIF-IE is less than UBF and UAF. This may be because Pol I used in the assays is contaminated with TIF-IE. However, it is still not clear whether TIF-IE is required for basal transcription since the Pol I used in the transcription assays is contaminated with TIF-IE even when highly purified. Even after multiple rounds of rate zonal sedimentation, a fraction of the TIF-IE remains associated with the polymerase.

Despite the functional similarities, TIF-IE differs from UBF and UAF in mechanism. TIF-IE does not bind to the rDNA promoter. UBF activity in rDNA transcription probably depends on its ability to bend and wrap DNA, a function that is mediated by the first few HMG box domains (Bazett-Jones *et al.*, 1994; Putnam *et al.*, 1994). In *A. castellanii*, it was previously shown that there is no significant wrapping or looping of the promoter DNA in the committed complex (Gong *et al.*, 1995). Therefore, it is unlikely TIF-IE is mechanistically homologous to UBF in the complex. Additionally, no UPE has been identified in *A. castellanii* (Paule, 1998b). It appears that TIF-IE interacts with TIF-IB, causing a conformational change that increases its binding affinity for the rDNA promoter. In addition, unlike UAF, TIF-IE alone is not sufficient for template commitment.

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TIF-IE also differs from UBF and UAF in structure. UBF purifies as a dimer (Bell *et al.*, 1990; Pikaard *et al.*, 1990b; Smith *et al.*, 1990; Bell *et al.*, 1988). In humans, rats, and mice, UBF is found in two isoforms referred to as UBF1 and UBF2 that have relative molecular weights of 97 and 94 kDa, respectively (Pikaard *et al.*, 1990a,b; Bell *et al.*, 1990; 1988). In *Xenopus laevis*, UBF1 and UBF2 have sizes of 82 and 85 kDa (Pikaard *et al.*, 1989). UBF consists of four to six copies of the HMG box, an amino-terminal domain that is required for dimerization, and a highly acidic carboxy-terminal domain. These structural features, including the primary sequences, are highly conserved from *Xenopus* to human (Jantzen *et al.*, 1992; 1990; Bacharov and Moss, 1991; McStay *et al.*, 1991a; O'Mahony *et al.*, 1991). UAF does not have any resemblance to UBF, consisting of six dissimilar subunits: three Pol I-specific subunits, Rrn5p, Rrn9p, Rrn10p of apparent molecular masses of 58, 50, 17 kDa, and histones H3 and H4 with relative molecular weights of 18 and 15 kDa respectively, and an uncharacterized 30 kDa protein, p30 (Keener *et al.*, 1997; Keys *et al.*, 1996). On the other hand, TIF-IE is a single 141 kDa polypeptide.

The general structural features of rDNA promoters are well conserved between the yeast promoter and the metazoan promoters from frog to human. It is noteworthy that *A. castellanii* is phylogenetically just above baker's yeast, Saccharomyces cerevisiae (Paule, 1998d). This conservation in the rDNA general promoter structure suggests a conservation of mechanisms of initiation used by Pol I transcriptional machinery. Thus, in view of the clear differences between TIF-IE, UBF, and UAF discussed above, a factor similar to TIF-IE may

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not have been discovered yet in other eukaryotes. Molecular cloning of the TIF-IE gene may help determine if any homologous genes exist in species whose genome sequences are known.

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CHAPTER 3

THE ISOLATION OF A RIBOSOMAL RNA PROMOTER-BINDING ACTIVITY ALONG WITH TIF-IE FROM RNA POLYMERASE I: ARE THESE TWO ACTIVITIES THE SAME?

This chapter describes the first attempts to purify TIF-IE. A rRNA promoter-binding activity was observed in TIF-IE samples and therefore was presumed to be the committing factor. However, the work presented in this chapter shows that it was not. Therefore, the data is not publishable at this time. I performed all the experiments and purified most of the proteins. Dr. Cathy Radebaugh and Dr. Gary Geiss provided me with TIF-IB and Pol I.

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THE ISOLATION OF A RIBOSOMAL RNA PROMOTER-BINDING ACTIVITY ALONG WITH TIF-IE FROM RNA POLYMERASE I: ARE THESE TWO ACTIVITIES THE SAME?

3.1 ABSTRACT

In *Acanthamoeba castellanii,* the formation of a stable committed complex during ribosomal RNA (rRNA) transcription initiation is achieved by the assembly of two trans-acting transcription initiation factors, TIF-IB and TIF-IE, on the rRNA core promoter. TIF-IE is required to stabilize the binding of TIF-IB to the promoter to commit the rDNA template. It is separated from heparin-Sepharose-purified RNA polymerase I (HS-purified Pol I) by rate zonal centrifugation in a glycerol gradient. A promoter-binding protein (BP) that binds specifically to the upstream region of the rRNA promoter from -83 to -63 relative to the transcription initiation site (tis) (+1) was observed in TIF-IE fractions. It was not clear whether this BP and TIF-IE are the same. A larger amount of this BP is present in early stages of TIF-IB purification and can be separated from TIF-IB by rate-zonal sedimentation in a glycerol gradient. After partial purification from TIF-IB, the BP was analyzed for the committing activity of TIF-IE. Results of template commitment assays show that it does not commit the rDNA template or stimulate transcription and therefore is different from TIF-IE.

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3.2 INTRODUCTION

In *Acanthamoeba castellanii,* specific initiation of ribosomal RNA (rRNA) transcription requires the accurate positioning of RNA polymerase I (Pol I) on the rRNA core promoter. This is achieved by the initial assembly of a stable committed complex between the two trans-acting transcription initiation factors, TIF-IB and TIF-IE, and the rRNA core promoter (Paule, 1998c; Radebaugh *et al.*, 1998; chapter 2 of this dissertation). Pol I is then recruited to the core promoter exclusively by protein-protein interactions with the committed complex to form the initiation complex (Bateman *et al.*, 1985; Kownin *et al.*, 1987; Bateman *et al.*, 1989; Paule, 1998c). Pol I alone preferentially binds nonspecifically to nicks, single-stranded gaps and ends with single-stranded extensions, and is therefore incapable of specific initiation (Paule *et al.*, 1984a; Paule *et al.*, 1984b).

Site-specific photo-cross-linking of the initiation complex near the transcription initiation site revealed that the addition of partially purified Pol I resulted in significantly increased proximity of TAF_I96 of TIF-IB to the DNA (Radebaugh *et al.*, 1997). In an attempt to relate this to commitment, Radebaugh *et al.* (1998) succeeded in separating a committing factor, TIF-IE, from heparin-Sepharose-purified RNA polymerase I (HS-purified Pol I). Geiss (1997) reported that the HS-purified Pol I alone protects the upstream portion of the rRNA promoter, from -81 to -65 relative to the transcription initiation site (tis) (+1), from MPE·Fe(II) cleavage. However, it was not clear whether this protection is due to the non-specific binding of Pol I to the promoter or the binding of a contaminating factor in the polymerase sample. It is noteworthy that this protected site slightly

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overlaps the region protected by the committed complex, which extends form -67 to -17.

In this work, we have separated a factor from the HS-purified Pol I by rate zonal sedimentation in a glycerol gradient that binds to the upstream portion of the rRNA promoter from -83 to -63. Interestingly, rate zonal sedimentation of HS-purified Pol I in a glycerol gradient also separates TIF-IE from Pol I (Radebaugh *et al.*, 1998). The sedimentation profile of the promoter-binding protein (BP) in the glycerol gradient is the same as TIF-IE. However, it was not clear whether these two proteins are the same.

TIF-IE is required for committing the rDNA template during rRNA transcription initiation (Radebaugh *et al.*, 1998). In this respect, it is similar to the upstream binding factor (UBF) in some metazoans and to the upstream activating factor (UAF) in yeast (Bell et al., 1990; Learned et al., 1986; Bell et al., 1988; McStay et al., 1991; Steffan et al., 1996; Keys et al., 1994; 1996). The ability of UBF or UAF to commit the rDNA template depends on their initial binding to an upstream promoter element (UPE) that extends 50-100 bp upstream of the core promoter element. Once bound to the UPE, they mediate the efficient recruitment and the stable binding of the core promoter-binding factor that leads to rDNA template commitment (Paule and White, 2000; Grummt, 1999; Reeder, 1999). Although a UPE and its cognate factor have never been found in *A. castellani* (Paule, 1998b), it is necessary to determine whether TIF-IE is the same factor that binds to the rRNA promoter from -83 to -63 and whether

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this site is required for its activity in template commitment. Therefore, we had to purify the BP and test it for TIF-IE activity.

3.3 MATERIALS AND METHODS

3.3.1 Protein purification from A. castellanii extracts

Purification of TIF-IB: TIF-IB was purified as described by Li (1995) or by Radebaugh *et al.* (1998). To initiate both purification approaches, TIF-IB was purified from a nuclear extract, using ammonium sulfate fractionation, DEAE Sepharose® Fast Flow and BioRex® 70 column chromatography. The Biorex 70 TIF-IB pool was divided into two, and one was fractionated by a heparin-ultrogel A4R column (Li, 1995) and the other by a single round of promoter-DNA SEPHAROSE CL-4B affinity chromatography followed by rate zonal sedimentation in a glycerol gradient (Radebaugh *et al.*, 1998).

Purification of Pol I and TIF-IE: Pol I was purified and its activity was assayed as described in chapter 2 (section 2.3.1). TIF-IE was separated from Pol I at the last step of purification, the rate zonal sedimentation in a glycerol gradient (chapter 2).

Purification of the promoter-binding protein (BP): The factor that binds to the upstream region of the rRNA promoter from -83 to -63 was purified from either Pol I or TIF-IB. It was separated from Pol I at the last step of purification, the rate zonal sedimentation in a glycerol gradient. Higher amounts of this BP were separated from TIF-IB that was purified by the method developed by Li (1995) (Fig. 3.1), by rate zonal sedimentation in a glycerol gradient.

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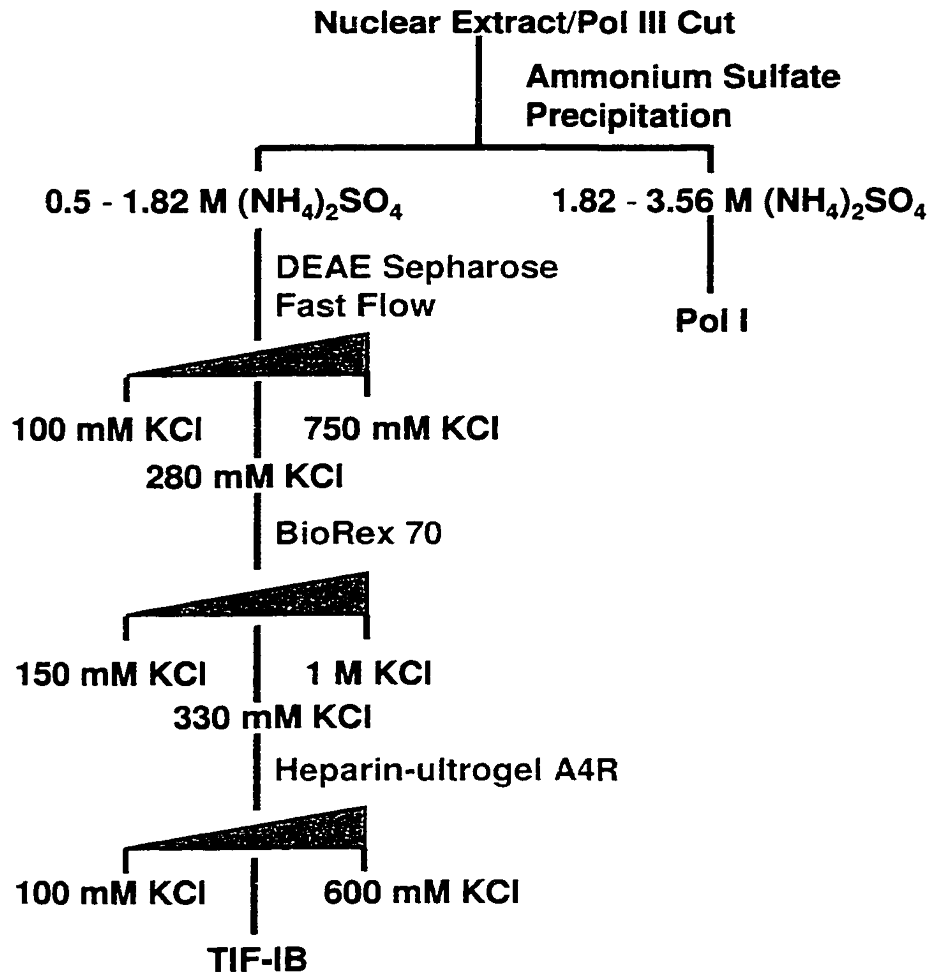

Fig. 3.1. Flow diagram for the purification of *A. castellanii* TIF-IB as described by Li (1995). Solid triangles indicate linear gradients, with the starting salt concentrations listed on the left and the final salt concentrations on the right.

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3.3.2 Plasmids and templates

The plasmids, pGG4C and pGG17C, contain a fragment of the *A. castellanii* rRNA promoter from -96 to +18 relative to the *tis* (+1) that is inserted in opposite orientations (Geiss *et al.*, 1997). For MPE·Fe(II) footprinting assays, the template strand and the RNA-like strand were prepared from pGG4C and pGG17C respectively as described in chapter 2 (section 2.3.2). The same ^{32}P-labeled template prepared from pGG17C was also used in the electrophoretic mobility shift assay (EMSA) and the binding competition assay.

In the binding competition assay, in addition to the ^{32}P-labeled probe, three unlabeled DNA templates were used. Two were used as specific competitor DNA:

- a 215-bp *NruI/SalI* fragment derived from pSBX60i (Paule *et al.*, 1984a), which contains the *A. castellanii* rRNA gene promoter from -55 to +19.
- a 143 bp *KpnI/SacI* fragment of pCR4 which contains the *A. castellanii* rRNA gene promoter from -91 to -50 subcloned into the *EagI* site of the pBluescript®SK (+/-) (Stratagene, La Jolla, CA) (Radebaugh, unpublished).

And one unlabeled DNA template was used as non-specific competitor DNA:

- a 156-bp *Eco*RV/*Hin*dIII fragment derived from pBR322.

For specific transcription run-off and template commitment assays, the templates were prepared from the plasmids, pEBH10 (Bateman *et al.*, 1985) and pAr6 (Radebaugh *et al.*, 1998), as described in chapter 2 (section 2.3.2). An additional template that produces a 307-nucleotide runoff RNA was prepared from pSBX60i by restriction digest with *SalI*.

