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NAT, a Human Complex Containing Srb Polypeptides that Functions as a Negative Regulator of Activated Transcription

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Summary

A complex that represses activated transcription and contains the human homologs of the yeast Srb7, Srb10, Srb11, Rgr1, and Med6 proteins was isolated. The complex is devoid of the Srb polypeptides previously shown to be components of the yeast Mediator complex that functions in transcriptional activation. The complex phosphorylates the CTD of RNA polymerase II (RNAPII) at residues other than those phosphorylated by the kinase of TFIIH. Moreover, the complex specifically interacts with RNAPII. The interaction is not mediated by the CTD of RNAPII, but is precluded by phosphorylation of the CTD. Our results indicate that the complex is a subcomplex of the human RNAPII holoenzyme. We suggest that the RNAPII holoenzyme is a transcriptional control panel, integrating and responding to specific signals to activate or repress transcription.

Introduction

Transcription is a highly regulated process. The first step involves the recognition of promoter DNA sequences and the formation of a transcription initiation complex. The minimal protein apparatus required for transcription of class II genes in vitro consists of the general transcription factors (GTFs) IIB, IID, IIE, IIF, IIH, and RNA polymerase II (RNAP II) (reviewed in Orphanides et al., 1996; Roeder, 1996). These factors support efficient transcription of naked DNA in vitro, but cannot respond to transcriptional activators without additional cofactors (reviewed in Guarente, 1995; Kaiser and Meisterernst, 1996).

RNAPII is a 12-subunit complex in which the largest subunit contains a carboxy-terminal domain (CTD) composed of a heptapeptide repeat sequence (YSPTSPS) rich in amino acids that can be phosphorylated (Young, 1991). Phosphorylation of the CTD is highly regulated and modulates the association of proteins with RNAPII (Neugebauer and Roth, 1997; Shuman, 1997). Only RNAPII that contains an unphosphorylated CTD (IIA form) is able to form transcription initiation complexes (Lu et al., 1991; Chesnut et al., 1992; Dahmus, 1996), whereas the elongating RNAPII contains a highly phosphorylated CTD (IIO form, Payne et al., 1989; O'Brien et al., 1994; Dahmus, 1996).

The CTD of RNAPII is essential for viability (Allison et al., 1988; Zehring et al., 1988). In yeast, the cold-sensitive growth phenotype associated with partial truncation of the CTD led to the isolation of intragenic mutations and to the SRB (*S*uppressor of *R*NA Polymerase *B*) family of genes (Nonet and Young, 1989). The Srb proteins are associated with RNAPII and a subset of general transcription factors to form the RNA polymerase II holoenzyme (Koleske and Young, 1994).

Different RNAPII complexes have been isolated from yeast (Carlson, 1997; Hampsey, 1998). Young and coworkers isolated an RNA polymerase II holoenzyme using antibodies against different Srb polypeptides and Western blot analyses (Koleske and Young, 1994). This RNAPII complex contains the entire set of Srbs (Srb2 and Srb4–Srb11) and most of the GTFs, except TBP and TFIIE. By contrast, Kornberg and coworkers (Kim et al., 1994) isolated the Mediator complex using a functional transcription assay analyzing for factors that mediate transcriptional activation. The Mediator is free of RNAPII and contains Srb2, Srb4–Srb7, Rox3 (Gustafsson et al., 1997), Gal11, Sin4, Rgr1 (Li et al., 1995), and other polypeptides referred to as Meds (Lee et al., 1997; Myers et al., 1998). This complex is devoid of Srb8–Srb11. Interestingly, disruption of the *SIN4* gene or truncation of the *RGR1* gene allowed the separation of the Mediator into two subcomplexes. One subcomplex is composed of Gal11, Sin4, Rgr1, and Med3 (Li et al., 1995; Myers et al., 1998), whereas the other subcomplex functionally resembles the Mediator. Another RNAPII complex has been described by Jaehning and coworkers (Shi et al., 1997). This complex appears functionally different from those described by Young and Kornberg and lacks Srb and Med polypeptides, but contains Paf1, Cdc73, Ccr1, and Hpr1 (Chang and Jaehning, 1997; Shi et al., 1997).

