

RNA Polymerase II Holoenzyme Components and Regulation

by

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Dedications

To,

Laura, for coming to Boston when everybody told you not to go; for your love, encouragement and sense of humor,

and

my parents, Jane and Philip, for their unwavering love and support.

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Abstract

The RNA polymerase II holoenzyme is thought to be the form of RNA polymerase II recruited to promoters of protein encoding genes. As a large mega-dalton sized complex, the yeast holoenzyme is composed of the core RNA polymerase subunits, the hallmark Srb and Med proteins, several general transcription factors and the nucleosome remodeling SWI/SNF complex. Chromatin presents a formidable challenge to polymerases and prevents access of critical factors to the DNA. As a holoenzyme sub-complex, the SWI/SNF proteins provide a mechanism to allow polymerase and other general transcription factors access to DNA at promoters; it also explains how SWI/SNF becomes targeted to specific promoters. Regulated transcription initiation may be achieved through a variety of mechanisms including activator interactions with holoenzyme components. Another mechanism of regulation is through the phosphorylation of RNA polymerase II CTD by the holoenzyme kinase-cyclin pairs, Kin28/Ccl1 and Srb10/Srb11. The timing of CTD phosphorylation appears to be a critical regulatory factor. Kin28 acts positively, phosphorylating CTD after initiation complex formation, whereas Srb10 act negatively, repressing transcription, by phosphorylating CTD prior to stable initiation complex formation.

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Chapter 1

RNA polymerase II holoenzyme and the regulation of transcription

RNA polymerase II must be regulated *in vivo* to transcribe messenger RNAs at proper levels in response to environmental and physiological signals. Eukaryotes contain between 3,000 and 100,000 protein encoding genes distributed throughout their genome. The levels of RNA molecules specified by these genes may vary up to four orders of magnitude within the cell. In the model organism *S. cerevisiae* transcripts can be present at high levels of 200-1000 copies per cell and at low levels of less than one transcript in every ten cell (0.1 copies / cell) (Holstege et al., 1998; Velculescu et al., 1997). Abrupt changes to the cellular environment can cause 100 fold increases or decreases in transcript levels (reviewed in (DeRisi et al., 1997; Holstege et al., 1998)).

Composed of 12 protein subunits, core RNA polymerase II is not capable of responding to the complex regulatory signals required for cell viability (Conaway and Conaway, 1993; Roeder, 1996; Young, 1991). Instead, other proteins and protein complexes associate with core RNA polymerase II, forming an extremely large entity called the RNA polymerase II holoenzyme. This fifty protein complex initiates transcription at nearly all promoters *in vivo* and can functionally respond to transcriptional activator proteins (reviewed in (Hampsey, 1998; Koleske and Young, 1995; Myer and Young, 1998; Parvin and Young, 1998)).

The holoenzyme's hallmarks are the SRB and Med proteins. SRBs are genes that when mutated can suppress truncations in the C-terminal domain

(CTD) of the largest subunit of pol II. Med proteins were cloned by sequencing components of the mediator, a Srb containing holoenzyme sub-complex. Several proteins discovered as Med's were previously identified in genetic selections and I have kept those names previously given to them (reviewed in (Hampsey, 1998), see table 1).

Koleske and Young discovered RNA polymerase II holoenzyme when they found that all of the Srb proteins in the cell co-purify with a sub population of core RNA polymerase II (Koleske and Young, 1994). This purified holoenzyme preparation also contained the general transcription factors TFIIB, TFIIF and TFIIH, the SWI/SNF complex and other mediator proteins (Kim et al., 1994; Koleske et al., 1996; Koleske and Young, 1994; Wilson et al., 1996). Quantitative western blots showed that representative proteins were stoichiometric to one another and present at levels equal to Rpb1 (Koleske et al., 1996). Purified holoenzyme is capable of responding to activators, an activity not seen *in vitro* using purified transcription factors (Kim et al., 1994; Koleske et al., 1996).

Mediator was originally purified by its ability to restore activator function to an *in vitro* transcription system (Flanagan et al., 1991; Kelleher et al., 1990; Kim et al., 1994). The mediator also enhances basal transcription and stimulates CTD phosphorylation by the TFIIH kinase (Kim et al., 1994). Biochemical purification of the mediator revealed that it was actually a sub-complex of the holoenzyme, bound to the RNA polymerase II CTD. Mediator can be separated from the holoenzyme using an anti-CTD antibody (Kim et

al., 1994), a CTD affinity column (Chao et al., 1996; Thompson et al., 1993; Wilson et al., 1996) and conventional chromatography (Myers et al., 1998).

Regulation of gene expression can be directed through the holoenzyme. DNA binding transcriptional activator proteins can increase the rate of transcription initiation by recruiting the RNA polymerase II holoenzyme to their target promoters (reviewed in (Ptashne and Gann, 1997)). Artificial tethering experiments demonstrate that recruiting the holoenzyme to a promoter can be a rate-limiting step in transcription activation (Barberis et al., 1995; Farrell et al., 1996; Gaudreau et al., 1997; Wu et al., 1996). Several holoenzyme components physically interact with activators and show activator specific defects when removed from holoenzyme (Gustafsson et al., 1998; Gustafsson et al., 1997; Hengartner et al., 1995; Koh et al., 1998; Lee and Kim, 1998; Lee et al., 1997; Myers et al., 1998; Myers et al., 1999). Struhl has proposed activators act through physically recruiting holoenzyme as well as TBP and TBP associated complexes (Struhl, 1996). Negative regulators can also act through holoenzyme components (reviewed in (Carlson, 1997)). For example Srb10/Srb11, a kinase-cyclin pair that can phosphorylate the CTD, are responsible for repressing greater than 150 genes in yeast cells grown in glucose at log phase (Hengartner et al., 1998; Holstege et al., 1998). Many other holoenzyme components appear to have both positive and negative role in transcriptional regulation although specific mechanisms have not yet been determined.

Chromatin, specifically nucleosomes, inhibits transcription by preventing the transcription apparatus from accessing promoter DNA (Knezetic et al., 1988; Knezetic and Luse, 1986; Lorch et al., 1992; Lorch et al., 1987; Lorch et al., 1988; Paranjape et al., 1994; Workman and Roeder, 1987). Several enzymatic complexes, like SWI/SNF, counteract this inhibition by remodeling the nucleosome structure, or, like SAGA, by chemically modifying histone proteins (Cote et al., 1994; Gregory et al., 1998; Ikeda et al., 1999; Imbalzano et al., 1994; Kwon et al., 1994; Utley et al., 1998). Other chromatin remodeling factors, which have similar catalytic ATPase subunits, have also been purified, including RSC, NURF, ACF, CHRAC and NRD/NURD. Likewise, many histone acetyltransferase complexes have recently been discovered, several of which were previously described as transcriptional coactivators, including SAGA (GCN5), TAF_{II} (TAF250), p300, p/CAF and CBP proteins (reviewed in (Workman and Kingston, 1998)). We have found that the SWI/SNF complex is physically associated with the holoenzyme providing a mechanism for holoenzyme components to gain access to promoters with repressive chromatin structures. It also explains how SWI/SNF becomes targeted to specific promoters (Wilson et al., 1996).

Although much of this chapter focuses on the yeast model systems, the RNA pol II holoenzyme paradigm has been extended to metazoans (reviewed in (Parvin and Young, 1998)). Several labs have purified holoenzyme complexes using affinity chromatography (Cho et al., 1998; Maldonado et al., 1996; Ossipow et al., 1995; Pan et al., 1997) as well conventional

chromatography (Chao et al., 1996; Cho et al., 1998; Maldonado et al., 1996; Neish et al., 1998; Scully et al., 1997). Others have purified mediator-like complexes that can affect activator dependent transcription (Gu et al., 1999; Jiang et al., 1998; Ryu et al., 1999; Sun et al., 1998; Xiao et al., 1999). These complexes all contain homologues to Srb or Med proteins, such as Srb10, Srb7, Med6 and Med7 and many contain other proteins that are transcriptional coactivators (Gu et al., 1999; Jiang et al., 1998; Ryu et al., 1999), chromatin remodeling factors (Cho et al., 1998; Neish et al., 1998) and that are implicated in carcinogenesis (Neish et al., 1998; Scully et al., 1997).

Several proteins and protein complexes are found in some yeast holoenzyme preparations but not others. These include TFIIB, the recessive SRBs (Srb8, Srb9, Srb10 and Srb11), SWI/SNF proteins and TFIIF (Cairns et al., 1996; Koleske and Young, 1994; Liao et al., 1995; Myers et al., 1998; Wilson et al., 1996). Differential composition of holoenzyme preparations probably reflect that RNA polymerase II holoenzyme is an extremely large megadalton sized complex being purified using chromatographic techniques designed to purify smaller protein complexes (Parvin and Young, 1998). As evidence to support this hypothesis, Myers et. al. have found that the SRB/MED mediator proteins, the quintessential hallmark proteins of the holoenzyme, can be separated from core polymerase with the same chromatographic techniques used to purify an intact holoenzyme entity (Myers et al., 1998; Myers et al., 1997). Growth and preparation of the source yeast cells also accounts for differences in holoenzyme preparations. For

example, cells collected during mid-log and late-log phase contain much more Srb10 and Srb11 protein than cells collected in stationary phase (Cooper et al., 1997; Holstege et al., 1998). Explaining why holoenzyme preparations from strains harvested in late log phase contain the recessive SRBs (Hengartner et al., 1995) and preparations made from cells in stationary phase cells contain none of the recessive SRBs (Myers et al., 1998).

The rest of this chapter aims to review all of the holoenzyme components in more detail as well as other proteins important for transcriptional regulation. I begin with a discussion of core RNA polymerase II and the general transcription factors. Then I progress to the CTD and the SRBs and their involvement in regulating gene expression. Finally, I finish the chapter discussing SWI/SNF nucleosome remodeling factors and histone acetylation /deacetylation complexes that regulate gene expression.

Core RNA Polymerase II. In eukaryotes there are three nuclear RNA polymerase complexes each responsible for transcribing a different type of RNA. The three polymerases were originally separated chromatographically and characterized by their differential α -amanitin sensitivity and optimal cation and salt concentrations (Roeder and Rutter, 1969; Ruet et al., 1978). *In vivo* the three polymerases have remarkably different functions. RNA polymerase I transcribes the large ribosomal RNAs (rRNA). RNA polymerase II transcribes the protein encoding messenger RNA (mRNA).

And, RNA polymerase III transcribes the all of the transfer RNA (tRNA) and the 5S rRNA (reviewed in (Sentenac, 1985)).

Core RNA polymerase II exists as a half mega-dalton multiple protein complex. In yeast, core RNA polymerase II is comprised of 12 proteins encoded by the genes *RPB1* to *RPB12* (Young, 1991). The entire complex is conserved throughout eukaryotes and six pol II subunits from humans can functionally replace their homologous subunits in yeast (Khazak et al., 1995; McKune et al., 1995; McKune and Woychik, 1994; Shpakovski et al., 1995). RNA polymerase II shares five subunits with RNA polymerase I and RNA polymerase III, they are Rpb5, Rpb6, Rpb8, Rpb10 and Rpb12 (Carles et al., 1991; Treich et al., 1992; Woychik et al., 1990; Woychik and Young, 1990). It is unclear what function these common subunits provide, however, several other large nuclear complexes also share subunits. For example, the SWI/SNF and RSC complexes have two common proteins (Cairns et al., 1998; Peterson et al., 1998) and the SAGA and TAF_{II} complexes share five proteins (Grant et al., 1998).

The three largest RNA polymerase II subunits, *RBP1*, *RPB2*, and *RPB3*, are similar to the *E. coli* RNA polymerase subunits, β' , β , and α , respectively. Several domains of high homology throughout Rpb1 and Rpb2 and the β' and β have been found and designated A-H in Rpb1/ β' and A-I in RPB2/ β (Allison et al., 1985; Sweetser et al., 1987). This sequence homology extends to functional homology as both β' /Rpb1 come in contact with the

DNA and RNA and β /Rpb2 bind nucleotides (Young, 1991). Moreover, the relative levels of the subunits are the same indicating that structural homology exists between the polymerases. *E. coli* polymerase is a 1:1:2 ratio of β' to β to α , likewise, yeast core polymerase II is a 1:1:2 ratio of Rpb1 to Rpb2 to Rpb3 (Kolodziej et al., 1990).

Although core RNA polymerase II is a very large protein complex, it is unable to selectively initiate transcription at promoters. Originally purified by Roeder and colleagues using non-specific chain elongation assays (Roeder and Rutter, 1969), attempts produce promoter specific transcripts were unsuccessful. A breakthrough occurred when a crude extract was developed that could selectively initiate transcription from a DNA template containing a promoter (Fire et al., 1981; Lue and Kornberg, 1987; Manley et al., 1980; Weil et al., 1979). This biochemical activity was used to purify and clone the General Transcription Factors that are necessary for promoter specific initiation *in vitro*. (Matsui et al., 1980; Samuels et al., 1982).

The General Transcription Factors (GTFs) were identified and cloned as proteins necessary and sufficient to reconstitute promoter specific transcription (reviewed in (Conaway and Conaway, 1993; Roeder, 1996)). This biochemical activity was separated into multiple subunits using standard biochemical chromatographic fractionation. The GTFs are TBP, TFIIB, TFIIA, TFIIE, TFIIF and TFIIH. Although originally purified from different sources such as yeast, rat and human cells, all eukaryotes appear to have homologous GTFs each having similar functional activities.

TBP. The core promoter elements of many genes include a TATAA sequence located 25-30 bp upstream from the start site in higher eukaryotes and 40-120 bp upstream in *S. cerevisiae* (Struhl, 1989). The TATAA-Binding Protein (TBP) specifically binds to this sequence (Buratowski et al., 1988) and induces an 80° kink in the DNA towards the major groove (Burley and Roeder, 1996). Using *in vitro* transcription systems, yeast TBP, a 27 kDA protein, can functionally substitute for the more complex mammalian TFIID (Buratowski et al., 1988; Hahn et al., 1989; Hahn et al., 1989). TBP was also cloned as *SPT15*, and when mutated can alter start site selectivity at Ty elements (Eisenmann et al., 1989). TBP does not copurify as a stoichiometric component of the yeast RNA polymerase II holoenzyme, however, it can be found in some mammalian holoenzyme preparations (Maldonado et al., 1996; Ossipow et al., 1995) and in some yeast preparations at sub-stoichiometric levels (Thompson et al., 1993).

TBP is a unique GTF because it is utilized at pol II promoters as well as pol I and pol III promoters (Geiduschek and Kassavetis, 1995; Hernandez, 1993). TBP is found in the pol I transcription factor SL1 (Comai et al., 1992) as well as the pol III transcription factor TFIIB (Kassavetis et al., 1992; Lobo et al., 1992; Taggart et al., 1992). Furthermore, mutations in yeast TBP diminish transcription at the promoters of all three polymerases (Cormack and Struhl, 1992; Schultz et al., 1992).

Several RNA polymerase II-specific regulatory protein complexes physically or genetically associate with TBP, including TAF_{II}s, TFIIA, TFIIF,

SAGA, MOT1, SNAP_c/PTF, NC2, TFIIB and the NOT complex (reviewed in (Lee and Young, 1998)). Alanine scanning has shown that the physical interactions with TFIIA, TFIIB, TFIIF involve small non-overlapping regions on TBP (Tang et al., 1996). However, physical and genetic data demonstrate that TBP also interacts with the larger and more complex TAF, SAGA and NOT factors. This raises the question, how does TBP interact with so many independent polymerase II regulatory proteins as well as polymerase I and polymerase II regulatory factors? Lee and Young have found that in yeast cells there is quantitatively more TBP protein present than all of the other TBP interacting factors combined. Implying that there is little competition between TBP interacting factors for TBP protein and that class II promoters are probably regulated by a diverse set of TBP containing protein complexes (Lee and Young, 1998).

Other experiments indicate that TBP plays a role in activator-dependent transcription. TBP physically interacts with many acidic activators, indicating that activators recruit TBP to promoters, thereby facilitating transcription initiation events (Wu et al., 1996). *In vivo* promoter tethering (Chatterjee and Struhl, 1995; Klages and Strubin, 1995; Xiao et al., 1995) and TBP specificity mutant experiments (Klein and Struhl, 1994) have been used to argue that TBP recruitment is a rate-limiting step in activator dependent transcription initiation. TBP is also associated with the TAF_{II}s, , TFIIB, SAGA and TFIIA, all of which have been implicated in activated transcription.

TFIIB is a monomeric 38 kDa protein in yeast. *SUA7* is the yeast gene encoding TFIIB and was originally cloned as a suppressor of start site selection mutants at a defective *CYC1* promoter (Pinto et al., 1992). TFIIB binds directly to the C-terminal stirrup of TBP (Geiger et al., 1996; Nikolov et al., 1995) and makes sequence specific DNA contacts downstream of the TATAA element, possibly explaining its involvement in start site selection (Lagrange et al., 1998). A physical interaction between RNA polymerase II and TFIIB has been established using affinity chromatography (Tschochner et al., 1992), surface-plasmon resonance (Bushnell et al., 1996) and low resolution crystal structure determination (Leuther et al., 1996). Genetic work also suggests a link between RNA polymerase II and TFIIB, as mutations in RPB9 can suppress start site selection defects in cells harboring *SUA7* mutations (Sun et al., 1996).

Like TBP, TFIIB physically interacts with several activators including VP16 (Lin et al., 1991). This interaction causes a conformational change in the TFIIB protein (Roberts and Green, 1994), and can increase the number of TFIIB molecules present at the promoter (Choy and Green, 1993; Lin and Green, 1991; Roberts et al., 1993).

TFIIA, although not required for basal transcription *in vitro*, performs an import role by stimulating activator function and stabilizing TBP and the pre-initiation complex. Yeast TFIIA has two protein subunits, TOA1 and TOA2 (Ranish and Hahn, 1991; Ranish et al., 1992), and mammalian TFIIA has three protein subunits encoded by two genes, one of which is cleaved into two proteins (De Jong and Roeder, 1993; Ma et al., 1993; Yokomori et al., 1993).

Originally, TFIIA was purified as a factor required for promoter specific reconstituted transcription (Reinberg et al., 1987), subsequently it was shown not to be required, but instead helps mediate activator enhanced transcription (Cortes et al., 1992; De Jong et al., 1995; De Jong and Roeder, 1993; Ozer et al., 1994; Sun et al., 1994). Yeast RNA polymerase II holoenzyme preparations contain no detectable TFIIA (Kim et al., 1994; Koleske and Young, 1994). The crystal structure of TFIIA-TBP-DNA shows that TFIIA binds to the TBP across from TFIIB and has contacts with the upstream DNA (Geiger et al., 1996; Tan et al., 1996). This TBP interaction is critical as it stabilizes the TBP-promoter DNA interactions (Buratowski et al., 1989; Imbalzano et al., 1994) and facilitates TBP recruitment by interacting with activators and coactivators like VP16, NTF-1, Sp1 (Ozer et al., 1994; Yokomori et al., 1994), PC4 (Ge and Roeder, 1994), HMG2 (Shykind et al., 1995) and topoisomerase I (Shykind et al., 1997). In yeast, activator specific TBP mutations are rescued by fusing TFIIA to TBP suggesting that the TFIIA-TBP interaction is critical for a subset of activators (Stargell and Struhl, 1996; Stargell and Struhl, 1995). TFIIA also seems to assist activators by inhibiting repressors of transcription (Ma et al., 1996), such as NC2 (Inostroza et al., 1992), HMG1 (Ge and Roeder, 1994), Mot1 (Auble et al., 1994) and some negative effects of the TAF_{II}s (Ozer et al., 1998).

TFIIF plays an important role in pre-initiation complex formation as well as polymerase II elongation. In mammals TFIIF is comprised of two proteins, RAP74 and RAP30, for RNA polymerase II Associated Factor (Flores et al., 1990). Yeast TFIIF has three subunits *TFG1*, *TFG2* and *TFG3* each being

105 kDa, 50 kDa and 30 kDa respectively (Henry et al., 1992). The two large yeast TFIIF subunits are homologous to the RAP74 and RAP30. The smallest TFIIF subunit, Tfg3, is weakly associated and is not required for TFIIF activity (Henry et al., 1992). Tfg3 is also found in the TAF_{II} and the SWI/SNF complexes, indicating that it performs a function common to all three or that there are dynamic associations between the three complexes (Cairns et al., 1996; Henry et al., 1994). RAP74/RAP30 appear to exist as a heterotetramer made up of two dimers (Flores et al., 1990). TFIIF physically interacts with RNA polymerase II (Burton et al., 1988; Sopta et al., 1985) and is present in most holoenzyme preparations (Kim et al., 1994; Koleske and Young, 1994; Neish et al., 1998; Parvin and Young, 1998; Scully et al., 1997). TFIIF can also interact with TFIIB (Fang and Burton, 1996; Ha et al., 1993), TFIID (Dubrovskaya et al., 1996; Ruppert and Tjian, 1995; Tang et al., 1996), and TFIIE (Maxon et al., 1994).

There is a functional similarity between bacterial sigma (σ) factors and TFIIF. Both make contacts with the promoter DNA and both can suppress nonspecific pol II DNA binding activity (Conaway and Conaway, 1993; Greenblatt, 1991). Recent promoter architecture studies using photocrosslinking demonstrate that TFIIF is required to wrap promoter DNA in a full turn around the pre-initiation complex (Kim et al., 1997; Robert et al., 1998). TFIIF is also part of an elongating pol II complex, stimulating the rate of chain elongation by pol II (Flores et al., 1989; Izban and Luse, 1992) and suppressing transient pausing (Bengal et al., 1991).

TFIIE is needed for open promoter formation. The two subunits, which make up TFIIE are conserved throughout eukaryotes; in yeast they are called Tfa1 and Tfa2 (Feaver et al., 1994; Ohkuma et al., 1992; Ohkuma et al., 1991; Peterson et al., 1991; Sumimoto et al., 1991). TFIIE is not found in yeast holoenzyme preparations (Kim et al., 1994; Koleske and Young, 1994) although some mammalian holoenzyme preparations do have TFIIE (Chao et al., 1996; Maldonado et al., 1996; Parvin and Young, 1998). TFIIE interacts with unphosphorylated RNA polymerase II (Maxon et al., 1994), TFIIH (Bushnell et al., 1996; Li et al., 1994) and single stranded DNA (Kuldell and Buratowski, 1997). TFIIE stimulates TFIIH phosphorylation of the pol II CTD (Lu et al., 1992; Ohkuma and Roeder, 1994). A zinc finger located in the largest subunit is essential for *in vitro* activity (Maxon and Tjian, 1994; Tijerina and Sayre, 1998) and viability in yeast (Kuldell and Buratowski, 1997; Tijerina and Sayre, 1998), however it does not play a role in ssDNA binding (Kuldell and Buratowski, 1997). TFIIE also physically interacts with the *Drosophila* repressor protein Krüppel (Sauer et al., 1995) and some homeodomain activators (Zhu and Kuziora, 1996) indicating that its activity may be regulated during transcription initiation.

TFIIE is not absolutely required for transcription initiation as studies using purified GTFs found that TFIIE and TFIIH are dispensable on some templates that are negatively supercoiled (Holstege et al., 1995; Parvin and Sharp, 1993; Parvin et al., 1992). Promoter elements, required for TFIIE/TFIIH dependence, map to 10 bp upstream of the initiation start site (Holstege et al.,

1997; Holstege et al., 1996). A mechanism has been proposed for promoter opening in which the TFIIH helicases generate the open promoter and, subsequently, TFIIE as well as core RNA polymerase II stabilize the open DNA structure (Holstege et al., 1997; Holstege et al., 1996). Negative supercoiling would effectively preopen the promoter reducing the need for TFIIE and TFIIH. Temperature sensitive mutations in yeast also reveal that inactivating TFIIE causes a large decrease in bulk mRNA production. However, a subset of genes continue to be synthesized, suggesting that this negative supercoiling affect may occur *in vivo* (Holstege et al., 1998; Kuldell and Buratowski, 1997; Sakurai et al., 1997; Tijerina and Sayre, 1998).

TFIIH is a large, complicated factor composed of nine proteins. Yeast TFIIH has a total mass of 500 kDa and is composed of: Ssl2 (95 kDa), Rad3 (85 kDa), Tfb1 (73 kDa), Tfb2 (59 kDa), Ssl1 (50 kDa), Ccl1 (45 kDa), Tfb4 (37 kDa), Tfb3 (33 kDa) and Kin28 (32 kDa) (Feaver et al., 1997). Mammalian preparations of TFIIH also contain 9 subunits and functional homologues for all of the proteins have been described (Feaver et al., 1997; Tirode et al., 1999). Some yeast holoenzyme preparations contain TFIIH and others do not; this situation is analogous to TFIIB and SWI/SNF. Yeast TFIIH can be separated chromatographically into three parts: Ssl2, TFIIK (containing Kin28, Ccl1 and Tfb3) and core TFIIH (the rest of the subunits) (Feaver et al., 1991; Feaver et al., 1994; Svejstrup et al., 1994). TFIIH is not only involved in transcription initiation, but also has a role in nucleotide excision repair (Drapkin et al., 1994; Friedberg et al., 1995; Seroz et al., 1995; Wang et al., 1994). The initial

cloning of the TFIIH helicase subunits generated a great deal of excitement because mutations in the genes encoding these proteins can cause disease in humans. *SSL2* is homologous to *XPB* (Park et al., 1992) and *RAD3* is homologous to *ERCC2* (Guzder et al., 1994), both of which, when mutated, cause increased sensitivity to light in the disease xeroderma pigmentosum (Schaeffer et al., 1993; Svejstrup et al., 1996).

Ssl2 and *Rad3* are both ATP dependent DNA helicases (Bardwell et al., 1994; Drapkin and Reinberg, 1994; Wade and Jaehning, 1996) needed to melt promoter DNA thus allowing the initiation complex to convert from closed to open formation (Goodrich and Tjian, 1994; Gralla, 1993; Holstege et al., 1997; Holstege et al., 1996; Ohkuma and Roeder, 1994; Tirode et al., 1999). They are also needed to open damaged DNA, allowing nucleotide excision repair to take place (Bardwell et al., 1994; Drapkin et al., 1994; Svejstrup et al., 1996; Wang et al., 1995). A temperature sensitive mutation in *Rad3* causes a reduction in bulk mRNA upon shifting to the non-permissive temperature, indicating that it is needed at most promoters *in vivo* (Guzder et al., 1994). *In vitro*, TFIIH, like TFIIE, is not needed when transcribing negatively supercoiled DNA templates or templates which have been artificial melted at the transcription start site (Holstege et al., 1997; Holstege and Timmers, 1997; Parvin and Sharp, 1993).

TFIIK, a subcomplex within TFIIH, contains a kinase-cyclin pair, that phosphorylates the pol II CTD (Feaver et al., 1994; Svejstrup and Feaver, 1996; Svejstrup et al., 1994). CTD phosphorylation is an important, regulated event

in transcription initiation and will be discussed later. In yeast, the kinase is Kin28 (Feaver et al., 1994), the cyclin is Ccl1 (Svejstrup and Feaver, 1996) and the third protein is Tfb3 (Feaver et al., 1997). In mammalian cells, the kinase Cdk7/MO15 can phosphorylate both the CTD and the cell-cycle kinase Cdc2 making it a Cdc2-Activating Kinase (CAK) (Fesquet et al., 1993; Roy et al., 1994; Serizawa et al., 1995; Shiekhattar et al., 1995). This suggests an interesting link between the cell cycle and transcription initiation because phosphorylation of Cdc2 is important regulatory step in cell cycle progression. However, genetic studies using a temperature sensitive yeast kin28 allele show that the TFIIH kinase does not phosphorylate Cdc28/Cdc2, does not have a cell cycle related phenotype, and is essential for transcription initiation (Cismowski et al., 1995). In yeast an alternative CAK candidate has been purified and cloned (Espinoza et al., 1996; Kaldis et al., 1996; Thuret et al., 1996). It is possible that metazoans have one kinase, Cdk7/MO15 that carries out two functions, although the true metazoan CAK may not yet have been discovered.

There is also evidence that TFIIH plays an important and sometimes unique role in transcriptional activation. Some activators, like VP16 and p53, can physically interact with mammalian TFIIH, indicating that they might recruit TFIIH and holoenzyme as a means of activating transcription (Xiao et al., 1994). HIV-Tat protein can stimulate CTD phosphorylation by TFIIH *in vitro* which, in turn, is thought to enhance transcriptional elongation (Cujec et al., 1997; Garcia-Martinez et al., 1997; Parada and Roeder, 1996).

CTD. Rpb1, the largest subunit of RNA polymerase II, contains a unique and conserved Carboxy-Terminal Domain (CTD). It is a hepta-peptide having the consensus sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser, which repeats multiple times. Intriguingly, as the complexity of the organism increases so does the repeat length of the CTD. For example, in yeast there are 27-28 repeats depending on the strain; the *C. elegans* CTD has 34 repeats; the *Drosophila* CTD has 43 repeats; and the mouse and human CTD has 52 repeats. CTD like sequences have not been found on any pol I or pol III subunits nor any of the bacterial RNA polymerase subunits. The CTD has been extensively studied both biochemically and genetically in yeast, *Drosophila*, and mammals (reviewed in Chao and Young, 1991; Young, 1991).

Truncation of the RNA polymerase II CTD revealed its important role in transcription initiation. Proteolytic removal of the CTD from Rpb1 has little or no effect in transcription systems using highly purified GTFs (Buratowski and Sharp, 1990; Li and Kornberg, 1994). However, CTD-less Rpb1 cannot replace an inactivated Rpb1 in a crude nuclear extracts (Li and Kornberg, 1994) and nuclear extracts made from yeast cells harboring CTD truncations show strong defects in activated transcription but not in transcript elongation (Liao et al., 1991). These studies indicate that other factors, that physically interact with polymerase via the CTD are missing from highly purified *in vitro* transcription systems. In similar experiments *in vivo*, CTD truncations have activated transcription defects in both yeast and

mammalian cells (Allison and Ingles, 1989; Gerber et al., 1995; Scafe et al., 1990). These activator dependent effects were seen at a subset of promoters suggesting that transcriptional activation involve CTD dependent and CTD independent mechanisms. *In vivo* the CTD is essential, as deletion causes inviability in yeast (Nonet et al., 1987), *Drosophila* (Zehring et al., 1988), and mammalian cells (Bartolomei et al., 1988).

The CTD can become heavily phosphorylated, enough so that the largest pol II subunit will show altered mobility on a SDS-PAGE gel. The three forms of the largest subunit of pol II have been designated: 1. **Ia**, unphosphorylated CTD, 2. **Ib**, CTD proteolytically removed and 3. **Io**, highly phosphorylated CTD. The Ia form of pol II is involved in transcription initiation, the Io form with elongation and mRNA processing, and the Ib form is probably a purification artifact (reviewed in (Dahmus, 1996)).

The conversion of a stable pre-initiation complex to an RNA producing elongation complex is associated with the phosphorylation of CTD and the conversion of pol II from the Ia form to the Io form. *Drosophila* promoters containing stalled initiation complexes contain unphosphorylated CTD and once released become elongating complexes with phosphorylated CTD (O'Brien et al., 1994). *In vitro*, stable initiation complexes are preferentially formed by the Ia form of pol II (Lu et al., 1991) and RNA polymerase II holoenzyme, which initiates transcription at nearly all promoters, contains unphosphorylated CTD (Koleske and Young, 1994; Thompson and Young, 1995). Also, purification of an elongating polymerase

II complex from yeast contains the Ilo form of polymerase (Svejstrup et al., 1997).

Many kinases have been identified that phosphorylate the CTD, however, the TFIIH kinase Kin28/Cdk7 appears to be responsible for phosphorylation of CTD at most promoters *in vivo*. Whole genome transcript analysis demonstrates that *akin28-ts* mutation inhibits transcription at rates comparable to the inactivation of a *ts-Rpb1* allele at the non-permissive temperature (Holstege et al., 1998). Cells with inactivated *Kin28* also have reduced levels of the Ilo form of pol II suggesting that transcription is dependent on CTD phosphorylation by Kin28 (Cismowski et al., 1995). *SRB10* is a cyclin-dependent kinase that can phosphorylate the CTD (Hengartner et al., 1998; Liao et al., 1995). Unlike TFIIH, however, it acts as a gene specific negative regulator as revealed by whole genome transcript analysis (Holstege et al., 1998). Mammalian Cdk8 is highly related to yeast *SRB10*, is found in some mammalian RNA polymerase II holoenzyme preparations (Chao et al., 1996; Cho et al., 1998; Gu et al., 1999; Scully et al., 1997) and also the NAT complex, a subcomplex of the pol II holoenzyme (Sun et al., 1998). Yeast Ctk1-Ctk2, a kinase-cyclin pair, was purified as a CTD kinase, however, it has never been shown to physically interact with any transcriptionally related complex (Lee and Greenleaf, 1991; Sterner et al., 1995). Genetic analysis indicates that *SRB10* and *CTK1* might have redundant functions at some TUP1/SSN6 regulated promoters but not others (Kuchin and Carlson, 1998). Metazoan P-TEFb (positive transcription elongation factor

b) contains another kinase-cyclin pair, CDK9 - cyclinT, that can phosphorylate the CTD (Marshall et al., 1996; Marshall and Price, 1992; Peng et al., 1998; Zhu et al., 1997). P-TEFb appears to be a regulatory kinase that can be recruited by HIV-Tat protein (Wei et al., 1998). Along with TFIIH, HIV-Tat stimulates phosphorylation of the CTD causing transcriptional activation via stimulation of RNA polymerase II elongation (reviewed in (Jones, 1997; Yankulov and Bentley, 1998)).

