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Structure/Function Analysis of the Eukaryotic Transcription Elongation Factor, TFIIS

by

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Dedicated to the memory of my mother, Mee-Tue Shimasaki, and
my grandmother, Lee Shee Wong
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Chapter One

Introduction
Introduction

An important point in the control of gene expression is the regulation of transcription elongation (Kane, 1994). The eukaryotic transcription factor, TFIIS, has been shown to modify the behavior of RNA polymerase II during transcription elongation (Reines, 1994). The focus of my project is to analyze the structure/function of TFIIS and to investigate the interactions of TFIIS with RNA polymerase II using a variety of biochemical and genetic methods. However, before discussing TFIIS in detail, several aspects which affect transcription elongation by RNA polymerase II will be addressed first.

Eukaryotic Transcription and Blocks to Elongation

Transcription is the process by which a cell converts the information coded in the deoxyribonucleic acid, DNA, of a gene into ribonucleic acid, RNA. The cellular machinery then uses the RNA as a template to make a protein. Because transcription is the first step in gene expression, control of transcription is a major means of regulation for the cell. Although in prokaryotic systems regulating transcription at the level of elongation has been well established, it has only been in the past decade or so that it has been studied intensely as a means of control of gene expression in viral and eukaryotic systems (Spencer and Groudine, 1990; Kane, 1994; Shilatifard et al., 1997a; Uptain et al., 1997). In vivo, gene expression must be coordinated to the changing needs of the cell, and modulation of the activity of RNA polymerase II, which produces mRNA and some small RNA's, is one point of control. Transcription regulation after the formation of the pre-initiation complex can occur during promoter escape, elongation, and termination. The growing list of genes that have been identified with blocks to elongation include those of both RNA and DNA viruses (human immunodeficiency viruses 1 and 2 and adenovirus) and a variety of cellular genes (Drosophila Hsp70, c-myc, c-fos, adenosine deaminase, human histone H3.3, and
α- and β-globin) [for a comprehensive list (Uptain et al., 1997)]. In several cases, nuclear run-on assays, in vivo UV crosslinking, and in vivo KMnO4 footprinting determined that transcription complexes were over represented at the 5' end of the gene which suggested that a block prevented the polymerases from transcribing the full length transcript (Shilatifard et al., 1997a; Uptain et al., 1997; Kane, 1994; Spencer and Groudine, 1990). In response to a variety of factors, such as environmental stress, cellular differentiation, tissue specificity, and development, the expression of these genes can be up or down regulated (Spencer and Groudine, 1990; Kane, 1994; Shilatifard et al., 1997a; Uptain et al., 1997). In order to understand how the cell is able to manipulate expression by affecting the elongation competence of the RNA polymerase II, much initial work used simpler, defined in vitro transcription systems.

In an effort to understand the mechanism of transcription elongation, considerable attention has focused on pause, arrest, and termination signals for the polymerase. A paused transcription complex is a complex that is halted but can resume transcription. An arrested complex remains stationary even in the presence of nucleotide substrates until the addition of accessory factors enables the polymerase to continue transcribing. A polymerase that has terminated transcription has released the RNA and dissociated from the template.

Intrinsic arrest sites are defined as sequences that purified RNA polymerase II can recognize when transcribing in vitro in a promoter independent fashion on poly dC-tailed templates (Kerppola and Kane, 1991; Kane, 1994; Edwards and Kane, 1996). Very often the arrest site consists of two spaced T-runs in the non-transcribed strand (Dedrick et al., 1987; Reines et al., 1987; Kerppola and Kane, 1990). The signal is orientation dependent and the resulting curvature caused by the T-rich sequence appears to be key to causing arrest (Kerppola and Kane, 1990; Wiest and Hawley, 1990). Sequence context also appears to have a role in signaling arrest. Deletion analysis of such a site in the human histone H3.3 gene identified the minimal signal as sequences -6 to +24 relative to the most
efficiently recognized site (Kerppola and Kane, 1990). What causes the polymerase to arrest remains unknown, but factors that affect the reaction have been identified. RNA polymerases that were allowed to transcribe to the end of linear templates were found to behave differently depending on the whether the end of the template was a 5'-overhang, 3'-overhang, or blunt ended. With 5'-overhang templates, the polymerase transcribed off the end of the template and released the transcript (Izban et al., 1995). Transcription of 3'-overhang or blunt ended templates resulted in the formation of two types of transcription complexes. One type of complex transcribed to within 5-10 bases of the end of the template and arrested. These complexes cleaved the RNA in response to a protein factor, TFIIS, that is the primary focus of this thesis (Izban et al., 1995). The second type of complex transcribed to within three bases of the template end and released the transcript (Izban et al., 1995). Because the arrested complexes were the ones which maintained contact with the template 5-10 bases downstream of the catalytic site, it was hypothesized that downstream sequences were involved in formation of an arrested complex (Izban et al., 1995).

Several other variables influence arrest. One effector is dwell time. The fraction of RNA polymerase II transcription complexes halted within an arrest site by withholding a nucleotide that were able to transcribe through this site upon addition of nucleotides decreased as a function of increasing incubation time (Gu and Reines, 1995a). It was also observed that transcription conditions that increased RNA polymerase II elongation rates, such as use of NH₄⁺, resulted in an increase in the proportion of polymerases that were able to read through a block to elongation (Gu and Reines, 1995a). With templates containing tandem arrest sites, it has also been observed that the fraction of polymerases transcribing through the first site is not the same as that transcribing through the second (Reines et al., 1993). It was concluded that a polymerase is not permanently elongation competent and that each individual encounter with a block to elongation involves some probability of arrest by polymerase (Reines et al., 1993).
Possibly arrest is the adoption of a changed conformation by polymerase that requires a certain amount of time to achieve. Kinetic studies with bacterial polymerase and transcription misincorporation suggest that the complex can undergo multiple conformations at each base along the template (Erie et al., 1993). It was proposed that the polymerase can exist in an activated state that binds substrate productively (if the nucleotide is correct) or nonproductively (if the nucleotide is incorrect). The activated complex is capable of rapid bond formation (that is, elongation) when the appropriate substrate is bound (Erie et al., 1993). If the polymerase has bound substrate nonproductively or has misincorporated the previous nucleotide, the activated conformation can change into an unactivated state that is incapable of bond formation (Erie et al., 1993). However, the unactivated state can convert to an activated one upon binding of the correct substrate whereupon chain extension occurs (Erie et al., 1993). Alternatively, the unactivated polymerase can transition into a third, inactive, "dead end" conformation that cannot transcribe even when high concentrations of nucleotides are present (Erie et al., 1993). It would seem reasonable then, that the use of factors which influence the elongation competence of polymerase (perhaps through maintaining an elongation competent conformation or preventing an arrest prone state of the complex) would be a potential means of regulating transcription elongation and gene expression in the cell.

Factors which Affect Transcription Elongation

*In vivo,* gene expression must be highly regulated in order for the organism to respond to a changing environment. RNA polymerase II must transcribe genes up to two million bases long (e.g. human dystrophin), and it has been estimated that the *in vivo* rate of transcription is on the order of 20-30 nucleotides/second (Izban and Luse, 1992a; Reines et al., 1996; Shilatifard et al., 1997a; Shermoen and Farrell, 1991; Sollner-Webb and Tower, 1986). However, *in vitro,* transcription rates by RNA polymerase enzymes range from 5-10 nucleotides/second (in metazoans) to over 20 nucleotides/second (in yeast) with
many instances of pausing (Spindler, 1979; Kadesch and Chamberlin, 1982; Edwards et al., 1991; Reines et al., 1996; Shilatifard et al., 1997a). It would be easy to speculate that the cell contains factors which enhance the ability of the polymerase to transcribe productively in vivo. Several factors have been characterized that have been shown to stimulate transcription by RNA polymerase. Some factors, such as ELL, Elongin, and TFIIF, appear to affect transcription by suppressing the amount of pausing by polymerase as it transcribes (Reines et al., 1996; Shilatifard et al., 1997a). Others, like P-TEFb, Tat, TFIH, SRB10/SRB11, and Fcp1 are involved in regulating the phosphorylation state of the heptapeptide repeats of the carboxy terminal domain of RNA polymerase II and possibly affect the transition of the polymerase from initiation to elongation (Chodosh et al., 1989; Marshall and Price, 1992; Chambers and Dahmus, 1994; Koleske and Young, 1994; Chambers et al., 1995; Maldonado and Reinberg, 1995; Marshall and Price, 1995; Chambers and Kane, 1996; Marshall et al., 1996; Archambault et al., 1997; Jones, 1997; Mancebo et al., 1997; Zhu et al., 1997; Archambault et al., 1998; Fujinaga et al., 1998; Jeang, 1998; Marshall et al., 1998; Peng et al., 1998a; Peng et al., 1998b). Another set of factors appear to mediate transcription elongation on chromatin templates, such as some SPT proteins, HMG14/17, and FACT (Ding et al., 1994; Bustin et al., 1995; John and Workman, 1998; LeRoy et al., 1998; Orphanides et al., 1998). N-TEF is another factor which appears to be a negative regulator of transcription elongation (Price et al., 1987; Xie and Price, 1996; Xie and Price, 1997; Xie and Price, 1998). Still others, like GreA, GreB in E. coli and TFIIS in eukaryotes, are anti-arrest factors that stimulate polymerase to cleave its nascent RNA and subsequently read through blocks to elongation (Reines, 1994; Uptain et al., 1997).

ELL, Elongin, and TFIIF

ELL, Elongin, and TFIIF stimulate in vitro transcription by RNA polymerase II by suppressing transient pausing by polymerase. The gene product of human 11-19 lysine-
*rich leukemia*, ELL, and the homologous protein, ELL2, are ubiquitously expressed and highly conserved proteins that can stimulate transcription by polymerase in promoter independent as well as promoter specific systems (Shilatifard et al., 1996; Shilatifard et al., 1997b). The ELL and ELL2 proteins are considered to act at the level of elongation because their effect occurs when the factors are added to artificially halted complexes or early elongation complexes before the complexes are incubated with nucleotides and allowed to resume transcription (Shilatifard et al., 1996). ELL is of particular interest because its gene undergoes translocations with the MLL gene, *trithorax-like mixed lineage leukemia*, in acute myeloid leukemias which suggests that there might be a connection between transcript elongation and carcinogenesis (Thirman et al., 1994; Mitani et al., 1995).

Elongin is another factor that suppresses pausing by RNA polymerase II *in vitro* and also may have a link to human disease. Elongin inhibits nonspecific pausing by RNA polymerase II, but its mechanism is clearly distinct from that of TFIIIS as discussed in detail below. Elongin does not stimulate readthrough or nascent RNA cleavage by an arrested polymerase (Bradsher et al., 1993b). Elongin is a heterotrimer (subunits A, B, and C) (Bradsher et al., 1993a). Transcription reactions with wildtype Elongin versus reactions with various combinations of Elongin A, B, and C subunits were compared in order to identify the contribution each subunit makes to the stimulatory effect of Elongin on RNA polymerase II (Aso et al., 1995). The greatest effect was seen when all three subunits were present (Aso et al., 1995). Subunit B, subunit C, individually or together had no effect (Aso et al., 1995). Subunit A alone had a very slight stimulatory effect on transcription (approximately 1/35 of the effect with Elongin ABC) suggesting that subunit A may have the pause suppression role in Elongin (Aso et al., 1995). Transcription with an Elongin AC complex had significantly more stimulatory activity than Elongin A alone, and it was concluded that subunit C most likely was a positive regulator of the Elongin ABC complex (Aso et al., 1995). Transcription with Elongin AB looked no different than transcription
with only subunit A (Aso et al., 1995). In the course of reconstituting the combinations of A, B, and C Elongin subunits using cation-exchange chromatography, it was observed that excess B and C would flow through the column while the rest eluted as the ABC complex (Aso et al., 1995). If only A and C were loaded on the column, an AC complex could be recovered (Aso et al., 1995). However, the presence of the B subunit in addition to Elongin C greatly increased the yield of Elongin A in a complex (in this case, as ABC) indicating that subunit B might serve to stabilize the complex (Aso et al., 1995). In support of the role of Elongin B as contributing stability to Elongin ABC, it was determined that the thermal stability of ABC was significantly greater than AC (Aso et al., 1995). The Elongin BC complex may have an additional function than that ofpause suppression as part of Elongin ABC. The BC subunits have been shown to bind to the von-Hippel-Lindau, VHL, tumor suppressor protein (Duan et al., 1995). Mutations in the VHL tumor suppressor proteins are believed to dispose one to tumors in such diseases as renal carcinoma and hemangioblastoma among others (Shilatifard et al., 1997a). A few of these mutant VHL proteins were cloned and found to be unable to bind to the Elongin BC complex suggesting that VHL related diseases may be a result of improperly regulated transcription (Kibel et al., 1995).

TFIIF is better known for its role in promoter specific initiation, but strong evidence exists that the factor has an effect on elongation also (Flores et al., 1989; Price et al., 1989; Chang et al., 1993; Kephart et al., 1994; Tan et al., 1994; Tan et al., 1995; Lei et al., 1998). In vitro transcription experiments with promoterless dC-tailed templates showed that the elongation rate of RNA polymerase II appeared to be enhanced upon the addition of TFIIF (Flores et al., 1989; Price et al., 1989). It was observed that longer transcripts were produced in shorter times and the extent of pausing by the polymerase along the template was reduced in reactions with TFIIF (Flores et al., 1989; Price et al., 1989). Unlike TFIIS, TFIIF could not stimulate an arrested complex to transcribe through a sequence specific block to elongation unless TFIIF was added before the polymerase had
fallen into an arrested state (Bengal et al., 1991). It has been proposed that during elongation TFIIF interacts with the polymerase and induces polymerase to change into an elongation competent state (Price et al., 1989).

Sopta et al. identified several RNA polymerase associating proteins called RAP's using an affinity column with calf thymus RNA polymerase II (Sopta et al., 1985). One RAP protein was TFIIS; the others, RAP30 and RAP74, were found to be TFIIF (Sopta et al., 1985; Flores et al., 1988). Further work lent support to the essential role of TFIIF in productive transcription by RNA polymerase II. In one set of studies, in vitro transcription was performed with a linear template containing the adenovirus major late promoter and HeLa nuclear extracts immunodepleted of RAP30 and RAP74 (Chang et al., 1993). The extract before immunodepletion could produce a full length run off transcript of 536 nucleotides (Chang et al., 1993). After depletion, the extract could not support transcription of full length RNA (Chang et al., 1993). The addition of either recombinant RAP30 or recombinant RAP74 was not sufficient for productive transcription (Chang et al., 1993). Only when both subunits of TFIIF were added to the reaction was full length transcript produced (Chang et al., 1993).

In an attempt to identify if the subunits of TFIIF were influencing transcription initiation or a post-initiation step, a Sarkosyl block assay was used (Chang et al., 1993). As described earlier, a linear template with the adenovirus major late promoter and RAP30 and RAP74 immunodepleted HeLa extracts were used (Chang et al., 1993). Transcription was initiated by adding ATP, CTP, and UTP to the extracts and template to form a 9-mer (Chang et al., 1993). Sarkosyl was then added to prevent re-initiation, and all four nucleotides were added to chase complexes out to the end of the template (Chang et al., 1993). Recombinant RAP30 or RAP74 was added before or after the addition of Sarkosyl (the subunit not being tested was added during initiation) (Chang et al., 1993). RAP30 needed to be added before the addition of Sarkosyl for the full length transcript to be produced (Chang et al., 1993). RAP74 could be added when the chase nucleotides were
added to the reaction (Chang et al., 1993). These results suggested that RAP30 functioned during initiation, and RAP74 was involved in post-initiation steps of transcription (Chang et al., 1993).

Work with more defined transcription systems provide evidence that both subunits of TFIIF influence RNA polymerase II during initiation and elongation (Tan et al., 1994). Using poly dC-tailed templates, purified polymerase, and recombinant RAP30 and RAP74, it was determined that the stimulatory effect of TFIIF on polymerase elongation required both subunits of TFIIF (Tan et al., 1994). A Sarkosyl challenge assay consisting of promoter specific initiation with purified polymerase and purified or recombinant general transcription factors was employed (rather than extracts as used by Chang et al.) (Tan et al., 1994). In order to support the production of full length transcript, both RAP30 and RAP74 needed to be added to the reaction before nucleotides were added to initiate transcription (Tan et al., 1994). These observations indicate that not only RAP30 but also RAP74 is required for transcription initiation (Tan et al., 1994).

Structure function analysis of RAP30 and RAP74 determined the regions of each subunit responsible for the stimulation of elongation by RNA polymerase II (Kephart et al., 1994; Frank et al., 1995; Tan et al., 1995; Lei et al., 1998). Studies with deletion mutants of RAP74 found that only the amino terminal half of RAP74 was needed (in the presence of RAP30) for the elongation stimulatory effect on polymerase (Kephart et al., 1994; Lei et al., 1998). Deletion mutagenesis with RAP30 indicate that a region just amino terminal to a putative RNA polymerase II binding region is important for the elongation effect of TFIIF (Tan et al., 1995). Also, a portion of the amino terminus of RAP30 appears important for elongation stimulation (Tan et al., 1995).

P-TEFb, Tat, TFIIF, SRB10/SRB11, and Fcp1

A growing body of research is leading to the conclusion that the phosphorylation of the carboxy terminal domain heptad repeats of RNA polymerase II is an important
regulatory target in shifting polymerase from transcription initiation to elongation (Dahmus, 1996). Factors which appear to act to regulate transcription by phosphorylating the RNA polymerase II CTD are P-TEFb, Tat, TFIH, SRB10/SRB11, and FCP1 (Chodosh et al., 1989; Marshall and Price, 1992; Chambers and Dahmus, 1994; Koleske and Young, 1994; Chambers et al., 1995; Liao et al., 1995; Maldonado and Reinberg, 1995; Marshall and Price, 1995; Chambers and Kane, 1996; Marshall et al., 1996; Archambault et al., 1997; Jones, 1997; Mancebo et al., 1997; Zhu et al., 1997; Archambault et al., 1998; Fujinaga et al., 1998; Jeang, 1998; Marshall et al., 1998; Peng et al., 1998a; Sun et al., 1998). Within the last few years, FCP1, a CTD phosphatase, has been purified, cloned, and characterized, and it most likely plays an integral part in regulating the phosphorylation state of the RNA polymerase II CTD (Chambers and Dahmus, 1994; Chambers et al., 1995; Chambers and Kane, 1996; Archambault et al., 1997; Archambault et al., 1998; Marshall et al., 1998).

Price and co-workers have isolated a DRB sensitive activity from *Drosophila* Kc nuclear extracts which stimulates the production of long transcripts, P-TEFb, positive transcription elongation factor b (Marshall and Price, 1992). Interest in isolating a P-TEFb activity arose from the observation that the majority of pre-initiated transcription complexes, which had been isolated and washed of factors before being allowed to begin transcription, did not produce long transcripts (Marshall and Price 1992). Instead, the complexes often paused and then terminated near the promoter after transcribing 30-200 nucleotides (Marshall and Price, 1992). This 'non-productive' elongation was defined as abortive elongation (Marshall and Price, 1992). However, if the nuclear extracts were added back to the washed complexes, then long transcripts would be produced (Marshall and Price, 1992). These observations indicated that a factor or factors in the extract affected the transcription complex and enabled the polymerase to elongate productively (Marshall and Price, 1992). A similar observation was seen with transcription in the presence of 5,6-dichloro-1-β-ribofuranosylbenzimidazole, DRB. DRB inhibited
productive elongation of long transcripts, but it did not inhibit the production of short transcripts (Chodosh et al., 1989). DRB also had been shown to affect elongation in previous studies (Sehgal et al., 1979; Tamm and Kikuchi, 1979). DRB, an analog of adenosine, was a known kinase inhibitor (Zandomeni et al., 1986). Because P-TEFb was inhibited by DRB and phosphorylation of the CTD of RNA polymerase II is involved in regulation of transcription elongation, it was hypothesized that P-TEFb may have a kinase activity that acted on the CTD of RNA polymerase II that shifted polymerase from an abortive elongation phase to an elongation competent phase. When P-TEFb was added to in vitro transcription assays with RNA polymerase II which had its CTD proteolytically digested, only the products from abortive elongation resulted indicating that P-TEFb acted either directly or indirectly through the CTD (Marshall et al., 1996). Furthermore, P-TEFb could phosphorylate purified polymerase, and this activity was inhibited by DRB (Marshall et al., 1996). Drosophila P-TEFb was found to be a heterodimer (Marshall and Price, 1995). One subunit is a cyclin dependent kinase, CDK9, and the other was a cyclin designated cyclin T (Zhu et al., 1997; Peng et al., 1998a). The human homolog of CDK9 is PITALRE, and three human cyclins T's have been cloned, T1, T2a, and T2b (Peng et al., 1998b; Wei et al., 1998). Recombinant CDK9 and any one cyclin T subunit are required for P-TEFb phosphorylation of the CTD of RNA polymerase II in vitro (Peng et al., 1998b). When the recombinant CDK9/cyclin T complexes were added back to HeLa nuclear extracts immunodepleted of endogenous CDK9, the recombinant P-TEFb's were able to support DRB sensitive transcription (Peng et al., 1998b).

Interestingly, it looks as if there is a connection between P-TEFb and another elongation factor, Tat. Tat is a focus of much research because it is a transactivator of transcription of the HIV long terminal repeat, LTR, of HIV-1 and HIV-2; transcription with Tat results in greater production of full length transcripts than are produced without Tat (Laspia et al., 1989; Marciniak et al., 1990; Marciniak and Sharp, 1991). Although the reaction mechanism is not understood, Tat acts through an RNA element, TAR, most likely
in conjunction with cellular factors such as P-TEFb/TAK, TFIH, Tat-SF1, TIP30, Tat-CT1, and Tat-CT2 (Cullen, 1990; Marciniak et al., 1990; Herrmann and Rice, 1995; Parada and Roeder, 1996; Yang et al., 1996; Zhou and Sharp, 1996; Cujec et al., 1997; Wei et al., 1998; Wu-Baer et al., 1998; Xiao et al., 1998). Because Tat transactivation, like that with P-TEFb, is inhibited by DRB and requires the CTD of RNA polymerase II, it was hypothesized that P-TEFb may be one of the cellular factors with which Tat interacts (Braddock et al., 1991; Marciniak and Sharp, 1991; Chun and Jeang, 1996; Parada and Roeder, 1996; Yang et al., 1996; Zhu et al., 1997). It was found that GST-Tat fusions could pull down PITALRE from HeLa nuclear extracts indicating that the two proteins could interact (Zhu et al., 1997). Additionally, Tat transactivation was not seen when performed in the presence of nuclear extracts that had been immunodepleted of PITALRE, but transactivation was seen when Drosophila P-TEFb was added back to the reaction (Zhu et al., 1997). In vivo transfection experiments with wildtype or PITALRE kinase knockouts determined that Tat transactivation resulting in reporter gene expression required P-TEFb (Mancebo et al., 1997). Other studies found that P-TEFb bound to the TAR element but only in the presence of Tat (Fujinaga et al., 1998). It was hypothesized that Tat transactivation involves Tat/P-TEFb complexes binding to TAR and thereby bringing PITALRE of P-TEFb in proximity to RNA polymerase. Then PITALRE would phosphorylate the CTD of RNA polymerase II and render the polymerase elongation competent (Fujinaga et al., 1998; Wei et al., 1998).

Recent work by Wei et al. supports the hypothesis that P-TEFb mediates the interaction of Tat with TAR (Wei et al., 1998). Recombinant cyclin T subunit of P-TEFb interacts with immobilized GST-Tat in pull down experiments (Wei et al., 1998). When defective activation domain mutant Tat proteins were used, cyclin T was not pulled down indicating that under the experimental conditions cyclin T binds with Tat through the activation domain of Tat (Wei et al., 1998). Gel mobility shift assays with radiolabeled TAR with or without Tat, with or without cyclin T suggest that cyclin T increases the
affinity of Tat for TAR (Wei et al., 1998). In these studies, wildtype Tat bound weakly to TAR compared to Tat/cyclinT bound to TAR (Wei et al., 1998). In agreement with the GST-Tat/cyclin T pull down experiments, when activation domain mutants of Tat were used in the gel mobility shift assays, the enhancement of binding to TAR by Tat with cyclin T present was lost (Wei et al., 1998). RNase footprinting experiments with TAR and Tat in the presence or absence of cyclin T further confirmed the positive effects of cyclin T on Tat binding to TAR (Wei et al., 1998). RNase digestion of TAR, TAR and Tat, and TAR and cyclin T were similar (Wei et al., 1998). However, RNase digestion of Tat/cyclin T with TAR showed regions of pronounced protection of TAR from nuclease digestion (Wei et al., 1998).

TFI IH is a multisubunit complex whose functions in the cell may involve nucleotide excision repair, cell cycle progression, and transcription (Maldonado and Reinberg, 1995). TFI IH is so far unique among the general transcription factors in that it possesses catalytic activities. The enzymatic activities are ATPase, helicase, and CTD kinase (Maldonado and Reinberg, 1995). Some subunits of TFI IH have roles in nucleotide excision repair. These subunits are XPB/ERCC3/Rad25, XPD/ERCC2/Rad3, and SSL1 (Maldonado and Reinberg, 1995). Both Rad3 and Rad25 from yeast have ATPase and helicase activities, but only Rad25 was shown to be essential for transcription (Feaver et al., 1993). The CTD kinase of TFI IH has been identified as MO15/Cdk7 (Maldonado and Reinberg, 1995). The kinase is a cyclin dependent kinase, and it is the catalytic subunit of Cdk-activating kinase or CAK (Maldonado and Reinberg, 1995). Cyclin H is also a subunit of TFI IH, and MO15/Cdk7 and cyclin H compose CAK (Maldonado and Reinberg, 1995). TFI IH may potentially have a connection to cell cycle control through CAK. CAK phosphorylates and activates cdc2, cdk2, and Cdk4 which are proteins involved with cell cycle progression (Maldonado and Reinberg, 1995).

The CTD kinase activity of TFI IH has been well characterized. It has been demonstrated in vitro that TFI IH itself or in association with the transcription preinitiation
complex can phosphorylate the CTD of RNA polymerase II (Lu et al., 1992). Various combinations of the hypophosphorylated form of RNA polymerase II (IIA form), general transcription factors, and +/- ATP were incubated and allowed to complex (Lu et al., 1992). The complexes were then evaluated on a non-denaturing gel. If the polymerase had been phosphorylated, then the mobility of the complex in the +ATP lane would be less than that in the -ATP lane. The mobility shift was only seen when the complex consisted of polymerase, TFIID (or TBP), TFIIA, TFIIB, TFIIF, TFIIE, TFIIH, and ATP (Lu et al., 1992). When the polymerase was recovered from the gel, the polymerase was identified as being in the hyperphosphorylated form (IIO form) by Western blot (Lu et al., 1992). The kinase activity resides in TFIIH. Only TFIIH, RNA polymerase IIA, and [γ-32P] ATP were required to label the polymerase (Lu et al., 1992; Serizawa et al., 1993b). The kinase activity is specific for the CTD. RNA polymerase II without the CTD (IIB form) was not phosphorylated by TFIIH (Lu et al., 1992). However, like P-TEFb, the kinase recognition requires only the C-terminal part of the largest subunit of RNA polymerase II; a bacterial fusion protein with the CTD was also labelled by TFIIH (Lu et al., 1992).