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3.3.3 *MPE·Fe(II) footprinting*

The 5'-end-labeled 150 bp *Bam*HI/*Sac*I fragments of pGG4C and pGG17C were used for footprinting on the template and the RNA-like strand of the rRNA promoter respectively. MPE·Fe(II) footprinting was carried out as described in chapter 2 (section 2.3.5). The binding reaction volume was sometimes scaled up to 40 μl instead of 20 μl, but the same footprinting conditions were maintained.

3.3.4 *Specific transcription run-off assay*

The assay conditions were the same as described for a template commitment assay in chapter 2 (section 2.3.4). Transcription reactions were initiated by the addition of individual fractions of TIF-IB (1-2 μl) and 20-30 milliunits of Pol I to linearized plasmid DNA (pEBH10/*Nde*I or pSBX60i/*Sal*I) in a final volume of 50 μl. Reactions were incubated for 2-30 min at 25°C and then terminated and processed as described in chapter 2.

3.3.5 *Electrophoretic mobility shift assay*

The 5'-end-labeled 150 bp *Bam*HI/*Sac*I fragment of pGG17C was used for EMSAs of TIF-IB and the BP. The binding conditions for the BP were the same as in EMSA of TIF-IB. EMSAs were carried out as described in chapter 2 (section 2.3.3).

3.3.6 *Binding competition assay*

In this assay, TIF-IB alone or with increasing amounts of unlabeled competitor DNA was preincubated for five minutes at 25°C. The binding

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conditions were the same as described for an EMSA. The addition of the 5'-end-labeled 150 bp *Bam*HI/*Sac*I fragment of pGG17C followed and incubation continued for another 20 min. Samples were analyzed by EMSA.

3.3.7 Template commitment assay

The assay conditions were the same as described in chapter 2 (section 2.3.4). The minimum amount of template required to bind all the available TIF-IB in the reaction was determined for pAr6/*Hind*III (DNA A) and pEBH10/*Nde*I (DNA B) and used in the following protocol. The first template(s) (DNA A and/or DNA B) was preincubated with TIF-IB alone or with either the BP or TIF-IE for 10 min. at 25°C. The second template (DNA B) or buffer was then added, and preincubation continued for another 10 min. RNA synthesis was initiated by the addition of Pol I and proceeded for 30 min. Transcription was terminated and the reactions were processed as described in chapter 2.

3.4 RESULTS

3.4.1 A rRNA promoter-binding protein (BP) is separated from heparin-Sepharose-purified Pol I by rate zonal sedimentation in a glycerol gradient.

MPE·Fe(II) footprinting of equal amounts (105 milliunits in a nonspecific assay) of each of the HS-purified and glycerol gradient-purified Pol I alone on the RNA-like strand of the rRNA promoter revealed a difference between the two preparations. HS-purified Pol I protects the upstream region of the promoter, from -83 to -63, from MPE·Fe cleavage (Fig. 3.2, lanes 3-5) while glycerol

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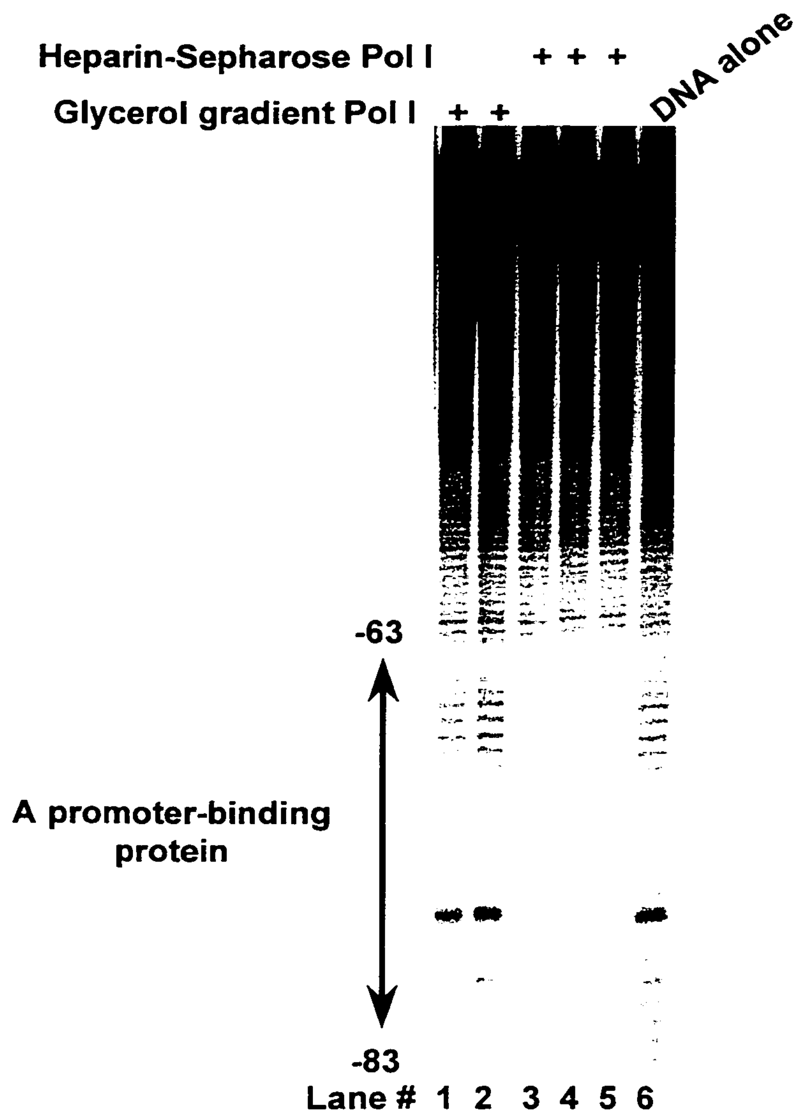

Fig. 3.2. Heparin-Sepharose-purified Pol I contains a promoter-binding component. MPE·Fe(II) footprints of equal amounts (105 milliunits) of glycerol gradient-purified Pol I (lanes 1 and 2) and heparin-Sepharose-purified Pol I (lanes 3-5).

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gradient-purified Pol I does not (lanes 1 and 2). Since the more homogeneous Pol I preparation did not protect the rRNA promoter at all, this either indicates that its binding to the promoter is unstable or that the footprint produced by the HS-purified Pol I fraction is not due to the non-specific binding of Pol I. To determine if a contaminating factor in the polymerase sample is binding to that upstream promoter region and is separated from Pol I by rate zonal sedimentation in a glycerol gradient, a total of 23 glycerol gradient fractions were assayed by MPE·Fe(II) footprinting on the RNA-like strand. As shown in Fig. 3.3, a factor that binds to the promoter from -83 to -63 sediments at the top of the gradient, in fractions 4-8 (lanes 4-8). These fractions do not exhibit any polymerase activity as detected by a non-specific transcription assay (Fig. 3.4). The peak activity of Pol I sediments in glycerol gradient fractions 15 and 16. These fractions do not protect the rRNA promoter from MPE·Fe (II) cleavage (Fig. 3.3, lanes 15 and 16). Therefore, this confirms that the footprint upstream of the rRNA promoter from -83 to -63 is not due to non-specific binding of Pol I. It is noteworthy that rate zonal sedimentation of HS-purified Pol I in a glycerol gradient also separates TIF-IE from Pol I (Radebaugh *et al.*, 1998). TIF-IE activity co-sediments[1] in the glycerol gradient with the BP.

[1] The sedimentation profile of TIF-IE in a glycerol gradient is shown in chapter two.

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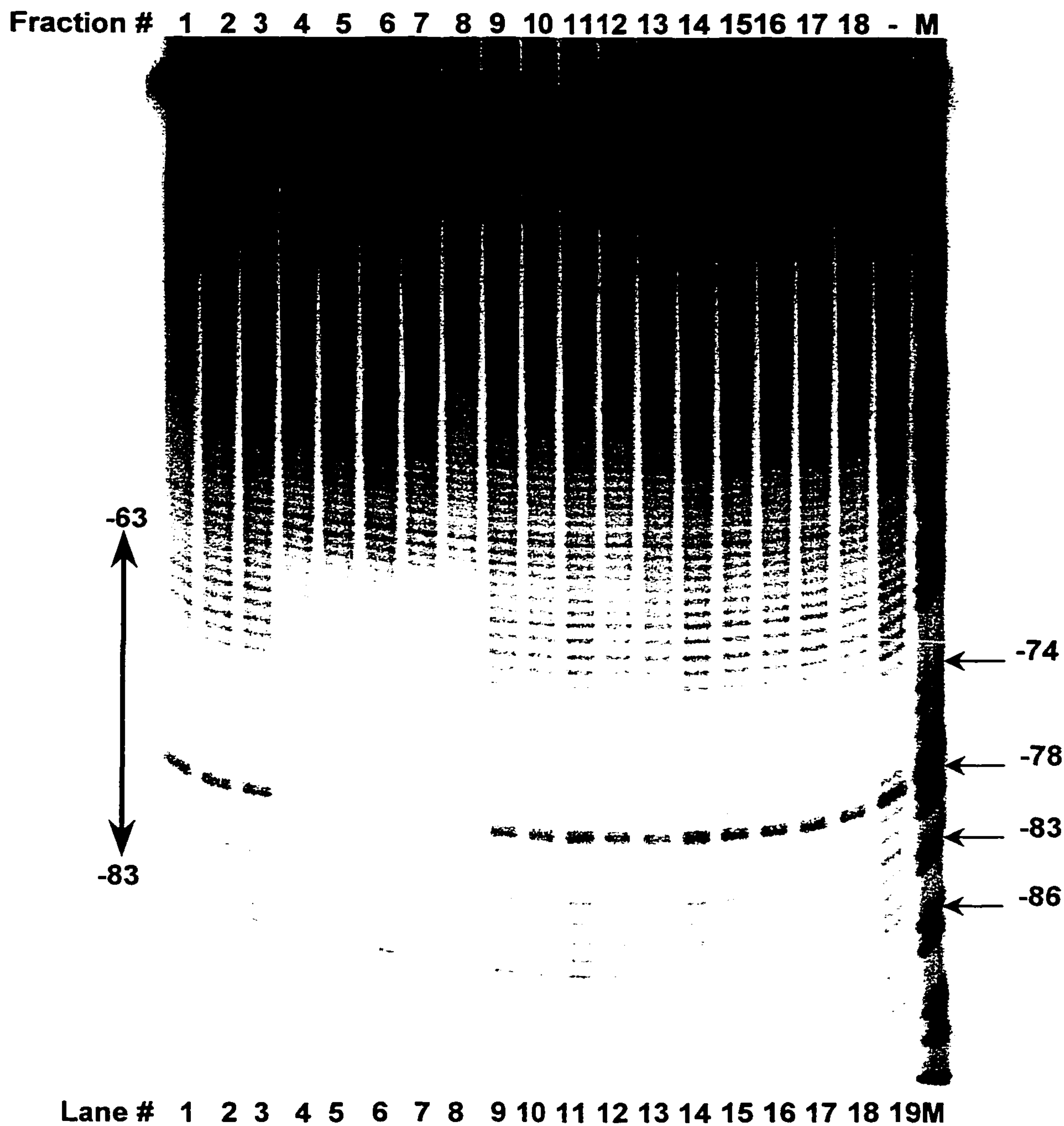

Fig. 3.3. A promoter-binding component is separated from the heparin-Sepharose-purified Pol I by rate zonal sedimentation in a glycerol gradient and it sediments at the top of the gradient. MPE·Fe(II) footprints of 8 μl of glycerol gradient fractions of Pol I (# 1-18) on the RNA-like strand of the rRNA promoter.

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Fraction #	Pol I activity
1	0.183615002
2	0.280501212
3	0.032816298
4	0.096886214
5	-0.023440213
6	0.113294363
7	0.178145619
8	0.228151406
9	0.01718949
10	0.084384767
11	0.053131149
12	0.614914921
13	2.352616044
14	12.8467994
15	35.08843616
16	32.12246788
17	17.85067543
18	7.141451557

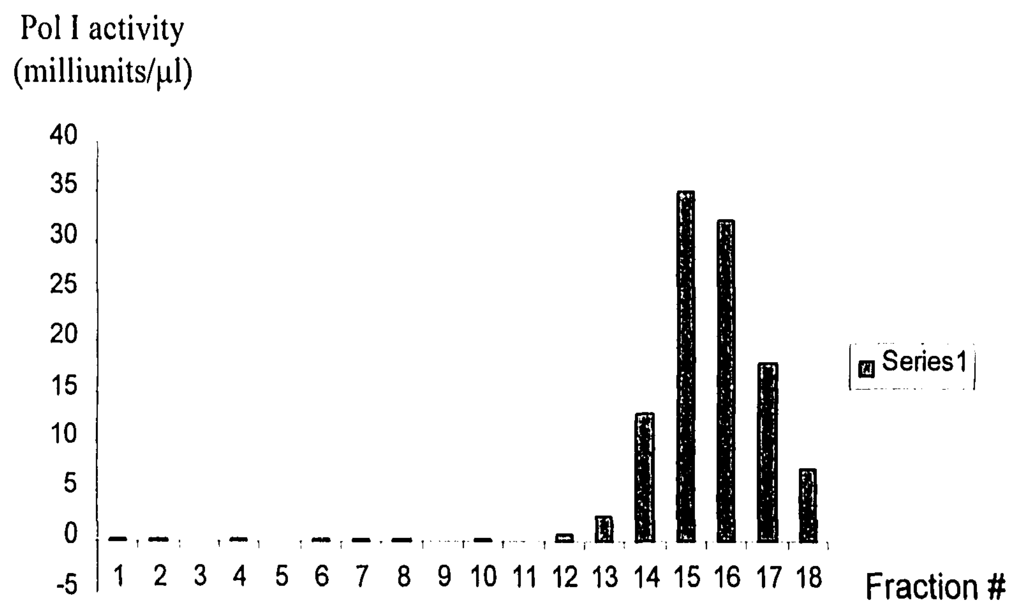

Fig. 3.4. Pol I sediments in glycerol gradient fractions 12-18 with the peak activity in fractions 15 and 16. 8 μl of glycerol gradient fractions (# 1-18) were assayed for Pol I activity by a non-specific transcription assay.

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3.4.2 The formation of the committed complex on the rRNA promoter causes a change in the footprint of the BP.