CTD phosphorylation can induce factors required for elongation, termination and mRNA processing to associate with RNA polymerase II and the newly synthesized RNA transcripts. Interactions between splicing factors and the CTD have been found using the two hybrid system and other biochemical assays (Du and Warren, 1997; Mortillaro et al., 1996; Yuryev et al., 1996). Furthermore, splicing can be inhibited *in vitro* (Yuryev et al., 1996) and *in vivo* (Du and Warren, 1997) by CTD peptides and anti-CTD antibodies. RNA 5'-capping enzymes and 3'- processing enzymes are also associated with the hyper-phosphorylated form of the CTD (Cho et al., 1997; McCracken et al., 1997; McCracken et al., 1997; Yue et al., 1997). A multi-subunit complex called the "elongator" was recently purified, that associates with elongating RNA polymerase II, possibly interacting through the hyper-phosphorylated CTD (Otero et al., 1999). These results suggest that hyper-phosphorylated CTD recruits proteins and enzymes needed for post-transcriptional processing of transcripts, unlike hypo-phosphorylated CTD which engages proteins essential for regulated transcription initiation.

SRBs. CTD truncation were used to genetically identify RNA polymerase II associated factors through a genetic selection. Yeast harboring the *RPB1* gene with fewer than 10 of the wild type 26 heptapeptide repeats are inviable but with greater than 13 repeats are fully viable (Nonet et al., 1987). Cells containing ten to twelve heptapeptide repeats exhibit multiple conditional phenotypes including cold and temperature sensitivity, inositol auxotrophy and the inability to utilize pyruvate as a carbon source (Nonet et al., 1987; Thompson et al., 1993). These conditional phenotypes associated with short CTDs were exploited to isolate extragenic suppressor mutations that restored cells to near wild-type growth states. Nine SRB genes (for Suppressor of RNA polymerase B) were cloned (Hengartner et al., 1995; Koleske et al., 1992; Liao et al., 1995; Thompson et al., 1993). Genetically there exists a clear relationship between the CTD and the SRBs.

The *SRB* genes can be grouped into three classes: the dominant suppressors, the recessive suppressors and *Srb7*. The dominant SRBs consist of *SRB2*, *SRB4*, *SRB5*, *SRB6* and the recessive SRBs are *SRB8*, *SRB9*, *SRB10*, *SRB11*. Biochemical work bolsters the groupings, as there are physical interactions within the dominant SRBs and also within the recessive SRB. Both dominant and recessive alleles of *SRB7* were isolated in the original CTD truncation selection. Physically, *Srb7* protein interacts with a distinct group of proteins none of which are *Srbs*, however, they form another subcomplex within the RNA polymerase II holoenzyme.

Characterization the **dominant SRBs** revealed that they are critical factors necessary for the initiation of transcription. The dominant SRBs are all undisputed components of the RNA polymerase II holoenzyme and mediator complex (Kim et al., 1994; Koleske and Young, 1994; Myers et al., 1998). Nuclear extracts made from strains with *SRB2* or *SRB5* deleted fail to transcribe *in vitro* unless *Srb2* or *Srb5* and *Srb2* proteins are added to the reaction (Koleske et al., 1992; Thompson et al., 1993). Promoter commitment assays and immobilized DNA template experiments demonstrate that the transcriptional defect results from an inability to form stable preinitiation complexes (Koleske et al., 1992; Ranish et al., 1999; Thompson et al., 1993). Lastly, a strain with temperature sensitive mutation in *SRB4* or *SRB6* shuts down transcription at virtually all promoters when shifted to the non-permissive condition. This argues that the dominant SRB proteins are essential for transcription initiation even though 80% of cellular core pol II is not associated with the SRBs (Koleske and Young, 1994) and the 20% that is in holoenzyme form initiates transcription at nearly all promoters (Holstege et al., 1998; Thompson and Young, 1995).

Med6 and Rox3 are components of the holoenzyme and are physically associated with the dominant SRB complex. Med6 was identified by peptide sequencing as a component of the mediator (Lee et al., 1997). Subsequently it was found in a genetic selection as a dominant suppressor of a *srb4* temperature sensitive allele (Lee et al., 1998). Conversely, a *med6-ts* mutation can be suppressed by an allele specific dominant mutation in *SRB4* (Lee and

Kim, 1998). Physically, Med6 protein interacts with Srb4, which interacts with Srb2 and Srb6 as determined by pairwise interaction studies using recombinant proteins (Koh et al., 1998; Lee et al., 1998). ROX3 was identified in numerous genetic selections and was characterized as a transcription factor, having both positive and negative influences at model promoters (Rosenblum-Vos et al., 1991; Song et al., 1996). Rox3 was a holoenzyme component by peptide sequencing and urea denaturation experiments revealed an association with the dominant SRB subcomplex (Gustafsson et al., 1997; Lee and Kim, 1998).

The dominant SRB subcomplex plays a role in proper activator response. A physical interaction exists between VP16 activator protein and holoenzyme and VP16 and mediator (Hengartner et al., 1995). A specific interaction between Gal4 activator protein and Srb4 has been demonstrated by several techniques. The region of Srb4 that interacts with Gal4 is essential *in vivo* and dominant mutations in *SRB4* partially suppress activator defects in *GAL4* (Koh et al., 1998). Holoenzymes purified from *med6Δ* strains do not respond to certain activators *in vitro* indicating that Med6 is also involved in the activator response. *In vivo* *MED6* is required for the transcriptional induction of several model promoters (Lee et al., 1997). Genome wide expression analysis of a *med6-ts* mutation shows that it plays an essential role at 10% or more of all yeast promoters (Holstege et al., 1998; Lee and Kim, 1998).

The recessive SRB complex contains a CTD kinase and negatively regulates transcription. Srb10 and Srb11 are a kinase-cyclin pair that phosphorylate the CTD when purified from recombinant and native sources (Hengartner et al., 1998; Koh et al., 1997; Liao et al., 1995). Srb8 and Srb9 are large proteins thought to physically interact with Srb10 and Srb11, however their function is unknown (Hengartner et al., 1995). All of the recessive SRBs were isolated independently in genetic selections and screens looking for genes regulating the repression at model promoters *SUC2*, *alpha2*, *FLO1* and meiotic promoters (Kuchin et al., 1995; Song et al., 1996; Surosky et al., 1994; Wahi and Johnson, 1995). Mutations and deletions within the four recessive SRBs produce identical phenotypes, including, flocculence, slow growth rates and partial derepression at several model genes (Carlson, 1997). Whole genome analysis shows that a kinase mutation in Srb10 causes derepression of ~170 genes (out of 6000). Many of them are involved in the diauxic shift, a transition that yeast undergo when changing from log phase growth to stationary phase (DeRisi et al., 1997; Holstege et al., 1998). These genes were depressed because there is less Srb10 protein in the cell during stationary phase. Other studies have found that destruction of Srb11, Srb10's cyclin pair, causes increased transcription at several model promoters, presumably by inactivating the SRB10 kinase (Cooper et al., 1997). A model for an SRB10 mechanism of repression has been described in Chapter 3. Briefly, Srb10 phosphorylates the CTD prior to the formation of a stable pre-initiation complex. Temporal regulation of the phosphorylation event is critical, as

phosphorylation is associated with elongation. Presumably, promoter proximal repressors can activate Srb10, which then phosphorylates the CTD prematurely causing local repression (Hengartner et al., 1998).

Srb7, Med1, Med2, Med4, Med7, Med8, Med9, Med11, Cse2, Nut1, Nut2, Gal11, Hrs1, Rgr1 and Sin4 proteins define another sub-complex in the mediator and holoenzyme. This complex was defined by limited urea denaturation / immuno-precipitation of mediator subunits under conditions that remove the dominant Srb subcomplex from the holoenzyme (Lee and Kim, 1998; Lee et al., 1997). *GAL11, HRS1, RGR1, MED1, MED2* and *SIN4* are similar because deletions and mutations in these genes produce overlapping positive and negative effects on gene expression (Balciunas et al., 1999; Covitz et al., 1994; Fassler and Winston, 1989; Gustafsson et al., 1997; Jiang et al., 1995; Jiang and Stillman, 1992; Jiang and Stillman, 1995; Li et al., 1995; Nishizawa et al., 1990; Piruat et al., 1997; Suzuki et al., 1988; Yu and Fassler, 1993).

Holoenzyme preparations produced from strains with deletions in one of these genes are missing all six proteins, suggesting that there are physical interactions between these six proteins (Gustafsson et al., 1997; Li et al., 1995).

Mediator preparations from a *med2Δ* strain increase basal *in vitro* transcription and stimulate the TFIIF kinase Kin28, but fail to restore activator dependent transcription. Suggesting that Med2 has a specific role in activator dependent transcription. Whole genome transcript analysis of the *med2* deletion strain showed 200 genes increased more than two-fold and 200 genes decreased more than two-fold demonstrating that Med2 has a broad

range of functions (Myers et al., 1999). *CSE2*, *MED11*, *NUT1*, and have activator specific defects when depleted from purified holoenzyme, but again their phenotypes suggest that they have important positive and negative functions (Gustafsson et al., 1998; Han et al., 1999; Tabtiang and Herskowitz, 1998; Xiao et al., 1993).

Mammalian homologues of SRB/mediator proteins have been identified and are found associated with pol II holoenzyme preparations. hSrb7 was the first mammalian Srb homologue to be cloned and antibodies raised against it were used to purify a mammalian holoenzyme (Chao et al., 1996; Maldonado et al., 1996). Srb10 and Srb11 homologues, Cdk7/CyclinC, were also cloned and can be found in other mammalian holoenzyme preparations (Cho et al., 1998; Pan et al., 1997) and mediator complexes (Gu et al., 1999; Sun et al., 1998; Xiao et al., 1999). Med6, Med7, Rgr1, and Nut2 homologues were discovered after peptide sequencing mammalian preparations of mediator (Gu et al., 1999; Jiang et al., 1998; Lee et al., 1997; Myers et al., 1998; Ryu et al., 1999; Sun et al., 1998). Two mediator like complexes, CRSP and SMCC, also contain several thyroid hormone receptor coactivator proteins called TRAPs (Gu et al., 1999; Ryu et al., 1999).

SWI/SNF complex is an ATP-dependent nucleosome remodeling machine comprised of 11 protein subunits. The protein subunits are Snf2/Swi2, Swi1, Swi3, Snf5, Snf6, Snf11, Arp9, Arp7, Swp73, Swp82 and Anc1 (Cairns et al., 1998; Cairns et al., 1996; Cairns et al., 1994; Cairns et al., 1996; Cote et al., 1994; Peterson et al., 1994; Peterson et al., 1998; Treich et al., 1995).

The Snf and Swi proteins were originally cloned in screens used to identify genes required for sucrose metabolism, *SUC2* gene expression (Neugeborn and Carlson, 1984), and mating type switching, *HO* gene expression (Stern et al., 1984). Mutant *SWI/SNF* genes all have similar phenotypes resulting from activator dependent transcriptional defects at multiple promoters *in vivo* (Carlson and Laurent, 1994; Laurent and Carlson, 1992; Laurent et al., 1993; Laurent et al., 1991; Laurent et al., 1990; Peterson and Herskowitz, 1992; Yoshinaga et al., 1992). Hirschhorn et al. were the first to directly demonstrate that *SWI/SNF* proteins functioned by antagonizing the repressive chromatin structure at promoters (Hirschhorn et al., 1992).

Soon after, *SWI/SNF* protein complexes were purified from yeast (Cairns et al., 1994; Cote et al., 1994), and mammals (Imbalzano et al., 1994; Kwon et al., 1994; Wang et al., 1996; Wang et al., 1996). *SWI2/SNF2*, the largest subunit of the *SWI/SNF* complex, is a DNA stimulated ATPase (Laurent et al., 1993) required for nucleosome remodeling (Cote et al., 1994). *In vitro* *SWI/SNF* is capable of altering the nucleosome such that the DNA is more accessible to DNA binding proteins and endonucleases (Cote et al., 1994; Imbalzano et al., 1994; Kingston et al., 1996; Kwon et al., 1994; Utley et al., 1996). Interesting, even after the removal of *SWI/SNF* complex, the remodeled nucleosome is stable (Cote et al., 1998; Imbalzano et al., 1996; Lorch et al., 1998; Owen-Hughes et al., 1996; Schnitzler et al., 1998). This remodeled nucleosome appears to be larger and can be converted back to its original

form by re-addition of the nucleosome remodeling complex and ATP (Lorch et al., 1998; Schnitzler et al., 1998).

The SWI/SNF complex is required for activator dependent transcription at some promoters that have repressive chromatin structure, however it is unclear how Swi/Snf becomes targeted to those promoters (Burns and Peterson, 1997; Hirschhorn et al., 1992; Peterson and Herskowitz, 1992; Ryan et al., 1998; Yoshinaga et al., 1992). Measurements of Swi/Snf's catalytic activity demonstrate that the complex remodels one nucleosome every four minutes (Logie and Peterson, 1997). Too slow, considering its low cellular abundance, if its effects were due to random remodeling of nucleosomes. SWI/SNF also binds DNA but this activity is nonspecific and does not appear to regulate its nucleosome remodeling activity (Quinn et al., 1996). Co-immunoprecipitation experiments suggests that the glucocorticoid activator suggest that it interacts with SWI/SNF proteins, although this interaction could be indirect and mediated through other proteins (Yoshinaga et al., 1992). The discovery that SWI/SNF is a component of the RNA polymerase II holoenzyme provides a simple model to explain how SWI/SNF becomes targeted to promoters, especially those that require the local remodeling of nucleosomes (Struhl, 1996; Wilson et al., 1996).

Other ATP-dependent chromatin remodeling complex have also been purified from yeast as well as other organisms. **Mammalian SWI/SNF** complexes have been purified that have two ATPase subunits, hBrm and Brg1 (Imbalzano et al., 1994; Kwon et al., 1994). Peptide sequencing of the Brg1

associated factors (BAFs) has shown that many of the proteins resemble yeast SWI/SNF components indicating that the complex has been conserved throughout evolution (Armstrong et al., 1998; O'Neill et al., 1999; Wang et al., 1996; Wang et al., 1996; Zhao et al., 1998). Recently it has been discovered that the human SWI/SNF complex is subject to cell-cycle regulation (Muchardt et al., 1996; Sif et al., 1998). Phosphorylation, possibly of Brg1 and hSwi3, and proteolytic destruction of hBrhm inactivates the nucleosome remodeling activity during G2-M phase of the cell-cycle (Sif et al., 1998).

The yeast **RSC** complex is a paralogue of the SWI/SNF complex that is more abundant in yeast cells and, like human SWI/SNF, appears to be cell cycle regulated (Cairns et al., 1996; Cao et al., 1997; Du et al., 1998). Two RSC subunits are shared with the SWI/SNF complex (Arp7 and Arp9). Interestingly, they are actin related proteins with ATPase motifs, and could have chaperone like activities (Cairns et al., 1998; Peterson et al., 1998).

Three complexes, all containing the ISWI ATPase, have been purified from *Drosophila* extracts. **NURF**, **CHRAC** and **ACF** are unique complexes, each having distinct protein subunit composition (Ito et al., 1997; Tsukiyama et al., 1995; Tsukiyama and Wu, 1995; Varga-Weisz et al., 1997). All three have nucleosome remodeling activity derived from ISWI, however, CHRAC and ACF, can also assemble nucleosomes into appropriately spaced chromatin (Ito et al., 1997; Tsukiyama et al., 1995; Tsukiyama and Wu, 1995; Varga-Weisz et al., 1997). NURF was identified as a factor that promotes GAGA binding to nucleosomal templates (Tsukiyama et al., 1994) and can

increase transcriptional output *in vitro* from chromatin templates (Mizuguchi et al., 1997). Recently, a human ISWI homologue, hSWF2h, was found as a subunit of RSF, a nucleosome remodeling complex, involved in transcriptional activation that also can assemble chromatin (LeRoy et al., 1998).

A novel nucleosome remodeling complex has been identified that contains histone deacetylase activity. Several labs have found that the Chd3 / Chd4 proteins, which have ATPase domains similar to Swi2/Snf2, are associated in complexes with the histone deacetylases HDAC1 and HDAC2. This complex, NRD/NURD/NuRD, can modestly increase its nucleosome deacetylation activity in the presence of ATP, indicating that nucleosome remodeling can enhance deacetylation (Tong et al., 1998; Xue et al., 1998; Zhang et al., 1998).

Targeted histone acetylation is thought to increase promoter accessibility and subsequently the frequency of transcription initiation; fortuitously many transcriptional coactivators have been discovered that are histone acetyltransferases (reviewed in (Brownell and Allis, 1996; Grunstein, 1997; Imhof and Wolffe, 1998; Struhl and Moqtaderi, 1998; Workman and Kingston, 1998). The **SAGA** complex contains a subset of the Ada proteins, Spt proteins and Taf_{II} proteins that have each been implicated in transcription regulation (Grant et al., 1997; Grant et al., 1998). ADAs were identified and cloned as genes that suppress toxicity associated with activator overexpression (Berger et al., 1990; Berger et al., 1992). GCN5 was found in this screen as an

ADA (Marcus et al., 1994). It was thought that the Ada proteins were coactivators, a bridging complex between the activator and the transcription machinery. *SPTs*, when mutated, alter the preferential start site for transcription, suppressing strains that have Ty insertions in reporter genes (Simchen et al., 1984; Winston and Carlson, 1992; Winston et al., 1984). Disruption of *GCN5*, the subunit of SAGA with acetyltransferase catalytic activity (Brownell and Allis, 1995; Brownell et al., 1996; Kuo et al., 1996; Wang et al., 1997), results in pleiotropic transcriptional defects *in vivo* (Gregory et al., 1998; Kuo et al., 1998; Wang et al., 1998; Wang et al., 1997). *In vitro*, targeted histone acetylation by SAGA, as well as other acetyltransferase complexes, can enhance activator directed transcription (Steger et al., 1998; Utley et al., 1998).

Several Taf proteins are found in the SAGA complex and, intriguingly, the largest subunit of **TFIID**, hTaf250/yTaf150 also has histone acetyltransferase activity (Mizzen et al., 1996). TFIID and Tafs were originally characterized as coactivators, and thought to be generally required for regulated transcription (Dynlacht et al., 1991; Sauer et al., 1996). However, experiments using yeast temperature sensitive mutations as well as *in vitro* transcription experiments using extracts depleted for Tafs, indicate that TFIID is not generally required for activated transcription (Apone et al., 1996; Holstege et al., 1998; Moqtaderi et al., 1996; Oelgeschlager et al., 1998; Walker et al., 1996; Walker et al., 1997). The TFIID subunits shared with SAGA are required at many promoters *in vivo* suggesting that the two complexes have overlapping functions required for acetylation activity or transcription at

most promoters (Apone et al., 1998; Holstege et al., 1998; Michel et al., 1998; Moqtaderi et al., 1998; Natarajan et al., 1998). Interestingly, many of these shared Tafs are structurally similar histone proteins (Birck et al., 1998; Hoffmann et al., 1996; Xie et al., 1996). A mammalian complex containing the histone acetyltransferase PCAF has been purified and it, like the yeast SAGA complex, has Taf protein subunits (Ogryzko et al., 1998). These findings argue that the functional activity of TFIID and SAGA is evolutionarily conserved.

Several other histone acetylases are also transcriptional coactivators. **P300** and **CBP** are acetyltransferases that were discovered as coactivators for CREB (Bannister and Kouzarides, 1996; Janknecht and Hunter, 1996; Ogryzko et al., 1996). Mutations in the catalytic domain of CBP and p300 remove coactivator activity *in vivo* for CREB as well as HIV-tat (Martinez-Balbas et al., 1998; Marzio et al., 1998). These complexes acetylate other proteins besides histones, including: p53 (Gu and Roeder, 1997; Gu et al., 1997), GATA-1 (Boyes et al., 1998), EKLF (Zhang and Bieker, 1998), TFIIE and TFIIF (Imhof and Wolffe, 1998; Imhof et al., 1997). **ACTR** is another acetyltransferase that acts in conjunction with CBP and p300 to enhance transcriptional activation by steroid receptors.

Targeted deacetylation of promoter proximal nucleosomes can repress transcription (Hassig and Schreiber, 1997). HDAC1/2 are histone deacetylases and were purified and cloned using the deacetylase inhibitor trapoxin (Taunton et al., 1996). Several studies show that recruiting HDAC1/2 or the yeast deacetylase Rpd3 to promoters can repress transcription. The

deacetylases do not interact directly with DNA bound repressors, instead they associate with Sin3 protein which acts as an intermediary, interacting with DNA binding repressor proteins like proteins pRB, Ume6, Mad/Max, methyl-CpG binding protein (Brehm et al., 1998; Hassig et al., 1997; Kadosh and Struhl, 1997; Laherty et al., 1997; Luo et al., 1998; Magnaghi-Jaulin et al., 1998; Nan et al., 1998; Zhang et al., 1997; Zhang et al., 1998). Chromatin immunoprecipitation experiments have demonstrated that the deacetylation of lysine 5 on histone H4 appears to be critical for repression (Rundlett et al., 1998).

Personal contributions to this work

When I joined the lab in the summer of 1994 there was great interest in the RNA polymerase II holoenzyme. Much of the energy focused around the unidentified components of holoenzyme and possible mechanisms of transcriptional regulation through the CTD. Within the Young lab, the SRB genes had been cloned and genetically characterized. Tony Koleske had purified an Srb complex which later turned out to be the RNA polymerase II holoenzyme. And Craig Thompson, using an SRB4-ts allele, was completing *in vivo* experiments demonstrating that RNA polymerase II holoenzyme is utilized at nearly all promoters *in vivo*.

At that time David Chao was using GST-CTD columns to purify SRB containing complexes. The complexes were extremely similar to mediator preparations purified in the Kornberg lab using the anti-CTD monoclonal antibody 8WG16. Before I arrived in the lab, David had taken eluates from the GST-CTD column, injected them into rabbits and made antibodies directed against CTD binding proteins.

I collaborated with David and using these antibodies screened a λ YES protein expression library made from yeast genomic DNA. Out of 10 million plaques screen, I identified, cloned and sequenced 82 positive plaques. Two genes were repeatedly cloned, they were HRS1 and SWI2/SNF2.

I obtained antibodies directed against both of these proteins as well as antibodies against Snf5, Snf6, Swi3 and Snf11 from the labs of Craig Peterson, Brehon Laurent, Marian Carlson and Andres Aguilera. Using recombinant proteins that I had made (Snf2, Snf5 and Snf6) and that I had received (Hrs1) I performed quantitative western blots using holoenzyme prepared by Tony Koleske and David Chao. I found that all of the SWI/SNF proteins were present and at levels approximately equal to that of Srb5, the standard used in the experiment. Hrs1 was present at the lowest stoichiometry at 4-8 times less abundant than Srb5.

Finding that Swi/Snf proteins were present in the holoenzyme directed further experiments. In the fall of 1995, the Kingston, Green and Peterson labs had all isolated Swi/Snf complexes, that had ATP-dependent chromatin remodeling activity. These results were extremely exciting because Swi/Snf had previously been described as a transcription co-activator. We collaborated with Bob Kingston and his lab, specifically Tony Imbalzano and Gavin Schnitzler. We showed, using several biochemical techniques, that RNA polymerase II holoenzyme contained Swi/Snf regulators. I performed immuno-precipitations from a semi-purified fraction using antibodies directed against Srb5 and Swi3. Both of these coprecipitated several established components of the holoenzyme as well as components of the Swi/Snf complex. We examined the elution of the holoenzyme from the last column in the purification and found that Swi/Snf proteins as well as nucleosome remodeling activity coelutes with pol II holoenzyme and

transcription activity. We examined the CTD complex which David Chao had purified and found that Swi/Snf proteins and as well as nucleosome remodeling activity coelutes with the Srb containing complex and a transcriptional activity. These results were novel and exciting because it was unclear how the Swi/Snf complex was targeted to promoters and it was also unclear how the holoenzyme might overcome nucleosome mediated repression at promoters. These results led to a model in which the simple association of Swi/Snf and holoenzyme could overcome both of these obstacles.

After this, in collaboration with Jun Qin of Brian Chait's lab, David and I tried to identify unknown CTD binding proteins using ion trap mass spectroscopy. We scaled up the CTD binding protein preparation approximately ten fold and using this preparation identified several proteins including Srb4, Srb5, Rgr1 and Sin4. The technique was rapid and sensitive but identified many genes that did not appear to be associated with the CTD in secondary western blot screening. Mass spec as well as scale up problems were also a factor as smaller scale preparation clearly had differing protein composition. Due to limited time availability we were only able to identify a subset of the CTD binding proteins (see appendix A).

Following this, I became interested in the Srb10/Srb11 kinase-cyclin pair and its functional role within the holoenzyme. As the Ptashne lab had shown, tethering a holoenzyme component to a promoter is sufficient to activate transcription. I made DNA binding fusions between lexA and Srb10

and *lexA* and a kinase dead *Srb10* mutant (*Srb10-3*). I found that *Srb10* does not significantly activate transcription, however, the *Srb10-3* fusion does activate transcription to levels equal to other holoenzyme component fusions. At the same time in the lab, Christoph Hengartner and Vic Myer had developed *in vitro* systems used to explore *Srb10* and *Kin28* function. Christoph found that *Srb10* and *Kin28* have nearly identical substrate specificity. Vic, using holoenzyme preparations with catalytically dead *Srb10*, discovered that *Srb10* could inhibit transcription by phosphorylating the CTD. Interestingly, this inhibition was only seen when the CTD was phosphorylated before polymerase entered the pre-initiation complex. Our data led to the model in which *Kin28* is the general CTD kinase, but *Srb10* specifically represses transcription by phosphorylating the CTD before PIC formation. CTD phosphorylation can therefore have opposite effects determined by timing of CTD kinase activity.

Presented in Appendix B is a technique developed by myself and Peter Murray, a postdoctoral fellow in the Young Lab, that increases the time and efficiency of mammalian targeting vector construction. This project grew out of lunchtime conversations, as Peter was looking for a better way to make mouse knockouts. I made critical plasmids and developed protocols essential for the production of the targeting vectors. Peter then went on to make IL-10R α and IL-10R β knockout cassettes and is in the process of making mice deficient for the IL-10 receptor loci.

Table I.

Factor	Gene	Size	Essential?	Features	References
RNA Polymerase II	<i>RPB1</i>	192 kDa	Y	Heptapeptide repeat	Young and Davis, 1983; Ingles et al, 1984; Allison et al., 1985 Sweetser et al., 1987 Kolodziej and Young, 1989 Woychik and Young, 1989 Woychik et al., 1990 Woychik et al., 1990 Woychik et al., 1990 Woychik et al., 1993a Woychik et al., 1990 Woychik et al., 1991 Woychik and Young, 1990 Woychik et al., 1993b Treich et al., 1992
	<i>RPB2</i>	139 kDa	Y		
	<i>RPB3</i>	35 kDa	Y		
	<i>RPB4</i>	25 kDa	N		
	<i>RPB5</i>	25 kDa	Y		
	<i>RPB6</i>	18 kDa	Y		
	<i>RPB7</i>	19 kDa	Y		
	<i>RPB8</i>	17 kDa	Y		
	<i>RPB9</i>	14 kDa	N		
	<i>RPB10</i>	8 kDa	Y		
	<i>RPB11</i>	14 kDa	Y		
<i>RPB12</i>	8 kDa	Y			
TFIIH	<i>TFB1</i>	73 kDa	Y	Nucleotide excision repair	Feaver et al., 1997 Feaver et al., 1997 Feaver et al., 1997 Feaver et al., 1997 Feaver et al., 1997 Feaver et al., 1997 Feaver et al., 1997 Feaver et al., 1997
	<i>TFB2</i>	59 kDa	Y		
	<i>TFB3</i>	32 kDa	Y		
	<i>TFB4</i>	37 kDa	Y		
	<i>RAD3</i>	90 kDa	Y		
	<i>SSL1</i>	52 kDa	Y		
	<i>SSL2/RAD25</i>	95 kDa	Y		
	<i>KIN28</i>	35 kDa	Y		
<i>CCL1</i>	45 kDa	Y	Kin28 cyclin pair		
TFIIE	<i>TFA1</i>	55 kDa	Y		Feaver et al., 1994 Feaver et al., 1994
	<i>TFA2</i>	37 kDa	Y		
TFIIF	<i>SSU1/TFG1</i>	82 kDa	Y	Shared with TFIID and TFIIH	Henry et al, 1994 Henry et al, 1994 Henry et al, 1994
	<i>TFG2</i>	47 kDa	Y		
	<i>ANCI/TFG3</i>	27 kDa	N		
TFIIB	<i>SUA7</i>	38 kDa	Y		Pinto et al., 1992
Srb5	<i>SRB2</i>	23 kDa	N	Cyclin dependant CTD kinase Srb10 cyclin pair	Nonet and Young, 1989; Koleske et al., 1992; Koleske and Young, 1994 Thompson et al, 1993; Koleske and Young, 1994 Thompson et al, 1993; Koleske and Young, 1994 Thompson et al, 1993; Koleske and Young, 1994 Hengartner et al., 1995 Hengartner et al., 1995 Hengartner et al., 1995 Liao et al., 1995 Liao et al., 1995
	<i>SRB4</i>	78 kDa	Y		
	<i>SRB5</i>	34 kDa	N		
	<i>SRB6</i>	14 kDa	Y		
	<i>SRB7</i>	16 kDa	Y		
	<i>SRB8</i>	166 kDa	N		
	<i>SRB9</i>	160 kDa	N		
	<i>SRB10</i>	63 kDa	N		
<i>SRB11</i>	38 kDa	N			
Meds	<i>MED1</i>	64 kDa	N		Balciunas et al., 1999 Myers et al., 1998 Myers et al., 1998 Myers et al., 1998 Myers et al., 1998 Myers et al., 1998 Myers et al., 1998 Gustafsson et al., 1998
	<i>MED2</i>	48 kDa	N		
	<i>MED4</i>	32 kDa	Y		
	<i>MED6</i>	33 kDa	Y		
	<i>MED7</i>	26 kDa	Y		
	<i>MED8</i>	25 kDa	Y		
	<i>MED11</i>	15 kDa	?		
	<i>CSE2</i>	17 kDa	N		
<i>GAL11</i>	120 kDa	N			
<i>NUT1</i>	129 kDa	N			
<i>NUT2</i>	18 kDa	Y			
<i>PGD1</i>	47 kDa	N			
<i>ROX3</i>	25 kDa	Y			
<i>RGR1</i>	123 kDa	Y			
<i>SIN4</i>	111 kDa	N			
Xtc1	<i>XTC1</i>	27 kDa	N		Emili et al., 1998
SWI/SNF	<i>SWI1</i>	148 kDa	N	DNA dependant ATPase	Cairns et al., 1994; Cote et al., 1995 Cairns et al., 1994; Cote et al., 1995 Cairns et al., 1994; Cote et al., 1995 Cairns et al., 1994; Cote et al., 1995 Cairns et al., 1994; Cote et al., 1995 Cairns et al., 1994; Cote et al., 1995 Cairns et al., 1994; Cote et al., 1995 Treich et al., 1995 Cairns et al., 1994; Cote et al., 1995; Cairns et al., 1998; Peterson et al., 1998 Cairns et al., 1994; Cote et al., 1995; Cairns et al., 1998; Peterson et al., 1998 Cairns et al., 1996A Cairns et al., 1994; Cote et al., 1995 Cairns et al., 1996B
	<i>SWI2/SNF2</i>	194 kDa	N		
	<i>SWI3</i>	93 kDa	N		
	<i>SNF5</i>	103 kDa	N		
	<i>SNF6</i>	38 kDa	N		
	<i>SNF11</i>	19 kDa	N		
	<i>ARP9</i>	53 kDa	Y / N		
	<i>ARP7</i>	54 kDa	Y / N		
	<i>SNF12</i>	64 kDa	N		
	<i>SWp82p</i>	~ 82 kDa	?		
	<i>ANCI/TFG3</i>	27 kDa	N		
			Shared with TFIID and TFIIH		

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Chapter 2

**RNA polymerase II holoenzyme contains SWI/SNF regulators involved in
chromatin remodeling**

Summary

The RNA polymerase II holoenzyme contains RNA polymerase II, a subset of general transcription factors and SRB regulatory proteins. We report here that *SWI* and *SNF* gene products, previously identified as global gene regulators whose functions include remodeling chromatin, are also integral components of the yeast RNA polymerase II holoenzyme. The SWI/SNF proteins are components of the SRB complex, also known as the mediator, which is tightly associated with the RNA polymerase II CTD. The SWI/SNF components provide the holoenzyme with the capacity to disrupt nucleosomal DNA, which may thus facilitate the stable binding of various components of the transcription initiation complex at promoters.