Several other features of the kinase which are associated with TFIIH are worth mentioning. The kinase activity of TFIIH from humans is stimulated by the presence of the other general transcription factors, but it is not in the case of TFIIH from rat liver (Lu et al., 1992; Serizawa et al., 1993b). The presence of DNA containing sequences of the promoter region of the adenovirus major late promoter was stimulatory, but poly (dI-dC) was not (Lu et al., 1992; Serizawa et al., 1993b). As with P-TEFb, kinase inhibitors H-8 and DRB inhibited the CTD kinase of TFIIH (Serizawa et al., 1993a; Yankulov et al., 1995). Phosphoamino analysis indicated that 90% of the residues phosphorylated were serines and the remainder were threonines (Lu et al., 1992). Phosphorylation of the CTD by human TFIIH appears to be processive in the presence of TFIIE. Timecourses of the kinase reaction with or without TFIIE showed that within five minutes essentially all of the IIA polymerase had been converted to IIO when TFIIE is present (Lu et al., 1992). In the
absence of TFIIE, even after 90 minutes, IIA and intermediates between IIA and IIO forms of the polymerase were detected (Lu et al., 1992). The total incorporation of $^{32}$P in IIA and IIO forms correlated with the amount of incorporation to the IIO in reactions with TFIIE suggesting that each polymerase was completely phosphorylated by the kinase before the kinase began on another polymerase (Lu et al., 1992). In reactions without TFIIE, the radioactivity in the IIO form was about 40% of the total radioactivity (Lu et al., 1992). These results suggest that the TFIIH kinase is processive only in the presence of other protein factors (Lu et al., 1992).

In vitro studies with reconstituted transcription systems have determined that TFIIH affects transcription after initiation (Goodrich and Tjian, 1994; Dvir et al., 1997; Kugel and Goodrich, 1998; Kumar et al., 1998). The development of an abortive initiation assay has defined the minimal requirements for formation of the first phosphodiester bond. In this assay a linear template with the adenovirus major late promoter was incubated with RNA polymerase II, differing mixtures of general transcription factors, dATP as an energy source, CpA as a primer, and [α-$^{32}$P] CTP (Goodrich and Tjian, 1994). The minimal requirements to make the trimer, CpApC, were RNA polymerase II, TBP, TFIIB, and TFIIF (Goodrich and Tjian, 1994). Similar experiments using a template containing five Gal4-binding sites upstream of the adenovirus major late promoter and the addition of Gal4-VP16, PC4, and TFIIB during the reaction, also determined that polymerase, TBP, TFIIB, and TFIIF were sufficient for initiation (Kumar et al., 1998). Another set of studies compared abortive initiation using linear templates that had the adenovirus major late promoter or an adenovirus major late promoter mutant sequence, Ad(-9/-1), that was pre-melted at the start site and did not require TFIIH for initiation (Dvir et al., 1997). It was found that formation of the first phosphodiester bond was comparable using either template (Dvir et al., 1997). From these results it was concluded that TFIIH and TFIIE were not required for initiation (Goodrich and Tjian, 1994; Dvir et al., 1997; Kumar et al., 1998).
The effect of TFIIH and TFIIE on elongation was then tested. The elongation assay consisted of promoter specific initiation with linear or supercoiled DNA having the adenovirus major late promoter followed by a G-less cassette, RNA polymerase II, subsets of general transcription factors, and nucleotides (Goodrich and Tjian, 1994). The reactions were then evaluated for their ability to produce a 390 nucleotide transcript. In the case of the supercoiled template, TFIIH and TFIIE were not required for production of the 390-mer (Goodrich and Tjian, 1994). However, when a linear template was used, TFIIH and TFIIE were necessary (Goodrich and Tjian, 1994). It was also observed that the substitution of a non-hydrolyzable ATP analog in the transcription reaction abolished the elongation effect of TFIIH and TFIIE on linear templates (Goodrich and Tjian, 1994). Further, it was also observed that GTP as a hydrolyzable energy source could not substitute for ATP (Goodrich and Tjian, 1994). Because GTP can be used as a substrate for the CTD kinase activity of TFIIH, this result ruled out the possibility of phosphorylation of the CTD as the post-initiation step affected by TFIIH and TFIIE under the experimental conditions tested (Serizawa et al., 1993b). Taking all of these observations into account, it was proposed that the helicase/ATPase activities of TFIIH were having the effect on elongation (Goodrich and Tjian, 1994). It was speculated that the post-initiation step being affected by TFIIH was promoter clearance (Goodrich and Tjian, 1994).

In order to distinguish between promoter clearance and elongation phases of transcription, the elongation assay was modified. Negatively supercoiled template was used to exclude the requirement of TFIIH and TFIIE for initiation. Transcription was initiated with the addition of Cpa, CTP, and UTP (Goodrich and Tjian, 1994). Based on the template sequence, transcription would only go to +16 where it was assumed that the transcription complex had cleared the promoter (Goodrich and Tjian, 1994). However, it should be noted that in other RNA polymerase II in vitro transcription systems after transcribing 16 nucleotides, the polymerase releases abortive products and has still not
cleared the promoter (Marshall and Price, 1992; Kumar et al., 1998). The template was then linearized by restriction enzyme digestion (Goodrich and Tjian, 1994). Nucleotides were added with or without the addition of TFIIB and TFIIE, and the production of full length transcript was evaluated (Goodrich and Tjian, 1994). It was found that full length transcript was made in the absence or presence of TFIIB and TFIIE (Goodrich and Tjian, 1994). It was concluded that TFIIB and TFIIE were not acting at the level of elongation, but instead, TFIIB and TFIIE were most likely acting on polymerase during promoter clearance (Goodrich and Tjian, 1994).

Work by others supports the hypothesis of the role of TFIIB at promoter clearance (Dvir et al., 1997; Kumar et al., 1998). In experiments using the Ad(-9/-1) template to eliminate the need for TFIIB for initiation, it was observed that complexes would prematurely arrest promoter proximally in the absence of TFIIB (Dvir et al., 1997). These reactions consisted of promoter specific initiation from the Ad(-9/-1) template, +/-TFIIB, subset of nucleotides, and O-methyl GTP to transcribe to +18 (Dvir et al., 1997). Transcription with TFIIB resulted in production of complexes transcribing to +18 (Dvir et al., 1997). Reactions in the absence of TFIIB resulted in no complexes making it to +18, but there were many transcripts recovered between +8 to +12 (Dvir et al., 1997). It was not determined whether the transcripts were still associated with an arrested complex or if the polymerases had terminated prior to transcribing to +18 (Dvir et al., 1997). It was also noted that for TFIIB to have its effect ATP and TFIIE were required (Dvir et al., 1997). If a different set of nucleotides were used to make artificially halted complexes at +5 and +7 before adding nucleotides to transcribe to +18 with or without TFIIB, it was found that TFIIB was able to facilitate transcription (Dvir et al., 1997). These results would suggest that TFIIB is able to enter the transcription cycle post-initiation and stimulate transcription by very early elongation complexes (Dvir et al., 1997).

In another set of studies, transcription was performed with a template containing five Gal4-VP16 binding sites upstream of the adenovirus major late promoter followed by a
50 base U-less cassette (Kumar et al., 1998). Pre-initiation complexes were formed with RNA polymerase II and general transcription factors (Kumar et al., 1998). For activated transcription, Gal4-VP16, PC4, and TFIIA were also included (Kumar et al., 1998). The template was biotinylated and immobilized on streptavidin coated magnetic beads enabling the pre-initiation complexes to be washed before incubation with ATP, CTP, and GTP to transcribe a 50 nucleotide transcript (Kumar et al., 1998). Further, the released, aborted transcripts could be distinguished from transcripts associated with the polymerase elongation complex by loading the supernatant or the bound fraction, respectively (Kumar et al., 1998). When TFIIB was not present, no complexes were recovered at +50, but many complexes had arrested or aborted at +12 to +17 (Kumar et al., 1998). When TFIIB was present, the majority of the polymerases transcribed to +50, and the amount of arrested complexes and aborts was reduced (Kumar et al., 1998). These results indicate that TFIIB is affecting promoter clearance in agreement with the observations of Dvir and co-workers. When the arrested complexes were isolated and TFIIB was added before resumption of transcription, no stimulatory effect was seen (Kumar et al., 1998). It appears that TFIIB acts to prevent arrest rather than acts on an already arrested complex. When the elongation assay was repeated except with the addition of activators, it was found that the proportion of complexes that were able to transcribe to +50 was significantly increased over that of reactions with TFIIB alone (Kumar et al., 1998). Also, the amount of complexes arrested promoter proximally and the amount of aborted products were reduced even more than with TFIIB alone (Kumar et al., 1998). The effect of the activators indicate that the effect of TFIIB on promoter clearance may be regulated in vivo (Kumar et al., 1998).

Although studies of promoter clearance suggest that the helicase/ATPase activities of TFIIB are involved, investigations indicating a possible connection between TFIIB and Tat suggest a role for the CTD kinase activity of TFIIB (Parada and Roeder, 1996; Cujec et al., 1997; Garcia-Martinez et al., 1997). Garcia-Martinez and co-workers purified from HeLa nuclear extracts a kinase activity capable of binding Tat, phosphorylating the CTD,
and stimulating Tat transcription activation \textit{in vitro} (Garcia-Martinez et al., 1997). A 600 kDa complex was isolated and microsequenced (Garcia-Martinez et al., 1997). Along with several uncharacterized polypeptides, subunits of TFIIH were identified including CAK (Garcia-Martinez et al., 1997). TFIIH and Tat interact \textit{in vitro} based on GST-Tat pull down experiments with HeLa nuclear extracts (Parada and Roeder, 1996). The kinase activity of TFIIH resides in a complex referred to as CAK that consists of subunits CDK7, cyclinH, and MAT1 (Maldonado and Reinberg, 1995). \textit{In vitro} pull down experiments with immobilized Tat and various combinations of the subunits of CAK showed that CDK7 alone could interact with Tat, but cyclinH and MAT1 could not (Cujec et al., 1997). Furthermore, Tat mutants defective in the activation domain did not bind CDK7 under similar experimental conditions (Cujec et al., 1997). \textit{In vivo}, TFIIH and Tat associate as determined by immunoprecipitation from extracts (Cujec et al., 1997). Hemagglutinin tagged Tat was expressed in COS cells, and total cell lysates were used to immunoprecipitate TFIIH with antibodies against ERCC3, the largest subunit of TFIIH (Cujec et al., 1997). Western blots of the proteins pulled down with TFIIH determined that Tat was present (Cujec et al., 1997). Similar experiments performed with activation domain mutants of Tat did not detect an interaction between TFIIH and the mutant Tat proteins (Cujec et al., 1997). These results suggest that TFIIH and Tat interact through the CDK7 subunit of TFIIH and the activation domain of Tat (Cujec et al., 1997).

Hela nuclear extracts immunodepleted of TFIIH were able to support a small amount of Tat transactivation, but the addition of purified TFIIH dramatically stimulated the Tat transactivation effect (Parada and Roeder, 1996). It was assumed that the small amount of Tat activation observed in the absence of TFIIH was due to residual TFIIH left in the immunodepleted extracts (Parada and Roeder, 1996). These results lent support to the involvement of TFIIH to Tat stimulation of transcription. Experiments to test the effect of Tat on the TFIIH CTD kinase activity consisted of recombinant GST-CTD incubated with purified TFIIH and \([\gamma^{32}\text{P}]\) ATP with or without Tat (Parada and Roeder, 1996). In the
presence of Tat, the GST-CTD was phosphorylated approximately 20 times more than in the absence of Tat (Parada and Roeder, 1996). It was also observed that the activation domain of Tat, amino acids 1-48, was sufficient to stimulate the CTD kinase of TFIIH, but non-functional mutants of the Tat activation domain were not (Parada and Roeder, 1996). Kinase studies with recombinant CAK, GST-CTD or purified RNA polymerase II, and \[\gamma^{32P}\] ATP incubated in the presence or absence of wildtype or activation domain mutant Tat proteins showed the same results (Cujec et al., 1997).

To test the possible link between the CTD kinase activity of TFIIH with the Tat transactivation effect on transcription, kinase inhibitors of TFIIH were used (Parada and Roeder, 1996; Cujec et al., 1997). In one instance, H-8 was used, and in the other, a mutant peptide sequence of CDK2 (CDK2 is a substrate for CAK) (Parada and Roeder, 1996; Cujec et al., 1997). Tat stimulation of transcription of HIV LTR templates was inhibited by the addition of the kinase inhibitors, H-8 and mutant CDK2 (Parada and Roeder, 1996; Cujec et al., 1997). To test the \textit{in vivo} requirement for CDK7 for Tat transactivation, COS cells were transfected with a plasmid coding for the a reporter gene under the control of the HIV LTR promoter sequences, a plasmid coding for Tat (or a negative control), and increasing amounts of the CDK2 mutant peptide (Cujec et al., 1997). RNase protection assays were performed to evaluate the amount of transcripts promoter proximal (+55 to 59 bases) and distal (+220 bases) (Cujec et al., 1997). As expected, in the absence of Tat, the majority of the transcripts were short, but in the presence of Tat, long transcripts were detected (Cujec et al., 1997). The levels of short transcripts were not affected by the CDK2 mutant peptide whether or not Tat was present (Cujec et al., 1997). However, Tat stimulation of long transcripts was inhibited by the presence of the CDK7 kinase inhibitor (Cujec et al., 1997). In related experiments a kinase deficient mutant of CDK7 was overexpressed in COS cells, and Tat transactivation was lost (Cujec et al., 1997). These results indicate that Tat transactivation is dependent on the CTD kinase activity of the CDK7 subunit of TFIIH \textit{in vivo} (Cujec et al., 1997). A model for the
mechanism of Tat transactivation proposed that Tat associates with the pre-initiation complex, and when RNA polymerase II transcribes TAR, Tat interacts with TAR and undergoes a change (perhaps in position or conformation). Tat can now stimulate the CTD kinase of TFIH, CDK7, and phosphorylation of the polymerase CTD results in a change in the elongation competence of the polymerase (Cujec et al., 1997). The observations of P-TEFb and TFIH involvement in Tat activation suggest that there may be multiple targets in the cell for Tat transactivation.

SRB10 and SRB11 form a complex that phosphorylates RNA polymerase II (Liao et al., 1995; Sun et al., 1998). They were isolated from a screen identifying extragenic suppressors of S. cerevisiae RNA polymerase II CTD truncation mutants (Suppressors of RNA polymerase II) (Thompson et al., 1993). Although the components of the complex have varied depending on the purification scheme, SRB proteins have been purified in association with various RNA polymerase II complexes (RNA polymerase II, subsets of general transcription factors, SRB proteins, and unidentified polypeptides) (Thompson et al., 1993; Koleske and Young, 1994; Koleske and Young, 1995; Liao et al., 1995; Maldonado et al., 1996; Sun et al., 1998). In some cases, SRB10/SRB11 has co-purified with one of these complexes, in others, it has not (Liao et al., 1995; Myers et al., 1998).

SRB10 and SRB11 most likely form a complex (Liao et al., 1995). GST-SRB11 pull down experiments with labeled SRB10 suggest that the two proteins interact, and a genetic interaction was indicated in a two hybrid screen (Liao et al., 1995). SRB10 is identical to UME5 which is involved in regulation of meiosis specific genes (Surosky et al., 1994; Liao et al., 1995). SRB11 has sequence homology to cycC from humans and Drosophila (Liao et al., 1995). SRB10 and SRB11 also are identical to SSN3 and SSN8, respectively (Kuchin et al., 1995; Song et al., 1996). SSN3 and SSN8 are members of a set of suppressors of snf1 (Kuchin et al., 1995; Song et al., 1996). SSN proteins may be involved in negative regulation of transcription (Kuchin et al., 1995; Song et al., 1996). It was found that mutations in SRB10 and SRB11 affected in vivo response to transcription
regulation (Liao et al., 1995). Using a plasmid with a GAL10 UAS containing promoter with a β-galactosidase reporter and strains with wildtype or mutant srb's, the response of the yeast to galactose was measured (Liao et al., 1995). It was found that srb10Δl and srb11Δl strains responded very poorly to galactose (Liao et al., 1995). A strain expressing srb10-3, an inactive kinase, was about 100-fold less responsive to galactose than wildtype (Liao et al., 1995). These results suggest that SRB10 and SRB11 may have roles in transcription regulation (Liao et al., 1995). The human homologs of SRB10 and SRB11 have been purified in a complex called NAT which represses activated transcription (Sun et al., 1998).

SRB10 and SRB11 proteins may regulate transcription by affecting the phosphorylation state of the RNA polymerase II CTD. SRB10 has sequence homology to cdc2 and CDC28 kinases (Liao et al., 1995). The kinase activity of SRB10/SRB11 is capable of phosphorylating the CTD of RNA polymerase II (Liao et al., 1995; Sun et al., 1998). In studies with the NAT complex, it was determined that the residues of the CTD phosphorylated were different than those phosphorylated by TFIH (Sun et al., 1998). In addition, NAT did not interact with the phosphorylated form of the polymerase (Sun et al., 1998).

If phosphorylation of the RNA polymerase II CTD is a point of regulation, it stands to reason that de-phosphorylation would also be a point of regulation (Dahmus, 1996). A phosphatase specific for the CTD of RNA polymerase II has been purified from both human (HeLa) and Saccharomyces cerevisiae, and the two are clearly homologous (Chambers and Dahmus, 1994; Chambers and Kane, 1996). The phosphatase activity from HeLa cells purifies with a fraction containing two polypeptides of apparent molecular mass of 205 kDa and 105 kDa as determined by SDS PAGE; the phosphatase activity is associated with the smaller peptide (Chambers and Dahmus, 1994; Marshall et al., 1998). The yeast phosphatase has an apparent molecular mass of 100 kDa and 103 kDa (Chambers and Kane, 1996). The phosphatase has been classified as a type 2C
phosphatase based on its requirement for Mg\(^{2+}\) and its resistance to okadaic acid (Chambers and Dahmus, 1994; Chambers and Kane, 1996). The phosphatase from yeast differs from that from humans in that the yeast phosphatase can use Ca\(^{2+}\), but the human phosphatase cannot (Chambers and Kane, 1996). The phosphatase acts processively. RNA polymerase II labelled with \([\gamma-^{32}P]\) ATP and TFIIH and then incubated with phosphatase produces the IIA form of the polymerase with no intermediates detected (Chambers and Dahmus, 1994). The phosphatase is specific for the RNA polymerase II CTD (Chambers and Kane, 1996). In this recognition, it contrasts with CTD kinases so far described. The phosphatase can not use phosphorylase a or the \(\alpha\) or \(\beta\) subunits of phosphorylase kinase as substrates (Chambers and Dahmus, 1994). The CTD phosphatase has a docking site on the RNA polymerase II, and it can complex with the polymerase in experiments using an RNA polymerase II affinity column (Chambers et al., 1995). Also, the phosphatase activity is competitively inhibited by the hypophosphorylated form of RNA polymerase II (Chambers et al., 1995; Chambers and Kane, 1996). The phosphatase binding site on the polymerase most likely is distinct from that of the CTD because RNA polymerase IIB (the form of polymerase lacking the CTD) also competitively inhibits the phosphatase (Chambers et al., 1995). In addition, phosphorylated, recombinant CTD cannot serve as a substrate for the phosphatase (Chambers et al., 1995).

The gene coding for an essential subunit of the CTD phosphatase has been named \(FCP1\) (TFIIF-associating component of CTD phosphatase) (Archambault et al., 1997; Archambault et al., 1998). Human \(FCP1\) was isolated from a two hybrid screen using the largest subunit of TFIIF, RAP74, as bait (Archambault et al., 1998). The gene coded for the 105 kDa polypeptide, and the recombinant Fcp1 had phosphatase activity (Marshall et al., 1998). In yeast, \(FCP1\) is an essential gene (Archambault et al., 1997). It had been demonstrated earlier that the phosphatase activity is stimulated in the presence of TFIIF (Chambers et al., 1995; Chambers and Kane, 1996). GST pull down experiments with GST- yeast RAP74 (residues 649-735) and labelled yeast Fcp1 also indicate that there is an
interaction between the two proteins (Archambault et al., 1997). When deletion mutants of yeast Fcp1 were used in the pull down studies, it was found that two regions of Fcp1 can complex with RAP74 in vitro (Archambault et al., 1997). The carboxy terminal TFIIIF binding domain of Fcp1 is dispensable in vivo and in vitro in yeast (Archambault et al., 1997).

The yeast and human CTD phosphatases also display species specificity. Yeast CTD phosphatase could not use mammalian RNA polymerase II0 as a substrate nor could human CTD phosphatase use yeast RNA polymerase II0 as a substrate (Chambers and Kane, 1996). As with the polymerases, the CTD phosphatases were species specific in their interactions with TFIIF. Mammalian TFIIIF could not stimulate yeast Fcp1 nor could yeast TFIIIF stimulate human Fcp1 (Chambers and Kane, 1996).

As with P-TEFb and TFIIH, Fcp1 may be regulated by Tat. Although Tat stimulates the CTD kinase activity of TFIIH, the opposite appears to be the case with the CTD phosphatase (Parada and Roeder, 1996; Cujec et al., 1997; Marshall et al., 1998). When Tat was incubated with human Fcp1 and labelled RNA polymerase II0, the phosphatase activity of Fcp1 was inhibited (Marshall et al., 1998). Further, activation domain mutants of Tat that are defective in stimulating Tat dependent transactivation were less effective at inhibiting Fcp1 (Marshall et al., 1998). However, a Tat mutant deleted of almost the entire activation domain was not able to inhibit Fcp1 (Marshall et al., 1998). It was proposed that Tat dependent inhibition of the CTD phosphatase may be a part of the mechanism of Tat transactivation (Marshall et al., 1998).

HMG14/17 and FACT

In the cell, RNA polymerase II must transcribe chromatin templates as opposed to the naked DNA used in many in vitro transcription systems. Within the past few years, proteins which facilitate transcription elongation on chromatin templates have been characterized (Crippa et al., 1993; Ding et al., 1994; Bustin et al., 1995; Hartzog and
Winston, 1997; John and Workman, 1998; Kadonaga, 1998; LeRoy et al., 1998; Orphanides et al., 1998). SPT4, SPT5, and SPT6 will be discussed below in the context of their relationship with TFIIS.

HMG14/17 are a subset of high mobility group proteins that may have a role in regulation of transcription elongation (Bustin et al., 1995). Hydroxy radical and DNaseI footprinting indicate that HMG14/17 bind to the 146 base pair nucleosomal core particle (Alfonso et al., 1994). Estimates of the in vivo amount of HMG14/17 suggest that the proteins are associated with only a subset of nucleosomes (Bustin et al., 1995). Although there is not yet consensus in the field, several observations indicate that HMG14/17 play a role in regulation of transcription. HMG14/17 appear to associate with regions of chromatin with actively expressed genes or with portions of chromatin with acetylated histones (Sandeen et al., 1980; Druckmann et al., 1986; Brotherton et al., 1990; Postnikov et al., 1991). In addition, antibodies against HMG14/17 inhibit transcription when injected in nuclei (Einck and Bustin, 1983). The antibodies have also been shown to bind to polytene chromosomes in areas that are transcribed (Westermann and Grossbach, 1984). In vitro transcription experiments with chromatin templates indicate that transcription is elevated on templates containing HMG14/17 (Crippa et al., 1993). In these studies, M13 single stranded DNA and Xenopus egg extracts were incubated with or without HMG14/17 (Crippa et al., 1993). Under the experimental conditions, DNA replication and chromatin assembly occurred (Crippa et al., 1993). Nucleotides were then added to initiate transcription, and the transcripts were isolated (Crippa et al., 1993). There was approximately four-fold more transcripts produced from reactions with templates that assembled in the presence of HMG14/17 than from those assembled in the absence of HMG14/17 (Crippa et al., 1993). The authors also noted that the effect of HMG14/17 on transcription did not occur if HMG14/17 were added to naked DNA or to pre-assembled nucleosomes (Crippa et al., 1993).
A second set of studies also showed that HMG14 enhanced transcription, and the rate of elongation by RNA polymerase II was stimulated by the presence of HMG14 (Ding et al., 1994). For these experiments, SV40 minichromosomes were assembled in vivo in CV-1 cell lines that either did or did not overexpress HMG14 (Ding et al., 1994). The investigators used a pulse-chase transcription assay consisting of the minichromosomes with or without HMG14 and HeLa whole cell extracts incubated with nucleotides (one of which was radioactive) followed by a chase with high concentrations of unlabelled nucleotides (Ding et al., 1994). There was 2.8 to 5.5-fold more transcription using HMG14 associated templates than with the negative control (Ding et al., 1994). In apparent contrast to results of Crippa et al., the addition of recombinant HMG14 to minichromosomes isolated from non-HMG14 expressing cells resulted in a dose dependent stimulation of transcription (Ding et al., 1994). The contradictory results may be due to the different conditions used to assemble the templates and perform in vitro transcription (Crippa et al., 1993; Ding et al., 1994). Ding and co-workers also found that transcription was enhanced when recombinant HMG14 was added during the chase phase of the transcription reaction after initiation (Ding et al., 1994). These results indicate that HMG14 was affecting polymerase post-initiation (Ding et al., 1994). When a timecourse was done with the pulse-chase experiments, it was found that the elongation rate of the polymerase was about 3.5 times greater when HMG14 was added to the reaction than when it was not (Ding et al., 1994). When recombinant HMG14 was added to naked DNA, no enhancement of transcription was observed suggesting that the transcription stimulation was likely due to HMG14 affecting the chromatin template rather than the transcriptional machinery (Ding et al., 1994). Another group has reported that association of HMG14/17 with nucleosomes during replication decreases the degree of compactness of the chromatin (Trieschmann et al., 1995). It has been proposed that the less compact HMG14/17 containing chromatin may provide better access for the transcriptional machinery in vivo (Hansen and Wolffe, 1994; Bustin et al., 1995).
FACT is an activity that facilitates chromatin transcription (John and Workman, 1998; LeRoy et al., 1998; Orphanides et al., 1998). The search for FACT arose from the observation that in vitro transcription with a reconstituted system consisting of recombinant or highly purified general transcription factors, pure RNA polymerase II, and co-activators PC4 and TFIIB could support productive transcription on naked templates, but not on chromatin templates (Orphanides et al., 1998). However, HeLa nuclear extracts could be used to produce long transcripts on chromatin templates (Orphanides et al., 1998). In these assays a plasmid template containing five GAL4 DNA binding sites, the adenovirus major late promoter, and a 390 base pair G-less cassette was used (Orphanides et al., 1998). The chromatin template was assembled by incubating the plasmid with Drosophila embryo S-190 extract and purified Hela cell core histones and ATP (Orphanides et al., 1998). The chromatin templates were then purified by gel filtration chromatography to remove almost all the excess proteins and incompletely assembled templates (Orphanides et al., 1998). It was estimated that the purified, chromatin templates were approximately 95% homogeneous (Orphanides et al., 1998). In some cases, the chromatin templates were assembled in the presence of GAL4-VP16 or GAL4(1–94) (the DNA binding region of GAL4) in order to make promoter proximal remodeled templates (Orphanides et al., 1998). For transcription, the templates were incubated with either the reconstituted system or the HeLa nuclear extracts and nucleotides to form a 390 nucleotide transcript (Orphanides et al., 1998).

When naked DNA was used as a template, both the reconstituted system and the nuclear extract were able to produce the full length transcript (Orphanides et al., 1998). When a non-remodeled chromatin template was used, full length RNA was not detected from reactions with either the reconstituted system or with the nuclear extract (Orphanides et al., 1998). When the remodeled chromatin template was used, the reconstituted system did not produce full length transcript, but reactions using HeLa extracts did (Orphanides et al., 1998). It was concluded from these results that the extract contains a factor or factors
that are necessary for productive transcription on remodeled chromatin templates (Orphanides et al., 1998).

It was determined that the factor in the extract affected transcription elongation (Orphanides et al., 1998). To investigate if the missing factor in the reconstituted system affected initiation, an abortive initiation assay was used (Orphanides et al., 1998). The assay was similar to that of the productive transcription assay except that only nucleotides needed to make the first phosphodiester bond (ATP and [α-32P]CTP) were used (Orphanides et al., 1998). The production of dimers was seen on both naked and chromatin remodeled templates (Orphanides et al., 1998). This result suggested that the missing factor stimulated productive elongation on chromatin templates by influencing a post-initiation event of transcription (Orphanides et al., 1998). A pulse-chase assay was used to take a closer look at the transcripts produced by the reconstituted system on the remodeled chromatin templates (Orphanides et al., 1998). The assay was similar to that used to make the 390-mer except that the transcripts were labeled with limiting amounts of radiolabeled CTP and then chased with a high concentration of CTP (Orphanides et al., 1998). The resulting 5'end labeled transcripts could then be detected even if they were short due to a block to elongation (Orphanides et al., 1998). It was found that little if any full length 390-mer was produced, but instead, most of the transcripts were less than 40 nucleotides long (Orphanides et al., 1998). When Sarkosyl was added during the chase in concentrations high enough to remove histones, full length transcripts were produced (Orphanides et al., 1998). These results suggested that the histones were the impediment to the polymerase as it elongated (Orphanides et al., 1998).

