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Mechanisms Of Factor Recruitment At Promoters During RNA Polymerase II Transcription

Natalya Yudkovsky

A dissertation submitted in partial fulfillment of the
requirements for the degree of

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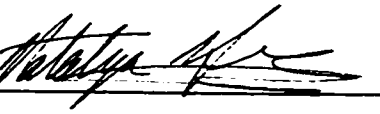
Signature 

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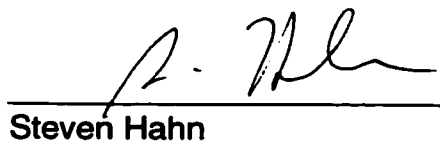

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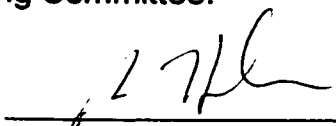

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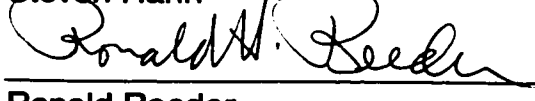

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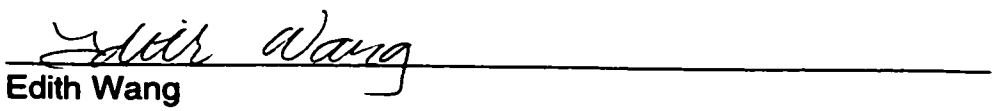

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Abstract

Mechanisms of Factor Recruitment at Promoters During RNA Polymerase II Transcription

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RNA Polymerase II transcription requires the concerted action of a large number of general transcription factors. These factors must assemble at promoters into a preinitiation complex in order to initiate transcription, and must continually reassemble to promote multiple rounds of transcription in a process termed reinitiation. In vivo, the assembly of these factors is frequently blocked by the packaging of DNA into chromatin, a high order protein/DNA structure. Chromatin remodeling factors must therefore be recruited to promoters to allow the transcription machinery to assemble. In this work I have used an in vitro *Saccharomyces cerevisiae* nuclear extract system to determine mechanisms by which chromatin remodeling factors and transcription factors can be recruited to promoters during transcription initiation and reinitiation. I found that some acidic transcription activators can recruit both the Swi/Snf ATP-dependent chromatin remodeling complex and the SAGA histone acetyltransferase chromatin remodeling complex to DNA. Interestingly, this recruitment occurs independently of the rest of the transcription machinery and promoter sequences. These results explain how activators can be used to target chromatin remodeling factors to specific genes. I also determined that, to form a preinitiaiton complex, transcription factors can be recruited in at least three cooperative steps. Importantly,

preinitiation complex formation requires the presence of a large complex of RNA Polymerase II interacting proteins called Mediator. Finally, I isolated a reinitiation intermediate that contains the majority of the general transcription machinery, including Mediator. Since transcription factor recruitment is a rate limiting step in transcription, this result helps to explain why reinitiation occurs more rapidly than initiation. In addition, the reinitiation intermediate is stabilized by some activators, suggesting a new role for activators in stimulating reinitiation. Taken together, this work begins to address how factor assembly is coordinated during RNA Polymerase II transcription.

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GLOSSARY

AA – acidic transcriptional activator

Ada2 – subunit of SAGA, a chromatin remodeling complex

AMPPNP – 5'-adenyl imidodiphoshate, a non-hydrolysable ATP analog

ATP – adenosine triphosphate

ATPγS – adenosine 5'-O-(thio)triphosphate, a hydrolysable ATP analog

bp – base pair

CAK – cyclin activating kinase; kinase/cyclin-containing subcomplex of TFIIH

Ccl1 – cyclin subunit of TFIIH

CDK complex – cyclin dependent kinase subcomplex of Mediator

CTD – carboxy-terminal domain of Rpb1, the large subunit of RNA Pol II

DNA – deoxyribonucleic acid

Gcn5 – HAT subunit of SAGA, a chromatin remodeling complex

GTF – RNA Pol II general transcription factor

GTP – guanosine triphosphate

HAT – histone acetyltransferase

HSF – heat shock factor transcriptional activator

Kin28 – kinase subunit of TFIIH

mRNA – messenger RNA

PIC – preinitiation complex

Pol II/Med – complex consisting of RNA Pol II and Mediator

Rad3, Rad25 – helicase subunits of TFIIH

RNA – ribonucleic acid

RNA Pol II – RNA Polymerase II

Rpb1 – large subunit of RNA Pol II

Rpb3 – subunit of RNA Pol II

Sth1 – subunit of RSC, a chromatin remodeling complex

Swi3, Snf5 – subunits of Swi/Snf, a chromatin remodeling complex

TAF_{II} – TBP-associated factor

TBP – TATA-box binding protein

TFIIA, B, D, E, F, H – RNA Pol II general transcription factors

Tfa1 – subunit of TFIIE

Tfb1 – subunit of core TFIIH

Tfg2 – subunit of TFIIF

Toa2 – subunit of TFIIA

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I thank all the friends who have been patient with and supportive of me for the last several years. I thank my husband Gary for keeping my life balanced with fun and for going all over the world with me. I thank my advisor Steve for teaching me how to be a scientist and for guiding me through my work. Finally, I thank my family for all their care of me. They are most responsible for all my successes because of the independence, inquisitiveness and love of knowledge they have instilled in me.

INTRODUCTION

A key process in all organisms from prokaryotes to humans is gene transcription, in which a gene sequence is read and converted to a messenger RNA (mRNA) molecule that can then be translated into a protein. This reaction is catalyzed by RNA Polymerase (RNA Pol) in prokaryotes and RNA Polymerase II (RNA Pol II) in eukaryotes. Years of work have revealed that there has been remarkable conservation of this process throughout evolution. These findings imply that what we learn about transcription in the yeast *Saccharomyces cerevisiae* can be readily applied to humans.

Although RNA Pol II is the enzyme that catalyzes mRNA formation, it cannot perform this reaction on its own. A large number of general transcription factors (GTFs) are required for RNA Pol II to perform its function. These GTFs assemble with RNA Pol II at a promoter to form a preinitiation complex (PIC) (Fig. 1). An in vitro transcription system minimally requires the

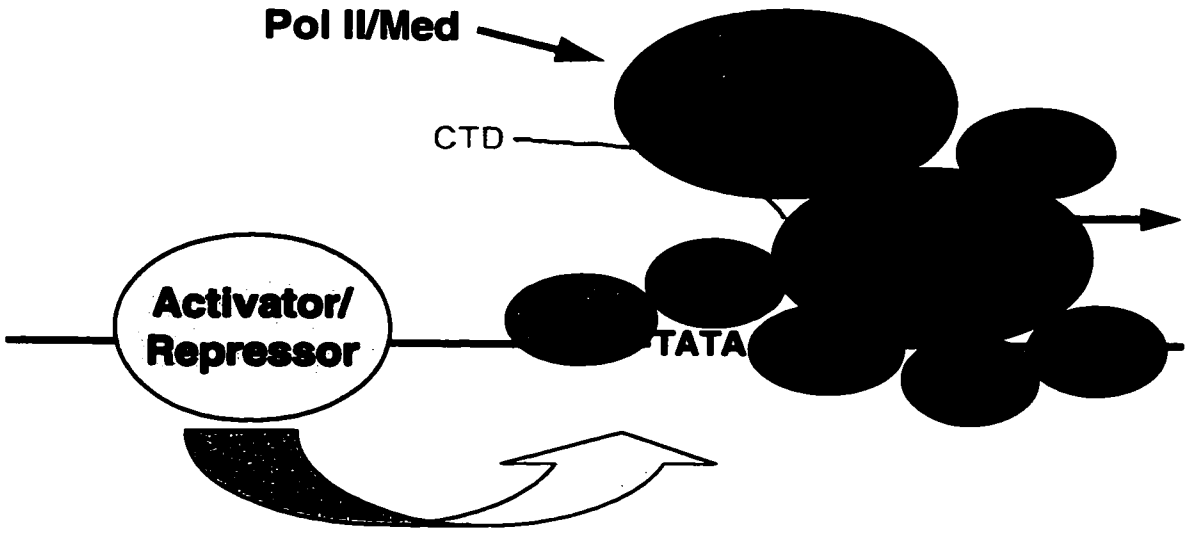


Figure 1. The RNA Pol II Preinitiation Complex. CTD – C-terminal domain of the large subunit of RNA Pol II (Rpb1).

GTFs TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH in addition to RNA Pol II (1-3). Each of these GTFs plays a unique role in transcription. TFIID is perhaps the most important architectural factor of the group. It is composed of TBP, a protein that binds to the TATA box in promoters, and 10-15 TBP associated factors (TAFs) (4, 5). TBP binds to the minor groove of the TATA box in a saddle shape and bends the DNA beneath it by 80° (6, 7). This bend allows TFIIB to associate with sequences both upstream and downstream of the TATA box (8). TFIIA stabilizes the binding of TFIID to DNA through several mechanisms. It destabilizes the dimerized form of unbound TFIID thereby unmasking its DNA binding domain (9, 10), and increases the affinity of TBP for DNA (11). TFIIA also prevents negative transcriptional regulators from interacting with TFIID and disrupting its association with DNA (12-16). TFIIB can also increase the affinity of TBP for DNA (11), but its main role is in helping RNA Pol II to select the correct transcription start site (17, 18). TFIIF is tightly bound to RNA Pol II in vivo and suppresses its nonspecific affinity for DNA (19). TFIIF has also been shown to suppress the transient pausing of RNA Pol II during transcription elongation (20). TFIIH is a large complex made up of nine subunits that has several functions in transcription (Fig. 2). It contains two ATP-dependent helicase activities, one of which (Rad3/XPD) is used in nucleotide excision repair (21-23) and the other (Rad25/XPB) in unwinding promoter DNA to form the open complex (21, 24, 25). Mutations in both these helicases are responsible for the human diseases xeroderma pigmentosum, Cockayne's syndrome, and trichothiodystrophy (26). Rad25/XPB has also been shown to prevent arrest of early elongation complexes (21, 25, 27-29). A subcomplex of TFIIH called the cyclin activating kinase (CAK) contains a kinase (Kin28/Cdk7) and cyclin (Ccl1/cyclin H) pair that phosphorylates RNA Pol II in vivo and in vitro (30-32). Virtually all genes in yeast require Kin28 activity for expression (33). Striking exceptions to this

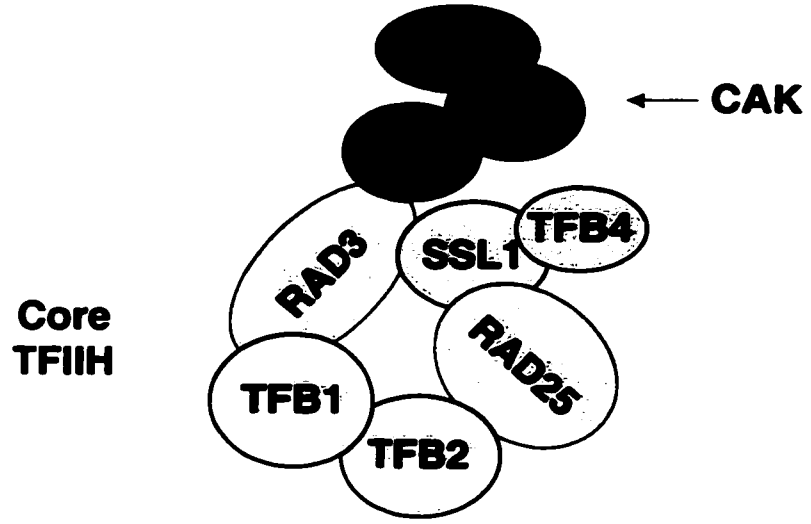


Figure 2. The yeast TFIIH complex.

are the heat shock and CUP1 genes, which can be induced in a Kin28 temperature sensitive mutant (34, 35). Interestingly, experiments in a purified mammalian transcription system have shown that the physical presence of CAK can stimulate transcription even in the absence of kinase activity (36). In mammalian cells CAK also regulates cell cycle transitions (37-42). In yeast these functions are performed by a different kinase, Cak1, which has been shown to phosphorylate Kin28 (43-45). These data suggest that TFIIH may serve as a link between cell cycle regulation and transcription. TFIIE has been shown to stimulate all these TFIIH functions (46-48).

RNA Pol II transcription can be divided into several distinct steps (Fig. 3). In the first step, the promoter must be made accessible to the transcription machinery. Promoter accessibility is reduced by the packaging of DNA into chromatin. In vivo, 146 bp of genomic DNA is wrapped around an octamer of four histone proteins (H3, H4, H2A, and H2B) in a structure termed the nucleosome (49-51). Nucleosomes are packaged together into higher order

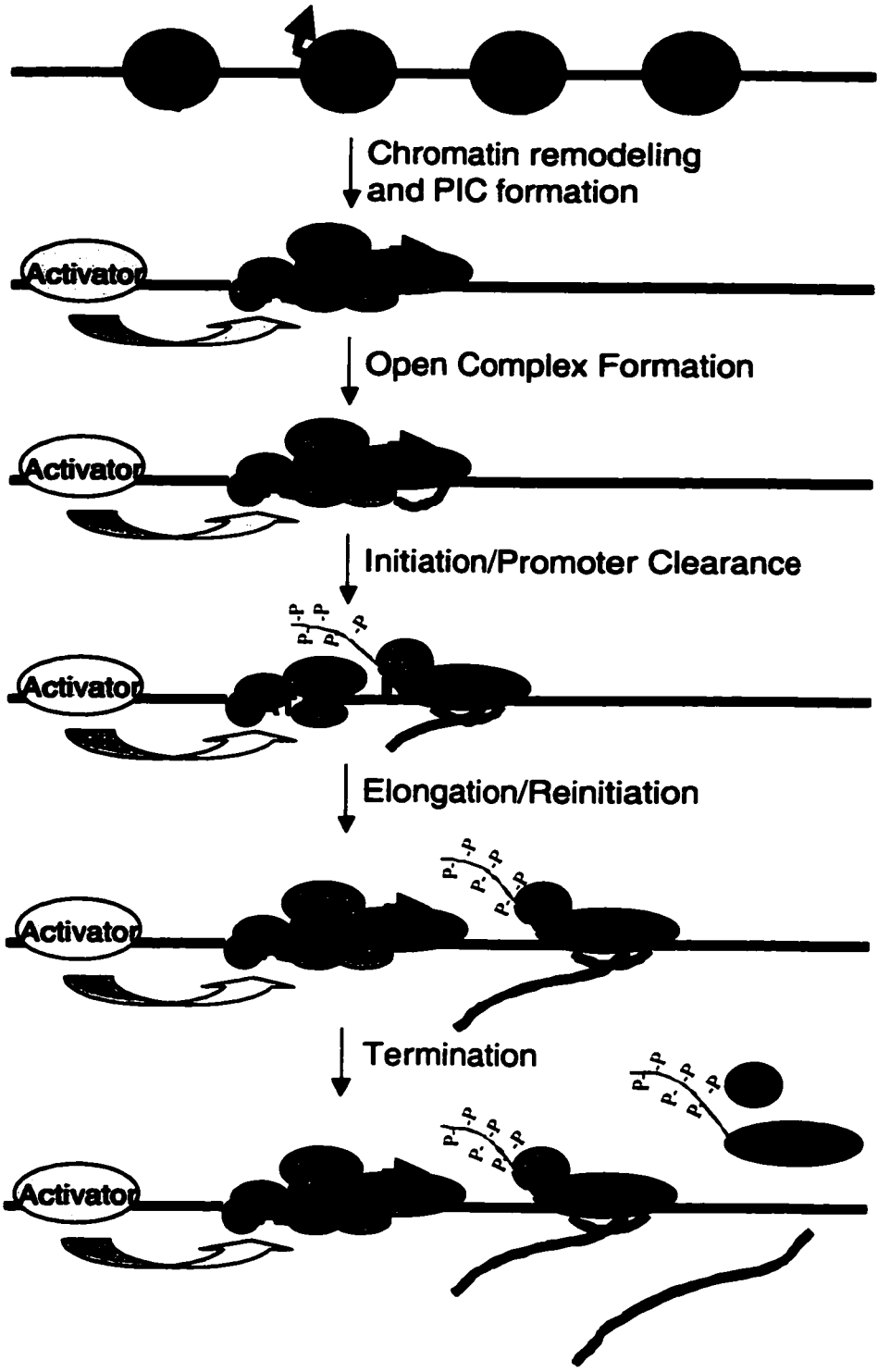


Figure 3. Steps in RNA Pol II transcription.

structures of chromatin. In vitro experiments have shown that when nucleosomal DNA templates are used, transcription initiation is inhibited (52, 53). Disruption of nucleosomes, on the other hand, enhances the binding of transcriptional activators and TBP (54-57). Once chromatin at the promoter has been remodeled, GTFs and RNA Pol II are recruited to form the PIC. This is followed by open complex formation, in which promoter DNA is unwound by the Rad25 helicase subunit of TFIIH. The C-terminal domain (CTD) of the largest subunit of RNA Pol II (Rpb1) is then phosphorylated by the Kin28 subunit of TFIIH, and RNA Pol II initiates transcription. Once polymerase clears the promoter, it enters the elongation phase and continues to synthesize mRNA until it reaches a termination signal and dissociates from the DNA. As soon as one polymerase has cleared the promoter, another promoter complex forms to allow for multiple rounds of transcription in a step called reinitiation.

A large number of factors have been discovered that can remodel chromatin to make promoters accessible to transcription factors. These factors can be divided into two major groups: ATP-dependent chromatin remodeling complexes and histone acetyltransferases (HATs). ATP-dependent chromatin remodeling complexes can further be divided into the Swi/Snf family and the ISWI family (3). Both of these families contain ATPase subunits that catalyze the reorganization of chromatin and increase chromatin fluidity (58). ATP-dependent chromatin remodeling complexes have been isolated from yeast, Drosophila, and humans, and many of the family members are conserved among these organisms (3, 58, 59). HATs are also found in all eukaryotes. They function by acetylating lysine residues in histone N-terminal tails. Since the nucleosome structure indicates that histone tails from one nucleosome contact adjacent nucleosomes, acetylation might remodel chromatin by interfering with these interactions, thereby disrupting

higher order chromatin structure (50, 51). In fact, hyperacetylated histones are generally associated with sites of transcriptional activity and accessible chromatin structure, while hypoacetylated histones are associated with transcriptionally silent regions (60-63). A recent in vivo analysis in yeast, however, has shown that this is not always the case, as decreases in histone acetylation were found to be associated with activation at some genes (64). Many different HAT-containing complexes have been identified in yeast (SAGA, ADA, NuA3, NuA4) and mammals (TFTC, STAGA, PCAF) (3). Several of these complexes contain homologs of Gcn5, a protein originally identified as a coactivator in yeast (65). Experiments have shown that stimulation of transcription by Gcn5 results in increased histone acetylation at responsive promoter regions, while mutations in its catalytic domain decrease both transcription and acetylation (66-68). In vivo analysis in yeast has revealed that both Swi/Snf and SAGA have roles in regulating mitotic gene expression, suggesting that both kinds of chromatin remodeling machines may be required for effective remodeling at some promoters (69). Interestingly, whole genome DNA array analysis in yeast has shown that Swi/Snf is required for both upregulation and downregulation of gene expression (33). Similar results have been seen with the ISWI family of remodeling complexes (70-72). In fact, chromatin remodeling complexes are important not only for activation of transcription, but also for repression (33). ATP-dependent chromatin remodeling complexes may move nucleosomes over promoter regions to block transcription factor binding. Many different types of histone deacetylases have also been discovered that likely reverse the open chromatin conformation set up by HATs (3).

In addition to chromatin remodeling complexes, several proteins have been discovered to phosphorylate, ubiquitinate, and methylate histones (73). The role of these histone modifications remains largely unknown. However,

they have been postulated to serve as a coding mechanism that tags histones for specific cellular processes (73, 74).

Many of the steps in transcription are influenced by transcription activators and repressors. Activators and repressors are sequence specific DNA binding proteins that bind to elements usually just upstream of the TATA box. They function through a variety of mechanisms. Activators are generally made up of two functionally separable domains, one for DNA binding and the other for activation. They have been shown to act primarily in the GTF and RNA Pol II recruitment phase of transcription (3). Specifically, activators have been shown to interact with TFIID, TFIIA, and TFIIB both biochemically and genetically (19). Recruitment of GTFs, particularly TFIID, is known to be rate limiting for transcription initiation (75, 76). This can be seen most clearly in in vitro experiments where PIC formation on naked DNA templates requires anywhere from 30-40 minutes to reach saturation, while transcription from preformed PICs can occur in 2 minutes (77, 78). In addition, in vivo artificial recruitment experiments in yeast and mammalian cells have shown that attaching DNA binding domains to TBP, TFIIB, and proteins associated with RNA Pol II is sufficient to activate reporter genes (76, 79-83). Some activators, such as HSF and VP16, have also been implicated in stimulating transcription elongation (84-86). This effect is most clearly seen in the case of HIV-1 transcription, where the Tat activator stimulates transcription by recruiting factors that increase the processivity of RNA Pol II (87). Many repressors of transcription have been shown to function by interacting with TBP. For example, Mot1 binds to TBP and causes it to dissociate from DNA (12, 88), while NC2 binds to TBP at promoters and prevents PIC formation (14, 89, 90). Other repressors work by binding to activators or competing for activator binding sites (3).