Introduction

Regulation of class II genes involves a complex interplay among gene-specific activators and cofactors, the general transcription apparatus, and chromatin. Gene-specific activators bind to promoters and stimulate transcription, at least in part, by binding and recruiting the general transcription apparatus (Chen et al., 1994; Hengartner et al., 1995; Ingles et al., 1991; Lin et al., 1991; Xiao et al., 1994; reviewed in Tjian and Maniatis, 1994; Sheldon and Reinberg, 1995; Emili and Ingles, 1995; Carey, 1995). Chromatin structure can affect the transcriptional activity of genes by blocking access of the transcription apparatus to promoters (Knezetic and Luse, 1986; Bresnick and Felsenfeld, 1993; Felsenfeld, 1992; Lorch et al., 1988; Workman and Roeder, 1987). The SWI and SNF proteins are global regulators that function by antagonizing repression mediated by nucleosomes, altering chromatin structure to facilitate binding of the transcription apparatus (Cote et al., 1994; Hirschhorn et al., 1992; Imbalzano et al., 1994; Kwon et al., 1994; reviewed in Carlson and Laurent, 1994; Peterson and Tamkun, 1995; Winston and Carlson, 1992). It is not yet clear how the SWI/SNF proteins are targeted to promoters, although some gene-specific activators may interact directly with these proteins (Yoshinaga et al., 1992).

Genetic and biochemical studies in yeast indicate that the form of the transcription initiation apparatus generally responsible for mRNA synthesis

in vivo is an RNA polymerase II holoenzyme (Barberis et al., 1995; Hengartner et al., 1995; Kim et al., 1994; Koleske and Young, 1994; Thompson and Young, 1995; reviewed in Carey, 1995; Emili and Ingles, 1995; Koleske and Young, 1995). This megadalton-sized complex contains RNA polymerase II, general transcription factors, and additional components called SRB regulatory proteins. The SRB proteins are a hallmark of the holoenzyme. The genes encoding the nine known SRB (Suppressor of RNA polymerase B) proteins were identified through a selection for factors involved in transcription initiation by RNA polymerase II in vivo, and all are required for normal yeast cell growth. Essentially all of the SRB protein in cells is tightly associated with the holoenzyme while approximately 80% of RNA polymerase II and general transcription factors are found independent of this complex (Koleske and Young, 1995). Experiments with temperature-sensitive *SRB* mutants indicate that the RNA polymerase II holoenzyme is the form of the transcription initiation apparatus employed at the majority of class II promoters in vivo (Thompson and Young, 1995). Other experiments have shown that recruiting a component of the SRB complex to promoters, presumably in association with the holoenzyme, suffices to obtain activated levels of transcription in vivo (Barberis et al., 1995).

The yeast *SWI* genes were first identified as positive regulators of HO transcription (Stern et al., 1984), and *SWI1*, *SWI2*, and *SWI3* were later shown to be required for the activation of a broad spectrum of inducible genes in vivo (Peterson and Herskowitz, 1992; Yoshinaga et al., 1992). Similarly, the

SNF genes were originally identified as positive regulators of *SUC2* (Neugeborn and Carlson, 1984), and *SNF2*, *SNF5*, and *SNF6* were subsequently found to be essential for activation of a diverse set of inducible genes (Laurent and Carlson, 1992; Laurent et al., 1991; Peterson and Herskowitz, 1992). Further study revealed that *SWI2* and *SNF2* are the same gene. Genetic evidence indicated that the *SWI* and *SNF* genes are involved in similar processes in gene activation (Carlson and Winston, 1992). Indeed, the discovery that *SWI1*, *SWI2/SNF2*, *SWI3*, *SNF5*, *SNF6* and *SNF11* proteins copurify in a large complex confirmed that the *SWI/SNF* gene products function together (Cairns et al., 1994; Cote et al., 1994; Peterson et al., 1994; Treich et al., 1995). Genetic and biochemical evidence implicated the *SWI/SNF* proteins in chromatin remodeling via nucleosome disruption (Cairns et al., 1994; Cote et al., 1994; Hirschhorn et al., 1992; Peterson et al., 1994).

Several lines of evidence led us to investigate whether *SWI* and *SNF* proteins are components of the RNA polymerase II holoenzyme, and furthermore, whether *SWI/SNF* proteins are components of the SRB-containing protein complex that is tightly associated with the CTD in the holoenzyme. First, genetic evidence suggests a functional relationship between the *SWI* and *SNF* gene products and the CTD. Strains containing mutations in *SWI* genes exhibit a large number of defects similar to those due to a truncation of the RNA polymerase II CTD (Nonet et al., 1987; Peterson and Herskowitz, 1992; Peterson et al., 1991). In addition, the CTD and the

SWI/SNF gene products show similar genetic interactions with mutations in *SIN1* and *SIN2*, genes that encode chromatin associated proteins (Peterson and Herskowitz, 1992; Peterson et al., 1991). Second, the *SRB* gene products have functional and physical interactions with the RNA polymerase II carboxyl terminal repeat domain (CTD) (Koleske and Young, 1995), which has been implicated in the response to activators in yeast and mammalian cells (Allison and Ingles, 1989; Gerber et al., 1995; Scafe et al., 1990). Third, the holoenzyme appears to be responsible for initiating transcription of most, if not all, class II genes in yeast, and the *SWI* and *SNF* gene products are required for transcriptional induction of a large number of genes in vivo (Thompson and Young, 1995; Peterson et al., 1991). Finally, there are perhaps a dozen polypeptides in purified yeast RNA polymerase holoenzyme that have yet to be identified.

We report here that the yeast RNA polymerase II holoenzyme contains *SWI2/SNF2*, *SWI3*, *SNF5* and *SNF11*. The *SWI/SNF* proteins are components of the *SRB* complex, also known as the mediator, which is tightly associated with the RNA polymerase II CTD. Both the holoenzyme and the *SRB/SWI/SNF* complex have ATP-dependent nucleosome disruption activities previously ascribed to the *SWI/SNF* complex. In addition, the holoenzyme facilitates the binding of TBP to nucleosomal DNA in an ATP-enhanced manner.

Results

Anti-SRB and anti-SWI antibodies coprecipitate holoenzyme

SRB regulatory proteins are found tightly and exclusively associated with other components of the RNA polymerase II holoenzyme in cell extracts. If SWI and SNF proteins are subunits of the RNA polymerase II holoenzyme, then antibodies against SRB5 should precipitate both the holoenzyme and SWI/SNF proteins from crude extracts. The results in Figure 1 show that this is indeed the case. SWI2/SNF2, SWI3 and SNF5 proteins coprecipitate with holoenzyme obtained through SRB5 immunoprecipitation. The fraction of SWI and SNF proteins immunoprecipitated from the crude extract appears to be the same as that of the SRB proteins. Control proteins introduced into the crude lysate did not coprecipitate, indicating that the immunoprecipitate was specific for the holoenzyme. When the immunoprecipitation experiment was carried out with antibody against SWI3, essentially identical results were obtained (Figure 1). The SWI/SNF and SRB proteins were immunoprecipitated from the crude extract with similar efficiency whether the immunoprecipitating antibody used was directed against SRB5 or SWI3. A control experiment with antibody against TGF β failed to precipitate SWI/SNF or SRB proteins. These results indicate that SRB and SWI/SNF proteins are tightly associated with one another.

Purified holoenzyme contains SWI/SNF proteins

The immunoprecipitation results led us to investigate whether SWI and SNF proteins are components of purified yeast RNA polymerase II holoenzyme. Antibodies against selected SWI and SNF proteins were used to determine whether these proteins coelute with the RNA polymerase II holoenzyme in the final purification step of the holoenzyme. The data in Figure 2A demonstrate that SNF2/SWI2, SNF5, SWI3 and SNF11 proteins coelute with other known components of the holoenzyme and with transcription activity.

The holoenzyme contains stoichiometric amounts of RNA polymerase II, SRB proteins, and general transcription factors. To ascertain whether the SWI/SNF proteins are stoichiometric components of the holoenzyme, the amounts of SNF2 and SNF5 were estimated by Western blot analysis with various amounts of recombinant proteins as standards (Figure 2B). These data indicate that the purified RNA polymerase II holoenzyme contains approximately equimolar amounts of SNF2, SNF5 and SRB5, the latter being a standard against which other holoenzyme components have previously been compared (Koleske and Young, 1994). Since yeast cells contain between 2000 and 4000 molecules of RNA polymerase II holoenzyme, it appears that there are at least this number of SWI2/SNF2 and SNF5 molecules per cell.

SWI/SNF proteins are components of CTD-binding SRB complexes

Genetic evidence indicates the SRB regulatory proteins and the RNA polymerase II C-terminal domain (CTD) have related functions in transcription initiation, and that these involve the response to transcriptional regulators (Allison et al., 1988; Gerber et al., 1995; Scafe et al., 1990; Koleske and Young, 1995). Since the SWI and SNF proteins are also involved in activation of a wide variety of genes, and mutations in SWI and SNF genes can produce phenotypes similar to those observed with mutations in SRB genes, we investigated whether SWI and SNF proteins are associated with the SRB complex. The SRB protein complex can be released from the holoenzyme when the latter is treated with monoclonal antibodies against the CTD, and this preparation has been called mediator (Kim et al., 1994). We previously prepared a mediator complex according to Kim et al. (1994), confirmed that it has the coactivator activity described by these investigators, and showed that the mediator contains all of the SRB proteins (Hengartner et al., 1995). When this mediator preparation was assayed for the presence of SNF2/SWI2, SNF5 and SWI3 proteins by Western blot, all three SWI/SNF proteins were found (Figure 3).

The SRB complex can also be isolated from crude extracts using a recombinant CTD column (Thompson et al., 1993). An SRB complex was purified extensively by using a recombinant GST-CTD column, followed by chromatography with mono S and mono Q columns (Figure 4A). The SRB, SWI, and SNF proteins bind to a GST-CTD column, but not to a control GST column, indicating that they bind specifically to the CTD (Figure 4B). Sliver

stain and western blot analysis confirm that a multiprotein complex containing SRB proteins and each of the three SWI/SNF proteins assayed comigrate from the mono Q column (Figure 4C and 4D). There are approximately 25 polypeptides in this complex and several correspond in size to previously identified SRB, SWI, and SNF proteins (Figure 5). No signals were obtained when western blots containing the SRB/SWI/SNF complex were probed with antibodies against RNA polymerase II, TBP, TFIIB, or the TFB1 subunit of TFIIF (data not shown). These results indicate that the SRB complex is in fact an SRB/SWI/SNF complex and furthermore, that the SWI and SNF proteins interact with the holoenzyme, at least in part through their association with RNA polymerase II CTD.

Nucleosome disruption activity in holoenzyme and SRB/SWI/SNF complex

Previous evidence that SWI1, SWI2, SWI3, SNF5, SNF6, and SNF11 gene products can be isolated as a large multisubunit complex capable of altering nucleosome structure led us to investigate whether the purified RNA polymerase II holoenzyme and the SRB/SWI/SNF complex were able to alter nucleosome structure. Mononucleosome particles were reconstituted from purified histone octamers and a DNA fragment containing two copies of an artificial phasing sequence (Shrader and Crothers, 1989). Digestion of the mononucleosomes with DNase I showed a 10 bp cleavage ladder typical of a rotationally phased nucleosome (Figure 6). Fractions in the last chromatographic step in the purification of the holoenzyme were mixed with

mononucleosomes and assayed for the ability to alter nucleosome structure, which can be visualized by changes in the accessibility of the nucleosome to DNase I cleavage. Figure 6A demonstrates that a nucleosome disruption activity coeluted with the RNA polymerase holoenzyme. The ability of the SRB/SWI/SNF complex to alter nucleosome structure was assayed in a similar experiment using fractions from the last step in the SRB/SWI/SNF purification (Figure 6C). The results show that nucleosome disruption activity coeluted with the SRB/SWI/SNF complex. Further analysis of the RNA polymerase II holoenzyme and SRB/SWI/SNF complex showed that the nucleosome disruption activity was ATP dependent (Figures 6B and 6D), as was previously shown for purified SWI/SNF complexes (Cote et al., 1994; Imbalzano et al., 1994; Kwon et al., 1994). In addition, purified core RNA polymerase II showed no nucleosome alteration capability (data not shown). These data indicate that the SRB/SWI/SNF complex contributes chromatin remodeling activity to the RNA polymerase II holoenzyme.

Purified holoenzyme and the SRB/SWI/SNF complex disrupts plasmid chromatin. To further characterize the nucleosome-disruption capabilities of the holoenzyme and the SRB/SWI/SNF complex we employed a supercoiling reduction assay (Figure 7). In this assay chromatin is assembled onto a relaxed closed-circular plasmid which is subsequently purified by glycerol gradient centrifugation. Each assembled nucleosome introduces approximately one negative supercoil to the plasmid, which can be resolved

by agarose gel electrophoresis after the removal of histones. When no protein is added to the nucleosome-assembled plasmid, it is highly supercoiled. Fractions from the last column of the holoenzyme purification (Figure 2A) were tested for their ability to disrupt nucleosome structure and thereby reduce supercoiling in the presence of added Topoisomerase I. As can be seen in Figure 7A, this activity coelutes with holoenzyme transcription activity, with the SRB and SWI/SNF proteins (Figure 2A), and with nucleosome-core disruption activity (Figure 6A). The supercoiling-reduction activity was dependent on ATP (Figure 7A, compare fraction 61 + and - ATP), as has been shown for the human SWI/SNF complex (Kwon et al., 1994). Repeating the experiment using fractions from the last column of the SRB/SWI/SNF complex shows that this complex also has an ATP-dependent supercoiling-reduction activity (Figure 7B).

Holoenzyme facilitates the binding of TBP to nucleosomes.

Previous work has shown that both yeast and human SWI/SNF complexes can facilitate transcription factor binding to nucleosomal DNA containing the relevant factor binding site (Cote et al, 1994; Imbalzano et al, 1994; Kwon et al, 1994). We tested whether the holoenzyme could increase the binding of TBP to a mononucleosome containing a TBP binding site. With holoenzyme and ATP present, TBP and TFIIA bound to the mononucleosome at TBP concentrations of 4×10^{-6} M (Figure 8A, lane 7),

while no TBP/TFIIA binding was observed in the absence of holoenzyme (Figure 8A, lane 6).

This holoenzyme-facilitated TBP binding might be caused by the stabilizing effects of the additional protein-protein and protein-DNA interactions that occur in the presence of RNA polymerase and general transcription factors, by the ATP dependent nucleosome disruption effects of SWI/SNF, or by a combination of both effects. To address this issue, we tested whether facilitated TBP binding was ATP dependent and observed partial protection of the TATA region on the mononucleosome when ATP is withheld or when ATP γ S is used instead of ATP (Figure 8B, lanes 4 and 6). However, addition of ATP enhanced the TBP binding as indicated by the increased protection from DNase I cleavage over the TATA box, the extension of the footprint in the 5' direction, and the appearance of a hypersensitive band in the 3' direction (Figure 8B, lane 5). Thus, it appears that the holoenzyme can partially stabilize binding of TBP and TFIIA to a mononucleosome in the absence of ATP. However, the full effect of holoenzyme-facilitated TBP binding requires ATP, presumably because it involves the ATP-dependent nucleosome disruption activity of the SWI/SNF proteins.

Discussion

The RNA polymerase II holoenzyme contains *SWI* and *SNF* gene products, previously identified as global gene coactivators. The *SWI* and *SNF* proteins are components of an *SRB/SWI/SNF* complex, also known as the mediator, which is tightly associated with the RNA polymerase II CTD. Both the holoenzyme and the *SRB/SWI/SNF* complex have nucleosome disruption activities previously ascribed to the *SWI/SNF* complex. In addition, the holoenzyme facilitates the binding of TBP to nucleosomal DNA in an ATP-enhanced manner.

Diverse transcriptional activators require *SWI/SNF* function in vivo

Mutations in *SWI1*, *SWI2/SNF2*, *SWI3*, *SNF5*, and *SNF6* cause a substantial reduction in the ability to activate transcription of a wide variety of well-studied genes in yeast cells, including *HO* (Stern et al., 1984), *SUC2* (Neigeborn and Carlson, 1984), *Ty* (Happel et al., 1991), *INO1* (Peterson et al., 1991), and *ADH1* and *ADH2* (Peterson and Herskowitz, 1992; Taguchi and Young, 1987). For example, *ADH1* and *SUC2* gene expression is reduced by about an order of magnitude in strains in which *SWI1*, *SWI2* or *SWI3* have been deleted (Peterson and Herskowitz, 1992). Experiments with reporter constructs have revealed that the *SWI* and *SNF* gene products are required for normal responses to a variety of gene-specific activators in yeast such as

GAL4, *Drosophila ftz*, mammalian glucocorticoid and estrogen receptors, and LexA-GAL4 and LexA-Bicoid fusion proteins (Peterson and Herskowitz, 1992; Laurent and Carlson, 1992; Yoshinaga et al., 1992).

Ptashne and colleagues have shown that recruiting a component of the SRB complex to promoters, presumably in association with the holoenzyme, suffices to obtain activated levels of transcription in vivo (Barberis et al., 1995; M. Ptashne, personal communication). Thus, evidence that LexA fusions with SWI2/SNF2, SNF5, SNF6 and SNF11 proteins are sufficient to activate transcription of a target gene in vivo (Laurent et al., 1990; Laurent et al., 1991; Treich et al., 1995) might now be interpreted in terms of holoenzyme recruitment to the target promoter.

We propose that recruitment of the holoenzyme to a specific promoter in vivo provides a means to facilitate TBP binding, regardless of the nucleosome structure at that promoter. The holoenzyme can enhance binding of TBP and TFIIA to a mononucleosome in vitro in the absence of ATP (Figure 8), a result compatible with evidence that the polymerase and general transcription factor components of the holoenzyme provide additional protein-protein and protein-DNA interactions that should stabilize TBP binding (Buratowski, 1994). Holoenzyme-facilitated TBP binding to a mononucleosome is greater in the presence of ATP, which presumably reflects the ATP dependent nucleosome disruption activity of the SWI/SNF proteins. These observations are consistent with the idea that

SWI/SNF protein function is necessary at the subset of promoters whose chromatin structure is particularly restrictive for TBP binding.

SWI/SNF in the holoenzyme accounts for previous genetic observations

The presence of SWI/SNF proteins in the RNA polymerase II holoenzyme and the observation that these proteins are components of a subcomplex that interacts with the RNA polymerase II CTD explains several previous observations. SWI/SNF proteins are necessary for transcription activation of many genes in yeast cells (reviewed in Winston and Carlson, 1992; Carlson and Laurent, 1994; Peterson and Tamkun, 1995); CTD truncation adversely affects the response to activators in yeast and mammalian cells (Allison and Ingles, 1989; Scafe et al., 1990; Gerber et al., 1995). Cells with RNA polymerase II CTD truncation mutations, cells with certain *SRB* mutations, and cells with *SWI1*, *SWI2* or *SWI3* mutations exhibit remarkably similar phenotypes (Peterson and Herskowitz, 1992; Thompson et al., 1993; Hengartner et al., 1995). The association of the SRB/SWI/SNF complex with the CTD accounts for the observation that cellular defects due to CTD mutations and *SWI* mutations can be alleviated by mutations in *SIN1* and *SIN2*, which encode an HMG1-related protein and histone H3, respectively (Kruger and Herskowitz, 1991; Peterson et al., 1991; Peterson and Herskowitz, 1992).

SRB/SWI/SNF complex is associated with the RNA polymerase II CTD

The SRB/SWI/SNF complex is tightly associated with the RNA polymerase II CTD. Independent attempts to purify various SRB proteins by column chromatography have always led us to purify the same multiprotein complex: the RNA polymerase II holoenzyme (Koleske and Young, 1994; Koleske et al., 1995; Hengartner et al., 1995; Liao et al., 1995). Only very small amounts of SRB protein can be detected that is not associated with the holoenzyme. Two different methods have been described which permit partial purification of an SRB subcomplex. An SRB complex can be isolated using a CTD affinity column (Thompson et al., 1993) or by releasing it from a holoenzyme preparation by using monoclonal anti-CTD antibodies (Kim et al., 1994). Because neither of these preparations is homogeneous, we further purified the SRB complex obtained by CTD-affinity chromatography (Figure 4). The SRB and SWI/SNF proteins coelute in the final step of the purification.

We also found that the SRB complex isolated by anti-CTD antibody release contains SWI and SNF proteins. Kim et al. (1994) demonstrated that reconstitution of the holoenzyme's response to activators required the presence of a subcomplex that could be isolated from holoenzyme with anti-CTD antibodies, which contained SRB2, 4, 5, and 6, and was called the mediator of activation. Our own studies with the mediator, which was purified precisely as described by Kim et al. (1994) and has chromatographic and transcriptional properties identical to those originally described for this subcomplex, revealed that it contained all nine of the known SRB proteins

(Hengartner et al., 1995). Thus, the mediator preparation and the SRB complex obtained by CTD-affinity chromatography contain very similar, if not identical, complexes.

We have shown that the RNA polymerase II holoenzyme, and its SRB/SWI/SNF subcomplex, contains SWI2/SNF2, SWI3, SNF5, and SNF11. Although we do not have direct biochemical evidence that SWI1 and SNF6 are present in the holoenzyme, other genetic and biochemical data indicate that it is highly likely that SWI1 and SNF6 are also subunits of these complexes (Cairns et al., 1994; Cote et al., 1994; Laurent and Carlson, 1992; Laurent et al., 1991; Peterson and Herskowitz, 1992).

Are there multiple SWI/SNF complexes?

Large multisubunit complexes containing yeast SWI and SNF proteins have been purified to varying extents (Cairns et al., 1994; Cote et al., 1994; Peterson et al., 1994). Characterization of two of these preparations by Western analysis did not reveal the presence of SRB proteins (Peterson et al., 1994; Cairns et al., 1994). This suggests that the purification procedures employed in these studies separated the SRB and SWI/SNF proteins or that SWI/SNF complexes can exist independent of the holoenzyme.

Since SWI2/SNF2 and SNF5 are stoichiometric components of the holoenzyme, and yeast cells contain 2000 - 4000 molecules of RNA polymerase II holoenzyme, there are at least 2000 molecules of SWI2/SNF2 and SNF5 molecules per cell. Based on their SWI/SNF complex purification,

Cote et al. (1994) estimated that there are between 50 and 150 copies of the SWI/SNF complex in yeast cells. One interpretation of these results is that most SWI/SNF protein resides in the RNA polymerase II holoenzyme, and the form of SWI/SNF complex purified by Cote et al. (1994) is the small amount of SWI/SNF protein that is in the process of assembly into holoenzyme or, alternatively, it represents a subcomplex that can be dissociated from the holoenzyme.

The ability to immunoprecipitate very similar holoenzyme complexes from crude yeast fractions using anti-SRB and anti-SWI antibodies suggests that most of the SWI/SNF protein in these fractions is associated with the holoenzyme. If the SRB and SWI/SNF proteins were in separate complexes, then the relative ratios of SRB and SWI/SNF proteins would differ in the anti-SRB and anti-SWI immunoprecipitates. However, the similar relative ratios of SRB and SWI/SNF proteins found in immunoprecipitates obtained with anti-SRB and anti-SWI antibodies (Figure 1) indicates that the SRB and SWI/SNF proteins are components of the same complex in the crude extract.

SWI/SNF function is highly conserved in eukaryotes

SWI/SNF proteins and their functions appear to be highly conserved in eukaryotes. Putative homologues of *SNF2/SWI2* include *Drosophila brahma* and human *hbrm* and *hBRG1*, which have been cloned and implicated in transcriptional regulation (Tamkun et al., 1992; Khavari et al., 1993; Muchardt and Yaniv, 1993). A mammalian homologue of *SNF5*, called

INI1, has also been cloned (Kalpana et al., 1994). A human SWI/SNF complex has been partially purified which has nucleosome disruption activities similar to those of the yeast SWI/SNF complex (Imbalzano et al., 1994; Kwon et al., 1994). The human SWI/SNF complex contains both hBRG1 and INI1 proteins (Kalpana et al., 1994; G.S., unpublished results), as would be expected based on the yeast results. Like the yeast SWI/SNF complex, the human SWI/SNF complex facilitates the binding of activators to nucleosomal DNA.

Implications for mechanisms involved in transcriptional activation

The presence of the SRB/SWI/SNF subcomplex in the RNA polymerase II holoenzyme has implications for the mechanisms involved in transcription activation in vivo. Dynamic competition between chromatin proteins and an activator for a specific DNA site could be resolved in favor of the activator once the SWI/SNF-containing holoenzyme was recruited to the promoter. In this model, the activator and the holoenzyme both contribute to stable transcription initiation complex formation; the activator recruits the holoenzyme by binding to a subset of its components and the SWI/SNF components of the holoenzyme enhance the stability of the activator-DNA interaction by destabilizing nucleosomes. This model is attractive because it provides a simple solution to the question of how SWI/SNF proteins are brought to promoters and it accounts for the coactivating and nucleosome disruption activities observed in vivo and in vitro for the SWI and SNF proteins.

Experimental Procedures

Immunoprecipitations

All immunoprecipitations were done as described (Hengartner et al., 1995). Briefly, 50 μ l of the DEAE(400) fraction was diluted 1:4 with modified transcription buffer (MTB) (50 mM HEPES KOH pH 7.3, 100 mM potassium acetate, 25 mM MgAc, 5 mM EGTA, 1 μ M DTT, 10% glycerol, 0.01% NP-40, 1 mM PMSF, 2mM benzamidine, 2 μ M pepstatin A, 0.6 μ M leupeptin, and 2 μ g/ml chymostatin) minus the potassium acetate. 4 μ g of ovalbumin, 4 μ g HA-GST, and 2 μ g BSA were added to each reaction prior to the addition of antibody. 0.4 μ g of affinity purified α -SRB5, ~0.15 μ g of affinity purified α -SWI3, or 1.5 μ g of affinity purified α -TGF β were added to the respective reactions and allowed to incubate 2 hours at 4°C. 15 μ l of goat anti-rabbit covalently linked to magnetic beads (Dynal) were then added and incubated for 1 hour at 4°C with constant agitation. Beads were precipitated with a magnet and washed three times in 200 μ l MTB buffer. The final wash contained no NP-40. Proteins were eluted of the magnetic beads by boiling in 20 μ l of sample buffer.

Western Blotting

All Western blots were performed as described (Koleske and Young, 1994). Proteins were detected with the following antibodies: SRB2, 4, 5, 6

(Thompson et al., 1993), SRB8, 9 (Hengartner et al., 1995), SRB10, 11 (Liao et al., 1995), SWI2/SNF2, SNF5 (gift of B. Laurent), SWI3 (gift of C. Peterson), SNF11 (gift of I. Treich and M. Carlson), TFIIE α and TFIIE β (C.J.W. and R.A.Y. unpublished). Quantitative Western blots were performed as described (Koleske and Young, 1994). Recombinant standards were SRB5 (Thompson and Young, 1995), GST-SNF2/SWI2₁₂₅₆₋₁₇₀₃ and GST-SNF5₁₋₁₉₃ (gifts of B. Laurent). GST proteins were purified as described (Smith and Johnson, 1988). Concentrations of recombinant proteins were determined using a colorimetric assay (BioRad) with bovine serum albumin as a standard.

Purification of holoenzyme and mediator

Holoenzyme was purified as described (Koleske and Young, 1994). Transcription assays for holoenzyme were done as described (Koleske and Young, 1994). Mediator was purified as described (Hengartner et al., 1995).

SRB/SWI/SNF Complex Purification

Whole cell extract was prepared from Red Star Yeast as described (Thompson et al., 1993). 1.2 L of the ammonium sulfate pellet was centrifuged for 30 minutes at 5,000 RPM. in an RC3B centrifuge (Sorvall). The pellet was resuspended in 900 ml of Buffer A (20 mM K-Hepes pH 7.6 1 mM EDTA 1 mM DTT 20% glycerol, and protease inhibitors (Thompson et al., 1993). The suspension was centrifuged again for 30 minutes at 5,000 RPM. in an RC3B centrifuge (Sorvall). The supernatant was mixed with 200 g (dry)

of BioRex 70 and stirred for 20 minutes. The suspension was packed into a column with a 5 cm diameter and washed with 1.5 l of Buffer A + 100 mM KOAc. Bound proteins were eluted with Buffer A + 600 mM KOAc. Fractions containing protein were pooled, frozen in liquid nitrogen and stored at -70°C until use. Eluates from 2 BioRex columns (320 ml, 1.0 g protein) were thawed and pooled. 320 ml of Buffer A + 2% Triton X-100 were added and the mixture was centrifuged for 30 minutes at 12,000 RPM. in a GSA rotor (Sorvall). The supernatant was loaded onto a 15 ml CTD affinity column prepared as described (Thompson et al., 1993) at a flow rate of 200 ml/hr. The column was washed with 100 ml of Buffer A + 300 mM KOAc + 1% Triton X-100, 100 ml or Buffer A + 300 mM KOAc. Bound proteins were eluted with Buffer A + 300 mM KOAc + 1 M Urea at a flow rate of 25 ml/hr. Fractions containing protein (3.7 mg) were pooled, frozen in liquid nitrogen, and stored at -70°C. The CTD column was equilibrated with Buffer A + 300 mM KOAc + 1% Triton X-100 and the flow through was loaded again. The column was washed and eluted as before. Fractions containing protein (1.8 mg) were pooled, frozen in liquid nitrogen, and stored at -70°C. The CTD eluates were pooled, diluted with 1.5 volumes of Buffer A + 0.01% NP-40, and centrifuged for 10 minutes at 17,000 RPM. in an SS-34 rotor (Sorvall). The supernatant was loaded onto a Mono S HR 5/5 (Pharmacia) at a flow rate of 0.3 ml/min. The column was washed with 3 ml of Buffer A + 120 mM KOAc + 0.01% NP-40. Bound proteins were eluted with a 20 ml gradient of Buffer A +0.01% NP-40 from 120 mM to 1000 mM KOAc. Fractions were frozen in

liquid nitrogen and stored at -70°C until use. Fractions containing SRB4 and SRB5 as assayed by Western blotting were pooled and diluted with 2 volumes of Buffer B (20 mM Tris OAc pH 7.6 + 20% glycerol + 1 mM DTT + 0.01% NP-40 + protease inhibitors). The mixture was centrifuged for 5 minutes in a microcentrifuge. The supernatant was loaded onto a Mono Q HRR 5/5 column (Pharmacia) at a flow rate of 0.3 ml/min. The column was washed with 1 ml of Buffer B + 200 mM KOAc. Bound proteins were eluted with a 40 ml gradient of Buffer B from 200 mM to 2000 mM KOAc. The yield of SRB complex was approximately 100 µg. 1 µl of each fraction was analyzed by silver staining. 7.5 µl - 10 µl of each fraction were analyzed by Western blotting.

Nucleosomal Disruption and Facilitated Transcription Factor Binding Assays

The PH MLT (figure 6) or PH MLT(+3) (figure 8) restriction fragments were assembled into rotationally phased mononucleosome particles, purified by glycerol gradient centrifugation, and assayed as described (Imbalzano et al, 1994). At the nucleosome concentrations and reaction conditions employed in this and previous studies, nucleosomes were determined to be stable on the basis of resistance to micrococcal nuclease, the appearance of a 10 bp repeat pattern upon DNase I digestion, and exhibition of reduced mobility upon electrophoresis in native polyacrylamide gels. We have not observed the appearance of free DNA due to nucleosome dissociation in any of our experiments.

In Figure 6 holoenzyme fractions were the same as those used in Fig 1A. In Fig 6A, 0.3 μ l of each fraction was assayed in the presence of 4 mM ATP. For the titration of holoenzyme, 0 μ l, 0.015 μ l, 0.05 μ l, 0.15 μ l, and 0.5 μ l of fraction 60 was used respectively with and without 4 mM ATP as indicated. SRB/SWI/SNF fractions were the as those used in Fig 3C and 3D. In Fig 6C, 1.7 μ l of each fraction was assayed in the presence of 4 mM ATP. For the titration in Fig 6D, 0 μ l, 0.07 μ l, 0.2 μ l, 0.7 μ l, and 2.0 μ l of fraction 24 was used respectively with and without 4 mM ATP as indicated.

For Figure 8 binding of yTBP and yTFIIA to nucleosomes containing the PH MLT(+3) restriction fragment was performed as previously described (Imbalzano et al, 1994). In figure 8A, all reactions contained 4 mM ATP. Following a 30 minute incubation at 30° C in the presence or absence of holoenzyme (as indicated), increasing amounts of yTBP in the presence of yTFIIA were added. TBP concentrations were 0 (lanes 1, 3, 10), 0.04 micromolar (lanes 2, 4, 9), 0.4 micromolar (lanes 5, 8), and 4 micromolar (lanes 6, 7). 1.5 micromolar yTFIIA was also added to all reactions. In figure 8B, reactions were treated with holoenzyme, alone (lanes 2,4), in the presence of 4 mM ATP (lanes 3,5) or in the presence of 4 mM ATP γ S (lane 6) for 30 minutes at 30° C, followed by addition of 4 micromolar yTBP in the presence of 1.5 micromolar yTFIIA.