Using the pulse-chase assay, an activity, FACT, which enabled the reconstituted system to synthesize full length transcript on remodeled chromatin templates was purified from HeLa nuclear extracts (Orphanides et al., 1998). The FACT activity co-eluted with 140 and 80 kDa polypeptides (Orphanides et al., 1998). FACT stimulation of elongation did not require ATP hydrolysis (Orphanides et al., 1998). Use of AMPPNP and
GMPPNP in the transcription chase had no inhibitory effect on FACT stimulation (Orphanides et al., 1998). FACT did not change the micrococcal nuclease digestion pattern on chromatin templates (Orphanides et al., 1998). These two observations suggest that FACT is not a nucleosome remodeling factor (Orphanides et al., 1998). The effect of FACT on elongation was not synergistic with the presence of either TFIIF or TFIIS (Orphanides et al., 1998). The elongation effect of FACT was somewhat inhibited by the addition of excess DNA in the transcription reactions suggesting that FACT may bind DNA (Orphanides et al., 1998).

N-TEF

N-TEF is a negative transcription elongation factor (Price et al., 1987). N-TEF is an activity opposite of P-TEF in that N-TEF suppresses the production of long transcripts (Price et al., 1987). Factor 2 is a 154 kDa component of N-TEF purified from Drosophila Kc nuclear extracts (Price et al., 1987). The inhibitory effect of factor 2 on productive transcription may be due to the ATP dependent transcript release activity of factor 2 (Xie and Price, 1996). The transcript release assay used promoter specific initiation with nuclear extracts on immobilized templates (Xie and Price, 1996). Early elongation complexes were formed by incubating the preinitiation complexes briefly with low concentrations of nucleotides (one of which was radiolabelled) (Xie and Price, 1996). The reaction was stopped with EDTA, and the isolated complexes were washed (Xie and Price, 1996). The complexes were then incubated with combinations of ATP and factor 2 (Xie and Price, 1996). The transcripts that remained associated with the complex ('bound' to the resin) and the transcripts that were released (free in the supernatant) were then analyzed (Xie and Price, 1996). Only when the complexes were incubated with both ATP and factor 2 were transcripts released (Xie and Price, 1996).

Several observations have been made about the activity of factor 2. Factor 2 has an ATPase activity (Xie and Price, 1997). Supercoiled DNA stimulated the ATPase activity.
better than linear DNA, but double stranded RNA, single stranded RNA, single stranded DNA, and RNA:DNA heteroduplex had no effect (Xie and Price, 1998). However, single stranded DNA could inhibit the stimulatory effect of double stranded DNA on the ATPase activity (Xie and Price, 1998). Additionally, the transcript release activity of factor 2 was stimulated by double stranded DNA and inhibited by single stranded DNA (Xie and Price, 1997; Xie and Price, 1998). Gel mobility shift assays demonstrated that factor 2 can complex with both double and single stranded DNA (Xie and Price, 1998). It was observed that ATP destabilized the factor 2 interaction with double stranded DNA but not with single stranded DNA (Xie and Price, 1998). Because factor 2 could not displace an oligo hybridized to single stranded M13 DNA, it was concluded that factor 2 did not have a helicase activity (Xie and Price, 1998). Xie and Price also found that factor 2 could facilitate transcript release from complexes formed with purified polymerase and a dC-tailed template (Xie and Price, 1997). These results suggest that no protein co-factors are required by factor 2 for its release activity (Xie and Price, 1997). Xie and Price speculate that factor 2 interacts with the elongation complex through the DNA template rather than through the RNA transcript based on the stimulatory effects of DNA on factor 2 and the lack of effect of RNA on factor 2 (Xie and Price, 1998). The destabilizing effect of ATP on factor 2:DNA interactions and the requirement for ATP hydrolysis for release activity (nonhydrolyzable analogs of ATP could not substitute for ATP in the release assays) suggest that the ATPase activity of factor 2 is key to the release activity of factor 2 (Xie and Price, 1997). However the mechanism by which factor 2 induces the polymerase to release its transcript has not been determined.

Gre Factors

As a proposed mechanism to increase productive transcription, GreA, GreB, and TFIIS are elongation factors that promote in vitro readthrough by inducing ternary elongation complexes of RNA polymerase to cleave the nascent transcript (Kassavetis and
Geiduschek, 1993; Reines, 1994). Although bacterial proteins GreA and GreB are functional homologs of TFIIS, they share no sequence or structural homology to the eukaryotic transcription factor (Hubert et al., 1983; Sparkowski and Das, 1990; Stebbins et al., 1995; Olmsted et al., 1998).

GreA was first identified as a high copy suppressor of a temperature sensitive mutant of the *E. coli* RNA polymerase β subunit (Sparkowski and Das, 1990; Sparkowski and Das, 1991). The Gre factors most likely stimulate an intrinsic cleavage activity of RNA polymerase (Orlova et al., 1995). GreA has no detectable nucleolytic activity when incubated with RNA or RNA:DNA hybrids (Borukhov et al., 1992). However, artificially halted transcription complexes are induced to cleave dimers from the 3'end of the nascent RNA when GreA is present (Borukhov et al., 1992). GreB is homologous to GreA, but GreB has a different effect on polymerase. Transcript cleavage products produced by RNA polymerase in the presence of GreB tend to be larger oligomers than the dimers from GreA induced cleavage (Borukhov et al., 1993).

GreA appears to act by preventing arrest rather than reactivating an already arrested complex. GreA facilitated readthrough of a block to elongation on an *rmbP1* promoter template when added before the complex had reached the block, but not after the polymerase had arrested (Borukhov et al., 1992). This observation suggests that the mechanism of readthrough is more complex than simply promoting the polymerase to cleave its transcript. Studies with TFIIS to be discussed later provide evidence that induction of cleavage by RNA polymerase II is necessary but not sufficient to facilitate readthrough. Kinetic experiments with bacterial polymerase led to the proposal that the polymerase can adopt several conformations of varying activity (Erie et al., 1993). Possibly GreA prevents arrest by keeping RNA polymerase in an elongation competent conformation. Unlike GreA, GreB does act on arrested complexes (Borukhov et al., 1993).
Structural studies have shown that GreA and GreB are closely related and each is composed of two domains. There is an N-terminal coiled coil domain and a C-terminal globular domain (Stebbins et al., 1995; Koulich et al., 1997). The amino terminal domain has a basic patch, and a site near this patch crosslinks to an 8-N3-AMP incorporated at the 3' end of the transcript (Koulich et al., 1997). Domain swapping studies between GreA and GreB show that the N-terminal domain is involved in the anti-arrest and cleavage function of the factors (Koulich et al., 1997). Work with the recombinant domains indicates that the coiled coil domain can induce cleavage by polymerase, and the globular domain can interact with the polymerase (Koulich et al., 1998). However, added either individually or together in trans, the domains were not able to alleviate arrest suggesting that proper orientation of the two domains may be critical for the wildtype activities (Koulich et al., 1998).

It is not yet known what in vivo function the Gre factors may provide. Through preventing arrest or re-activating arrested transcription complexes, the Gre proteins may increase productive elongation. Similar to that for TFIIIS, it has been proposed that the cleavage event may provided a mechanism for increasing transcription fidelity (Erie et al., 1993; Jeon and Agarwal, 1996; Thomas et al., 1998). The effect of GreA on RNA polymerase transcribing templates containing the his or trp leader pause sites was tested, and no significant change in the half-life of pausing by polymerase was seen when GreA was present (Feng et al., 1994). Also the effect of GreA on the efficiency of termination by RNA polymerase at several ρ-independent terminators was tested, and no significant effect was seen (Feng et al., 1994). However, GreA and GreB did have an effect on abortive initiation and promoter clearance in vitro (Hsu et al., 1995). In addition, in vivo, expression of a reporter gene under the control of a weak promoter was increased upon overexpression of GreA and GreB in their respective deletion strains (Hsu et al., 1995).
TFIIS

Transcription factor TFIIS is the most extensively characterized of all the eukaryotic transcription elongation factors. Most of what is known about TFIIS is based on in vitro biochemical studies. TFIIS was first identified from mouse cell extracts as an activity, termed SII, that stimulated RNA polymerase II in nonspecific transcription assays (Natori et al., 1973; Sekimizu et al., 1976). Originally purified from Ehrlich ascites tumor cells, it was determined that the protein responsible for the stimulatory activity was a 40 kDa basic protein (pI=8.7) (Sekimizu et al., 1979; Sekimizu et al., 1981). At the same time it was observed that the protein could be purified in at least two states, one of which was phosphorylated (Sekimizu et al., 1981). Later work with the yeast homolog revealed that the factor was species specific and could not stimulate wheat germ or calf thymus RNA polymerase II (Sawadogo et al., 1980a). Roeder's group working with human TFIIS and Weinmann's group working with calf thymus TFIIS hypothesized that TFIIS may act at the level of transcription elongation (Rappaport et al., 1987; Reinberg and Roeder, 1987). During single round, promoter specific transcription from the AdML promoter, the stimulatory effect of TFIIS was the same whether TFIIS was added before or after transcription was initiated (Rappaport et al., 1987; Reinberg and Roeder, 1987). When the transcripts were visualized on gels, full sized transcripts appeared earliest in reactions with TFIIS present. It was also noted that when TFIIS was present, fewer polymerases "paused" at two sites on the template which mapped to the in vivo transcription termination sites of adenovirus (Reinberg and Roeder, 1987). It was proposed that TFIIS stimulated the rate of transcription by decreasing the pausing of the polymerase as it traversed the DNA template (Rappaport et al., 1987; Reinberg and Roeder, 1987).

TFIIS was also purified to apparent homogeneity from S. cerevisiae as a DNA strand transfer protein (strand-transfer protein α, STPα) that catalyzed the transfer of a DNA strand from linear duplex DNA to a complementary circular single strand (Sugino et al., 1988). It was proposed that STPα may be involved in meiotic recombination (Sugino
et al., 1988). The gene encoding STPα, DST1 (DNA strand transferase 1), was identical to PPR2, the gene originally identified as encoding yeast TFIIS (Hubert et al., 1983; Sugino et al., 1988; Clark et al., 1991; Kipling and Kearsey, 1993). Disruption of DST1 by URA3 resulted in a viable strain (Clark et al., 1991). Sporulation by dst1-1/dst1-1 diploid strains was like that of wildtype strains indicating that DST1 is not necessary for meiosis and sporulation (Clark et al. 1991). The dst1-1/dst1-1 diploid strain did show reduced intragenic meiotic recombination of his1 heteroalleles as compared to a dst1/DST1 strain (Clark et al., 1991). The significance of the apparent strand transfer activity is unclear. E. coli strains expressing high levels of DST1 had no enhanced strand transfer activity (Clark et al., 1991). The authors speculate that post-translational modification may be required to activate DST1 during meiosis (Clark et al., 1991).

Although the major body of work on TFIIS has been in vitro biochemical studies, the in vivo work with TFIIS may potentially reveal the greatest understanding of its role in the cell. TFIIS in yeast was originally identified in a screen designed to look for yeast mutants that were sensitive to the drug 6-azauracil (Hubert et al., 1983). In the screen was found a mutant in the gene, PPR2, which codes for TFIIS (Hubert et al., 1983). Such mutants were expected to be regulatory for the pyrimidine biosynthetic pathway (Hubert et al., 1983). The drug, 6-azauracil, competitively inhibits orotidyl acid decarboxylase and IMP dehydrogenase (Handschumacher, 1960; Exinger and Lacroute, 1992). Yeast grown in the presence of this drug show a 2-3-fold drop in UTP pools and a 10-fold drop in GTP pools (Exinger and Lacroute, 1992). ppr2Δ strains were found to be more sensitive to 6-azauracil than wildtype strains (Exinger and Lacroute, 1992). In addition, the ppr2Δ strain was more sensitive to mycophenolic acid, an inhibitor of IMP dehydrogenase (Exinger and Lacroute, 1992). Because it has been observed in vitro that transcription with low concentrations of nucleotides causes polymerase to be more prone to pause and arrest, it has been hypothesized that when yeast are exposed to 6-azauracil, the UTP and GTP pools drop creating a situation where the polymerase now needs the help of an elongation factor,
such as TFIIS, in order to transcribe productively (Uptain et al., 1997). However, the pleasing simplicity of this hypothesis cannot explain all phenotypes observed in ppr2Δ strains, as discussed below.

TFIIS has been found in all eukaryotic systems examined for its gene or activity ranging from humans to yeast (Hubert et al., 1983; Chen et al., 1992 and references therein). Sequences closely related to PPR2 have been seen in archaea and pox viruses (Ahn et al., 1990; Langer and Zillig, 1993; Rosales et al., 1994). TFIIS proteins are conserved across species with the most highly conserved region encompassed by a carboxy-terminal Zn ribbon motif and the most varied portion in the amino-terminus. The Zn ribbon motif shares significant sequence similarity with RPB9, A12.2, and C11 (Woychik et al., 1991; Nogi et al., 1993; Zhu et al., 1996; Chedin et al., 1998; Wang et al., 1998). Mutagenesis studies with TFIIS determined that the Zn ribbon was very important for TFIIS stimulation of cleavage and readthrough by RNA polymerase II (Agarwal et al., 1991; Jeon et al., 1994; Cipres-Palacin and Kane, 1995; Nakanishi et al., 1995). The function of the varied amino portion of TFIIS is less clear. There is evidence that this region is phosphorylated in vivo, but this region is dispensable for the in vitro TFIIS stimulatory activities and suppression of 6-azauracil sensitivity in a ppr2Δ strain (Sekimizu et al., 1981; Horikoshi et al., 1985; Supta et al., 1985; Nakanishi et al., 1995). However, the amino-terminus is required for the genetic interaction between TFG3 and PPR2 (Davie, 1998). A null allele of TFG3 was isolated from a synthetic lethal screen designed to identify mutant alleles that require PPR2 for viability (Davie, 1998). A tfg3Δ strain was mated with a ppr2Δ strain containing either a plasmid coding for full length PPR2 or the truncated gene (sequence coding for amino acids 131-309) (Davie, 1998). The diploids were sporulated, and the tfg3Δ ppr2Δ strains were checked for the presence of the plasmid bearing either the full length PPR2 or the truncated one (Davie, 1998). From the diploid bearing the plasmid with full length PPR2, viable tfg3Δ ppr2Δ segregants all contained the plasmid (Davie, 1998). No viable tfg3Δ ppr2Δ segregants

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were obtained from the diploid with the plasmid coding for the truncated TFIIS protein (Davie, 1998). TFG3 is a yeast protein found in TFIID, the SWI/SNF complex, and in the TFIIF complex (Henry et al., 1994; Cairns et al., 1996; Kadonaga, 1998).

There is a TFIIS gene family within mammals, and tissue specificity in expression (Kanai et al., 1991; Xu et al., 1994; Ito et al., 1996; Plant et al., 1996; Weaver and Kane, 1997). It has been observed that TFIIS from a mammal is able to stimulate an RNA polymerase II from Drosophila, but neither TFIIS from S. cerevisae nor S. pombe is capable of functioning with an RNA polymerase II from a metazoan (Kane, unpublished results; Sawadogo et al., 1980a; Williams and Kane, 1996; Shimoaraiso et al., 1997). Furthermore, the proteins from the two fungi do not function cross species either (Williams and Kane, 1996).

TFIIS Facilitates Readthrough by RNA Polymerase II

Since the original purification of TFIIS, much work has been devoted to investigating the nature of the stimulatory property of the elongation factor. Initial in vitro transcription systems used mammalian promoter-specific initiation with a template containing sequences from the human histone H3.3 gene containing blocks to elongation (Reines et al., 1989). Investigators found that the arrested elongation complexes were stable and had not terminated. Upon addition of partially purified human TFIIS or purified calf thymus TFIIS, the polymerases elongated their transcripts and read through the sequence-specific transcription block (Reines et al., 1989). Later work used even simpler in vitro transcription systems. These reactions used dC-tailed templates (Kadesch and Chamberlin, 1982) that allowed promoter independent initiation with purified RNA polymerase II. These experimental conditions eliminated any contribution initiation factors or promoter sequences may have on polymerase elongation. It was demonstrated that reading through a sequence-specific block to elongation required only RNA polymerase II and TFIIS (Sluder et al., 1989; SivaRaman et al., 1990). The readthrough effect could be
seen when TFIIS was added to transcription complexes after they had been stalled at the block (Bengal and Aloni, 1989; Sluder et al., 1989). Because TFIIS could act on an already arrested complex, it was concluded that TFIIS acted at the level of transcription elongation ((Sluder et al., 1989; Bengal et al., 1991). The readthrough effect in the presence of TFIIS was lost if 0.25% Sarkosyl was added even though RNA polymerase II itself is unaffected during elongation by this concentration of detergent (Reines et al., 1989). Apparently the Sarkosyl was disrupting the interaction between TFIIS and polymerase. It was also found that readthrough was abolished by α-amanitin, a potent inhibitor of RNA polymerase II supporting the possibility that TFIIS was acting directly through RNA polymerase for the stimulatory effect (Reines et al., 1989).

Besides sequence specific blocks, other blocks to transcription by RNA polymerase II include DNA lesions, DNA minor groove binding proteins, drugs, or dyes, and nucleosomes. One potential transcriptional block that the polymerase might encounter \textit{in vivo} would be a lesion in the DNA. Donahue, et al., investigated the transcription-coupled DNA repair relationship and the possible role that TFIIS could have (Donahue et al., 1994). Using templates with a cyclobutane pyrimidine dimer either in the template strand or the nontemplate strand, this group first determined which situation blocked transcription by RNA polymerase II. In addition, the investigators wished to determine if TFIIS could help polymerase to read through the lesion. If it were found that TFIIS could not help promote readthrough, could TFIIS instead help the polymerase back up and allow photolyase access to the lesion so that the lesion could be repaired? They found that a lesion in the nontranscribed strand had no effect on transcription. However, the cyclobutane pyrimidine dimer was an effective block when it was located on the template strand, and the addition of TFIIS did not enable the polymerase to read through (Donahue et al., 1994). In addition, photolyase was unable to repair the lesion with the arrested polymerase present possibly due to steric hindrance by the polymerase (Donahue et al., 1994). When TFIIS was incubated with the arrested polymerase before photolyase was
added, photolyase was still unable to repair the DNA (Donahue et al., 1994). Most likely, in the cell there are other factors that aid the repair machinery to access the lesion, and it is also possible that TFIIS is not involved in DNA repair. A variety of yeast strains defective in nucleotide excision repair (e.g. rad26, rad7, and rad7 rad26) have been disrupted for PPR2. None of the ppr2Δ RAD+, ppr2Δ rad26, ppr2Δ rad7, or ppr2Δ rad7 rad26 strains had any altered sensitivity to UV indicating that TFIIS is not essential for repair in these strains (Verhage et al., 1997). Further, cells containing a PPR2 disruption show no altered viability following exposure to UV (Jin et al. in preparation; Verhage et al., 1997).

Another experiment tested the ability of RNA polymerase to transcribe past a DNA binding protein, a minor groove binding drug, or intercalating dye. Reines and Mote tested the ability of TFIIS to facilitate polymerase transcription through a block consisting of lac repressor bound on a DNA template (Reines and Mote, 1993). The lac repressor served as a physical barrier to polymerase elongation. The transcription complexes were stable and active, and when incubated with IPTG to remove the lac repressor, the polymerases resumed transcription without TFIIS present (Reines and Mote, 1993). When lac repressor was bound to the DNA, TFIIS enabled more polymerases to transcribe the template despite the hinderance of the lac repressor (Reines and Mote, 1993). In another variation on a similar theme, it was found that distamycin, a drug that preferentially binds A+T rich DNA in the minor groove, would cause polymerases to arrest along the length of the template presumably where the drug was binding; upon addition of TFIIS, the polymerases were able to further elongate the transcript and produce full length RNA (Mote et al., 1994). Similar results were found using DAPI, a dye which binds A+T rich DNA in the minor groove (Mote et al., 1994). It was hypothesized that TFIIS was not facilitating transcription by physically removing the block, but instead, TFIIS was allowing the polymerase multiple attempts to transcribe through the arrest site by stimulating the polymerase to cleave the RNA and then resume transcription (Mote et al., 1994).
Another group looked at transcription after the template was treated with neotropsin, an A+T rich minor groove binding protein, and found that TFIIS was unable to stimulate polymerase to elongate through blocks caused by this drug (Ueno et al., 1992). The experiments with distamycin and DAPI were done with promoter specific initiation and general transcription factors, and the work done with neotropsin was performed with a dC-tailed template for promoter independent transcription with purified polymerase and TFIIS. It is possible that the elongating ternary complexes are different in the two systems and so responded differently. Alternatively, the binding of each of the blocking agents may be fundamentally different. The complexes arrested with distamycin and DAPI were stable; however, it was claimed that the complexes that encountered neotropsin were more likely to terminate (Ueno et al., 1992; Mote et al., 1994).

Izban and Luse studied the effect of TFIIS as RNA polymerase II transcribed purified DNA templates or DNA containing nucleosomes (Izban and Luse, 1992a). Mammalian RNA polymerase II alone can not transcribe pure DNA templates at physiological rates estimated at 20-30 nucleotides/second (Spindler, 1979; Kadesch and Chamberlin, 1982; Sollner-Webb and Tower, 1986; Shermoen and O'Farrell, 1991; Izban and Luse, 1992a; Reines et al., 1996; Shilatifard et al., 1997a). The rate of in vitro transcription elongation increased to approximately 25 nucleotides/second in the presence TFIIF but not with TFIIS (Izban and Luse, 1992a). This result was not unexpected. As mentioned above, TFIIF stimulates transcription by reducing pausing and increasing the elongation rate. In contrast, TFIIS has no effect on elongation rate, but TFIIS does stimulate arrested polymerases to resume productive elongation.

On templates treated with *Xenopus* oocyte extracts in order to reconstitute nucleosomes, elongation by RNA polymerase was severely hampered even when incubated with either TFIIS or TFIIF. However, incubating the complexes with TFIIF, TFIIS, and an uncharacterized component referred to as TFIIX significantly enhanced the ability of the polymerases to transcribe the nucleosome containing template (Izban and Luse, 1992a).
The X factor is likely to be one of the more recently characterized proteins that facilitate transcription on chromatin templates (see above and below).

In a more recent study, Chang and Luse did similar experiments except that the nucleosomes were reconstituted with purified histones (Chang and Luse, 1997). Contrary to the results in the previous report, they found that elongation was poor on the nucleosome containing templates even in the presence of TFIIS and TFIIF. The arrested complexes were not inactive because after washing with Sarkosyl to remove the nucleosomes, the polymerases transcribed productively. The same was true for transcription on templates with just the H3/H4 tetramers (Chang and Luse, 1997). The difference in results may be due to the possible presence of nucleosome remodeling factors in the Xenopus oocyte extracts used in the first study that in conjunction with TFIIS and TFIIF were able to enhance transcription. Such remodelling factors are discussed further below.

TFIIS Stimulates Transcript Cleavage by RNA Polymerase II

Key to the mechanism by which TFIIS facilitates readthrough by RNA polymerase II is the stimulation of polymerase by TFIIS to cleave the nascent RNA. This reaction was first reported for E. coli RNA polymerase (Surrett et al., 1991). The cleavage reaction for RNA polymerase II was first reported by Reines who was studying arrested complexes on sequences of the human histone H3.3 gene (Reines, 1992). When the arrested complexes were incubated with TFIIS after first removing unincorporated nucleotides, shortened RNA's could be detected over time (Reines, 1992). The transcription reactions were performed such that the transcripts were radioactively labeled on the 5' end (Izban and Luse, 1992b; Reines, 1992). If the RNA was shortened at the 5' end, the labeled region would have been removed and the remaining transcript could have not been detected under the experimental conditions used. Therefore it was deduced that the cleavage event was occurring at the 3' end (Izban and Luse, 1992b; Reines, 1992). A divalent cation was required for cleavage, and both Sarkosyl and α-amanitin were inhibitory (Izban and Luse,
1992b; Reines, 1992). Adding back nucleotides to the complexes resulted in elongation of the transcript and subsequent readthrough of the original block to elongation (Reines, 1992). From this it was concluded that the 5' portion of the shortened RNA remained associated with the polymerase (Reines, 1992). The cleavage reaction was hydrolytic rather than pyrophosphorolytic because the transcript could be elongated. Therefore it contained a 3' hydroxyl (Reines, 1992). By adding only a subset of nucleotides after allowing cleavage, it was determined that the resumption of transcription was template dependent (Izban and Luse, 1992b). Incubation of arrested complexes with inorganic pyrophosphate produced a pattern of cleavage products the same as that when the complexes were incubated with TFIIS (Rudd et al., 1994). This result indicated that it is not TFIIS which is hydrolyzing the RNA, but instead, it is the polymerase (Rudd et al., 1994). Like that of bacterial RNA polymerase, RNA polymerase II possesses an intrinsic cleavage activity (Weilbaecher and Kane; Rudd et al., 1994; Orlova et al., 1995).

The nature of the cleavage product appeared to be a reflection of the elongation competence of the polymerase. It was observed that the cleavage products were different if the ternary complex had been artificially halted by nucleotide starvation or by an intrinsic arrest site (Izban and Luse, 1993a; Izban and Luse, 1993b). Complexes that had been stalled due to a lack of a nucleotide and that could resume elongation with only the addition of that nucleotide were defined as elongation competent and TFIIS independent (Izban and Luse, 1993b). These complexes produced primarily dinucleotides when incubated with TFIIS (Izban and Luse, 1993b). Complexes that had arrested at an intrinsic arrest site are unable to elongate without TFIIS even if nucleotides were present. These were defined as elongation incompetent complexes, and they produced oligomers of approximately 6-14 nucleotides depending on the complex (Izban and Luse, 1993a). Because previous work indicated that particular T-rich sequences in the non-template strand created intrinsic arrest signals (Kerppola and Kane, 1990), complexes were artificially halted on templates just after transcribing successively longer U runs (Izban and Luse, 1993a). An elongation
competent complex that had three 3' U's produced both dimers and larger oligomers when incubated with TFIIS. Complexes that were halted after transcribing four or five 3' U's and incubated with TFIIS cleaved off only large oligomers. A proportion of the complexes also became arrested over time and now required TFIIS before resuming elongation (Izban and Luse, 1993a).

Studies with artificially halted RNA polymerase III elongation complexes indicate that this enzyme, too, carries out transcript cleavage (Whitehall et al., 1994; Bobkova and Hall, 1997) and that there are preferred cleavage sites (Bobkova and Hall, 1997). Cleavages tended to occur on the transcript 5' of phosphoryl groups of uridine residues beginning with the 3' most uridine and moving in the 5' direction (Bobkova and Hall, 1997). Further, RNA polymerase I also carries out this reaction (Tschochner, 1996), and under some conditions TFIIS stimulates this polymerase as well (Schnapp et al., 1996).

Further study of the cleavage reaction indicated that an arrested RNA polymerase II must be stimulated to cleave before it can leave its arrested state and become elongation competent (Reines et al., 1992; Izban and Luse, 1993a). This conclusion arose from studies of arrested complexes that had been purified away from unincorporated nucleotides and then stimulated to cleave by TFIIS. Upon addition of subsets of nucleotides, sometimes including a chain terminator, the complexes were then allowed to resume transcription. The nucleotides were chosen such that if a complex had undergone cleavage and then restarted elongation, the RNA could not be polymerized to that of the original arrested complex. The behavior of the complexes that remained at the arrest site could then be distinguished from those that had undergone cleavage. It was found that only those complexes which had cleaved their transcript were able to resume transcription (Reines et al., 1992; Izban and Luse, 1993a).