Recently, genetic analysis of numerous regulatory systems has converged with analysis of RNA polymerase II CTD function to reveal regulatory roles for the Srb/mediator proteins associated with the CTD. Carlson and coworkers identified the *SNF* family of genes based on their requirement for *SUC2* expression (Neigeborn and Carlson, 1984). A subset of the *SNF* genes encode subunits of the chromatin remodeling Swi/Snf complex (*SNF2*, *SNF5*, *SNF6*, and *SNF11*), whereas *SNF1* encodes a protein kinase. Some suppressors of *snf1* mutations (SSN) encode components of the RNAPII holoenzyme complex (Kuchin et al., 1995; Song et al., 1996). These *SSN* genes encoding components of the RNAPII complex include *SSN2* (*SRB9*), *SSN3* (*SRB10*), *SSN4* (*SIN4*), *SSN5* (*SRB8*), *SSN7* (*ROX3*), and *SSN8* (*SRB11*) and are negative regulators of transcription (Carlson, 1997; Hampsey, 1998).

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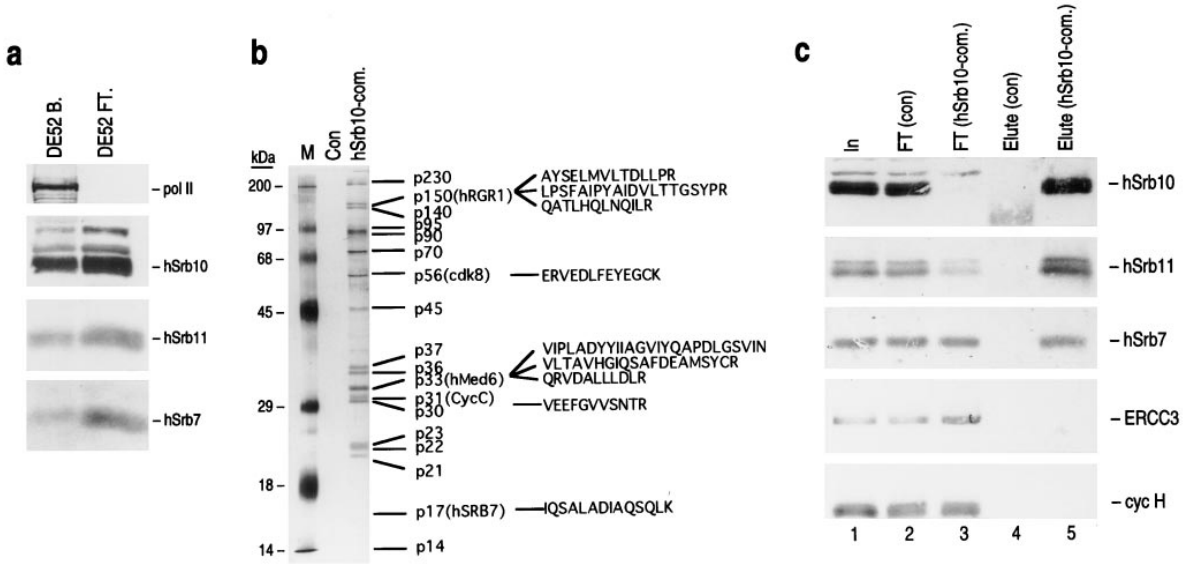

Figure 1. Isolation of a Human Srb-Containing Complex

(a) Western blot analysis of Srbs and RNAPII present in the DEAE-cellulose flowthrough and bound fractions. An aliquot of the flowthrough (10 μg) and bound (10 μg) fractions were separated by electrophoresis on a polyacrylamide–SDS gel followed by Western blot analysis using antibodies against human Srbs and RNAPII as indicated on the side of the panel.

(b) Silver stain of an 11% polyacrylamide–SDS gel containing an aliquot of samples derived from the control column and the anti-hSrb10 affinity column. The DEAE-cellulose flowthrough fraction was used as input for the affinity purification procedure. The identity of the polypeptides was based on the results from Western blot analysis and/or ion trap MS/MS analysis; some peptide sequences obtained from some of the bands are shown on the side. We have also identified 12 more peptide sequences for CDK8 (including REFLTEEEPDDKGDK, DLKPANILVM GEGPER, and others), 4 more peptide sequences for Cyclin C (including SIDPVLMAPTCVFLASK, QWFAELSVDMEK, and others), 2 more peptide sequences for hSrb7 (including DQPANPTEEYAQLFAALIAR, GDMLLEK), and 8 more peptide sequences for hMed6. All of them match perfectly with the corresponding protein sequences. The other major bands were named according to their apparent molecular weight. Among these, we identified P140 as the human homolog of the SUR-2 protein in *C. elegans*, and P14 (stained better in Figure 3a) as the human homolog of the yeast Med10 protein (Y.-J. Kim, personal communication).