Recombinant yTBP was purified as described (Hoey et al, 1990), except that the heparin peak was further purified on a Mono S HR5/5 FPLC column

(Pharmacia). Recombinant γ TFIIA was purified as described (Ranish et al, 1992).

Supercoiling Reduction Assay

Plasmid chromatin was assembled and purified as described (Kwon et al., 1994). Reactions, total volume 12.5 μ l, contained chromatin (2ng DNA), 1 U Topoisomerase I (Promega), 2.5 μ l ~30% Glycerol Gradient Buffer, 7 μ l Buffer A minus KCl, 7 mM MgCl₂, 50-100 mM KOAc (final), 4 mM ATP where indicated, and 2 μ l holoenzyme mono S fractions or 1 μ l SRB/SWI/SNF complex mono Q fractions. Reactions were stopped after 90 minutes at 30°C by addition of 6 μ l stop buffer (3% SDS, 100mM EDTA, 50 mM Tris HCl pH 8.0, 25% Glycerol, 2 mg/ml proteinase K). Reactions were incubated for 90 minutes at 37° and resolved on a 2% agarose gel (50 mM Tris-Phosphate pH 7.3, 1mM EDTA) for 40 hrs, at 40V. Gels were dried and exposed to film.

Acknowledgments

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Figure 1 Immunoprecipitation of RNA polymerase II holoenzyme from crude extracts using anti-SRB5 and anti-SWI3 antibodies.

Immunoprecipitations were from a crude DEAE fraction prepared as described in Hengartner et al. (1995). Immunoprecipitations were carried out with affinity-purified antibodies against SWI3, SRB5, or TGF β . Ovalbumin and HA-tagged GST were added to each reaction prior to precipitation to serve as controls for specific immunoprecipitation. 1/50 of the Onput and Flow Through, and 1/5 of the Final Wash and Eluate were subjected to SDS-PAGE and analyzed by Western blot using specific antibodies.

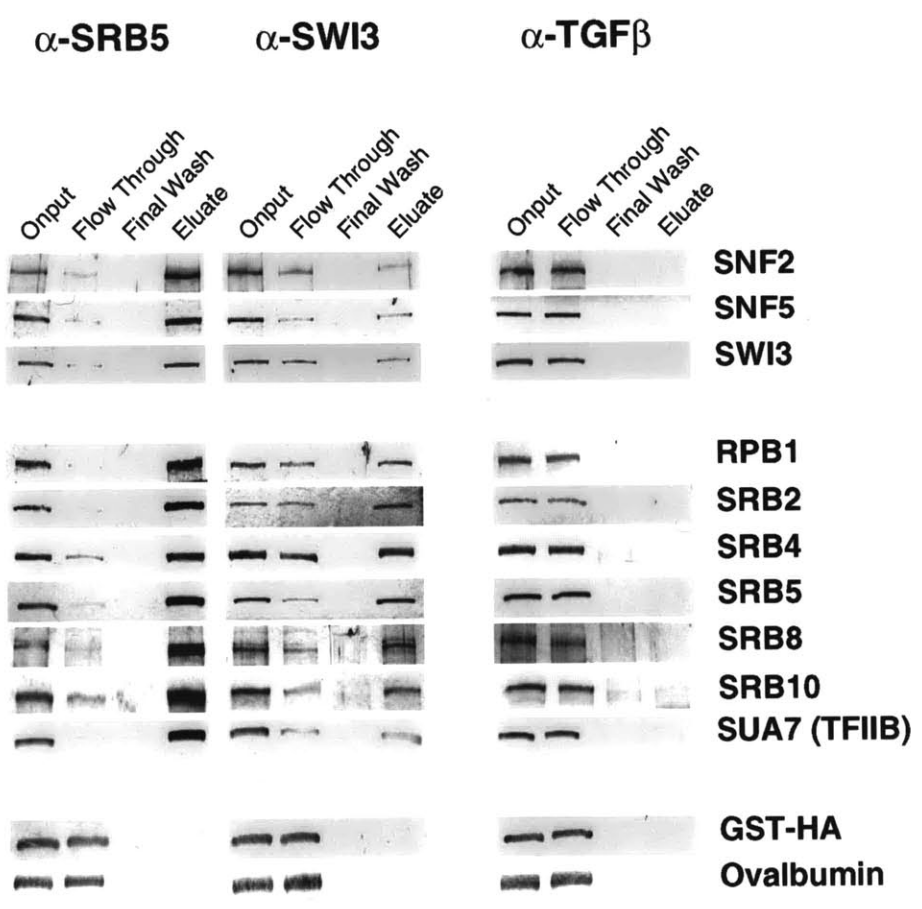
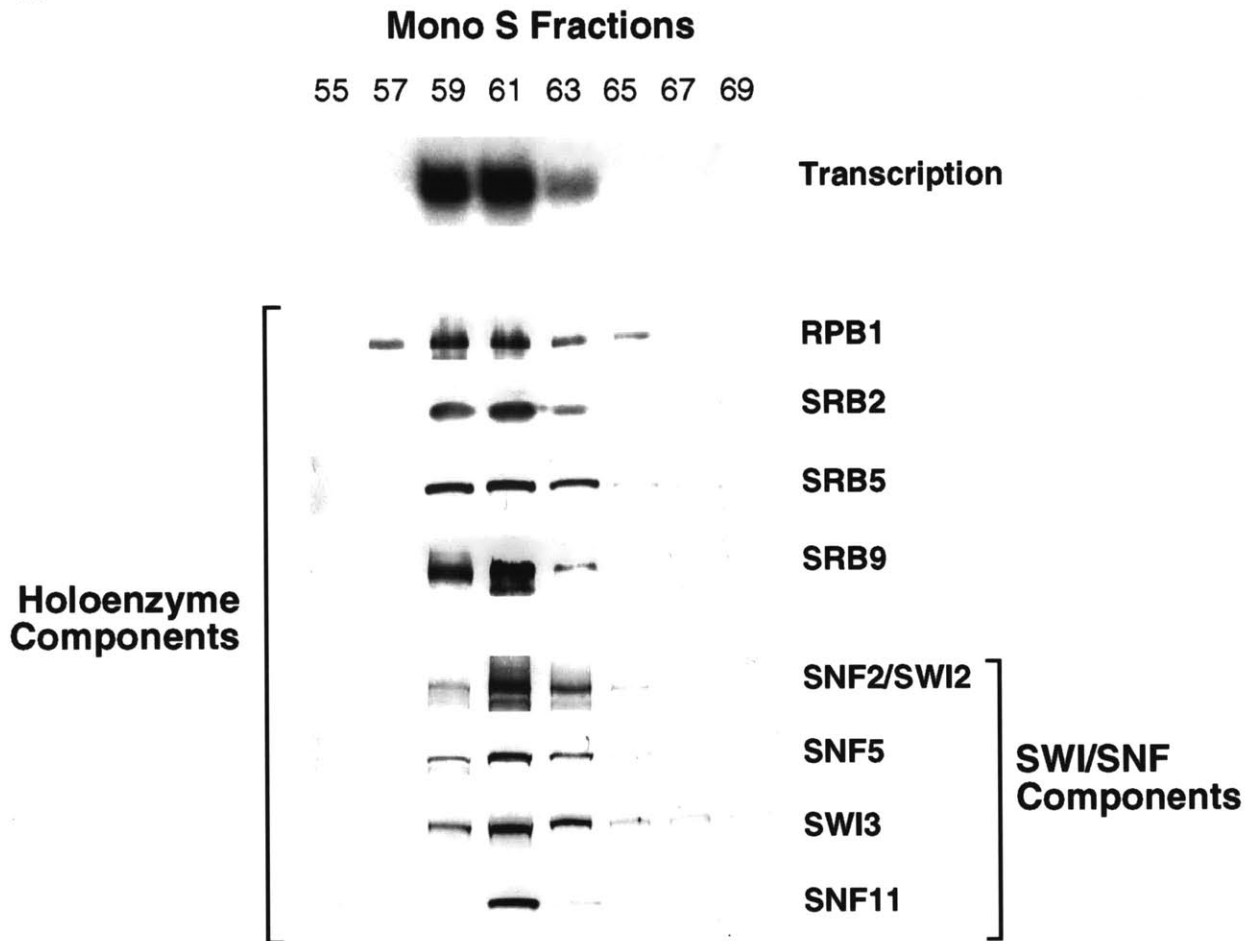


Figure 2 SWI/SNF proteins are components of purified RNA polymerase II holoenzyme.

(A) RNA polymerase II holoenzyme eluted from a Mono S column, the last chromatographic step in the purification procedure (Koleske and Young, 1994), was analyzed for transcriptional activity and for the presence of SRB and SWI/SNF proteins by Western blot.

(B) Quantitative western blots used to determine the relative amounts of SRB5 and SWI/SNF proteins in the holoenzyme. Known amounts of recombinant GST-SNF2/SWI2₁₂₅₆₋₁₇₀₃, GST-SNF5₁₋₁₉₃ and SRB5 were subject to SDS-PAGE and Western blot analysis along with 2.5 μ l and 0.5 μ l of purified holoenzyme. There are similar levels of SNF2/SWI2, SNF5 and SRB5 in the purified holoenzyme. Previous studies have shown that RBP1 and other SRB proteins are equimolar in purified holoenzyme (Koleske and Young, 1994).

A



B

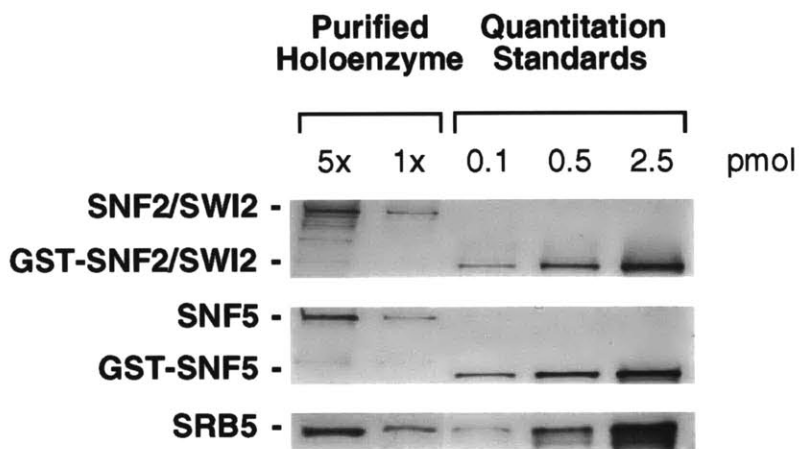


Figure 3 SWI/SNF proteins are present in mediator purified using 8WG16 monoclonal antibodies.

Mediator was western blotted along with holoenzyme and core polymerase and probed for the presence of SWI/SNF proteins. The mediator preparation was previously assayed (Hengartner et. al., 1995) and shown to have all transcriptional activities previously described (Kim et. al., 1994).

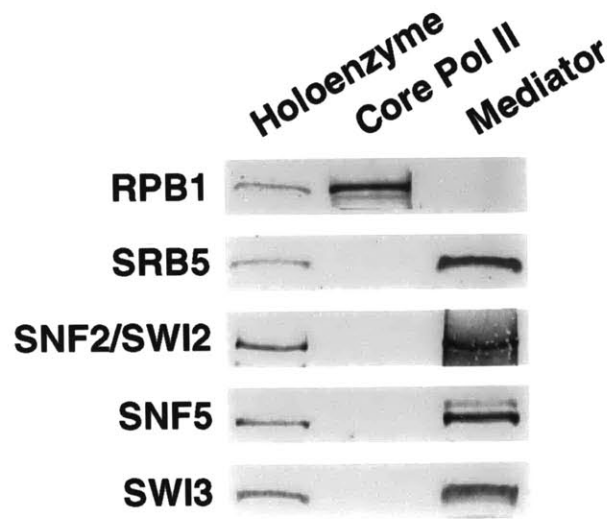


Figure 4 An SRB/SWI/SNF complex purified using CTD-affinity chromatography.

(A) Schematic diagram of the purification.

(B) SRB, SWI, and SNF proteins bind specifically to a GST-CTD column.

Western blot analysis of proteins eluted from a GST column and from a GST-CTD column. TFIIE was a negative control for specific retention, as it does not bind GST or GST-CTD.

(C) Silver stain of fractions across the final mono Q column.

(D) Western blot analysis of SRB and SWI/SNF proteins across the final mono Q column.

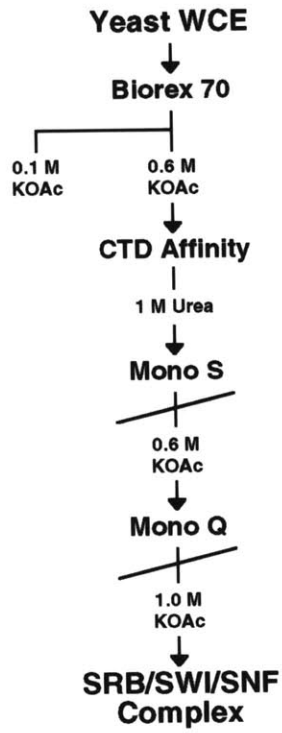
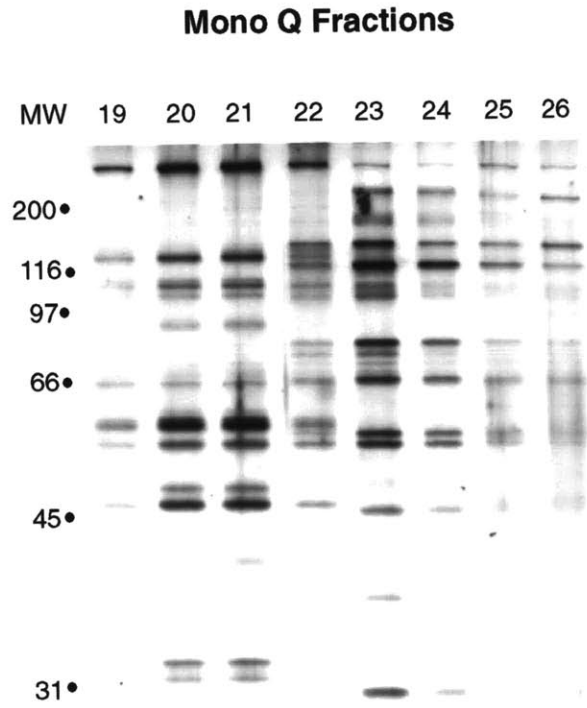
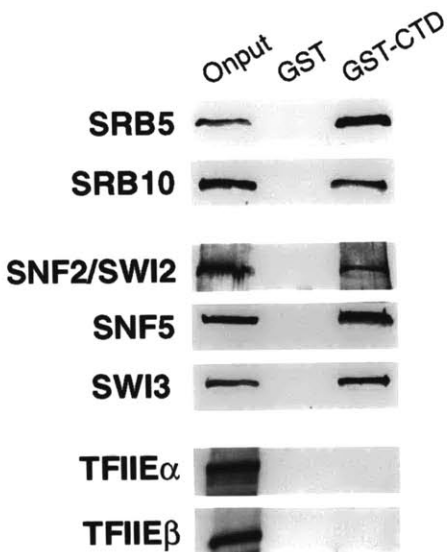
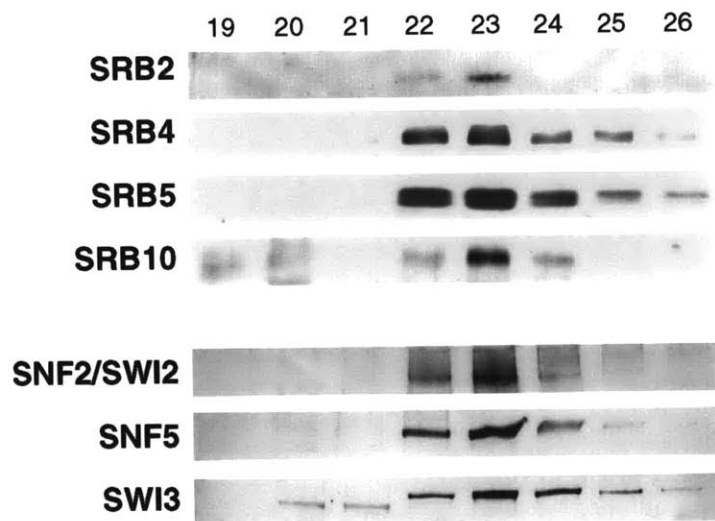
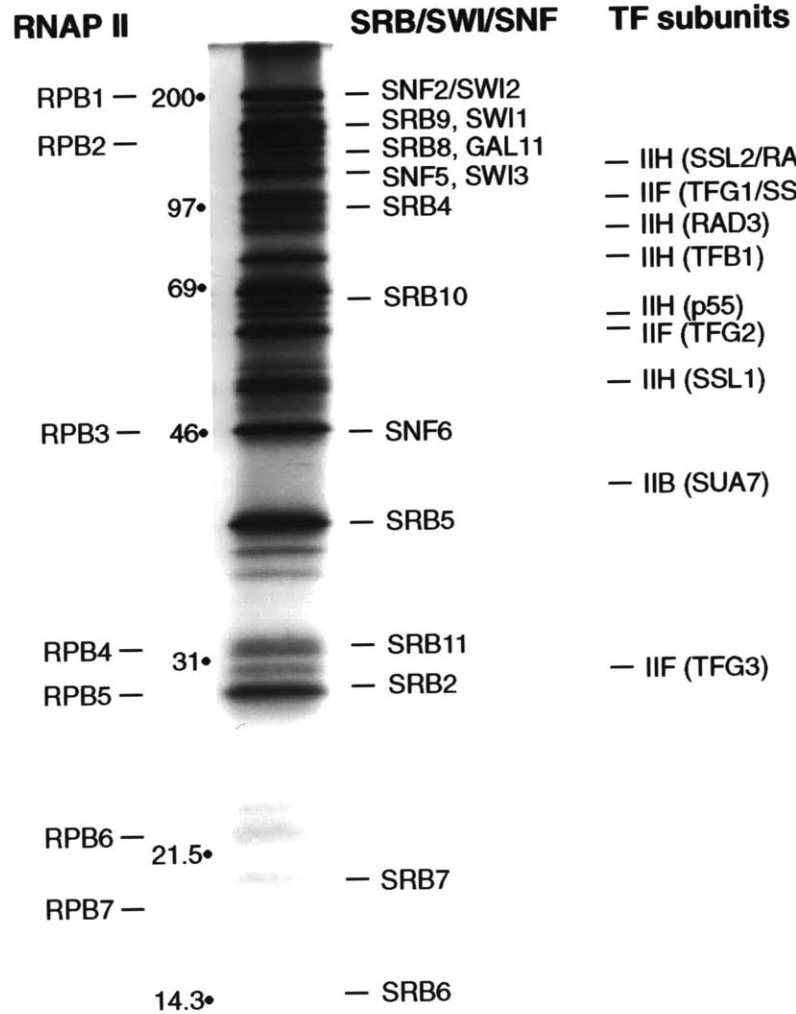
A**C****B****D**

Figure 5 Components of the RNA polymerase holoenzyme and the SRB/SWI/SNF complex

(A) Silver stain of purified RNA polymerase II. Bands which correspond in size to RNA polymerase core subunits, SRB, SWI and SNF proteins, and general transcription factor IIB, IIF, and IIH subunits are indicated.

(B) Silver stain of the SRB/SWI/SNF complex. Bands which correspond in size to SRB, SWI, and SNF proteins are indicated.

A Holoenzyme



B SRB/SWI/SNF Complex

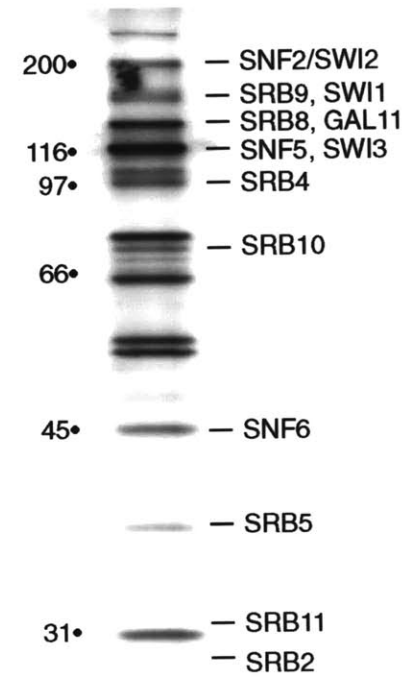


Figure 6 An ATP-dependent nucleosomal disruption activity coelutes with the holoenzyme and the SRB/SWI/SNF complex.

(A) Fractions from the last column of holoenzyme purification (Figure 2A) were assayed for nucleosomal disruption. The peak of nucleosomal disruption activity is in fractions 59-63, coincident with the peak of transcriptional activity.

(B) Purified RNA polymerase II holoenzyme (fraction #60) was titrated for activity with and without 4 mM ATP, as indicated.

(C) Fractions from the final column of the SRB/SWI/SNF complex purification (Figure 4) were assayed for nucleosomal disruption. The peak of nucleosomal disruption activity is in fractions 23 and 24, which is also where the bulk of SRB and SWI/SNF proteins elute.

(D) The SRB/SWI/SNF complex (fraction #24) was titrated for activity with and without 4 mM ATP, as indicated.

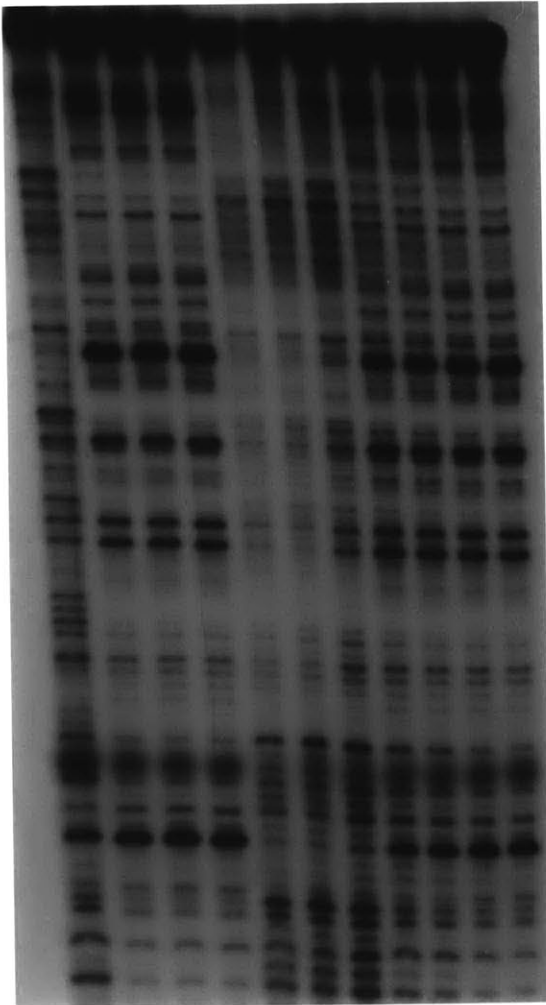
A

**Holoenzyme
Mono S Fractions**

+ ATP



N - 55 57 59 61 63 65 67 69 -



B

**Holoenzyme
Mono S Fraction 60**

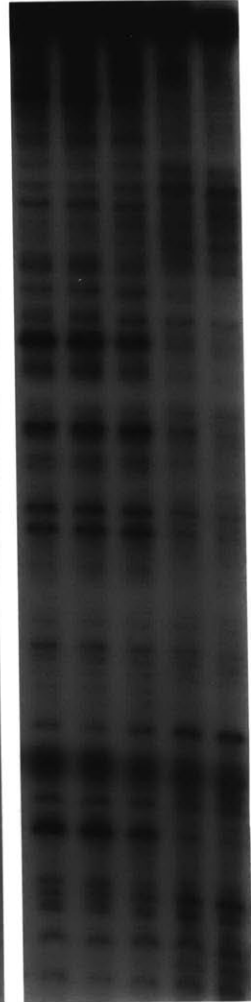
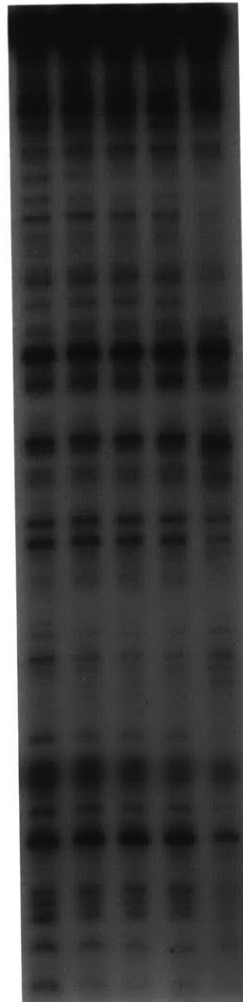
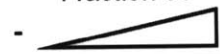
- ATP

+ ATP



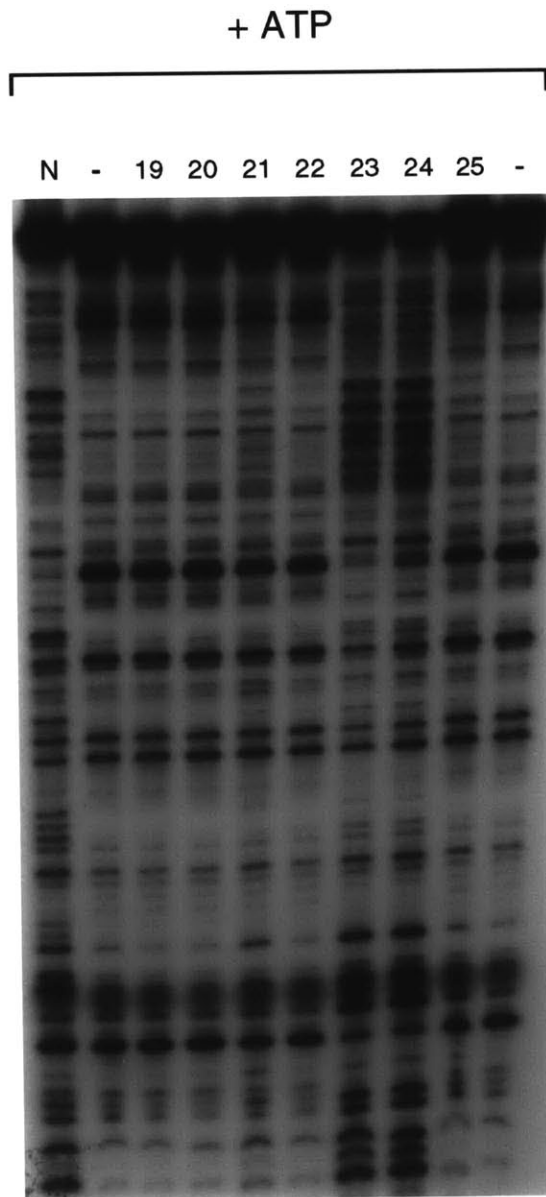
Fraction 60

Fraction 60



C

**SRB/SWI/SNF Complex
Mono Q Fractions**



D

**SRB/SWI/SNF
Complex Mono Q**

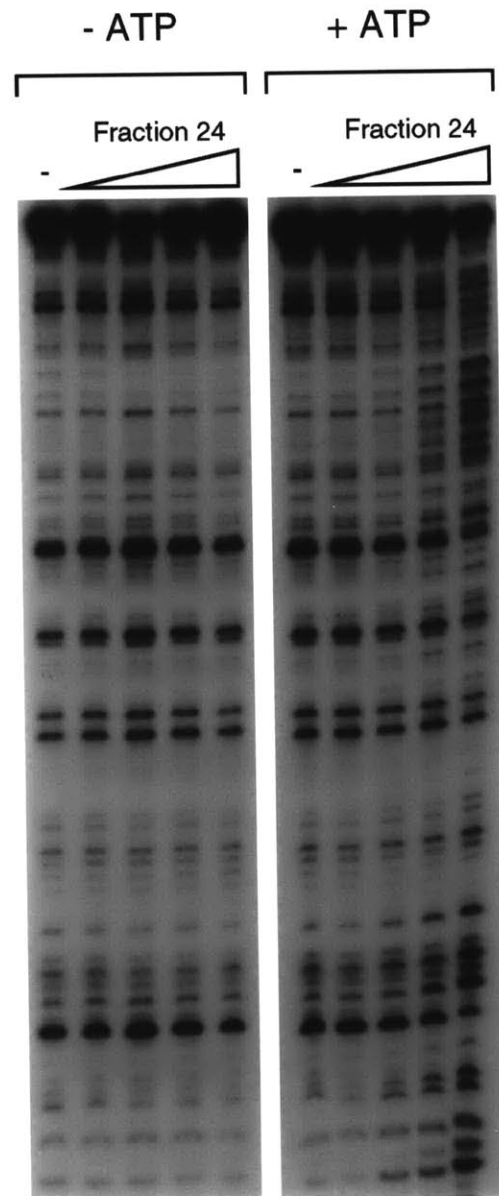
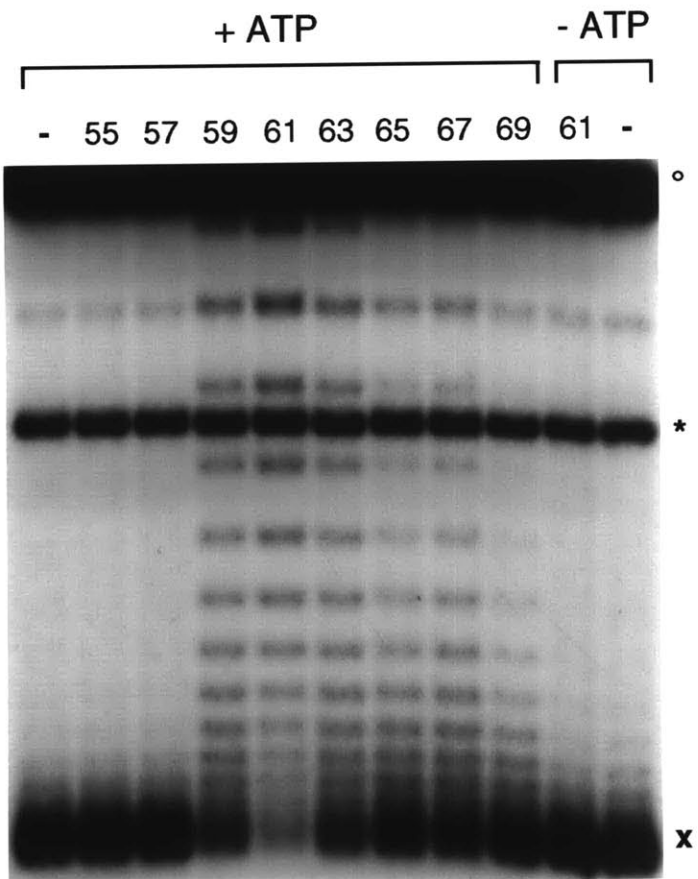


Figure 7 The holoenzyme and the SRB/SWI/SNF complex reduce the superhelical density of chromatin assembled plasmids in an ATP-dependent manner.

Fractions from the last column of holoenzyme purification (A) and from the last column of SRB/SWI/SNF complex purification (B) were assayed in the presence of 4 mM ATP. Peak fractions of purified holoenzyme and SRB/SWI/SNF complex were assayed with and without 4 mM ATP present as described in experimental procedures. The symbols "o", "*" and "x" indicate nicked circular plasmid DNA, linear DNA, and highly supercoiled circular DNA, respectively.