TFIIS Interaction with RNA Polymerase II
RNA polymerase II is a multi-subunit complex (Woychik and Young, 1994). The three largest subunits of RNA polymerase II share homology with the largest subunits of RNA polymerases I and III (Woychik and Young, 1994). In addition, the two largest subunits of RNA polymerase II (RPB1 and RPB2) share sequence homology with the β and β' subunits of E. coli RNA polymerase (Woychik and Young, 1991). As with β and β', RPB1 and RPB2 most likely comprise the active site of the eukaryotic polymerase (Woychik and Young, 1991). The structure of RNA polymerase II has been solved to 16 angstrom resolution (Darst et al., 1991). Similar to that of the bacterial RNA polymerase, the yeast polymerase has a structure reminiscent of a partially closed hand (Darst et al. 1991; Darst et al., 1989). A large groove on the RNA polymerase II (formed by the "palm" and "fingers") has dimensions that would accommodate double-stranded nucleic acids (Darst et al., 1991). The large groove branches off to a narrower channel that has dimensions that would accommodate single-stranded nucleic acids (Darst et al., 1991).

Several groups using a variety of methods have determined that TFIIS physically interacts with RNA polymerase II. Glycerol gradient centrifugation assays showed that TFIIS co-sedimented with polymerase (Sawadogo et al., 1980b; Horikoshi et al., 1984; Sopta et al., 1985; Reinberg and Roeder, 1987). Affinity chromatography with calf thymus RNA polymerase II pulled down TFIIS as well as TFIIF from whole cell extracts from murine erythroleukemia cells (Sopta et al., 1985). Binding assays with purified yeast polymerase and recombinant, labeled yeast TFIIS also confirmed these interactions (Wu et al., 1996; Awrey et al., 1997; Awrey et al., 1998). Affinity chromatography with a GST fusion of TFIIS pulled down an RNA polymerase II holoenzyme that contained the general transcription factors as well as CDK8 and other unidentified polypeptides from HeLa whole cell extracts (Pan et al., 1997). Interestingly, a GST fusion to an amino terminal portion (amino acids 1-103) of TFIIS, for which the functional significance is unknown (see below), also pulled down the same proteins as with the full length TFIIS GST fusion, but a carboxy terminal fragment (amino acids 102-280) of TFIIS, which can interact with
purified polymerase (Awrey et al., 1998), only brought down RNA polymerase II itself (Pan et al., 1997). Perhaps *in vivo*, the carboxy terminal portion of TFIIS interacts with RNA polymerase II, while the amino terminal region interacts with factors associated with one of the RNA polymerase II complexes. RNA polymerase II incubated with an antibody specific for the sixth largest subunit of RNA polymerase II was less responsive to stimulation by TFIIS (Sawadogo et al., 1980a). However, it was found that pre-incubation of TFIIS with RNA polymerase II reduced the transcription inhibitory effect of the RPB6 antibody suggesting that TFIIS might also be interacting with polymerase in proximity to the sixth largest subunit (Sawadogo et al., 1980a). Using an arrested RNA polymerase II ternary complex and TFIIS with a photoaffinity label incorporated at the 3'end of the RNA, TFIIS became crosslinked to the two largest subunits of polymerase indicating that TFIIS interacts with the polymerase in close proximity to the catalytic site of the enzyme (Powell et al., 1996). Similarly, crosslinking studies position the bacterial Gre proteins near the catalytic center of the polymerase in elongation complexes (Koulich et al., 1997).

*In vitro* and *in vivo* evidence indicates that TFIIS interacts with the largest subunit of RNA polymerase II. A β-galactosidase fusion with amino acids 191-273 of the largest subunit of RNA polymerase II inhibited the stimulatory activity of TFIIS in a non-specific transcription assay, but β-galactosidase alone did not (Rappaport et al., 1988). In addition, using promoter specific transcription from the AdML promoter and HeLa whole cell extracts, the β-galactosidase/ΔRBP1 fusion protein reduced the amount of full length RNA's produced. Based on primer extension experiments, the fusion did not affect the amount of complexes initiated (Rappaport et al., 1988). Other experiments with antibody that reacted with the same portion of the largest subunit of RNA polymerase II used to make the fusion protein had similar results again suggesting that TFIIS has an interaction with the largest subunit of RNA polymerase II (Rappaport et al., 1988). Glycerol gradient sedimentation with TFIIS and native RNA polymerase II or the proteolyzed, IIB form
suggested that TFIIIS had a preference for the native polymerase with an intact CTD (Sawadogo et al., 1980b). However, in vitro, TFIIIS functions with polymerase with or without the CTD in a quantitatively indistinguishable fashion (Christie et al., 1994).

Genetic interactions between the largest subunit of RNA polymerase II and TFIIIS have been reported. Linker insertions and random point mutations were made in the gene coding for the largest subunit of RNA polymerase II of yeast and tested for sensitivity to 6-azauracil (Archambault et al., 1992). The 6-azauracil sensitive mutants (rpo21-6, -7, -17, -18, -19, -23, -24, and -25) were clustered between conserved regions G and H (Fig. 1). Increasing the dosage of TFIIIS resulted in suppression of the 6-azauracil sensitivity phenotype in the seven strains tested (rpo21-6, -7, -17, -18, -19, -23, and -24) (Archambault et al., 1992). The rpo21 (rpo21-6, -7, -17, -18, -19, -23, and -24), ppr2 strains were viable (Archambault et al., 1992). Two mutants, rpo21-18 and rpo21-24, were further characterized (Wu et al., 1996). Polymerases from these mutants were purified and found to have similar in vitro elongation properties as that of wildtype polymerase (Wu et al., 1996). However, it was observed that the mutant polymerases were much less responsive to TFIIIS (Wu et al., 1996). As a possible explanation, it was determined that the ability of the mutants to bind TFIIIS was approximately 50-fold less than that of wildtype indicating that the largest subunit of polymerase is involved in binding TFIIIS (Wu et al., 1996). This result lent support to in vitro evidence previously reported that TFIIIS interacted with the largest subunit of RNA polymerase II (Sawadogo et al., 1980b; Rappaport et al., 1988).

A set of mutants of the second largest subunit of RNA polymerase II were studied in the context of their relationship with TFIIIS because they also displayed a 6-azauracil sensitivity phenotype (Scafe et al., 1990a; Scafe et al., 1990b; Powell and Reines, 1996). The in vitro elongation properties of the mutant polymerases were characterized. The mutant polymerases were more likely to arrest when transcribing, and all were weakly responsive to stimulation of readthrough by TFIIIS (Powell and Reines, 1996). The rpb2-
10 mutant was the least sensitive to 6-azauracil and not sensitive to mycophenolic acid, but it was the most arrest prone of the polymerases and had a slower elongation rate than that of wildtype (Powell and Reines, 1996). The weak drug sensitivity of this mutant is in apparent contradiction to predictions based on the hypothesis of the effect of 6-azauracil on nucleotide pools and transcription (Uptain et al., 1997). However, RNA polymerase isolated from rpb2-10 was the most responsive to stimulation by TFIIS which may explain the mild drug phenotypes (Powell and Reines, 1996). The double mutant, ppr2Δ, rpb2-10, was viable but much more sensitive to 6-azauracil than either single mutant (Lennon et al., 1998). Because it had been observed that increasing the dwell time of the polymerase at a potential arrest site increased the likelihood of arrest, the slower elongation rate of the rpb2-10 mutant may be an explanation for its greater tendency to arrest (Gu and Reines, 1995a).

A third mutant of RNA polymerase II functionally related to TFIIS has also been characterized. This mutant, rpb9Δ, is minus one of the smaller subunits of RNA polymerase II (Woychik et al., 1991). Strains without RPB9 are viable, mildly temperature sensitive, and have relatively normal transcription levels in vivo (Woychik and Young, 1990; Woychik et al., 1991). Further, these strains initiate transcription upstream from the normal start sites (Furter-Graves et al., 1991; Furter-Graves et al., 1994; Hull et al., 1995). The rpb9Δ strain is marginally more sensitive to 6-azauracil than wildtype strains, but it is exquisitely sensitive to mycophenolic acid (Weilbaecher, 1997). The RPB9 subunit shares homology to TFIIS within a Zn ribbon motif (Woychik et al., 1991; Chen et al., 1992). A comparison of the purified wildtype and Δrpβ9 polymerases revealed that they had the same maximal elongation rates and similar rate and extent of intrinsic cleavage activities (Awrey et al., 1997). However, the Δrpβ9 polymerase transcribed through an intrinsic block to elongation with higher efficiency than wildtype polymerase (Awrey et al., 1997). Those Δrpβ9 polymerase complexes that did arrest were less responsive to stimulation of cleavage and readthrough when incubated with TFIIS
even though the ΔrpB9 polymerase bound TFIIS comparably to that of wildtype polymerase (Awrey et al., 1997). Adding back the RPB9 protein returned the recognition of an intrinsic arrest site and promotion of readthrough by TFIIS to levels similar to wildtype polymerase (Awrey et al., 1997). These results reinforce that the mere binding of TFIIS is not sufficient to facilitate cleavage and readthrough by RNA polymerase II.

Structure/Function of TFIIS

Based on protease digestion, TFIIS consists of three structural domains (Morin, et al., 1996). Deletion analysis has determined that the amino terminal third of the protein (amino acids 2-130) is not needed either for in vitro promotion of cleavage and readthrough nor for complementation in vivo (Guo and Price, 1993; Christie et al., 1994; Nakanishi et al., 1995). It has been reported that this amino-terminal portion of TFIIS can be phosphorylated in vivo although the significance is not yet known (Sekimizu et al., 1981; Horikoshi et al., 1985; Sopta et al., 1985). This region of TFIIS also associates with a complex of proteins that includes RNA polymerase II (Pan et al., 1997). The first known discovery of a function for the amino terminal portion of TFIIS resulted from a synthetic lethal screen with strains disrupted for the gene encoding TFIIS (Davie, 1998). A genetic interaction between PPR2 and TFG3, a component of TFIID, the SWI/SNF complex, and TFIIIF, was identified (Cairns et al., 1996; Davie, 1998; Kadonaga, 1998). The tfg3Δ strain was viable only with the full length TFIIS and not the amino truncated protein (Cairns et al., 1996).

The second domain of TFIIS, residues 131-240 in yeast, is α-helical in nature (Fig.2) (Morin et al., 1996; Olmsted et al., 1998). Deletion analysis as well as site directed mutagenesis have shown that this region of the protein is important for binding to the polymerase (Agarwal et al., 1991; Awrey et al., 1998). Alanine scanning studies with human TFIIS characterized several mutants, including, K187A/K189A, in the domain II region (Cipres-Palacin and Kane, 1994). This mutant had the unusual phenotype of
stimulating an arrested mammalian polymerase to cleave the nascent transcript, but not to read through a block to elongation (Cipres-Palacin and Kane, 1994). This result indicated that although cleavage is necessary for readthrough, it is not sufficient (Cipres-Palacin and Kane, 1994).

As determined by NMR, the second and the third domains of TFIIS are connected by an unstructured, but not fully flexible linker, residues 238-263 in yeast (Fig.2) (Olmsted et al., 1998). Alanine scanning and site directed mutagenesis studies have identified several residues in the linker region that are critical to the function of TFIIS (Cipres-Palacin and Kane, 1995; Awrey et al., 1998). Deletions, insertions, and replacement by an eight amino acid random coil in the linker region all resulted in an inactive protein (Agarwal et al., 1991; Awrey et al., 1998). These results suggest that along with possible contacts with the polymerase, there may be a geometrical function provided by the linker (Agarwal et al., 1991; Awrey et al., 1998).

The third domain of TFIIS, amino acids 279-309 in yeast, has three anti-parallel \( \beta \)-sheets (Qian et al., 1993; Olmsted et al., 1998). It is the most highly conserved region of the protein across species (Fig.3). This domain consists of a Zn ribbon motif, and shares homology to RPB9, the ninth largest subunit of RNA polymerase II (Woychik et al., 1991; Qian et al., 1993; Olmsted et al., 1998), C11 of RNA polymerase III (Chedin et al., 1998), and A12.2 of RNA polymerase I (Nogi et al., 1993). A portion of this domain will bind single stranded nucleic acid though the full length protein does not (Agarwal et al., 1991; Qian et al., 1993). Site-directed mutagenesis and deletion analysis have shown that there are several residues in this region that are vital to the proper functioning of TFIIS (Agarwal et al., 1991; Jeon et al., 1994; Cipres-Palacin and Kane, 1995; Nakanishi et al., 1995; Awrey et al., 1998).
TFIIS and Chromatin Remodeling Factors

Recent work has revealed that there is a genetic connection between TFIIS and chromatin remodeling. Increasingly, it is becoming clear that regulation of transcription and nucleosome architecture are intertwined (Hartzog and Winston, 1997; Pazin and Kadonaga, 1997; Tsukiyama and Wu, 1997; Kadonaga, 1998). Genes encoding the SWI/SNF complex were first identified for their requirement for wildtype transcription of selected genes. In support of the transcription connection, the SWI/SNF complex can be purified in association with one of the RNA polymerase II complexes and the mediator complex (Wilson et al., 1996; Kadonaga, 1998). In addition, it was found that mutations in SWI/SNF genes were suppressed by mutations in core histone genes, and in vitro, purified SWI/SNF complex can disrupt reconstituted nucleosomes linking SWI/SNF function with chromatin remodeling (Kadonaga, 1998). It has been hypothesized that a chromatin remodeling complex may serve to destabilize nucleosomes in order to provide the transcriptional machinery access to binding sites in the cell (Hartzog and Winston, 1997).

A synthetic lethal screen with TFIIS has yielded results supporting the connection of nucleosome remodeling with transcription in vivo (Davie, 1998). Genes with a genetic interaction with PPR2 included SNF2, SWI1, SNF5, and TFG3 (Davie, 1998). SNF2, SWI1, SNF5, and TFG3 have been characterized as components of the SWI/SNF complex (Cairns et al., 1996; Kadonaga, 1998). TFG3 is a component of several complexes associated with transcription. It has been identified as a loosely associated component of TFIIF from S. cerevisiae (Henry et al., 1994). This non-essential gene encodes TAF30 that is identical to ANC1 was identified in a genetic a screen investigating the actin cytoskeleton (Henry et al., 1994). The tfg3Δ strain has decreased transcription in vivo, and in vitro the protein also has been purified as part of the mediator complex and has been shown to interact with SNF5 (Henry et al., 1994; Cairns et al., 1996).
Genetic interactions between \textit{PPR2} and \textit{SPT4}, \textit{SPT5}, and \textit{SPT6} have also been demonstrated (Hartzog et al., 1998). \textit{SPT4}, \textit{SPT5}, and \textit{SPT6} were originally identified as suppressors of transcriptional defects caused by \( \delta \) and Ty insertions in the 5' regions of \textit{HIS4} and \textit{LYS2} (Winston et al., 1984; Fassler and Winston, 1988). \textit{SPT4} is not essential but \textit{SPT5} and \textit{SPT6} are (Clark-Adams and Winston, 1987; Neugeboren et al., 1987; Swanson et al., 1991). Several pieces of information link the function of the proteins encoded by these \textit{SPT} genes to chromatin remodeling. \textit{SPT4}, \textit{SPT5}, and \textit{SPT6} share phenotypes with \textit{SPT11/HTA1} and \textit{SPT12/HTB1} that encode histones H2A and H2B, respectively (Clark-Adams et al., 1988). Mutants of \textit{spt4}, \textit{spt5}, and \textit{spt6} suppress defects in transcription of \textit{SUC2} caused by \textit{snf2} mutants (Neugeboren et al., 1986; Swanson and Winston, 1992). Mutants of \textit{spt6} have also been shown to suppress transcriptional defects of \textit{SUC2} caused by \textit{snf5} and \textit{snf6} mutations (Neugeboren et al., 1986). Overexpression of H3 alone or H3 and H4 core histones can suppress the lethality of a disruption of \textit{spt6} (Bortvin and Winston, 1996). A GST-SPT6 fusion bound to H3-H4 tetramers with higher affinity than to H2A-H2B dimers (Bortvin and Winston, 1996). The H3-H4 tetramers eluted from the GST-SPT6 affinity column at 0.7M NaCl, but the H2A-H2B dimers eluted from the column at 0.5M NaCl (Bortvin and Winston, 1996). The \textit{spt6} mutants caused changes in chromatin structure \textit{in vivo} (Bortvin and Winston, 1996).

\textit{SPT6} was able to assemble chromatin \textit{in vitro} as determined by a plasmid supercoiling assay (Bortvin and Winston, 1996). GST-SPT6 incubated with relaxed plasmid, human histones, and topoisomerase I resulted in negatively supercoiling 50\% of the plasmid (Bortvin and Winston, 1996). When the DNA was digested with micrococcal nuclease, the regions of protection were about 150 basepairs which correspond to a nucleosome core complex (Bortvin and Winston, 1996). It has also been reported that \textit{SPT4} and \textit{SPT6} have a role in chromatin segregation (Basrai et al., 1996).

\textit{SPT4}, \textit{SPT5}, and \textit{SPT6} also may have functions involved with regulation of transcription elongation. \textit{In vitro}, human homologs of \textit{SPT4} and \textit{SPT5} were purified as
factor DSIF (DRB sensitive inducing factor) which was required for DRB inhibition of productive elongation (Wada et al., 1998). The human homolog of SPT5 was also purified as a component required to support Tat transactivated transcription of a template containing the HIV LTR, and this polypeptide was named Tat co-transactivator or Tat-CT1 (Wu-Baer et al., 1998). The human homolog of SPT6 was dubbed Tat-CT2, because like Tat-CT1, extracts immunodepleted of either Tat-CT1 or Tat-CT2 were found to no longer be able to support Tat transactivation (Wu-Baer et al., 1998). Reminiscent of ppr2Δ strains, mutant strains of spt4Δ, spt5, and spt6 were found which were 6-azauracil sensitive (Hartzog et al., 1998). Cold sensitive mutants of spt5 were synthetically lethal with ppr2Δ, and double mutants of spt4 or spt6 with ppr2Δ became Cs− (Hartzog et al., 1998). Two suppressors of the spt5 Cs− mutants were mutants in the largest subunit of RNA polymerase II which themselves were 6-azauracil sensitive and very sick or inviable in a ppr2Δ strain (Hartzog et al., 1998). It has been hypothesized that growth in the presence of 6-azauracil, which lowers nucleotide pools, could cause the polymerase to be more arrest prone and more likely to require a transcription elongation factor (Uptain et al., 1997). Although it has not yet been determined, it may be speculated that TFIIS may enable the polymerase to productively transcribe various genes (perhaps even the same ones as the SPT proteins). The loss of function of either TFIIS or the SPT proteins may cripple the cell by reducing gene expression, but there remains sufficient expression for viability. However, when both PPR2 and an SPT are mutants, the effect is cumulative and more deleterious (perhaps more genes have decreased expression or perhaps one gene regulated by both PPR2 and SPT is expressed even less in the double mutant) than that seen with the single mutant. At least two 6-azauracil sensitive mutants of the largest subunit of RNA polymerase II (rpo21-18 and rpo21-24) have been shown to have a decreased affinity for TFIIS (Wu et al., 1996). A possible explanation for the finding that 6-azauracil sensitive mutants of the largest subunit of RNA polymerase II were suppressors of spt5 Cs− mutants suggests that in order to achieve balance of gene expression,
expression of genes regulated by TFIIS needed to be decreased in the spt5 Cs^- mutants by compromising the interaction between TFIIS and RNA polymerase.

Mechanism of Transcription Elongation

Biochemical studies with transcription elongation factors TFIIS and GreA and GreB as well as additional experiments with the eukaryotic and prokaryotic RNA polymerases have led to the development of a model for the mechanism of transcription elongation by RNA polymerase (Kane, 1994; Chamberlin, 1995; Landick, 1997; Uptain et al., 1997). DNA footprinting studies done with both mammalian and bacterial RNA polymerases indicate that the transcribing polymerase is dynamic and assumes many different conformations as it moves along the template (Linn and Luse, 1991; Krummel and Chamberlin, 1992a; Krummel and Chamberlin, 1992b; Borukhov et al., 1993; Rice et al., 1993; Feng et al., 1994; Nudler et al., 1994; Nudler et al., 1995; Wang et al., 1995; Zaychikov et al., 1995). In addition, the response of the polymerases to induction of cleavage by TFIIS or the Gre factors varies depending on the elongation competence of the polymerases suggesting again that the polymerases are capable of establishing multiple conformations. Artificially stalled, but elongation-competent eukaryotic polymerases can be induced to cleave 2-mers by TFIIS, but arrested complexes have been observed to cleave much larger oligos (Izban and Luse, 1993a). Similarly, with GreB and bacterial polymerases, small dimers were cleaved from active transcription complexes, and larger fragments were removed from arrested complexes (Komissarova and Kashlev, 1997). Kinetic studies with bacterial RNA polymerase after misincorporation also indicate that the ternary complex can assume multiple forms (Erie et al., 1993). RNAse protection assays with mammalian and bacterial polymerases determined that the transcript could be digested up to three nucleotides from the 3' end of the RNA, and the remaining fragment of RNA was still associated with the polymerase in an active complex because it could be elongated upon resumption of transcription (Rice et al., 1991; Milan, 1995). These results led to the
hypothesis that the stability of the transcription complex was due to protein:nucleic acid interactions rather than nucleic acid:nucleic acid interactions like that of a DNA:RNA hybrid.

In similar experiments with the *E. coli* polymerase at lower concentrations of RNAse, partial protection in two regions of the transcript was observed (S. Milan, in press, Biochemistry). Each region extended about 15 nucleotides, one at the 3' end of the RNA and the other approximately 30-40 nucleotides from the 3' end (S. Milan, in press, Biochemistry). This would suggest that the polymerase might possess two RNA binding sites. These results were consistent with RNA binding studies with the yeast and bacterial polymerases (Altmann et al., 1994; Johnson and Chamberlin, 1994). Also, it is believed that RNA polymerase possesses two DNA binding sites; the most compelling evidence lies in the observation that bacterial polymerase can transcribe to the end of a linear template and then can switch to a different blunt ended template, (Nudler et al., 1996).

A model has been proposed by Chamberlin that RNA polymerase has an upstream and a downstream nucleic acid binding site which could bind tightly or loosely independently of the other (Chamberlin, 1995). In this model the catalytic site moves in concert with the upstream binding site (Fig. 4). Reminiscent of that of an inchworm, the polymerase undergoes a cycle of contraction and expansion. First, the upstream edge of the protein moves down the template as the downstream binding site becomes filled with newly polymerized RNA while the downstream edge remains fixed (Fig. 4a-c). Then the upstream edge becomes fixed while the downstream edge translocates further down the template emptying the downstream nucleic acid binding site of RNA whereupon the cycle begins again (Fig. 4d).

It should be noted that the inchworm model does not agree with all of the known data about polymerase elongation. Several footprinting studies involving different transcription complexes show that the polymerase moves monotonically along the template with the movement of the upstream and downstream boundaries of the protein moving in
concert with that of the catalytic site (Feng et al., 1994; Nudler et al., 1994; Wang et al., 1995). Interestingly, ExoIII footprinting work with the eukaryotic polymerase transcribing a template with a known arrest site and bacterial polymerase transcribing a template with a ρ independent terminator indicate that the polymerase translocates monotonically until it encounters the block to elongation whereupon it begins a cycle of discontinuous movement (Nudler et al., 1995; Samkurashvili and Luse, 1996). This would suggest that the dynamic conformational changes during an "inchworming" cycle may be targets for regulation.

Based on the model proposed by Chamberlin, it can be speculated that when polymerase encounters a block to elongation, the polymerase is able to fill the downstream binding site but is unable to translocate forward along the template properly to relieve the contracted conformation (Fig. 4e). The polymerase may then slip into an elongation incompetent conformation that would result in the catalytic site being out of register with the 3’ end of the transcript leading to arrest. Footprinting experiments with eukaryotic polymerase as it was walked through an intrinsic arrest site showed that when the transcription complex encountered the arrest site, downstream translocation stopped while the RNA continued to be elongated, and the footprint became compressed (Samkurashvili and Luse, 1996). Other experiments with bacterial polymerase walked through a ρ independent terminator, indicated that the complex also became contracted as it encountered the site (Nudler et al., 1995). RNase digestion of isolated eukaryotic transcription complexes showed that the region of RNA protected increased from 18 nucleotides before the complex encountered the arrest site to 27 nucleotides after the complex encountered the block clearly indicating a conformational change in the complex (Gu et al., 1996). When RNA polymerase II complexes were walked through a well defined arrest site and stimulated to cleave the transcript in the presence of TFIIS, it was found that there was a preferred cleavage site in the RNA sequence as if the catalytic site was moving to a default "home" position in an arrested complex (Gu and Reines, 1995b). Studies with an isolated E. coli polymerase complex also suggested that the location of the catalytic site with respect
to polymerase changes depending on whether the complex is active or not (Markovtsov et al., 1996). In these experiments a cross linkable nucleoside analog was incorporated at the 3' end of the RNA. Upon activation of the analog, linkages were made to the β and β' subunits in the active complex. When the complex was allowed to become inactivated before the crosslinking reaction, a single new linkage to the β' subunit was formed indicating that the orientation of the catalytic site had changed with respect to the polymerase in an active versus inactive enzyme (Markovtsov et al., 1996). It was hypothesized that the role, in part, for TFIIS or the Gre factors to re-activate polymerase would be to induce cleavage by the polymerase, removing the "extra" RNA to empty the downstream RNA binding site and re-align the catalytic site to the new 3'end of the RNA. This could also explain the observations that the TFIIS or GreB induced cleavage products from elongation competent polymerases are only dimers, but those from arrested complexes are much larger (Izban and Luse, 1993a; Komissarova and Kashlev, 1997).

An alternative model for transcription elongation has been proposed (Landick, 1997; von Hippel, 1998). This model has been dubbed the "sliding clamp" model (Fig. 4) (Landick, 1997). In this model, the conformation of the RNA polymerase is unchanging as it elongates. The stability of the complex is determined in part by a duplex DNA binding site that serves as a clamp. An approximately 8-9 base pair DNA:RNA hybrid is involved in stabilizing the complex and in maintaining the 3'end of the RNA in register with the catalytic site. Another aspect of this model is that the transcription complex can slide upstream along the DNA (Landick, 1997; von Hippel, 1998). The RNA:DNA hybrid and the transcription bubble shift upstream in concert with the polymerase, and the 3'end of the transcript is extruded out the downstream end of the polymerase (Fig. 4f&g). Resistance to sliding by the polymerase is dependent upon the strength of the RNA:DNA hybrid. It has been hypothesized that the polymerase may oscillate back and forth (Landick, 1997). An elongation active complex would be in the "forward" position with its catalytic site in alignment with the 3'end of the RNA, and an inactive complex (paused or arrested) would
be in the "backward" position after sliding upstream. A paused complex could simply shift back downstream into the elongation active state and continue polymerization of the RNA. Alternatively, if the RNA:DNA hybrid were destabilized (by the formation of an RNA hairpin, for example), then the paused polymerase would dissociate resulting in termination. An arrested complex would irreversibly slide backward and would require the induction of cleavage of the extruded RNA (by a transcription elongation factor like TFIIS, for instance) in order for the catalytic site to once again be in correct register with the 3'end of the transcript (Fig. 4i) (Landick, 1997).

Several pieces of evidence support the sliding clamp model. The duplex DNA binding clamp of the polymerase is based on structural and biochemical information. The structure of the bacterial holoenzyme contains a cleft of dimensions that could accommodate duplex DNA (Polyakov et al., 1995). In the structure of the core enzyme, the elongation form of polymerase, the cleft is closed (Polyakov et al., 1995). Bacterial polymerase has the ability to transcribe to the end of a template and then switch to a second template (Nudler et al., 1996). A variety of second templates were used to identify factors which contributed to the elongation complex stability (Nudler et al., 1996). It was found that a halted complex needed about 7-9 base pairs of duplex DNA downstream of the 3'end of the transcript in order to be resistant to a high salt wash (Nudler et al., 1996). The existence of the hybrid is based on crosslinking studies with the bacterial polymerase (Nudler et al., 1997). A UMP analog was incorporated at various positions in the transcript proximal to the 3'end. Crosslinking occurred between the analog and DNA when the analog was no more than nine bases from the growing end of the RNA (Nudler et al., 1997). When the analog was located more than nine bases from the 3'end of the RNA, the analog primarily crosslinked to the polymerase (Nudler et al., 1997).