Geiss (1997) reported that the protection of the upstream region of the rRNA promoter, from -83 to -63, from MPE·Fe(II) cleavage by HS-purified Pol I alone was reduced significantly upon the formation of the committed complex. It was interpreted that the unknown factor that binds to that upstream region has a lower affinity for its site than the transcription initiation factors involved in the committed complex formation. This line of reasoning was further examined by MPE·Fe(II) footprinting (Fig. 3.5). The results showed that the committed complex[2] alone protects the promoter from -67 to -17 (compare lane 1 with lanes 4 and 7) and that increasing amounts of the BP alone binds from -83 to -63 (lanes 2 and 3). Therefore, the committed complex binding site overlaps with the binding site of the BP by 5 base pairs. When the committed complex is formed in the presence of the BP (lanes 5 and 6; 8 and 9), the protection of the upstream region of the promoter from -83 to -63 from MPE·Fe(II) cleavage is not only significantly reduced but also different. If enough of the BP is present along with promoter-DNA affinity purified TIF-IB, the resulting upstream footprint persists but becomes smaller (lanes 6 and 9). Instead of a 20 bp footprint, from -83 to -63, the footprint becomes only about 11 bp, from -81 to -70. In addition to the explanation suggested by Geiss (1997) that the committed complex has a higher

[2] Promoter-DNA affinity-purified TIF-IB is presumably still associated with TIF-IE and therefore can form the committed complex by itself as discussed in chapter 2.

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Promoter-binding protein (BP) (μl) 8 16 8 16 8 16

Promoter-DNA affinity TIF-IB (μl) 1 .5 1 .5 1 .5 1 1 1 M

-17

-63

-67

-70

-78

-81

-83

-83

Lane # 1 2 3 4 5 6 7 8 9 10

Fig. 3.5. The formation of the committed complex causes a change in the promoter-binding protein (BP) footprint. MPE·Fe(II) footprints of the BP (lanes 2 and 3), promoter-DNA affinity-purified TIF-IB alone (lanes 4 and 7), or with the BP (lanes 5, 6, 8 and 9) on the RNA-like strand of the rRNA promoter. The region from -71 to -77 is not cleaved by MPE·Fe(II) efficiently (lane 1).

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affinity to its binding site than the BP, the footprinting results here can be interpreted as follows. TIF-IB in the committed complex might be interacting with the BP causing a conformational change that results in the smaller upstream footprint. If this interaction is real, it could be very significant in instigating the formation of a stable committed complex leading to RNA template commitment. This notion may be supported by the inconclusive results in Fig. 3.5 (lanes 8 and 9); as more of the BP is added to the affinity-purified TIF-IB, the level of promoter protection from MPE·Fe(II) cleavage increases. These preliminary results motivated us to pursue our goal to purify the BP and examine it for TIF-IE activity.

3.4.3 The BP can be separated from TIF-IB.

Based upon the hypothesis that TIF-IE is the factor that binds to the upstream region of the rRNA promoter, we attempted to purify the BP to homogeneity and test it for the committing activity of TIF-IE. Higher amounts of the BP can be separated from TIF-IB when purified as described by Li (1995) (Fig. 3.6). The BP is separated from TIF-IB at the last step of purification, the heparin-ultrogel A4R column chromatography. It elutes from the column at 220-330 mM KCl while TIF-IB elutes at 430 mM. Li (1995) showed that it produces a DNase I footprint from -60 to -90 on the upstream region of the rRNA promoter. This is very similar to the MPE·Fe(II) footprint of the BP present in TIF-IE fractions.

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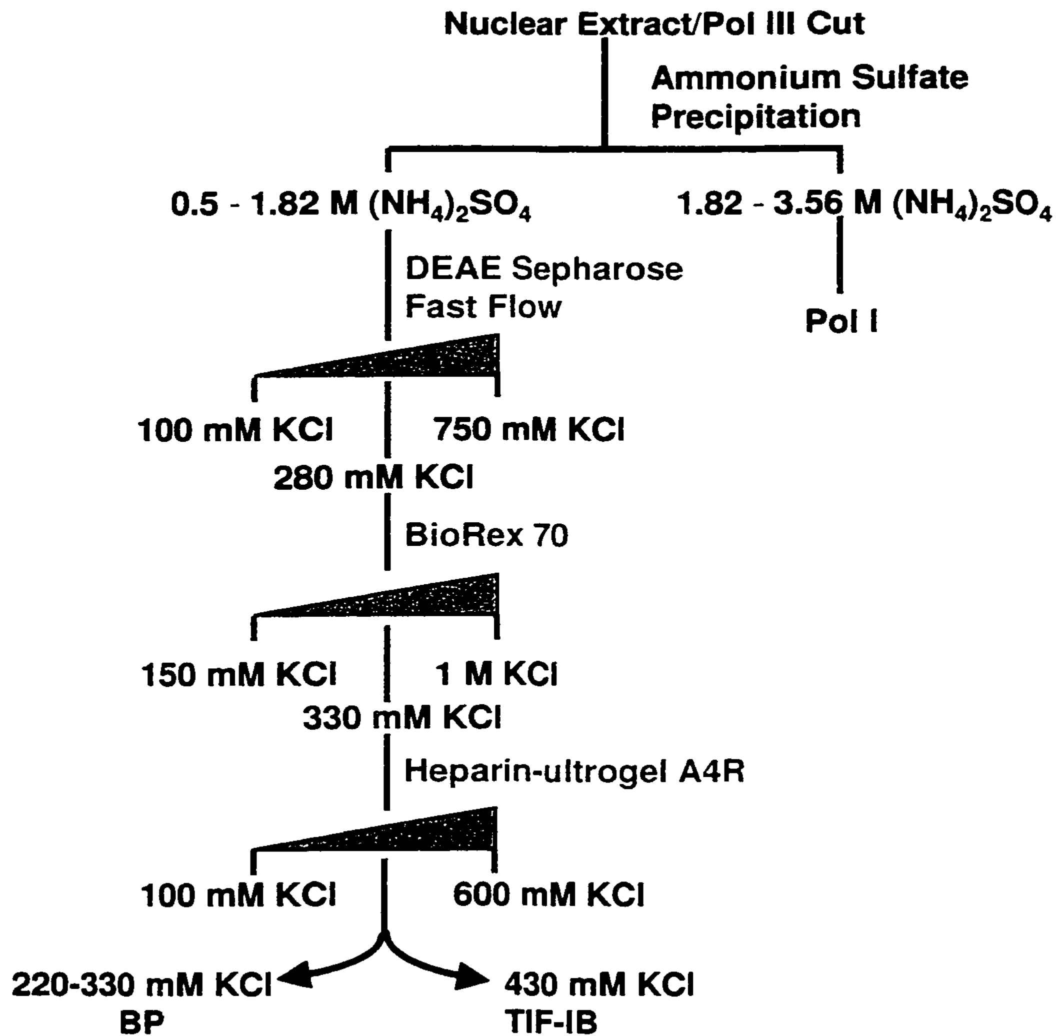

Fig. 3.6. Flow diagram for the purification of *A. castellanii* promoter-binding protein (BP) from TIF-IB. Solid triangles indicate linear gradients, with the starting salt concentrations listed on the left and the final salt concentrations on the right.

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Implementing Li's purification method, TIF-IB activity, as detected by a specific transcription run-off assay, eluted from the heparin-ultrogel column between 314 to 414 mM KCl in fractions 31-35 (Fig. 3.7, lanes 9-11). MPE·Fe(II) footprinting was used to detect BP in the eluate of this column. The BP elution starts at 300 mM KCl (Fig. 3.8, lane 12). However, the peak of BP activity overlapped with TIF-IB, in fractions 29-32 (Fig. 3.8, lanes 15-18). It should be noted here that the footprint of the BP is reduced because these fractions have TIF-IB activity as shown in Fig. 3.7. However, the protection of the promoter at –78 was indicative of the BP activity in fractions 25-32. Rate zonal sedimentation in a glycerol gradient separated the BP from TIF-IB. The BP sediments in fractions 8 to 12 at the top of the glycerol gradient (Fig. 3.9, panel B, lanes 5-7) while most of the TIF-IB sediments in fractions 20 to 22 at the bottom of the gradient (Fig. 3.9, panel A, lanes 10 and 11). The sedimentation profile of the BP in the glycerol gradient appears different from the one shown in Fig. 3.3, where it sediments in fractions 4-8, because a total of 38 fractions were obtained instead of 23.

3.4.4 The BP binds specifically to the upstream region of the rRNA core promoter.

An EMSA was developed to assay for the BP (Fig. 3.10). The BP produces a doublet shift (lane 3) that is lower in the gel than the committed complex shift (lane 2). This doublet shift is also noticeable in the affinity-purified TIF-IB lane. To determine if the doublet shift in the TIF-IB sample binds specifically to the upstream region of the promoter from -83 to -63, a binding

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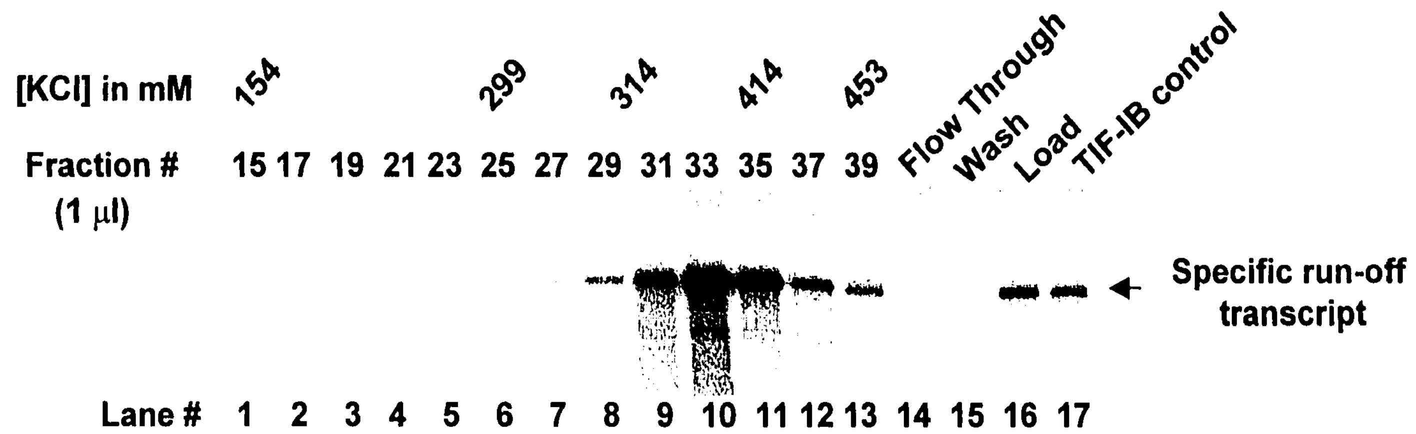

Fig. 3.7. Elution profile of TIF-IB from the heparin-ultrogel A4R column. The specific run-off transcription product phosphor image and corresponding fraction number and its [KCl], flow-through, wash, load, and a positive TIF-IB control are indicated. Transcription is initiated by adding individual TIF-IB fractions and 30 milliunits of Pol I to 50 ng of the linearized plasmid pEBH10/*Nde*I. The RNA synthesis phase proceeded for 30 min.

Fig. 3.8. The promoter-binding protein (BP) is still associated with TIF-IB. MPE·Fe(II) footprints of 8 μl of fractions15-32 (lanes 1-18) eluted from the heparin-ultrogel A4R column. The BP elution starts in fraction 25 (lane 11) but it is still associated with TIF-IB in fractions 29-32 (lanes 15-18).

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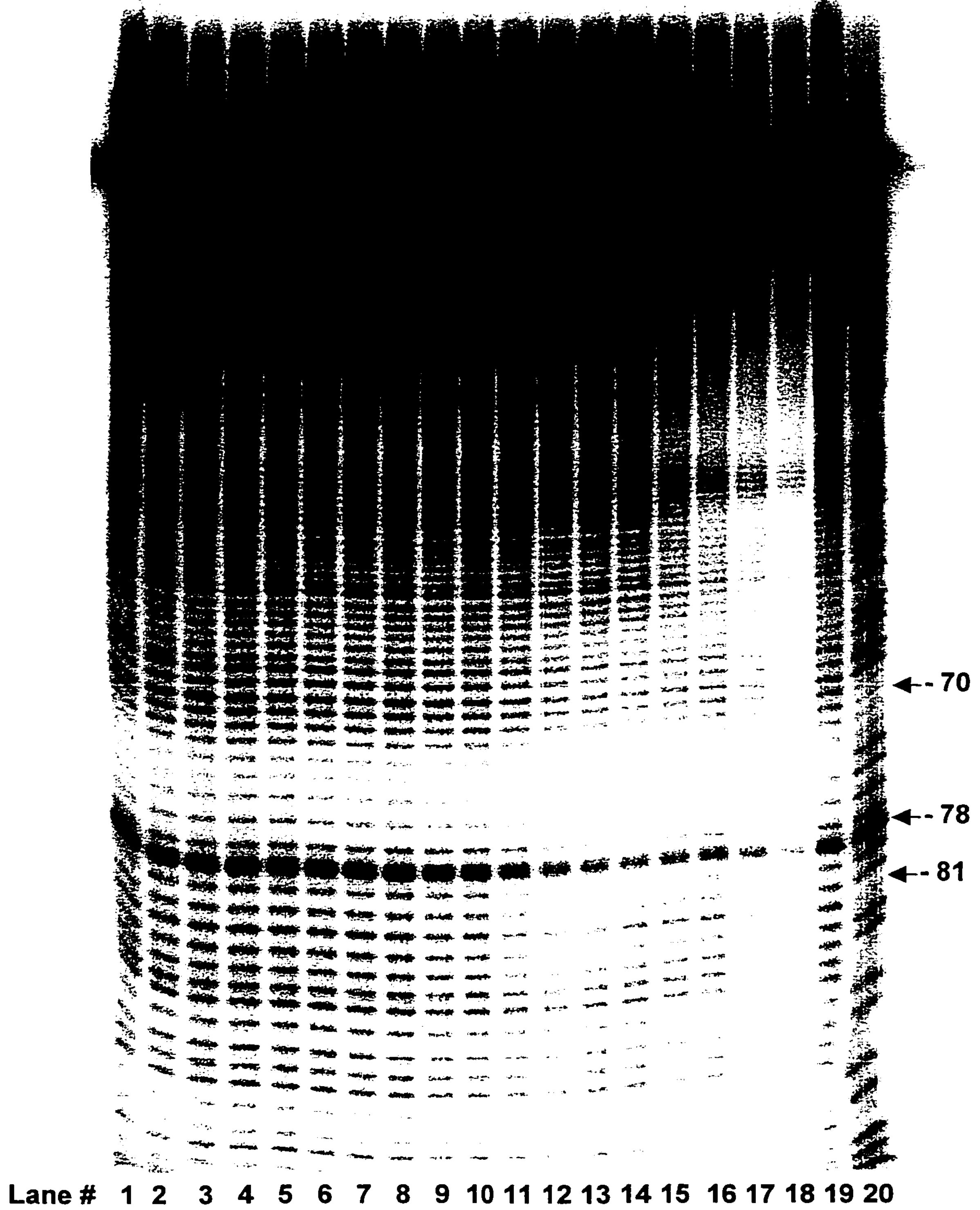

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Fig. 3.9. Rate zonal sedimentation in a glycerol gradient separated the promoter-binding protein (BP) from TIF-IB. Panel (A) shows the specific run-off transcription product phosphor image and the corresponding fraction number. 2 μl of individual glycerol gradient fractions of TIF-IB and 30 milliunits of Pol I were added to 50 ng of pEBH10/*Nde*I to start the transcription phase which proceeded for 30 min. Panel (B) shows the MPE·Fe(II) footprints of 16 μl of every other glycerol gradient fraction (# 2-20) (lanes 2-12) and 8 μl of fractions 20 and 22 (lanes 13 and 14). The BP sediments in fractions 8-12 (lanes 5-7).