(c) Western blot analysis of the fractions derived from the control (lane 4) and the anti-hSrb10 affinity (lane 5) columns. Lane 1 is the input used in affinity purification; lanes 2 and 3 are the flowthrough from the control and the anti-hSrb10 affinity columns, respectively. The blot was probed with different antibodies, as indicated on the side of the panel.

Complexes similar to the yeast RNAPII holoenzyme have been isolated from mammalian cells; however, the mammalian complexes show greater heterogeneity than those derived from yeast (for review, see Greenblatt, 1997).

The kinase-cyclin pair CDK8/cyclin C are the human homologs of the yeast Srb10/Srb11 complex (Liao et al., 1995). Srb10 and Srb11 are components of the RNA polymerase II complex described by Young and coworkers, but are not components of the mediator complex described by Kornberg and coworkers. In this study, we report the isolation of a human Srb10/(CDK8)-containing complex that functions as a negative regulator of activated transcription.

Results

Isolation of a Human Srb10/(CDK8)-Containing Complex

Fractionation of HeLa cell nuclear extracts on a phosphocellulose column followed by chromatography on a DEAE-cellulose column resulted in the resolution of two fractions containing hSrb10, as detected by Western blot analysis. One fraction was found in the flowthrough of the DEAE-cellulose column, whereas the other fraction bound to the column. Western blot analysis demonstrated the presence of hSrb7, hSrb10, and hSrb11 in both fractions. However, the flowthrough fraction was devoid of RNAPII (Figure 1a). The RNAPII complex derived from the DEAE-cellulose bound fraction has been characterized previously (Maldonado et al., 1996; Cho et al., 1997, 1998). In the present study, we have characterized the DEAE-cellulose flowthrough fraction. We used antibodies against CDK8 (hSrb10) to affinity-purify hSrb10-containing complexes.

Silver-staining analysis of a polyacrylamide–SDS gel containing the hSrb10 affinity-purified complex resulted in the identification of ≈20 polypeptides that were specifically retained by the anti-CDK8 affinity column, but not by the control column, which was made from anti-CDK8-depleted serum (Figure 1b). The presence of hSrb7, hSrb10, and hSrb11 in the anti-CDK8 affinity-purified complex, but not in the control column, was demonstrated by Western blot analysis using antibodies specific for each of the polypeptides (Figure 1c). The isolated complex was free of GTFs, RNAPII, and other polypeptides previously described to be part of the human RNAPII complex (Figure 1c, and data not shown).

Characterization of the Polypeptides Present in the hSrb10 Affinity-Purified Complex

Peptide sequencing by capillary HPLC ion trap mass spectrometry of each of the polypeptides present in the

a

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  1  MAAVDIRDNL LGISWVDSSW IPILNSGSVL DYFSERSNPF YDRTCNNEVV

 51  KMQRLTLEHL NQMVGIEYIL LHAQEPILFI IRKQQRQSPA QVIPLADYYI

101  IAGVIYQAPD LGSVINSRVL TAVHGIQSAF DEAMSYCRYH PSKGYWWHFK

151  VHEEQDKVRP KAKRKEEPSS IFQRQRVDAL LLDLRQKFPP KFVQLKPGEK
           *
201  PVPVDQTKKE AEPIPETVKP EEKETTKNVQ QTVSAKGPPE KRMRLQ
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b

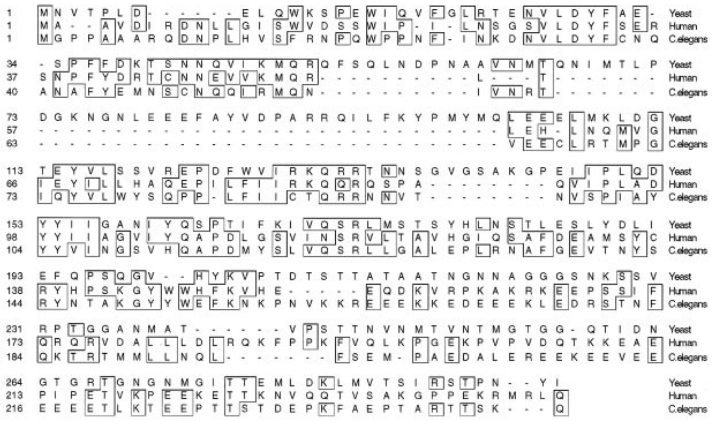

Figure 2. The Amino Acid Sequence of the Human Med6 Polypeptide Present in the hSrb10 Complex