Early studies using purified transcription systems that contained TBP,

TFIIA, TFIIH, TFIIE, TFIIF, and RNA Pol II revealed that these systems were unresponsive to activators (1, 19). Subsequent biochemical purification and genetic analysis in *S. cerevisiae* discovered a new complex of proteins called Mediator that now allowed purified yeast systems to be responsive to activators (91-94). Yeast Mediator consists of 25 proteins and has been found tightly associated with ~5-10% of RNA Pol II through its CTD in a complex called Pol II/Med (Fig. 4) (92, 95). Some isolations of Pol II/Med also contain

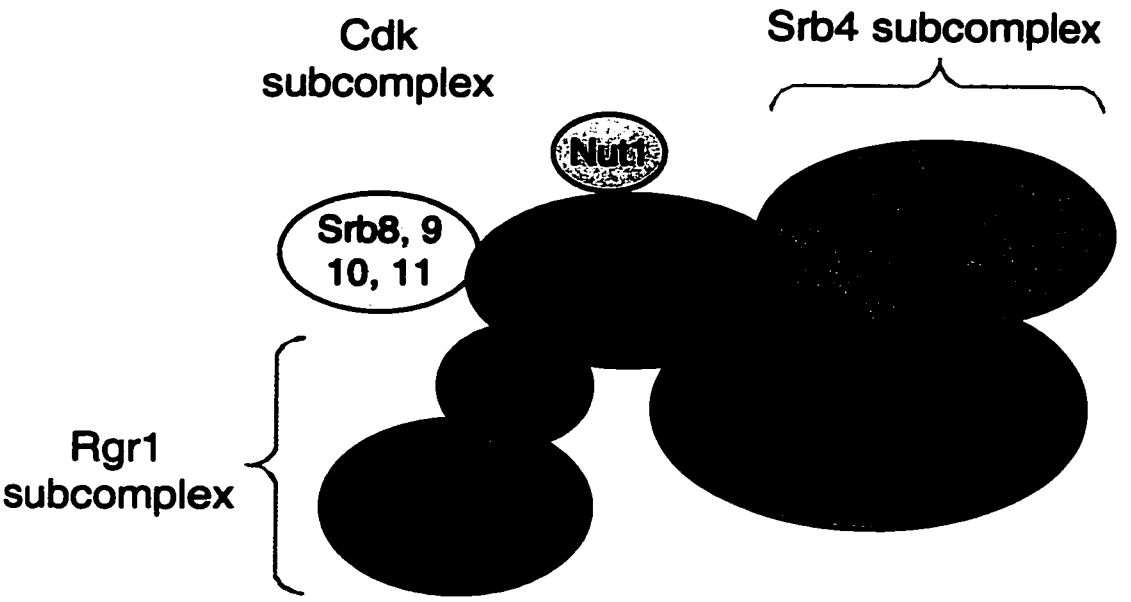


Figure 4. The Pol II/Med complex.

TFIIF, TFIIA, TFIIB, and TFIIH (92). The heterogeneity of Pol II/Med preparations probably reflects the difficulty of stably isolating such large complexes. Yeast Mediator itself can be biochemically broken down into three subcomplexes: the Srb4 subcomplex, which contains Srb2, Srb4-6, Med6, Med8, Med11, and Rox3; the Rgr1 subcomplex, which contains Rgr1, the Gal11 module (Gal11, Sin4, Med2, Pgd1), Med1, Med4, Med7, Med9, Srb7,

and Nut2; and the CDK complex, which contains Srb8-11 (96-103). The Srb proteins were first identified genetically as suppressors of a cold sensitive CTD truncation mutant (94). The RNA Pol II CTD is conserved among eukaryotes and is essential for viability of yeast and metazoan cells (3). It has since been shown that Mediator can directly interact with a CTD peptide (93, 103), and that the Srb4 and Rgr1 subcomplexes are probably directly associated with the CTD, as seen in a low resolution electron micrograph structure (104). In purified transcription systems, Pol II/Med stimulates basal transcription, mediates activated transcription, and stimulates CTD phosphorylation by TFIIH (91-93). Whole yeast genome DNA array analysis has also shown that Srb4 and Srb6 are required for transcription of almost all genes in yeast, with a phenotype nearly identical to that seen with a mutation that inactivates RNA Pol II (33). These data suggest that Pol II/Med is responsible for transcription of virtually all genes in yeast. A striking exception to this has been seen in experiments with stress and copper inducible promoters. Transcription with the activators ACE1 and HSF was shown to be independent of Srb4, but dependent on Rgr1 (34, 105). However, since the Rgr1 subcomplex has never been isolated apart from the entire PolII/Med complex (95), it is likely that these activators still require PolII/Med for transcription. Interestingly, these activators have also been shown to act independently of the Kin28 CTD kinase activity, suggesting a link between CTD phosphorylation and Srb4-dependent transcription (34).

In support of Mediator's function in relaying activation signals to polymerase, several interactions have been seen between Mediator components and activators. Yeast Mediator has been shown to bind to the activators GCN4 and VP16 in vitro, but not to the activation defective forms of these activators (106, 107). More specifically, direct interactions between the activator Gal4 and Srb4 have been seen (96). In these experiments,

mutations that disrupted the activation function of Gal4 in vivo also disrupted its interaction with Srb4. An Srb4 gain of function mutant could suppress the activation defect and partially restored binding to Gal4. Most activator interactions, however, have been detected within the Gal11 module of the Rgr1 subcomplex. Pol II/Med complexes purified from Gal11 module mutants generally retain the rest of the Rgr1 subcomplex and the Srb4 subcomplex, and show no defects in basal transcription, in contrast to those purified from an Srb5 deletion strain (102, 108). These Gal11 module mutant complexes have been shown to be defective in interactions with VP16 and GCN4 in vitro and GCN4 in vivo (102, 108, 109).

Interestingly, Mediator has also been shown to play a role in repression of transcription. Several of the genes isolated biochemically as Mediator subunits were previously isolated genetically as repressors. Sin4 is a negative regulator of HO and IME1 gene transcription (110, 111). Rgr1 is a repressor of glucose regulated genes and also the IME1 gene (111, 112). In addition, Pgd1 has been shown to be a target of the Cyc8-Tup1 corepressor (113, 114). Finally, whole yeast genome array analysis has revealed that Srb10 is required for repression of genes expressed during nutrient deprivation (33). These data show that the Mediator complex relays both positive and negative signals to the transcription machinery.

Mediator complexes have also been isolated from Drosophila, mice, and humans. These Mediators contain some homologs and orthologs of the yeast Mediator including Med6, Med7, Nut2, Soh1, Srb7, Srb10, Srb11, and Rgr1 (115-126). Some of the mammalian Mediators were isolated in a complex with RNA Pol II and contain virtually all GTFs except TFIID (116, 120, 122). The rest of the complexes were isolated solely as coactivators or corepressors and do not contain any detectable RNA Pol II or GTFs. SMCC was isolated from human cells by affinity purification of Srb7, Srb10, and

Srb11 (117). SMCC was found to be identical to TRAP, which was purified based on its function as a coactivator for the thyroid hormone receptor in vitro (118, 127). TRAP has since been shown to interact with other nuclear receptors, such as the estrogen receptor (128, 129). ARC was purified as a cofactor required by the Sp1 and SREBP-1a activators (121). It was subsequently found to be identical to DRIP, a cofactor complex isolated through specific binding to the ligand-bound vitamin D receptor (124). CRSP was also purified through its ability to stimulate Sp1 activity, and contains many subunits similar to ARC/DRIP (125). NAT, a corepressor complex, was isolated from human cells by affinity purification of Cdk8, the human homolog of Srb10 (126). Since these complexes were all identified biochemically, it has been important to determine their biological relevancy. Srb7 has been shown to be essential for mouse viability and is expressed in many mouse tissues (130). Two Drosophila Mediator subunits, dTRAP240 and dTRAP80, are also essential for viability, and have been shown to be important for segment identity specification and cell viability, respectively (131). Experiments in mice have shown that TRAP is required for viability and proper embryonic development (132). These data, and the fact that different mammalian Mediators share core subunits, suggest that Mediators may be tailored to function at specific times and in specific tissues during development.

The fact that a number of Mediators exist in mammalian cells has led to the search for other Mediators in yeast. A non-Srb/Med-containing Pol II complex has been isolated using affinity purification against the RNA Pol II CTD and TFIIF (133, 134). This Pol II complex contains the proteins Paf1, Cdc73, Ccr4, Hpr1, Gal11, TFIIB, and TFIIF (133, 135). Whole genome DNA array and genetic analysis shows that this complex is involved in upregulating expression of cell wall biosynthesis genes and genes involved in the PKC MAP kinase cascade (135). Interestingly, an Srb5 and Ccr4 deletion mutant

shows synthetic lethality, suggesting that these Pol II complexes act in parallel pathways (135). However, the genes downregulated in a Cdc73/Paf1 Pol II complex mutant have also been shown to be downregulated in an Srb4 mutant (33). Since the Cdc73/Paf1 Pol II complex has not yet been shown to have transcriptional activity, it is hard to determine whether this complex acts apart from Pol II/Srb-Med or simply at another step in the transcription cycle. A third form of Mediator unassociated with RNA Pol II has also been isolated using Srb5 affinity purification (95). This Mediator, termed Medc, is similar to Srb/Mediator, but lacks the Gal11 module and the Srb10/CDK subcomplex. The Medc complex has very low in vitro transcriptional activity and its function in vivo remains to be determined.

A key protein domain involved in a variety of interactions at all steps in transcription is the RNA Pol II CTD. The CTD consists of the peptide sequence YSPTSPS repeated anywhere from 26 times in yeast to 52 times in humans. It is essential for viability in yeast and metazoans (108). The CTD can be phosphorylated on Ser2 and Ser5 of each repeat. RNA Pol II with a hypophosphorylated form of the CTD is seen at promoters (136-138), while a hyperphosphorylated form predominates in elongating complexes (139-141), suggesting that phosphorylation occurs during the transition from initiation to elongation. In fact, hypophosphorylated RNA Pol II is preferentially recruited into PICs (137). In vitro transcription experiments with purified factors have shown that phosphorylation is not required for either initiation or elongation at the adenovirus major late promoter, but is required for elongation at the DHFR promoter (31, 36, 142, 143). CTD phosphorylation is also important for regulating the exchange of cofactors that bind the CTD. The Srb/Mediator associates with the hypophosphorylated CTD, while capping enzymes, elongation factors, and other RNA processing factors are associated with the hyperphosphorylated CTD (91, 92, 94, 144-147). Importantly, CTD

phosphorylation does not always have a positive effect on transcription. At the initiation of mitosis, the CTD is phosphorylated to block its association with PICs, thereby inhibiting transcription (148, 149).

Five CTD kinases have been identified to date. The Kin28/Cdk7 subunit of TFIIH has been shown to be responsible for most CTD phosphorylation in vivo in yeast, as well as in vitro (31, 32, 36). Kin28 phosphorylates Ser5 of the CTD in vitro, and can thus aid in binding of mRNA capping enzymes in vivo (145, 150). Its CTD kinase activity is stimulated by Srb/Mediator, TFIIE, and Gal11 (91, 93, 151). Kin28 is also required for transcription of virtually all yeast genes (33). The Srb10/Cdk8 subunit of Pol II/Med has also been identified as a Ser5 CTD kinase (100, 120, 150). Gene expression analysis has determined that Srb10 acts mainly as a repressor, and experiments have shown that its kinase activity is required for repression by Ssn6-Tup1 (33, 152). Two experiments have resulted in different models for Srb10 repression. The Young group has shown that phosphorylation of purified yeast RNA Pol II prior to PIC formation inhibits its transcriptional activity (150). This effect is likely due to the poor ability of hyperphosphorylated RNA Pol II to be recruited into PICs. Experiments from Reinberg's group have shown that the Cdk8-containing NAT complex represses transcription in vitro by phosphorylating TFIIH, thereby inhibiting its CTD kinase activity (153). Further in vivo experiments will need to be performed in order to differentiate between these two models. A third CTD kinase, Ctk1, was isolated from yeast and found to be involved in increasing transcription elongation efficiency (154, 155). A Ctk1 deletion results in decreased expression of genes induced during the diauxic shift, a decrease in Ser2 phosphorylation during the diauxic shift, and an increase in Ser5 phosphorylation in log phase (156). These results suggest that Ctk1 acts by phosphorylating Ser2 and blocking phosphorylation of Ser5. Interestingly,

Ser5 phosphorylated CTD was localized to regions near promoters, while Ser2 phosphorylation was localized further downstream, suggesting that the role of Ctk1 in elongation may be to effect this change in phosphorylation state (144). A fourth CTD kinase, P-TEFb, was originally purified from Drosophila as a stimulator of transcription elongation. It was subsequently identified in humans, and found, along with TFIIH, to be required for activation by Tat, a HIV transcription activator protein. A possible ortholog of P-TEFb, Bur1, has also been identified in yeast (157). It phosphorylates Ser5, interacts genetically with CTD truncations, and has an in vivo elongation phenotype (158). Of course, a CTD phosphatase has also been isolated from both yeast and humans, which can recycle phosphorylated RNA Pol II to ensure a steady supply of the hypophosphorylated version. The phosphatase Fcp1 associates with and is stimulated by TFIIF, and this stimulation is blocked by TFIIB (159-163).

The RNA Pol II transcription machinery is a complex network of proteins that must dynamically interact throughout the transcription process. Chromatin remodeling factors must reorganize nucleosomes to make promoters accessible for factor binding. The PIC must then assemble and initiate transcription, forming and breaking protein-protein and protein-DNA contacts in the process. A large number of mRNA processing factors must associate with elongating RNA Pol II to ensure the production of a stable and mature mRNA. And this process must be reinitiated many times over to produce multiple transcripts. Finally, this process must be regulated by activators and repressors so that only specific genes are transcribed at specific times. Although we know most of the factors involved in RNA Pol II transcription, the mechanisms by which they come together and fall apart remain to be determined.

CHAPTER 1: Steps in Preinitiation Complex Formation

Introduction

One of the first steps in transcription by RNA Pol II is the recruitment of the transcription machinery to a promoter to form the PIC. PIC formation is a highly regulated process that is stimulated by activators and blocked by repressors (3, 164). TBP recruitment is one of the rate limiting steps in PIC formation that is stimulated by activators (75, 76). The promoter binding of the TBP-containing complex TFIID is influenced by the TATA box sequence, and promoter elements such as the initiator and downstream promoter element (19). Strong TATA consensus sequences in particular have been linked to high levels of transcription in vivo and in vitro (165, 166). However, in vitro experiments show that TATA mutant promoters, although transcriptionally inactive, still allow robust PIC formation when the activator Gal4-VP16 is used (167, 168). This is likely due to the fact that VP16 can bind to many PIC components in vitro (106).

Two models of PIC formation have been proposed. The stepwise recruitment model is based on in vitro binding studies and proposes that transcription factors are recruited to promoters one at a time (1). In this model, TBP binds first to the TATA box. This is followed by TFIIB binding, which sets the stage for RNA Pol II and TFIIF recruitment. TFIIB acts as a bridge due to its interactions with the TFIID/DNA complex, TFIIF, and RNA Pol II (8, 169, 170). The last factors to assemble into the PIC are TFIIH and TFIIE. TFIIA can enter the complex at any point when TBP alone is used, but is required for optimal TFIID binding (1, 19). The holopolymerase model proposes that RNA Pol II and GTFs are recruited in one step after TFIID and TFIIA are recruited (171). This model relies on the fact that holopolymerases have been purified that contain Mediator and almost all the GTFs, except

TFIID and TFIIA (92, 94, 116, 120, 122). In addition, almost all of Mediator is associated with about 5-10% of RNA Pol II in vivo (92, 95), and Mediator subunits are required for transcription of virtually all genes in yeast (33). However, many holopolymerase preparations do not contain GTFs, suggesting that GTFs may only be loosely associated with RNA Pol II during the purification process (91, 93, 95, 172).

Ranish and colleagues (168) have performed experiments to look at PIC formation using yeast nuclear extracts and DNA templates immobilized on magnetic beads. They found that TFIID and TFIIA bound promoters cooperatively and in the absence of the rest of the transcription machinery. Specifically, no GTFs bound the template when a TBP or TFIIA mutant extract was used. A TFIIB mutant, however, still allowed TFIID/TFIIA binding, but abrogated binding of TFIIB, the Pol II/Med complex, TFIIF, TFIIH, and TFIIE. In addition, TFIIA was found to stimulate both the rate and extent of PIC formation. These data begin to support the holopolymerase model of PIC formation, although they do not address the role of the Pol II/Med complex in the process.

In this chapter I have continued the use of the immobilized template system to dissect the steps in PIC formation. I show that PIC formation occurs in a series of cooperative, rather than individual, steps and that Pol II/Med is required for this process.

Results

An immobilized template assay was used to analyze PIC formation in a variety of yeast nuclear extracts made from transcription factor mutant strains. The immobilized template consists of a HIS4 core promoter with a single Gal4 DNA binding site upstream linked to a magnetic bead (Fig. 5). A unique PstI restriction site is positioned 20bp upstream of the Gal4 site to allow elution of

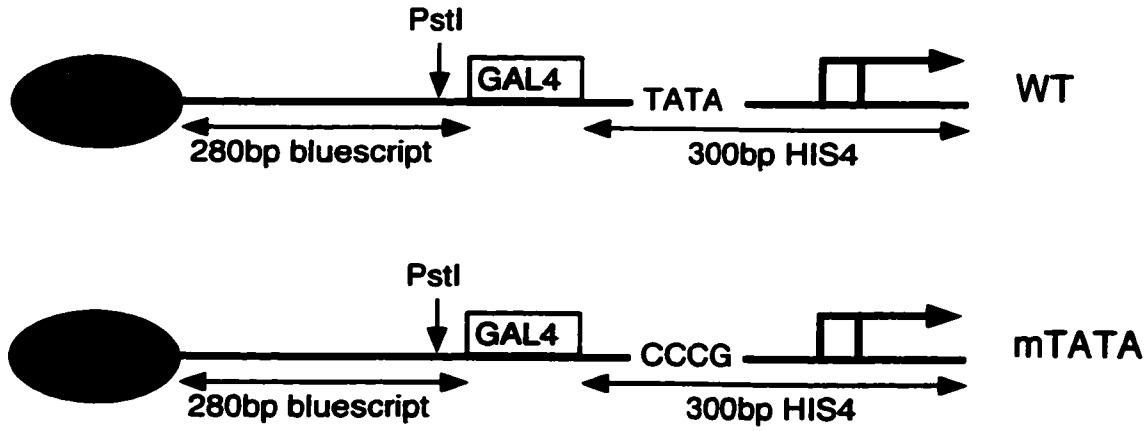


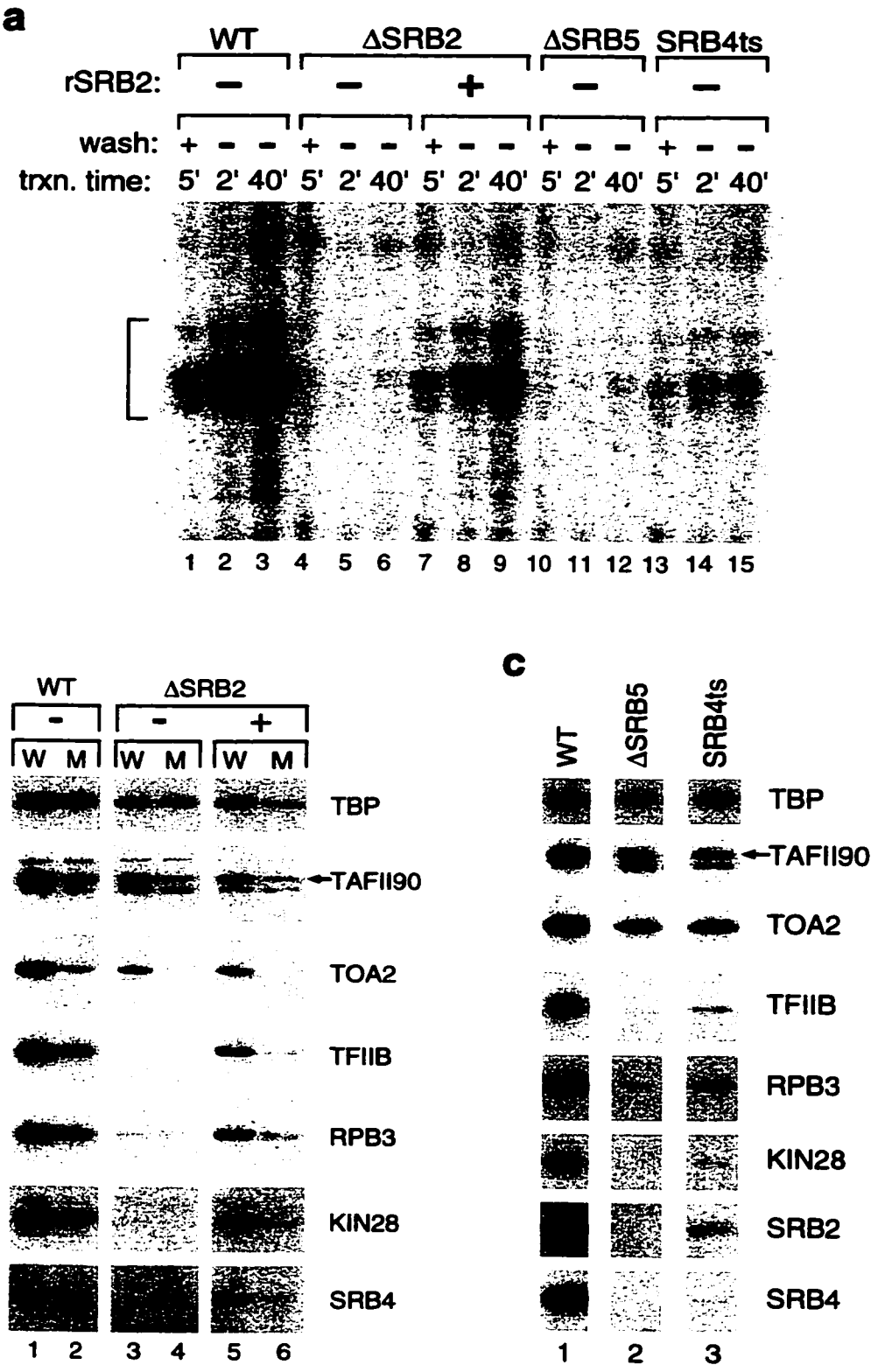
Figure 5. Immobilized templates used in this assay. The wild type template (WT) contains the HIS4 core promoter and transcription start sites. mTATA is identical to wild type, except that the TATA box is replaced with a GC-rich sequence.

the template from the bead. To show that efficient PIC formation is TATA-box dependent, a template with a TATA-box mutation (mTATA) was also used. The assay is performed by incubating nuclear extract with the immobilized templates to allow PIC formation, washing the templates to remove unbound proteins, and digesting with PstI to elute template bound factors. The eluted proteins are assayed by SDS-PAGE and Western blot. A representative PIC formation experiment is shown in Figure 6b. Several lines of evidence indicate that this assay can specifically isolate authentic PICs (168). First, PIC formation occurs in a TBP-, TFIIA-, and promoter-dependent manner. Second, the eluted transcription factors are present in stoichiometric amounts, suggesting that they are all part of the same complex. Finally, activators were found to stimulate PIC formation and transcription on immobilized templates to similar extents. Interestingly, while the TATA box is required for transcription, its effect on PIC formation is small. Reactions with the mTATA template show a 2-4 fold decrease in all PIC components, suggesting a role for the TATA box

in a step after PIC formation ((168); and Fig. 6b).