In vitro transcription studies with mammalian RNA polymerase II suggested that the pathway leading to arrest involves an upstream translocation of the polymerase (Reeder and Hawley, 1996). The proportion of the complexes that arrested at a block to elongation
in the adenovirus transcription unit could be significantly reduced when oligos complementary to the transcript sequence predicted to be 5' of the upstream edge of the polymerase were added in trans to the reaction (Reeder and Hawley, 1996). It was hypothesized that the oligo hybridized to the transcript and prevented the polymerase from sliding back, extruding the RNA from the downstream portion of the polymerase, and falling into a state of arrest (Reeder and Hawley, 1996).

Other studies were done with an isolated, artificially halted bacterial polymerase complex that was active, but then incubated until it became inactive and unable to resume transcription upon addition of nucleotides. ExoIII footprinting revealed that the boundaries of the inactive complex had shifted upstream relative to that of the active form (Komissarova and Kashlev, 1997; Nudler et al., 1997). In addition, KMnO4 sensitivity of the template had moved upstream in the inactive complex (Komissarova and Kashlev, 1997). This result lends support to the proposal that the transcription bubble moves upstream in conjunction with the backwards sliding of the polymerase. Further, RNase digestion indicated that in the inactive complex versus the active one, the 3'end of the RNA in the inactive complex was sensitive to RNase, but the RNA in the active form was protected (Komissarova and Kashlev, 1997). The interpretation of this result was that the inactive polymerase had not only translocated backwards on the template, but it had also extruded the transcript from the 3' end (Komissarova and Kashlev, 1997).

The contention that the strength of the RNA:DNA hybrid effects the sliding movement of the polymerase is supported by work with bacterial RNA polymerase. In these experiments, artificially halted complexes were formed that when incubated at 37°C would rapidly become inactive (Nudler et al., 1997). When the complexes were made with nucleotide analogues predicted to stabilize the hybrid, a larger proportion of the complexes were able to resume transcription after the incubation at 37°C than the complexes made with normal nucleotides (Nudler et al., 1997). In contrast, when the complexes were formed with nucleotide analogs expected to destabilize the hybrid, more of the complexes
were inactive than the control complexes (Nudler et al., 1997). In addition, when a hybrid destabilizing analog was incorporated in the transcript in a position not within the proposed 8-9 base pair hybrid, the complexes remained slightly more active than the controls (Nudler et al., 1997).

However, not all of the data is explained by the sliding clamp model (Reynolds et al., 1992; Reynolds and Chamberlin, 1992). A key aspect of the model is that the elongating polymerase is capable of sliding backwards and forwards along the template (Landick, 1997; Nudler et al., 1997). It is speculated that populations of polymerases at forward and backward positions at a given a site along a DNA template are what have given rise to the compressed and expanded footprints interpreted as inchworming (Nudler et al., 1997). The direct evidence for upstream translocation of "arrested" RNA polymerase transcription complexes consists of ExoIII and RNase footprinting experiments on artificially halted complexes (Komissarova and Kashlev, 1997; Nudler et al., 1997). These complexes were initially active, and then incubated under conditions such that they became unable to resume transcription upon addition of nucleotides (Komissarova and Kashlev, 1997; Nudler et al., 1997). Rather than RNA polymerases that arrest at site specific blocks to elongation, these complexes more closely resemble the "dead end" complexes studied by Krummel and Chamberlin (Krummel and Chamberlin, 1992b). The "dead end" complexes were also formed artificially by walking the polymerase along a template with subsets of nucleotides (Krummel and Chamberlin, 1992b). It was observed that if a "dead end" complex were made, the polymerase could not be walked further (Krummel and Chamberlin, 1992b). For the particular "dead end" complexes studied, it was found that substitution of ITP for GTP resulted in an active complex (Krummel and Chamberlin, 1992b). DNaseI footprints of the "dead end" complexes were found to be different than the ITP active equivalent of the "dead end" complexes (Krummel and Chamberlin, 1992a). This would suggest that the structure of the polymerase was different in the two transcription complexes. This raises the possibility that the inactive, backward sliding
complexes studied by Komissorova and Kashlev and Nudler and co-workers, may not be representative of an elongating polymerase.

Another component of the sliding clamp model is that an 8-9 base pair RNA:DNA hybrid contributes to the stability of the transcription complex (Landick, 1997; Nudler et al., 1997). As mentioned above in the discussion of the inchworm model, Rice and co-workers and Milan and Chamberlin working with mammalian and bacterial RNA polymerases, respectively, concluded that an RNA:DNA hybrid was at most 2-3 bases long in the complexes that they studied (Rice et al., 1991; Milan, 1995). Single strand specific RNAse digestion of halted transcription complexes revealed that the transcript was subject to cleavage to within 2-3 bases of the 3'end of the transcript (Rice et al., 1991; Milan, 1995). Furthermore, the undigested 3' fragment of RNA remained in association with the polymerase because addition of nucleotides resulted in elongation (Rice et al., 1991; Milan, 1995).

The requirement of the sliding clamp model for an RNA:DNA hybrid also does not sufficiently explain reports involving RNA polymerase transcription complexes and DNA replication (Liu et al., 1993). These experiments investigated the fate of a halted bacterial transcription complex after a replication fork had passed through the transcription complex in the same direction as transcription (Liu et al., 1993). It was found that the transcription complex remained bound to the DNA and active to resume transcription (Liu et al., 1993). It is difficult to imagine how the RNA polymerase would maintain an RNA:DNA hybrid under these circumstances.

Clearly the mechanism of transcription elongation by RNA polymerase is still not fully understood. A greater understanding of polymerase arrest and rescue by transcript cleavage may help to further elucidate the mechanism.
The goal of my project was to do structural/functional analysis of TFIIS using various mutagenesis techniques to investigate the interactions between TFIIS and RNA polymerase II. In Chapter Two, using site-directed mutagenesis, charged to alanine substitutions, I have identified residues, K196, R198, and R200, which form a basic patch on the surface of TFIIS that are important for the binding of TFIIS to RNA polymerase II. In Chapter Three, through structural domain swaps between human and yeast TFIIS, I have isolated the region, amino acids 240-270 in yeast TFIIS, involved in determining species specificity between TFIIS and RNA polymerase II.
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Figure Legends

Fig. 1 Regions of RPB1 that are similar to the $\beta'$ subunit of bacterial polymerase.

The regions of homology are shown in black, and the heptad repeat of the CTD is represented with diagonal lines.
Figure 1

RPB1

β'

A B C D E F G H

100 amino acids
Fig. 2 Ribbon structure of AyTFIIS (amino acids 131-309).

The linker region has no defined structure and is arbitrarily drawn. As a result, domain II and domain III have no precise orientation with respect to each other (from Olmsted et al., 1998).
Fig. 3  Amino acid sequence comparison of TFIIS proteins.

An amino acid sequence alignment comparing TFIIS proteins from humans (HeLa), human kidney (HK1), mouse (MEh-murine Erhlich ascites), mouse liver pL121 (MLi1), mouse liver pL122 (MLi2), Drosophila (Dm), Saccharomyces cerevisiae (Sc), and vaccinia (V) (from Chen et al., 1992). Identical and conserved residues are underlined. Residues of domain II are indicated by an '*'. Residues of the linker region are indicated by a '^'. Residues of domain III are indicated by a '+'.

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Fig. 4 Models for the mechanism of transcription elongation.

Each diagram represents hypothesized structural features of RNA polymerase II. The subunits associated with these features are unknown.

The inchworm model. a) The downstream nucleic acid binding site (rectangle) remains fixed while the upstream nucleic acid binding site (rectangle) translocates along the template (thick black line) in concert with the catalytic site (*) and growing 3' end of the RNA (dashed line). The hash mark on the DNA represents the original position of the catalytic site along the template at the beginning of the cycle. b) The polymerase contracts as the downstream nucleic acid binding site fills with RNA. c) When the downstream nucleic acid binding site is full of newly transcribed RNA, the upstream nucleic acid binding site locks while the downstream nucleic acid binding site unlocks. The downstream nucleic acid binding site can now translocate along the DNA emptying its site of RNA. d) The polymerase is now able to resume the transcription cycle at step "a". e) It is proposed in this model that the polymerase may enter a state of arrest and undergo a conformational change such that the catalytic site is no longer in register with the 3' end of the transcript. In order to re-align the catalytic site, the RNA must be cleaved by the polymerase.

The sliding clamp model. f) The polymerase (large rectangle) moves monotonically along the template (thick black line) as RNA is polymerized (dashed line). The hash mark on the DNA represents the original position of the catalytic site along the template at the beginning of the cycle. g&h) At each position along the template, the polymerase may slide back and forth. When in position "h", the 3' end of the RNA is extruded out of the downstream end of the polymerase. i) When in the forward, productive position "g", the polymerase can elongate the transcript "i". j) Alternatively, the polymerase could irreversibly slide backwards and require a transcript cleavage event to re-align the catalytic site with the 3' end of the RNA before being able to resume transcription.
Figure 4

Inchworm model

Sliding clamp model
Chapter Two

Structure/Function Analysis of TFIIS Identifies a Region that Interacts with RNA Polymerase II

A portion of the results presented in this chapter has been published in: Donald E. Awrey, Nell Shimasaki, Chris Koth, Rod Weilbaecher, Valerie Olmsted, Sophia Kazanis, Xi Shan, Jerry Arellano, Cheryl H. Arrowsmith, Caroline M. Kane, Aled M. Edwards.
Introduction

*In vivo*, RNA polymerase II could encounter a variety of impediments as it transverses a gene. These blocks to transcription elongation could be nucleosomes, DNA lesions, and specific DNA sequences to name just a few. Modulating the ability of RNA polymerase to overcome these blocks may likely serve as a point of regulation of gene expression in the cell (Spencer and Groudine, 1990; Kane, 1994; Shilatifard et al., 1997; Uptain et al., 1997). One regulatory activity is carried out by TFIIS. TFIIS is a well characterized eukaryotic transcription elongation factor which enables RNA polymerase II to transcribe through sequence specific arrest sites *in vitro* (Kerppola and Kane, 1991; Kassavetis and Geiduschek, 1993; Reines, 1994; Shilatifard et al. 1997; Uptain et al., 1997).

As described in detail in Chapter One, when arrested at a block to elongation, RNA polymerase II can be stimulated by TFIIS to cleave the nascent transcript and read through an arrest site (Kerppola and Kane, 1991; Kassavetis and Geiduschek, 1993; Reines, 1994; Shilatifard et al., 1997; Uptain et al., 1997). TFIIS induces transcript cleavage by RNA polymerase II that occurs in the 3’ to 5’ direction (Izban and Luse, 1992; Reines, 1992). Because the polymerase is able to resume transcription after cleaving the transcript, the 5’ fragment must remain associated with the elongation complex (Reines, 1992).

An arrested polymerase must cleave the nascent transcript before it becomes elongation competent (Reines et al., 1992; Izban and Luse, 1993). It has been proposed that when RNA polymerase falls into a state of arrest, the polymerase undergoes a conformational change. In this model, the 3’ end of the transcript slips out of register with the catalytic site requiring a cleavage event to restore the proper alignment (Chamberlin, 1995; Reeder and Hawley, 1996; Landick, 1997; Nudler et al., 1997). However, work with mutant human TFIIS proteins demonstrates that TFIIS stimulation of cleavage by the polymerase is not sufficient for reversing the polymerase from an arrested state (Cipres-
Palacin and Kane, 1994). It has been hypothesized that TFIIS must also induce RNA polymerase II to shift from an arrested to elongation competent state in order to stimulate polymerase to read through a block to elongation (Awrey et al., 1997).

Although not sequence homologs, bacterial transcription elongation factors, GreA and GreB, share functional homology with TFIIS (Kassavetis and Geiduschek, 1993). Both GreA and GreB can stimulate the intrinsic cleavage activity of bacterial RNA polymerase (Borukhov et al., 1992; Borukhov et al., 1993; Reines, 1994; Orioza et al., 1995). Like TFIIS, the Gre proteins are able to promote readthrough of RNA polymerase (Borukhov et al., 1992; Borukhov et al., 1993). Although GreB can act similarly to TFIIS on arrested complexes, GreA must be present before the polymerase arrests in order to facilitate transcription through a block to elongation (Borukhov et al., 1992; Borukhov et al., 1993). The 'anti-arrest' effect of GreA would suggest that the factor might keep the polymerase in an elongation competent state as the polymerase transcribes.

Structurally, the Gre factors are not homologous to TFIIS but there are some similarities. The Gre proteins consist of two domains, specifically, an amino-terminal coiled-coil domain and a carboxy-terminal globular domain linked by a 12 amino acid loop (Stebbins et al., 1995; Koulich et al., 1997). The coiled-coil domain of the Gre factors has a basic patch that is in close proximity to the polymerase based on crosslinking studies (Koulich et al., 1997). It has been speculated that the basic patch of GreA (Stebbins et al., 1995) may in part determine GreA interaction with negatively charged regions of the bacterial polymerase. Site-directed mutagenesis of domain II of TFIIS shows that residues K196, R198, R200, and K209 are important for binding to RNA polymerase II (Awrey et al., 1998). These residues form a positively charged patch on TFIIS, and mutations in an acidic residue of RNA polymerase II interfere with binding to TFIIS (Wu et al., 1996; Olmsted et al., 1998). Work with the individual recombinant Gre domains demonstrated that when added in trans, to an arrested complex, the two domains could not function like the native Gre factor even though each domain folded independently (Koulich et al., 1998).
This observation would suggest that the geometry of the two domains with respect to each other may be important for function (Koulich et al., 1998). Investigations involving species specificity determination by TFIIS in Chapter Three conclude that the linker between domain II and III of TFIIS is important for function (this work). It is proposed that the linker may serve to orient the domains of TFIIS for proper interaction with the polymerase (this work).

Both *in vivo* and *in vitro* evidence suggests that subunits 1, 2, and 9 of RNA polymerase II are involved with TFIIS stimulated cleavage and readthrough (Sawadogo et al., 1980; Rappaport et al., 1988; Archambault et al., 1992; Powell et al., 1996; Wu et al., 1996; Awrey et al., 1997; Lennon et al., 1998). Mutants in RPB1 and RPB2 have been isolated with 6-azauracil sensitivity phenotypes characteristic of *ppr2Δ* yeast strains (Scafe et al., 1990a; Scafe et al., 1990b; Archambault et al., 1992; Powell and Reines, 1996). Overexpression of TFIIS in the *rpo21* strains (*rpo21-6, -7, -17, -18, -19, -23, and -24*) suppressed the 6-azauracil sensitivity phenotype (Archambault et al., 1992). Crosslinking experiments with TFIIS and RNA polymerase II demonstrated that TFIIS is spatially close to both RPB1 and RPB2 in an arrested elongation complex (Powell et al., 1996).

RPB9 contains a Zn ribbon motif homologous in sequence and structure to that of TFIIS (Woychik et al., 1991; Chen et al., 1992; Wang et al., 1998). Δrbp9 polymerase is less likely to arrest at an intrinsic site than wildtype (Awrey et al., 1997). However, the Δrbp9 polymerase was comparable to wildtype in elongation rates, intrinsic cleavage activity, and affinity for TFIIS (Awrey et al., 1997). In addition, the Δrbp9 polymerase was less responsive to TFIIS induced cleavage and readthrough than wildtype polymerase (Awrey et al., 1997). These results suggest that RPB9 may serve to shift polymerase between arrest prone and elongation competent states. The lack of response to TFIIS by arrested Δrbp9 polymerases may indicate a requirement for TFIIS to stimulate read through by way of interaction with RPB9. However, attempts to show a direct interaction between

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TFIIS and RPB9 in vitro have so far been unsuccessful (A. Edwards, personal communication).

As discussed previously in Chapter One, TFIIS has been the focus of several structure/function analyses. The NMR structure for the minimally active yeast TFIIS (residues 131-309) has been solved (Olmsted et al., 1998). The ΔyTFIIS consists of two domains (II and III) connected by a flexible linker (Olmsted et al., 1998). Domain II (amino acids 135-209) is a three helix bundle and interacts with the polymerase (Agarwal et al., 1991; Morin et al., 1996; Awrey et al., 1998). Domain II is connected to Domain III by a linker region (residues 238-263) that has no regular tertiary structure yet is not fully flexible (Olmsted et al., 1998). Mutagenesis studies show that residues within the linker region are important for TFIIS promotion of cleavage and readthrough by RNA polymerase II (Agarwal et al., 1991; Cipres-Palacin and Kane, 1995; Awrey et al., 1998). Work presented in Chapter Three demonstrates that the linker also contributes to determination of species specificity (this work). Domain III has a Zn ribbon motif that shares homology to RPB9, A12.2, and C11 (Woychik et al., 1991; Chen et al., 1992; Nogi et al., 1993; Chedin et al., 1998; Wang et al., 1998). Across species domain III is the most highly conserved portion of TFIIS (Chen et al., 1992). Both site-directed and deletion mutagenesis studies determined that the domain III region is key to TFIIS function (Agarwal et al., 1991; Jeon et al., 1994; Cipres-Palacin and Kane, 1995; Nakanishi et al., 1995; Awrey et al., 1998).

Since TFIIS activity was originally observed in mammalian systems, the initial studies with TFIIS consisted of in vitro biochemistry. The advantage of studying the yeast TFIIS protein is the ability to evaluate function in vivo as well as in vitro. In addition, determination of the NMR structure of yeast ΔTFIIS provided the opportunity to map the location of the residues found to be important for function on the folded protein. Combining both the structural and functional information might could lead to insight on how TFIIS works with RNA polymerase II to stimulate cleavage and readthrough.
With this goal in mind, I carried out site-directed mutagenesis of charged to alanine substitutions of yeast TFII S. The resulting mutants were evaluated in vitro for their ability to facilitate cleavage and readthrough of yeast RNA polymerase II arrested at a sequence specific block to elongation. The mutants were also tested for their ability to compete with wildtype TFII S for interaction with RNA polymerase II. In vivo, the mutants were expressed in prpr2Δ yeast and examined for their ability to rescue 6-azauracil sensitivity. The experiments focus attention on residues K196, R198, and R200. Changes made to these residues affected the ability of TFII S to bind to polymerase and promote cleavage and readthrough. When mapped onto the structure of TFII S, these residues formed a basic patch that may comprise a surface for interaction with RNA polymerase II.
Materials and Methods

*Mutagenesis* Mutagenesis was performed as described Kunkel, et al., with the following modifications (Kunkel et al., 1987). Single stranded template was isolated from *E. coli* strain CJ236 [dutl ung1 thi1 relA1/pCJ105 (Cm')] that had been transformed with pKC16(1-309) (Christie, 1995) and grown overnight at 37°C with shaking in LB containing 50μg/ml ampicillin and 33μg/ml chloramphenicol. 200 μl of overnight culture was used to inoculate 10 ml LB and incubated 1.5 hours at 37°C. This culture was then inoculated with approximately 2x10^9 pfu of R408 helper phage and grown an additional 5-8 hours at 37°C (Russel, et al., Gene 1986). Cells then were heated at 65°C for 15 minutes and centrifuged for two minutes at 13,600xg. The supernatant was centrifuged again for 5 minutes at 13,600xg. To the supernatant was added NH₄OAc to a final concentration of 0.75M NH₄OAc and PEG 8000 to a final concentration of 5%. This was incubated at room temperature for 15 minutes and then centrifuged at 13,600xg for 15 minutes at room temperature. The pellet was resuspended in 200 μl TE and extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1) and once with chloroform:isoamyl alcohol (24:1). The DNA was ethanol precipitated with 1/10 volume 3M NaOAc and 2 1/2 volume of 100% ethanol, and then the pellet was washed with 70% ethanol and resuspended in TE to an approximate concentration of 0.05pmol/μl of template.

Prior to the primer extension reaction, primers synthesized with the desired mutant sequence were phosphorylated with polynucleotide kinase (Table I). 1mM ATP, 20pmol primer, and 3U polynucleotide kinase (USB 30U/μl diluted 1/10 with USB dilution buffer) were incubated in a total volume of 40 μl in 1X Kinase/Ligase buffer (50mM TrisCl pH 7.8, 7 mM MgCl₂, 1mM DTT) for 30 minutes at 37°C. Incubating the mix for 10 minutes at 70 °C stopped the reaction.

For the primer extension reaction, the primer was annealed to the template by mixing 10 pmol of the phosphorylated primer with 0.25 pmol of the single stranded
pKC16(1-309), heating at 65-70°C for 5 minutes, and cooling slowly to room temperature. To this was added to a final concentration: 125µM dATP, dGTP, dCTP, and dTTP; 1µM ATP; 1U T4 DNA polymerase (Boehringer Mannheim Biochemicals); and 1U T4 DNA ligase (USB 1U/µl) in a total volume of 40 µl in 1X Kinase/Ligase buffer. This mix was incubated for 10 minutes on ice, 30 minutes at room temperature, and two hours at 37°C to allow for extension and ligation. The reaction was brought to a final concentration 0.27M NaCl and 18mM EDTA. Phenol:chloroform extracting, and ethanol precipitating isolated the DNA. The DNA pellet was resuspended in 10 µl TE, and 2.5 µl was used to transform E. coli TG1 cells [Δ(lac, pro) supI, thi, hsd D5, F', tra 036, pro A,B, lac IQ, Zdc1 M15].

The mutagenesis was confirmed by dideoxy chain termination sequencing (Table II for plasmids) (Mytelka and Chamberlin, 1996).

Purification of mutant proteins Expression constructs of mutant TFIIS proteins were made as follows. Mutant TFIIS open reading frames were cut out of the pKC16 based plasmids with BamHI and NdeI and ligated into pET15b-HMK (Wu et al., 1996) which had been similarly digested. Expression of these new open reading frames included an aminoterminal His6 tag and heart myosin kinase site.

Over expression of mutant proteins was as described by the manufacturer of the pET vector (Novagen) with the following modifications. BL21 cells were transformed with an expression plasmid. LB with 100 µg/ml ampicillin was inoculated with a transformant. The culture was grown at 37°C with shaking to an OD600 0.6-0.8. Overexpression was induced with 1mM IPTG for 3 hours at 30°C with shaking. Cells were harvested by centrifuging for 5 minutes at 4°C at 5000xg. Cell pellets were then flash frozen in a dry ice/ethanol bath.

Purification of proteins was as described by Novagen with the following modifications. One to two grams of cells were resuspended in 6 ml ice cold Binding buffer (5mM imidazole, 0.5M NaCl, 20mM Tris pH 7.9) with 0.1% Triton X-100 (Sigma) and
1mM PMSF. The cells were then sonicated with a microtip at settings #4-6 (Sonifier Cell Disrupter Model W185D, Heat Systems-Ultronics, Inc., Plainview L.I., N.Y.) for 4 minutes total in 15 second pulses alternating with 45 second incubations on ice. The sonicant was clarified by centrifugation for 20 minutes in a microfuge at 4°C (~13,600xg). The supernatant was passed through a 0.22μm filter and loaded on a 1-2 ml NTA Ni²⁺ agarose column (Qiagen) equilibrated with Binding buffer. The column was washed with 10X volume of Binding buffer, 6X volume of Wash buffer (30mM imidazole, 0.5M NaCl, 20mM Tris pH 7.9), and 6X volume Elute buffer (250mM imidazole, 0.5M NaCl, 20mM Tris pH 7.9). The eluted protein was dialyzed into 50mM Tris pH 7.9, 10% glycerol, 50 mM NaCl, 1mM DTT.

Yeast Transformations The method for low efficiency yeast transformation was a modification of (Gietz et al., 1992). Five ml of YPD medium (yeast extract-peptone-dextrose) (Guthrie and Fink, 1991) was inoculated with a colony from a freshly streaked yeast culture and grown overnight at 30°C with shaking. Transformants from 1-1.5 ml of culture were collected by centrifugation. The cell pellet was washed with 1 ml 1X TE LiAc (10mM Tris pH 7.5, 1mM EDTA, 100mM LiAc) and then resuspended with less than 50 μl residual supernatant. To the resuspended cell mixture was added: 8 μg of single stranded carrier DNA (2mg/ml stock) that had been boiled for five minutes and set on ice before using; approximately 1 μg of transforming plasmid (2-4 μl); and 300 μl of 1X PEG TE LiAc (40%PEG 3350 MW, 10mM Tris pH 7.5, 1mM EDTA, and 100mM LiAc). After mixing gently by pipetting, the yeast were incubated for 4-6 hours at room temperature, heat shocked for 15 minutes at 42°C, washed twice with 1 ml water, resuspended in 250 μl of water, and plated on synthetic complete -ura -trp medium (Guthrie and Fink, 1991).

Growth Assay with 6-azauracil The yeast strains used were CH1305 that was a gift form Connie Holm (MATα ade2 ade3 his3 leu2 ura3) (Kranz and Holm, 1990) and CMKY5 which was derived form CH1305 (MATa ade2 ade3 leu2 ura3 lys2 ppr2Δ::hisG
trp1Δ::hisG URA3 hisG). Freshly streaked cells grown for three days at 30°C were suspended in approximately 1 ml of sterile water and diluted to an OD600 of 0.1 and 0.01. Five μl of each dilution was placed on synthetic complete -ura -trp medium also containing 0, 60, or 100 μg/ml 6-azauracil. The yeast were then incubated at 30°C and monitored for growth. The plates were prepared as described by Sherman (Guthrie and Fink, 1991). 6-azauracil (Sigma) was dissolved in water at 6mg/ml and added to the medium at a final concentration of 60 or 100μg/ml.

Readthrough Assay In vitro transcription was performed as described (Christie et al., 1994) with the following modifications. Purified polymerase was incubated with a 2-fold molar excess dC-tailed template over polymerase, ATP, UTP, GTP, and [α-32P] CTP in order to initiate transcription and label the RNA 5' proximally. Then high concentrations of all four nucleotides were added to allow the polymerase to elongate along the template and form an arrested complex at one of the histone H3.3 arrest sites. Then each reaction was incubated at 30°C with TFIIS storage buffer (50mM Tris pH7.9, 10% glycerol, 50mM NaCl, 1mM DTT) or TFIIS wildtype or mutant proteins at 5:1, 100:1, and 500:1 TFIIS:polymerase molar ratios. Aliquots were taken at 5, 10, and 30 minutes. Each aliquot was treated as described (Christie et al., 1994) and resolved on a 6% acrylamide, 7M urea gel. Quantitation of bands was done with a Molecular Dynamics Phosphorimager system. The fraction of polymerases that transcribed through the T1A site was calculated as (cpms run off RNA)/(cpms run off RNA + cpms T1A RNA). Relative activity of mutant to wildtype TFIIS was calculated as (fraction readthrough_{mutant} - fraction readthrough_{buffer only})/(fraction readthrough_{wt} - fraction readthrough_{buffer only}).

Cleavage Assay The cleavage assay was performed as described in (Christie et al., 1994) with the following modifications. After being passed through two Biospin 30 columns (BioRad) equilibrated with chase buffer (Christie et al., 1994), the transcription complexes were incubated at 30°C with either TFIIS storage buffer (50mM Tris pH7.9, 10% glycerol, 50mM NaCl, 1mM DTT) or with wildtype or mutant TFIIS proteins at 5:1,
100:1, or, 500:1 TFIIS:polymerase molar ratios. Aliquots were taken at 1, 5, and 10 minutes. An additional aliquot was incubated with 1mM ATP, UTP, GTP, and CTP for 10 minutes at 30°C and denoted as the "chase." All samples were stopped as described (Christie et al., 1994) and resolved on 6% acrylamide, 7M urea gels.