(a) The full-length amino acid sequence of human Med6 polypeptide. Peptide sequences identified by capillary HPLC ion trap mass spectrometry of the sample p33 in the hSrb10 complex (Figure 1b) are underlined. The amino acids that are different from the previous report start from the position marked with an asterisks (amino acid number 156).

(b) Amino acid sequence comparison of yeast (*S. cerevisiae*), human, and the *C. elegans* Med6 homologs. The alignment was accomplished using DNA STAR software.

affinity-purified complex confirmed that three of these polypeptides were identical to hSrb7, hSrb10, and hSrb11 (Figure 1b). Amino acid sequences of peptides from p150 led us to identify EST (expressed sequence tag) clones with significant homology to the N terminus of the yeast Rgr1 protein (20.3% identity, 28.8% similarity), previously shown in yeast to be present in a subcomplex of the Mediator. Peptides derived from p33 demonstrated that it is a human homolog of the yeast Med6 polypeptide (Figure 1b, and see below). Most of the other polypeptides appear to be novel and without a significant counterpart in *Saccharomyces cerevisiae* (data not shown, see Figure 1b legend).

The Human Med6 Polypeptide

Of particular interest was the human Med6 polypeptide, as studies in yeast have demonstrated that it is a subunit of the Mediator complex (Kim et al., 1994; Lee et al., 1997). Moreover, yeast Med6 is necessary to mediate the response to transcription activators in a reconstituted system, and to be required for expression of a subset of yeast genes in vivo (Lee et al., 1997).

Some of the peptides derived from p33 were present in the previously reported human Med6 sequence (Lee et al., 1997). However, other peptides were absent from the reported human Med6 sequence and from a Med6-EST clone we obtained from the data bank (dbest). By screening a HeLa cell cDNA library, we were able to isolate a longer cDNA clone that includes all of the peptides derived from p33 (Figure 2a). Sequence alignment of the human, *S. cerevisiae*, and *C. elegans* Med6 polypeptides is shown in Figure 2b.

The cDNA encoding the full-length human Med6 protein was expressed in bacteria, and the purified recombinant polypeptide was used in functional transcription assays and for antibody production. Human Med6 did not affect basal transcription, nor did it function as a coactivator using either in vitro reconstituted system or HeLa nuclear extract (data not shown). Thus, hMed6 protein does not appear to function in transcription as an isolated polypeptide.

Isolation of a Human Med6-Containing Complex

We next asked whether a complex could be isolated using antibodies against the recombinant human Med6 protein. The DEAE-cellulose flowthrough fraction described above was used in the affinity purification procedure. Silver staining analysis of a polyacrylamide–SDS gel containing proteins isolated by affinity chromatography on columns containing anti-CDK8 or anti-hMed6 antibodies revealed a similar pattern of polypeptides (Figure 3a). However, the relative abundance of some of the polypeptides varied between the complexes. The presence of hSrb7, hSrb10, and hSrb11 in the Med6 affinity-purified complex was demonstrated by comigration of polypeptides on a polyacrylamide–SDS gel and by Western blot analysis (Figure 3b). Like the anti-CDK8 affinity-purified complex, the anti-Med6 affinity-purified complex was devoid of GTFs and RNAPII (data not shown). The fact that these two affinity-purified complexes are almost identical strongly suggests that hSrb10

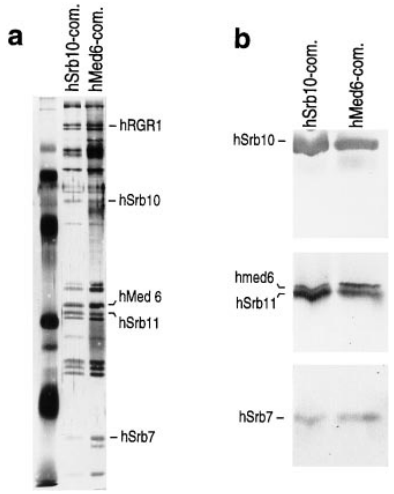

Figure 3. Comparison of the Polypeptides Present in Samples Generated by Affinity Purification on Columns Containing Anti-hSrb10 and Anti-hMed6 Antibodies