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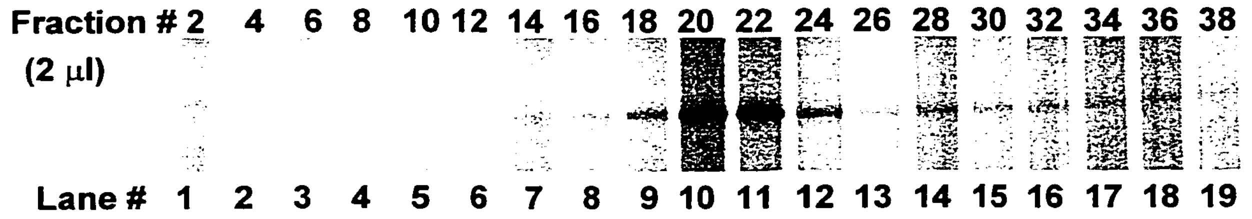
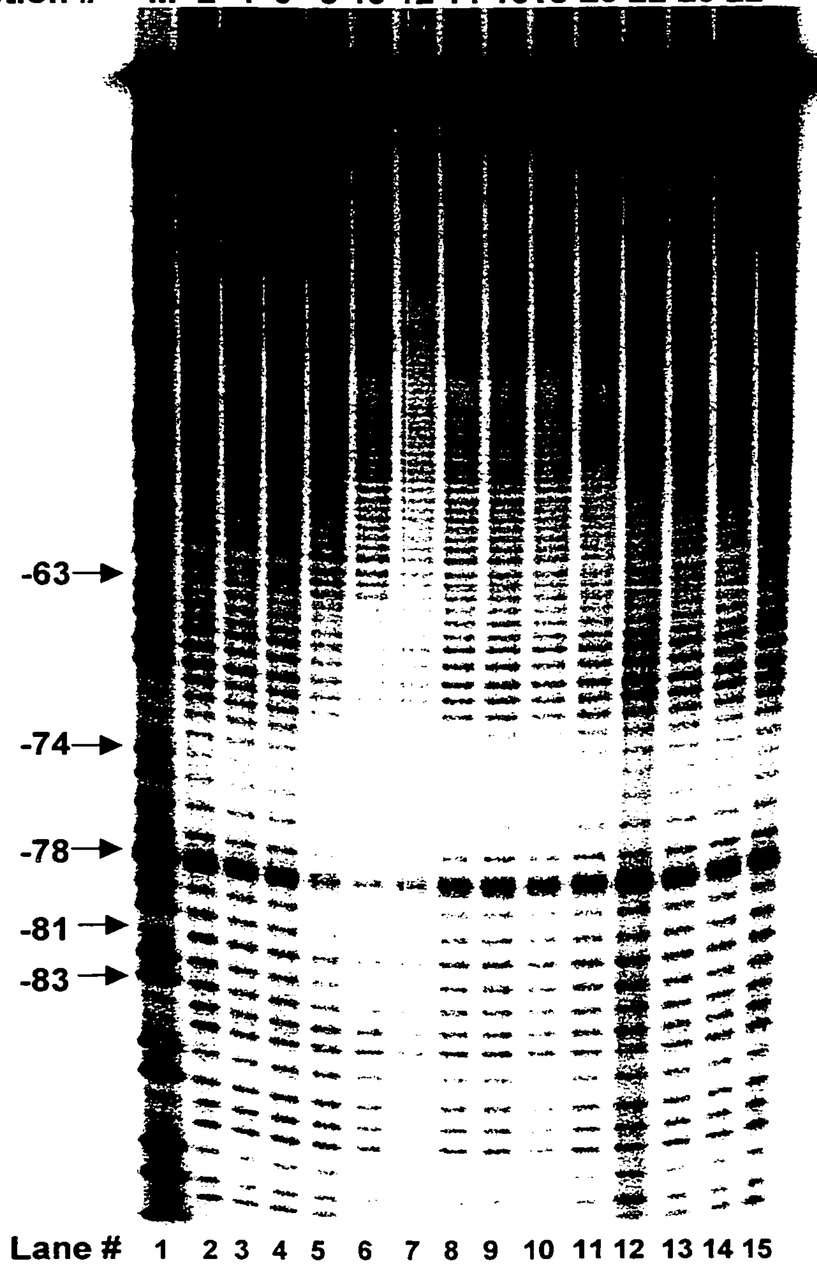
(A)



(B)



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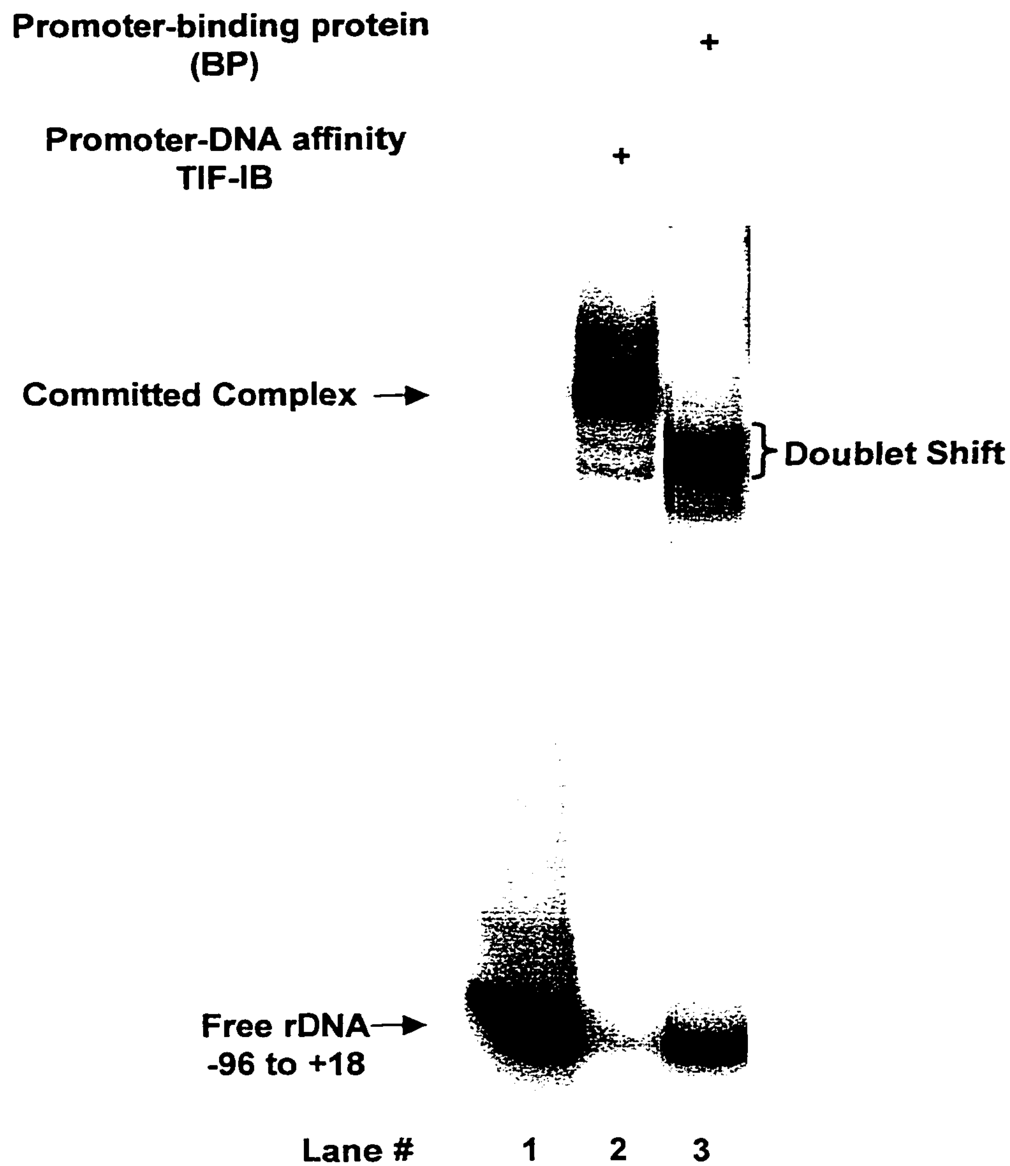

Fig. 3.10. An EMSA was developed to assay for the BP activity. EMSAs of promoter-affinity-purified TIF-IB (2 μl) (lane 2) and 4 μl of the BP that is separated from TIF-IB (lane 3). The BP produces a doublet shift.

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competition assay was used (Fig. 3.11). Preincubation of TIF-IB sample with increasing amounts of an unlabeled DNA competitor that contains the promoter region from -91 to -50, competed for binding of the factor that produces the doublet shift to the ^{32}P-labeled DNA but not the committed complex (lanes 3 and 4). Preincubation with increasing amounts of an unlabeled DNA competitor that contains the promoter region from -55 to +19 competed for binding of TIF-IB/TIF-IE to the ^{32}P-labeled DNA, but not the BP doublet (lanes 7 and 8). Preincubation with random competitor DNA did not affect the formation of any of the complexes (lanes 5 and 6). We conclude that the binding of the factor to the upstream region of the promoter from -83 to -63 is specific. It is noteworthy that the BP produces the same doublet shift in an EMSA when complexed with a ^{32}P-labeled fragment of the rRNA promoter containing only the region extending from -91 to -50 (data not shown). Some TIF-IE fractions contain a promoter-binding contaminant (chapter 2) that produces a different complex (Complex 2) than the BP doublet.

3.4.5 Is the BP the same as the committing activity (TIF-IE).

After partially purifying the BP, it was analyzed for TIF-IE activity in a template commitment assay (Fig. 3.12). The minimum amount of a promoter-containing DNA template[3] (DNA A and/or DNA B) needed to bind all the available glycerol gradient-purified TIF-IB was determined. This DNA was first incubated with glycerol gradient-purified TIF-IB either alone or with added BP. The DNA was given the opportunity to form a complex with the proteins. This complex was

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[3] DNA A and DNA B are pAr6/*Hind*III and pEBH10/*Nde*I, respectively.

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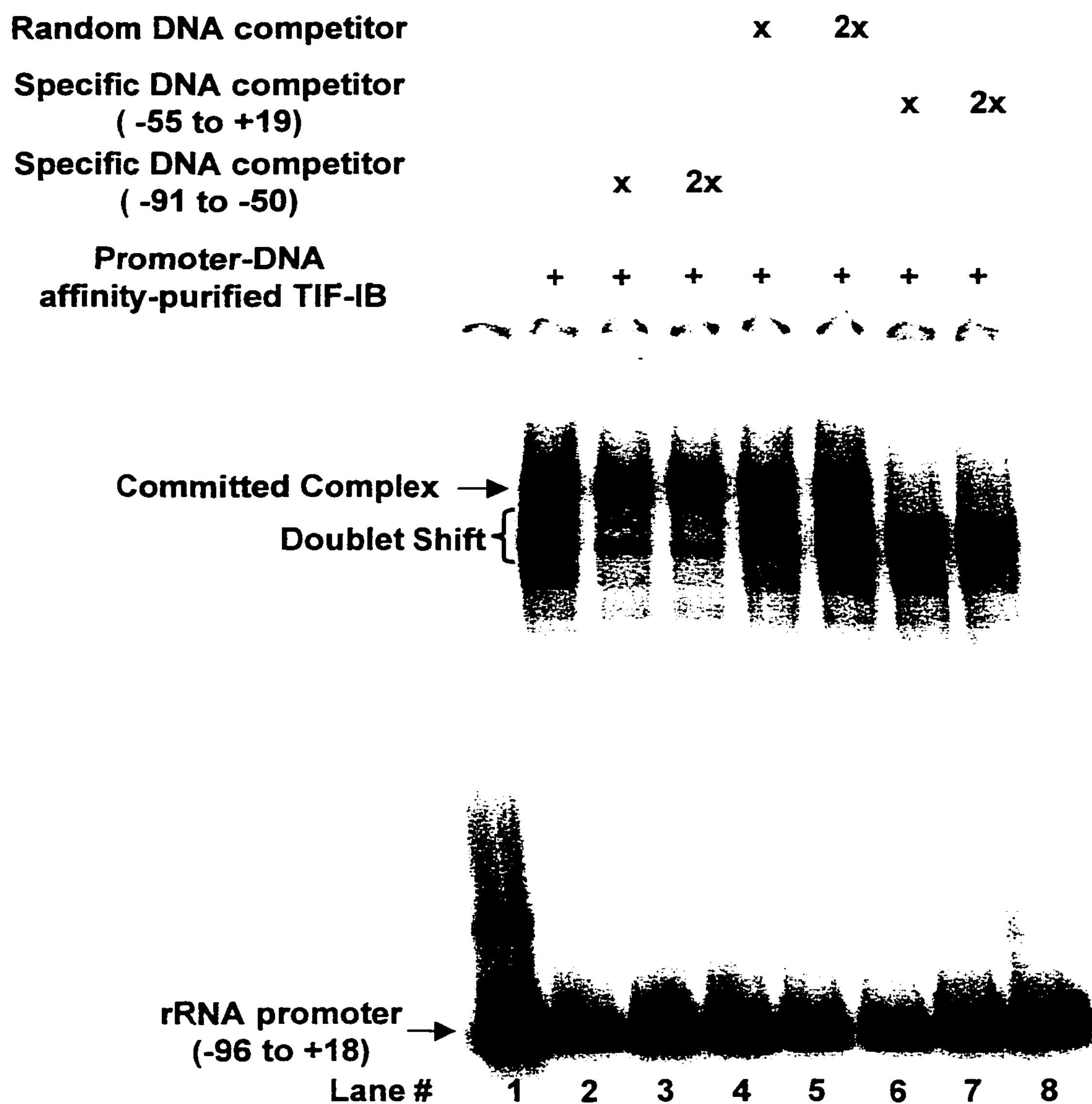

Fig. 3.11. The promoter-binding protein (BP) binds specifically to the upstream region of the rRNA promoter. EMSAs of 2 μl of TIF-IB (lanes 2-8), competed with an unlabelled specific competitor DNA (lanes 3 and 4; 7 and 8) or competed with an unlabelled random competitor DNA (lanes 5 and 6).