Initial immobilized template experiments using the activator Gal4-AH showed that mutations in either TBP or TFIIA completely prevented PIC formation, while mutations in TFIIB only allowed TFIID and TFIIA to be recruited to the promoter. These data suggested that the first step in PIC formation is the cooperative recruitment of TFIID and TFIIA, and that TFIIB is required for subsequent stable recruitment of all other factors. However, these experiments did not address the role of Pol II/Med in PIC formation. In order to test this, nuclear extracts were made from strains that contained mutations in the Mediator components Srb2, Srb5, and Srb4. All of the extracts were defective in both single- and multi-round transcription (Fig. 6a). The Srb2 deletion (ΔSrb2) and Srb5 deletion (ΔSrb5) extracts were the most severely defective, with at least a 20-fold decrease in single-round transcription as compared with the wild type extract (Fig. 6a, lanes 1 and 2 vs. 4, 5, 10, and 11). Addition of recombinant Srb2 (rSrb2) to the ΔSrb2 extract restored transcription to levels that were at least 50% of wild type (Fig. 6a, lanes 1 and 2 vs. 7 and 8). The Srb4 temperature sensitive extract (Srb4ts) showed an approximately 6-fold reduction in single-round transcription as compared with the wild type extract (Fig. 6a, lanes 1 and 2 vs. 13 and 14). I then tested whether these defects in transcription could be due to a failure to form stable PICs. All of the extracts showed essentially wild type levels of TBP, $TAF_{II}90$ and Toa2 (a TFIIA subunit), but reduced levels of all other components probed for (Fig. 6b, lanes 1, 3 and 5, and 6c). Specifically, the ΔSrb2 and ΔSrb5 extracts showed decreases of greater than 16-fold in levels of RNA Pol II, TFIIB, Kin28, Srb2, and Srb4. The Srb4ts extract showed decreases of between 5- and 20-fold in these same components. Addition of rSrb2 did not affect levels of TBP, $TAF_{II}90$ and Toa2, but did restore the stable assembly of Pol II/Med components, TFIIB, and TFIIH into PICs (Fig. 6a, lane 3 vs. 5). The

Figure 6. The role of Mediator in PIC formation. **a.** Transcription activity of PICs formed in a wild type extract, and in Srb2, Srb4, and Srb5 mutant extracts. The indicated extracts were incubated with the wild type immobilized template and Gal4-AH for 40 min. rSrb2 (100 ng) was added to reactions in lanes 7-9. For multi-round measurements, PICs were incubated with NTPs for 40 min (lanes 3, 6, 9, 12, 15). Single-round measurements were obtained by incubating PICs with NTPs for 2 min (lanes 2, 5, 8, 11, 14). Activity of washed complexes was measured by resuspending PICs in transcription buffer containing NTPs for 5 min (lanes 1, 4, 7, 10, 13). Brackets indicate specific transcription signal as detected by primer extension. **b** and **c.** PIC assembly on immobilized templates in wild type, ΔSrb2, ΔSrb5, and Srb4ts mutant extracts. PICs were formed on either wild type (W) or mTATA (M) immobilized templates as described in **a**. rSrb2 (200 ng) was included where indicated. PICs were isolated and analyzed by Western blot with antibodies against the components indicated at right. $TAF_{II}90$ is a TFIID component, TOA2 is a TFIIA component, RPB3 is an RNA Pol II component, KIN28 is a TFIIH component.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relative levels of PICs formed corresponded well with the levels of transcription seen with each extract. In addition, the levels of all PICs were enhanced by the presence of a functional TATA box (Fig. 6b, lanes 1, 3, and 5 vs. 2, 4, and 6).

The above results suggest that Pol II/Med is recruited as a whole to PICs, and is required for recruitment of all subsequent factors, including TFIIB and TFIIH. However, it remains a possibility that Mediator is recruited to promoters separately from RNA Pol II, as has recently been seen in vivo at SBF regulated promoters (173, 174). In order to test this possibility, I made a nuclear extract from a strain containing a temperature sensitive mutation in Rbp1, the largest subunit of RNA Pol II (Rpb1-1). This extract was severely defective in transcription, even at room temperature (Fig. 7a). When the extract was used in the immobilized template assay, TBP levels were essentially like those seen in wild type, whereas Srb2, Srb4, TFIIB, and Rpb3 decreased to undetectable levels at high temperatures (Fig. 7b). These results show that, in this assay, Mediator cannot be recruited to promoters separately from RNA Pol II. This conclusion is also supported by identical results seen with a CTD truncation mutant (168). It is possible that the separate recruitment seen previously is an activator specific event, and, therefore, cannot be detected when the activator Gal4-AH is used.

To further dissect the steps in PIC formation, a nuclear extract was made from a strain with a temperature sensitive mutation in Kin28 (Kin28ts16), the kinase subunit of TFIIH, and Rad3 (Rad3ts), a helicase subunit of TFIIH. These extracts were severely defective in transcription at room temperature (Fig. 8a, and S. Hahn, unpublished). When the extracts were used in the immobilized template assay they showed identical phenotypes (Fig. 8b). Levels of RNA Pol II, TFIIB, TFIIA, TBP, TFIIE, and Mediator subunits in the mutants were comparable to those in wild type, while levels of the TFIIH

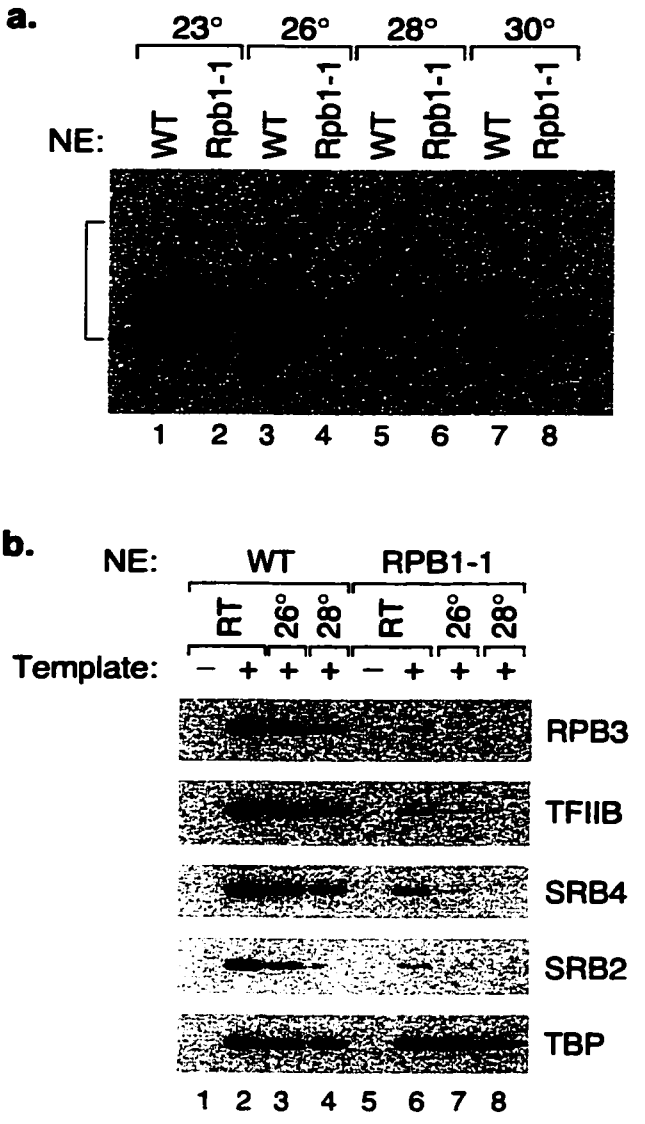

Figure 7. The role of RNA Pol II in PIC formation. **a.** Rpb1-1 nuclear extract is defective in transcription. The indicated extracts were incubated with the wild type immobilized template and Gal4-AH for 40 min at the temperatures indicated. PICs were then washed in transcription wash buffer and resuspended in transcription buffer with NTPs for 2 min. Brackets indicate specific transcription signal as detected by primer extension. **b.** PIC assembly in wild type and Rpb1-1 nuclear extracts. PICs were formed on wild type templates as described in **a**. PICs were isolated and analyzed by Western blot with antibodies against the components indicated at right. The reaction in lane 1 was performed without template as a control for non-specific binding to Dynabeads.

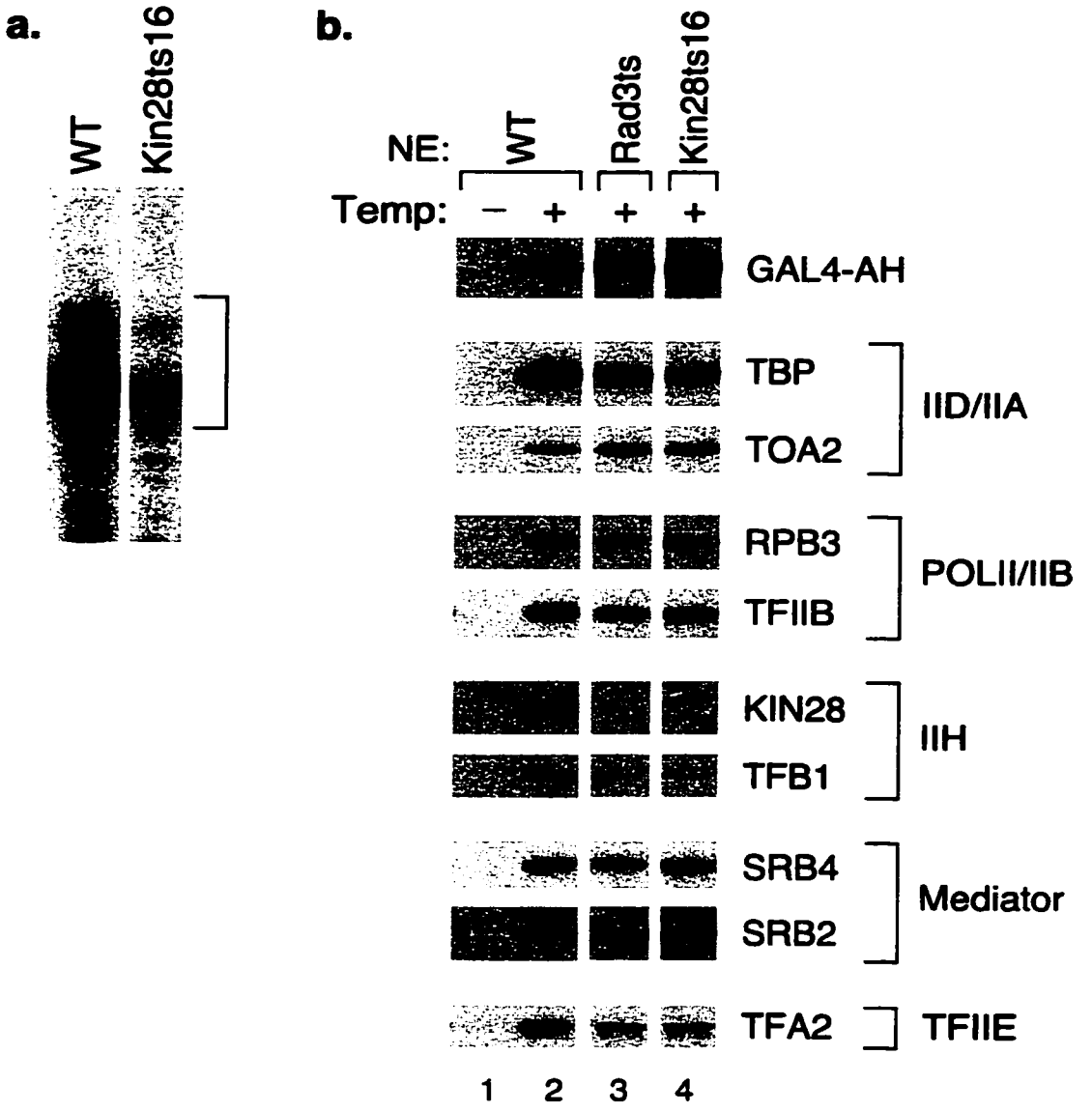

Figure 8. The role of TFIIH in PIC formation. **a.** Kin28ts16 nuclear extract is defective in transcription. Wild type or Kin28ts16 nuclear extract was incubated with the wild type immobilized template and Gal4-AH for 40 min. PICs were then washed in transcription wash buffer and resuspended in transcription buffer with NTPs for 2 min. Brackets indicate specific transcription signal as detected by primer extension. **b.** PIC assembly in wild type, Rad3ts, and Kin28ts16 nuclear extracts. PICs were formed as described in **a**. PICs were isolated and analyzed by Western blot with antibodies against the components indicated at right. The reaction in lane 1 was performed without template as a control for non-specific binding to Dynabeads.

subunits Kin28 and Tfb1 were low or undetectable. Since Kin28 is a subunit of the CAK subcomplex, while Tfb1 is a subunit of core TFIIH, this suggests that the Rad3ts and Kin28ts16 mutants prevent the entire TFIIH complex from being stably recruited to the promoter. These data, therefore, show that in the absence of TFIIH, the rest of the transcription machinery can be stably recruited to promoters, thus suggesting that TFIIH recruitment is one of the final steps in PIC formation.

Finally, to test the role of TFIIE in PIC formation a nuclear extract was made from a strain containing a temperature sensitive mutation in the Tfa1 subunit of TFIIE (Tfa1ts). This mutant showed a 4-fold decrease in transcription at room temperature, but its transcriptional activity could be rescued by the addition of recombinant TFIIE (rTFIIE) (Fig. 9a). When the Tfa1ts extract was used in the immobilized template assay, levels of all the factors probed for were reduced as compared to wild type (Fig. 9b, lane 1 vs. 2). However, levels of TBP, Toa2, and Kin28 were not stimulated by addition of rTFIIE, whereas levels of the TFIIE subunit Tfa2 were stimulated greater than 4-fold. Thus the lower levels of TBP, TFIIA, and TFIIH are probably due to a lower specific activity of the Tfa1ts nuclear extract and not to the decrease in TFIIE. These results indicate that recruitment of TFIID, TFIIA, and TFIIH to promoters does not require TFIIE (Fig. 9b, lane 2 vs. 3). Interestingly, levels of Rbp3, Srb4, and TFIIB were stimulated up to 2-fold by addition of rTFIIE. Given that this level of stimulation is not as great as that of TFIIE, and that Kin28 levels were unaffected by these lower levels of Pol II/Med components, this suggests that TFIIE does not play a significant role in the stable recruitment of Pol II/Med and TFIIB to promoters.

Taken together, these results lead to a 3-step model of PIC formation (Fig. 10). The first step is the cooperative recruitment of TFIID and TFIIA (168). This is followed by cooperative recruitment of TFIIB and Pol II/Med. In

the final step, TFIIE and TFIIH are recruited to form the complete PIC. Since the Srb/Mediator is required for stable recruitment of RNA Pol II, TFIIB, TFIIE, and TFIIH, these results also support studies indicating that the Srb/Mediator form of holopolymerase is required for transcription initiation at virtually all yeast genes (33).

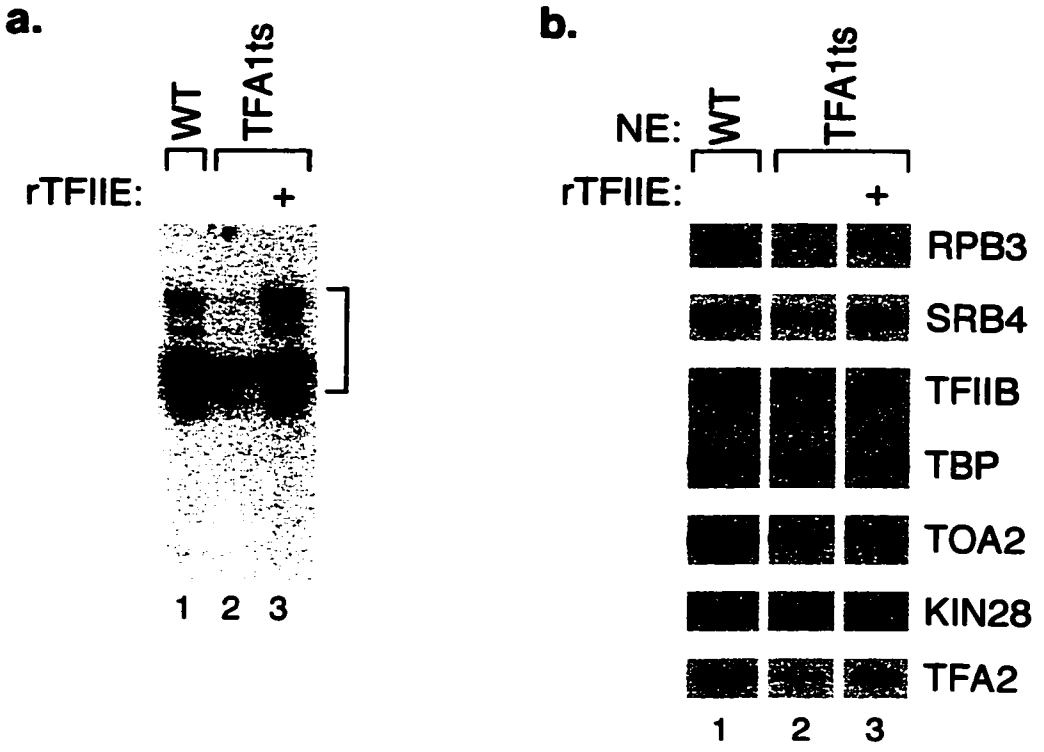

Figure 9. The role of TFIIE in PIC formation. **a.** Tfa1ts nuclear extract is defective in transcription. Wild type of Tfa1ts nuclear extract was incubated with the wild type immobilized template and Gal4-AH for 40 min. rTFIIE (80 ng) was added in lane 3. PICs were then washed in transcription wash buffer and resuspended in transcription buffer with NTPs for 2 min. Brackets indicate specific transcription signal as detected by primer extension. **b.** PIC assembly in wild type and Tfa1ts nuclear extracts. PICs were formed as described in **a.** rTFIIE (160 ng) was added in lane 3. PICs were isolated and analyzed by Western blot with antibodies against the components indicated at right. TFA2 is a TFIIE component.