A

**Holoenzyme
Mono S Fractions**

**B**

**SRB/SWI/SNF Complex
Mono Q Fractions**

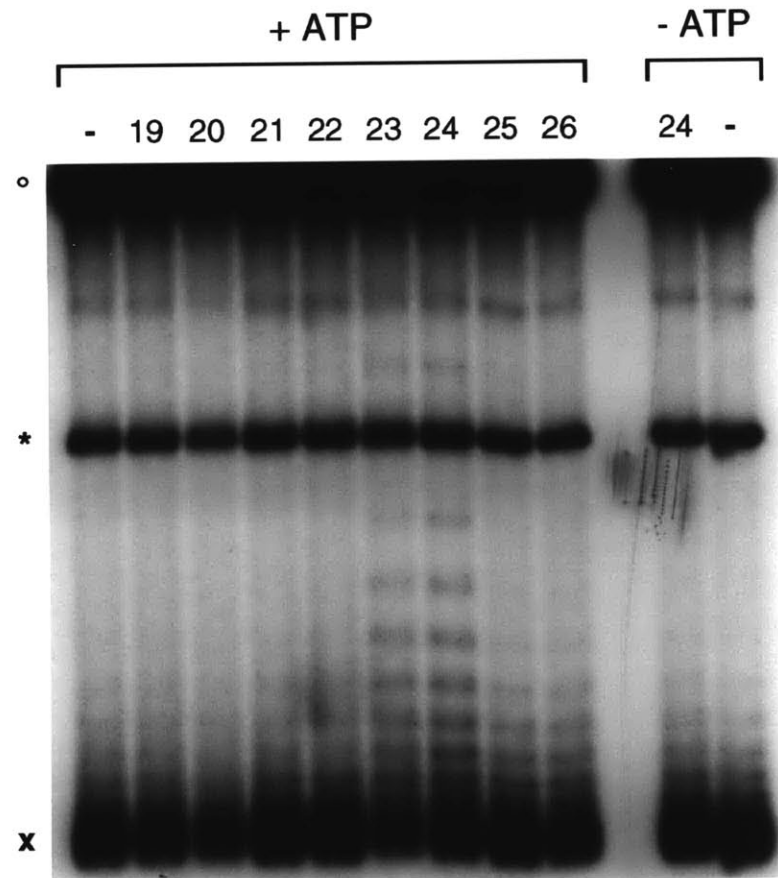
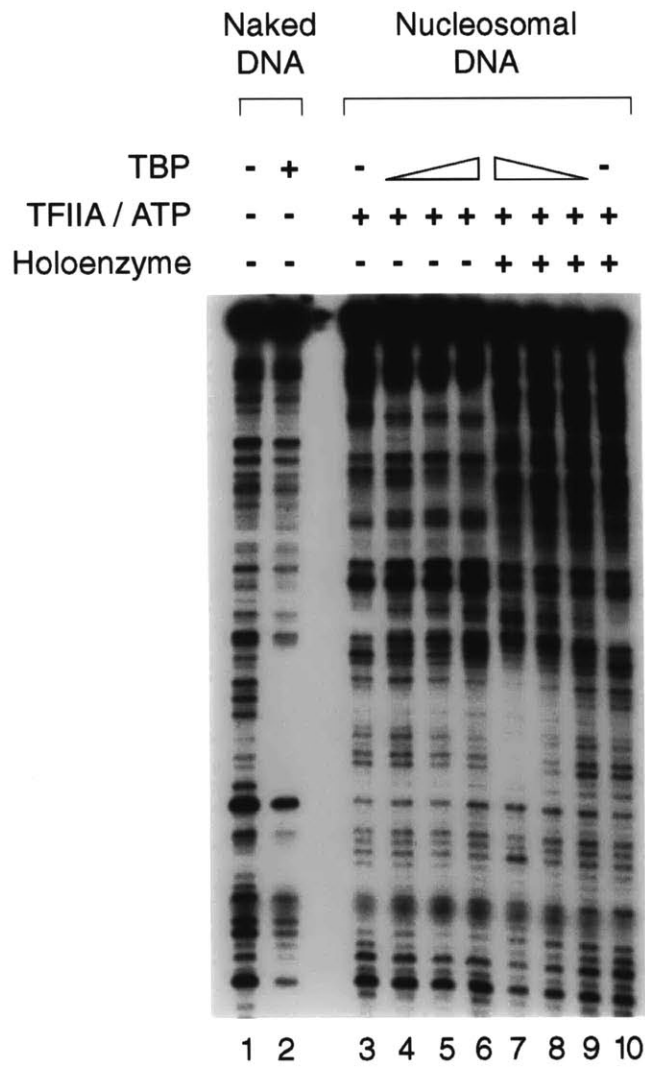
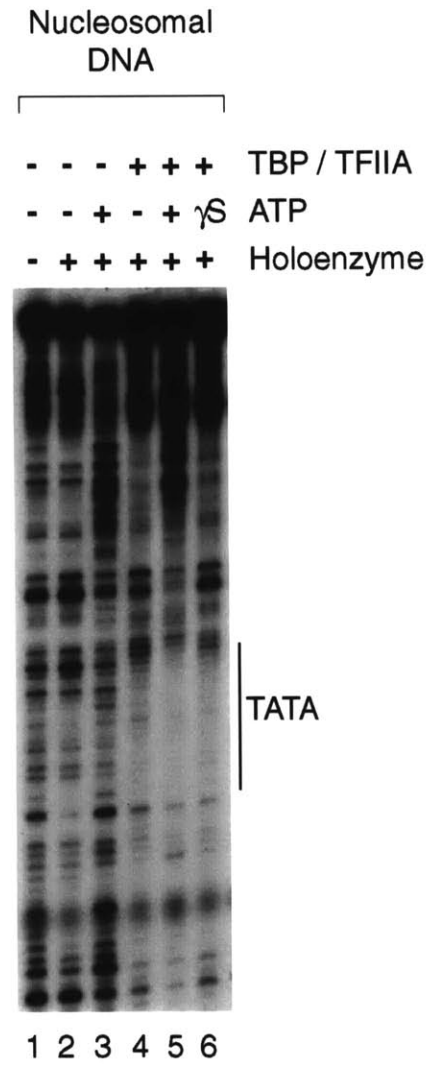


Figure 8 Holoenzyme facilitates binding of yeast TBP and yeast TFIIA to a nucleosome containing a TATA box in an ATP enhanced manner.

(A) Increasing amounts of yTBP in the presence of yTFIIA and 4 mM ATP were tested for the ability to bind to a TATA box containing nucleosome with and without holoenzyme present.

(B) Nucleosomes were incubated with and without holoenzyme, 4 mM ATP or 4 mM ATP γ S, yTBP and yTFIIA, as indicated.

A**B**

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Chapter 3

Temporal regulation of RNA polymerase II by Srb10 and Kin28 cyclin-dependent kinases

Summary

Two cyclin-dependent kinases have been identified in yeast and mammalian RNA polymerase II transcription initiation complexes. We find that the two yeast kinases are indistinguishable in their ability to phosphorylate the RNA polymerase II CTD, yet in living cells one kinase is a positive regulator and the other a negative regulator. This paradox is resolved by the observation that the negative regulator, Srb10, is uniquely capable of phosphorylating the CTD prior to formation of the initiation complex on promoter DNA, with consequent inhibition of transcription. In contrast, the TFIIF kinase phosphorylates the CTD only after the transcription apparatus is associated with promoter DNA. These results reveal that the timing of CTD phosphorylation can account for the positive and negative functions of the two kinases and provide a model for Srb10-dependent repression of genes involved in cell type specificity, meiosis, and sugar utilization.

Introduction

Cyclin-dependent kinases (CDKs), originally described as cell cycle regulators, also have roles in transcription (reviewed in Dynlacht, 1997). Two distinct cyclin-dependent kinases are associated with eukaryotic RNA polymerase II transcription initiation complexes (Liao et al., 1995; Maldonado et al., 1996; Pan et al., 1997; Scully et al., 1997). The yeast kinase Kin28, and its mammalian homologue Cdk7, are subunits of the general transcription factor TFIIF, which phosphorylates RNA polymerase II subsequent to formation of the pre-initiation complex (PIC) on promoter DNA (Feaver et al., 1994; Roy et al., 1994; Serizawa et al., 1995; Shiekhattar et al., 1995). Yeast Srb10, and its mammalian homolog Cdk8, are subunits of the RNA polymerase II holoenzyme, but their functions are not yet understood (Liao et al., 1995; Maldonado et al., 1996; Pan et al., 1997; Scully et al., 1997).

The C-terminal domain (CTD) of the large subunit of eukaryotic RNA polymerase II (pol II) contains a repeated heptapeptide which is phosphorylated in a portion of pol II molecules in the cell (Cadena and Dahmus, 1987; Kolodziej, et al., 1990; reviewed in Dahmus, 1996). Several lines of evidence indicate that PIC formation involves RNA polymerase II molecules with unphosphorylated CTDs, and that these molecules become phosphorylated during or after the transition to active elongation. The form of pol II found in holoenzymes lacks phosphate on its CTD (Koleske and

Young, 1994; Kim et al., 1994). The unphosphorylated form of pol II preferentially assembles into a PIC reconstituted with purified transcription factors (Bartholomew et al., 1986; Laybourn and Dahmus, 1990; Lu et al., 1991; Chesnut et al., 1992; Usheva et al., 1992; Kang et al., 1993; Maxon et al., 1994). Since the phosphorylated CTD has a role in recruiting the mRNA capping enzyme to the nascent transcript, and mRNA capping occurs soon after promoter clearance (Coppola et al., 1983; Cho et al., 1997; McCracken et al., 1997a; McCracken et al., 1997b; Yue et al., 1997), CTD phosphorylation most likely occurs during the transition from transcription initiation to elongation. pol II molecules in the midst of elongation contain CTDs which are highly phosphorylated (Bartholomew et al., 1986; Cadena and Dahmus, 1987; Weeks et al., 1993; O'Brien et al., 1994).

The TFIIF kinase is apparently responsible for CTD phosphorylation subsequent to PIC formation (Laybourn and Dahmus, 1990; Ohkuma and Roeder, 1994; Akoulitchev et al., 1995; reviewed in Dahmus, 1996). Consistent with such a role, TFIIF has been found as one of two CTD kinases stimulated by the viral transactivator Tat (Parada and Roeder, 1996; Garcia-Martinez et al., 1997; Cujec et al., 1997). The HIV-1 Tat protein enhances transcription elongation by interacting with either TFIIF or P-TEFb CTD kinases to stimulate CTD phosphorylation (reviewed in Jones, 1997; Yankulov and Bentley, 1998). Loss-of-function mutations in the yeast TFIIF kinase subunit cause a general defect on class II transcription in vivo

(Cismowski et al., 1995; Valay et al., 1995), confirming the positive role in transcription inferred from in vitro studies.

RNA polymerase II holoenzymes have been purified from yeast and mammalian cells which contain a second CDK implicated in CTD phosphorylation. Genes encoding the Srb10 kinase, and its cyclin partner Srb11, were initially identified in a yeast genetic screen designed to reveal factors involved in CTD function; subsequent analysis revealed that their protein products copurify with the other SRB proteins in the RNA polymerase II holoenzyme (Nonet and Young, 1989; Liao et al., 1995). Holoenzymes with a catalytically inactive Srb10 subunit have substantially reduced CTD kinase activity, suggesting that Srb10 is a CTD kinase (Liao et al., 1995), but this has yet to be directly demonstrated. Mammalian Cdk8 and cyclin C are apparently homologues of Srb10 and Srb11, as they share strong sequence similarity and are both found in mammalian holoenzymes (Maldonado et al., 1996; Pan et al., 1997; Scully et al., 1997; D. Chao and R. Young, unpublished data).

While the function of the TFIIF kinase has been thoroughly studied, the function of Srb10 is poorly understood. It is clear, however, that yeast Kin28 and Srb10 CDKs are not functionally redundant. Substantial genetic and biochemical evidence indicates that Kin28 plays an essential, general and positive role in transcription. In contrast, the evidence suggests that Srb10 is essential for regulation of a subset of genes which are involved in cell type specificity (Wahi and Johnson, 1995), meiosis (Surosky et al., 1994), and sugar

utilization (Kuchin et al., 1995). How Srb10 contributes to the regulation of these important genes is not yet clear.

Here we describe evidence that the two holoenzyme CDKs are indistinguishable in their ability to phosphorylate the CTD, yet in living cells Kin28 functions as a positive regulator and Srb10 as a negative regulator. The different regulatory consequences appear to be due to the fact that the Srb10 kinase is able to phosphorylate the CTD prior to holoenzyme binding to promoter DNA, with consequent inhibition of transcription. In contrast, Kin28 is active only after PIC formation, and plays a positive role through CTD phosphorylation. These results support a novel model for transcriptional regulation in which the negative and positive roles of the two kinases, which act on the same substrate, are a consequence of the time at which they are activated. This model describes how Srb10 contributes to repression of yeast genes involved in cell type specificity, meiosis, and sugar utilization.

Results

Srb10 is a negative regulator of transcription in vivo

The *Srb10* gene was identified in a genetic screen designed to reveal genes whose products interact functionally with the RNA polymerase II carboxy-terminal domain (CTD) (Liao et al., 1995). Cells containing a CTD truncation mutation exhibit conditional lethality, and extragenic suppressors (SRBs) were identified which restore the ability of these cells to grow at the nonpermissive temperature (reviewed in Koleske and Young, 1995). We have found that the suppressing phenotype of the *srb10-1* allele, the original recessive suppressor obtained in the selection, can be duplicated by altering a single amino acid residue in *Srb10* which is critical for its kinase function (*srb10-3*; *Srb10(D290A)*) or by deleting the entire *SRB10* gene (*srb10Δ1*) (Figure 1 A). The observation that loss-of-function mutations in *SRB10* can restore viability to yeast cells with a CTD truncation indicates that the *Srb10* kinase normally has a negative role in transcription in vivo.

The effect of *SRB10* mutations on yeast cells with a spectrum of CTD truncation mutations (Figure 1B) supports a negative role for *Srb10* in transcription. We previously demonstrated that progressive truncation of the RNA polymerase II CTD produced cells with increasingly severe growth phenotypes, and that these phenotypes were due to functional defects rather than reduced stability of the pol II molecules (Nonet et al., 1987; Scafe et al., 1990). The phenotypes exhibited by each of eighteen different strains, which

differ only in pol II CTD length, were classified into three categories: nonviable (N) strains which failed to grow under any condition, conditional lethal (C) strains which were cold sensitive, and viable (V) strains which exhibited essentially wild-type growth phenotypes. The wild-type *SRB10* gene was replaced with the *srb10-1* or the *srb10Δ1* allele, and the growth phenotypes of these cells were examined. The results, summarized in Figure 1B, demonstrate that the loss of Srb10 function restores full viability to CTD-mutants which exhibited conditional lethal phenotypes in the presence of wild-type Srb10 kinase activity. In addition, the loss of Srb10 function rescues N15 cells, which were inviable with wild-type Srb10. Thus, the loss of this putative CTD kinase increases the viability of cells whose pol II molecules have shortened CTDs. In contrast, the loss of Srb2 or Srb4, both positive acting transcription factors, decreases the viability of these cells (Figure 1B). These results provide strong evidence that Srb10 is a negative regulator of transcription in vivo.

An artificial holoenzyme recruitment assay (Barberis et al., 1995; Farrell et al., 1996; reviewed in Ptashne and Gann, 1997) provides another in vivo test of the hypothesis that Srb10 is a negative regulator. Tethering of a holoenzyme component (such as Gal11, Srb2, etc.) to a sequence specific DNA-binding domain (LexA) is sufficient to activate transcription from a promoter containing the appropriate upstream element, as the tethered holoenzyme component apparently recruits the remaining transcription apparatus to the promoter. If the kinase activity of Srb10 has a negative

function in vivo, a mutation which eliminates kinase activity but does not alter the ability of the protein to interact with the holoenzyme should be a better artificial activator than its wild-type counterpart because it would recruit holoenzyme as efficiently as its wild-type counterpart, but would have no inhibitory effect on transcription (Figure 2A). Strains were constructed which contain the LexA DNA binding domain fused with either the wild-type Srb10 sequence or the Srb10(D290A) sequence. The D290A mutation renders Srb10 protein catalytically inactive but fully capable of being incorporated into the holoenzyme (Liao et al., 1995). The results of the experiment (Figure 2B) demonstrate that LexA-Srb10 has substantially less activity in artificial recruitment than LexA-Srb10(D290A). This result supports the model that the Srb10 kinase is a negative regulator of transcription in vivo.

CTD Phosphorylation by Srb10 and Kin28 CDKs

Although two CDKs have been identified in yeast and mammalian holoenzyme preparations (Liao et al., 1995; Maldonado et al., 1996; Pan et al., 1997; Scully et al., 1997), only the TFIIF kinases have been demonstrated to phosphorylate the CTD directly (Feaver et al., 1991; Lu et al., 1992; Serizawa et al., 1992). The Srb10 kinase has been proposed to be a CTD kinase based upon genetic evidence for a interaction with the CTD and evidence that holoenzyme preparations lacking Srb10 activity have substantially reduced CTD kinase activity (Liao et al., 1995). We tested whether purified Srb10 has

CTD kinase activity and, if so, how it compares to Kin28 CTD kinase activity. Epitope-tagged recombinant Srb10/Srb11 and Kin28/Ccl1 cyclin-dependent kinases were expressed in a baculovirus expression system and purified in a one-step affinity purification (Figure 3). Catalytically inactive recombinant Srb10(D290A)/Srb11 and Kin28(D147A)/Ccl1 cyclin-dependent kinases were also produced and purified as controls. Both Srb10/Srb11 and Kin28/Ccl1 were found to be capable of phosphorylating recombinant glutathione-S-transferase-CTD (GST-CTD) (Figure 4A). The recombinant kinase-cyclin pairs phosphorylated rGST-CTD and pure yeast RNA polymerase II with similar efficiencies (data not shown). Neither kinase could phosphorylate GST alone, calf thymus histone H1, the other kinase-cyclin pair, or general transcription factors (GTFs) (Figure 4A and data not shown). The activity of Srb10/Srb11 and Kin28/Ccl1 could be directly attributed to the highly purified kinases, since the catalytically inactive CDK mutant kinases were unable to phosphorylate GST-CTD at any level (Figure 4B). These results demonstrate that Srb10 and Kin28 CDKs are both capable of phosphorylating the CTD.

Genetic evidence presented here and elsewhere (Cismowski et al., 1995; Valay et al, 1995) indicate that Kin28 and Srb10 contribute positive and negative functions, respectively, to transcription *in vivo*. We investigated the possibility that differential phosphorylation of the CTD by the two CDKs might account for their different functions. The amino acid residues of the CTD phosphorylated *in vitro* by the two CDKs were identified by two

dimensional thin layer chromatography. The results demonstrate that both Srb10 and Kin28 phosphorylate serine residues (Figure 4C).

To investigate further the substrate specificity of the two CTD kinases, a battery of synthetic peptides were used as substrates to determine which amino acid residues in the heptapeptide consensus repeat are critical for CTD phosphorylation (Figure 4D). The results show that the activities of Srb10 and Kin28 on these peptide substrates are indistinguishable. Substitution of Ser₂, Thr₄ or Ser₇ with alanine did not significantly affect the ability of the peptide to act as a substrate for either kinase. In contrast, substitution of Ser₅ with alanine led to a dramatic loss in peptide phosphorylation, suggesting that Ser₅ is the principal phosphoacceptor in the heptapeptide repeat. Substitutions of Tyr₁, Pro₃ or Pro₆ reduced phosphorylation of the synthetic peptides, probably due to the effects such alterations have on their structure. These results indicate that Srb10 and Kin28 CDKs are indistinguishable in substrate specificity and activity in these CTD phosphorylation assays.

Srb10 Dependent Inhibition of Transcription In Vitro

The existence of two CDKs in the holoenzyme with similar biochemical specificity and activity, yet opposite in vivo function, led us to entertain the possibility that the timing of CTD phosphorylation in the holoenzyme could determine whether the event had a negative or a positive consequence. Although both kinases are capable of CTD phosphorylation as purified, recombinant kinase-cyclin pairs, it is possible that they can function

only at certain times when assembled in the holoenzyme. We therefore considered a temporal model for the action of these kinases, in which Srb10 is uniquely capable of CTD phosphorylation prior to initiation complex formation by the holoenzyme, thereby repressing transcription. In contrast, Kin28, when assembled into the holoenzyme, is capable of CTD phosphorylation only after preinitiation complex formation, when such activity would not interfere with transcription.

This temporal model predicts that holoenzymes with catalytically active Srb10 should be transcriptionally inhibited when the kinase functions prior to association with template DNA. RNA polymerase II holoenzymes containing Kin28 and either wild-type Srb10 or catalytically inactive Srb10(D290A) were purified in parallel and assayed for kinase and transcriptional activities (Figure 5). The two purified holoenzymes contained comparable amounts of Rpb1, Srb2, Srb4, Srb5, Srb10 and Kin28 (Figure 5A). To determine whether Srb10 kinase activity can inhibit transcription by acting prior to PIC formation, we performed an *in vitro* transcription experiment in which both wild-type and mutant Srb10 containing holoenzymes were preincubated with ATP prior to addition of template DNA, additional GTFs and nucleoside triphosphates (NTPs) (Figure 5B). Preincubation with ATP produced a significant inhibition of transcription with the wild-type holoenzyme, but not with the holoenzyme lacking Srb10 catalytic activity (Figure 5B; compare lanes 2 and 4). These data show that transcription by

RNA polymerase II holoenzyme is inhibited when the Srb10 kinase is allowed to function prior to PIC formation.

Pol II CTD phosphorylation was monitored in the holoenzymes that were subjected to preincubation with and without ATP (Figure 5B). CTD phosphorylation occurred only in holoenzymes containing catalytically active Srb10 (Figure 5B, compare lanes 2 and 4). Kin28 is apparently not active in the holoenzyme prior to PIC formation, because the Srb10 mutant holoenzyme exhibits essentially no CTD phosphorylation activity during the preincubation. The Srb10-dependent phosphorylation of the CTD was highly efficient; most of the pol II molecules in the wild-type holoenzyme became phosphorylated in this reaction (Figure 5B, compare lanes 1 and 2). A control experiment confirmed the specificity of the antibodies used to detect unphosphorylated and phosphorylated CTDs (Figure 5C). Thus, Srb10-dependent CTD phosphorylation is coincident with repression of transcription, a result consistent with previous evidence that formation of a functional preinitiation complex is impaired if the pol II molecules contain phosphorylated CTDs (Lu et al., 1991; Chesnut et al., 1992; Usheva et al., 1992; Kang et al., 1993; Maxon et al., 1994).

The temporal model also predicts that RNA polymerase II holoenzymes which are allowed to bind template DNA prior to addition of nucleoside triphosphates should not be transcriptionally inhibited by Srb10 activity. The experiment shown in Figure 5D shows that the wild-type and Srb10 mutant holoenzymes are in fact equally active in transcription under

these conditions, confirming the prediction. The state of CTD phosphorylation was also assayed after the transcription reaction, revealing that CTD phosphorylation occurs in RNA polymerase II molecules from holoenzymes with or without functional Srb10, albeit the levels are three-fold less in holoenzymes lacking catalytically active Srb10 kinase. These results indicate that Srb10-independent CTD phosphorylation occurs during the *in vitro* transcription reaction, as would be expected from the action of Kin28.

We and others have found that Srb10 is critical for regulation of a subset of genes in yeast cells, including those involved in cell type specificity (Wahi and Johnson, 1995), meiosis (Surosky et al., 1994), and sugar utilization (Liao et al., 1995; Kuchin et al., 1995). Srb10 is not a general repressor of transcription, as a variety of genes are expressed normally in Srb10 mutant cells (Surosky et al., 1994, Liao et al., 1995), and the levels of active holoenzyme are similar in wild-type and Srb10 mutant cells (S.S.K. and R.Y., unpublished). The observation that Srb10 is not a general repressor of protein-coding genes suggests that in living cells, where there is abundant ATP, Srb10 activity in holoenzymes must be inhibited in order to prevent constitutive inactivation of the general transcription initiation apparatus. To test this idea, we produced nuclear extracts from wild-type and Srb10(D290A) mutant strains and investigated whether the wild-type extract showed an ATP-dependent inhibition of transcription prior to PIC formation. We previously showed that the transcriptional activity in these extracts is entirely

dependent on components of the Srb/mediator complex (Koleske et al., 1992; Thompson et al., 1993), which are tightly associated with pol II holoenzymes (Koleske and Young, 1994). The results, shown in Figure 6, demonstrate that transcription in nuclear extracts is not inhibited by preincubation with ATP, suggesting that these extracts contain an Srb10 inhibitory activity which is lost during holoenzyme purification.

Discussion

Yeast and mammalian RNA polymerase II holoenzymes have been described which contain two cyclin-dependent kinases. Previous studies established that Kin28 is a CTD kinase with a positive role in transcription, that of producing a phosphorylated form of the enzyme which is associated with active elongation. Genetic and biochemical evidence described here reveals that the Srb10 kinase is a CTD kinase with a negative role in transcription. Srb10 is uniquely capable of phosphorylating the CTD in purified holoenzymes prior to template binding, and this phosphorylation inhibits subsequent transcription by the holoenzyme. Srb10 does not appear to inhibit transcription after formation of a stable preinitiation complex. Thus, the transcription initiation apparatus can be regulated positively or negatively via modification of the CTD, depending on the timing of the phosphorylation event (Figure 7).

In arriving at this temporal model, we first examined the two most obvious models which could account for differential regulation by the two CDKs. It was possible that the Srb10 and Kin28 kinases could act on other substrates in the transcription initiation apparatus, but we did not detect phosphorylation of general transcription factors or histones, nor did we find that either kinase could phosphorylate the other. It was also possible that the two kinases phosphorylated different residues on the CTD, but our

experiments indicate that they exhibit very similar substrate recognition and modification behaviors. The one clear difference in behavior was the unique ability of Srb10 to phosphorylate the pol II CTD prior to initiation complex formation when a component of the holoenzyme. We conclude that the temporal regulation of transcription by CDKs is an instance where a specific phosphorylation event, carried out at different times, can produce opposite regulatory effects in the cell.

Negative Regulation by Srb10 in vivo

Progressive truncation of the RNA polymerase II CTD produces cells with increasingly severe growth phenotypes (Nonet et al., 1987). The greater the truncation of the CTD, the larger the number of genes affected, accounting for the increasingly severe growth phenotypes (Scafe et al., 1990). The *SRB* genes were originally identified as suppressors of defects due to CTD truncation. A subset of these genes, for example those encoding Srb4 and Srb6, are essential for expression of most protein-coding genes (Thompson and Young, 1995). In contrast, Srb8, Srb9, Srb10 and Srb11 are not essential for expression of protein coding genes generally, but are critical for normal regulation of a subset of genes (Surosky et al., 1994; Kuchin et al., 1995; Liao et al., 1995; Wahi and Johnson, 1995).

Genetic and biochemical evidence indicates that Srb2, Srb4, Srb5 and Srb6 all contribute positively to holoenzyme function (Koleske and Young, 1995; Hengartner et al., 1995). For example, mutations which reduce the

function of the Srb2, Srb4, Srb5, Srb6 or Srb7 proteins cause reduced cell viability, and this is exacerbated in cells with CTD truncation mutations (Koleske et al., 1992; Thompson et al., 1993; Hengartner et al., 1995). In contrast, mutations which eliminate Srb10 or Srb11 function actually restore viability to cells with CTD truncation mutations. This, and additional genetic evidence, indicates that Srb10 is a negative regulator of transcription. Highly repressed genes such as *SPO13*, *GAL1*, *SUC2*, *PHO5*, and *MFA2* are derepressed in strains lacking Srb10 activity (Kuchin et al., 1995; Liao et al., 1995; Wahi and Johnson, 1995; S.-M. L. and R. Y., unpublished). Since Srb10 represses only a subset of genes, there must be a mechanism to activate the kinase solely at these genes. We suggest that promoter specific factors repress transcription at these genes by stimulating an otherwise quiescent Srb10 prior to stable association of the holoenzyme with promoter DNA (Figure 7).

Phosphorylation of CTD by Srb10 and Kin28

Previous studies demonstrated that the kinase activity of purified TFIIF could phosphorylate the CTD (Feaver et al., 1991; Lu et al., 1992; Serizawa et al., 1992). Previous reports also indicated that Srb10 is involved in CTD phosphorylation, as the *SRB10* gene was identified in genetic selection for suppressors of a CTD truncation defect, and holoenzymes with catalytically inactive Srb10 protein have markedly reduced CTD phosphorylation activity (Liao et al., 1995). The use of highly purified recombinant forms of the two yeast CDKs allowed us to demonstrate that

they phosphorylate the CTD, to identify the residues of the heptapeptide which are modified, and to compare and contrast their activities. Srb10/Srb11 and Kin28/Ccl1 phosphorylate the CTD with similar efficiency and are indistinguishable in their specificity towards recombinant full length CTD or synthetic heptapeptide repeats, down to the specific residue they modify, Ser₅. These results suggest that the positive and negative regulatory functions of the two CDKs are not due to differences in substrate specificity.

Temporal Regulation via CTD Phosphorylation

The form of pol II found in RNA polymerase II holoenzyme preparations lacks phosphate on its CTD (Koleske and Young, 1994; Kim et al., 1994). Several experimental observations led us to postulate that the timing of CTD phosphorylation in the holoenzyme determines whether the event has a negative or a positive consequence. The two holoenzyme CDKs have very similar biochemical specificity and activity, yet opposite *in vivo* function. In an assay designed to measure transcriptional activity subsequent to template binding, wild-type and Srb10(D290) mutant holoenzymes are indistinguishable. However, previous studies have shown that the phosphorylation state of the CTD affects PIC formation; formation of such a complex is impaired if the pol II molecules contain phosphorylated CTDs (Lu et al., 1991; Chesnut et al., 1992; Usheva et al., 1992; Kang et al., 1993; Maxon et al., 1994). If one of the holoenzyme kinases phosphorylates the CTD prior to template association, it could inhibit subsequent transcription.

We carried out an experiment designed to identify an effect of kinase activity in the holoenzyme prior to pre-initiation complex (PIC) formation on template DNA. This experiment revealed that CTD phosphorylation and transcription inhibition does occur when holoenzymes are provided with ATP prior to template association, but only if Srb10 is catalytically active. In contrast, Kin28 kinase activity in these holoenzymes is not evident prior to template association, but is evident later in the transcription reaction. Thus, the positive and negative roles of the two kinases can be attributed to the time at which they act during the process of transcription initiation. In this model, Srb10-dependent CTD phosphorylation prior to stable PIC formation at specific promoters inhibits transcription initiation, accounting for the negative regulatory activity observed for Srb10 *in vivo*. In contrast, Kin28 phosphorylation of the pol II CTD subsequent to PIC formation has a positive role, that of producing the phosphorylated RNA polymerase II molecule which recruits mRNA capping enzyme and which is associated with efficient elongation of the nascent transcript (Cho et al., 1997; McCracken et al., 1997a; McCracken et al., 1997b; Yue et al., 1997).

Regulation of CDKs

Cyclin-dependent kinases were first described as cell cycle regulators. These kinases are themselves regulated in a temporal fashion through pairing with various cyclins, through phosphorylation events which can have positive or negative effects, and through interactions with CDK

inhibitors. The two holoenzyme CDKs are paired with cyclin molecules, but are not typically activated, since holoenzyme preparations contain RNA polymerase II molecules with unphosphorylated CTDs. Furthermore, our experiments suggest that Srb10 kinase activity is inhibited in nuclear extracts. The future identification of this CDK regulator should reveal important new insights into the molecular mechanisms involved in regulation of cell type specificity, meiosis, sugar utilization and other important cellular processes under the control of Srb10.

Experimental Procedures

Genetic Analysis

To examine the ability of various *SRB10* alleles to suppress the conditional phenotypes caused by a truncated CTD (*rpb1Δ104*), yeast strains Z768 and Z769 (Table 1) were transformed with plasmids containing *SRB10* (pRY2973), *srb10-1* (pRY7091), *srb10Δ1* (pRY2966), and *srb10-3* (pRY7096). Growth conditions were assayed as described (Nonet et al., 1987).

Growth phenotype analysis of yeast cells containing CTD truncation mutations was performed as described (Nonet et al., 1987). The various *SRB10* background strains used were N418 (*SRB10*), Z741 (*srb10Δ1*) and Z735 (*srb10-1*) (Table 1). The viability of cells containing CTD truncations in those backgrounds were assayed by plasmid shuffle, and surviving strains were tested for cold sensitivity.

In Vivo Recruitment Assays

β-galactosidase assays were performed as described (Rose and Botstein, 1983). The strains are derivatives of Z719 transformed with the reporter pSH18-34 and the appropriate LexA fusion. To make the LexA fusions, *SRB10*, *srb10-3*, and *SRB6* open reading frames were subcloned into the LexA fusion plasmid pEG202 (Ausubel et al., 1997).

Recombinant CDK/cyclin Production and Purification from Insect Cells

Recombinant CDK/cyclin pairs were produced using a baculovirus expression system. For expression of CDKs, genes for Srb10 and Kin28 were amplified by polymerase chain reactions (PCR) and cloned into baculoviral transfer vectors pSK277 or pSK278 (Koh et al., 1997) to produce recombinants with FLAG epitope-tag at their N-termini. For expression of cyclins, genes for Srb11 and Ccl1 were amplified by PCR and cloned into baculoviral transfer vectors pBacPAK8 or pBacPAK9 (Clontech). PCRs were performed with Vent DNA polymerase (New England Biolabs). All the PCR clones were verified by DNA sequencing. Mutant CDK clones, Srb10(D290A) and Kin28(D147A), were produced by site-directed in vitro mutagenesis (Kunkel et al., 1987) using oligonucleotides CAAAACCTAAAGCACCAATTTT and CCTTGCTAGACCGAAAGCTGCTACTTTTATCTG, respectively. All mutations were verified by DNA sequencing.