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DNA A &/or DNA B
TIF-IB
+/- BP

DNA B

Pol I
20 milliunits

STOP

Initial 10' Incubation

Secondary 10' Incubation

30'

Initial Incubation							
BP (μl)					0.25	0.5	0.75
DNA A	+		+	+	+	+	+
DNA B		+	+				
Secondary Incubation							
DNA B				+	+	+	+
Lane Number	1	2	3	4	5	6	7

DNA B transcript

DNA A transcript

Fig. 3.12. The promoter-binding protein (BP) does not commit the rDNA promoter. DNA A and DNA B produce 240 and 309 nucleotide runoff RNAs respectively (lanes 1, 2, and 3). Template commitment assays of glycerol gradient-purified TIF-IB (1 μl) (lane 4), with the addition of increasing amounts of the BP (lanes 5-7). DNA A and DNA B are pAr6/*Hind*III and pEBH10/*Nde*I, respectively.

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then challenged with a second promoter-containing DNA template (DNA B) that was allowed to form a complex with the proteins during a secondary incubation period. Pol I was then added to start the RNA synthesis phase, which proceeded for 30 min. Recall that Pol I contains TIF-IE, so once it is added, committed complexes can form and transcription can proceed. DNA A and DNA B produced 240 and 309 nucleotide runoff RNAs respectively (lanes 1 and 2). A pause product at about 270 nucleotides was also produced from DNA B (lane 2). DNA A and DNA B were transcribed equally when they were both present in the initial incubation period (lane 3). When only DNA A was present with TIF-IB in the initial incubation period and DNA B was added during the secondary incubation period (lane 4), transcription levels from both templates were similar to when both templates were present simultaneously in the initial incubation period (compare lanes 3 and 4). This result was expected since glycerol gradient-purified TIF-IB is unable to form a stable complex on the first DNA template during the initial incubation period (Radebaugh *et al.*, 1998). Therefore, stable complexes form on both DNA after Pol I is added. The addition of increasing amounts of the BP along with TIF-IB to DNA A in the initial incubation period (lanes 5-7) did not result in the exclusion of transcription of the template (DNA B). This exclusion only takes place whenever the transcription initiation factor(s) forms a stable complex on the first DNA template during the initial incubation period. Therefore, the BP did not confer commitment upon TIF-IB, as does TIF-IE. In addition to the committing activity, TIF-IE stimulates transcription about 3 fold. The BP did not affect transcription levels at all. Therefore, we conclude that the BP is not TIF-IE.

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Several other observations provided evidence to support this conclusion. First, similar transcription stimulation levels by TIF-IE were observed from rRNA promoter templates containing or lacking the upstream region of the promoter from -83 to -63 indicating that TIF-IE stimulating activity does not need that site (Fig. 3.13). Second, later preparations of TIF-IE separated from Pol I by rate zonal sedimentation in a glycerol gradient showed no binding to the upstream region of the promoter. Glycerol gradient fractions 3, 6, and 9 exhibit the committing and stimulating activities of TIF-IE (Fig. 3.14, lanes 6-8). However, these same fractions do not produce the upstream footprint on the RNA-like strand of the rRNA promoter (Fig. 3.15, lanes 5, 8, and 11). Moreover, 105 milliunits of the HS-purified Pol I, from which the TIF-IE was obtained, does not footprint in that upstream region at all (lane 16). These overall observations are sufficient to conclude that the unknown protein that binds to the upstream region of the rRNA promoter is not the same as TIF-IE.

3.5 DISCUSSION

Purification of RNA polymerase I to homogeneity separates it from TIF-IE and a DNA-binding factor whose function is unknown. MPE·Fe(II) footprinting assays revealed that impure preparations of Pol I, purified through the heparin-Sepharose column, protect the rRNA promoter between –63 and –83. However, further purification of Pol I by rate zonal sedimentation in a glycerol gradient results in the loss of this footprinting activity. This is due to the loss of an unidentified protein (BP) from Pol I that binds specifically to the

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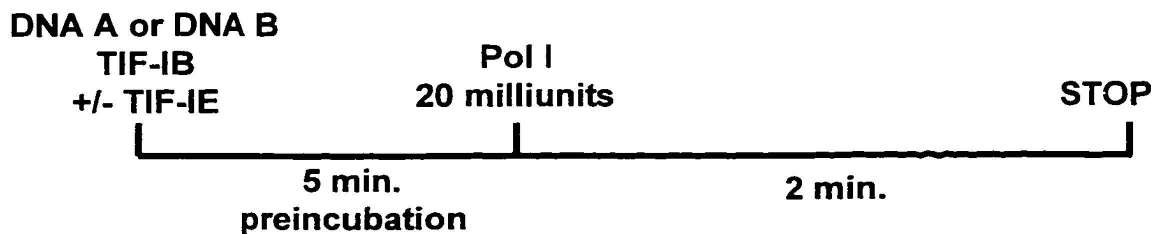

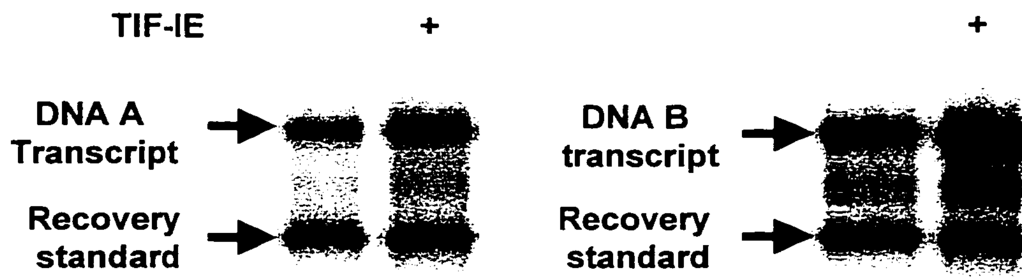

Fig. 3.13. TIF-IE stimulates transcription from rRNA promoter templates containing or lacking the -83 to -63 site. The specific run-off transcription product phosphor image from DNA A and DNA B are indicated. Transcriptions were initiated by the addition of glycerol gradient-purified TIF-IB (1 μl) alone or with 0.25 μl TIF-IE and 20 milliunits of Pol I to equimolar amounts of DNA A or DNA B. The RNA synthesis phase proceeded for 2 min. DNA A and DNA B are pSBX60i/*Sal*I and pEBH10/*Nde*I, respectively.

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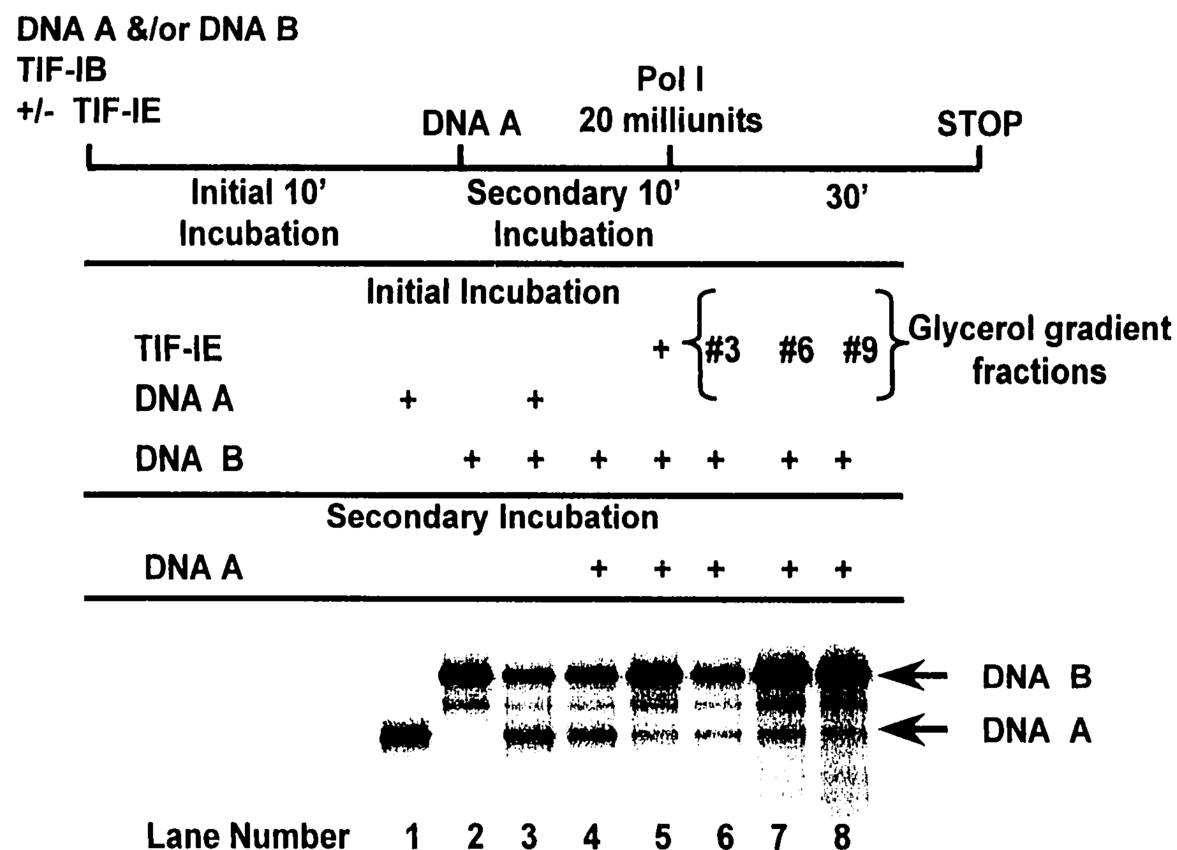

Fig. 3.14. TIF-IE is separated from HS-purified Pol I by rate zonal sedimentation in a glycerol gradient. DNA A and DNA B produce 240 and 309 nucleotide runoff RNAs respectively (lanes 1, 2, and 3). Template commitment assays of glycerol-gradient TIF-IB alone (2.5 μl) (lane 4), or with TIF-IE (0.25 μl) (lane 5), or with 0.5 μl of glycerol gradient fractions of Pol I # 3, 6 and 9 (lanes 6-8). DNA A and DNA B are pAr6/*Hind*III and pEBH10/*Nde*I, respectively.

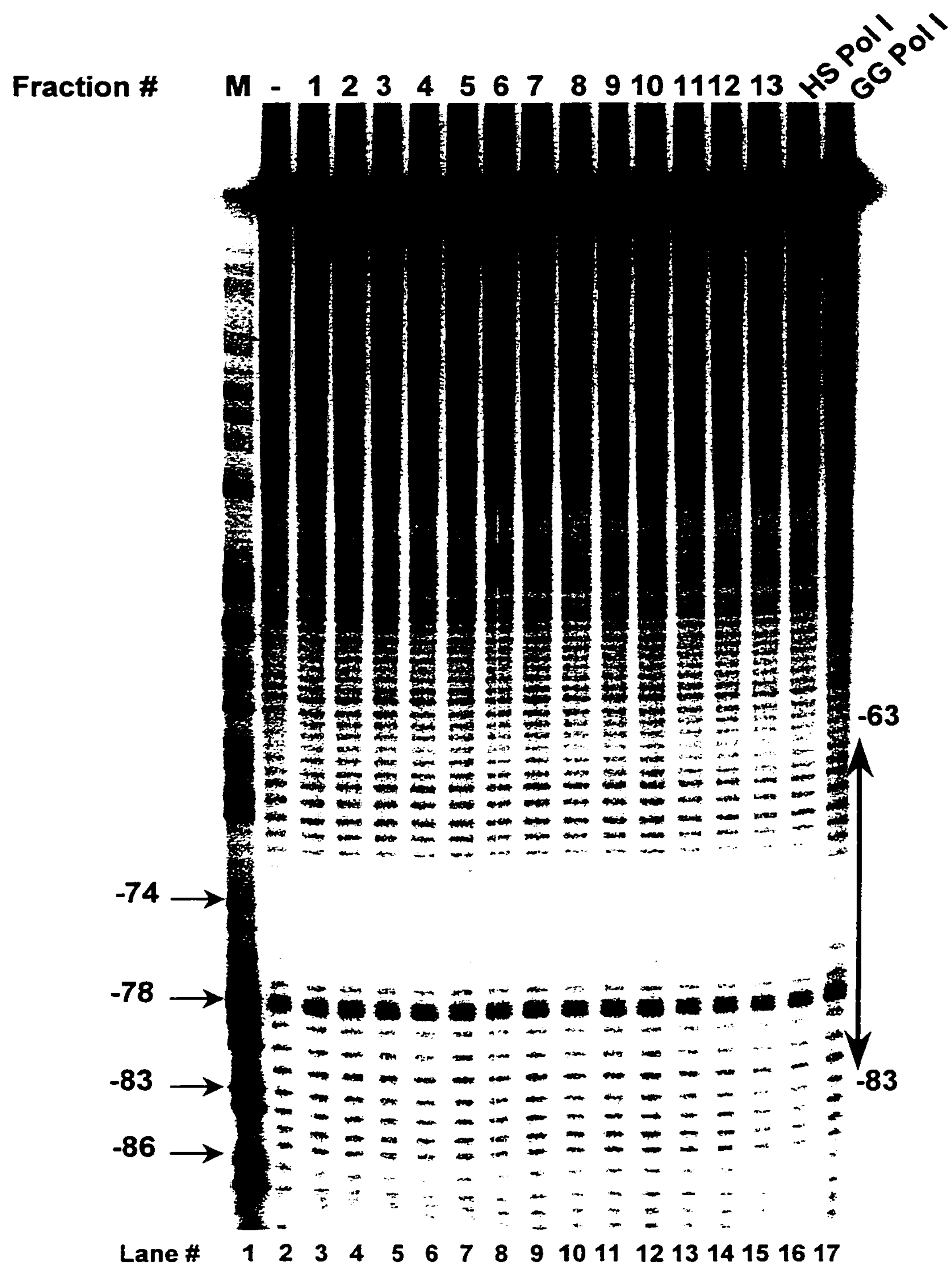

Fig. 3.15. TIF-IE does not bind to the upstream region of the rRNA promoter from -83 to -63. MPE. Fe(II) footprints of 8 μl of glycerol gradient fractions of Pol I # 1-13 (lanes 3-15), 105 milliunits of heparin-Sepharose-purified Pol I (lane 16), and 105 milliunits of glycerol gradient-purified Pol I (lane 17).