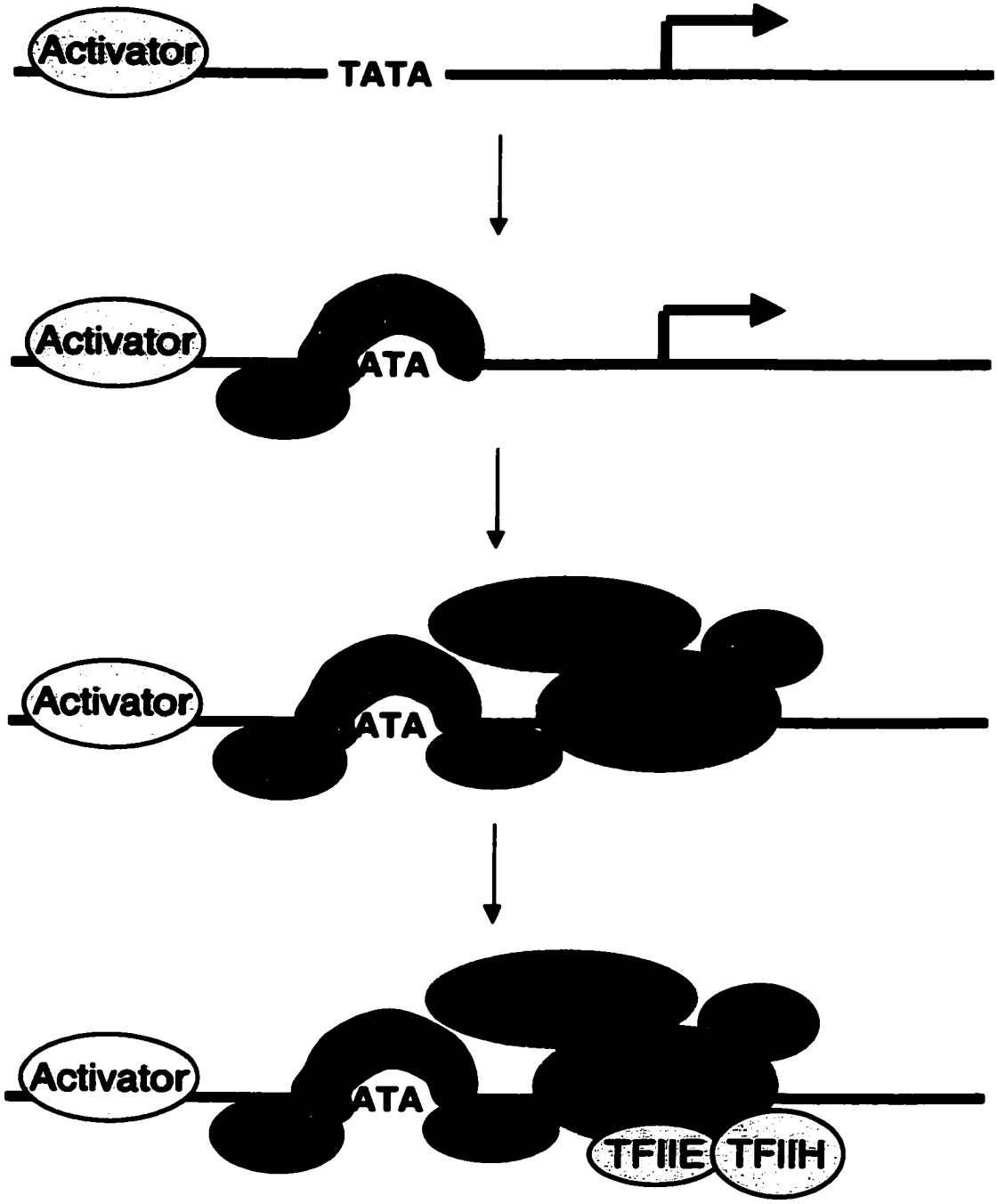

Figure 10. PIC assembly model. The first step in PIC assembly is the cooperative binding of TFIID and TFIIA. In the next step, Pol II/Med, TFIIB, and TFIIF bind cooperatively. Finally, TFIIH and TFIIE bind in one step. This entire process can be stimulated by activators.

Materials and Methods

Yeast strains

Yeast strains are listed in Table 1.

Table 1. Strain list

Strain	Name	Genotype	Reference
Z561	wild type	*MATa his3Δ200 leu2-3,112 lys2-801 ura3-52*	(175)
Z525	ΔSrb2	*MATa his3Δ200 leu2-3,112 lys2-801 ura3-52 trp1Δsrb2Δ1::HIS3*	(89) and pers. comm.
Z562	ΔSrb5	*MATa his3Δ200 leu2-3,112 lys2-801 ura3-52 srb5Δ1::HISG*	(89) and pers. comm.
Z628	Srb4ts	*MATa his3Δ200 leu2-3,112 lys2-801 ura3-52 srb4Δ2::HIS3* pRY2882 (*srb4-138 LEU2* CEN)	(89) and pers. comm.
Z649	wild type	*MATa his3Δ200 leu2-3,112 ura3-52*	(89) and pers. comm.
Z111	Rpb1-1	*MATα ura3-52 his3Δ200 leu2-3,112 rpb1-1 ade2*	N. Woychik, pers. comm.
37-1.1a	Kin28ts16	*ade2-1 ade3-22 his3-11,15 leu2 trp1-1 ura3-1 can1-100 Δkin28::LEU2* (ARS CEN TRP1 *kin28ts*)	(30)
RSY11	Rad3ts	*MATta ura3-52 leu2 trp5-27 ade2-40 ilv1-92 arg4-3* pRS11:HIS3 *rad3 ts-14* integrated at chromosomal locus	(176)
HS46	Tfa1ts	*MATα ade2-1 leu2 trp1-1 ura3-1 can1-100 tfa1::ADE2* (pSK492 ARS CEN TRP1 pADH1-HA-*tfa1-21*)	(177)
HS33	wild type	same as HS46 but with plasmid pSK492 ARS CEN TRP1 pADH1-HA-TFA1	(177)

Nuclear extract preparation

Yeast nuclear extracts were prepared from 2 liter cultures of the indicated strains grown at permissive temperature as described previously

(178) and on the World Wide Web (www.fhcrc.org/labs/hahn).

Recombinant and purified proteins

rSrb2 was provided by S. Miller. Purified yeast CAK was provided by S. Hahn. rTFIIE was provided by S. Hurst.

Plasmid template

For transcription assays on a plasmid template, pSH515 was used. pSH515 contains 144bp of the HIS4 core promoter (-141 to +3 with respect to the translation start site), including the TATA box and transcription start sites, cloned downstream of a single Gal4 binding site. In addition, the bases at four positions around the TATA box were changed to eliminate potential cryptic TATA sequences.

Biotinylated templates

Biotinylated templates were prepared by PCR with either pSH515 or pSH514 as templates. pSH514 is identical to pSH515 except that the TATA box (TATATAATA) was replaced with the sequence TACCCGGGA. The biotinylated upstream primer p965 (5'-biotin-TAATGCAGCTGGCACGACAGG-3'), located ~280bp upstream from the Gal4 site, and the downstream primer pNot (5'-GGCCGCTCTAGCTGCATTAATG-3') were used with these templates to produce 594bp products. PCR products were extracted with phenol:chloroform (2:1), ethanol precipitated, purified with S300 columns (Pharmacia), and quantitated by a fluorescent dye binding assay (PanVera Corp.).

Immobilized templates

M-280 Streptavidin Dynabeads (Dynal) were concentrated with a

magnetic particle concentrator (MPC) (Dynal) and washed twice in buffer T [10mM Tris (pH 7.5), 1mM EDTA, 1M NaCl] at 80 µl buffer T/µg beads. The beads were then resuspended in buffer T with 0.003% NP-40 at 10 mg/ml. Dynabeads were incubated with 20 fmol biotinylated template/µg beads in buffer T for 30 min at room temperature with constant shaking. The Dynabeads were then washed in buffer T at 80 µl buffer T/µg beads, and blocked in block buffer at 1 ml/mg beads for 15 min at room temperature. Block buffer consists of transcription buffer [10mM HEPES (pH 7.6), 100mM potassium glutamate, 10mM magnesium acetate, 5mM EGTA, 3.5% glycerol], containing 60 mg/ml casein (Sigma), 5 mg/ml polyvinylpyrrolidone (USB), and 2.5mM DTT. The immobilized templates were then washed three times in transcription buffer, and resuspended in transcription buffer with 0.003% NP-40 at 10 mg/ml.

In vitro transcription with immobilized templates

Reactions were scaled up twofold from the standard transcription reaction to 50 µl (179). On ice, 120-180 µg of nuclear extract was mixed with 20 µl of 2.5X transcription mix (2.5X transcription buffer, 38.4 mg/ml phosphocreatine, 6.3mM DTT, 0.02 mg/ml creatine phosphokinase), 0.05% NP-40, and transcription buffer to a final volume of 44 µl. After a 10 min incubation at room temperature, the nuclear extract mix was spun at 9K rpm for 2 min in an Eppendorf microfuge at 4°C to remove insoluble material. The supernatant was then transferred to a new tube on ice, and recombinant factors were added where indicated, along with 500 ng HaeIII digested *E. coli* DNA as nonspecific competitor. Immobilized templates were preincubated with no activator, 60 ng Gal4-AH, or 48 ng Gal4-VP16 for 10' at room temperature. 2.5 µl of immobilized template was then added to each nuclear extract mix, and the reactions incubated at room temperature for 40 min with occasional

shaking to form PICs. PICs were washed three times with 400 µl of wash buffer (transcription buffer containing 0.05% NP-40 and 2.5mM DTT), and resuspended in 50 µl transcription buffer containing 12mM phosphocreatine, 400 ng creatine phosphokinase, 2.5 mM DTT, and 20 units of ribonuclease inhibitor. Transcription was then initiated by addition of 100µM of each NTP. Reactions were stopped at the times indicated by addition of 400 µl of stop buffer. The supernatant was removed from the beads, phenol:chloroform (2:1) extracted, and ethanol precipitated. Primer extension was performed on the RNA samples as described previously (179), except that actinomycin C_1 was included (15 µg/ml) during the extension reaction. Primer extension products were resolved on a 6% Quickpoint sequencing gel according to the manufacturers protocol (NOVEX).

Immobilized template assay

PIC formation experiments were performed as described above for immobilized template transcription, except that reactions were scaled up 2-fold to 100 µl. After washing, templates were isolated from the beads by resuspension in NE Buffer 3 (NE Biolabs) and digestion with 60 units PstI (NE Biolabs) for 30 min at 37°C with constant shaking. The reactions were run on a 4-12% NuPAGE gel (NOVEX), and transferred to Immobilon membranes (Millipore). Proteins were detected by use of Pierce ECL kits. Quantitation was performed with IQMAC v1.2 software (Molecular Dynamics).

CHAPTER 2: Swi/Snf is Recruited to Promoters by Acidic Activators

Introduction

Chromatin structure inhibits transcription initiation by packaging DNA so that it is inaccessible for transcription factor binding (52-57). A number of chromatin remodeling complexes have been identified that perturb chromatin structure to allow transcription to occur (3). Swi/Snf is a member of the ATP-dependent chromatin remodeling family of complexes. It was originally identified in yeast, but Swi/Snf homologs were subsequently found in Drosophila and humans (180). Yeast Swi/Snf is a 2 MDa, 11 subunit complex that binds to the minor groove of DNA with nM affinity (181-183). Although ATP hydrolysis is not required for its DNA binding, it is required for its ability to remodel chromatin. Continued binding and ATP hydrolysis is required to persistently remodel nucleosomal arrays, but only in the absence of activators, which can stabilize the remodeled state (57, 184). Swi/Snf has been observed to use two different mechanisms to remodel chromatin. It can displace nucleosomes in trans in the presence of the activator Gal4 (185). It can also slide histone octamers in cis by spooling or twisting the DNA, a mechanism that is preferred over displacement in trans (184, 186).

Although Swi/Snf has been shown to stimulate transcription in vitro, it is not clear how it is able to influence transcription of specific genes in vivo. Three models have been postulated for Swi/Snf action at specific targets (180). In the catalytic model, Swi/Snf acts to transiently remodel chromatin throughout the genome. This transiently remodeled state is then stabilized at specific promoters by activators. This model is supported by evidence that nucleosome disruption by Swi/Snf enhances activator binding in vitro (54, 56, 185, 187), and in vivo to low affinity Gal4 activator binding sites (188). Activators have also been shown to stabilize remodeled chromatin states (57,

184). However, given that less than 5% of genes are influenced by Swi/Snf in vivo (33), and that Swi/Snf is a rare enzyme existing at only 100-500 copies per cell (180), it is hard to imagine that Swi/Snf can perform its remodeling functions transiently with any sort of efficiency. In the holopolymerase model, Swi/Snf is recruited to promoters through its association with Pol II/Med. This model is supported by experiments in which Swi/Snf has been purified as part of yeast and human holopolymerases (172, 189, 190). However, many Pol II/Med preparations do not contain Swi/Snf, and Swi/Snf preparations do not contain any Pol II/Med components (54, 93). In the activator model, Swi/Snf is specifically recruited to promoters by activators. This model is supported by a variety of evidence for activator Swi/Snf interactions. Yeast Swi/Snf has been shown to associate with glucocorticoid receptor (GR) in yeast whole cell extracts, and GR can stimulate human Swi/Snf dependent chromatin remodeling in vitro (191, 192). Human Swi/Snf can also bind the viral transactivator EBNA2, and the estrogen receptor in a ligand dependent manner (193, 194). Swi/Snf has also been shown to interact with c-myc in vitro, and mildly stimulate c-myc activation (195). Finally, the binding of the Swi5 activator at the HO promoter in vivo in yeast is required for Swi/Snf recruitment (196). This model, however, requires that activators be able to bind sites buried in chromatin. In fact, the activators Gal4, Gal4-VP16, and Swi5 have been shown to bind to their DNA sites in chromatin in vitro and in vivo in the absence of chromatin remodeling (196-198).

In order to begin to determine which of these models of Swi/Snf action at specific promoters is most likely, I have analyzed Swi/Snf recruitment to the HIS4 promoter in vitro, using a yeast nuclear extract immobilized template system. I show here that Swi/Snf is recruited to DNA independently of GTFs and Pol II/Med. This recruitment requires the binding of acidic activators, thus supporting the activator recruitment model of Swi/Snf action.

Results

To directly investigate the targeting of Swi/Snf to a promoter, I used yeast nuclear transcription extracts in combination with promoter templates immobilized on magnetic beads. This system was initially used to examine steps involved in PIC assembly as described in Chapter 1. Briefly, the wild type immobilized template consists of a modified HIS4 promoter linked to a magnetic bead with a single Gal4 DNA binding site upstream of the HIS4 TATA box (Fig. 11). PICs were formed by incubating yeast nuclear extract with the wild type template, followed by washing and liberation of the PIC with PstI restriction enzyme digestion. As described previously and in Chapter 1,

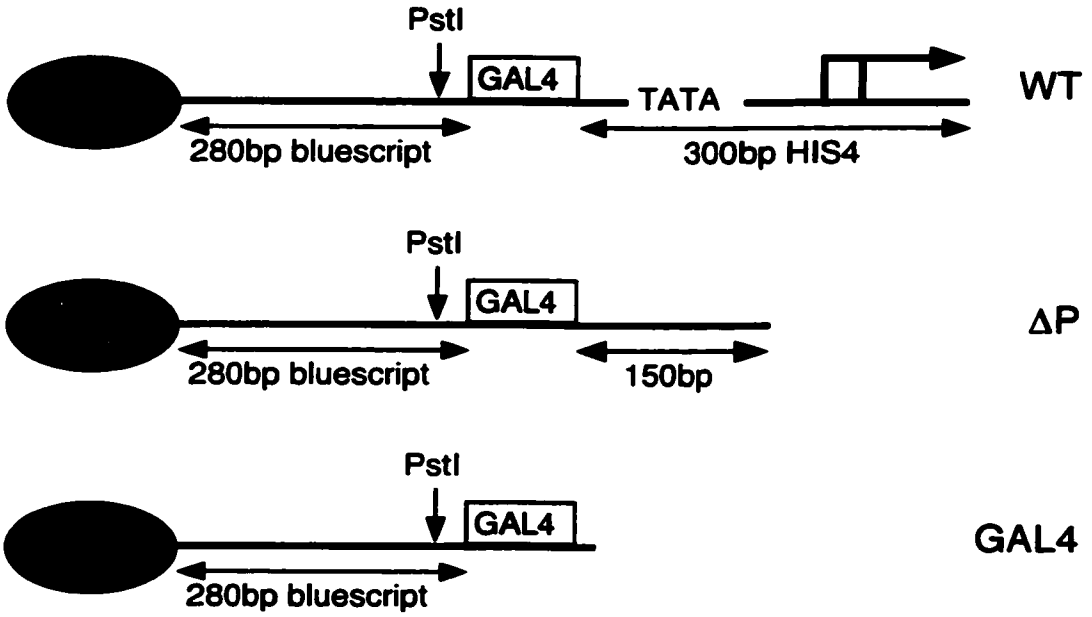

Figure 11. Immobilized templates used in this study. The wild type template contains the HIS4 core promoter and transcription start sites. The core promoter, including the TATA box, has been deleted and replaced by downstream non-promoter sequences to create the ΔP template. The GAL4 template was created by digesting the WT promoter sequences to leave just 14bp downstream of the Gal4 binding site.

PIC assembly in this system depends on the presence of a promoter, TATA box, TBP, TFIIA, and Pol II/Med subunits and is modestly stimulated by activators (168).

Figure 12 shows a Western blot of a PIC assembly experiment using the acidic activator Gal4-AH. Two subunits of the Swi/Snf complex, Swi3 and Snf5, were recruited to wild type templates from wild type yeast extracts (lane 1). To determine whether Swi/Snf is recruited to PICs as part of the Pol II/Med

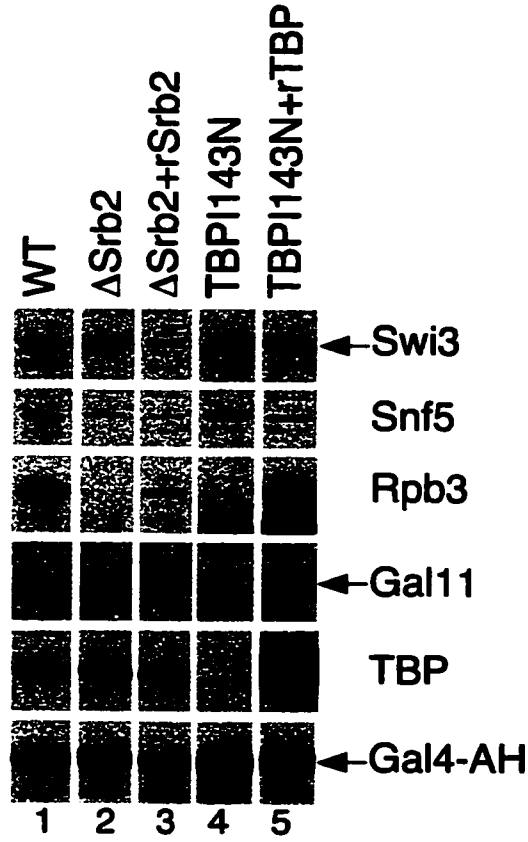

Figure 12. Recruitment of Swi/Snf in wild type and mutant NEs. PICs were formed with wild type (WT), ΔSrb2, or TBPI143N nuclear extract as described in Fig. 6a. PICs were isolated and analyzed by Western blot using antibodies against the components indicated at right. All reactions included the activator Gal4-AH. rSrb2 (200ng) and rTBP (400ng) were added where indicated. Swi3 and Snf5 are Swi/Snf components. Gal11 is a Mediator component.

complex, a nuclear extract made from an Srb2 deletion (ΔSrb2) yeast strain was used in the immobilized template assay. As shown in Chapter 1, this extract is defective in transcription, but its transcriptional activity can be restored by the addition of rSrb2. In addition, deletion or mutation of Mediator components such as Srb2 prevents the recruitment of the entire Pol II/Med complex to the promoter, but allows normal recruitment of TFIID and TFIIA in vitro (see Chapter 1). Figure 12 shows that levels of Rpb3, an RNA Pol II subunit, and Gal11, a Pol II/Med subunit, are severely decreased in the case of the ΔSrb2 nuclear extract, as compared to wild type (lane 1 vs. 2). Importantly, the levels of both components are increased upon the addition of recombinant Srb2 (rSrb2) (Fig. 12, lane3). Although the levels of Swi3 and Snf5 were slightly lower in the case of the ΔSrb2 extract, as compared to wild type, their binding was not stimulated by the addition of rSrb2 (Fig. 12C, lanes 1-3). Thus, the lower binding of Swi/Snf in this extract is probably due to a lower specific activity of this extract, and is not a consequence of the Srb2 deletion. To test whether Swi/Snf was recruited to the templates by a GTF not in the Pol II/Med complex, such as TFIID, an extract made from a TBP temperature sensitive mutant strain (TBPI143N) was used in the immobilized template assay. The I143N mutation in TBP abolishes TBP-DNA binding, and disrupts all PIC formation ((168, 199) and Fig. 12, lane 4). This TBP mutation did not affect recruitment of either Swi3 or Snf5 to the promoter, and addition of recombinant TBP (rTBP) did not stimulate the levels of these factors as it did Rpb3 and Gal11 (Fig. 12, lanes 4 and 5). Therefore, in this transcription system, recruitment of Swi/Snf to the promoter occurs independently of Pol II/Med and other GTFs.