**Competition assay** TFIIS was labeled with $^{32}$P as described (Awrey et al., 1998) with the following modifications. After the kinase reaction to label TFIIS, free [$\gamma^{32}$P] ATP was removed from the reaction by passing the mix over three Biospin 6 (BioRad) columns under conditions recommended by BioRad. The columns were equilibrated with 20mM Hepes pH 7.5, 10% glycerol, 100mM NaCl, 0.01% NP-40, 1mM DTT, and centrifuged for two minutes in an IEC clinical centrifuge at a setting of 4 (~1000 relative centrifugal force).

The competition assay was performed as follows. Yeast RNA polymerase II was de-salted with a Biospin 30 micro column (BioRad) equilibrated with 50mM Tris pH 8, 10% glycerol, 50mM NaCl, 2mM DTT. To form the labeled TFIIS (TFIIS*)/polymerase complex: 0.17µM polymerase, 0.17µM TFIIS*, and 17mM DTT in 20mM TrisOAc pH 7.5, 10% glycerol were incubated on ice for 15 minutes. Then 3µl of this binding reaction was added to 2µl of 12.5pmol/µl mutant TFIIS (about 50-fold molar excess over labeled TFIIS*) or simply 2µl of TFIIS storage buffer with an equivalent amount of BSA as a negative control (50mM Tris pH 7.9, 10% glycerol, 50 mM NaCl, 1mM DTT). This mixture was incubated for an additional 20 minutes on ice. The complexes were resolved on 5% acrylamide, 1% glycerol, 50mM TrisBorate pH 8.3 gels (at 4°C) with 50mM TrisBorate pH 8.3 (at 4°C), 1mM DTT running buffer. Gels were electrophoresed at 100V for two hours.

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Results

Selection of Mutants

In order to investigate the structure/function of yeast TFIIS, a variety of charged to alanine substitutions were made in the TFIIS protein. The mutants were evaluated for their ability to stimulate readthrough and cleavage by yeast RNA polymerase II, to compete with wildtype TFIIS for binding to RNA polymerase II, and to complement a ppr2Δ yeast strain \textit{in vivo}. The majority of charged residues targeted for alanine substitution in yeast TFIIS were chosen based on previous work done by Cipres-Palacin and Kane with human TFIIS (Cipres-Palacin and Kane, 1994; Cipres-Palacin and Kane, 1995). Homologous residues in the yeast TFIIS were chosen based upon phenotypes of mutants in the human TFIIS. Human TFIIS mutant K187A/K189A was found to have the ability to stimulate cleavage by mammalian polymerase, but it could not promote readthrough (see Chapter One, Fig. 3) (Cipres-Palacin and Kane, 1994). The rate of cleavage was reduced 10-fold (Cipres-Palacin and Kane, 1994). But if cleavage were sufficient to promote readthrough, this mutant should have been effective in a readthrough assay. Human TFIIS mutant, R191A/R193A/R195A, was inactive and unable to promote readthrough or cleavage by calf thymus polymerase suggesting that these residues were important for TFIIS function (Cipres-Palacin and Kane, 1995). Thus, a variety of mutants were made in the yeast TFIIS protein in the homologous residues either singly (K196A, R198A, and R200A) or in combination (K196A/R198A, K196A/R200A, R198A/R200A, and K196A/R198A/R200A).

Several other yeast mutants were made, guided either by human mutants or by sequence homologies. Human TFIIS mutants, E173A/E174A and R297A/K300A, were defective in stimulating polymerase (Cipres-Palacin and Kane, 1995); so yeast TFIIS E181A and R305A were made. Another yeast TFIIS mutant, D235A, was made because a similar residue was conserved among several species (see Chapter One, Fig. 3). K147A
was made because it is highly conserved across species, and alanine scanning mutagenesis with human TFIIS indicated that this residue might be important for function (see Chapter One, Fig. 3) The tested mutants are found in Table III.

**Stimulation of Readthrough**

The ability of TFIIS to promote readthrough by RNA polymerase II is a well studied reaction (Reines, 1994). Such readthrough assays were performed with a dC-tailed template containing sequences from the human histone H3.3 gene that has well characterized intrinsic arrest sites (T1A, T1B, and TII) for RNA polymerase II (Reines et al., 1987; Kerppola and Kane, 1990). The T1A arrest site is the most efficiently recognized of all of the three arrest sites on the template by RNA polymerase II, and the transcription complexes arrested at this site will be the ones studied in the following experiments. The RNA transcripts were labeled proximal to the 5' end as described in Materials and Methods. Then the initiated complexes were chased to form a population of complexes arrested at the T1A site in the human histone H3.3 gene by the addition of high concentrations of all four nucleotides. Heparin was present to prevent new initiation from occurring. The complexes were subsequently incubated with TFIIS, TFIIS mutants, or TFIIS storage buffer. Readthrough and transcript cleavage were monitored and quantified.

Each of the mutants was tested for its ability to facilitate readthrough by polymerase arrested at blocks to elongation. The transcription reactions were done at 5:1, 100:1, and 500:1 TFIIS:polymerase molar ratios. It has been roughly approximated that there is 1:1 TFIIS:polymerase in vivo in mammalian cells (Sopta et al., 1985). When arrested elongation complexes are incubated with wildtype TFIIS, full length transcripts accumulate. This result demonstrates that wildtype TFIIS can stimulate efficient readthrough by polymerase at a 5:1 TFIIS:polymerase molar ratio (Fig. 1). Mutants K147A, E181A, D235A, and R305A were qualitatively like wildtype (data not shown). Mutants K196A, R198A, and R200A were somewhat defective at stimulating readthrough
at 5-fold excess over polymerase, but they were essentially like wildtype at 100-fold excess over polymerase (Figs. 2-4). The fraction of transcripts remaining at the T1A band after 30 minutes incubation of 5-fold excess wildtype TFIIS was approximately 12% as compared to 57% for K196A, 35% for R198A, and 42% for R200A. At 100-fold excess over polymerase the fraction of T1A transcripts after 30 minutes incubation was 15% with K196A, 13% with R198A, and 17% with R200A. Double or triple mutations in these three residues resulted in more severe effects. K196A/R198A was less able to promote readthrough than each individual mutant at a 5:1 molar ratio with polymerase, but like the single mutants, at a higher molar excess stimulation of readthrough by K196A/R198A was comparable to that of wildtype TFIIS (Fig. 5). K196A/R198A had only 0.19 the stimulatory activity of wildtype TFIIS when present in 5-fold excess over polymerase, but K196A/R198A had 0.99 the stimulatory activity of wildtype TFIIS when present in 100-fold excess over polymerase. K196A/R200A was less functional. K196A/R200A had no measurable stimulatory activity at 5-fold molar excess over polymerase. Only at the highest ratio of TFIIS:polymerase, 500:1, was any readthrough detected, and even then K196A/R200A had only 0.26 the stimulatory activity of wildtype TFIIS under the same conditions (Fig. 5). Mutant R198A/R200A was essentially inactive as was the triple mutant, K196A/R198A/R200A (Fig. 6). These results suggest the importance of R200 in the ability of TFIIS to stimulate readthrough.

Stimulation of Transcript Cleavage

The ability of TFIIS to stimulate the arrested polymerase to cleave the nascent transcript as a prelude to promoting readthrough is well documented (Reines, 1994). For the cleavage assay, arrested complexes are formed like those for the readthrough assays (Reines, 1992; Christie et al., 1994). After the arrested elongation complexes are formed, they are separated from unincorporated nucleotides before the addition of TFIIS or mutant proteins (Reines, 1992; Christie et al., 1994). Therefore, if the RNA is cleaved, the
polymerase cannot immediately resume transcription, and the shortened transcripts can be detected by gel electrophoresis. The evaluation of cleavage activity was qualitative and based on the accumulation of cleavage products as visualized on an RNA denaturing gel (indicated by arrows in the figures).

Since mutants K196A, R198A, and R200A were slightly defective in facilitating readthrough of RNA polymerase II at a 5:1 TFIIS:polymerase molar ratio, it was expected that these mutants would also be defective in inducing cleavage by polymerase. As can be seen in Fig. 7, shortened transcripts can be detected within the first minute of incubation of the arrested polymerase with wildtype TFIIS at a 5-fold excess over polymerase. The single mutants, K196A and R200A, were not able to induce detectable cleavage by RNA polymerase II at a 5:1 TFIIS:polymerase molar ratio in the same timeframe (Figs. 8 & 9). Cleavage was stimulated by R198A, but the extent of cleavage promoted by R198A was less than that detected with wildtype TFIIS (Fig. 9). By increasing the amount of mutant protein twenty times to 100-fold molar excess over polymerase, K196A and R198A were able to begin to approach wildtype levels of cleavage stimulation, but R200A required a 500-fold molar excess to evince the same cleavage activity (Fig. 10). Out of all of the multiple mutants, K196A/R198A was the most robust at stimulating transcript cleavage. However, even at 500:1 TFIIS:polymerase molar ratio, K196A/R198A did not promote cleavage to the extent of that of wildtype TFIIS (Fig. 11). An even smaller amount of cleavage could be detected at a 500-fold excess over polymerase with K196A/R200A, but no cleavage could be detected in reactions with R198A/R200A or K196A/R198A/R200A (Figs. 12-14).

Binding to RNA Polymerase II

A simple explanation for the defect in the ability of the mutants to promote readthrough and cleavage is that the K196, R198, and R200 residues might be important for binding to the polymerase. Initial binding studies with RNA polymerase II and TFIIS
were comprised of labeled wildtype or mutant TFIIS proteins complexed to increasing amounts of purified polymerase (Awrey et al., 1998). During the course of this project, it was found that the conditions used for binding assay described in Awrey et al. resulted in precipitation of the proteins. The aggregation of the complexes affected reproducibility of this assay. At least two experimental factors that caused precipitation were the presence of Zn\textsuperscript{2+} in the binding buffer and the absence of 1mM DTT in the gel running buffer. A competition assay was developed for this study to evaluate the ability of each mutant TFIIS to compete with wildtype TFIIS for binding to polymerase. The competition assays were done in the absence of Zn\textsuperscript{2+} in the binding buffer and in the presence of 1mM DTT in the gel buffer to eliminate the aggregation seen using the protocol developed by Awrey et al. (Awrey et al., 1998).

In these assays, an active construct was made so that wildtype TFIIS could be labeled on an engineered heart myosin kinase site. This fusion protein was as active as the non-derivatized protein (Wu et al., 1996). Complexes were formed at a 1:1 ratio with RNA polymerase II. Then, a 50-fold excess of mutant TFIIS was added to the complexes, the complexes were further incubated, and the products of the reaction were evaluated by native gel electrophoresis. In the gel system used, free TFIIS would migrate toward the negative pole and not into the gel. If the mutant protein was able to compete for binding, there should be less labeled wildtype TFIIS complexed to the polymerase as compared to the control. When the competition assay was done with full length TFIIS proteins, the TFIIS*/PolII complexes appeared heterogeneous when run on a native gel (Fig. 15). The heterogeneity of the complexes most likely is due to the presence of the amino terminus of the TFIIS proteins. In a similar assay with the truncated TFIIS proteins, the TFIIS*/PolII complexes remained as a discrete band when challenged by 50-fold competitor TFIIS (see Fig. 3, Chapter Three). All of the radioactive counts in the gel detected by the phosphorimager within a given lane were considered to represent TFIIS*/PolII complexes.
The mutants K147A, E181A, D235A, and R305A competed as well as the wildtype protein (Fig. 15). However, changing the basic residues, K196A, R198A, R200A, altered the binding (Fig. 15). In lane 2, the control experiment, a 50-fold molar excess of unlabeled wildtype TFIIS readily competed for binding (compare lanes 2 and 14). Based on the activity assays, one might expect that R198A would compete best among the mutants followed by K196A and then R200A. Mutant K196A was tested at 12-fold molar excess, and under these conditions, it did not significantly compete with wildtype TFIIS for polymerase binding. By quantification with a phosphorimager, R198A was able to compete about half as well as wildtype TFIIS for interaction with polymerase (lane 9). Mutant R200A and all of the multiple mutants were unable to compete detectably with wildtype TFIIS for binding to polymerase (lanes 10-13). Thus the reduced (or abolished in some cases) ability of the mutants to stimulate polymerase to cleave and read through a block to elongation is likely due to the compromised ability of the mutants to interact with the polymerase. Although mutants K196A, R200A, K196A/R198A, and K196A/R200A were not able to compete for binding, stimulation of polymerase by these mutants was detected. However, the transcription stimulatory activity of these mutants was observed at TFIIS:polymerase ratios higher than the 50-fold excess over polymerase used for the competition assays. It is likely that the experimental conditions used for the competition assay were not sensitive enough to detect interference due to weak polymerase binding by these mutants.

**In vivo Function**

A major advantage of studying the TFIIS protein from yeast rather than mammalian cells is that the yeast TFIIS mutants can be tested for function *in vivo*. There is approximately 23% amino acid identity between the full length yeast and human proteins (see Chapter One, Fig. 3). Yeast strains deleted for the gene encoding TFIIS (*ppr2Δ*) are more sensitive to the drug, 6-azauracil, than wildtype strains (Exinger and Lacroute, 1992).
Each of the eleven yeast TFIIS mutants was patched onto synthetic complete -ura-trp medium with 0, 60, or 100 μg/ml 6-azauracil and then grown for three days at 30°C. As expected, the wildtype cells grew on 6-azauracil, and the ppr2Δ cells did not grow on the drug containing media. Yeast harboring TFIIS mutations K147A, E181A, D235A, or R305A were able to grow essentially like the wildtype cells on 6-azauracil containing media indicating that all of these mutants were able to function sufficiently to complement the absence of TFIIS (data not shown). Mutants K196A, R198A, and R200A were also able to complement in vivo (Fig. 16). Of the multiple mutants, only K196A/R198A was able to confer nearly wildtype growth on drug containing media (Fig. 16). Thus, although mutants K196A, R198A, R200A, and K196A/R198A were defective in stimulating polymerase in in vitro transcription assays, they could still substitute in vivo for TFIIS in whatever functions are assayed by growth on 6-azauracil.

A summary of in vitro and in vivo assays is presented in Table III.
Discussion

Several charged to alanine substituted mutants of yeast TFIIS were made in this study with the purpose of gaining insight into the structure/function relationships for TFIIS. The residues targeted were selected because they were either homologous to those found to be important in mutagenesis studies with human TFIIS (Cipres-Palacin and Kane, 1994; Cipres-Palacin and Kane, 1995) or highly conserved among TFIIS proteins. Many of the mutants made in the yeast protein appeared to have wildtype activity within the sensitivity of the assays used to define function: stimulation of cleavage and readthrough by the polymerase, binding to polymerase, and suppression of 6-azauracil sensitivity. However, residues K196, R198, and R200 in yeast TFIIS were found to be important for function, a result expected based on previous studies with human TFIIS. A mutant made in human TFIIS, which was equivalent to K196A in yeast, had the unusual phenotype of enabling it to stimulate transcript cleavage but not readthrough by mammalian RNA polymerase II (Cipres-Palacin and Kane, 1994). Deletion mutants of human TFIIS containing residues equivalent to K196, R198, and R200 in yeast TFIIS showed that the region was important for interacting with the polymerase (Agarwal et al., 1991). Single alanine substituted mutants of K196, R198, and R200 in yeast TFIIS resulted in a reduction of ability by each mutant to facilitate cleavage and readthrough of RNA polymerase II arrested at a sequence specific block to elongation.

Based on the competition binding assays, these residues appear important for TFIIS interaction with polymerase as these mutants were unable or reduced in their ability to compete with wildtype TFIIS for binding. Impaired binding to polymerase might result in a need to increase the concentration of the mutant in order to see stimulation similar to that of wildtype during in vitro transcription assays. Indeed, this prediction was satisfied.

In addition, although defective in vitro, these mutants could suppress the 6-azauracil sensitivity of a ppr2Δ yeast strain. For the complementation assays, the alanine
substituted mutants were expressed on low copy number plasmids under the control of the genomic PPR2 promoter. The gene encoding TFIIS exists as a single copy in yeast, and the expression of mutant proteins was comparable to expression of wildtype proteins as assayed by Western analysis (data not shown). These results suggested that cells naturally have more TFIIS than is needed for in vivo function. There are at least two ways to identify more subtle effects of these mutants in vivo. First, determining the growth curves of mutant strains in drug containing liquid media might reveal differences between mutant and wildtype strains not detected with the plate assay. Second, co-cultures of wildtype and mutant strains with different auxotrophic markers might reveal a selective advantage. Either procedure might help determine what threshold of activity is needed in vivo for complementation of the drug sensitivity phenotype. Alternatively, there may exist other factors in the cell which have overlapping functions with TFIIS [possibly SPT4, SPT5, and SPT6 (Hartzog et al., 1998)]. The synthetic phenotypes between the ppr2 and spt4, spt5, and spt6 double mutants suggest that such a functional overlap exists (Hartzog et al., 1998). Even though K196A, R198A, and R200A were defective, there could still be sufficient activity provided by these other factors to enable wildtype growth on the 6-azauracil plates. Analysis of the TFIIS mutants in spt mutant strains may reveal phenotypes that were not seen in the SPT strains.

Multiple substitutions in residues K196, R198, and R200 resulted in further reduction in readthrough and cleavage stimulatory activity. In particular, multiple mutations with R200 were the most deleterious. The K196A/R200A mutant could not complement the drug sensitivity in vivo, and only at the highest molar excess tested was stimulation of readthrough and cleavage detected in vitro. The other two mutants, R198A/R200A and K196A/R198A/R200A were essentially inactive in vivo and in vitro.

Work done in collaboration with A. Edwards and C. Arrowsmith has helped to put the biochemical and phenotypic results with the alanine substituted mutants in context with the three-dimensional structure of TFIIS. Previous NMR analysis and protease digestion
studies had determined that TFIIS consists of three structural domains (Morin et al., 1996). Domain II, residues 131-240, is a three-helix bundle (Morin et al., 1996) that binds to RNA polymerase II with the same affinity as the truncated, minimal active region of TFIIS (residues 131-309) (Awrey et al., 1998). Consistent with this observation is that deletion mutants in the domain II region of human TFIIS had a reduced affinity for mammalian polymerase (Agarwal et al., 1991). Further, alanine scanning mutants of human TFIIS revealed that there are residues in this region important for stimulation of cleavage and readthrough including K186A/K188A which is equivalent to K196A in yeast (see Chapter One, Fig. 3) (Cipres-Palacin and Kane, 1994). Several other yeast TFIIS mutants in domain II were also defective in interacting with RNA polymerase II (Awrey et al., 1998). The most seriously compromised mutants (K154A/D158A, K196A, R198A, R200A, and K209A) bound at least 10-fold less well than wildtype TFIIS (Awrey et al., 1998). When mapped onto the NMR structure, residues K196, R198, and R200 clustered together forming a basic patch (Awrey et al., 1998) (Fig. 17).

It has been reported that TFIIS promotion of readthrough by polymerase is reduced under conditions of high ionic strength (SivaRaman et al., 1990). Further, polymerase and TFIIS will not co-sediment in glycerol gradients under conditions of high salt (Horikoshi et al., 1984). Also, affinity purification of TFIIS with polymerase attached to a solid support was found to be salt dependent (Sopta et al., 1985). These observations strongly suggest that the interaction between polymerase and TFIIS is ionic in nature. Additionally, a mutant of the largest subunit of polymerase, which has an acidic residue altered to a basic one, has a 6-azauracil sensitivity phenotype, which can be suppressed by increasing the dosage of TFIIS (Archambault et al., 1992). This polymerase mutant has been found to be defective in binding to TFIIS (Wu et al., 1996). The results with the alanine mutants of TFIIS and the mapping of the residues to form a basic patch would lead one to hypothesize that TFIIS binds through this positively charged region to polymerase through this negative residue in its largest subunit (Awrey et al., 1998).
Interactions of TFIIS with eukaryotic polymerase may be analogous to interactions of GreA, a functional homolog of TFIIS, with bacterial polymerase. Like TFIIS, GreA has a basic patch (Stebbins et al., 1995). An adduct incorporated at the 3' end of RNA in an elongation complex crosslinked to GreA near this basic patch (Koulich et al., 1997). The isoelectric point of core bacterial polymerase is acidic (pI=5.34), and the basic patch of GreA may in part serve to orient GreA properly towards the elongation complex and/or the negatively charge phosphates of the RNA transcript (Stebbins et al., 1995).

TFIIS may bind to the polymerase through the two largest subunits. In vitro crosslinking studies indicate that TFIIS is located close to the active site in an elongation complex (Powell et al., 1996). RPB1 and RPB2 are believed to comprise the active site for RNA polymerase II (Woychik and Young, 1990). Biochemical and genetic experiments have shown that TFIIS functionally interacts with the two largest subunits of RNA polymerase II (Rappaport et al., 1988; Archambault et al., 1992; Powell and Reines, 1996; Wu et al., 1996).

Most likely, the mechanism by which TFIIS promotes cleavage and readthrough of an arrested polymerase is more complex than simply binding of TFIIS to the two largest subunits of RNA polymerase II near the active site. Evidence supports a role for RPB9 in arrest site recognition and/or elongation competence as well as function with TFIIS (Awrey et al., 1997). The binding affinity between TFIIS and Δrbp9 polymerase was like wildtype strongly suggesting that RPB9 is not needed for the polymerase:TFIIS binding interaction (Awrey et al., 1997). Additionally, Cipres-Palacin and Kane studied a mutant TFIIS that was able to promote cleavage but not readthrough (Cipres-Palacin and Kane, 1994). All these results would suggest that the mechanism of TFIIS stimulation of arrested polymerase consists of multiple steps. TFIIS may first need to bind to the arrested complex through RPB1 near the active site. TFIIS induces the polymerase to cleave the nascent transcript and thereby re-align the 3' terminus of the RNA with the catalytic site of the polymerase. Then, perhaps by acting through RPB9, TFIIS may induce or stabilize a
conformational change in the polymerase that converts an elongation incompetent state to an elongation competent state resulting in readthrough.
References


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RGW = R.G. Wellbaecher  
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KRC=K.R. Christie; RGW=R.G. Weilbaecher; NBS=N.B. Shimasaki
### Table III Results

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</table>

* TFIIS:PolII molar ratio

# all TFIIS mutant proteins were at 50-fold molar excess over labelled wildtype TFIIS with the exception of K196A that was tested with a 12-fold molar excess
Figure Legends

Fig. 1  Wildtype TFIIS stimulates RNA polymerase II to transcribe through the human histone H3.3 T1A block to elongation.

Arrested transcription complexes were formed (lane 1) and then incubated with either TFIIS storage buffer ("buffer") or TFIIS at 5:1, 100:1, or 500:1 TFIIS:polymerase molar ratios. In the presence of wildtype TFIIS, more RNA polymerase II molecules are able to transcribe past the T1A arrest site and produce full length transcripts (RO) than in the absence of TFIIS (compare lanes 4&7). The bands labeled T1B and TII are transcripts from complexes arrested at the T1B and TII sites also present on the template.
Fig. 2  TFIIS mutant, K196A, is defective at promoting readthrough by RNA polymerase II arrested at a block to elongation.

A higher molar excess (100:1) of K196A is required to stimulate readthrough by polymerase to produce the effects seen with only 5:1 wildtype TFIIS (compare Fig. 1 lanes 5-7 and 8-10 with lanes 5-7 and 8-10 of this figure).
Fig. 3  **Mutant R198A is less effective at stimulating readthrough by RNA polymerase II than wildtype TFIIS.**

Like K196A, 20-fold more R198A than wildtype TFIIS is needed to facilitate a comparable amount of full length transcription by polymerase (compare Fig. 1 lanes 5-7 and 8-10 with lanes 5-7 and 8-10 of this figure).
Fig. 4  R200A is as defective as K196A and R198A at promoting readthrough by RNA polymerase II of the T1A arrest site. A 100-fold molar excess of R200A over polymerase is required to observe a comparable amount of stimulatory activity seen with only 5-fold molar excess of wildtype TFIIS over polymerase (compare Fig. 1 lanes 5-7 and 8-10 with lanes 5-7 and 8-10 of this figure).
**Figure 4**

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</table>

- **TFII-S-Poll**
- **R200A**

*Note: The table represents different time points and concentrations for TFII-S-Poll and R200A.*
Fig. 5  Mutant K196A/R198A has a similar stimulatory activity as mutants K196A, R198A, and R200A, but K196A/R200A is even more defective than these mutants. Even at a 500-fold molar excess over polymerase, K196A/R200A cannot stimulate readthrough by polymerase to the extent of wildtype TFIIS at 5:1 TFIIS:polymerase ratio (compare Fig. 1 lanes 5-7 and lanes 16-18 of this figure).
<table>
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Fig. 6 R198A/R200A and K196A/R198A/R200A are unable to promote readthrough by RNA polymerase II.

No stimulation of readthrough was detected in reactions with R198A/R200A or K196A/R198A/R200A even at the highest TFIIS:polymerase ratio tested of 500:1.
Fig. 7 Wildtype TFIIS induces RNA polymerase II to cleave its transcript when the polymerase is arrested at a block to elongation.

Arrested RNA polymerase complexes were formed and isolated from unincorporated nucleotides (lane 1). The complexes were then incubated with either TFIIS storage buffer ("buffer") or TFIIS at 5:1, 100:1, or 500:1 TFIIS polymerase molar ratios. After ten minutes of incubation, nucleotides were added to the reaction, and the complexes were incubated for an additional 10 minutes (lane "C"). At a 5:1 TFIIS:polymerase molar ratio, arrested polymerases cleave their transcripts after only one minute of incubation with wildtype TFIIS (compare lanes 2&6). Arrows indicate cleaved transcripts.
### Figure 7

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Fig. 8  K196A is unable to stimulate transcript cleavage by RNA polymerase II arrested at the T1A site at a 5:1 TFII-S:polymerase molar ratio. K196A is defective at promoting transcript cleavage by polymerase at 5-fold excess over polymerase (compare lanes 2-4 with 6-8). However, transcript cleavage was induced in reactions with a 100-fold K196A molar excess over polymerase (lanes 10-12). In this gel, the "chase" lanes ("C") were over loaded.
Figure 8

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1234567891011121314151617
Fig. 9  **Mutant R198A less active than wildtype TFIIS at stimulating transcript cleavage by RNA polymerase II.**

At a 5:1 R198A:polymerase ratio, polymerase is induced to cleave its transcript, but the extent of cleavage is less than that observed in reactions with wildtype TFIIS (compare Fig. 7, lanes 6-8 with lanes 6-8 of this figure). In this gel, the "chase" lanes ("C") were overloaded.
### Figure 9

<table>
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Fig. 10  **R200A is the most defective of the single mutants at promoting transcript cleavage by an arrested RNA polymerase II.**

In reactions with R200A, only a small amount of cleavage activity is detected at the highest molar ratio of TFIIS:polymerase tested of 500:1. In this gel, the "chase" lanes ("C") were over loaded.
Fig. 11  K196A/R198A is able to promote transcript cleavage by an arrested polymerase at a 5:1 TFIIS:polymerase molar ratio.

RNA polymerase II cleaves its transcript when incubated with a 5-fold excess of K196A/R198A, but the cleavage is not to the extent observed with wildtype TFIIS (compare Fig.7 lanes 6-8 with lanes 6-8 of this figure).
Fig. 12  K196A/R200A is severely defective at stimulating transcript cleavage by RNA polymerase II.

At the highest molar ratio tested of 500:1 TFIIS:polymerase, polymerase is induced to cleave its transcript after a ten minute incubation with K196A/R200A (compare lane 4&16).
Fig. 13  

**R198A/R200A is unable to promote transcript cleavage by an arrested RNA polymerase II.**

Even at the 500:1 TFIIS:polymerase ratio, no cleavage activity by arrested transcription complexes was detected after 10 minutes of incubation with R198A/R200A.
Figure 13

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145
Fig. 14  K196A/R198A/R200A is unable to promote transcript cleavage by an arrested RNA polymerase II.