Recombinant baculoviruses were generated from the recombinant transfer plasmids containing CDKs or cyclins by cotransfection of the plasmids with wild-type viral DNA as recommended by the manufacturer (Clontech). *Spodoptera frugiperda* (*Sf21*) cells were coinfecting with recombinant baculoviruses expressing CDKs and their cyclin partners at a multiplicity of infection (m.o.i.) of 5-10. The cells from 200 mL of culture (approx. 3×10^8) were collected 60-72 hr post-infection and lysed by sonication. The lysates were then clarified by centrifuging for 3 hr at $100,000 \times g$ yielding 20-40 mg of total protein in 10 mL. CDK/cyclin pairs were purified from the

lysates as described (Koh et al., 1997) using 1 mL of anti-FLAG M2 affinity gel and 73 µg/mL of FLAG peptide in the elution buffer. Typical yields were 0.2-0.4 % of total protein from cell lysate.

Kinase Assay

Kinase assays were performed using 1 µg of protein substrate (GST, recombinant GST-CTD, or calf thymus histone H1) or 15 µg of synthetic CTD peptide substrate with 100 ng of pure recombinant CDK/cyclin pairs in 15 µl reaction containing 20 mM Hepes-KOH, pH 7.3, 10% glycerol, 2.5 mM EGTA, 15 mM magnesium acetate, 1 mM DTT, 100 mM potassium acetate, 200 µM ATP, 10 µCi [γ -³²P] ATP (NEN, 6000 Ci/mmol, 10 mCi/ml), a mixture of phosphatase inhibitors (1 mM NaN₃, 1 mM NaF, 0.4 mM NaVO₃, 0.4 mM Na₃VO₄), a cocktail of protease inhibitors (0.5 mM PMSF, 1 mM benzamidine, 1 µM pepstatin, 0.3 µM leupeptin and 1 µg/ml chymostatin) and 0.5 mg/ml of acetylated BSA. Reactions were assembled on ice and initiated with the addition of ATP. After 60 min at 25°C, the reactions were terminated by adding 15 µl of Stop Buffer (2X SDS-PAGE loading buffer supplemented with 100 mM Tris-HCl, pH 6.8, and 40 mM EDTA) and then resolved by 4-20% acrylamide gradient SDS-PAGE. The dried gels were exposed directly to autoradiographic films.

Kinase substrates GST and GST-CTD were purified from bacteria as described (Thompson et al., 1993) and purified calf thymus histone H1, purchased (Boehringer Mannheim). Triple CTD heptapeptide consensus

repeats were synthesized (Research Genetics) and were provided in the form H-YSPTSPSYSPTSPSYSPTSPS-Amide. CTD peptide variants where one or more amino acids of the consensus sequence have been systematically replaced by alanine were also synthesized by Research Genetics.

Phosphoamino Acid Analysis

Using GST-CTD as a substrate, a kinase reaction was performed. After SDS-PAGE, the samples were transferred to a PVDF membrane and the labelled phosphorylated GST-CTD band, localized and cut out after a short film exposure. Two-dimensional electrophoretic analysis of phosphoamino acid content was performed subsequent to acid hydrolysis as described in Coligan et al. (1997). The phospholabelled phosphoamino acids were visualized by autoradiography.

Nuclear Extract Transcription

Nuclear extracts from Z719 and Z690 were prepared according to Lue et al. (1991) with the modifications described by Liao et al. (1991), yielding a final protein concentration of 85 and 75 mg/ml, respectively. In vitro transcription was carried out essentially as described (Liao et al., 1991). Each reaction contained 90 µg of Z719 protein or 120 µg of Z690 protein with 250 ng of template.

Transcription and Western Blot Analysis

Holoenzyme was purified according to Liao et al. (1995). In vitro transcription reactions were performed essentially as described (Gadbois et al., 1997) with the following modifications. Preincubations (19 μ l total) contained all reaction components except TBP, TFIIE, TFIIB, nucleotides and DNA template; ATP containing reactions were brought to a final ATP concentration of 1mM with 100 mM stock (Pharmacia). After a fifteen minute preincubation at room temperature, GTFs (3 μ l) and NTP mix (4 μ l; 5 mM ATP, CTP, 0.156 mM UTP, 0.25 mM 3'-O-Me GTP and 10 μ Ci [α -³²P] UTP 3000 Ci/mmole) containing 100ng DNA template (pGAL Δ) were added for a final reaction volume of 26 μ l. After allowing transcription to proceed for 30 minutes at room temperature, reactions were stopped by addition of 125 μ l stop buffer (10 mM Tris-HCl, pH 7.5, 20 mM EDTA, 2 M ammonium acetate, and 10 μ g/ml glycogen) and 150 μ l isopropanol. Samples were placed on dry ice for 10 min., microcentrifuged at 14K RPM for 10 min., pellets resuspended in 6 μ l formamide loading dyes and electrophoresed on a 4% Urea-containing denaturing polyacrylamide gel. Gels were dried and exposed to Kodak X-AR film at -80°C with an intensifying screen.

Samples for western blot analysis were fractionated on 5% SDS-PAGE gels and transferred according to standard procedures. 8WG16 monoclonal antibody (Babco) and Srb4 rabbit anti-serum were used at 1:1000, H5 monoclonal antibody (Babco) was used at 1:250. HRP-conjugated anti-mouse (Pierce) and anti-rabbit (Amersham) secondary antibodies were used at 1:2000.

Detection was performed by ECL according to the manufacturers directions (Amersham).

Acknowledgments

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Table 1. Yeast Strains

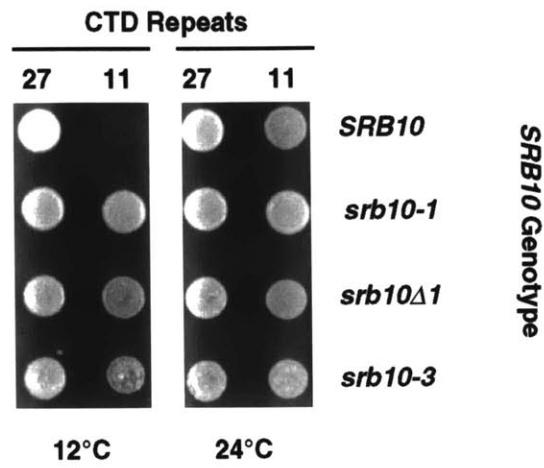
Strain	Alias	Genotype	Reference
Z768	SLY67	Mata <i>ura3-52 his3Δ200 leu2-3,-112 rpb1Δ187::HIS3 srb10Δ1::hisG</i> [L14 (<i>LEU2 CEN RPB1</i>)]	This study
Z769	SLY69	Mata <i>ura3-52 his3Δ200 leu2-3,-112 rpb1Δ187::HIS3 srb10Δ1::hisG</i> [C6 (<i>LEU2 CEN rpb1Δ104</i>)]	This study
N418		Mata <i>ura3-52 his3Δ200 leu2-3,-112 rpb1Δ187::HIS3 SRB10</i> [pRP112 (<i>URA3 CEN RPB1</i>)]	Nonet et al., 1989
Z741	SLY37	Mata <i>ura3-52 his3Δ200 leu2-3,-112 rpb1Δ187::HIS3 srb10Δ1::hisG</i> [pRP112 (<i>URA3 CEN RPB1</i>)]	This study
Z735	SLY26	Mata <i>ura3-52 his3Δ200 leu2-3,-112 rpb1Δ187::HIS3 srb10-1::hisG</i> [pRP112 (<i>URA3 CEN RPB1</i>)]	This study
Z687	SLY7	Mata <i>ura3-52 his3Δ200 leu2-3,-112 RPB1 srb10Δ1::hisG</i>	Liao et al., 1995
Z690	SLY96	Mata <i>ura3-52 his3Δ200 leu2-3,-112 RPB1 srb10-3::hisG</i>	Liao et al., 1995
Z719	SLY3	Mata <i>ura3-52 his3Δ200 leu2-3,-112 RPB1 SRB1</i>	Liao et al., 1995

Figure 1 Srb10 is a negative regulator in vivo.

(A) Loss-of-function mutations in *SRB10* rescue the conditional lethality of a CTD truncation mutant. Strains with a truncated CTD (11 heptapeptide repeats) are inviable when grown at 12°C. Three different loss-of-function mutations in the *SRB10* gene restore viability to the CTD truncation strain. *srb10-1*, the original *SRB* suppressor, is a C-terminal truncation of the kinase, *srb10Δ1* is a deletion of the entire *Srb10* coding sequence and *srb10-3* is an engineered point mutation (D290A) which renders the kinase catalytically inactive. CTD repeat length is indicated on the left, growth temperature on the right and *SRB10* genotypes across the top.

(B) *SRB10* loss-of-function alleles suppress growth defects across a spectrum of CTD truncation mutations. The effect of loss-of-function mutations in *SRB2*, *SRB4*, and *SRB10* was investigated in strains containing progressively truncated CTDs. The number of CTD repeats is shown on the horizontal axis, and the plasmid carrying each CTD truncation allele is indicated (i.e. pN51). The growth phenotypes exhibited by each CTD truncation mutant in the presence of wild-type *SRB* genes or with mutations in *SRB2*, *SRB4* or *SRB10* is shown. Non-viable (N) cells are indicated by a dashed line, conditional (C) cells that are inviable at 12°C but can grow at 24°C are indicated with a thin line, and viable (V) cells that exhibit wild-type growth characteristics under all conditions tested are indicated by a heavy line. The loss of *Srb10* increases the viability of CTD truncation mutants, whereas the loss of *Srb2* or *Srb4* decreases the viability of the CTD mutants.

A



B

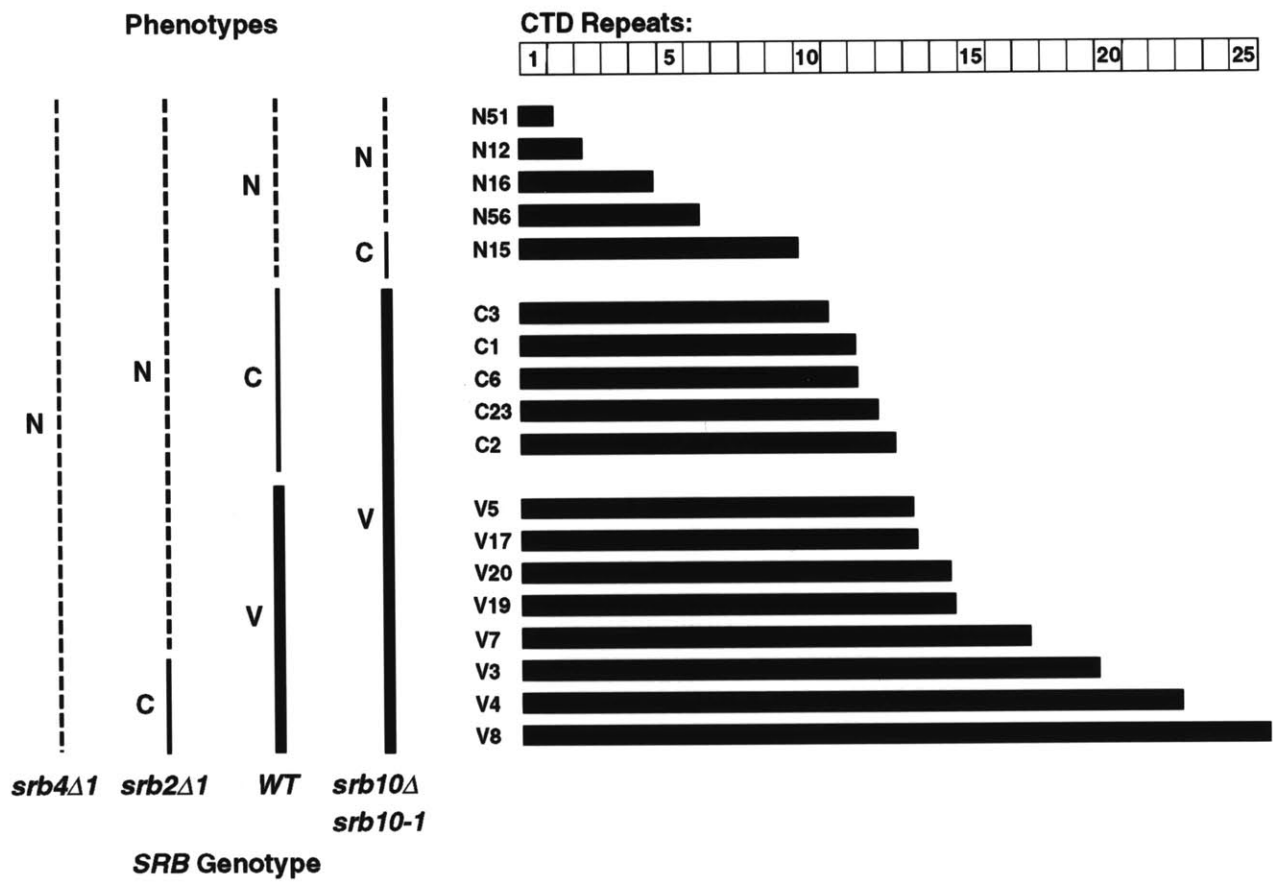


Figure 2 Artificial recruitment of holoenzyme with LexA-Srb fusion proteins.

(A) Diagram of the experimental concept. If Srb10 is a negative regulator of the transcription initiation complex, then a LexA-Srb10 fusion protein should recruit the transcription apparatus, yet repress transcription. In contrast, a LexA-Srb10(D290A) fusion protein, in which the kinase is catalytically inactive, should recruit the apparatus and produce levels of transcription similar to those observed with Srb proteins which have positive roles in the holoenzyme.

(B) A wild-type strain containing the LexA-lacZ reporter plasmid pSH18-34 was transformed with plasmids expressing LexA alone, or LexA fused to Srb6, Srb10, or Srb10(D290A) as indicated. The specific activity of β -galactosidase is expressed in nmoles of *o*-nitrophenol produced/min/mg of total protein assayed. As predicted by the model in (A), the LexA-Srb10 protein is a very poor artificial activator whereas the LexA-Srb10(D290A) fusion protein is a good activator. The LexA-Srb10(D290A) fusion activates as well as LexA-fusions with Srb proteins which have positive functions; LexA-Srb6 is shown as an example. The Srb10 protein is active in vivo when fused to LexA; the LexA-Srb10 expression plasmid complements all phenotypes associated with a *srb10* Δ strain (data not shown). Western blots of whole cell extracts probed

with λ -LexA antibodies show that LexA-Srb10, LexA-Srb10(D290A), and LexA-SRB6 are all expressed at similar levels (data not shown).

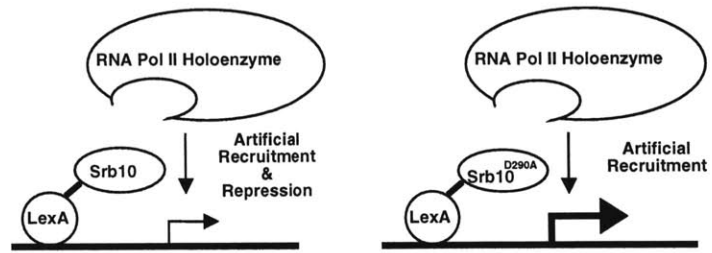
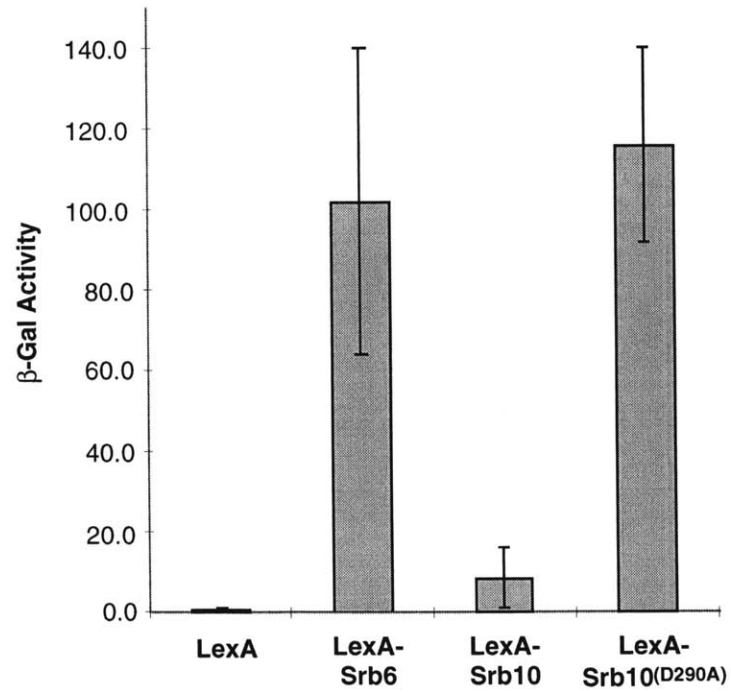
A**B**

Figure 3 Purification of recombinant Srb10/Srb11 and Kin28/Ccl1 cyclin-dependent kinases (CDKs).

(A) Scheme for production and purification of recombinant holoenzyme CDKs from Sf21 cells co-infected with baculovirus encoding a kinase (Srb10 or Kin28) and the corresponding cyclin partner (Srb11 or Ccl1, respectively). FLAG-epitope tagged recombinant Srb10/Srb11 and Kin28/Ccl1 and their inactive mutant derivatives, Srb10^{D290A}/Srb11 and Kin28^{D147A}/Ccl1 were purified in a single step from whole cell extracts of baculovirus infected insect cells using an anti-FLAG affinity column.

(B) Purity of recombinant kinase-cyclin pairs. Onput (OP), Flowthrough (FT), Wash (W) and Eluate (E) fractions of the anti-FLAG affinity column were subjected to SDS-PAGE electrophoresis followed by Coomassie (upper panel) or silver staining (lower panel). The identities of the kinase and cyclin subunits and position of the molecular weight markers (MW) in kiloDaltons is shown.

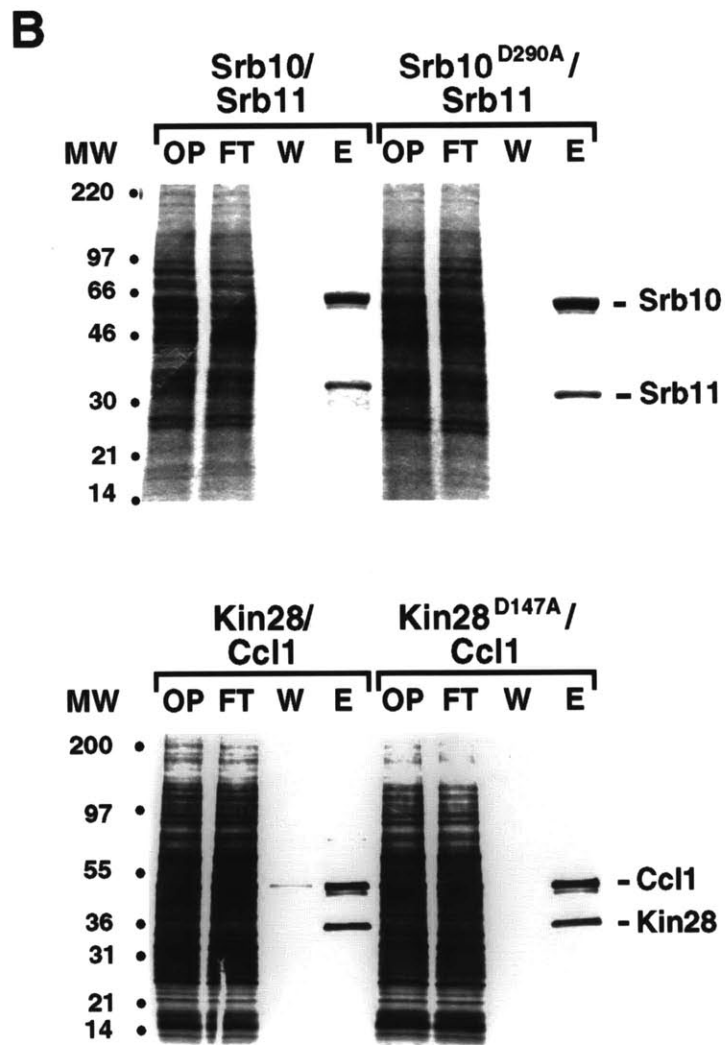
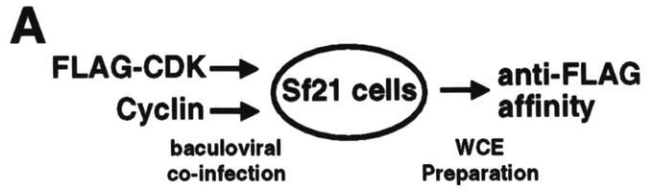


Figure 4 Recombinant CDKs are indistinguishable in CTD phosphorylation activity.

(A) Holoenzyme associated CDKs phosphorylate the CTD, but not histone H1 *in vitro*. Recombinant GST, GST-CTD, or calf thymus H1 substrates were incubated with pure recombinant CDK/cyclin pairs in the presence of γ -³²P-ATP, separated by SDS-PAGE and visualized by autoradiography. Label is transferred only to the GST-CTD fusion and not to GST or histone H1, a well studied kinase substrate.

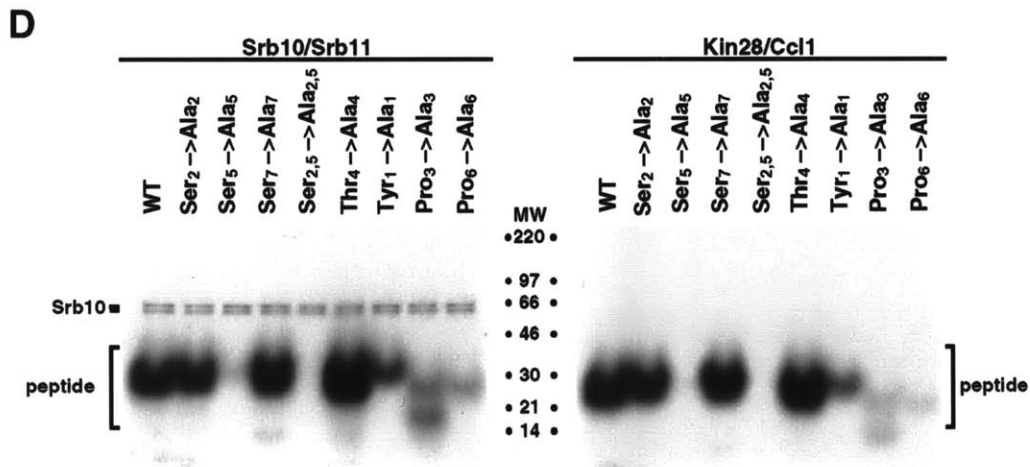
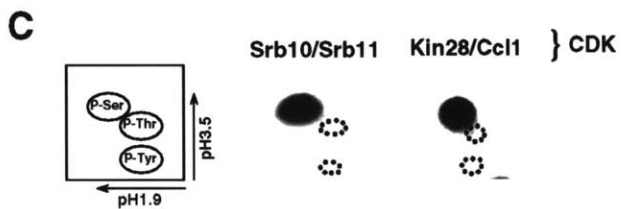
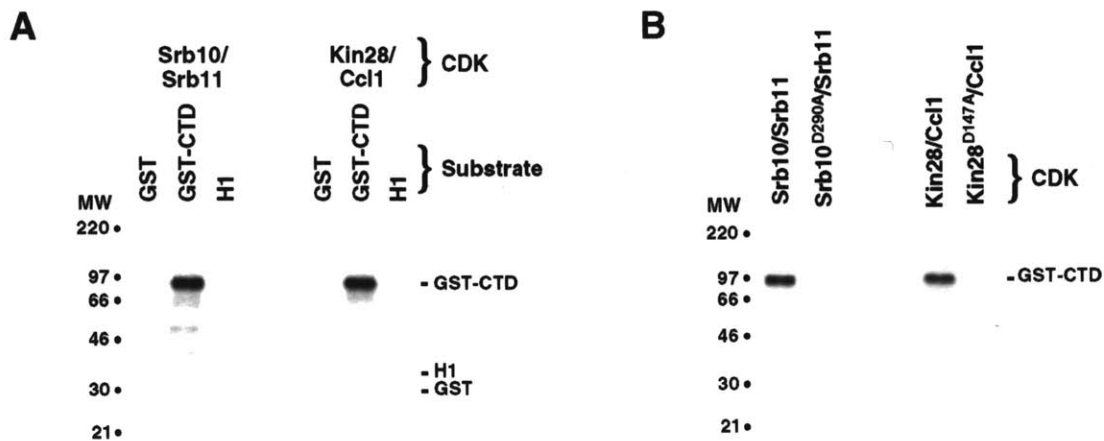
(B) Purified mutant recombinant CDKs exhibit no kinase activity. Wild-type and mutant CDKs were incubated with GST-CTD in the presence of γ -³²P-ATP as in panel A. The inactive CDK/cyclin pairs contained a point mutation at a highly conserved aspartate residue critical for catalytic activity. The absence of a labeled product in the mutant CDK/cyclin preparations suggests the observed activity is not due to a contaminating kinase.

(C) Phosphoamino acid analysis of *in vitro* phosphorylated CTD. Recombinant GST-CTD was incubated with recombinant CDK/cyclin pairs in the presence of γ -³²P-ATP. After SDS-PAGE and transfer to a PVDF membrane, the labeled CTD band was cut out and subjected to acid hydrolysis. The phosphoamino acids were separated by two-dimensional thin layer electrophoresis. Amino acid standards were visualized by ninhydrin and their mobilities shown on the left, while the labeled phosphoamino acids

were visualized by autoradiography as shown in the middle and right panel.

Serine is the primary phosphoacceptor on the GST-CTD substrate for both Srb10/Srb11 and Kin28/Ccl1 kinases.

(D) Holoenzyme CDKs show identical specificity for synthetic CTD peptide substrates. Synthetic peptides consisting of three heptapeptide consensus repeats, or mutant variations thereof, were used as substrates for recombinant holoenzyme CDKs. The wild-type (WT) heptapeptide consensus sequence as well as the amino acid numbering used in describing various mutants is shown at the bottom of the figure. After SDS-PAGE, the phosphorylated peptides were visualized by autoradiography. The Ser₅ to Ala mutant peptide was unable to serve as a substrate for either CDK, strongly suggesting that Ser₅ is the primary substrate labeled by the CDKs.



CTD triple heptapeptide repeat substrate:

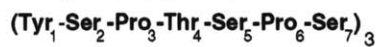


Figure 5 Catalytically active Srb10 can inhibit transcription by RNA polymerase II holoenzyme in vitro.

(A) Purified holoenzymes contain similar amounts of Rpb1, Srb2, Srb4, Srb5 and the kinases Srb10 and Kin28. Wild-type and Srb10 (D290A) mutant holoenzymes were purified in parallel and analyzed by western blot. Monoclonal antibodies specific to unphosphorylated CTD (α -P⁻ CTD; 8WG16) were used to detect Rpb1.

(B) Holoenzymes containing either wild-type Srb10 kinase (lanes 1-2) or catalytically inactive Srb10(D290A) kinase (lanes 3-4) were preincubated with or without ATP prior to PIC formation and analyzed as diagrammed. Only holoenzymes containing functional Srb10 are inhibited for transcription when kinases are allowed to function before PIC formation (compare lanes 2 and 4). In vitro transcription is performed in the presence of α -³²P-UTP resulting in internal labeling of a 400 nucleotide transcript derived from a G-less cassette driven by the *CYC1* promoter. The state of CTD phosphorylation after ATP preincubation was monitored by western analysis using monoclonal antibodies specific to unphosphorylated (α -P⁻ CTD; 8WG16) or Ser-phosphorylated CTD (α -P⁺ CTD; H5). CTD phosphorylation occurs during preincubation only in holoenzymes containing functional Srb10 kinase (compare lanes 2 and 4). Control experiments indicate that the Srb10

CTD kinase activity is largely restricted to the holoenzyme in which it resides (data not shown). Srb4 is probed as a loading control.

(C) Changes in mAb reactivity to RPB1 is due to phosphorylation.

Holoenzyme containing functional Srb10 was incubated with ATP (lanes 1-2).

The signal obtained when the phosphorylated preparation is probed with mAb 8WG16 (α -P⁻ CTD) is reduced and the mobility of RPB1 is retarded (lane

3). The same preparation then reacts with the phospho-serine CTD specific

H5 mAb (α -P⁺ CTD). Subsequent treatment of the sample with protein

phosphatase eliminates the H5 reactive band and restores 8WG16 reactivity and mobility to that seen prior to ATP incubation (compare lanes 1 and 4).

This indicates the mAbs are accurately probing the phosphorylation state of the CTD.

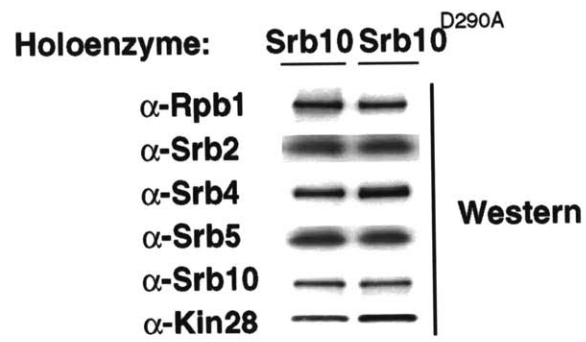
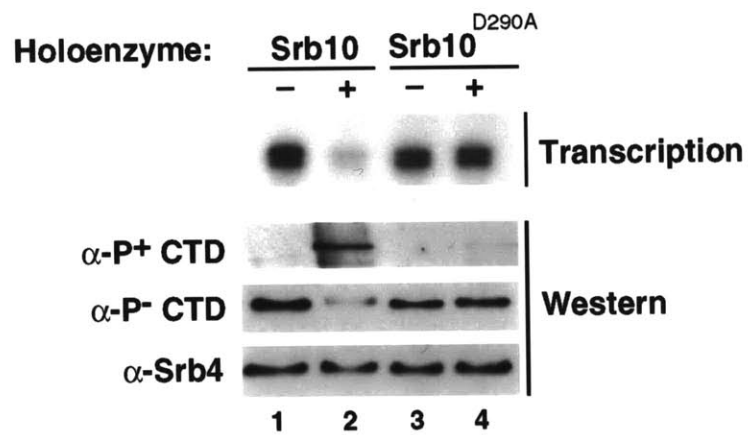
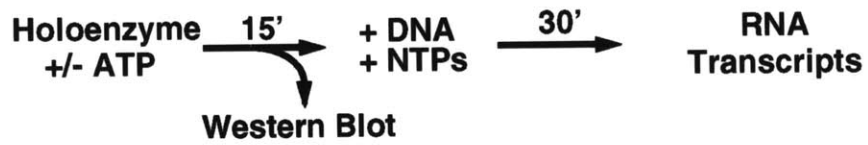
(D) Srb10 kinase does not affect transcription post PIC formation.

Holoenzymes containing either wild-type Srb10 kinase (WT) or catalytically inactive Srb10(D290A) kinase, preincubated with template DNA and GTFs prior to addition of NTPs, exhibit identical transcriptional activity (top panel).

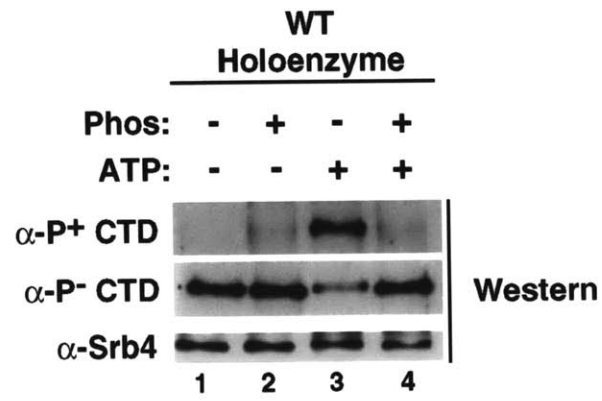
The state of CTD phosphorylation after transcription was monitored with the phospho-serine specific H5 mAb (α -P⁺ CTD). Both wild-type and

srb10(D290A) containing holoenzymes are able to phosphorylate the CTD.

Srb4 is probed as a loading control (bottom panel).

A**B**

C



D

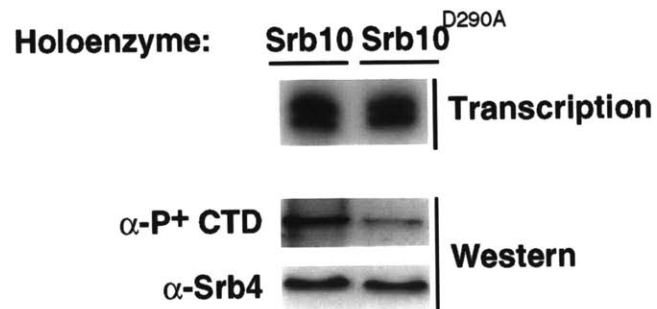


Figure 6 Nuclear extracts show no Srb10-dependent inhibition of transcription by RNA polymerase II holoenzyme.