Since previous work suggested that Swi/Snf was recruited to promoters after activator binding and that Swi/Snf can interact with some activators, I tested directly whether Swi/Snf was recruited to the templates by activator in

this assay. Immobilized template assays were performed without activator, with the Gal4(1-94) DNA binding domain, or with the activators Gal4-AH or Gal4-VP16, using both wild type and TBPI143N nuclear extracts. In the case of the wild type extract, Gal4-AH stimulated recruitment of Rpb3, Srb4, TFIIB, and Toa2 4-10 fold, as compared to recruitment in the absence of activator (Fig. 13, lane 2 vs. 6). As has been reported previously, Gal4-AH did not significantly recruit TBP (168). Gal4-VP16, however, stimulated recruitment of all these components 2-20 fold (Fig. 13, lane 2 vs. 8). Interestingly, recruitment of Swi3 and Snf5 by these activators was stronger than recruitment of Pol II/Med components. Gal4-AH increased Swi3 and Snf5 levels greater than 13-fold, while Gal4-VP16 increased Swi3 and Snf5 levels greater than 21-fold; both Swi3 and Snf5 were barely detectable in the absence of activator (Fig. 13, lanes 2, 6, and 8). To show that this recruitment was dependent on the activation domains, the Gal4(1-94) DNA binding domain was used as a control (Fig.13, lane 4). Although it weakly stimulated binding of Rpb3, Srb4, TFIIB, and Toa2, there was no stimulation of either Swi3 or Snf5 recruitment. Both Gal4-AH and Gal4-VP16 stimulated Swi3 and Snf5 recruitment to the same extent in TBPI143N nuclear extract as in wild type (Fig. 13, lanes 10, 12, and 14). Importantly, Swi3 and Snf5 recruitment was unaffected by addition of rTBP to restore PIC assembly (Fig. 13, lanes 16, 18, and 20). In the case of Gal4-AH, Swi/Snf recruitment was clearly occurring in the absence of recruitment of other PIC components. However, Gal4-VP16 was also able to recruit nearly wild type levels of Rpb3, Srb4, and TFIIB in the absence of rTBP, as noted previously (Fig. 13, lane 14 and (168)). This is because Gal4-VP16, unlike Gal4-AH, can interact with and recruit Pol II/Med in the absence of TBP (106, 168). By using recombinant GST-Swi3 as a standard in Western blots, it was determined that approximately 5 fmol of Swi/Snf were recruited to the promoter by Gal4-AH (data not shown). This is

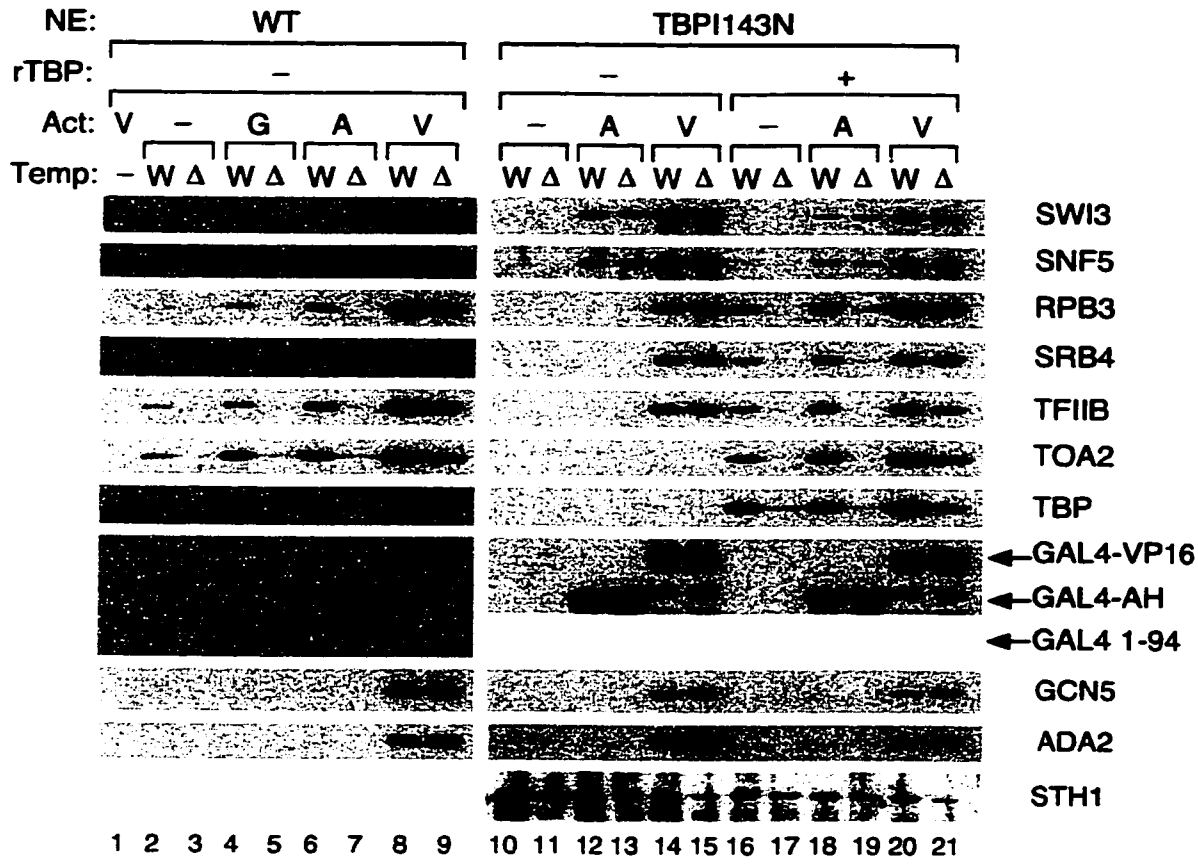

Figure 13. Swi/Snf is recruited to DNA by activators independently of promoter sequences. PICs were assembled on both wild type (W) and ΔP (Δ) templates as described in Fig. 6a, using the nuclear extracts (NE) indicated. Reactions were performed with no activator (-), the Gal4(1-94) DNA-binding domain (G), or the activators Gal4-AH (A) and Gal4-VP16 (V), as indicated. rTBP (400ng) was added where indicated. PICs were analyzed by Western blot using antibodies against the components indicated at right. The reaction in lane 1 was performed without templates as a control for non-specific binding to Dynabeads. GCN5 and ADA2 are SAGA components. STH1 is a RSC component.

significantly lower than the ~40 fmol of TFIIA, TFIIB, and TFIIE and ~200 fmol of TBP recruited by Gal4-AH in this assay (J. Ranish, unpublished). Since only ~10% of the templates are occupied by PICs in this assay (J. Ranish, unpublished), it is possible that Swi/Snf is not recruited to promoters as part of the PIC, but actually interacts with the unoccupied templates. These data are

consistent with the idea that Swi/Snf is recruited to promoters independently of Pol II/Med.

Next, I determined whether Swi/Snf recruitment by activators required the presence of promoter sequences. The HIS4 promoter sequences from the wild type template were deleted and replaced by downstream non-promoter DNA to form the ΔP template (Fig. 11). This template was then tested in immobilized template assays using a wild type nuclear extract. Swi3 and Snf5 recruitment by Gal4-AH and Gal4-VP16 was completely unaffected by deletion of the promoter sequences (Fig. 13, lanes 3, 7, and 9). As expected, only small to undetectable amounts of the other general factors probed for were present when Gal4-AH was used with this template, while nearly wild type amounts of Pol II/Med components were recruited by Gal4-VP16. Identical results were seen when the TBPI143N mutant extract was used (Fig. 13, lanes 11, 13, and 15). As before, the recruitment of Swi3 and Snf5 was unaffected by the addition of rTBP (Fig. 13, lanes 17, 19, and 21). These data show that recruitment of Swi/Snf by activators occurs independently of both GTFs and promoter sequences.

I also performed immobilized template assays using a template that terminated 14 bp downstream of the Gal4 site (Fig. 11). Digestion of this template with PstI after incubation with nuclear extract produced a 53 bp fragment (Fig. 14). As expected, Rpb3 and TBP are not recruited to this template. Interestingly, although ~2-fold less Gal4-AH bound to this template, there was no recruitment of Swi/Snf. These data, together with evidence that Swi/Snf can only bind pieces of DNA greater than 80 bp non-specifically (181), suggest that the interaction between Swi/Snf and activator requires DNA for stability, but not specific promoter sequences.

In a parallel series of experiments, Logie and Peterson analyzed the recruitment of Swi/Snf activity to a chromatin template consisting of 11 repeats

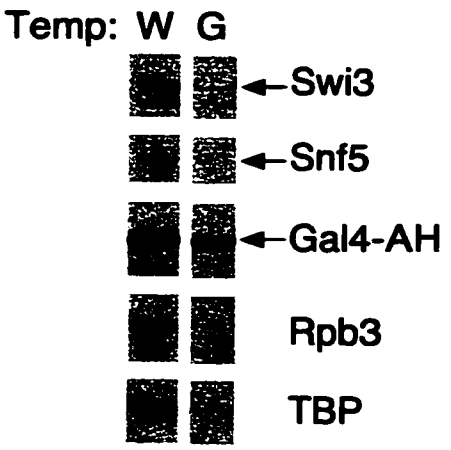

Figure 14. Swi/Snf requires DNA for activator recruitment. PICs were assembled on both wild type (W) and Gal4 (G) templates as described in Fig. 6a, using the wild type nuclear extract. PICs were analyzed by Western blot using antibodies against the components indicated at right.

of a *L. variegatus* 5S rRNA gene (200). Five Gal4 DNA binding sites were located in the middle of this nucleosomal array. Swi/Snf nucloesomal remodeling activity was measured by the accessibility of a restriction enzyme to a site just downstream of the Gal4 DNA binding sites. In agreement with my results, Gal4-AH and Gal4-VP16 recruited Swi/Snf activity to the 5S rRNA nucleosomal template, while the Gal4 (1-94) DNA binding domain did not. In addition, the Gal4-proline activator was not able to recruit Swi/Snf activity, suggesting that only acidic activators can perform this function.

I also tested the recruitment of two additional chromatin remodeling complexes. RSC, a member of the ATP-dependent chromatin remodeling family of complexes, is significantly more abundant in cells (1000-10,000 copies/cell) than Swi/Snf (181). Unlike Swi/Snf, RSC is essential for mitotic growth, suggesting that it may act on a more global scale, thus fitting the catalytic model of activity. In support of this, I found that the RSC subunit Sth1 bound to immobilized templates non-specifically and did not require the presence of activators, GTFs, Pol II/Med, or promoter sequences (Fig. 13,

lanes 10-21). The decrease in Sth1 levels seen on the ΔP template is most likely due to the fact that this template is ~150 bp shorter than the wild type template, coupled with the likelihood that multiple RSC complexes are associated with each template. The second complex analyzed in the immobilized template recruitment assay was SAGA, a member of the histone acetyltransferase (HAT) family of complexes. SAGA has already been shown to stimulate transcription of chromatin templates as a result of direct interaction with the acidic activators Gal4-VP16 and Gcn4, but not proline-rich activators (201, 202). In agreement with this, the SAGA subunits Gcn5 and Ada2 were recruited to immobilized templates by Gal4-VP16 in both wild type and TBPI143N mutant extracts (Fig. 13, lanes 8, 9, 14, 15, 20, and 21). As with Swi/Snf, this recruitment required the activation domain, but not GTFs, Pol II/Med, or promoter sequences (Fig. 13). In contrast to Swi/Snf, however, Gcn5 and Ada2 were not recruited by Gal4-AH. Gal4-AH was not previously used in assays where SAGA recruitment by other acidic activators was demonstrated. These results suggest that differences exist between acidic activators in their abilities to recruit SAGA.

The data presented here show that Swi/Snf is recruited to promoters by the acidic activators Gal4-AH and Gal4-VP16, supporting the activator recruitment model of Swi/Snf specific activity. This recruitment is independent of GTFs, the Pol II/Med complex, and promoter sequences, showing that in these nuclear extracts, as in other studies, yeast Swi/Snf is not associated with Pol II/Med, (54, 93). These data, along with those of Logie and Peterson described above, support a model in which acidic activators recruit Swi/Snf chromatin remodeling activity to promoters to allow PIC formation (Fig. 15). SAGA recruitment likely works by a similar mechanism, as it was also recruited to promoters in a GTF- and promoter-independent manner by Gal4-VP16. However, SAGA was not recruited by Gal4-AH, suggesting that not all

acidic activators can perform this function. RSC, on the other hand, associated non-specifically with immobilized templates, suggesting that it works through the catalytic model of chromatin remodeling. Taken together, these results suggest that a variety of mechanisms are used to target different chromatin remodeling complexes to specific promoters.

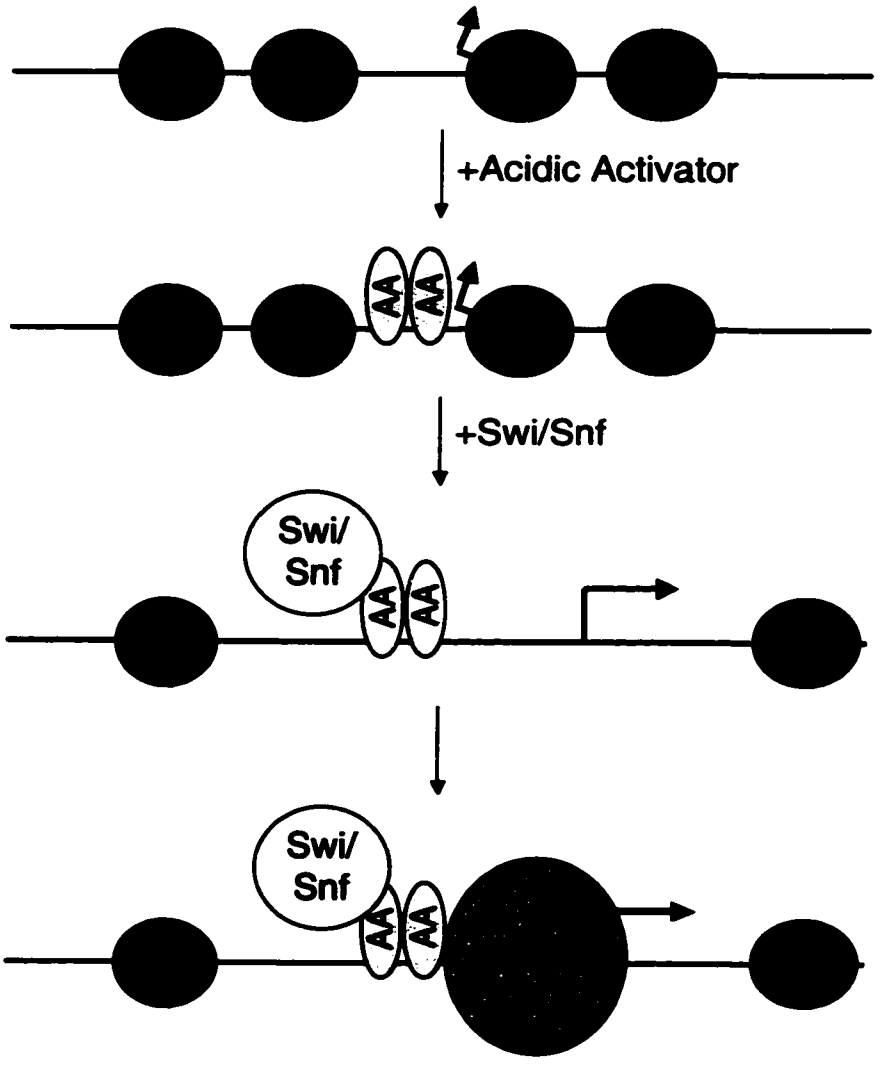

Figure 15. Swi/Snf is recruited to promoters by acidic activators. Acidic activators (AA) bind to activator binding sites in chromatin and recruit Swi/Snf. Swi/Snf remodels chromatin around the promoter, thus allowing PIC formation.

Materials and Methods

Yeast strains

The yeast strains used are described in Chapter 1, except TBPI143N (168).

Immobilized templates

Wild type template was prepared and biotinylated by PCR from pSH515 as described in Chapter 1. The ΔP template was made by PCR from pSH515ΔP. pSH515ΔP was created by digesting pSH515 with XhoI (NE Biolabs) and BamHI (Boehringer Mannheim) to remove a ~150 bp HIS4 promoter fragment. The cut plasmid was then purified, filled in with Klenow fragment (GibcoBRL), and religated. The ΔP template was prepared and biotinylated by PCR from pSH515ΔP using the same primers and reaction conditions as for pSH515. The Gal4 template was made by PCR using plasmid pSH515, primer p965 (see Chapter 1), and primer BKS7 (5'-TACCGAGCTCGAATTCGGAGG-3'). The PCR reaction resulted in a 360 bp fragment that ended 14 bp downstream of the Gal4 site. The templates were purified and immobilized on M-280 Streptavidin Dynabeads (Dynal) as described in Chapter 1.

Immobilized template assay

The immobilized template assay was performed in 100 μl reactions as described in Chapter 1. Each reaction contained 480 μg wild type, 480 μg ΔSrb2, or 360 μg TBPI143N nuclear extract, as indicated.

CHAPTER 3: Dissecting the Mechanism of Reinitiation

Introduction

High levels of gene transcription by RNA Pol II depend on high rates of transcription initiation and reinitiation. I have already described that initiation occurs by the stepwise, yet cooperative, recruitment of the complete transcription machinery to a promoter (see Chapter 1). Activators and chromatin remodeling factors facilitate this process. Although reinitiation likely involves the same complement of transcription factors, evidence suggests that it occurs through a different pathway (4).

Studies have shown that the rate of reinitiation is higher than that of initiation. Specifically, Jiang and Gralla (1993) looked at rates of open complex formation during initiation and reinitiation, and found that reinitiation occurred 3-4 times faster (77). A strong TATA box consensus sequence was also found to be important for maintaining high reinitiation rates (78). Interestingly, the initiator promoter element did not stimulate reinitiation, and, therefore, initiation and reinitiation at the TATA-less, initiator-containing DHFR promoter occurred at the same rate. Since TBP binds to TATA boxes, whereas TAFs bind to initiators, these studies suggest that the affinity of TBP for DNA is a more important factor for reinitiation than the affinity of TAFs for DNA. Some, but not all, activators have also been found to stimulate reinitiation. Heat shock factor stimulates reinitiation on both naked and chromatin templates (203), while estrogen receptor only does so on chromatin templates (204), suggesting that chromatin may also attenuate transcription by blocking or slowing reinitiation. The activators Gal4-AH and SP1, on the other hand, increase reinitiation rates very little or not at all (78, 205). Experiments with these activators, however, were performed only on naked DNA templates leaving unanswered their effects on chromatin templates. Interestingly,

several experiments have shown that continuous binding of activators at promoters is required to maintain high levels of transcription in vitro and in vivo (206, 207). In a particularly elegant series of in vivo experiments, Crabtree and colleagues separately fused DNA binding and activation domains to receptors. They could then activate transcription of a reporter gene by expressing a dimeric ligand that would bind to the receptors, thus linking the DNA binding and activation domains together. When the link was broken through overexpression of the monomeric ligand, transcription was found to decrease with the same kinetics as ligand-receptor dissociation.

The experiments described above, and others, suggested that some transcription factors remain stably associated with promoters during reinitiation (78, 208). Further study has in fact revealed that activators, TFIID, and TFIIA remain bound to transcription templates after transcription initiation (203, 207, 209). All of the studies have used an assay in which transcription templates fused to magnetic beads are used to isolate PICs. The factors that remain associated with the templates after initiation can then be isolated and assayed for reinitiation. Some of these assays were performed with cell extracts, but only monitored the binding of activators and select transcription factors, such as TFIID, TFIIB, TFIIA, and TFIIF (203, 207). In a more methodical study, Reinberg and colleagues used a purified transcription system consisting of TBP, TFIIB, TFIIF, TFIIE, TFIIH, and RNA Pol II to track release of factors from immobilized templates at various times after initiation (209). They found that TBP remained bound at the promoter after initiation, while all other factors were released. Interestingly, TFIIH was only released after RNA Pol II had reached +30, consistent with its role in promoter clearance (21, 27, 29, 210), while TFIIF could reassociate with elongating RNA Pol II at any time, consistent with its role in elongation (20, 211, 212).

A more thorough analysis of the transcription machinery during

reinitiation is required in order to understand the mechanism by which reinitiation occurs. In this chapter, I describe the use of the immobilized template assay and yeast nuclear extracts to isolate a reinitiation intermediate. Such an approach allowed me to monitor the entire transcription machinery in a crude Mediator-dependent transcription system, rather than one using purified factors. It also allowed for analysis of the role of activators in reinitiation through their effects on this intermediate.