Even at the 500:1 TFIIS:polymerase ratio, no cleavage activity by arrested transcription complexes was detected after 10 minutes of incubation with K196A/R198A/R200A.
Figure 14

K196A/R198A/R200A

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Fig. 15  Competing with wildtype TFIIS for binding to RNA polymerase II.
Wildtype TFIIS was radioactively labeled (TFIIS*) and incubated at a 1:1 molar ratio with RNA polymerase II to form TFIIS*/polymerase complexes. The complexes were then challenged with the addition of 50-fold excess mutant TFIIS. After a second incubation, the complexes were resolved on a native gel as described in Materials and Methods. The arrow indicates the location of the well, and the asterisk indicates the TFIIS*/polymerase complexes. 50-fold excess wildtype TFIIS, K147A, D235A, R305A, or E181A can compete for binding with TFIIS* (compare lanes 2-6 with lane 14). 12-fold excess of K196A was not able to compete with wildtype TFIIS* for binding to polymerase (lane 7). R198A and R200A could not compete as well as wildtype TFIIS for binding to polymerase, and the multiple mutants were unable to compete at all for binding to polymerase (compare lanes 8-13 with lanes 2&14).
Fig. 16 Testing for the ability of the mutant TFIIS proteins to suppress the 6-azauracil sensitivity phenotype of a ppr2Δ yeast strain.

Wildtype (CH1305) or ppr2Δ (CMKY5) yeast strains were transformed with marker plasmids (pRS314 or pRS316) or in the case of CMKY5, with plasmids encoding for wildtype and TFIIS mutants. The yeast were plated on SC-ura-trp medium with or without 60 or 100µg/ml drug and grown for three days at 30°C. Both the wildtype strain and the ppr2Δ strain with a plasmid encoding for TFIIS, pKC16(1-309), are less sensitive to 6-azauracil than the ppr2Δ strain (CMKY5+pRS314). Mutants K196A, R198A, R200A, and K196A/R198A are able to complement in vivo. Mutants K196A/R200A, R198A/R200A, and K196A/R198A/R200A are not able to suppress the drug sensitivity phenotype.
### Figure 16

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**no drug** | **60 µg/ml** | **100 µg/ml**

Diagram illustrating the effect of different drug concentrations on cell growth.
Fig. 17 Surface potential of TFIIS domain II.

On the left is the α-carbon backbone of residues 131-220 of TFIIS. On the right is a surface representation of this region. Blue indicates a positively charged surface, and red indicates a negatively charged one. Amino acids K196, R198, and R200 that were found to contribute to the interaction of TFIIS with RNA polymerase II are labeled (from Awrey et al., 1998).
Chapter Three

Identification of a Species Specificity Determinant of TFIIS
Introduction

Transcription elongation factor, TFIIS, has been found in every examined eukaryotic system, and it has been cloned in organisms ranging from *Saccharomyces cerevisiae* to *Drosophila* to humans (Chen et al., 1992; Kugawa et al., 1996; Williams and Kane, 1996; Weaver and Kane, 1997). Sequence homology has also been found in archaea and pox viruses (Ahn et al., 1990; Langer and Zillig, 1993; Rosales et al., 1994).

Across species, TFIIS proteins share a high degree of conserved sequences (see Chapter One, Fig. 3). The greatest degree of conservation exists between TFIIS proteins from metazoans such as human, *Drosophila*, and mouse (see Chapter One, Fig. 3). The amino-terminal portion of TFIIS (amino acids 1-130 in yeast) shows the least homology across species (Fig.1). This region is dispensable for *in vitro* stimulation of transcript cleavage and readthrough by RNA polymerase II (Nakanishi et al., 1995). *In vivo*, the amino-terminus is not required for suppression of 6-azauracil sensitivity in a *ppr2A* yeast strain, but it is needed for a genetic interaction between *PPR2* and *TFG3* (Nakanishi et al., 1995; Davie, 1998).

The most highly conserved region of TFIIS lies within domain III (residues 279-309) (see Chapter One, Fig. 3) (Olmsted et al., 1998). As mentioned previously in Chapter Two, mutagenesis has determined that domain III is critical for TFIIS function (Jeon et al., 1994; Cipres-Palacin and Kane, 1995; Nakanishi et al., 1995; Awrey et al., 1998). This region possesses a Zn ribbon motif that shares similarity to RPB9 of RNA polymerase II, C11 of RNA polymerase III, and A12.2 of RNA polymerase I (Nogi et al., 1993; Qian et al., 1993; Chedin et al., 1998; Wang et al., 1998).

Domains II and III are connected with a linker region [residues 238-263 in the yeast TFIIS (Olmsted et al., 1998)]. NMR analysis indicated that although this region possessed no defined conformations, it was not fully flexible either suggesting that the linker may be more than simply a tether anchoring domains II and III together (Olmsted et al., 1998).
Furthermore, mutational studies demonstrated that there were residues in this region that were also key to the function of TFIIS. Alanine substitutions in this region did not perturb the structure as evaluated by NMR; nevertheless, they caused a reduction in the ability of the mutants to promote cleavage and readthrough by polymerase (Awrey et al., 1998). Alanine scanning mutagenesis with the human TFIIS also identified residues in the linker region that resulted in reduced function when altered (Cipres-Palacin and Kane, 1995). With the yeast protein, a deletion mutant, Δ240-245, as well as a mutant that had a tandem repeat of these residues were defective (Awrey et al., 1998). In another study, the linker region of the human protein was deleted and replaced with an eight amino acid random coil, and the mutant was found to be inactive (Agarwal et al., 1991).

However, despite the high degree of conservation between TFIIS proteins from different organisms, the proteins are not interchangeable in every instance. *Drosophila* and mammalian TFIIS proteins are able to function cross species, but TFIIS from neither *S. cerevisiae* nor *S. pombe* is able to substitute for the metazoan TFIIS (Kane, unpublished results; Sawadogo et al., 1980; Williams and Kane, 1996). Also, human TFIIS is unable to complement a *ppr2Δ* yeast strain *in vivo* (this work). In addition, the *S. cerevisiae* and *S. pombe* TFIIS proteins are not interchangeable with each other (Williams and Kane, 1996).

TFIIS is not the only transcription factor to distinguish between species. Human TFIIF can stimulate transcription by *Drosophila* RNA polymerase II, but yeast TFIIF can not substitute for mammalian TFIIF in an *in vitro* mammalian transcription system (Conaway and Conaway, personal communication). It has also been reported that the CTD phosphatase from yeast can not de-phosphorylate the CTD on mammalian RNA polymerase II (Chambers and Kane, 1996). Further, the stimulatory effect of TFIIF on the phosphatase activity is also species specific. Mammalian TFIIF does not stimulate yeast phosphatase (Chambers and Kane, 1996).
Structure/function studies with the bacterial GreA protein suggest that domain orientation is important for function (Koulich et al., 1998). GreA consists of two domains tethered together by a 12 amino acid loop (Stebbins et al., 1995). As had been observed with TFIIS and RNA polymerase II, recombinant GreA domains added in trans separately or together could not stimulate transcription by bacterial RNA polymerase (Koulich et al., 1998). This result suggests that the two domains of GreA may require precise orientation with respect to each other in order to interact appropriately with an elongation complex (Koulich et al., 1998).

In order to determine the region of species specificity of TFIIS and to further elucidate the interactions of TFIIS with RNA polymerase II, human/yeast chimeric TFIIS mutants were constructed and tested for their ability to function with the mammalian and yeast RNA polymerase II enzymes. For these studies, the minimal active region (amino acids 131-309 in yeast) was used (Nakanishi et al., 1995). This region consists of structural domain II (residues 135-209 in yeast), a flexible linker (amino acids 238-263 in yeast), and domain III (residues 279-309 in yeast) as determined by NMR (Olmsted et al., 1998). Structural domain swaps between human and yeast TFIIS proteins were made. Because domain II is involved in binding with the polymerase, it was initially hypothesized that domain II of TFIIS also determined species specificity (Agarwal et al., 1991; Awrey et al., 1998). Shimoaraiso et al. reported that residues 132-270 in yeast TFIIS lent species specificity in mouse/yeast TFIIS chimeras indicating that domain II and the linker are involved in distinguishing between polymerases (Shimoaraiso et al., 1997). Work presented in this chapter shows that a chimera that contains all human sequence except for a yeast region encompassing the linker (residues 241-270 from yeast) is sufficient to stimulate yeast RNA polymerase II.

The linker of TFIIS may serve to provide critical interactions with RNA polymerase that lead to the induction of transcript cleavage and readthrough by an arrested polymerase. Alternatively, the linker may determine the proper orientation for domain II and domain III.
interactions with polymerase. The linker is not highly conserved between human and yeast TFIIS (only 20% identity) suggesting that the interactions and/or orientation might be unique between the two species.
Materials and Methods

*Construction of human/yeast TFIIS mutants* The chimeric human/yeast TFIIS sequences were made by using the appropriate oligos and standard PCR methods (Table I) to amplify the desired fragments (Sambrook et al., 1989). The PCR products were then phosphorylated with polynucleotide kinase and digested with NdeI and BamHI (as described in Chapter Two). pKC16 is derived from pRS15, and it contains the upstream and downstream sequences flanking the genomic TFIIS ORF (Sikorski and Hieter, 1989; Christie, 1995). The PCR products were then ligated into pKC16 that had been also digested with NdeI and BamHI. Specifically (Table II): ΔyTFIIS was amplified with oligos NdeI131SII and BamHI309SII using pKC16(1-309) as a template and ligated into pKC16 creating pNS-ΔyTFIIS. yII/hIII was made from fragments yII (oligos NdeI131SII and yP240 with pET15bHMKΔwt as a template) and hIII (oligos hL233 and hBamHI301 with HMKpETΔhTFIIS as a template) which were ligated into pKC16 creating pNS-yII/hIII. hII/yIII was made from fragments hII (oligos hNdeI131 and hE232 with HMKpETΔhTFIIS as a template) and yIII (oligos yL241 and BamHI309SII with pET15bHMKΔwt as a template) which were ligated into pKC16 creating pNS-hII/yIII. yII*/yZn was made from fragments yII* (oligos NdeI131SII and hT262 with pET15bHMKyII/hIII as a template) and yZn (oligos yC271 and BamHI309SII with pET15bHMKhII/yIII as a template) which were ligated into pKC16 creating pNS-yII*/yZn. hII+/hZn was made from fragments hII+ (oligos hNdeI131 and yT270 with pET15bHMKhII/yIII as a template) and hZn (oligos hC263 and hBamHI301 with pET15bHMKyII/hIII as a template) which were ligated into pKC16 creating pNS-hII+/hZn. yT270/hZn was made from fragments yT270 (oligos yT270 and NdeI131SII with pNS-ΔyTFIIS as a template) and hZn (oligos hC263 and hBamHI301 with pET15bHMKyII/hIII as a template) which were ligated into pKC16 creating pNS-yT270/hZn. hT262/yZn was made from fragments hT262 (oligos hT262 and hNdeI131
with pET15bHMKΔhTFIIS as a template) and yZn (oligos yC271 and BamHI309SI with pET15bHMKhII/yIII as a template) which were ligated into pKC16 creating pNS-hT262/yZn. ΔhTFIIS was made with fragments hII (oligos hNdeI131 and hE232 with HMKpETΔhTFIIS as a template) and hIII (oligos hL233 and hBamHI301 with HMKpETΔhTFIIS as a template) which were ligated into pKC16 creating pNS-ΔhTFIIS.

Purification of mutant proteins Expression constructs of mutant TFIIS proteins were made as follows. Mutant TFIIS open reading frames were cut out of the pKC16 based plasmids with BamHI and NdeI and ligated into pET15b-HMK which had been similarly digested. Expression of these new open reading frames included an amino-terminal His6 tag and heart myosin kinase site (Wu et al. 1996 JBC). Overexpression and purification of mutant proteins was as described in Chapter Two.

Yeast Transformations The method for low efficiency yeast transformation was as described in Chapter Two.

Growth Assay with 6-azauracil Growth on 6-azauracil was carried out as described in Chapter Two.

Readthrough Assay with yeast RNA polymerase II In vitro transcription was performed as described in Chapter Two.

Readthrough Assay with calf thymus RNA polymerase II In vitro transcription was performed as described in (Cipres-Palacin and Kane, 1994) with the following modifications. The template was pGemTerm that contained the histone H3.3 arrest sites (Christie et al., 1994). The transcription buffer used was 70mM Tris HCl pH 8.0, 150mM NH4Cl, 20% (vol/vol) glycerol, 6mM MgCl2, 5mM spermidine, and 0.15mM DTT (Kerppola and Kane, 1988). Purified polymerase was incubated with a dC-tailed template, ATP, UTP, GTP, and [α-32P] CTP in order to initiate transcription and label the RNA proximal to the 5' end. Then high concentrations of all four nucleotides were added ("chase" buffer) to allow the polymerase elongate along the template and form an arrested complex. Each reaction was incubated with TFIIS storage buffer (50mM Tris pH7.9, 10%
glycerol, 50mM NaCl, 1mM DTT) or TFIIS or mutants at 5:1, 100:1, and 500:1 TFIIS:polymerase molar ratios at 30°C. Timepoints were taken at 5, 10, and 30 minutes. Each aliquot was treated as described (Christie et al., 1994) and resolved on a 6% acrylamide 7M urea gel.

Cleavage Assay with calf thymus RNA polymerase II The cleavage assay was performed as described (Cipres-Palacin and Kane, 1994) with the following modifications. The H3.3 template was the same as for the readthrough assays. The transcription buffer used was 70mM Tris HCl pH 8.0, 150mM NH₄Cl, 20% (vol/vol) glycerol, 6mM MgCl₂, 5mM spermidine, and 0.15mM DTT (Kerppola and Kane, 1988). Arrested complexes were passed through two Biospin 30 columns (BioRad) equilibrated with chase buffer (minus nucleotides) as described in Chapter Two. Then the transcription complexes were incubated with TFIIS storage buffer (50mM Tris pH 7.9, 10% glycerol, 50mM NaCl, 1mM DTT), or with wildtype or mutant TFIIS protein at 5:1, 100:1, or, 500:1 TFIIS:polymerase molar ratios. Timepoints were taken at 1, 5, and 10 minutes. An additional aliquot was incubated with 1mM ATP, UTP, GTP, and CTP for 10 minutes at 30°C and denoted as the "chase". All samples were stopped as described (Christie et al., 1994) and resolved on 6% acrylamide 7M urea gels.

Competition assay TFIIS was labeled as described (Awrey et al., 1998) with the following modifications. As described in Chapter Two, after the kinase reaction to label TFIIS, free [γ-32P] ATP was removed from the reaction by passing the mix over 3 Biospin 6 (BioRad) columns equilibrated with 20mM Hepes pH 7.5, 10% glycerol, 100mM NaCl, 0.01% NP-40, 1mM DTT.

The competition assay was performed as follows. RNA polymerase II was desalted with a Biospin 30 micro column (BioRad) equilibrated with 50mM Tris pH 8, 10% glycerol, 50mM NaCl, 2mM DTT as described in Chapter Two. To form the labeled TFIIS (TFIIS*)/polymerase complex: 0.17µM polymerase, 0.17µM TFIIS*, and 17mM DTT in 20mM TrisOAc pH 7.5, 10% glycerol were incubated on ice for 15 minutes. Then
3µl of binding reaction was added to 2µl of 12.5pmol/µl mutant or TFIIIS storage buffer (50mM Tris pH 7.9, 10% glycerol, 50 mM NaCl, 1mM DTT) with an equivalent amount of BSA as a negative control. The final concentrations of proteins were 0.10µM polymerase, 0.10µM TFIIIS*, and 5.0µM mutant TFIIIS. This mixture was incubated for an additional 20 minutes on ice. The complexes were resolved on 5% acrylamide, 1% glycerol, 50mM TrisBorate pH 8.3 (at 4°C) gels with 50mM TrisBorate pH 8.3 (at 4°C), 1mM DTT running buffer. Gels were electrophoresed at 100V for two hours, and the position of TFIIIS* was determined by phosphorimager and autoradiography.
Results

In order to identify the region of TFIIS that determines the functional species distinction between yeast and mammalian RNA polymerase II, domain swaps of human and yeast TFIIS proteins were made. The resulting chimeras were tested for their ability to function in vivo in yeast, to stimulate yeast or mammalian RNA polymerase in vitro, and to bind to yeast or mammalian RNA polymerase in vitro. The mutants are described in Table III. Previous work with alanine substituted mutants determined that the region of TFIIS that interacted with RNA polymerase II was domain II (residues 135-209 in yeast) (this work; Awrey et al., 1998). Therefore, it was originally hypothesized that domain II of TFIIS was the primary if not sole determinant of species specificity.

The first two human/yeast TFIIS chimeras tested were yII/hIII and hII/yIII. Mutant yII/hIII consisted of residues 131-240 of yeast TFIIS (encompassing structural domain II and denoted "yII") fused to human sequences (residues 233-301, denoted "hIII") homologous to the carboxy terminal portion of yeast TFIIS (encompassing the linker and domain III) (Table III). Chimera hII/yIII was made of residues 131-232 of human TFIIS (denoted "hII") and carboxy terminal region of yeast TFIIS (amino acids 241-309, denoted "yIII"). As controls, ΔyTFIIS (yII/yIII) and ΔhTFIIS (hII/hIII) were constructed. If the ability to bind to the polymerase through domain II was solely what determined specificity, the expectation was that yII/hIII would be able to function in yeast in vivo to complement the drug sensitivity of a ppr2Δ yeast strain, and stimulate yeast RNA polymerase II in vitro. The opposite would be true for hII/yIII. However, these expectations were not observed.

Complementation in a ppr2Δ Yeast Strain

The species chimeric mutants were tested for their ability to rescue the 6-azauracil sensitivity phenotype of a ppr2Δ yeast strain (Fig. 1). As expected, the yII/yIII construct
(i.e., wildtype ΔyTFIIS) allowed the cells to grow on 60 and 100 μg/ml 6-azauracil comparable to that of the wildtype strain. The strain containing a plasmid coding for hII/hIII (i.e., wildtype ΔhTFIIS) remained sensitive to the drug. However, mutant hII/yIII and not mutant yII/hIII was also able to complement in vivo. From this result, it appeared that the region encompassed by amino acids 131-240 in yeast TFIIS (and 131-232 in human TFIIS) was not the determinant of species specificity, but instead, it was amino acids 241-309 in yeast TFIIS.

Consistent with this observation, a report published by Shimoaraiso, et al. concluded that residues 132 to 270 in yeast TFIIS and residues 132 to 262 in mouse TFIIS conferred specificity (Shimoaraiso et al., 1997). However, these authors concluded that RNA polymerase binding was the primary species specificity determinant (Shimoaraiso et al., 1997). I hypothesized that the overlapping region, amino acids 240-270 in yeast TFIIS, most likely was the region that conferred species specificity. This region corresponded to the flexible linker that tethered domain II and domain III of TFIIS (Olmsted et al., 1998).

In order to test this hypothesis, additional chimeras were constructed (Table III). Chimera yII*/yZn consisted of yeast domain II, human linker region (the putative species specificity region, denoted "*"), and yeast domain III that contains the Zn ribbon motif (denoted "yZn"). Chimera hII+/hZn consisted of human domain II, yeast linker region (denoted "+"), and human Zn ribbon domain III (denoted "hZn"). As controls, yT270/hZn and hT262/yZn were constructed. Chimera yT270/hZn was yeast domain II and yeast linker (residues 131-270, denoted "yT270") fused to the human domain III. Mutant hT262/yZn was human domain II and human linker (residues 131-262, denoted "hT262") fused to the yeast domain III.

If the linker region truly determined species specificity, mutant yII*/yZn would not be expected to complement in vivo or stimulate yeast polymerase in vitro. However, yII*/yZn should stimulate calf thymus polymerase in vitro. For hII+/hZn, it would be
predicted that hII+/hZn would be able to complement in vivo and that it would stimulate yeast RNA polymerase II rather than mammalian RNA polymerase II. YT270/hZn and hT262/yZn were constructed to confirm that the highly conserved domain III of the proteins was essentially interchangeable (Table III).

I first tested for the ability of the chimeras to complement the TFIIS disruption strain in vivo. As expected, domain III from either human or yeast TFIIS was interchangeable (Fig. 1) (Shimoaraiso et al., 1997). Mutant YT270/hZn was able to suppress the 6-azauracil sensitivity phenotype of a ppr2Δ yeast strain as well as wildtype ΔyTFIIS, yII/yIII (Fig. 1). Meanwhile hT262/yZn behaved similarly to that of wildtype ΔhTFIIS, hII/hIII, and was unable to complement in vivo (Fig. 1). This was not surprising based on the extremely high homology between the two proteins in this region of TFIIS (70% amino acid identity) and the similar result reported by Natori’s group with yeast and mouse TFIIS proteins (see Chapter One, Fig. 3) (Shimoaraiso et al., 1997). As predicted, mutant yII*/yZn that possessed the mammalian linker region did not rescue (Fig. 1). However despite expectations, chimera hII+/hZn that contained the yeast linker, hypothesized to confer specificity, also did not rescue (Fig. 1). These results indicated that although the linker region might be essential for species specificity, it was not sufficient.

**Readthrough Assays with Yeast RNA Polymerase II**

Work with the site directed mutants had suggested that the in vitro assays were quite sensitive and could more readily distinguish between mutants’ activities than the in vivo drug sensitivity assay. Thus, the chimeras were tested for their ability to stimulate either yeast or mammalian RNA polymerase II to cleave the nascent transcript and read through a block to elongation. Promoter independent transcription was performed with a dC-tailed template containing sequences of the human histone H3.3 gene with well characterized blocks to elongation (Kadesch and Chamberlin, 1982; Reines et al., 1987; Kerppola and Kane, 1990).
If linker residues 240-270 in yeast TFIIS contribute species specificity, then proteins ΔyTFIIS, hII/yIII, hII+/hZn, and yT270/hZn should be able to stimulate yeast RNA polymerase II to read through a block to elongation. As can be seen in figure 2 and as previously reported, wildtype ΔyTFIIS readily stimulates readthrough by yeast RNA polymerase II (Christie et al., 1994). When ΔyTFIIS was present, there was a noticeable decrease in RNA representing polymerases arrested at the T1A site and an increase in the production of full length run off at the lowest TFIIS:polymerase ratio tested of 5:1 (Fig. 2). As expected, mutant hII/yIII that complemented in vivo also was able to stimulate yeast polymerase, albeit not as well as wildtype ΔyTFIIS even at 100-fold excess over polymerase (Fig. 3). hII/yIII had only about 0.23 the stimulatory activity of wildtype TFIIS at 100-fold molar excess over polymerase and about 0.83 the stimulatory activity of wildtype at 500-fold excess over polymerase. Mutant hII+/hZn, which was entirely human TFIIS sequence except for the theoretical specificity region, was able to facilitate detectable readthrough of the yeast polymerase at 500:1 molar excess over polymerase (Fig. 4). Even at 500-fold excess over polymerase, hII+/hZn had less stimulatory activity (approximately 79%) than wildtype TFIIS. Although this mutant was not able to rescue the 6-azauracil sensitivity phenotype of a ppr2Δ yeast strain, it is possible that the in vivo assay requires a threshold of function that this mutant did not possess. It would be interesting to see whether a high copy number plasmid coding for hII+/hZn would result in complementation of a ppr2Δ strain. Mutant yT270/hZn, which was able to complement in vivo, was able to promote readthrough by yeast RNA polymerase II almost to the extent of that of wildtype ΔyTFIIS (88% the stimulatory activity of wildtype TFIIS at 100:1 molar excess of TFIIS:polymerase) (Fig. 5), again confirming that the highly conserved domain III of the human and the yeast TFIIS proteins was functionally interchangeable.

TFIIS proteins ΔhTFIIS, yII/hIII, yII*/yZn, hT262/yZn could not carry out in vivo complementation. Therefore, they are not expected to be able to stimulate yeast polymerase in vitro. This prediction held for all except yII*/yZn (Figs. 3-6). For this chimera, at 500:1
molar excess over polymerase, there was a slight but measurable amount of readthrough stimulation (Fig. 4). It is possible that the activity is more a reflection of calculated error rather than actual stimulation. The results from the cleavage assays lead to this conclusion and will be discussed further in the next section.

**Cleavage Assays with Yeast RNA Polymerase II**

It has been demonstrated that transcription cleavage by RNA polymerase II is a necessary prerequisite for readthrough of blocks to elongation (Reines et al., 1992; Izban and Luse, 1993). In addition, Cipres-Palacin and Kane showed that although cleavage was necessary for readthrough, it was not sufficient (Cipres-Palacin and Kane, 1994). Therefore, it was expected that the effects of using chimeric mutants to stimulate cleavage with yeast polymerase would parallel effects observed with the readthrough assays. Thus, wildtype ΔyTFIIS, hII/yIII, hII+/hZn, and yT270/hZn were predicted to stimulate transcript cleavage by yeast RNA polymerase II. The cleavage assays were performed under the same conditions as the readthrough assays except that unincorporated nucleotides were removed from the arrested elongation complexes before incubation with the TFIIS proteins. As a result, any complexes that were stimulated to cleave the nascent RNA would not be able to re-extend the transcript. The positive control, wildtype ΔyTFIIS, efficiently promoted cleavage by the arrested complex as can be seen by the shortening of the RNA over time (Fig. 7 compare lanes 1-4 with lanes 6-9). Chimera hII/yIII that was able to promote readthrough by yeast RNA polymerase II also stimulated transcript cleavage by yeast polymerase (Fig. 8). Chimera hII+/hZn was able to promote transcript cleavage by yeast RNA polymerase II although not as well as that of wildtype (Fig. 9). And in concert with the results of the readthrough assays, yT270/hZn also stimulated cleavage (Fig. 10). It should be noted that the transcript cleavage pattern of yeast RNA polymerase II induced by the chimeric TFIIS proteins is that same as the pattern seen with wildtype ΔyTFIIS.
Those TFIIS proteins that did not have the putative yeast RNA polymerase II species specificity region (ΔhTFIIS, yII/hIII, yII*/yZn, and hT262/yZn) did not promote readthrough and were not predicted to stimulate transcript cleavage by yeast RNA polymerase II. As expected, no evidence of transcript cleavage was detected in reactions with ΔhTFIIS (Fig. 11). Mutant yII/hIII which did not complement in vivo and did not stimulate readthrough, also did not facilitate transcript cleavage even at the highest molar ratio of factor to polymerase of 500:1 (Fig. 12). No production of cleavage products was observed upon incubation of arrested ternary complexes with mutant yII*/yZn which did not rescue in vivo, but did very slightly stimulate readthrough by yeast RNA polymerase II (Fig. 13). Because the cleavage assay is more sensitive than that of the readthrough assay and cleavage is required before readthrough, the apparent contradiction of detecting readthrough but not cleavage stimulation most likely was due to the propagation of error in the calculation of readthrough rather than actual stimulatory activity by the protein. In agreement with the results of the readthrough assay with yeast polymerase, chimera hT262/yZn did not induce any detectable transcript cleavage activity by yeast RNA polymerase II (Fig. 14).

Taken together the results of the readthrough and cleavage assays with yeast RNA polymerase II suggest that the region, encompassed by residues 240-270 in yeast TFIIS, is necessary to confer species specificity. In addition, with one exception, all of the chimeras that contained residues 240-270 of yeast TFIIS were able to complement a ppr2Δ yeast strain. It is possible that the minimal stimulatory activity observed in vitro with hII+/hZn is not sufficient to suppress 6-azauracil sensitivity in vivo.