(a) Silver staining of a polyacrylamide–SDS gel containing the samples derived from the anti-hSrb10, and the anti-hMed6 antibody affinity columns. The polypeptides known are indicated on the side.
(b) Western blot analysis of the samples used in Figure 3a. Antibodies used are indicated on the side.

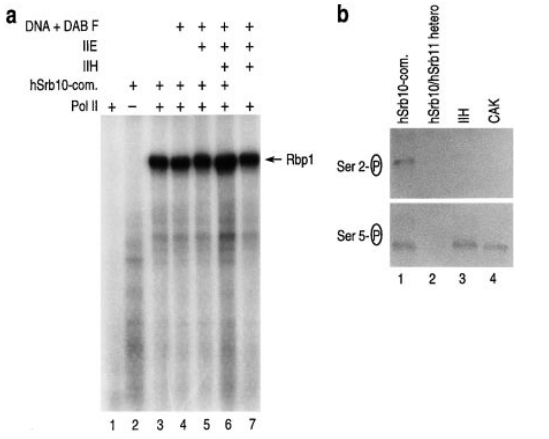

Figure 4. Phosphorylation of RNAPII by hSrb10-Containing Complex

(a) Human Srb10 complex phosphorylates RNA polymerase II. Reactions were performed as described in the Experimental Procedures. Components added to the reactions are indicated on top of the panel. Human RNA polymerase II (100 ng) [γ-^{32}P]-ATP (3 μCi), and ATP (100 μM) were used. After incubation at 37°C for 60 min, the reactions were spanned and an aliquot of the supernatant was separated on a 6% polyacrylamide–SDS gel. The heavy band represents the largest subunit of RNAPII.
(b) Western blot analysis of the residues phosphorylated by the hSrb10 complex on the CTD of RNA polymerase II. The kinase activity derived from the hSrb10 complex, human Srb10/11 heterodimer, TFIIH, and human rCAK complex are indicated at the top of the panel. Bacterially expressed GST-$(CTD)_{26}$ (300 ng) and ATP (2 mM) were present in the reaction. The CAK complex as well as the human Srb10/Srb11 heterodimer were produced in baculovirus, and the complexes were near to homogeneity as judged by polyacrylamide–SDS gel followed by silver staining. The products of the reaction were resolved on a 6% polyacrylamide–SDS gel, transferred to nitrocellulose membrane, and analyzed by Western blot using monoclonal antibodies against phosphorylated Ser-2 and Ser-5 as indicated on the side of the panel.

complex is a bona fide complex, and hMed6 is a component of the complex.

Functional Analysis of the hSrb10/(CDK8)-Containing Complexes

The hSrb10 complex contains kinase activity that can phosphorylate RNAPII (Figure 4a, lane 3). In the absence of RNAPII, the complex generated a series of polypeptides that were phosphorylated, yet in the presence of RNAPII, its largest subunit, Rbp1, was the major phosphorylated species. Phosphorylation of Rbp1 by the Srb10-containing complex was not affected by other GTFs, nor was the activity affected by the association of RNAPII with promoter sequences (in the presence of promoter DNA, TBP, TFIIB, TFIIF, and TFIIA), as is the case with the TFIIH-associated kinase (Lu et al., 1992) (Figure 4a, lane 3 versus lane 4). The hSrb10-containing complex did not stimulate the TFIIH-kinase activity, as observed with the Mediator complex (Kim et al., 1994). However, an additive effect on RNAPII phosphorylation was observed when TFIIH was added to the hSrb10 complex (Figure 4a, lane 6). This finding prompted us to investigate the residues within the CTD of RNAPII that are phosphorylated by the hSrb10-containing complex, the hSrb10/hSrb11 heterodimer, TFIIH, and the rCAK complex of TFIIH. This analysis was performed by Western blot analysis using monoclonal antibodies that are specific for the CTD of RNAPII phosphorylated at serine 2 or at serine 5 residues (Patturajan et al., 1997). In agreement with previous findings (Gebara et al., 1997), Ser-5, but not Ser-2 was phosphorylated by TFIIH or the CAK complex (Figure 4b, lanes 3 and 4). In contrast, the hSrb10-containing complex phosphorylated the CTD at Ser-2 and at Ser-5 (lane 2). When the kinase activity of the hSrb10-containing complex was compared to that of the hSrb10/hSrb11 heterodimer, we observed that the heterodimer was much less efficient using RNAPII as a substrate (Figure 4 and data not shown), although both the hSrb10-containing complex and the hSrb10/hSrb11 heterodimer phosphorylated CTD peptides with apparent similar specific activities (data not shown). However, the specificity of the heterodimer was similar to that of the hSrb10-containing complex and phosphorylated Ser-2 and Ser-5 (data not shown). These findings, together with those presented in Figure 4a, suggest that the additive effect on RNAPII phosphorylation observed with saturating amounts of TFIIH and the hSrb10-containing complex is likely due to the phosphorylation of different residues within the CTD.