Results

The immobilized template assay described in Chapter 1 was modified in order to isolate proteins that remained bound to the templates after a single round of transcription. The HIS4 promoter immobilized templates were incubated with nuclear extract and the activator Gal4-AH to allow PIC formation. The PICs were then washed, and transcription initiated by addition of nucleotides for two minutes. This procedure allows only a single round of transcription to occur, since the transcription signal detected is equivalent to that seen after incubation of PICs with NTPs, followed by addition of sarkosyl after one minute to block reinitiation (J. Ranish and S. Hahn, unpublished). Also, addition of NTPs to washed PICs for times longer than two minutes does not result in an increase in transcription, thus showing that washed PICs can undergo only a single round of transcription (J. Ranish and S. Hahn, unpublished). After nucleotide addition, proteins still bound to the templates were isolated. As expected from previous studies (203, 207, 209), RNA Pol II, TFIIB, and TFIIF dissociated from the templates, while activator, TBP, the TFIID subunit $TAF_{II}90$, and TFIIA remained bound to the promoters (Fig. 16, lane 2 vs. 3). Surprisingly, the Mediator complex (Srb4, Srb2, Med6, and Gal11 subunits), and substantial amounts of TFIIH and TFIIE also remained at the promoter. Specifically, the level of RNA Pol II was reduced 13-fold, the

level of TFIIB was reduced 24-fold, and the level of TFIIF was reduced 14-fold, while the levels of all other components were reduced less than 2.5-fold.

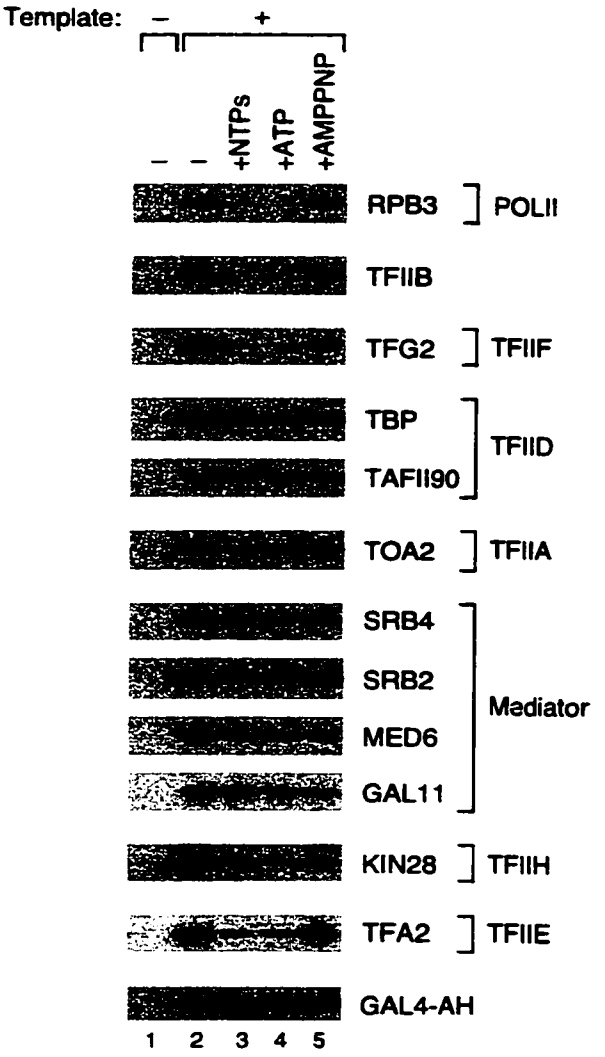

Figure 16. Scaffold contains activator, TFIID, TFIIA, Mediator, TFIIH, and TFIIE. PICs were formed on a wild type immobilized template (515 template) using the activator Gal4-AH and a wild type nuclear extract as described in Fig. 6a. Templates were washed and nucleotides added for 2 min as indicated. Templates were washed again, and bound proteins were isolated by PstI digestion and detected by Western blot. As a control for non-specific binding to the Dynabeads, the reaction in lane 1 was performed without template.

I then investigated whether this complex of activator, TFIID, TFIIA, TFIIH, TFIIE, and Mediator could function as a reinitiation intermediate, by acting as a scaffold upon which a functional transcription complex would reassemble. The scaffolds were formed and washed as described above. A second nuclear extract was then added along with nucleotides to determine whether a second round of transcription could occur (Fig. 17a). As a second extract, extracts made from strains with mutations in Mediator components (ΔSrb2 or Srb4ts), TFIIB (G41E), TFIIH (Kin28ts16), or TFIIE (Tfa1ts) were used. All of these extracts are defective in PIC assembly (see Chapter 1 and (168)) and transcription (Fig. 17b, lanes 1-7; Fig. 17c, lanes 1-3). As a control to show that few active PICs remained after the first round of transcription, very little RNA was produced when nucleotides were added to the scaffolds in the absence of a second extract (Fig. 17b, lane 8; Fig. 17c, lane 4). When supplemented with extracts from ΔSrb2, Srb4ts, and Kin28ts16 mutants, the scaffolds supported a second round of transcription, confirming the presence of these components in a functional reinitiation intermediate (Fig. 17b, lanes 8-12 and 15). However, little transcription was seen with the IIB(G41E) extract, confirming that TFIIB is not part of the scaffold (Fig. 17b, lanes 13 and 14). Importantly, while recombinant Srb2 restored transcriptional activity to the ΔSrb2 extract, it had no effect on transcription when the scaffold was used (Fig. 17b, lanes 2 and 3 vs. 10 and 11). Recombinant TFIIE stimulated transcription from the Tfa1ts extract 4-fold (Fig. 17c, lanes 2 and 3), compared to a 2-fold stimulation when scaffold templates were used (lanes 6 and 7). From this, and from Fig. 16, I conclude that the scaffold contains some functional TFIIE. From these data it is apparent that TFIIE is the least stable component of the scaffold, and that TFIIH also dissociates to some extent upon NTP addition. Similar experiments were performed with TBP and TFIIA mutant extracts that were also defective in transcription and PIC formation

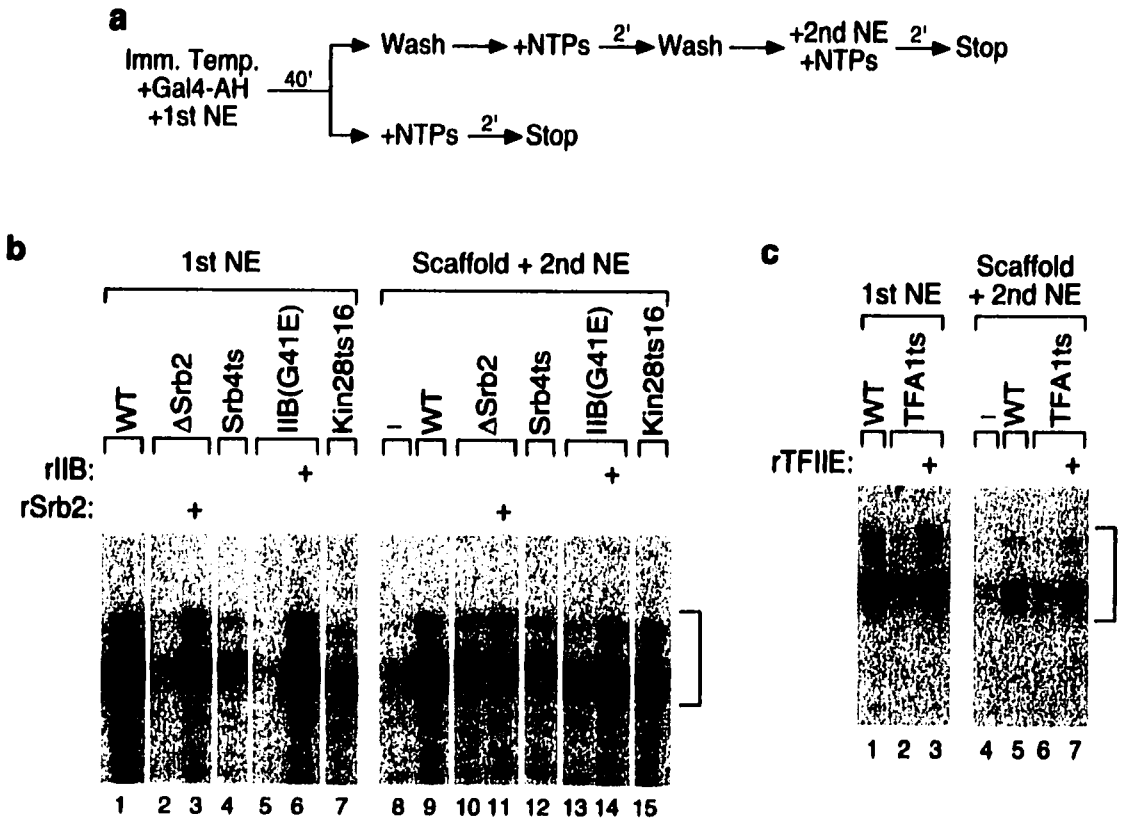

Figure 17. Scaffold supports reinitiation. **a.** The scaffold reinitiation assay. **b.** In lanes 1-7, nuclear extracts (NEs) were incubated with wild type immobilized templates for 40 min to form PICs. NTPs were added and reactions stopped after 2 min to allow for a single round of transcription. Reactions in lanes 8-15 were performed as described in **a**. NTPs were added along with the 2nd NE for 2 min. As a control for residual active complexes, no 2nd NE was added in lane 8. rTFIIB (30ng) and rSrb2 (100ng) were added where indicated. **c.** Reactions were performed as described in **b**. rTFIIE (80 ng) was added where indicated. Reactions were assayed by primer extension. Brackets indicate the transcription signal.

(205). The scaffolds supported a second round of transcription with these extracts, showing that TBP and TFIIA are present in a functional reinitiation intermediate.

The interpretation of the above results relies on the second round of transcription originating from scaffold templates, rather than newly formed PICs. This is a sound assumption given that new PIC formation needs 40 minutes to achieve saturation (J. Ranish and S. Hahn, unpublished), whereas the second round of transcription is only allowed to proceed for 2 minutes after addition of the second extract. However, to more clearly show that most of the transcription observed in the second round of initiation originated from scaffolds, rather than newly formed PICs, a competition assay was performed (Fig. 18a). Scaffolds were formed using the ΔSrb2 nuclear extract supplemented with rSrb2. A second competitor template was then added along with more ΔSrb2 extract either without rSrb2, to allow competition to occur, or with rSrb2 as a control. Competition was allowed to proceed for either 10 or 20 minutes, followed by addition of NTPs for 2 minutes to allow a single round of transcription to occur. Transcription from the competitor template was seen in the absence of recombinant Srb2, suggesting some instability of the scaffold complex (Fig. 18b, lanes 11 and 12). However, transcription from the scaffold template was not significantly affected by the addition of competitor (Fig. 18b, lanes 7, 8 vs. 11, 12). In addition, while transcription from the second template was stimulated 2-fold by the addition of rSrb2, transcription from the scaffold template was not significantly stimulated (Fig. 18b, lanes 7-10 vs. 11-14). Taken together, these data show that the majority of scaffold complexes are stable to competition, and that the majority of second round transcription originates from the scaffold template. Therefore, these results support the conclusion that the scaffold is a functional reinitiation intermediate.

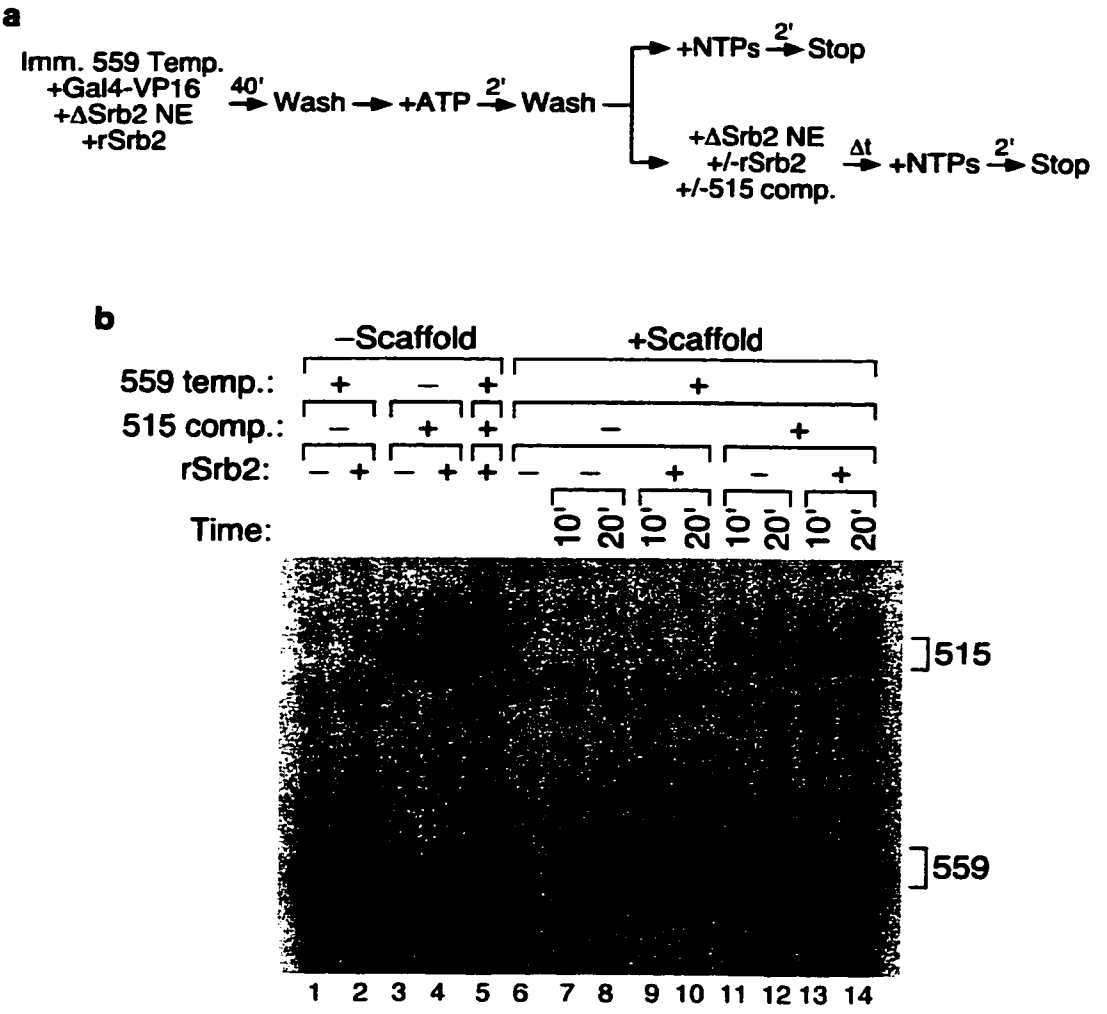

Figure 18. The majority of scaffolds remain stably associated with the template during competition. **a.** Competition assay. Imm. 559 Temp. = immobilized 559 template. 515 comp. = immobilized 515 template competitor. The 559 template is identical to the wild type 515 template except that it contains a 50bp deletion in the transcribed region. **b.** In lanes 1-5 the stated immobilized templates were incubated with ΔSrb2 NE either in the absence or presence of rSrb2 (100ng) for 40 min to form PICs. NTPs were then added for 2 min to allow a single round of transcription to occur. Reactions performed in lanes 6-14 used scaffolds formed on the 559 immobilized template as described in **a**. Scaffolds were incubated with ΔSrb2 NE in either the absence or presence of 515 competitor for either 10 or 20 min. As a control for residual active complexes no NE was added in lane 6. NTPs were then added for 2 min to allow a single round of transcription to occur. Reactions were assayed by primer extension. Brackets indicate the transcription signals.

I was able to isolate this functional reinitiation intermediate despite the low percentage of active PICs formed in this assay. The number of active PICs was determined by measuring the amount of RNA produced in a single round of transcription. Comparing this number to the total number of PICs formed showed that only 5-10% of PICs were active in transcription (J. Ranish and S. Hahn, unpublished, and discussed is Discussion section). These data suggest that the scaffold complexes isolated by this assay are the result of dissociation of both active and inactive PICs. Since these complexes can support reinitiation, these results imply that both active and inactive PICs dissociate by the same mechanism upon nucleotide addition.

I also analyzed the effects of activator on scaffold formation and stability. Although activators are known to act by stimulating PIC formation through transcription factor recruitment, there is evidence that they also play a role in reinitiation. The heat shock factor (203) and estrogen receptor (204) transcription activators, as well as the HIV-1 enhancer (213) stimulate reinitiation in vitro. Other in vitro (207) and in vivo (206) experiments showed that the presence of activator at the promoter is required for continued high levels of transcription. Since PIC dissociation can occur in the absence of activator (Fig. 19, lanes 1 and 2), scaffold stability was measured in either the absence of activator, or the presence of the activators Gal4-AH or Gal4-VP16. The scaffold was formed as described in Fig. 16 and analyzed by Western blot after incubation in transcription buffer for up to 40 minutes (Fig. 19). In either the absence of activator or the presence of Gal4-AH the levels of TBP, TFIIA, Srb4, Srb2, and Med6 decreased by 3- to 5-fold after 40 minutes. In contrast, when Gal4-VP16 was used, the levels of all of these factors remained steady after 40 minutes. These results show that although the scaffold reinitiation intermediate can be formed without activator, it is more stable in the presence of Gal4-VP16. Since studies have shown that some activators can interact

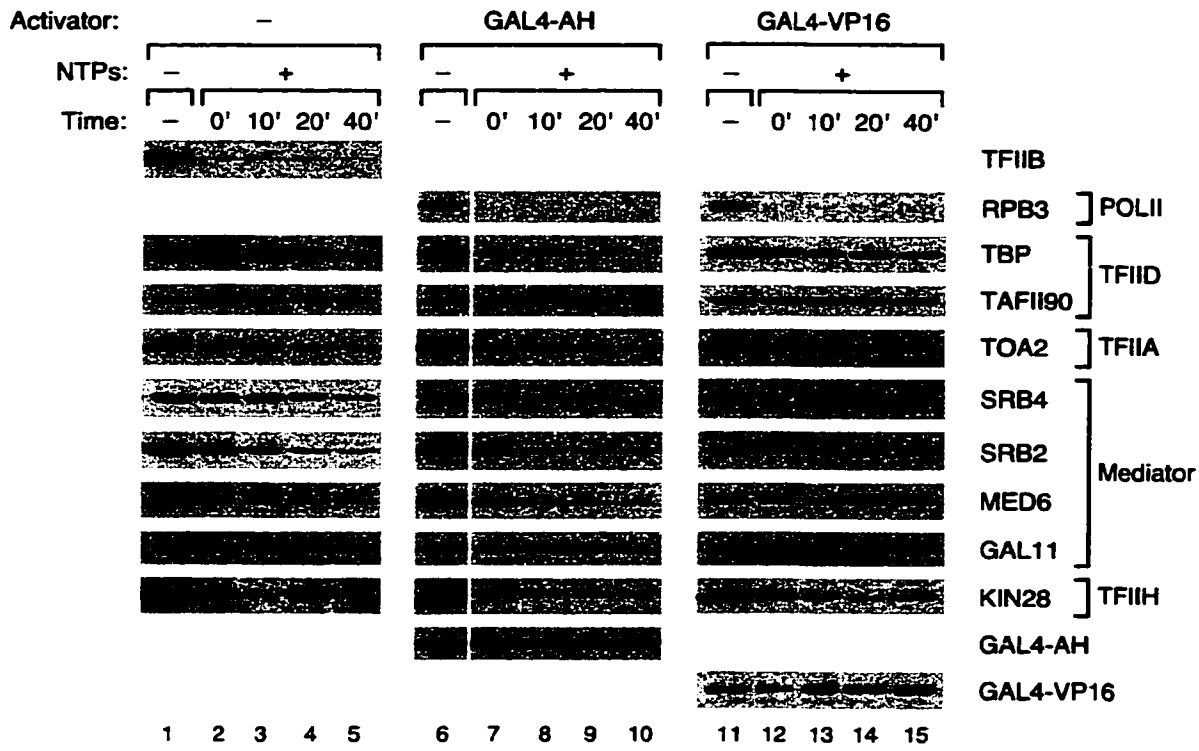

Figure 19. Gal4-VP16 promotes scaffold stability. **a.** 515 wild type immobilized templates were preincubated with Gal4-AH, Gal4-VP16, or no activator. Scaffolds were formed using wild type NE as described in Fig. 16. Scaffolds were incubated in transcription buffer for the times indicated, washed for 1 min, and bound proteins analyzed by Western blot. As controls, PICs are shown in lanes 1, 6, and 11.

with TFIID (214), TFIIA (215), and various Mediator components (96, 108), this stabilization is likely due to interactions between activators and scaffold components. In experiments performed in parallel with these, transcription rates were measured following saturating PIC formation in the absence of activator and in the presence of Gal4-AH and Gal4-VP16 (205). RNA was rapidly produced from the preformed PICs followed by a slower rate of RNA synthesis resulting primarily from reinitiation events. The results showed that with Gal4-VP16, the rate of transcription after the first round was 10-fold

higher than with no activator and 3-fold higher than with Gal4-AH. These data indicate a correlation between scaffold stability and the rate of reinitiation, and support a role in scaffold stability for some activators in reinitiation.