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promoter from -83 to -63 during purification. Rate zonal sedimentation in a glycerol gradient also separates TIF-IE from Pol I. TIF-IE is needed for homogeneous TIF-IB to form a stable committed complex on the promoter. The BP co-sediments with TIF-IE, suggesting they might be the same protein. In addition, MPE·Fe(II) footprinting suggested that the factor(s) in the committed complex may be interacting with the BP. Since we speculated that the BP and TIF-IE were the same, this interaction could be significant in stabilizing TIF-IB binding to the promoter and therefore committing the rDNA template. However, the studies presented in this chapter argue against this.

The promoter-binding activity and TIF-IE are most likely not the same factor. The one time co-purification of BP and TIF-IE from Pol I made it necessary to investigate whether these two activities are the same. The notion that these two factors may be the same was intriguing since other committing factors, UBF and UAF in some vertebrates and yeast respectively, had a DNA-binding activity. UBF and UAF bind to the UPE in order to commit the rDNA template (Bell *et al.*, 1990; Learned *et al.*, 1986; Bell *et al.*, 1988; McStay *et al.*, 1991; Steffan *et al.*, 1996; Keys *et al.*, 1994; 1996; Paule and White, 2000; Grummt, 1999; Reeder, 1999).

Results of template commitment and footprinting assays clearly indicated that the protein that binds specifically to the promoter from –83 to –63 is not capable of committing the rDNA template and that TIF-IE does not bind to that upstream region of the promoter. In addition, TIF-IE can stimulate transcription from the rDNA promoter lacking the upstream region.

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The BP was separated from Pol I and TIF-IB but its function, if any, in rRNA transcription is still unknown. Li (1995) found that it can inhibit or stimulate rRNA transcription depending on its concentration. However, further studies are required to characterize this factor.

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CHAPTER 4

ADDITIONAL OBSERVATIONS AND FUTURE DIRECTIONS

After the partial characterization of *Acanthamoeba castellanii* TIF-IE, several interesting questions remain to be answered. i) Is TIF-IE a novel RNA Polymerase I (Pol I) transcription factor? ii) Is it required for basal ribosomal RNA (rRNA) transcription or is its activity only stimulatory? iii) Is it identical to the *A. castellanii* rRNA enhancer binding factor (EBF)? iv) Is TIF-IE a homologue to yeast Rrn3p? To answer these questions, the following studies are proposed.

4.1 Is TIF-IE a novel transcription factor?

Molecular cloning of the gene encoding TIF-IE may facilitate the search for potential homologues of this factor based on similarities in their genes' primary sequence. In order to clone the TIF-IE gene, identification of its internal peptide sequence is required. TIF-IE fractions, separated from Pol I by rate zonal sedimentation, are not homogeneous and therefore cannot be used for protein sequence analysis. Instead, the 141 kDa polypeptide of TIF-IE was

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excised from a 6% SDS polyacrylamide gel and sent to *The Rockefeller University Protein/DNA Technology Center* for sequencing. The microchemistry and protein sequencing procedures implemented were: In-gel protein digestion with trypsin, tentative protein identification by MALDI-TOF MS[1] analysis of the intact digestion mixture, HPLC purification of the peptides and MALDI-TOF MS to determine the best peptides to sequence, then finally Edman sequence analysis. The MALDI-TOF MS and the protein sequencing data are included in appendix I. The MS data did not match anything in the database and therefore TIF-IE could be a novel protein. Attempts to clone the TIF-IE gene by RT-PCR, using the data obtained from the internal peptide sequencing, is currently underway. The identification of the TIF-IE gene would make it possible to determine if homologous factors have been found in other systems. By using a similar approach, we have found that one of the *A. castellanii* TIF-IB subunits, TAF_I96, is a homologue of the yeast core factor Rrn7p (Radebaugh, unpublished).

4.2 TIF-IE, an essential or stimulatory activity?

TIF-IE stimulates rRNA transcription about 3-fold in specific run-off transcription assays (chapter 2 of this thesis). However, Pol I used in those assays was contaminated with TIF-IE even when highly purified. Therefore, the full effects of TIF-IE on rRNA transcription could not be determined. To overcome this problem, it may be possible to immunodeplete TIF-IE from transcription reactions. In this case, if transcription is completely inhibited and the inhibition is

[1] Matrix-Assisted-Laser-Desorption-Ionization Time-of-Flight Mass Spectrometry

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relieved by the addition of TIF-IE, this indicates that TIF-IE is required for basal levels of rRNA transcription. If transcription is only reduced by TIF-IE immuno-depletion, this indicates that its effect on rRNA transcription is more likely stimulatory. In order to carry out these experiments, antibodies against TIF-IE can be raised using synthetic peptides derived from the protein sequencing data, and following the immunization procedure described by Harlow and Lane (1988).

4.3 Are the two transcription factors, TIF-IE and EBF, identical?

A rRNA enhancer binding factor has been identified in *A. castellanii* and was referred to as EBF (Enhancer Binding Factor). Several features of this factor suggested that it is a functional homologue of the vertebrate Upstream Binding Factor (UBF) (Yang *et al.*, 1995). Although UBF has been found in human (Jantzen *et al.*, 1990), rat (O'Mahony and Rothblum, 1991), mouse (Pikaard *et al.*, 1990; Hisatake *et al.*, 1991), and *X. laevis* (McStay *et al.*, 1991), it has not been identified in lower eukaryotes (Paule, 1998c). EBF is similar to UBF in two aspects. First, it binds to the rRNA repeated enhancer sequences and to several other sequences within the intergenic spacer. However, it does not bind to a consensus recognition sequence but appears to recognize some structural variation in the DNA. Second, EBF stimulates the assembly of the committed complex on the rRNA core promoter. However, while UBF functions as an assembly factor by binding to the core promoter and the upstream promoter element prior to the general transcription initiation factors and Pol I, EBF does

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not bind to the core and an UPE has not been identified in *A. castellanii* (Paule, 1998e; Yang *et al*, 1995).

It is intriguing that EBF shares a lot of similarities with TIF-IE (discussed below) and it is very possible that these two *A. castellanii* factors are the same. However, differences between the two have also been observed and therefore further studies will be required to elucidate whether or not these two factors are truly identical.

4.3.1 Similarities between TIF-IE and EBF

Committed complex formation: EBF stimulates binding of TIF-IB to the rRNA core promoter. Under dilute conditions, neither TIF-IB nor EBF can bind to the core promoter by themselves. DNaseI footprinting and EMSAs have shown that EBF and TIF-IB are both required for the formation of the committed complex on the core promoter (Yang *et al*., 1995). Geiss (1997) reported that the effect of EBF on the stabilization of TIF-IB complex on the core promoter was non-specific since heat treatment of EBF did not affect its activity. However, it was recently found that TIF-IE is very heat-stable (Fig. 4.1). Heat treatment of the factor for 10 minutes at 95°C did not significantly reduce its activity. If TIF-IE and EBF are the same factor, this could explain why EBF was still active after the same heat treatment.

rRNA transcription stimulation: Similar to TIF-IE, Yang *et al.* (1995) reported that EBF has a stimulatory effect on rRNA transcription (3-fold) and that it is not required for transcription initiation. This conclusion was due to the

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TIF-IE + + TIF-IE heated at 95℃ for 10min

Promoter-DNA affinity TIF-IB +

Glycerol Gradient TIF-IB + + +

Committed complex

Lane # 1 2 3 4 5

Fig. 4.1. TIF-IE is very heat stable. EMSAs 1 μl of glycerol gradient-purified TIF-IB alone (lane 2), with 0.5 μl of TIF-IE (lane 3), with 0.5 μl of heat-treated TIF-IE (lane 4), and EMSA of 2 μl of promoter-DNA affinity-purified TIF-IB alone (lane 5).

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observation that TIF-IB and Pol I were sufficient to get basal transcription of the rRNA gene. However, new evidence has emerged showing that Pol I is tightly associated with TIF-IE. Even when highly purified, Pol I remains contaminated with TIF-IE, therefore not allowing for complete characterization of the "basal" transcriptional machinery. If TIF-IE and EBF are actually the same factors, this could explain why we get basal rRNA transcription in the absence of added EBF. Further investigation is needed to determine the exact requirement of TIF-IE for rRNA transcription (discussed above).

Enhancer binding: EBF binds to the rRNA repeated enhancer sequences and to several other sequences in the intergenic spacer. However, it does not bind to a consensus recognition sequence but appear to recognize some structural variation in the DNA (Yang *et al.*, 1995). In chapter two of this dissertation, it was shown that partially purified TIF-IE samples contain a DNA-binding component. This component binds to an enhancer element that extends from -1801 to –1675. It also binds to other DNA sequences such as the core promoter region from -96 to +18 and to an upstream region of the promoter from -91 to -50 (Fig. 4.2). Yang *et al.* (1995) showed that EBF does not bind to the core promoter. Moreover, we have concluded, in chapter 2, that the promoter-binding activity in TIF-IE samples (lane 2) must be a contaminant factor since the TIF-IE 141 kDa polypeptide did not bind to the promoter. It seems that this contaminating factor either binds to the DNA non-specifically or does not bind to a consensus recognition sequence but rather to a certain DNA structure. In the

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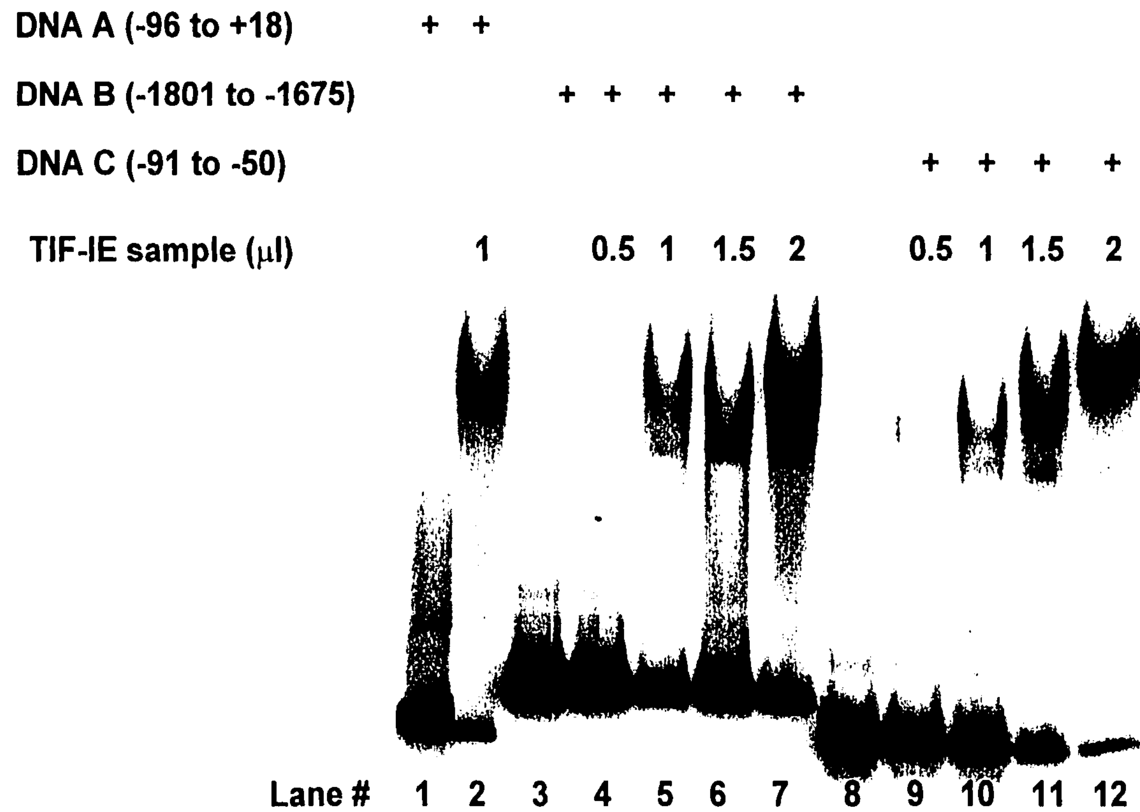

Fig. 4.2. A DNA-binding component in TIF-IE samples binds to several DNA sequences. EMSAs of TIF-IE sample on the *A. castellanii* rRNA core promoter region from -96 to +18 (lane 2), on an enhancer element (lanes 4-7), and on the *A. castellanii* rRNA core promoter region from -91 to -50 (lanes 9-12).

latter aspect, it is similar to EBF and UBF. Therefore, further studies are necessary to investigate the function of this promoter-binding protein.

Sedimentation profile: EBF has similar sedimentation profiles in a glycerol gradient (17.5% to 35%) (Geiss, 1997) as TIF-IE.

4.3.2 Differences between TIF-IE and EBF

Purification: EBF is purified from a whole cell extract TIF-IB fraction as described by Yang *et al.* (1995) (Fig. 4.3). TIF-IE, on the other hand, was purified from Pol I by rate zonal sedimentation in a glycerol gradient.

Subunit composition: According to Yang (1992), EBF is a 125 kDa polypeptide as identified by its immunological cross-reaction with rat anti-UBF serum. However, Geiss (1997) found that EBF does not cross-react with either *Xenopus* or rat anti-UBF sera when it is purified further by glycerol gradient centrifugation. Therefore, attempts to identify its polypeptide composition were not successful.

These differences between TIF-IE and EBF do not exclude the possibility that they are identical. However, the specific binding of TIF-IE to enhancer repeats and other DNA sequences in the intergenic spacer should be confirmed before declaring that these two factors are the same. In addition, immunological cross-reactivity of EBF with anti-TIF-IE serum could help clarify this matter.