**Competition Assays with Yeast RNA Polymerase II**

Competition assays were performed with the mutants and wildtype TFIIS to test for the ability of the chimeras to bind yeast RNA polymerase II. In these experiments, wildtype ΔyTFIIS, was radioactively labeled and incubated with yeast polymerase at a 1:1
ratio in order to form complexes. Then the complexes were incubated with a 50-fold molar excess of cold competitor wildtype or mutant TFIIS protein. The reaction mix was then loaded onto a native gel and resolved under conditions such that free TFIIS would not enter the gel, but the TFIIS:polymerase complex would (Wu et al., 1996). If the mutant protein were able to compete with wildtype ΔyTFIIS, then it would be expected that the amount of labeled, wildtype ΔyTFIIS*/yeast RNA polymerase II complex detected would be less than the unchallenged control. As can be seen in figure 15, wildtype ΔyTFIIS readily competed with labeled ΔyTFIIS* (compare lane 2 with lane 3). Not surprisingly, any mutant that contained yeast TFIIS domain II, the polymerase binding region, was able to compete (Fig. 15). This included mutants, yII/hIII and yIII*/yZn, which were not able to complement in vivo or stimulate yeast RNA polymerase II in vitro suggesting that simply binding to polymerase is not sufficient for function. At 100-fold excess, even hII/yIII can compete partially with wildtype yeast TFIIS for binding to yeast polymerase (Fig. 15). Approximately 60% of the original ΔyTFIIS*/polymerase complexes remained after incubation with 100-fold excess of hII/yIII. Within the levels of detection of this assay, hII+/hZn was not seen to compete with wildtype ΔyTFIIS* (Fig. 15). This result may explain why 500-fold excess of hII+/hZn over yeast RNA polymerase II was needed to see promotion of readthrough and transcript cleavage in vitro and why no complementation was observed in vivo.

Readthrough and Cleavage Assays with Mammalian RNA Polymerase II

The results of the in vitro transcription assays with yeast RNA polymerase II would indicate that residues 240-270 of yeast TFIIS are involved in determining species specificity (summarized in Table III). Reciprocal assays were also carried out with mammalian RNA polymerase II, and the results suggest that the species specificity determinants may be more complex. If residues 240-270 of yeast TFIIS contributed to species specificity, then the same would be expected of the homologous residues, 232-262,
in human TFIIS. *In vitro* readthrough assays with the chimeric mutants and calf thymus RNA polymerase II did not confirm this expectation (summarized in Table III). Wildtype human TFIIS promoted readthrough by mammalian RNA polymerase II, as expected (Fig. 16) (Reines et al., 1989; SivaRaman et al., 1990; Cipres-Palacin and Kane, 1994; Cipres-Palacin and Kane, 1995). However, no stimulation of readthrough was detected when calf thymus polymerase was incubated with ΔyTFIIS, even at a 500-fold molar excess (Fig. 17). Chimeras that did not have the putative specificity region of human TFIIS (hII/yIII, hII+/hZn, and yT270/hZn) did not promote readthrough by mammalian polymerase (Figs. 18&19). Those mutants that contained the human equivalent of the TFIIS linker, residues 232-262 of human TFIIS (yII/hIII, yII*/yZn, and hT262/yZn) might have been predicted to have stimulatory activity with the mammalian polymerase in analogy to results presented above. However, only hT262/yZn had detectable stimulation of readthrough by calf thymus polymerase and only at the highest molar excess of factor over polymerase, 500:1 (Figs. 18&20). With the more sensitive cleavage assay, the same results were seen. Only wildtype ΔhTFIIS and hT262/yZn were able to facilitate cleavage by the mammalian polymerase (compare Figs. 21&22 to Figs. 23-28; summarized in Table III). These results would indicate that both the polymerase binding domain (encompassed by residues 131-232 in human TFIIS) and the putative specificity region (amino acids 232-262 in human TFIIS) are needed for mammalian polymerase to functionally interact with TFIIS. It is also possible that the flexible linker can position regions II and III in the correct orientation at a very low frequency captured only when the equilibrium of the reaction is driven by the high concentration present in the *in vitro* reaction. Alternatively, and not readily testable, those TFIIS proteins with no or very limited detectable activity may be less stable during purification and inactive when added to these reactions.
Competition Assays with Mammalian RNA Polymerase II

Competition assays were also done with calf thymus RNA polymerase II and the TFIIS proteins. In this case, ΔhTFIIS was labeled and complexed with the mammalian polymerase (Fig. 29). As with the previous experiments with ΔyTFIIS and yeast polymerase, only those mutants possessing domain II (including those that could not stimulate mammalian RNA polymerase II), this time from human TFIIS, were able to compete (Fig. 29). These results support the previous observations with yeast RNA polymerase II that binding of TFIIS to polymerase is not the sole determinant of function. It is notable that the two proteins (ΔhTFIIS and hT262/yZn) that were able to stimulate mammalian RNA polymerase II in the transcription assays were most able to compete with ΔhTFIIS* for binding to the polymerase (Fig. 29, compare lanes 3&9 to lanes 5&8).

The ΔhTFIIS*/mammalian RNA polymerase II complexes appear more heterogeneous than the yeast complexes possibly due to the presence of multiple forms of the carboxy terminal domain (CTD) of the largest subunit of calf thymus polymerase (Dahmus, 1996). The purified fraction of mammalian polymerase consists of the hyperphosphorylated, hypophosphorylated, and proteolyzed forms of the CTD which all have unique electrophoretic mobilities. The yeast polymerase is more homogeneous than the mammalian polymerase because the yeast polymerase is purified based on the presence of its CTD, but calf thymus polymerase is not.

The results of the in vitro assays with yeast RNA polymerase II indicate that the linker region (residues 240-270 of yeast TFIIS) is necessary to confer species specificity although full function requires other determinants that apparently are also species specific, such as domain II. However, work with mammalian polymerase suggests that both domain II and the linker are required for any functional interaction in mammals (this work, Shimoaraiso et al., 1997). There are several possible explanations for the disparity in results between the yeast and the mammalian polymerases. One could be a trivial one in that the in vitro assays were more sensitive with the yeast polymerase than with the calf
thymus polymerase due to the more robust transcription seen with the yeast protein. It is formally possible that the little stimulation of cleavage and readthrough by the chimeras of TFIIS with calf thymus polymerase was below the experimental threshold of detection. However, transcription reactions with the calf thymus polymerase were increased in volume considerably in order to increase signal to noise and to compensate for the lower number of active molecules present.

Another explanation could be that the recombinant chimeric proteins (yII*/yZn and yII/hIII) that were expected to stimulate mammalian polymerase were improperly folded and therefore were inactive. However, yII*/yZn had partial function with yeast polymerase, indicating that at least some molecules folded properly. In addition, NMR structural analysis of domain III of human TFIIS, domain II of yeast TFIIS, and the minimal active truncated yeast TFIIS (consisting of domain II, linker region, and domain III) demonstrated that the individual domains could fold accurately and independently of each other (Qian et al., 1993; Morin et al., 1996; Olmsted et al., 1998).

A more interesting reason could be that yeast polymerase is less stringent than the mammalian polymerases in what constitutes a functional interaction with TFIIS. Although the TFIIS proteins from *S. cerevisiae* to HeLa are well conserved, TFIIS proteins from metazoans are more homologous to each other than to TFIIS from yeast (Chen et al., 1992) with most of the amino acid identity occurring in the Zn ribbon motif of domain III (see Chapter One, Fig. 3). The TFIIS proteins from *D. melanogaster*, mouse, and human share much more identity with each other and are interchangeable in their ability to stimulate the RNA polymerase II of each species (Kane, unpublished results; Sawadogo et al., 1980; Shimoaraiso et al., 1997). Since only hT262/yZn was able to promote readthrough and cleavage by calf thymus polymerase, and yII*/yZn was not, perhaps there are residues in TFIIS human domain II (and *Drosophila* and mouse domain II, too) that must interact with mammalian polymerase that do not exist in TFIIS yeast domain II. Furthermore, alanine scanning mutagenesis studies with human TFIIS by Cipres-Palacin and Kane have revealed
several charged residues within domain II that are important for function (Cipres-Palacin and Kane, 1995). Taking those residues identified as critical in the human TFIIS that are also conserved in mouse and *Drosophila* proteins but not in yeast TFIIS domain II, there are only nine amino acids within domain II that meet these criteria. If these residues are involved in interacting with mammalian polymerase and were changed accordingly in chimera yII*/yZn, the resultant mutant might be expected to now stimulate calf thymus RNA polymerase II to cleave and read through.
Discussion

It was the purpose of this study to identify the portion of TFIIS that was the determinant of species specificity. TFIIS from *Saccharomyces cerevisiae* is unable to stimulate transcript cleavage and readthrough by mammalian RNA polymerase II, and human TFIIS is unable to complement a *ppr2Δ* yeast strain (this work; Kane, unpublished results; Sawadogo et al., 1980; Shimoaraiso et al., 1997). Structurally, TFIIS consists of three independently folding domains (see Chapter One, Fig. 2) (Agarwal et al., 1991; Morin et al., 1996; Olmsted et al., 1998).

Domain swaps between human and yeast TFIIS proteins have indicated that the region from residues 240-270 in yeast TFIIS was involved in species specificity. Domain II, amino acids 131-240 in yeast, although responsible for binding to polymerase could not on its own cause a switch in specificity because mutants yII/hIII and yII*/yZn were unable to stimulate yeast RNA polymerase II and could not complement a disruption strain for TFIIS *in vivo*. Assays with chimeric mutants competing with wildtype TFIIS bound to polymerase showed that in order to compete with binding the chimera only needed to have the species specific domain II that corresponded its cognate polymerase. This result was true whether or not the mutant was actually able to stimulate the polymerase in cleavage and readthrough.

The Zn ribbon motif in domain III, residues 270-309 in yeast, was also ruled out as a distinguishing between species because the Zn ribbon motif from the two proteins was found to be essentially interchangeable. This result was not unexpected due to the fact that there is 70% identity between these TFIIS proteins in this area.

Mutant hII+/hZn, which contained human TFIIS sequence except for residues 240-270 of yeast TFIIS was able to stimulate yeast RNA polymerase II although a high molar ratio of chimera to polymerase was required. Clearly this portion of TFIIS is involved in species specificity determination. However, this chimera was not able to rescue the 6-
azauracil sensitivity phenotype in vivo in a ppr2Δ yeast strain. This may be due to the
mutant sequence being on a low copy number plasmid and not being expressed at a high
enough dosage in vivo to reach a threshold of activity that would result in
complementation. Based on the NMR structure, amino acids 240-270 span the linker
region, 240-260, and the first ten residues before the beginning of the first β-sheet of
domain III (Olmsted et al., 1998).

The species specificity and site directed mutagenesis studies from this project as
well as mutagenesis studies from others have provided much information about the
function of TFIIS (Agarwal et al., 1991; Cipres-Palacin and Kane, 1994; Jeon et al., 1994;
Cipres-Palacin and Kane, 1995; Nakanishi et al., 1995; Awrey et al., 1998). This
knowledge in combination with the recently reported NMR structure of minimal active
region of yeast TFIIS provides a picture of how TFIIS interacts with RNA polymerase II in
order to stimulate transcript cleavage and readthrough of the polymerase through a block to
elongation.

Binding by TFIIS to polymerase is necessary for stimulation. Alanine substituted
mutants in structural domain II, specifically residues K196A, R198A, R200A, and
K209A, were defective in binding to the polymerase and required much higher
concentrations of protein to promote readthrough of polymerase as compared to that of
wildtype TFIIS (this work; Awrey, et al. 1998). Multiple mutations of residues 196, 198,
and 200 resulted in proteins that were so defective that not only could they no longer
compete with wildtype TFIIS in binding to polymerase, but also they could no longer
complement in vivo, nor stimulate polymerase to cleave its transcript and read through
transcription blocks even at 500-fold excess over polymerase (this project).

The NMR structure indicates that these three residues constitute a basic patch on the
outer face on the third helix of the three helix bundle of domain II (Awrey et al., 1998).
TFIIS likely interacts with at least the largest subunit of RNA polymerase II. There are
mutants in the largest subunit that are 6-azauracil sensitive and overexpression of TFIIS
results in the loss of this sensitivity (Archambault et al., 1992). In addition, two of the mutant polymerases were purified (rpo21-18 and rpo21-24), and it was found that they had wildtype transcription elongation properties, but that they had 50-fold reduced binding to TFIIS (Wu et al., 1996). Because one of the polymerase point mutants was an acidic residue changed to a basic one (E1230K) and since it was observed that the interaction between polymerase and TFIIS was sensitive to high ionic strength, it was proposed that TFIIS binds through its basic patch on domain II to polymerase at this acidic residue (Sopta et al., 1985; Archambault et al., 1992; Awrey et al., 1998).

As discussed previously in Chapter Two, the interactions of TFIIS functional homologs, GreA and GreB, with bacterial RNA polymerase may shed some light on TFIIS interactions with eukaryotic RNA polymerase II. There are several parallels between TFIIS and the Gre factors. (1) They can promote transcript cleavage and readthrough (Kassavetis and Geiduschek, 1993; Reines, 1994; Uptain et al., 1997). (2) The crystal structure of GreA reveals that GreA has a basic patch (Stebbins et al., 1995). (3) A point near this basic region of GreA crosslinks close to the 3'end of the transcript (Koulich et al., 1997). Crosslinking studies also have placed TFIIS close to the catalytic site of an elongation complex (Powell et al., 1996). (4) GreA consists of two domains linked by a 12 amino acid loop (Stebbins et al., 1995). Work with individual, recombinant GreA domains demonstrated that the domains were incapable of facilitating readthrough when added in trans (Koulich et al., 1998). The authors hypothesized that the orientation of the two domains with respect to each other in the native protein likely contributed to the function of the protein (Koulich et al., 1998).

The stimulation of readthrough of polymerase by TFIIS is most likely more than a one step process of simply binding to an arrested polymerase. Work with the human/yeast TFIIS chimeras demonstrated that the mutants only needed to possess yeast domain II to compete with wildtype yeast TFIIS for binding to yeast polymerase regardless of the ability or inability of the mutant to promote readthrough by yeast polymerase (this work).
Additionally, work with a Δrbp9 polymerase showed that although the mutant polymerase could bind TFIIS like the wildtype polymerase, the Δrbp9 polymerase responded very poorly to TFIIS again indicating that binding is not sufficient (Awrey et al., 1997).

Once TFIIS binds to an arrested polymerase, it is possible to speculate that another interaction needs to occur between the two proteins. TFIIS binds through domain II to RNA polymerase II. Then the linker region would help to orient domain III, which is critical for TFIIS function, and establish key interactions between polymerase and domain III. In this model, the polymerase would be induced to undergo a conformational change from one that was elongation incompetent to one that is now elongation competent. The importance of the linker region of TFIIS is highlighted by the result with chimeric mutant, hII+/hZn, which only requires the yeast linker in order to be able to stimulate yeast polymerase (this work). Additionally, when the two domains are added in trans or when the linker is replaced with an eight amino acid random coil no activity is seen (Agarwal et al., 1991; Awrey et al., 1998). One way to interpret these results would be to conclude that the linker serves to properly orient domain III of TFIIS to interact with polymerase once TFIIS has bound to polymerase through domain II (Olmsted et al., 1998). Alternatively, there may be critical contacts that must be made between the linker of TFIIS and polymerase that results in facilitation of readthrough by polymerase.
References


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Table II Plasmids

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183
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<th>Binding with Yeast RNA Polymerase II</th>
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# TFIIS:PolII molar ratio
Figure Legends

Fig. 1  **Testing for the ability of the mutant TFIIS proteins to suppress the 6-azauracil sensitivity phenotype of a ppr2Δ yeast strain.**

Wildtype (CH1305) or ppr2Δ (CMKY5) yeast strains were transformed with marker plasmids (pRS314 or pRS316) or in the case of CMKY5, with plasmids encoding for wildtype and TFIIS mutants. The yeast were plated on SC -ura -trp medium with or without 100μg/ml drug and grown for three days at 30°C. Both the wildtype strain and the ppr2Δ strain with a plasmid encoding for TFIIS, pNS-ΔyTFIIS, are less sensitive to 6-azauracil than the ppr2Δ strain (CMKY5+pRS314). Mutants hII/yIII and yT270/hZn were able to suppress the drug sensitivity phenotype, but yII/hIII, ΔhTFIIS, yII*/yZn, hII+/hZn, and hT262/yZn could not.
### Figure 1

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**no drug**  

**100 µg/ml**
Fig. 2  ΔyTFIIS stimulates yeast RNA polymerase II to read through a block to elongation. Arrested yeast RNA polymerase II complexes are formed (lane 1) and incubated with either TFIIS storage buffer ("buffer") or ΔyTFIIS at 5:1, 100:1, or 500:1 TFIIS:polymerase molar ratios. After 30 minutes incubation with 5-fold excess of ΔyTFIIS, stimulation of readthrough is indicated by the increase in full length transcripts produced (compare lanes 4 & 7).
Fig. 3 Chimera yII/hIII is unable to promote readthrough by yeast RNA polymerase II, but mutant hII/yIII is able to stimulate readthrough of yeast polymerase. Even after 30 minutes of incubation with 500-fold excess of yII/hIII over yeast polymerase, no stimulation of readthrough is detected (compare lanes 7-9 with lanes 2-4 of Fig. 2). However, hII/yIII promotes readthrough at 100-fold excess over yeast RNA polymerase II although not to the extent of wildtype ΔyTFIIS (compare lanes 13-15 with lanes 8-10 of Fig. 2).
Fig. 4 Mutant hII+/hZn with the yeast TFIIS linker region has stimulatory activity with yeast RNA polymerase II, but mutant yII+/yZn with the human TFIIS linker region has little if any stimulatory activity.

After 30 minutes of incubation with 500-fold molar excess of hII+/hZn over yeast RNA polymerase II, a small amount of readthrough is detected (lane 9).
Figure 4

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</tr>
<tr>
<td>500:1</td>
<td>5 10 30</td>
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</table>

RO
TIA
TIB
TII
Fig. 5  The Zn ribbon motifs of TFIIS proteins are essentially interchangeable.
Mutant hT262/yZn behaves like ΔhTFIIS and is unable to promote readthrough by yeast RNA polymerase II (lanes 1-9). Chimera yT270/hZn, like wildtype ΔyTFIIS, is able to stimulate readthrough by yeast RNA polymerase II (lanes 10-18).
Fig. 6 ΔhTFIIS shows species specificity and has no stimulatory activity with yeast RNA polymerase II.
ΔhTFIIS does not promote readthrough of yeast RNA polymerase II complexes arrested at the T1A site.
Figure 6

<table>
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Legend:
- RO
- TIA
- TIB
- TII

ΔhfIIIS
Fig. 7  ΔyTFIIS stimulates yeast RNA polymerase II complexes arrested at a block to elongation to cleave the nascent transcript.

Transcripts are shortened when yeast polymerase is incubated with ΔyTFIIS but not in the absence of ΔyTFIIS (compare lanes 2-4 with lanes 6-8). Arrows indicate cleaved products.
Figure 7

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<tr>
<td>T1B</td>
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</tr>
<tr>
<td>TII</td>
<td></td>
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</table>
Fig. 8 Chimera, hII/yIII, that contains the yeast TFIIS linker and Zn ribbon motif is able to stimulate transcript cleavage by yeast RNA polymerase II.

Transcripts are cleaved by arrested yeast RNA polymerase II complexes in the presence of hII/yIII, but not to the extent of that seen in the presence of wildtype ΔyTFIIS (compare Fig. 7&8).
Figure 8

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RO

T1A

T1B

TII
Fig. 9 A mutant containing the yeast TFIIS linker region, hII+/hZn, is able to promote transcript cleavage by yeast RNA polymerase II.

Cleavage products are produced after incubation with hII+/hZn.
Figure 9

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RO

T1A

T1B

TII

1 2 3 4 5 6 7 8 9 10 11 12 13
Fig. 10 Chimera YT270/hZn is able to induce transcript cleavage by yeast RNA polymerase II.

Mutant, YT270/hZn, that contains the yeast TFIIS domain II and linker region fused to that human TFIIS Zn ribbon can stimulate yeast RNA polymerase II to cleave its transcript.
Figure 10  yT270/hZn

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RO

T1A

T1B

TII
Fig. 11  ΔhTFIIS is species specific and does not stimulate yeast RNA polymerase II to
cleave its transcript.

No cleavage is detected even when arrested yeast RNA polymerase II complexes are
incubated with a 500-fold excess of ΔhTFIIS over polymerase (compare lanes 2-4 with
lanes 10-12).
## Figure 11

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206
Fig. 12 A chimera containing yeast TFIIS domain II fused to human TFIIS linker and Zn ribbon is unable to stimulate transcript cleavage by yeast RNA polymerase II. Mutant, yII/hIII does not induce transcript cleavage by yeast RNA polymerase II even when incubated at 500-fold excess over polymerase (compare lanes 2-4 with lanes 10-12).
Figure 12

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RO
T1A
T1B
TII

208
Fig. 13  A mutant consisting of all yeast TFIIS sequences except for the human TFIIS linker region cannot promote transcript cleavage by yeast RNA polymerase II. Chimera yII*/yZn does not stimulate yeast RNA polymerase II to cleave its nascent transcript even at the highest molar excess tested of 500:1 TFIIS:polymerase.
Fig. 14 Chimera hT262/yZn that is human TFIIS domain II and linker region fused to the yeast TFIIS Zn ribbon motif is not able to induce transcript cleavage by yeast RNA polymerase II.

Even at 500-fold molar excess over polymerase, no cleavage products are detected when yeast polymerase is incubated with hT262/yZn.
Fig. 15 TFIIS mutants that possess yeast TFIIS domain II are able to compete with wildtype ΔyTFIIS for binding to yeast RNA polymerase II. Lane 2 represents the ΔyTFIIS*/yeast RNA polymerase II complexes before being incubated with 50-fold excess of competitor TFIIS mutant proteins (lanes 3-10) or 100-fold hII/yIII (lane 11). TFIIS proteins that have yeast domain II (lanes 2, 5, 6, and 9) can compete with ΔyTFIIS* for binding to yeast RNA polymerase II. Those chimeras without yeast TFIIS domain II are not able to compete (lanes 4, 7, 8, and 10). However, at 100-fold excess, hII/yIII is able to partially compete with ΔyTFIIS* for binding to yeast RNA polymerase II. The arrow indicates the bottom of the well and the asterisk indicates the ΔyTFIIS*/polymerase complex.
Fig. 16 ΔhTFIIS stimulates readthrough by mammalian RNA polymerase II arrested at a block to elongation. ΔhTFIIS promotes readthrough by calf thymus RNA polymerase II complexes past the T1A site of the human histone H3.3 gene.
Fig. 17 ΔyTFIIS displays species specificity and does not facilitate readthrough by mammalian RNA polymerase II at a block to elongation.

Even when ΔyTFIIS is incubated for 30 minutes at 500-fold excess over calf thymus RNA polymerase II, no stimulation of readthrough is detected.
Figure 17

ΔyTFII S

<table>
<thead>
<tr>
<th>TFII S:ctPol I</th>
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<th>100:1</th>
<th>500:1</th>
</tr>
</thead>
<tbody>
<tr>
<td>time (min)</td>
<td>5 10 30</td>
<td>5 10 30</td>
<td>5 10 30</td>
</tr>
</tbody>
</table>

RO

TIA

TIB

TII
Fig. 18  Neither yII/hIII or hII/yIII is able to promote readthrough by mammalian RNA polymerase II.

Stimulation of readthrough is not observed when either yII/hIII or hII/yIII is incubated at 500-fold excess over calf thymus RNA polymerase II.
Fig. 19 Neither hII+/hZn or yT270/hZn is able to promote readthrough by mammalian RNA polymerase II.

Stimulation of readthrough is not observed when either hII+/hZn or yT270/hZn is incubated at 500-fold excess over calf thymus RNA polymerase II.
Fig. 20 Chimera yII*/yZn does not stimulate readthrough by mammalian RNA polymerase II, but hT262/yZn does.

Mutant hT262/yZn that consists of human TFIIS domain II and linker fused to the yeast TFIIS Zn ribbon motif is able to facilitate readthrough by calf thymus RNA polymerase II although not to as well as wildtype ΔhTFIIS.
Figure 20

<table>
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<th>TIB</th>
<th>TII</th>
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<tr>
<td>1:1</td>
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</table>

hT262/yZn

yII*+/yZn
Fig. 21  ΔhTFIIS induces transcript cleavage by mammalian RNA polymerase II arrested at a block to elongation.

Within the first minute of incubation with ΔhTFIIS, calf thymus RNA polymerase II arrested at the T1A site is stimulated to cleave its nascent transcript (compare lanes 2-4 with lane 6). Arrows indicate cleaved products.
Figure 21

ΔhTFIIS

TFIIS:ctPolII

buffer 100:1 500:1

time (min)

0 1 5 10 C 1 5 10 C 1 5 10 C

RO

T1A

T1B

1 2 3 4 5 6 7 8 9 10 11 12 13
Fig. 22  Mutant hT262/yZn that contains human TFIIIS domain II and linker region fused to the yeast Zn ribbon motif can stimulate mammalian RNA polymerase II to cleave its transcript.

Although not as well as wildtype ΔhTFIIIS, transcript cleavage is detected when calf thymus RNA polymerase II is incubated with hT262/yZn.
Fig. 23  A mutant, hII+/hZn, that is all human TFIIS sequence except for the yeast TFIIS linker region is not able to stimulate mammalian RNA polymerase II.

Even at 500-fold excess over calf thymus RNA polymerase II, yII*/yZn does not induce arrested polymerase to cleave its transcript.
Fig. 24  Chimera yII/hIII does not promote transcript cleavage by mammalian RNA polymerase II.

Even at 500-fold excess over calf thymus RNA polymerase II, yII/hIII does not induce arrested polymerase to cleave its transcript.
Figure 24

<table>
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<tr>
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<td>C 1 5 10</td>
<td>C 1 5 10</td>
</tr>
</tbody>
</table>

RO

T1A

T1B
Fig. 25  **Mutant hII/yIII does not facilitate transcript cleavage by mammalian RNA polymerase II**

Calf thymus RNA polymerase II complexes arrested at the T1A site are not induced to cleave their transcripts by hII/yIII even when incubated at 500-fold excess of TFIIS:polymerase.
Figure 25

<table>
<thead>
<tr>
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234
Fig. 26 Chimera yII*/yZn that is all yeast TFIIS sequences except for the human TFIIS linker region does not stimulate transcript cleavage by mammalian RNA polymerase II. Incubation with 500-fold excess of yII*/yZn was not sufficient to stimulate detectable transcript cleavage by calf thymus RNA polymerase II complexes.
Figure 26  yll*/yZn

<table>
<thead>
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<th>500:1</th>
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<tbody>
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<td>5</td>
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<tr>
<td></td>
<td>C</td>
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<td>RO</td>
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<tr>
<td>T1A</td>
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</tr>
<tr>
<td>T1B</td>
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</tbody>
</table>

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Fig. 27 ΔyTFIIS displays species specificity and does not promote transcript cleavage by mammalian RNA polymerase II arrested at a block to elongation. Even at 500-fold excess over calf thymus RNA polymerase II, ΔyTFIIS does not induce arrested polymerase to cleave its transcript.
Figure 27

<table>
<thead>
<tr>
<th>TFIIS:ctPolII</th>
<th>buffer</th>
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<tbody>
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</table>

| RO            |       |       |       |
| T1A           |       |       |       |
| T1B           |       |       |       |
| TII           |       |       |       |

238
Fig. 28 Chimera yT270/hZn is unable to stimulate transcript cleavage by mammalian RNA polymerase II.

Incubation with 500-fold excess of yT270/hZn was not sufficient to stimulate detectable transcript cleavage by calf thymus RNA polymerase II complexes.
Figure 28  yT270/hZn

<table>
<thead>
<tr>
<th>TFIIIS:ctPolII</th>
<th>buffer</th>
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</thead>
<tbody>
<tr>
<td>time (min)</td>
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<td>1 5 10 C</td>
<td>1 5 10 C</td>
</tr>
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</table>

RO
T1A
T1B
TII
Fig. 29 The chimeras are tested for their ability to compete with wildtype ΔhTFIIS for binding to mammalian RNA polymerase II. Lane 2 represents the ΔhTFIIS*/calf thymus RNA polymerase II complexes before incubation with 50-fold excess of mutant TFIIS proteins. Mutants that possess human TFIIS domain II can compete with wildtype ΔhTFIIS* for binding to polymerase (lanes 3-10). The two TFIIS proteins, ΔhTFIIS and hT262/yZn, that are able to stimulate transcript cleavage and readthrough by mammalian RNA polymerase II compete the best with ΔhTFIIS* for binding to polymerase (lanes 3&9).