Nuclear extracts from cells containing either wild-type Srb10 kinase or catalytically inactive Srb10(D290A) kinase were preincubated with or without ATP prior to PIC formation, as diagrammed (the experimental design is identical to that in Figure 5B). In vitro transcription is performed in the presence of α -³²P-UTP resulting in internal labeling of a 400 nucleotide transcript derived from a G-less cassette driven by the *CYC1* promoter. No inhibition of transcription was observed after ATP preincubation with either extract.

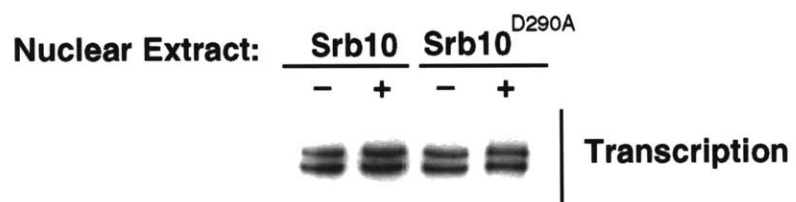
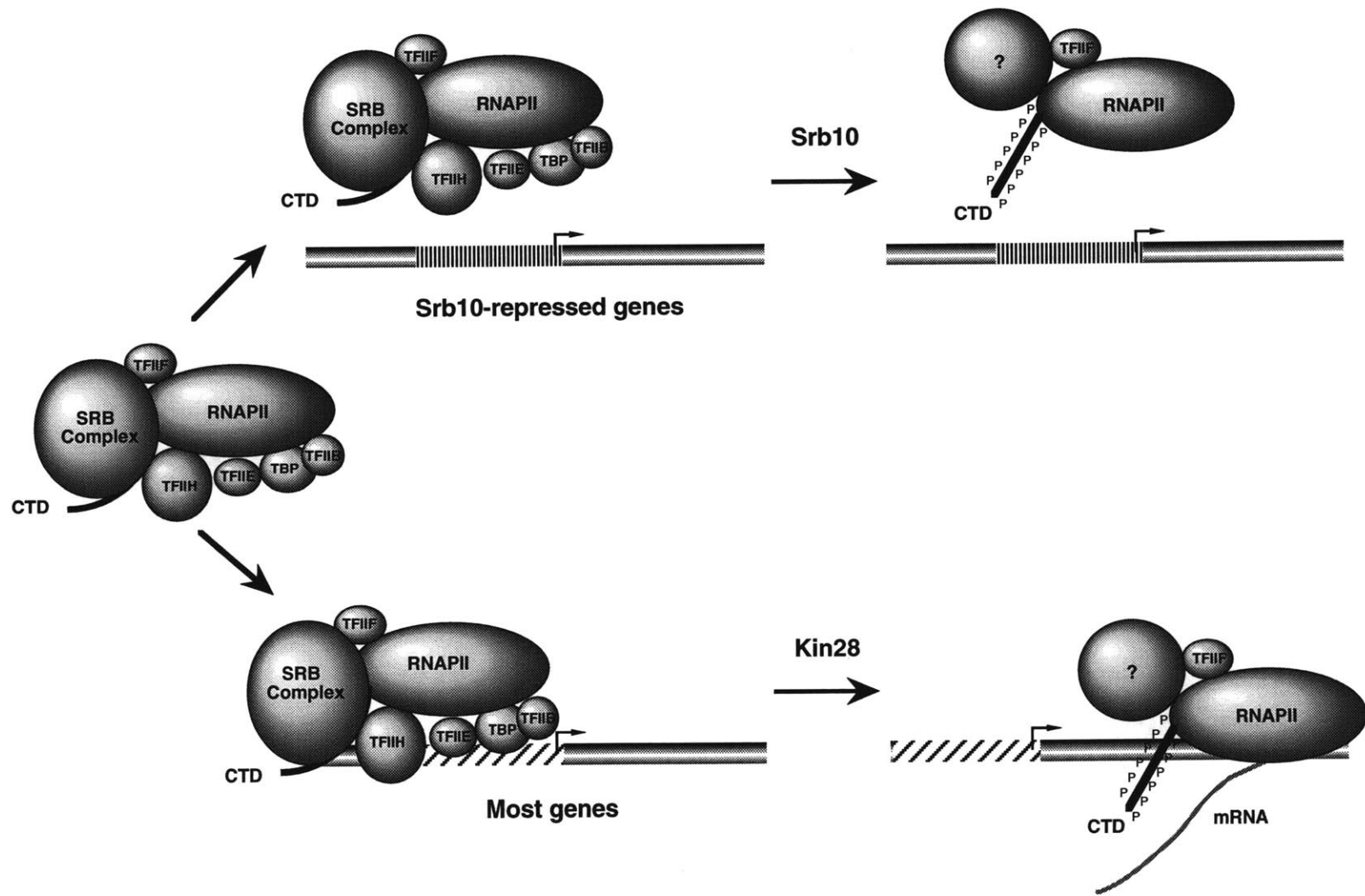


Figure 7 Model for temporal function of holoenzyme CDKs in transcription initiation.

The two holoenzyme cyclin-dependent kinases are CTD kinases which function at different times. Srb10-dependent CTD phosphorylation can occur prior to stable preinitiation complex (PIC) formation at a subset of promoters, presumably activated by factors associated with these promoters, with consequent inhibition of transcription. The Kin28 kinase functions after stable PIC formation at promoters generally, producing the hyperphosphorylated form of pol II associated with productive elongation.



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Appendix A

A strategy for rapid, high-confidence protein identification

A Strategy for Rapid, High-Confidence Protein Identification

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A Strategy for Rapid, High-Confidence Protein Identification

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A procedure is described for rapid, high-confidence identification of proteins using matrix-assisted laser desorption/ionization tandem ion trap mass spectrometry in conjunction with a genome database searching strategy. The procedure involves excision of copper-stained bands or spots from electrophoretic gels, in-gel trypsin digestion of the proteins, single-stage mass spectrometric analysis of the resultant mixture of tryptic peptides, followed by tandem ion trap mass spectrometric analysis of selected individual peptides, and database searching of the relevant genomic database using the program PepFrag. The scheme provides sensitive, real-time protein identification as well as facile identification of modifications. A single operator can unambiguously identify 5-10 proteins/day from an organism whose genome is known at a level of >0.5 pmol of protein loaded on a gel. The utility of the technique was demonstrated by the identification and characterization of a band from a human HTLV-I preparation and 11 different proteins from a yeast RNA polymerase II C-terminal repeat domain-affinity preparation. The technology has great potential for postgenome biological science, where it promises to facilitate the dissection and anatomy of macromolecular assemblages, the definition of disease state markers, and the investigation of protein targets in biological processes such as the cell cycle and signal transduction.

Genome sequencing projects are producing an unprecedented information resource for biologists. Efficient utilization of this remarkable resource demands the development of new tools for rapidly analyzing mature proteins and for correlating them with their genes and ultimately their functions. One particularly powerful new set of tools for rapidly identifying and characterizing proteins utilizes mass spectrometric techniques such as matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) mass spectrometry (MS) in combination with genome database searching strategies.¹⁻¹⁵

Two general strategies have been developed for identifying proteins by MS. In both, the proteins of interest are separated (e.g., by gel electrophoresis) and individually subjected to proteolysis with an enzyme of known specificity (e.g., trypsin), and the molecular masses of the resulting peptides accurately and rapidly determined by MS. In the first strategy, these experimentally determined masses (i.e., the tryptic map) are compared with the calculated masses of all tryptic peptides that can be theoretically produced from sequences corresponding to all of the proteins in the genomic database of the organism under study.^{1-5,12} The protein yielding the best match between the experimental and theoretical peptides is identified. MALDI time-of-flight MS has been the preferred technique for this peptide mapping approach because it is sensitive and allows for the measurement of the component peptides resulting from the digest without prior HPLC separation or extensive cleanup. Although this method is fast and simple, its success can be compromised by the presence of more than one protein in the gel spot or by extensive posttranslational modifications of the protein of interest and errors in the database sequence. In addition, the observation of too few peptides in the MS map from a given protein may preclude its identification.

A second strategy, involving tandem mass spectrometry (MS/MS),¹⁶ has been developed to circumvent these difficulties.⁶⁻¹⁵ Here, a particular tryptic peptide is selected and dissociated in the mass spectrometer to produce a fragmentation mass spectrum

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that is characteristic of the sequence of the peptide. The database search for the protein uses the molecular mass (M_r) of the tryptic peptide together with its fragmentation spectrum. Although the M_r of the peptide by itself only moderately constrains the search—and can lead to large numbers of possible proteins—a good match of the fragmentation spectrum often identifies a unique protein. The identification can be verified by checking how many of the remaining tryptic peptides have measured masses that are in accord with hypothetical tryptic peptides from the putative protein. To further increase the confidence of the call, fragmentation mass spectra of one or more additional tryptic peptides are obtained. Because protein identification incorporating mass spectrometric fragmentation and database searching requires only single (or at most a few) tryptic peptides from any given protein, this strategy can confidently identify multiple proteins in mixtures^{14,17} and is highly tolerant of posttranslational modifications or errors in the database. Nanospray ionization¹⁸ (i.e., low flow rate electrospray ionization) combined with triple-stage quadrupole MS has been shown to work well for this latter strategy.^{10,11,13–15} Alternatively, postsorce decay in MALDI time-of-flight MS analysis¹⁹ has been applied for this purpose.^{20–24}

Although protein identification by simple peptide mapping using MALDI time-of-flight MS is sensitive and fast, the confidence level of the identification may be insufficient for an assured call. The additional constraints obtained through ESI tandem MS provides more reliable identification than simple peptide mapping but suffers from reduced sample throughput. Alternatively, additional constraints may be obtained through the use of postsorce decay in MALDI time-of-flight mass spectrometry.^{19–24}

In this paper, we present a procedure that combines the positive virtues of the above two strategies for the identification of proteins. This procedure utilizes unique properties of our newly developed MALDI ion trap mass spectrometer^{25–27} and provides sensitive, confident protein identification with high throughput.

EXPERIMENTAL SECTION

Isolation of HTLV-1 Proteins. The HTLV-I transformed lymphocyte cell (N5-CR) was maintained in RPM I medium containing 10% fetal calf serum. Supernatants forming large-scale cultures (5000 mL) were clarified by centrifugation at 60000g for 90 min. Pellets were resuspended and centrifuged on linear 25–65% sucrose gradients. Purified virus bands were collected and pelleted. The latter were resuspended in NTE buffer (0.01 M Tris-HCl pH 7.4, 0.001 M EDTA, 0.1 M NaCl).

Isolation of CTD-Binding Proteins. Details of the isolation of the CTD-binding proteins are described in ref 28.

Procedures for Sample Preparation. Protein samples were resuspended in 1× Bio-Rad Tris–glycine sample buffer (Bio-Rad Laboratories, Hercules, CA) plus 1/10 vol of 10% (w/v) SDS solution. The proteins were separated on a 4–15% gradient Bio-Rad Ready Gel and electrophoresed at 200 V. After protein separation, the gel was soaked in deionized H₂O for 1 min and then 1× Bio-Rad copper stain solution for 5 min, with constant shaking. The stained gel was washed for 1 min in H₂O. Because the protein bands are negatively stained, the gel appears opaque greenish blue and the protein bands relatively clear. The bands are most easily visualized on a black background.

The protein bands were cut out using a stainless steel scalpel and transferred to 0.5 mL Eppendorf tubes. To each tube was added 0.4 mL of 1× Bio-Rad copper destain solution (Bio-Rad Laboratories), and the tubes were vortexed for 5 min. After discarding the wash liquid, this step was repeated for 2 min. By this stage, the gel pieces have turned from faint greenish blue to clear. H₂O (0.4 mL) was added, the mixture vortexed for 1 min, and the liquid discarded. Digestion buffer (0.4 mL 50 mM Tris-HCl, pH 8.0) was added, the mixture vortexed for 1 min, and the liquid discarded. Modified trypsin (20 μL of 25 ng/μL in 50 mM Tris-HCl, pH 8.0, Boeringer Mannheim, Indianapolis, IN) was added to each tube (i.e., to each gel piece). The gel pieces were squashed with a plastic pipet tip that had been sealed closed using heat from an open flame. Proteins in the squashed gel pieces were digested for 2 h at 37 °C.

Extraction of the peptides from the gel pieces was facilitated by sonication for 3 min, followed by removal of the liquid with a gel-loading tip. The liquid was transferred to a fresh Eppendorf tube, taking care not to inadvertently transfer any small gel pieces into the tube. An extraction solution (20 μL of ACN–0.5% TFA/H₂O 1:1 (v/v)) was added to the original crushed gel pieces, the mixture sonicated for 3 min, and the liquid removed and pooled with the first extract. Finally, 10 μL of 100% ACN was added to the crushed gel pieces, the mixture sonicated for 2 min, and the liquid removed and pooled with the first two extracts. The pooled solutions were evaporated to dryness (SpeedVac, Savant, Farmingdale, NY) at medium heat. For the MS measurements, the dried samples were redissolved in 5–10 μL of acetonitrile–0.5% TFA/H₂O 1:1 (v/v).

Matrix-Assisted Laser Desorption Ion Trap MS. The design and performance of our custom MALDI ion trap mass spectrometer has been described.^{25–27} It is composed of an external MALDI ion source and a modified Finnigan ITMS electronics kit. Laser desorption/ionization was carried out at wavelength of 355 nm with 10 ns duration pulses. MS and MS/MS spectra were taken as described previously.²⁵ The matrix solution consisted of 2,5-dihydroxybenzoic acid (DHB) in 1:1 (v/v) ACN/H₂O (2× dilution of a saturated solution of DHB in 1:1 (v/v) ACN/H₂O). The MS samples were prepared by mixing on the sample probe 1 μL of sample solution with 1 μL of matrix solution. The instrument was mass calibrated once a week.

Procedure for Protein Identification. (1) The peptide mixture produced by in-gel trypsin digestion of a protein was analyzed directly by MALDI ion trap MS without prior chromatographic separation or further treatment. (2) After the MS peptide

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map was inspected, one peptide ion species was isolated and fragmented by collision-induced dissociation to obtain an MS/MS spectrum. (3) The masses of the precursor and fragment ions were searched against a database using the program PepFrag²⁹ (see below), and candidate proteins were identified. The search was carried out with constraints that include the cleavage specificity of the digesting enzyme, the originating species of the protein, and the systematics of MALDI ion trap collision-induced dissociation of peptides.^{30,31} (4) Other peptides in the measured MS peptide map were assigned to the identified candidate protein. (5) Of these newly assigned peptides, one was chosen and fragmented, and the fragmentation pattern checked against the candidate protein sequence to verify the identification. (6) For those peptides that could not be assigned to the identified protein, one peptide was selected and fragmented, and steps 3–5 were repeated until the majority of intense peaks were assigned. Steps 1–6 were all performed in real time.

The Protein Identification Program PepFrag. The program PepFrag,²⁹ which was developed in our laboratory, allows for the searching of protein or nucleotide sequence databases (SWISS-PROT, PIR, GENPEPT, OWL, or dbEST) using a combination of information from MS peptide maps and MS/MS spectra of proteolytic peptides. The databases have been taxonomically divided to allow for faster searches and to minimize the number of unrelated hits. The experimental conditions (enzyme specificity, approximate protein mass, place in phylogenetic tree of the species, and modifications of amino acids) can be specified in the search. The result of the search is a list of proteins, each of which contains a peptide that matches the measured mass of a proteolytic peptide as well as the measured masses of MS fragments of the peptide. In addition, other search constraints can be specified, if such information is available. These constraints include specification of the MS fragmentation systematics, masses of other proteolytic peptides that are assumed to belong to the protein of interest, and partial amino acid composition. PepFrag is publicly available over the Internet at URL <http://chait-sgi.rockefeller.edu>.²⁹

RESULTS AND DISCUSSION

Preferential Cleavage of Peptide Ions in Tandem MALDI Ion Trap MS: (1) A Highly Effective Constraint for Protein Identification and (2) High Sensitivity. We found previously that fragmentation of peptide ions by tandem MALDI ion trap MS is highly selective and that Arg-containing peptides undergo facile, preferential cleavage adjacent to amino acid residues with acidic side chains,^{25,30,31} producing exclusively b- and/or y-type ions.^{32,33} Lys-containing peptides also undergo preferential fragmentation adjacent to Asp/Glu although the selectivity is not as high as for Arg-containing peptides.³¹ Because most tryptic peptides contain an Arg/Lys residue at the C-terminal, we have included such preferential cleavages as a selectable constraint in our search program, PepFrag²⁹ (see Experimental Section) and have found that application of this constraint greatly facilitates the unambiguous identification of proteins. For example, in an experiment

designed to test the present methodology, we separated proteins from a human T cell leukemia virus type 1 (HTLV-I) preparation by SDS-PAGE and obtained a MS tryptic peptide map (Figure 1a) for a prominent band with an apparent M_r of ~22 kDa (Experimental Section). Some 20 peaks appear in the m/z range between 1200 and 2800 (Figure 1a). To identify the protein in the band, we obtained an MS/MS spectrum of the ion with m/z 2147.6 Da (Figure 1b). Two product ions that arise from preferential cleavage at the C-termini of Asp/Glu residues, dominate the MS/MS spectrum (in addition to noninformative fragment ions produced by the facile loss of small neutral molecules, e.g., H₂O, NH₃, and CO₂). We therefore input into PepFrag (Figure 1c) the M_r of the precursor peptide and the m/z values of the two informative fragment ions, together with the constraint that the fragments were b- and/or y-type generated at the C-terminal of Asp/Glu residues. We also specified the type of enzyme used (trypsin) and allowed for partial enzymatic degradation of the protein (with up to two internal Arg/Lys residues retained in the fragments). We did not specify the species from which the protein originated to allow for the possibility of adventitious impurities from the host cell and did not constrain the search with the apparent SDS-PAGE M_r to allow for the possibility of proteolytic processing of the protein.

The search results (using the program PepFrag) summarized in Figure 1c identify a single gene product—the gag polyprotein of HTLV-I (albeit from three different strains of the virus). The specificity of the cleavage reaction is seen to provide a highly effective constraint for protein identification. The results are especially impressive considering that the search was carried out for all proteins in the SwissProt database using a conservative mass tolerance (± 2 Da) for both the precursor and product ions. We have subsequently found, through an analysis of more than 200 different protein bands, that it is nearly always possible to identify a protein (whose sequence is present in the database) with just two fragment ions generated at the C-termini of Asp/Glu in a single tryptic peptide and that it is usually possible to identify a protein with more than four fragments generated at unspecified amino acid residues. In the latter case, the fragment ions need not constitute a sequence tag as long as they are b- and/or y-type ions—i.e., gaps are well tolerated. Because b- and y-type ions dominate the MALDI ion trap MS/MS spectra,^{25,30,31} the intense fragmentation peaks normally correspond to these ion species. One potentially complicating factor in the interpretation of the fragmentation spectra is the occurrence of b*- and y*-type fragment ions—i.e., b and y ions that have undergone loss of a H₂O/NH₃ moiety. This additional fragmentation produces little ambiguity in practice because its occurrence is readily recognized by the presence of pairs of fragment peaks spaced 17–18 Da apart (see, e.g., the pair at m/z 1975.9 and 1959.1).

The average abundance of Asp/Glu residues in proteins is ~12%.³⁴ Thus, tryptic peptides with mass of >1000 Da often contain an Asp/Glu residue and the probability for the presence of Asp/Glu increases as a function of increasing peptide mass. For the purpose of protein identification, we therefore find it advantageous to obtain MS/MS spectra of tryptic peptide ions with higher masses ($1500 < m/z < 3500$). We have found that singly protonated peptide ions in this mass range can be efficiently fragmented in the MALDI ion trap.²⁵ An added benefit of

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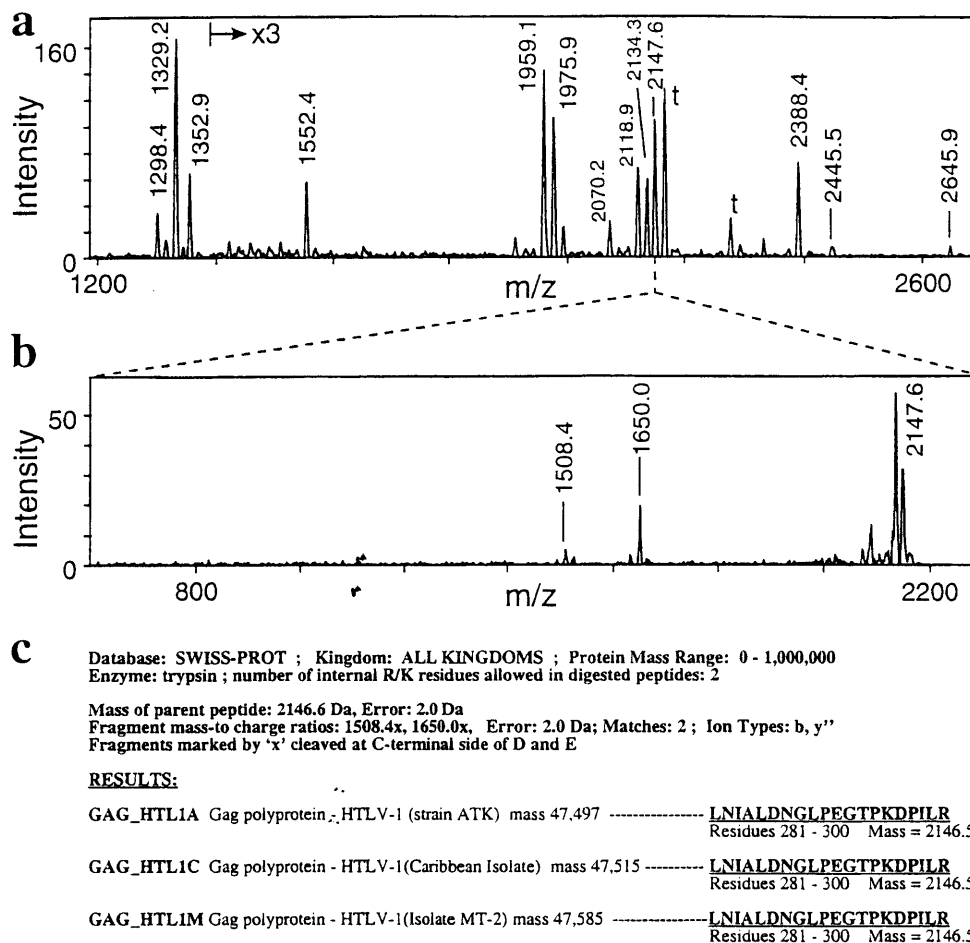


Figure 1. (a) MALDI ion trap mass spectrum of the products of in-gel trypsin digestion of the 22 kDa SDS-PAGE protein band from the HTLV-I preparation (Experimental Section). (b) Tandem MALDI ion trap mass spectrum of the peptide ion with m/z 2147.6. (c) Protein identification search results obtained with the program PepFrag using the information in Figure 1b. The protein is identified as the gag polyprotein from HTLV-I.

analyzing higher mass tryptic peptide ions derives from their reduced statistical occurrence relative to lower mass tryptic peptides.

Selective fragmentation at Asp/Glu also significantly enhances the sensitivity of the MS/MS measurement. Because relatively few dissociation channels are open, signal dilution effects normally experienced in ESI triple quadrupole or MALDI postsource decay tandem MS is avoided. This concentration of fragmentation is particularly important when only small amounts of sample are available—i.e., where the number of ions available for fragmentation is severely limited. It can be seen from Figure 1b that dilution of the fragmentation spectrum into additional channels would quickly compromise our ability to observe the fragmentation peaks. The selectivity at Asp/Glu appears especially strong for singly charged ions (unpublished observations) and is most obvious in the slow (ms) decomposition measurements made in the ion trap. Finally, in contrast to ESI, MALDI yields mainly singly charged peptide ions, leading to an additional reduction in signal dilution losses and easier mass spectral interpretation.

Protein Identification with MALDI Tandem Ion Trap MS with High Confidence and High Throughput. The protein identification procedure outlined in the Experimental Section ensures the identification of proteins with a high level of confidence. After the protein is tentatively identified using the MS/MS data (Figure 1b), the measured peptide map is compared with the map calculated for this putative protein and an attempt

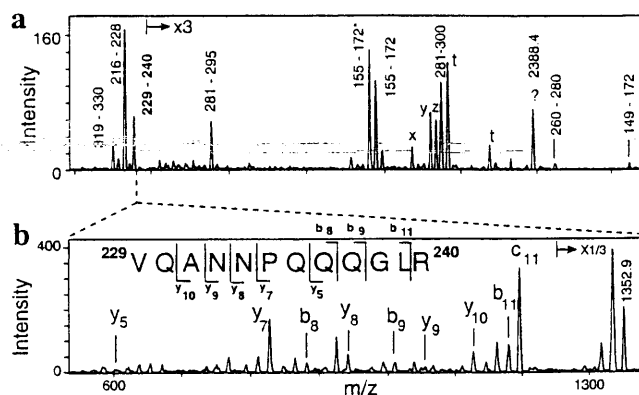


Figure 2. (a) Assignment of the observed MS peaks to peptides from the identified gag polyprotein of HTLV-I. (b) Tandem MALDI ion trap mass spectrum of the peptide ion with m/z 1352.8, confirming its assignment as gag 229–240 and the progenitor protein as gag.

is made to assign the various peptide peaks (Figure 2a). Ions that cannot be assigned could arise either from other proteins present in the band or from modifications of the already identified protein. In cases where most of the ions can be assigned to the putative protein, the likelihood is high that the identification is correct. However, to further increase the confidence level of the call, we test the hypothesis that the protein has been correctly identified by obtaining an additional MS/MS spectrum from a second peptide that has been assigned to the putative protein

(Figure 2b). Two MS/MS spectra plus a peptide map normally yield an unambiguous identification.

Our utilization of copper staining/destaining (see Experimental Section) and the elimination of the need for chromatographic separation make the sample preparation procedure fast. The whole process—from staining the gel to the point when samples are inserted into the mass spectrometer—requires < 4 h. For convenience, we typically process 10 proteins at a time. The initial MS and first MS/MS measurements require <30 min. Another 30 min is needed to search the database, to interpret the data, and to obtain the second MS/MS spectrum. In this way, protein identification is performed at an average rate of 1 protein/h by a single operator. Such high throughput is crucial for large-scale biological research projects (see below). The present protocols allow us to unambiguously identify real-world protein samples at a rate of 5–10/day from genomes that have been fully sequenced (e.g., *Saccharomyces cerevisiae*).

Protein Identification with MALDI Tandem Ion Trap MS in Real-Time. The extremely low sample consumption and pulsed nature of MALDI allow us to stop the experiment at any time, analyze the data in real-time, and design the next experiment by following leads provided by the previous experiment(s). It is not necessary to attempt to obtain MS/MS spectra for all peptide ions in the map as may be optimum for a continuous ionization technique like electrospray or nanospray, where the spray time is limited. The ability to continue to measure a single sample for as long as is needed to obtain an unambiguous result is crucial for the success of the present iterative protein identification procedure. For example, after we obtained the peptide map shown in Figure 1a, we took the MS/MS spectrum of the peptide with M_r 2146.6 Da (Figure 1b) and stopped the experiment to search the database. The search (<1 min) identified the gag protein (Figure 1c). After assigning several of the observed peptides in the peptide map to the gag protein (Figure 2a), we resumed the experiment and took another MS/MS spectrum of the assigned peptide with M_r 1351.9 Da to confirm the putative identification (Figure 2b).

To assure that we have identified all of the abundant proteins present in a band, we attempt to assign every intense peak in the peptide map. Inspection of Figure 2a shows that we were unable to assign several of the peaks to the gag protein, including the ion with m/z 2118.9. To investigate the origin of this ion, we obtained its MS/MS spectrum (data not shown). The subsequent database search failed to return an identification. The failure to identify a protein indicates that the peptide may be posttranslationally modified. However, no suggestion of the most commonly occurring posttranslational modifications (including phosphorylation and glycosylation) was apparent from the MS and MS/MS spectra (see below). We thus deduced that the protein may have been processed proteolytically. A relaxed search allowing non-specific processing returns a peptide that belongs to the same gag gene product but is proteolytically processed between Leu-130 and Pro-131. This finding is in concert with *in vivo* processing of the HTLV-1 gag polyprotein, which has been observed to yield three mature proteins—i.e., gag P19 (residues 1–130), gag P24 (residues 131–344), and gag P15 (residues 345–429).^{35–37} The peptide with m/z 2118.9 arises from the processed N-terminal peptide of gag P24.

Amino Acid Modifications Identified by MALDI Ion Trap MS. Posttranslational modifications or chemical modifications introduced in gel electrophoresis can be problematic for protein identification because modification information is not inherent to the DNA sequence database. We have found that commonly occurring posttranslational modifications such as phosphorylation and glycosylation can be readily identified in MALDI ion trap MS because phosphopeptides and glycopeptides each have clear signatures in the MALDI ion trap mass spectra.^{31,38} Protonated phosphopeptides undergo facile loss of ~98 Da in single-stage MALDI ion trap MS so that peaks separated by ~98 Da indicate the presence of a phosphopeptide. The presence of a suspected phosphopeptide can be readily confirmed by acquiring its MS/MS spectrum. The observation of a dominant product ion with a mass ~98 Da less than the precursor ion confirms the presence of a phosphate group on the peptide. A similar phenomenon occurs for glycopeptides, which readily dissociate at the glycosidic bonds, generating a series of ions in the single-stage spectrum with mass differences of 162 (Hex), 203 (HexNAc), or 291 Da (sialic acid). The observation of these ions indicates the presence of a glycopeptide. Again, the hypothesis can be readily tested by MS/MS.

A commonly occurring chemical modification is the oxidation of Met during electrophoresis or storage of the dried gels. Peptide ions containing oxidized Met readily lose the methyl sulfoxide moiety in the ion trap to give a signature pair of peaks 64 Da apart.^{38,39} If the Met residue is not completely oxidized to the sulfoxide, a triplet of peaks can be observed separated by -48 and +16 Da from the unmodified peptide ion. In Figure 2a, the ions at m/z 2070.2 (labeled x), 2117.8 (labeled y), and 2134.3 (labeled z) constitute such a triplet. Again, the peptide containing oxidized Met can be readily verified by an MS/MS experiment, in which the product spectrum shows a dominant product ion with a mass 64 Da less than the precursor ion (data not shown). The ability to readily identify modifications of proteins by MALDI ion trap MS further increases the success rate and confidence of protein identification.

Protein Identification with MALDI Tandem Ion Trap MS: A Rapid and Effective Tool for Identifying Protein Components of Complex Macromolecular Assemblies. We have applied the above-described methodology to identify proteins interacting with the RNA polymerase II C-terminal repeat domain (CTD) (reviewed in ref 40). The CTD has been shown to interact with components of the RNA polymerase II holoenzyme.

A highly purified preparation of CTD-binding protein complex was prepared by a combination of CTD affinity and ion exchange chromatography. Protein components of this CTD-binding fraction were separated by SDS-PAGE, subjected to proteolysis with trypsin, and analyzed by MS. As an example, Figure 3a shows the MS tryptic map from a gel band that migrated with an apparent M_r of 35 kDa. The MS/MS spectrum of the m/z 2445.7 ion (Figure 3b) unambiguously identifies its progenitor protein as Srb5, a component of a functional preinitiation complex that has

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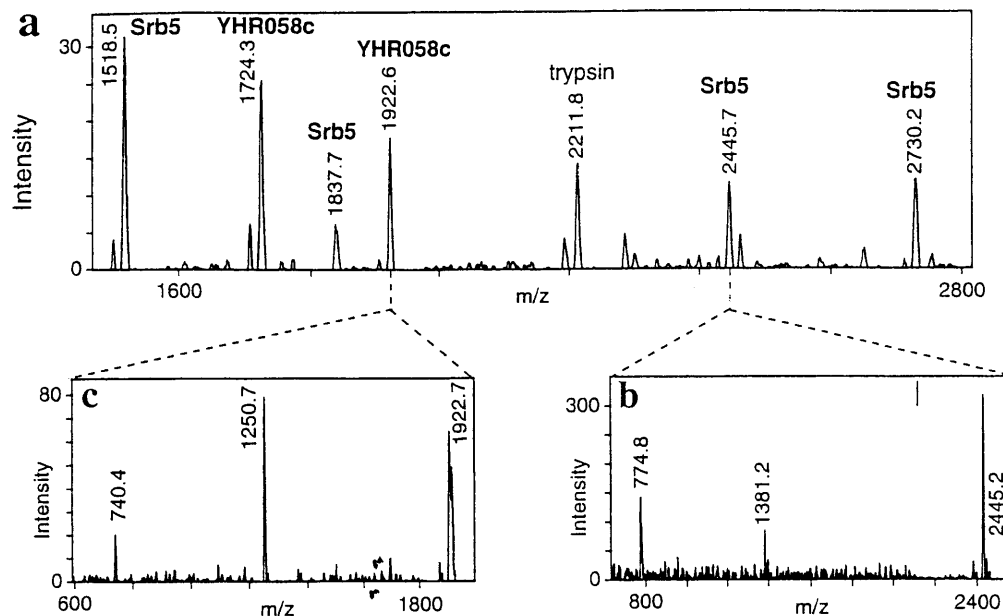


Figure 3. (a) MALDI ion trap mass spectrum of the products of in-gel trypsin digestion of an electrophoretic band with an apparent M_r of 35 kDa from a purified preparation of yeast RNA polymerase II CTD-binding proteins (Experimental Section). (b) Tandem MALDI ion trap mass spectrum of the peptide ion with m/z 2446, identifying its progenitor protein as SrB5. (c) Tandem MALDI ion trap mass spectrum of peptide ion with m/z 1923, identifying its progenitor protein as the open reading frame yielding the gene product YHR058c.