Interestingly, I found that adding only ATP to PICs had the same effect as adding all four nucleotides, with both resulting in PIC dissociation and loss of active PICs (Fig. 16 and data not shown). Further analysis revealed that neither the ATP analog AMPPNP nor GTP promoted PIC dissociation, while ATPγS did (Fig. 16, lane 5; Fig. 20). These results suggest that ATP hydrolysis, rather than transcription, is necessary for PIC dissociation. I therefore attempted to identify a PIC component with ATP-dependent activity

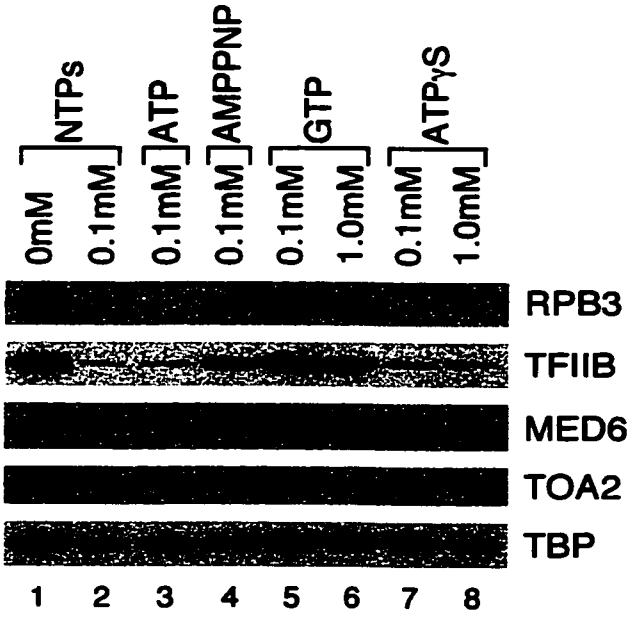

Figure 20. PIC dissociation is ATP-dependent. PICs were formed on 515 wild type immobilized templates using the activator Gal4-AH and wild type nuclear extract as described in Fig. 6a. Templates were washed and nucleotides (NTPs, ATP, GTP) or nucleotide analogs (AMPPNP, ATPγS) added for 2 min as indicated. Templates were washed again, and bound proteins were isolated by PstI digestion and detected by Western blot. MED6 is a Mediator component.

that could be responsible for PIC dissociation. Three subunits of TFIIH were good candidates: the helicases Rad25 and Rad3, and the CTD kinase Kin28. Nuclear extracts were made from transcriptionally defective strains that contained temperature sensitive mutations in either Rad3 or Kin28, and tested for PIC dissociation. As shown in Chapter 1, these extracts form identical PIC intermediates that likely lack the entire TFIIH complex (Fig. 21, lanes 2, 4, and 6). After ATP addition, PICs lacking TFIIH were not able to dissociate into scaffolds, indicating that PIC dissociation is ATP- and TFIIH-dependent (Fig. 21, lanes 3, 5, 7). Because PICs formed with both TFIIH mutant extracts probably lack the entire TFIIH complex, it was impossible to determine which of the TFIIH subunits is necessary for PIC dissociation.

Some evidence implicates Kin28 as a probable candidate for PIC dissociation. Kin28 can use ATPγS as a substrate, while the TFIIH helicases cannot (28, 29, 216). In addition, studies have shown that phosphorylated, elongating RNA Pol II is not associated with Mediator (147), suggesting that CTD phosphorylation may be required for the dissociation of Mediator from RNA Pol II during scaffold formation. To further analyze the role of Kin28 in PIC dissociation, I prepared a nuclear extract from a second Kin28 temperature sensitive mutant (Kin28ts3). This extract was defective in in vitro transcription assays, but its activity was restored by the addition of purified CAK (L. Warfield and S. Hahn, unpublished). When the Kin28ts3 extract was used in the immobilized template PIC formation assay, levels of all components probed for, except Kin28, were similar to wild type and were not stimulated by the addition of CAK (Fig. 22, lanes 1, 9, and 11). Importantly, while Kin28 was absent from these PICs, wild type levels of Tfb1 were present. These results suggest that PICs formed with the Kin28ts3 mutant extract lack CAK, but not the helicase-containing core TFIIH. Addition of ATP to these mutant PICs did not result in PIC dissociation, but this activity could

be restored by CAK, suggesting that CAK is required for PIC dissociation (Fig. 22, lanes 9-12).

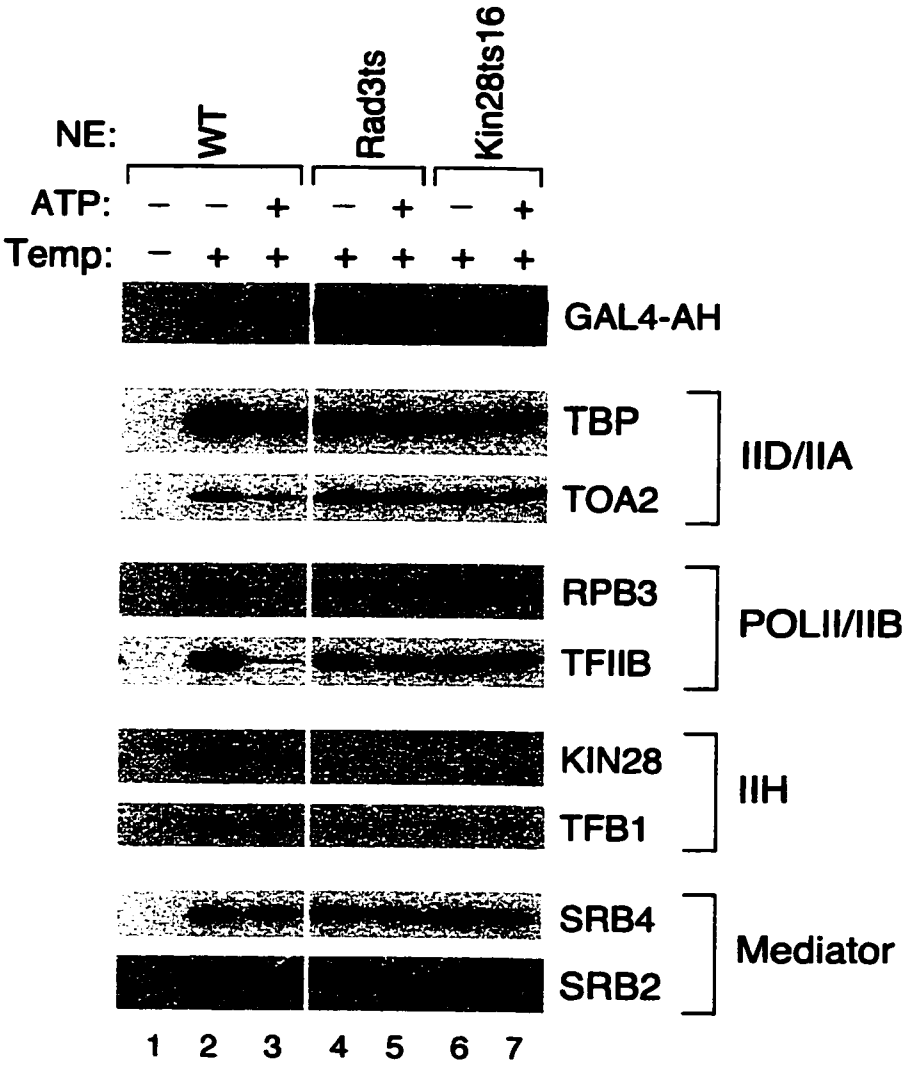

Figure 21. PIC dissociation is TFIIH-dependent. PICs were formed on 515 wild type immobilized templates using Gal4-AH and the indicated NEs as described in Fig. 6a. Templates were washed and ATP was added to the reactions where indicated. After washing again, factors bound to the templates were assayed by Western blot. A typical wild-type PIC and a typical wild-type scaffold are shown in lanes 2 and 3 respectively. As a control for non-specific binding to Dynabeads, the reaction in lane 1 was performed without template.

I next investigated CTD phosphorylation in both wild type and Kin28ts3 nuclear extracts during scaffold formation. The yeast CTD consists of 26 repeats of the peptide YSPTSPS, and is phosphorylated on Ser5 by Kin28 (150). I therefore monitored CTD phosphorylation by adding $^{32}P\gamma ATP$ to the reactions, as well as using an antibody specific for the Ser5 phosphorylated form of the CTD. Since the majority of RNA Pol II is released from PICs after ATP addition, both supernatants and template bound fractions were analyzed in these experiments. ATP addition to a wild type PIC resulted in CTD phosphorylation, whereas ATP addition to the Kin28ts3 mutant PIC did not (Fig. 22, lane 2 vs. 10). Addition of purified CAK to the mutant PIC restored its CTD phosphorylation activity, showing that CAK is required for CTD phosphorylation (Fig. 22, lane 10 vs. 12). Taken together, these results show that PIC dissociation requires CAK and is correlated with CTD phosphorylation. Interestingly, although the Rpb3 signal indicates that the large majority of RNA Pol II was released from templates after ATP addition, the ^{32}P-labeled Rpb1 signal indicates that a significant amount of phosphorylated Rpb1 remained on the template and only 2-fold more was released (Fig. 22, lane 2). In addition, equivalent amounts of Ser5 phosphorylated Rpb1 were seen in both the template bound and supernatant fractions, suggesting that a significant amount of Ser2 phosphorylated Rpb1 was released. It is also possible that phosphorylation does not immediately result in release of Rpb1 from the template, but simply makes it less stable, thus allowing for undetected release during the template washes. The fact that a large majority of the CTD is phosphorylated after ATP addition is supported by experiments using an antibody specific for the unphosphorylated form of Rpb1, in which this unphosphorylated signal decreases dramatically after ATP addition (data not shown). Paradoxically, in experiments where ATP was added to mutant PICs formed with Kin28ts3 extract, addition of CAK

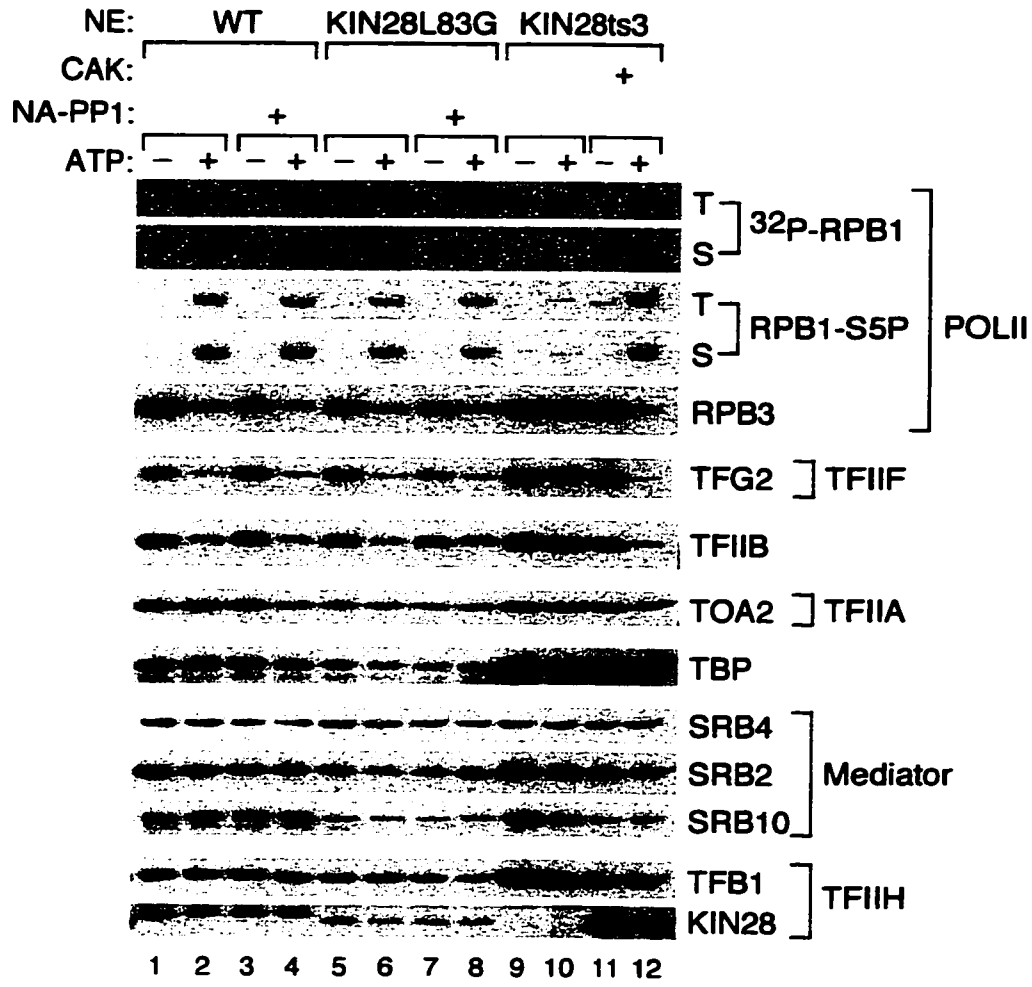

Figure 22. PIC dissociation is CAK-dependent. PICs were formed on 515 wild type immobilized templates using Gal4-VP16 and the indicated NEs as described in Fig. 6a. NA-PP1 (200nM) and 1 µl purified CAK were added where indicated. Templates were washed, and ATP, along with ^{32}P-γATP (10 µCi of 3000 Ci/mmol, NEN), was added for 2 min where indicated. The supernatants were removed, TCA precipitated, resuspended in 1X NuPAGE sample buffer (NOVEX), and analyzed by autoradiography and Western blot in panels labeled S. The templates (T and all other panels) were washed, and bound proteins isolated and analyzed by autoradiography and Western blot.

resulted in significantly more ^{32}P-Rpb1 release into the supernatant, while the amount of Ser5 phoshorylated Rpb1 remained identical in both template and supernatant fractions (Fig. 22, lane 2 vs. 12). Since Kin28 has been shown to

predominantly phosphorylate Ser5 of the CTD (150), one would expect that the addition of purified CAK would lead to an increase in Ser5 phosphorylation in the supernatant. Some of this disparity can be explained by the fact that the two methods used to detect CTD phosphorylation produce signals that can be interpreted in different ways. The ^{32}P-Rpb1 signal is an indicator of the amount of phosphate incorporated into the CTD. The Ser5-phosphorylation signal, on the other hand, is the result of an antibody interaction with the hyperphosphorylated CTD, suggesting that it is an indicator of the number of hyperphosphorylated CTD molecules. Since the yeast CTD consists of 26 repeats of the peptide YSPTSPS, hyperphosphorylation at Ser5 could lead to an increase in the ^{32}P signal without a concomitant increase in the amount of Ser5 phosphorylated Rpb1. Therefore, the addition of CAK to the Kin28ts3 mutant PIC likely resulted in preferential release of hyperphosphorylated Rpb1 into the supernatant, while the total amount of Ser5 phosphorylated Rpb1 in the supernatant was similar to that which remained associated with the templates (Fig. 22, lane12).

In order to analyze the role of Kin28 kinase activity in CTD phosphorylation and PIC dissociation more specifically, the ATP binding pocket of Kin 28 was enlarged by mutation of leucine 83 to glycine (S. Hahn, unpublished). Such mutations have been made in other cyclin dependent kinases, and shown to have little effect on kinase activity (217). However, inhibitors, such as NA-PP1, can be created which fit in these enlarged binding pockets and inhibit kinase activity with nanomolar specificity (217). Therefore, the Kin28 L83G mutation would allow me to inhibit kinase activity while Kin28 remains associated with the PIC. The Kin28 L83G mutant yeast strain grew normally, and nuclear extracts made from this strain showed wild type levels of transcription (S. Hahn, unpublished). Surprisingly, addition of NA-PP1 maximally inhibited transcription by only 2-fold (S. Hahn, unpublished). PICs

formed on immobilized templates were similar to wild type, and showed wild type levels of Rpb3, TFIIF, and TFIIB dissociation upon ATP addition (Fig. 22, lanes 1, 2 vs. 5, 6). Addition of the inhibitor NA-PP1 along with ATP resulted in a 2-fold decrease of the ^{32}P-Rpb1 signal, but did not affect levels of Ser5 phosphorylated Rpb1 or PIC dissociation significantly (Fig. 22, lanes 5, 6 vs. 7, 8). This result suggests that NA-PP1 may inhibit only the extent of CTD phosphorylation, and not the total amount of Ser5 phosphorylated Rpb1.

Since the extent to which NA-PP1 inhibits Kin28 L83G kinase activity has not been directly determined, these results can be interpreted in three ways. First, if NA-PP1 completely inhibits Kin28 L83G kinase activity, then Kin28 activity is not required for PIC dissociation. However, the results with the Kin28ts3 extract show that the presence of CAK is required for PIC dissociation. This interpretation does not rule out the possibility that CTD phosphorylation is required for PIC dissociation, since the amount of Ser5 phosphorylated Rpb1 is unaffected by Kin28 inhibition. It is therefore possible that another CTD kinase, whose association with the PIC requires the presence of CAK, is responsible for CTD phosphorylation and PIC dissociation in this system. Three such CTD kinase candidates are Bur1, Ctk1, and Srb10. Srb10 can be ruled out as a candidate, however, since it remains associated with the Kin28ts3 mutant PIC and its levels are not stimulated by the addition of CAK (Fig. 22, lane 9 vs. 11). The importance of Bur1 and Ctk1 in CTD phosphorylation and PIC dissociation in this system remains to be determined. A second interpretation of these results is that NA-PP1 does not completely inhibit Kin28 L83G kinase activity, and this residual activity is sufficient for CTD phosphorylation and PIC dissociation. This interpretation is supported by evidence that Kin28 is responsible for most of CTD phosphorylation in yeast in vivo (32). Finally, it is also possible that the CTD is not the only target of Kin28 kinase activity, and that phosphorylation of this other target is

responsible for PIC dissociation. Indeed, when $^{32}P\gamma ATP$ is used in PIC dissociation experiments, a number of proteins besides Rpb1 are detected (data not shown). Further analysis of the effect of NA-PP1 on Kin28L83G kinase activity, however, is required to distinguish between these interpretations.

Taken together, these results suggest a model for reinitiation in which activator, Mediator, TFIID, TFIIA, TFIIH, and TFIIE remain at the promoter after RNA Pol II initiates transcription (Fig. 23). These factors are components of a scaffold upon which other factors can assemble to form a reinitiation complex. Since the binding of TFIID to promoters has been shown to be a rate-limiting step in transcription initiation in vivo (75, 76), such a model can account for the observation that rates of reinitiation are higher than those of initiation (77). In fact, a prediction of this model is that the rate of transcription initiation on a scaffold template would be higher than that on a naked template. To test this prediction, transcription was monitored from both a naked (pSH515) and a scaffold-containing (pSH559) plasmid template without prior PIC formation (Fig. 24a). Plasmid templates were used, because immobilized templates were unable to undergo more than two rounds of transcription, and therefore did not allow the reliable calculation of a transcription rate (data not shown). The fact that immobilized templates do not reinitiate well suggests that something about the Dynabead inhibits transcription. This, however, does not invalidate the reinitiation intermediate isolated with the immobilized template, since it can support reinitiation when provided with a mutant nuclear extract (Fig. 17). As predicted by the reinitiation model, initial rates of transcription from the scaffold plasmid template were 2- to 3-fold higher than those from the naked plasmid template (Fig. 24b). These data are consistent with results showing that previously transcribed templates are preferentially transcribed (208).