4.4 Is TIF-IE a homologue to yeast Rrn3p?

Rrn3p plays a central role in Pol I recruitment to rDNA promoters by bridging the enzyme to the core factor (Peyroche *et al.*, 2000). It is an essential

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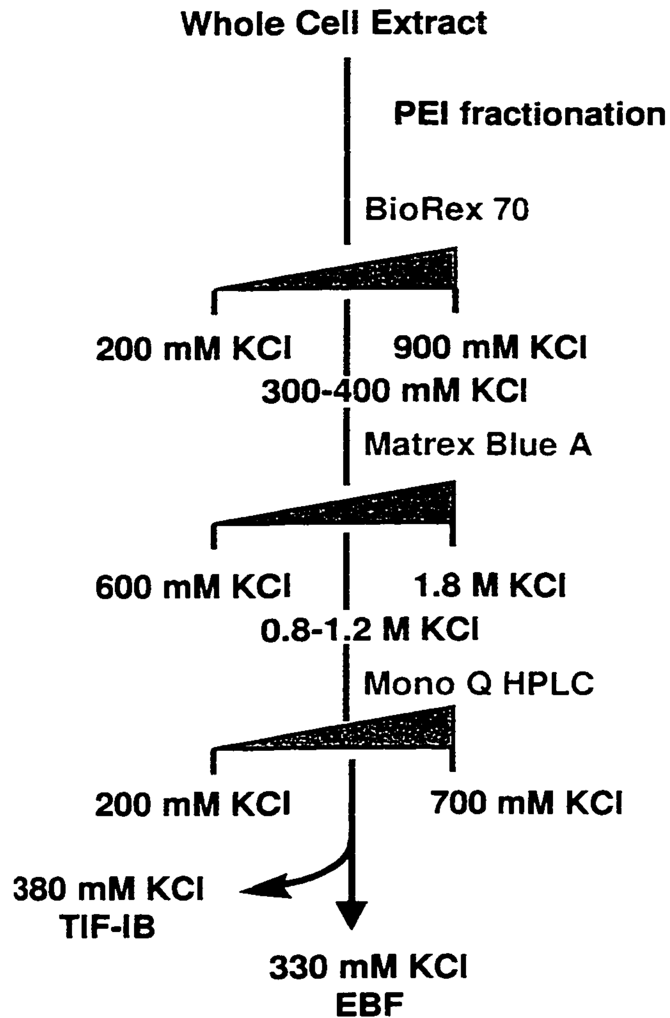

Fig. 4.3. Flow diagram for the purification of *A. castellanii* EBF.
Solid triangles indicate linear gradients, with the starting salt concentrations listed on the left and the final salt concentrations on the right

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Pol I transcription initiation factor that interacts with Pol I in the absence of DNA template leading to transcription stimulation. This interaction increases the amount of a transcription-competent initiation complex. However, Rrn3p is not part of the stable committed complex (Yamamoto *et al.*, 1996).

In *Acanthamoeba castellanii,* Pol I is recruited to the promoter by its interaction with the committed complex (Kownin *et al.*, 1987). Methidiumpropyl-EDTA·Fe (II) [MPE·Fe(II)] footprinting showed that the addition of Heparin-Sepharose-purified Pol I to the committed complex, which protects the template strand of the rRNA core promoter from -67 to -17 relative to the *tis* (+1) (Fig. 4.4, lane 3), results in the extension of the protected site by only two small regions: one of 3 base pairs (bp) located at -16, -15, and -14, and another region surrounding the *tis* (lane 4). Pol I alone does not bind to these two regions which indicates that its positioning there is mediated by its interaction with the committed complex. This confirms the earlier findings by Geiss (1997) and Kownin *et al.* (1987). However, the addition of an equal amount of the glycerol-gradient-purified Pol I to the committed complex (lane 5) does not show the same pattern of protection. These results suggest that the homogeneous Pol I in the initiation complex either does not bind or binds in an unstable way to the rRNA promoter. However, since it is capable of mediating accurate transcription from the rRNA promoter in transcription run-off assays (data not shown), it is most likely that the glycerol gradient-purified Pol I binds to the rRNA promoter but in an unstable way. Therefore, it is possible that during the purification, a factor is partially separated from Pol I that is required for its recruitment and stable

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Glycerol gradient Pol I +
Heparin Sepharose Pol I +
Promoter-DNA affinity TIF-IB (1.5 μl) + + +
M M
-67
-17
-15
-13
tis (+1)
Lane # 1 2 3 4 5 6

Fig. 4.4. Glycerol gradient-purified Pol I does not show the same pattern of protection from MPE·Fe(II) cleavage as the heparin Sepharose-purified Pol I in the initiation complex. MPE·Fe(II) footprints of the committed complex (lane 3), with 105 milliunits of heparin Sepharose-purified Pol I (lane 4), with 105 milliunits of glycerol gradient-purified Pol I (lane 5) on the template strand of the rRNA promoter.

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binding to the promoter. Rate zonal sedimentation partially separates TIF-IE from HS-purified Pol I. Therefore, it is possible that TIF-IE, similar to Rrn3p, is required for recruiting Pol I to the promoter. However, attempts to restore the stable binding of the glycerol gradient-purified Pol I to the promoter by adding TIF-IE failed (data not shown). This can be interpreted as follows: TIF-IE is either not required for Pol I recruitment to the promoter or is unable to successfully reassociate with Pol I.

In yeast, Rrn3p is also the regulated factor. The initiation-competent form (Pol I-Rrn3p) is lacking in transcriptionally inactive extracts from stationary phase yeast cells despite the substantial amounts of Rrn3p and Pol I (Milkereit and Tschochner, 1998). Therefore, formation and disruption of Rrn3p-Pol I complex appear to constitute a molecular switch for regulating rDNA transcription.

In *A. castellanii* stationary cells, rDNA transcription is down-regulated as a result of the functional inactivation of Pol I. This inactivation is caused by a modification of Pol I itself or a factor tightly associated with it that prevents Pol I from binding to the promoter (Bateman and Paule, 1986). The modified factor could be TIF-IE. However, further studies are necessary to investigate if TIF-IE is required for Pol I recruitment and if it is the regulated component of the Pol I transcriptional machinery in *A. castellanii*. Immunological cross-reactivity of TIF-IE with anti-Rrn3p serum would provide preliminary evidence for this hypothesis. To prove that TIF-IE is the regulated factor, it should be inactive when purified from stationary cells. The requirement of TIF-IE for Pol I recruitment can be determined by run-off transcription assays. Pol I should be unable to mediate

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accurate rRNA transcription when it is completely separated from TIF-IE. This approach is possible only if TIF-IB is capable of minimal binding to the promoter by itself. In fact, it was shown in chapter 2 that TIF-IB can bind to the promoter by itself but in a weak and unstable manner.

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APPENDIX

Preface: This section includes the Matrix-Assisted-Laser-Desorption-Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) and the protein sequencing data of TIF-IE provided by *The Rockefeller University Protein/DNA Technology Center* for sequencing. The MS data did not match anything in the database and therefore TIF-IE could be a novel protein.

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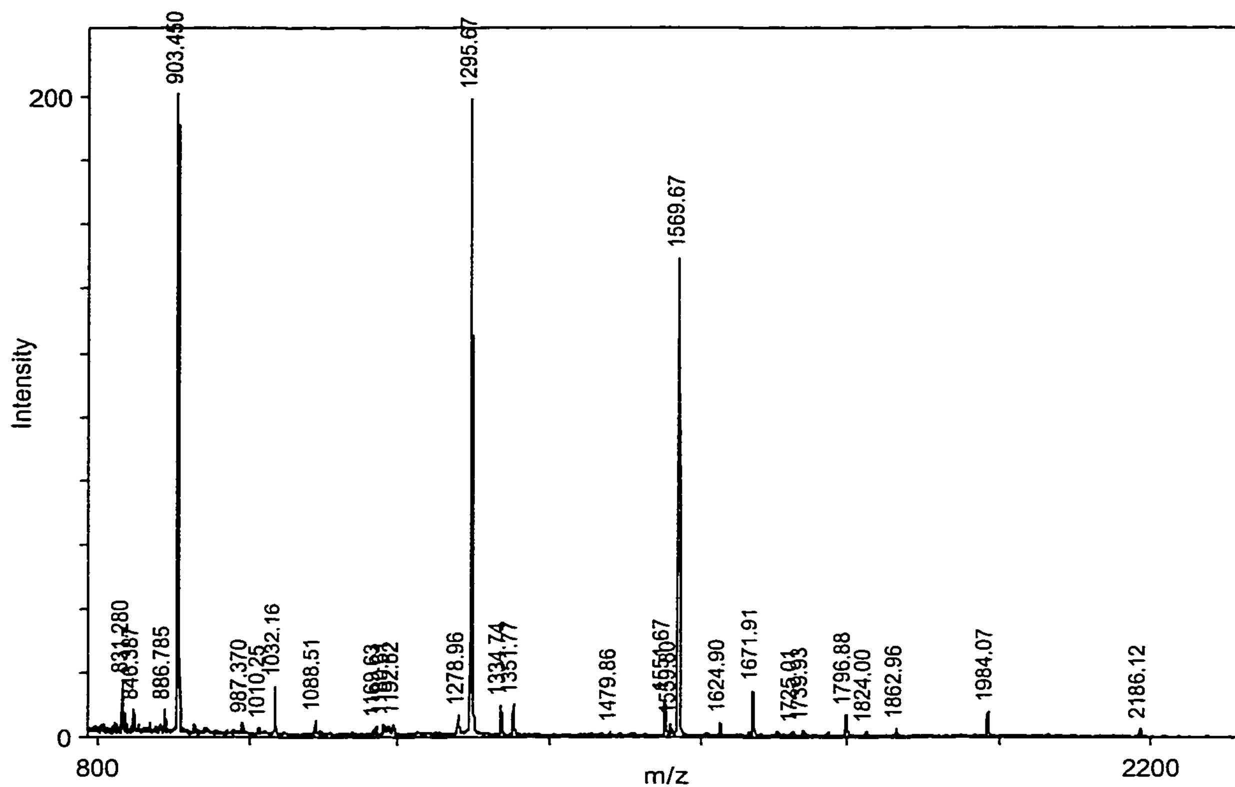

F:\MS Data\SEP00\0921\J0921001.ms (09:41 09/22/00)
Description: T00151
Spectrum Notes: TIF-1E

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Sample: TIF-1E
ID Number: 00331
Sequencer Run F101200

Quantity Loaded:
N-terminal

Raw Data Tabulation ... (in pmols)

	A	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y	eu	Major	Minor
1	2.2	7.9	1.5	0.3	5.8	0.7	0.3	0.7	0.8	0.1	9.0	0.2	3.3	1.5	11.7	5.1	0.5	0.0	0.5	0.7	X	
2	0.4	0.8	0.3	0.1	2.3	0.2	0.2	0.6	0.3	0.0	0.7	0.1	0.4	0.6	1.7	0.5	0.2	0.0	0.2	0.0	(K)	
3	0.2	0.6	0.2	0.1	1.0	0.1	0.3	0.8	0.2	0.0	0.9	0.1	0.1	0.4	0.5	0.1	0.1	0.0	0.1	0.0	K	N
4	0.2	0.6	0.2	0.1	0.7	0.1	0.1	0.2	0.4	0.0	0.6	0.4	0.3	0.3	0.6	0.0	0.1	0.0	0.1	0.0	P	L/Q
5	0.2	0.4	0.3	0.1	0.6	0.1	0.2	0.2	0.2	0.0	0.8	0.1	0.2	0.3	0.3	0.1	0.2	0.1	0.1	0.0	N	E/V
6	0.2	0.4	0.2	0.1	0.6	0.0	0.2	0.2	0.2	0.0	0.6	0.3	0.2	0.3	0.6	0.1	0.1	0.0	0.1	0.5	P	(S)
7	0.2	0.4	0.2	0.1	0.6	0.0	0.1	0.1	0.2	0.0	0.5	0.2	0.3	0.4	0.7	0.1	0.1	0.1	0.1	0.0	S	
8	0.2	0.3	0.1	0.1	0.7	0.0	0.1	0.1	0.2	0.0	0.2	0.1	0.2	0.2	0.5	0.0	0.1	0.0	0.1	0.0	G	
9	0.2	0.3	0.1	0.1	0.7	0.0	0.1	0.1	0.2	0.0	0.3	0.2	0.2	0.3	0.5	0.1	0.1	0.0	0.1	0.0	P	
10	0.2	0.6	0.2	0.1	0.7	0.0	0.1	0.1	0.2	0.0	0.7	0.1	0.3	0.2	0.7	0.0	0.1	0.1	0.1	0.0	E	
11	0.2	0.3	0.2	0.1	0.7	0.0	0.1	0.1	0.2	0.0	0.3	0.1	0.2	0.2	0.5	0.0	0.1	0.0	0.1	0.4	(G)	
12	0.1	0.3	0.2	0.1	0.7	0.0	0.2	0.1	0.2	0.0	0.5	0.1	0.2	0.2	0.5	0.1	0.2	0.0	0.1	0.0	V	
13	0.2	0.3	0.2	0.1	0.7	0.0	0.1	0.1	0.2	0.0	0.5	0.1	0.3	0.3	0.7	0.2	0.2	0.0	0.1	0.0	E	
14	0.2	9.1	0.6	0.1	3.4	0.0	0.2	0.1	0.2	0.0	10.9	0.1	3.8	0.2	8.7	4.4	0.1	0.0	0.1	0.0	(S)	
15	0.1	0.3	0.1	0.1	0.7	0.0	0.1	0.2	0.2	0.0	0.4	0.1	0.0	0.3	0.3	0.0	0.1	0.1	0.1	0.0	K	
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Observed Mass = 1671.91
Calculted Mass = ______
Difference = ______

Deduced amino acid = at cycle #

When including this data for publication, please use the following acknowledgement: "Protein sequence analysis was provided by The Rockefeller University Protein/DNA Technology Center, which is supported in part by NIH shared instrumentation grants and by funds provided by the U.S. Army and Navy for purchase of equipment."