Table 1. List of Identified Proteins

PAGE	M_r		gene name	NCBI identifiers	protein description
	calcd	obsd			
97	78.5		SRB4	172693	RNA polymerase II suppressor protein SrB4
35	34.3		SRB5	172536	RNA polymerase II suppressor protein SrB5
200	191.0		RPB1	1419221	RNA polymerase II subunit 1
130	139.0		RPB2	1293711	RNA polymerase II subunit 2
114	123.3		RGR1	218472	glucose repression regulatory protein Rgr1
58	54.8		ACT3	436808	actin-like protein Act3
56	60.4		RRN7	567927	RNA polymerase I specific initiation factor Rrn7
31	25.2		YBR193c	311669	protein of unknown function
35	32.8		YHR058c	487956	protein of unknown function
70	64.2		YPR070w	805050	protein of unknown function
150	110.0		YDR359c	849181	protein of unknown function

previously been found to be required for efficient transcription initiation.⁴¹ Although three other peaks in the peptide map were also identified as arising from SrB5 (Figure 3a), the peaks at m/z 1922.6 and 1724.3 could not be assigned to this protein. MS/MS of the ion at m/z 1923 (Figure 3c) identified the presence of a second protein in the band—i.e., the open reading frame yielding the hypothetical gene product YHR058c. This identification was confirmed by MS/MS of a second peptide at m/z 1724.3 from the same gene product. This example demonstrates that two proteins can be readily identified in a single electrophoretic band, even when relatively few peptides are available for MS/MS analysis.

In this manner, 11 proteins were identified over a period of two days by a single operator (Table 1). Five (RPB1, RPB2, RGR1,⁴² SRB4, SRB5) are known components of the RNA polymerase II holoenzyme, confirming the efficacy of the technique. Proteins corresponding to four open reading frames (with unknown function) and ACT3 and RRN7 were also identified (Table

1). ACT3 is an actin-related protein for which genetic evidence suggests a role in transcriptional regulation.⁴³ The identification of ACT3 as a CTD-binding protein provides biochemical evidence for a link between class II transcription and actin-related functions. The identification of RRN7, a regulatory protein known to be involved in class I transcription,⁴⁴ suggests a common regulatory mechanism that is conserved between class I and class II transcription. Further work will be required to test these hypotheses.

CONCLUSIONS

We have devised a procedure for identifying proteins that combines the robustness, simplicity, and high sensitivity of MALDI-MS and the specificity and efficiency of ion trap tandem MS. The scheme provides fast, sensitive, high-confidence real-time protein identification as well as facile identification of modifications. A single operator can currently identify unambiguously 5–10 proteins/day (from an organism whose genome has been sequenced) at a sensitivity level of >0.5 pmol of protein

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loaded on a gel. The utility of the technique was demonstrated by the rapid identification of 11 proteins in CTD-affinity preparations. The technology has great potential for postgenome biological science where it promises to facilitate the dissection and anatomy of macromolecular assemblages^{14,17} (e.g., we are currently defining the total complement of proteins in the yeast nuclear pore complex), the definition of disease state markers, and the investigation of protein targets in biological processes such as cell cycle and signal transduction.

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Appendix B

Rapid construction of targeting vectors using YACS

Summary

Construction of targeting vectors for gene replacement or modification is often a laborious task. We sought to make the bulk of this process obsolete, by the application of a rapid screening procedure in mouse YAC clones followed by the precise insertion of a selection cassette that can be used in bacteria, yeast and mammalian cells. YAC clones are screened by PCR for a segment of the gene of interest, followed by preliminary characterization of the local intron-exon structure by PCR. A cassette containing the *HIS3* gene and the zeocin resistance gene are amplified by PCR with extended overhanging ends homologous to the region in which the HIS-zeo cassette will be inserted. Yeast strains containing the YAC are transformed and selected for growth on his-deficient plates. The targeted locus is excised by restriction digestion and cloned into a plasmid vector and selected for zeocin resistance. Vectors to delete the IL-10 receptor α or β chain genes were successfully created in ~ 1 month. This procedure can generally be applied to introduce precise mutations in any gene. In addition, vectors for targeting genes in human tissue culture cells can be rapidly constructed using human YAC libraries as a starting point.

Introduction

Each year, targeted mutations in the mouse (knockout mice) prove increasingly valuable to biomedical research both as elegant tools to investigate the mechanics of biological systems and as models of human diseases. The number of knockout mice produced yearly is also steadily increasing, as the methodology and sophistication for production increases. There is, however, a fundamental bottleneck in the creation of knockout mice: the rapid establishment of the vector which will carry the targeting mutation into the mouse genome. Traditionally, this has been a time-consuming and often frustrating process which relies upon the isolation of a genomic clone, its detailed characterization and addition of a selectable marker, such as the neomycin gene, into the relevant region of the locus to be disrupted. The production of vectors relies heavily on the knowledge of the appropriate restriction sites within the locus (for insertion of the selection cassette). In many cases, this is not an straightforward task, particularly if there are no convenient restriction sites present. This report describes a technique which seeks to alleviate two major problems with vector construction. We describe a process which greatly increases speed of clone isolation and vector construction and we also demonstrate that there need be little reliance on a detailed knowledge of restriction sites in order to quickly make a vector. Although other procedures have been described which use

yeast (Storck et al., 1996) or lambda-based mutagenesis (Tsuzuki and Rcouanrt, 1998) our methodology combines the power of yeast genetics with the burgeoning knowledge of the mammalian genome projects to decrease to the time and effort required to produce targeting vectors. We estimate that our procedure will reduce the time of vector construction from ~4-8 months to ~1 month.

Results & Discussion

Overview of the Procedure

Our procedure utilizes YACs (Yeast Artificial Chromosome) as the starting point for clone isolation. YACs carry very large segments of chromosomes (~0.2-1 Mbp) as a pseudo-chromosome in yeast. The first step is to identify a portion of the gene of interest by PCR-based screening. This may constitute an important part of the translated protein product (e.g. an exon encoding the kinase domain in a protein that is considered essential for activity) and will be partially removed in the knockout mouse. The PCR assay is designed to amplify part of the gene and is initially tested on genomic DNA. A YAC containing the locus of interest is then isolated by PCR-based screening a pooled YAC library (such as the commercially available Whitehead/MIT C57BL/6 mouse YAC library used here) with the primers (Fig. 1, Primers A and B) designed to identify the exon of interest. Alternatively, if the gene of interest has already been mapped to a contig (via the mouse genetic and physical mapping project: www-genome.wi.mit.edu/cgi-bin/mouse/index), YAC clones overlapping the region can simply be purchased and then screened to ensure that the gene is present.

Another set of PCR primers is used to amplify a unique HIS-zeo cassette. The *HIS3* gene is used to select for the targeting event in yeast and the *zeo* gene will be used to select bacterial clones and targeted ES cells. In principle, any of the common yeast auxotroph genes could be used, but *HIS3* was chosen because the gene is small

making the PCR reaction of the cassette easier to perform, and the yeast strain harboring the YAC clone is *his3-2* (a null allele). Zeocin is an antibiotic which was chosen because it can be used in bacteria, yeast and mammalian cells. For this crucial step, the primers are designed with extended overhanging ends which are homologous to a region internal to primers A and B. This need not necessarily be within the region of interest; but could be designed to delete a larger segment utilizing a neighboring exon (the methodology was used in the examples given below). This will be the region of the locus in which the selection cassette is inserted. The PCR product is transformed into the yeast carrying the YAC and colonies selected which are *his+*.

Clones are initially screened for the correct targeting event with the PCR primers A or B and primers complementary to the *zeo* or *HIS3* genes. As a further test, genomic DNA is prepared from the *his+* strains and also tested by Southern blotting for the correct targeting event.

The next step is to isolate the targeted locus from the yeast and have it propagated in bacteria for transfection into ES cells. In order to achieve this, an enzyme must be identified which will excise the locus as a 6-15 kb piece of DNA. This can be done earlier as a preliminary investigation of the locus by Southern blotting or after the *HIS-zeo* cassette has been inserted. A genomic DNA preparation is digested with the enzyme (X in the figure) and ligated into a bacterial vector such as pbluescript. The library is transformed into *E. coli* and clones are selected on LB agar for amp and zeo resistance. In principle, colonies which arise

will contain only the fragment of interest (due to the use of zeo in the targeting cassette).

The final step is to isolate a small fragment from the end of the clone. This will serve as a probe for identification of the correctly targeted event in ES cells. This is represented as removing the X-X' fragment from the clone in Fig.1. The targeting construct is now ready for transfection into ES cells. For this step, ES cells are transfected with a linearized construct and selected with zeocin. Zeo+ colonies are screened with the primers described earlier and then analyzed by Southern blotting with the small end probe. This entire procedure should take only a few weeks. In addition, several different clones can be worked on simultaneously.

Targeting constructs for the IL-10R α and IL-10R β genes

Primers were designed to amplify part of an exon in the putative ligand binding domains of the IL-10R α or IL-10R β genes. In the case of the human IL-10R β gene, Lutfalla *et al.* (Lutfalla et al., 1993; Lutfalla et al., 1995) had already characterized a significant portion of the gene, from which we inferred the likely structure of the mouse homologue. Each primer set successfully amplified a small fragment (~150 bp) from each gene. Further primers were then designed to localize adjacent exons (see Fig. 2 for primer locations). It was important to design the recombination primers such that they were internal to the location of another primer set so that correct recombination events could be scored with the primers within the HIS-zeo cassette (see insets in Fig.2). Thus, for the IL-10R α gene, the recombination

event will take place between PM229 and PM231, which can then be used in combination with the oCW240 or zeo primers.

Following PCR of the HIS-zeo cassette (see table 1 for the primer design), several colonies were obtained which were his⁺. These were initially scored by PCR and several colonies were found to have a correct recombination event (5 of 21 for the IL-10R α gene and 12 of 29 for the IL-10R β gene). Genomic DNA was prepared from these strains and analyzed by Southern blotting to test for illegitimate recombination events (using either a full-length HIS or zeo probe), correct targeting in the locus of interest (using a full-length IL-10R α or β cDNA probe) and to determine a suitable restriction enzyme to excise each targeted locus. The results showed that each locus was correctly targeted (Fig. 3) and that Eco RI excised the targeted IL-10R α locus as a ~10 kb fragment while Sal I excised the IL-10R β locus as a ~13 kb fragment.

Genomic DNA was digested with the enzymes listed above and cloned into pbluescript or pGEM3zf. Ligations were transformed into Genehogs and plated onto LB agar containing ampicillin and zeocin. Although very few colonies grew (5 colonies for the IL-10R α gene and 2 for the IL-10R β gene), each contained the correctly targeted locus, which was further analyzed by Southern blotting of the plasmid DNAs (data not shown). Probe fragments were prepared from the ends of the clones (to assay for recombination in ES cells). Kpn I was used to isolate a 0.5 kb or 2.2 kb fragment from the end of the IL-10R α or IL-10R β targeted clones, respectively. Each plasmid was then re-

sealed using DNA ligase. Kpn I could then be conveniently used to linearize each clone

Conclusions

The procedure described in this paper significantly decreases the time and effort required to construct targeting vectors. There are three obvious advances made above existing technologies:

1. Screening. Commercially available YAC libraries can be rapidly screened by PCR to the clonal level or single YAC clones can be isolated based on existing genetic maps. This obviates the need to screen lambda or BAC libraries and then isolate and characterize the DNA.
2. Decreased reliance on restriction enzyme mapping. Little knowledge of the restriction map is need to perform a simple loss-of-function mutagenesis, other than the enzyme required to excise the targeting fragment from the YAC. For the introduction of more sophisticated mutations (e.g. knock-ins, subtle mutations, lox sites etc), more information will be required. However, the ease of performing the manipulations in yeast should facilitate this procedure.
3. Speed and generality to both mouse and human systems. The speed of clone isolation and mutation introduction is very rapid. In addition, multiple clones can be worked on simultaneously. Importantly, for the

disruption of genes in human cell lines, similar procedures can be performed using the existing human YAC libraries.

In the post-genomic era, the reliance on testing gene function in an unambiguous way makes the production of knockout mice and cells paramount. The technology described here greatly facilitates this process by utilizing the information from the mouse and human genome projects, as well as the rapid and simple genetic tools available in yeast. Finally, it is apparent that as the sequence of the mouse genome becomes more complete, it should be possible to employ the system described here to systematically knock-out all genes in the mouse. This can be accomplished by a combination of the complete YAC library covering the entire genome along with the synthesis of ~65, 000 targeting primer pairs (assuming that is approximately the number of genes present) to amplify HIS-zeo cassettes robotically. Following systematic recombination and excision, targeting vectors for all genes could be constructed rapidly. A bank of knock-out mice for all known genes would be an investment with enormous potential for understanding biological systems.

Experimental Procedures

Construction of the HIS-zeo cassette

The HIS3 gene was amplified by PCR using pRS313 (Sikorski and Hieter, 1989) as a template and primers oCW276 and oCW277 (see table 1) which contain overhanging restriction sites. The 1.2kbPCR product was purified and digested with Bam HI and Sal I. The 1.2kb CMV/Zeo cassette was cut out of pCMV/Zeo (Invitrogen) with Eco RI and Xho I. pBluescript was cut with Eco RI and Bam HI. The cut HIS3, CMV/Zeo, and pBluescript DNA fragments were mixed in a 3-way ligation reaction which was subsequently transformed into DH5 α cells. The plasmid (pCW296) was confirmed to be correct by extensive restriction analysis.

Screening the YAC library

PCR primers (see Table 1) were designed to amplify a segment of an exon encoding part of the ligand binding domain in either the IL-10R (Ho et al., 1993; Ho et al., 1995) or IL-10R β (Lutfalla et al., 1993; Lutfalla et al., 1995) gene. PCR conditions were first established using mouse genomic DNA (from C57BL/6 mice) to ensure that the PCR product corresponded to the segment of the exon. The PCR assay was applied to the Whitehead/MIT C57BL/6 YAC library (WI/MIT-820 YAC library) according to the manufacturer's instructions (Research Genetics, Huntsville, AL), beginning

with the superpool and progressively isolating smaller fractions of the pooled library. Eventually, two YAC clones containing the IL-10R α or IL-10R β genes (or parts thereof) were isolated in clonal form. The genotype of the yeast strain (*S. cerevisiae* J57D) carrying the YAC library is: *MAT α leu3-3,112 ura3-52 trp1 his3-2,-15 ade2 can1*. YAC strains were maintained on minimal media lacking uracil (ura) and tryptophan (trp).

Rapid analysis of the intron/exon structure

The intron/exon structure in the neighboring region was determined by a PCR-based assay using oligonucleotides with regions complementary to cDNA sequences of the IL-10R α or IL-10R β (see Figure 2 for the locations and Table 1 for the sequences). Previous reports had identified intron-exon boundaries of the human IL-10R β gene (Lutfalla et al., 1993; Lutfalla et al., 1995). We had independently cloned the mouse IL-10R β cDNA (PJM, unpublished) and used this sequence as the basis for primer design. PCR reactions were performed and the resulting product sizes used to determine a rough outline of the structure of the gene which would be relevant for the design of homologous integration of the HIS-zeo cassette.

PCR of the HIS-zeo cassette

PCR primers (Table 1) with overlapping ends homologous to regions of the gene to be deleted were designed (see Figure 2 and Table 1). The HIS-zeo cassette was amplified with these primers using Taq polymerase in a set

of 20x100 μ l reactions. Initial experiments suggested that it was crucial to perform a titration of Mg^{2+} for each PCR reaction. The size of the each PCR product was \sim 2.6 kb. DNA was phenol/chloroform extracted, ethanol precipitated and resuspended at a final concentration of $1\mu\text{g}/\mu\text{l}$.

Transformation and initial assay of yeast strains

The amplified HIS-zeo cassette was transformed into the YAC-containing strains using the conventional lithium acetate/PEG method. A titration of DNA was performed and the number of colonies obtained was DNA-dependent, $10\mu\text{g}$ giving the most number of colonies in each case. Yeast were plated onto synthetic complete media lacking *ura*, *trp* and *his*. His⁺, *ura*⁺, *trp*⁺ colonies were picked three days later and re-streaked onto minimal media lacking *ura*, *trp* and *his*. Colonies were immediately subjected to PCR assays to detect the correct integration of the HIS-zeo cassette into the IL-10R α or IL-10R β locus (see insets in Fig. 2). The oligos used were homologous to regions within the HIS gene (oCW240) or the zeo gene (*zeo*) and were designed to amplify relatively short fragments in combination with the initial screening PCR primers. PCR analysis was performed directly on yeast, using the TaqGold enzyme (Applied Biosystems) according to the manufacturer's instructions. A small sample of yeast was picked with a sterile toothpick and placed in 100 μ l water. This was diluted 1:20 with water and 1 μ l used per 50 μ l PCR reaction. This assay is highly dependent on the

amount of yeast added to the PCR reaction, and it may be necessary to titrate down the quantity of cells added to the reaction.

Southern analysis of the integrated locus

Genomic DNA was purified from yeast strains which had the expected profile based upon PCR and subjected to Southern analysis. Initially, we digested genomic DNA from each strain with a battery of restriction enzymes which cut infrequently within mouse genomic DNA (e.g. Sal I, Xho I, Not I etc.). Southern blots were probed with either the entire IL-10R α or IL-10R β cDNA probes, or probes consisting of the HIS or zeo genes. This step serves two purposes: a further check that the locus has been correctly targeted and identification of enzymes which can excise a large enough fragment of the targeted locus to use in ES cells.

Isolation of the targeted locus

The targeted locus was isolated by taking advantage of the zeo resistance gene added to the targeting vector. Total genomic DNA from each strain was digested with a restriction enzyme as described above which could excise a large segment of the locus. Digested DNA was cloned into pbluescript (Stratagene) or pGEM (Promega) vectors and transformed into *E. coli*. It was important to titrate in the amount digested DNA in the reactions to ensure the maximum ligation efficiency. Ligations were initially transformed into DH5 α and plated onto LB agar with 50 μ g ampicillin to determine the

approximate size of the library (we aimed for ~10-fold coverage of the yeast genome). Libraries were then transformed into Genehogs (Research Genetics), an *E. coli* preparation specifically designated for the uptake of larger DNA fragments. Libraries were plated onto LB agar containing 50 µg/ml ampicillin and 25 µg/ml zeocin (Invitrogen). The LB agar was prepared in the low-salt version for full zeocin activity as specified by Invitrogen. Amp⁺, zeo⁺ clones were picked into low-salt LB broth and plasmid DNA analysed by restriction digest and Southern blotting as described above.

Isolation of a probe from the end of each clone

Each plasmid containing the correct targeted locus was analyzed by restriction digest to obtain a small fragment from the end of each clone. This would serve as a probe for a region outside the targeted locus following transfection into ES cells. For the IL-10R α or IL-10R β locus, Kpn I digest excised 400 bp or 2.3 kb fragments, respectively, from the end of the clone. The fragments were cloned into pbluescript and the remaining targeting plasmids re-ligated giving rise to the final form of the targeting vector.

Transfection into ES cells

In order to transfect each construct into an isogenic cell line, ES cells from C57BL/6 mice (Bruce 4 cells) were used (Kontgen et al., 1993). These were maintained on STO SNL2 cells (STO embryonic fibroblasts transfected with a construct expressing the murine LIF gene; obtained from the ATCC)

which were transfected with the CMV-zeo plasmid (Invitrogen). Linearized targeting constructs were electroporated in the Bruce 4 cells and selected with zeo.

Table 1: Oligonucleotides used for screening and targeting vector construction

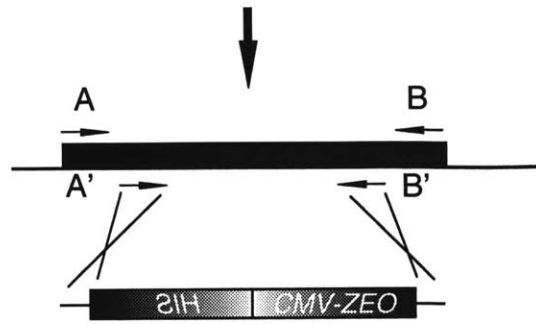
<u>Name</u>	<u>Sequence</u>
PM201	TCAACCTGGAATGACATCCATAT
PM203	CCAGTTGGAGTACTGACTGTTGTC
PM204	CAGGTCTTTCCAAGATCACTGC
PM205	GGCAGAAGGTGACATTGACCC
PM224	CTATCTGCATCTCAGGAGGTC
PM225	CTCTGTAAGCCCATGAGTCAT
PM226	CTTGAATGCAGTAAGTTGTCC
PM227	AGCGCACTGCCTCGACTCAGTGCGACTTCTCTCATCTTTCTAAATACGGAGACTCCCAGGTCTGTGCGGTATTTAC
PM228	TGTTCTTCAAGGTCCACGTCTCAGGCTCATTCTCAATTTGTGGGGCTGAGAAACGAGACATGATAAGATACATTG
PM229	CTATGTGTGGTTTGAAGCCAG
PM230	GAGCACCTACTATGAAGTGGC
PM231	AGAATCACTTCATCCACTGTG
PM232	AGATCCTTGAAGACTTGTTTCG
PM233	CTCACCCCTATGGGGACCGTG
PM250	TTTCCAGCACATCCTCCACTGGAAACCTATCCCAAACAGTCTGAGAGCACCTACTACCCAGGTCTGTGCGGTATTTAC
PM251	CGAGTCTCAGTGGTGGTCCAGTTGGAGTACTGACTGTTGTCCACTGCCCCGACTCTGGAGACATGATAAGATACATTG
oCW240 (his)	GTGGTGCCGGACAACACCCT
oCW357 (zeo)	GTGGTGCCGGACAACACCCT
oCW276	CGCGGTGACCCCCGGGGCAGATTGTACTGAGAGTGC
oCW277	CGGCGGATCCCCCGGGTCTGTGCGGTATTTACACC

Figure 1. Schematic representation of the targeting procedure.

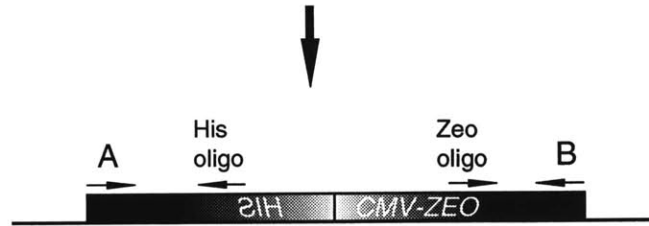
- Identify single exon by PCR
- Isolate YAC clones
- Southern blot to find enzymes which will excise locus of interest



- Design PCR primers to amplify his-zeo cassette
- Transform yeast
- Select for his+ colonies



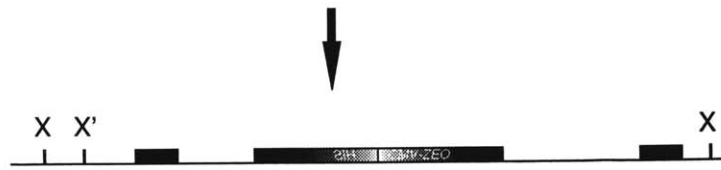
- Test integration by PCR
- Test integration by Southern



- Digest YAC gDNA with enzyme X
- Clone into plasmid vector and select for amp+, zeo+



- Identify probe fragment
- Re-ligate to remove X-X' fragment



- Linearize and transform ES cells
- Select for zeo+ colonies
- Test for integration by PCR/Southern

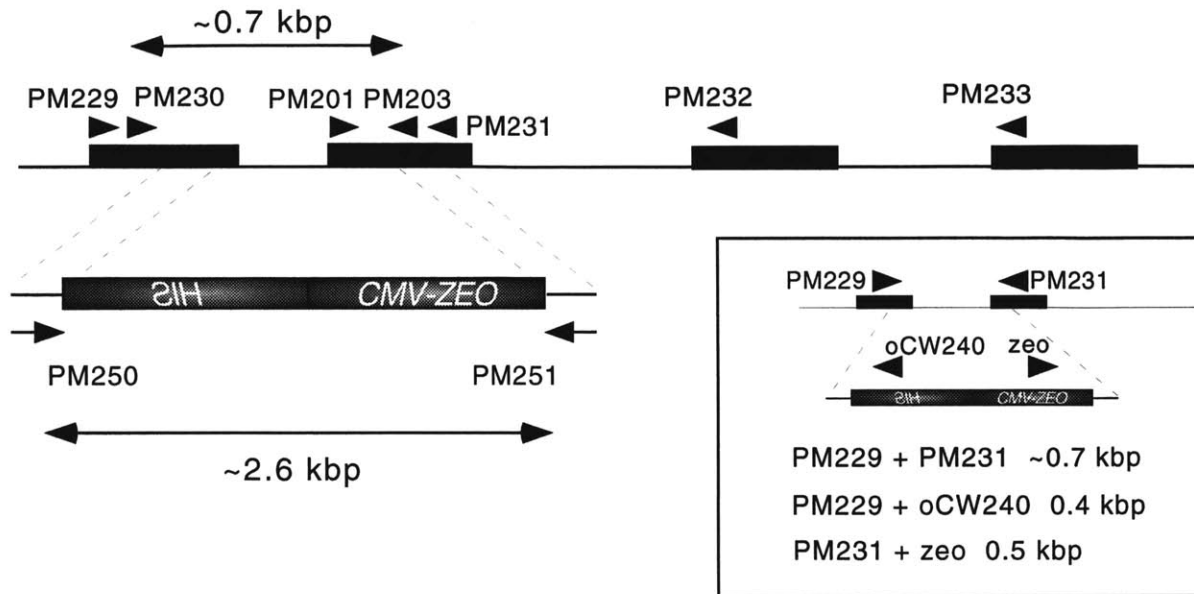


Figure 2. Diagrammatic representation of the targeting strategy for the IL-10R α or IL-10R β loci.

For each locus, PCR was used to identify the location of neighboring exons. The initial oligonucleotides used in the PCR reactions were PM201 and PM203 for the IL-10R α locus and PM204 and PM205 for the IL-10R β locus. Additional oligonucleotides are shown in each diagram. The designed recombination sites are shown with broken lines. The oligonucleotides used to PCR the HIS-zeo cassette are shown (PM250 and PM251 for the IL-10R α and PM227 and PM228 for the IL-10R β). The PCR screening strategy for each event is diagrammed in the boxes. For the IL-10R α , a ~0.7 kb fragment is replaced with a 2.6 kb HIS-zeo cassette. For the IL-10R β , a ~3.0 kb fragment is replaced.

A

IL-10R α locus



B

IL-10R β locus

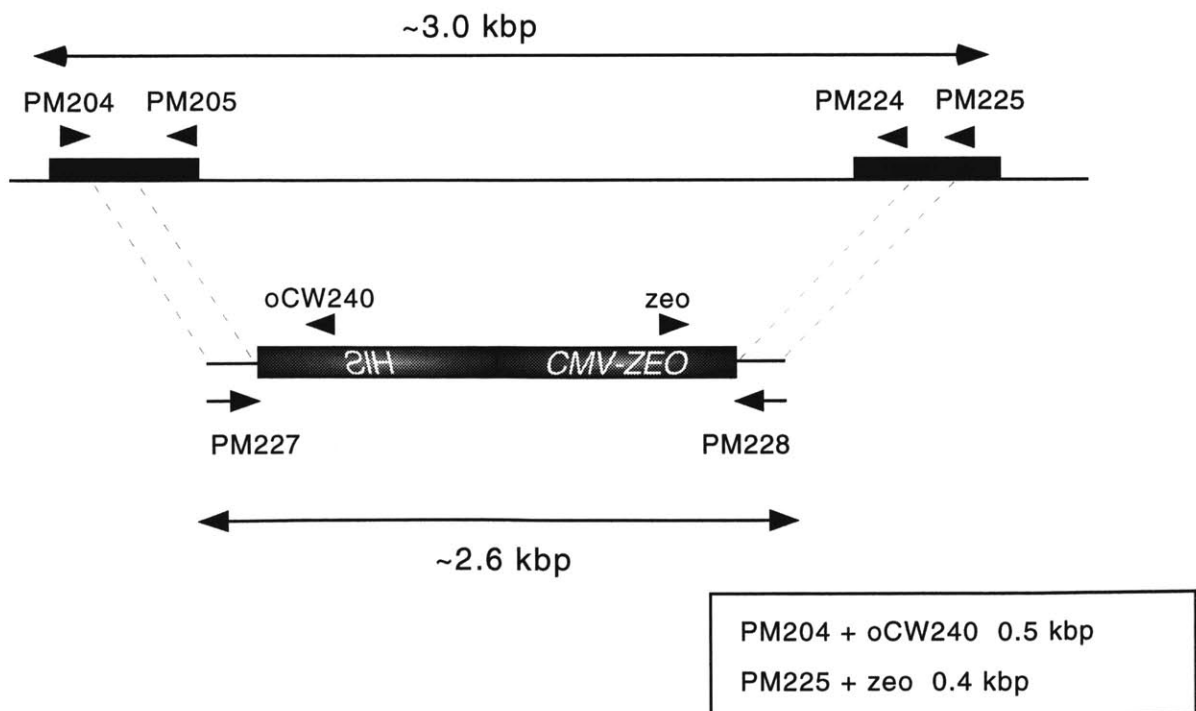
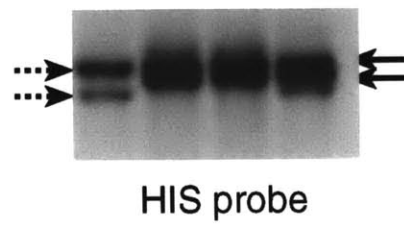
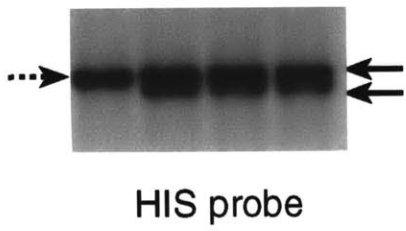
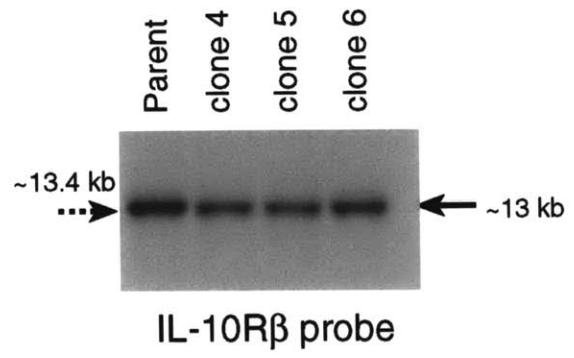
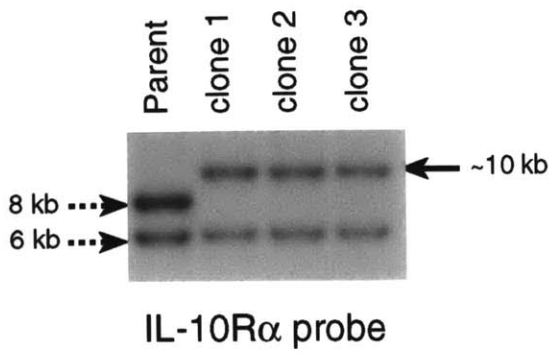


Figure 3. Southern analysis of targeting events.

Panel A. Disruption of the IL-10R α locus. Genomic DNA from 3 independent his⁺ clones was digested with Eco RI and analysed by Southern blotting. The parent YAC strain is shown on the left. When gDNA is probed with a full-length IL-10R α cDNA (upper blot), two bands are identified. The lower band corresponds to Eco RI fragment at the 3' end of the gene (the Eco RI site is present in the cDNA sequence). The targeting event increases the size of the 7 kb Eco RI fragment by 2.6 kb corresponding to the insertion of the HIS-zeo cassette along with the appearance of an additional band hybridizing to the HIS probe (lower blot)

Panel B. Disruption of the IL-10R β locus. Genomic DNA from 3 independent his⁺ clones was digested with Sal I and analysed by Southern blotting. The parent YAC strain is shown on the left. When gDNA is probed with a full-length IL-10R β cDNA (upper blot), two bands are identified. The cDNA hybridizes to a ~13 kb Sal I fragment. The targeting event decreases the size of the 13 kb Sal I fragment by 0.4 kb corresponding to the insertion of the HIS-zeo cassette along with the appearance of an additional band hybridizing to the HIS probe (lower blot). This targeting event was further verified by extensive analysis of the plasmid DNAs isolated (data not shown). See a diagram of the targeting events in Fig.2.



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