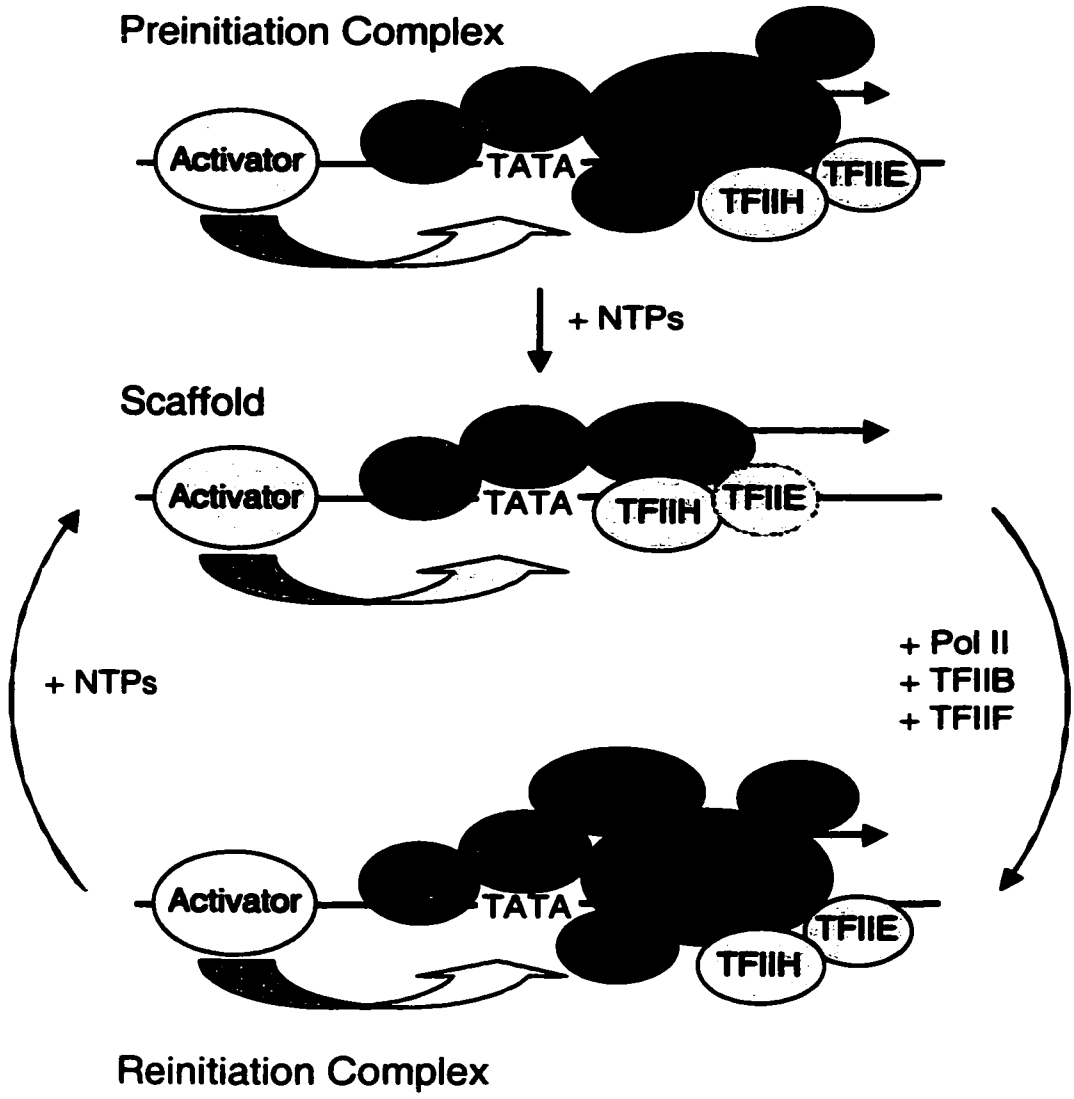

Figure 23. Reinitiation model. When NTPs are added to a preinitiation complex, RNA Pol II is phosphorylated and initiates transcription. Whereas TFIIB and TFIIF dissociate from the promoter, activator, TFIID, TFIIA, TFIIH, TFIIE, and Mediator are left behind in a scaffold complex. RNA Pol II, TFIIF, and TFIIB then reassemble onto the scaffold to form a complex capable of reinitiating transcription. TFIIE is shown as being the least stable scaffold component.

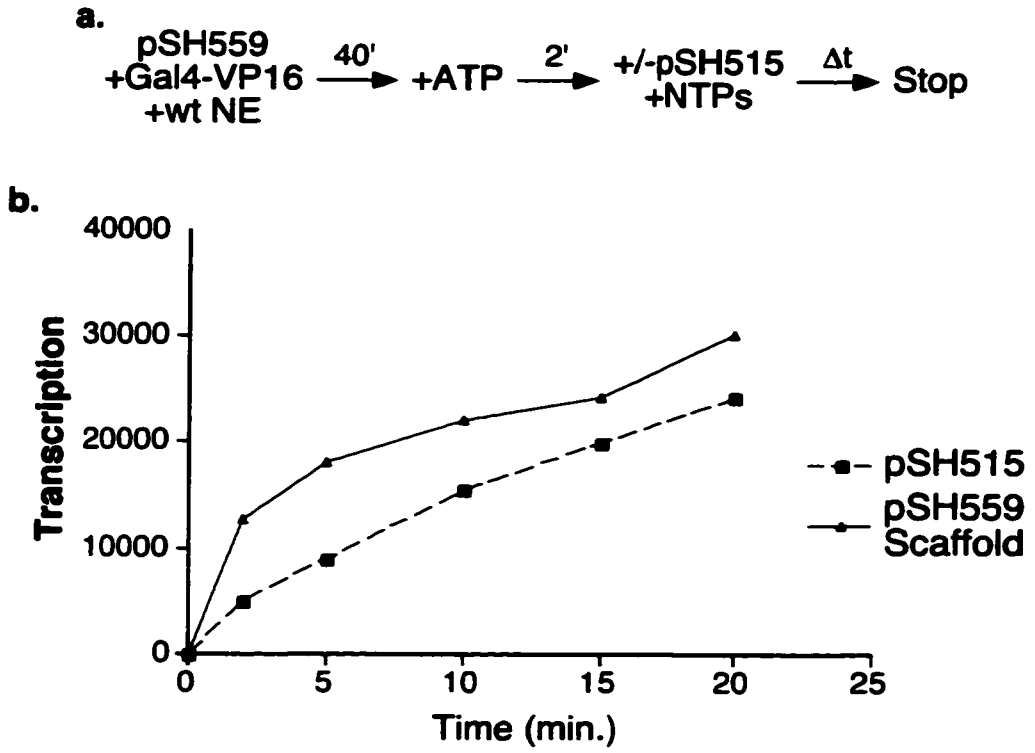

Figure 24. Transcription of a scaffold template occurs at a higher initial rate than that of a naked template. **a.** The pSH559 plasmid template was incubated with wild type NE to form PICs. ATP was added to allow PICs to dissociate into scaffolds. NTPs were then added for various times either with or without the pSH515 naked plasmid template. Reactions were assayed by primer extension. The pSH559 plasmid template is identical to the pSH515 plasmid template except that it contains a 50bp deletion in the transcribed region. **b.** The results of the experiment described in **a** were plotted as transcription units vs. time. The primer extension signals were quantitated using the Phosphorimager, and then normalized to each other.

The results described here also show that the scaffold reinitiation intermediate is stabilized by the activator Gal4-VP16, and that this stability is correlated with a high rate of reinitiation, suggesting a new role for activators in transcription. In addition, the formation of this reinitiation intermediate is correlated with CTD phosphorylation and requires the Kin28-containing CAK subcomplex of TFIIH, suggesting that CTD phosphorylation may allow RNA Pol II to dissociate from Mediator, and thus initiate transcription.

Materials and Methods

Yeast strains

Wild type, ΔSrb2, Srb4ts, Kin28ts16, and Tfa1ts strains were described in Chapter 1. All other strains are described in Table 2.

Table 2. Strain list

Strain	Name	Genotype	Reference
BY4705	wild type	*MATα Δade2::HISG his3Δ200 leu2Δ0 met15Δ0 trp1Δ63 ura3Δ0 lys2Δ0*	(218)
SHY245	IIB(G41E)	*MATα* Ade- *leu2-3,112 ura3-52 his4-519 sua7Δ::HIS4* pRK68.30 (ARS CEN *sua7G41E*)	(168)
JGV4	Kin28ts3	*MATα leu2 trp1 ura3 his3 kin28ts3*	(32)
SHY442	Kin28L83G	*ade2-1 ade3-22 his3-11,15 leu2 trp1-1 ura3-1 can1-100 Δkin28::LEU2 TRP1:kin28L83G*	S. Hahn, pers.comm.
NYY6	wild type	*ade2-1 ade3-22 his3-11,15 leu2 trp1-1 ura3-1 can1-100 Δkin28::LEU2* pNY4(ARS CEN URA3 HA-KIN28)	this work

Preparation of yeast nuclear extracts

Yeast nuclear extracts were prepared from 2 liter cultures as described previously (178) and on the world wide web (www.fhcrc.org/labs/hahn).

Immobilized templates assay

Immobilized templates were prepared and PIC formation was performed as described in Chapter 1. Scaffold isolation was performed similarly, except that after washing, PICs were resuspended in 100 μl transcription mix, and incubated with 1 μg HaeIII digested *E. coli* DNA competitor, and the stated amounts of NTPs, ATP, AMPPNP, GTP, or ATPγS for 2 min at room temperature. The templates were washed once with wash

buffer, isolated by digestion with 60 units PstI for 30 min at 37°C, and processed as described in Chapter 1. For the scaffold stability experiment, scaffolds were isolated as described above, except that after being washed, they were resuspended in transcription mix with 1 μg HaeIII digested *E. coli* DNA competitor. Aliquots of 100 μl were removed at the indicated times, washed once with wash buffer, and isolated and processed as described in Chapter 1.

Transcription

Plasmid transcription was performed by incubating wild type nuclear extract with the HIS4 promoter-containing plasmid, pSH515 or pSH559, as described previously (179) and on the world wide web (www.fhcrc.org/labs/hahn). pSH559 was made by digesting pSH515 with BamHI and SfoI to delete 50 bp of transcribed sequence. In experiments where both pSH515 and pSH559 were used, the Cyc1 primer (5'-GAGAGGCGGTTTGCGTATTGGG-3') was used for primer extension. Transcription on immobilized templates was performed as described in Chapter 1. The RNA was isolated by phenol:chloroform (2:1) extraction and ethanol precipitation. Primer extension was performed on the RNA samples as described previously using either the LacI primer or the Cyc1 primer (179). For scaffold functional assays, scaffolds were formed as described above using wild-type nuclear extracts. After washing, scaffolds were resuspended in transcription mix containing 120–180 μg of a second nuclear extract and 500 ng HaeIII digested *E. coli* DNA competitor. NTPs were added to 100μM immediately. Reactions were stopped after 2 min and analyzed by primer extension. For the scaffold competition experiment, scaffolds were formed on the 559 immobilized template as described above using ΔSrb2 nuclear extract with 100 ng rSrb2. After washing, an equivalent amount of 515 immobilized

competitor template was added, and the reactions were resuspended in transcription mix containing ΔSrb2 nuclear extract either with or without 100 ng rSrb2. Reactions were incubated for either 10 or 20 min at room temperature. NTPs were then added for 2 min, and the reactions were stopped and analyzed by primer extension using the Cyc1 primer. All transcription signals were quantitated by PhosphorImager (Molecular Dynamics).

DISCUSSION

The process of transcription requires the coordinate binding of a large number of proteins to DNA, which must then act on each other and RNA Pol II to begin to catalyze the formation of an mRNA. This factor assembly must occur many times in order to generate multiple transcripts. Activators can stimulate this process by interacting with transcription factors, while repressors can block factor assembly. In addition, the initial formation of a PIC frequently requires the aid of chromatin remodeling factors that can loosen DNA-histone contacts, making DNA more accessible. In this work, I have described how some chromatin remodeling complexes can be recruited to promoters, a mechanism by which PIC formation can occur, and a mechanism by which transcription factors can reassemble at promoters during reinitiation.

To analyze how chromatin remodeling factors are targeted to specific promoters, I focused on the ATP-dependent chromatin remodeling complex Swi/Snf. Using an immobilized template assay, I showed that Swi/Snf can be recruited to promoters by acidic activators in a process that is independent of GTFs and promoter sequences. These results have since been supported by new evidence that Swi/Snf remodeling activity is recruited to chromatin by activators to stimulate transcription both in vitro (200, 219) and in vivo (107, 196, 220, 221). The SAGA histone acetyltransferase complex has also been shown to be recruited to promoters by some acidic activators in vitro (201, 202) and in vivo (222, 223). Interestingly, yeast gene expression analysis showed that 25% of genes expressed during mitosis require both Swi/Snf and SAGA for activation (69). In vivo chromatin immunoprecipitation (ChIP) analysis at mitotic genes has shown that Swi/Snf binding is required for SAGA recruitment (69, 196), suggesting that in densely packed mitotic chromatin Swi/Snf is needed to loosen histone-DNA contacts to make histone tails

accessible for acetylation. However, the reverse was seen at the human IFN-β promoter, where SAGA binding is required for Swi/Snf recruitment (222). At this promoter acetylation of histone tails may be required for Swi/Snf to bind stably to nucleosomes, since the Swi/Snf subunit Swi2 contains a bromodomain that can interact with acetylated lysines (224). In support of this hypothesis, recent experiments have shown that histone acetylation increases the retention of Swi/Snf on promoters in vitro (225). Although Swi/Snf and SAGA are both required for mitotic gene expression, they work independently at most promoters during interphase (33). Therefore, it is possible that local chromatin structure dictates whether one or both of these remodeling complexes are required for transcription.

Although all of my Swi/Snf experiments were performed in the yeast system, recent evidence suggests that mammalian Swi/Snf can also be recruited to promoters by activators. The glucocorticoid receptor has been shown to interact with purified human Swi/Snf and this interaction stimulates both chromatin remodeling and transcription in vitro (192, 226). Human Swi/Snf can also interact with the estrogen receptor in the presence of estrogen in vitro (193), and is recruited to estrogen responsive genes in vivo, where it is required for activation (227). Mammalian Swi/Snf can interact with beta-catenin in vitro, and thereby promotes activation of beta-catenin/Tcf complex responsive reporter genes in vivo (228). Finally, human heat shock factor has been shown to bind Swi/Snf in vitro and recruit it to chromatin templates (229). Thus the activator recruitment model of Swi/Snf action at specific promoters applies to mammalian systems as well as to yeast.

It is likely that not all chromatin remodeling complexes work by recruitment, however. My results with the significantly more abundant RSC complex show that it is not recruited by acidic activators, and can bind DNA very well on its own. This suggests that RSC may act more globally by

changing chromatin fluidity, thus making DNA transiently more accessible to transcription factors (58). Interestingly, recent ChIP results in yeast showed that TBP and RNA Pol II bound to SIR generated heterochromatic regions in the absence of any chromatin remodeling activity (230). These results imply that transcription factors can use methods other than chromatin remodeling to associate with promoters.

I have also used the immobilized template assay to follow transcription factor assembly at promoters during initiation and reinitiation. As mentioned previously, only 10% of the immobilized templates are occupied by PICs and only 10% of PICs are actually active in transcription, suggesting that PIC formation and transcription in this system are inefficient (J. Ranish, unpublished). Such low transcription efficiency has also been detected in other yeast nuclear extract transcription systems (231, 232), as well as in HeLa and Drosophila nuclear extracts (233-235). The reasons for such inefficiencies are not known. Detailed analysis of the PICs formed on immobilized templates suggests that active and inactive PICs are indistinguishable in that they both require promoter sequences, TBP, and other GTFs for assembly (168). In addition, the PICs contain stoichiometric amounts of TFIIB, TFIIA, and TFIIE (J. Ranish and S. Hahn, unpublished). The level of PIC formation also correlates well with transcription activity (168). Additionally, no transcripts shorter than the 100 nucleotides detected by primer extension were detected by direct labeling, suggesting that inactive PICs are completely defective in transcript synthesis (J. Ranish and S. Hahn, unpublished). Taken together, these data suggest that inactive PICs are defective in a step after PIC formation, but before transcript synthesis. My results have also shown that both active and inactive PICs dissociate upon NTP and ATP addition (see Chapter 3). These data imply that inactive PICs become unstable during initiation, but still form the reinitiation intermediate

because they are able to support reinitiation by a second extract. Therefore, even though active PIC formation is inefficient in the immobilized template assay, the observation that active and inactive PICs behave similarly prior to initiation makes the assay useful in studying transcription factor assembly during initiation and reinitiation.

My analysis of PIC formation in the in vitro immobilized template system has led to a three step model for PIC assembly (Fig.10). In the first step, TFIID and TFIIA bind to the promoter cooperatively. In the second step, Pol II/Med, TFIIB, and TFIIF are cooperatively recruited. Finally, TFIIH and TFIIE bind to complete PIC formation and begin transcription. This model is a combination of the stepwise recruitment pathway originally proposed, in which each GTF is recruited individually (1), and the holopolymerase model, in which all components except TFIID and TFIIA are recruited with RNA Pol II (171). Although my results show that Pol II/Med is required for PIC formation, only 5-10% of yeast RNA Pol II is found associated with Mediator (92, 95). In addition, purified systems do not show a requirement for Mediator in PIC formation (1). Therefore, my results imply that the stepwise assembly pathway is blocked in the more crude yeast nuclear extract system. It is possible that the extract system contains repressors that can destabilize PICs formed through stepwise assembly. Mediator may therefore be necessary to counteract these repressors by providing a larger platform for GTF interactions. More work will need to be done to identify these repressors and their mechanism of action.

Recent in vivo experiments suggest that there are other mechanisms of PIC recruitment, and that the mechanism used may be promoter-dependent. Studies in yeast have classified transcription at some promoters as either TAF-dependent or TAF-independent (236, 237). TAF-dependent promoters show high levels of TFIID binding, while TAF-independent promoters show

high levels of TBP binding and little TAF recruitment. Interestingly, these studies show that TBP recruitment at TAF-independent promoters is Srb4- and TFIIB-dependent, whereas recruitment at TAF-dependent promoters is not (237). Taken together, these studies suggest that at TAF-dependent promoters, TFIID can bind independently because TAFs may act to stabilize TBP binding, while at TAF-independent promoters, TBP binding now requires Mediator and TFIIB for stability. The fact that my results show TFIID binding to promoters independently of TFIIB and Mediator suggests that TAFs may stabilize TBP binding in my yeast nuclear extract system. PIC formation experiments performed in the absence of TAFs may determine whether this is the case. A new series of studies has looked more closely at PIC recruitment in vivo in yeast by performing ChIPs on Swi5 regulated cell cycle promoters. These studies have identified PIC intermediates by either formaldehyde crosslinking at various times after Swi5 binding, or blocking PIC formation at discrete steps. The results have shown that Swi/Snf is recruited immediately after Swi5, followed by SAGA, and then by GTFs (174, 196). Surprisingly, closer scrutiny revealed that Mediator is recruited well before, and in the absence of, RNA Pol II at these promoters (173, 174). The interpretation of these results remains unclear given that most Mediator in yeast is associated with RNA Pol II (92, 95), and that the likely role of Mediator is to transmit signals from activators and repressors to RNA Pol II (238). Experiments using a mutation that precludes RNA Pol II binding, such as Rpb1-1, may be helpful in validating these results.

Although I have shown that PIC formation can occur in at least three steps, I have also shown evidence for a model in which the formation of a reinitiation-competent complex occurs very differently. After RNA Pol II initiates transcription, a large part of the PIC remains at the promoter, including activator, TFIID, TFIIA, TFIIH, TFIIE, and Mediator (Fig. 23). This

scaffold reinitiation intermediate requires only the recruitment of RNA Pol II, TFIIF, and TFIIB to reinitiate transcription. Given that such a large part of the transcription machinery remains as part of the scaffold, and that transcription from a scaffold template occurs more rapidly than from a naked template, this model can account for the observation that reinitiation occurs more rapidly than initiation (77). In addition, the fact that Gal4-VP16 remains with and can stabilize the scaffold, suggests a dual role for activators in promoting high levels of reinitiation. First, after initiation, activator may directly promote the recruitment of the missing components of the transcription machinery. Second, some activators such as VP-16 can directly stabilize the scaffold complex, thus promoting reinitiation. This reinitiation model is supported by in vivo artificial recruitment assays in which high levels of transcription are achieved by fusing scaffold components, such as TBP and Mediator subunits, to DNA binding domains (76, 82, 239). Although these high levels of transcription have been interpreted as resulting from an increase in factor recruitment, my model suggests that they could also result from an increase in reinitiation due to greater scaffold stability.

Importantly this reinitiation model suggests that Pol II/Med is not involved in reinitiation. Although there is evidence that Mediator subunits are responsible for transcription of most genes in yeast (33), the presence of Mediator in the scaffold suggests that Pol II/Med participates only in initiation and not reinitiation. Instead, reinitiation may involve the recruitment of free RNA Pol II, or RNA Pol II in a distinct complex, to the scaffold. Since the bulk of transcription occurs during reinitiation, this may explain why such a small percentage of RNA Pol II is found associated with Mediator (92, 95).

The models for PIC formation and reinitiation mechanisms that I have described here are a result of experiments using the yeast model system. However, it is reasonable to predict that similar mechanisms are used by

higher eukaryotes due to the conservation of the general transcription machinery between these organisms. In support of my reinitiation model specifically, experiments with both Drosophila and mammalian systems have shown that part of the transcription machinery, including TFIID and TFIIA, remains at promoters after initiation (203, 209). Unfortunately, no experiments using higher eukaryotes have been done which monitor the binding of Mediator at promoters throughout transcription. Many different types of metazoan Mediators have been identified, and studies suggest that they may be used by different activators at different times in development (3, 130-132). However, all of these Mediators contain homologs and orthologs of yeast Mediator subunits (3), suggesting that an essential Mediator structure has been evolutionarily conserved, but also adapted to serve species-specific functions. Thus, it is likely that the mechanisms of transcription initiation and reinitiation, and the role of Mediator in these processes, have also been evolutionarily conserved.

Further analysis of how transcription factor complexes assemble and reassemble in vitro and in vivo may shed light on how these processes are regulated at specific promoters. In vitro immobilized template experiments on chromatin templates can identify how different chromatin remodeling complexes interact to remodel chromatin, and whether they are required only initially or throughout transcription. In vitro immobilized template experiments with a variety of activators and a variety of promoter elements can elucidate activator- and promoter-specific mechanisms of PIC assembly. Finally, ChIP experiments can be used to validate these results in vivo and provide a picture of how the general transcription machinery cooperates during RNA Pol II transcription.

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