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Sample: T00151 TIF-1E
ID Number: 00332
Sequencer Run F100600

Quantity Loaded:
N-terminal

Raw Data Tabulation ... (in pmols)

	A	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y	eu	Major	Minor
1	0.5	0.5	0.3	1.4	2.0	0.2	0.3	0.2	0.2	0.0	0.1	0.1	0.2	0.4	1.1	0.2	0.2	0.0	0.2	0.9	F	
2	0.3	1.3	0.4	1.2	2.0	0.1	0.2	0.1	0.6	0.0	1.2	0.1	0.8	0.3	1.9	1.0	0.2	0.0	0.2	0.0	F	L
3	0.3	0.0	0.4	0.2	2.0	0.0	0.1	0.0	0.5	0.2	1.7	0.1	0.9	0.3	2.5	1.1	0.1	0.2	0.2	0.0	S	L
4	0.3	1.1	0.2	0.2	1.5	0.0	0.2	0.1	0.2	0.0	1.1	0.7	0.7	0.2	1.5	0.7	0.1	0.0	0.2	0.0	P	
5	0.3	1.2	0.2	0.2	1.5	0.0	0.2	0.1	0.2	0.0	1.1	0.3	0.7	0.3	1.4	1.1	0.2	0.0	0.2	0.0	T	
6	0.3	0.0	0.1	0.3	1.0	0.0	0.2	0.2	0.3	0.0	0.7	0.4	0.3	0.3	0.6	0.4	0.4	0.3	0.2	0.7	P	V
7	0.4	0.0	0.2	0.1	1.2	0.0	0.4	0.2	0.3	0.0	1.3	0.3	0.6	0.3	1.4	0.6	0.2	0.1	0.2	0.0	I	A
8	0.3	0.0	0.6	0.2	3.9	0.0	0.3	0.2	0.3	0.0	8.5	0.2	4.4	0.3	9.4	5.4	0.2	0.1	0.1	0.0	Q	K
9	0.3	0.0	0.2	0.2	0.9	0.0	0.2	0.2	0.2	0.0	0.5	0.2	0.4	0.3	0.5	0.1	0.2	0.1	0.2	0.0	E	
10	0.3	0.0	0.2	0.1	0.9	0.0	0.2	0.3	0.3	0.0	0.6	0.1	0.4	0.3	0.6	0.1	0.2	0.1	0.1	0.0	K	
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Observed Mass = 1192.62
Calculted Mass = 1192.61
Difference = 0.01

Deduced amino acid = at cycle #

When including this data for publication, please use the following acknowledgement: "Protein sequence analysis was provided by The Rockefeller University Protein/DNA Technology Center, which is supported in part by NIH shared instrumentation grants and by funds provided by the U.S. Army and Navy for purchase of equipment."

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Sample: T00151
ID Number: 00333
Sequencer Run F101900

Quantity Loaded:
N-terminal

Raw Data Tabulation ... (in pmols)

	A	D	E	F	G	H	I	L	M	N	P	Q	R	S	T	V	W	Y	eu		Major	Minor
1	0.4	0.4	0.2	0.2	0.9	0.1	0.2	0.1	0.0	0.0	0.1	0.1	0.3	0.7	0.1	0.1	0.0	0.1	0.7	1	S	
2	0.1	0.2	0.1	0.1	0.6	0.0	0.5	0.2	0.0	0.0	0.0	0.1	0.2	0.1	0.0	0.1	0.0	0.1	0.0	2	I	L
3	0.1	0.1	0.1	0.1	0.4	0.0	0.5	0.1	0.0	0.0	0.0	0.1	0.1	0.1	0.0	0.1	0.0	0.1	0.0	3	I	
4	0.1	0.1	0.1	0.1	0.4	0.1	0.1	0.1	0.0	0.0	0.0	0.1	0.1	0.1	0.3	0.2	0.0	0.1	0.0	4	T	V
5	0.1	0.1	0.1	0.1	0.4	0.0	0.1	0.5	0.0	0.1	0.1	0.1	0.1	0.1	0.0	0.1	0.0	0.1	0.0	5	L	
6	0.1	0.1	0.1	0.1	0.4	0.0	0.3	0.2	0.0	0.1	0.1	0.1	0.1	0.1	0.0	0.1	0.0	0.1	0.6	6	I	P
7	0.1	0.0	0.1	0.1	0.4	0.0	0.1	0.1	0.0	0.1	0.1	0.4	0.1	0.2	0.0	0.1	0.0	0.0	0.0	7	Q	
8	0.0	0.1	0.1	0.1	0.4	0.2	0.1	0.1	0.0	0.1	0.0	0.2	0.1	0.2	0.1	0.1	0.0	0.0	0.0	8	H	
9	0.1	0.1	0.1	0.1	0.4	0.0	0.1	0.2	0.0	0.1	0.2	0.1	0.2	0.1	0.0	0.1	0.0	0.1	0.0	9	P	
10	0.3	0.1	0.1	0.1	0.5	0.0	0.1	0.1	0.0	0.1	0.1	0.1	0.2	0.1	0.0	0.1	0.1	0.1	0.0	10	A	
11	0.2	0.1	0.1	0.1	0.4	0.0	0.1	0.2	0.0	0.1	0.1	0.1	0.0	0.2	0.0	0.1	0.1	0.1	0.4	11	S	
12	0.1	0.1	0.1	0.1	0.4	0.0	0.1	0.2	0.0	0.1	0.0	0.2	0.2	0.2	0.0	0.1	0.1	0.1	0.0	12	R	
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Observed Mass = 1334.74
Calculted Mass = 1334.76
Difference = 0.03

Deduced amino acid = at cycle #

When including this data for publication, please use the following acknowledgement: "Protein sequence analysis was provided by The Rockefeller University Protein/DNA Technology Center, which is supported in part by NIH shared instrumentation grants and by funds provided by the U.S. Army and Navy for purchase of equipment."

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Sample: T00151 TIF-1E
ID Number: 00334
Sequencer Run F110100

Quantity Loaded:
N-terminal

Raw Data Tabulation ... (in pmols)

	A	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y	eu		Major	Minor
1	0.2	0.5	0.3	0.4	1.3	0.0	0.5	0.2	0.4	0.1	2.3	0.1	0.3	0.3	1.0	0.4	0.2	0.0	0.2	0.6	1	X	
2	0.0	0.6	0.2	0.4	1.2	0.2	0.1	0.1	1.1	0.0	0.7	0.1	0.1	0.3	0.3	0.1	0.2	0.0	0.2	0.5	2	L	
3	0.1	0.6	0.2	0.8	0.9	0.2	0.4	0.1	0.4	0.0	0.3	0.1	0.2	0.2	0.3	0.1	0.1	0.0	0.2	0.6	3	F	
4	0.1	0.6	0.2	0.3	0.9	0.1	0.3	0.1	0.4	0.1	0.2	0.1	0.1	0.2	0.3	0.1	0.1	0.0	0.5	0.5	4	Y	
5	0.1	0.6	0.1	0.3	0.7	0.1	0.2	0.1	0.5	0.0	0.2	0.1	0.1	0.2	0.2	0.1	0.2	0.3	0.1	0.6	5	(L)	
6	0.1	0.6	0.2	0.3	0.8	0.0	0.3	0.0	0.4	0.1	0.2	0.2	0.1	0.2	0.2	0.1	0.2	0.3	0.1	0.6	6	P	
7	0.1	0.6	0.1	0.3	0.7	0.1	0.2	0.0	0.4	0.1	0.2	0.1	0.2	0.1	0.2	0.0	0.1	0.0	0.1	0.6	7	(Q)	
8	0.1	0.6	0.1	0.3	0.6	0.1	0.4	0.1	0.4	0.0	0.2	0.1	0.1	0.3	0.2	0.1	0.1	0.1	0.1	0.5	8	R	
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Observed Mass = 1796.88
Calculted Mass = ______
Difference = ______

Deduced amino acid = at cycle #

When including this data for publication, please use the following acknowledgement: "Protein sequence analysis was provided by The Rockefeller University Protein/DNA Technology Center, which is supported in part by NIH shared instrumentation grants and by funds provided by the U.S. Army and Navy for purchase of equipment."

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Sample: T00151
ID Number: 00375
Sequencer Run F100200

Quantity Loaded:
N-terminal

Raw Data Tabulation ... (in pmols)

	A	D	E	F	G	H	I	K	L	M	N	P	Q	R	T	V	W	Y	eu	Major	Minor
1	1.3	0.2	0.3	0.1	1.9	0.3	0.1	0.4	0.5	0.0	0.2	0.1	0.1	0.4	0.6	0.3	0.0	0.2	0.6	X	
2	0.3	0.1	0.2	0.0	1.0	0.2	0.1	0.1	0.2	0.0	0.1	0.1	0.0	0.2	0.2	0.2	0.0	0.0	0.4	X	
3	0.2	0.1	0.1	0.1	0.7	0.1	0.1	0.0	0.2	0.0	0.2	0.0	0.0	0.2	0.1	0.2	0.0	0.0	0.5	L	
4	0.3	0.1	0.1	0.2	0.5	0.1	0.1	0.0	0.1	0.0	0.2	0.0	0.1	0.2	0.2	0.0	0.0	0.0	0.4	F/A	
5	0.2	0.1	0.2	0.0	0.4	0.0	0.1	0.0	0.1	0.0	0.0	0.1	0.1	0.1	0.1	0.1	0.0	0.1	0.4	E/A	
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Observed Mass =
Calculted Mass = ______ AA -
Difference = ______

Deduced amino acid = at cycle #

When including this data for publication, please use the following acknowledgement: "Protein sequence analysis was provided by The Rockefeller University Protein/DNA Technology Center, which is supported in part by NIH shared instrumentation grants and by funds provided by the U.S. Army and Navy for purchase of equipment."

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The Rockefeller University Protein/DNA Technology Center

Microchemistry ID # T00151 | Sample TIF-1E
Investigator Anna Al-Khouri | Molecular Weight 146 kDa
Species/organism:

PROTEIN IDENTIFICATION RESULTS

(Please see attached MALDI-TOF MS results)

Tentative Protein Identification (Peptide Masses) None
Mass Tolerance: 0.1Da | Masses Matched (#/#): | % Coverage
Cysteine Derivative used: None
MET oxidized option used? Yes | Mass Data entered as: M
Program (s) Used for searching ProFound | Est'd Z

Definitive Protein Identification (Peptide Sequence)
Sequence ID number used for Search
Actual Sequence used for search

Comments Sequence data summary continued on next sheet.

SEQUENCE RESULTS

(Please use data on sequence report as this is only a summary)

Sequence ID	00331		00332		00333	
Observed Mass	1671.91		1191.62		1334.74	
	Major	Minor	Major	Minor	Major	Minor
1	X		F		S	
2	(K)		F	L	I	L
3	K	N	S	L	I	
4	P	L/Q	P		T	V
5	N	E/V	T		L	
6	P	(S)	P	V	I	P
7	S		I	A	Q	
8	G		Q	K	H	
9	P		E		P	
10	E		K		A	
11	(G)				S	
12	V				R	
13	E					
14	(S)					
15	K					
16						
17						
18						
19						
20						

Masses Used for Search	
2186.12	1725.95
1984.07	1771.91
1862.96	
1824.00	
1796.88	
1739.93	
1624.90	
1559.80	
1551.67	
1479.86	
1351.77	
1334.74	
1278.96	
1192.62	
1180.61	
1169.63	
1088.51	
1032.16	
1010.25	
987.37	
886.79	
846.39	
831.28	

This data sheet is a summary of the best interpretation the Center can make with the available data. Mass Spectra of the digestion mixture used for peptide mass identification, HPLC profile of the peptide isolation run, and mass spectra of the purified peptides that were sequenced are attached. Database search results for both mass spec data and primary sequence data are NOT included, and are the responsibility of the investigator to get printouts. Conditions necessary for the database search are given above, and the ProFound and MS-FIT sites can be accessed throught the centers home page (http://pdtc.rockefeller.edu/pdtcmain/pseq/pseqinfo) . A final report of the peptide sequence data is also included. PTH chromatograms are not supplied but are available upon request. **Please note, final protein assignment and identification as well as scientific implications of the findings are the responsibility of the investigator.** Any questions involving the data or if additional explanations are requested please contact Joseph Fernandez at 212-327-8869 (fernaj@rockvax.rockefeller.edu) or Brian Imai 212-327-8487 (imaib@rockvax.rockefeller.edu).

If and when this data is published, please be kind enough to acknowledge the Center's participation in this work by citing the following reference: "Routine Identification of Proteins from SDS-PAGE Gels or PVDF membranes using Matrix-Assisted-Laser-Desorption-Ionization Time-of Flight Mass Spectrometry", Fernandez, J., F. Gharahdaghi, and S.M. Mische (1998) *Electrophoresis* **19**, 1036-1045.

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The Rockefeller University Protein/DNA Technology Center

Microchemistry ID # T00151 Part B Sample TIF-1E
Investigator Anna Al-Khouri Molecular Weight 146 kDa
Species/organism:

PROTEIN IDENTIFICATION RESULTS

(Please see attached MALDI-TOF MS results)

Tentative Protein Identification (Peptide Masses) None
Mass Tolerance: 0.1Da Masses Matched (#/#): % Coverage
Cysteine Derivative used: None
MET oxidized option used? Yes Mass Data entered as: M
Program (s) Used for searching ProFound Est'd Z

Definitive Protein Identification (Peptide Sequence)
Sequence ID number used for Search
Actual Sequence used for search

Comments Sequence data summary continued on next sheet.

SEQUENCE RESULTS

(Please use data on sequence report as this is only a summary)

Sequence ID	00334		00375			
Observed Mass						
	Major	Minor	Major	Minor	Major	Minor
1	X		X			
2	L		X			
3	F		L			
4	Y		F/A			
5	(L)		E/A			
6	P		X			
7	(Q)					
8	R					
9						
10						
11						
12						
13						
14						
15						
16						
17						
18						
19						
20						

Masses Used for Search			
2186.12	1725.95		
1984.07	1771.91		
1862.96			
1824.00			
1796.88			
1739.93			
1624.90			
1559.80			
1551.67			
1479.86			
1351.77			
1334.74			
1278.96			
1192.62			
1180.61			
1169.63			
1088.51			
1032.16			
1010.25			
987.37			
886.79			
846.39			
831.28			

This data sheet is a summary of the best interpretation the Center can make with the available data. Mass Spectra of the digestion mixture used for peptide mass identification, HPLC profile of the peptide isolation run, and mass spectra of the purified peptides that were sequenced are attached. Database search results for both mass spec data and primary sequence data are NOT included, and are the responsibility of the investigator to get printouts. Conditions necessary for the database search are given above, and the ProFound and MS-FIT sites can be accessed throught the centers home page (http://pdtc.rockefeller.edu/pdtcmain/pseq/pseqinfo) . A final report of the peptide sequence data is also included. PTH chromatograms are not supplied but are available upon request. **Please note, final protein assignment and identification as well as scientific implications of the findings are the responsibility of the investigator.** Any questions involving the data or if additional explanations are requested please contact Joseph Fernandez at 212-327-8869 (fernaj@rockvax.rockefeller.edu) or Brian Imai 212-327-8487 (imaib@rockvax.rockefeller.edu).

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