Next, we analyzed the function of the hSrb10-containing complex in transcription. The addition of the complex to a reconstituted assay measuring basal transcription (i.e., in the absence of activator and cofactors required for activation) resulted in an approximately 4-fold stimulation of transcription (Figure 5a, lanes 1–4). The observed effect was specific to the Srb10-containing complex, as control beads failed to stimulate basal transcription (Figure 5a, lane 5).

Activators require coactivators to exert their function (Guarente, 1995; Kaiser and Meisterernst, 1996). We

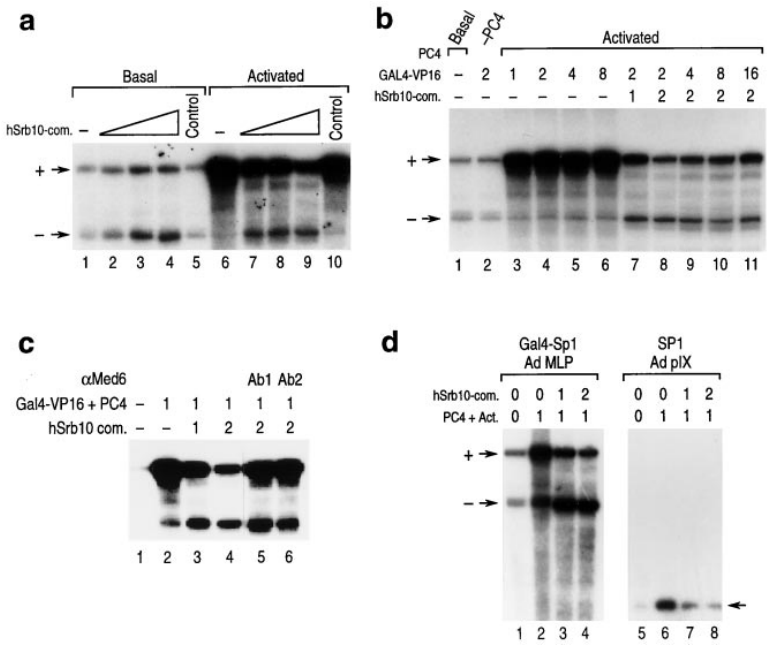

Figure 5. Repression of Activated Transcription by the NAT Complex

(a) The effect of the NAT complex on basal and activated transcription. Reconstituted transcription reactions were as described in the Experimental Procedures. Activator, coactivator, and the NAT complex were added as indicated on the top of the panel. Control stands for the beads without the NAT complex. The top template contains five Gal4 binding sites upstream of the TATA motif of the Ad-MLP as described by Ma et al. (1996). The activator used was Gal4-VP16, and the cofactors were TFIIA and PC4.

(b) Addition of excess amounts of the activators cannot overcome the repression activity NAT. Transcription reactions were performed as described above. The relative amount of the activators added is indicated in the panel.

(c) Antibodies against human Med6 alleviate the repression activity of the NAT complex. Anti-hMed6 antibodies, derived from two different rabbits, were incubated with the NAT complex for 20 min at 30°C prior to the addition to the transcription reaction. Reactions were performed as described above.

(d) The NAT complex represses transcription activation mediated by Gal4-Sp1 and Sp1 on two different promoters. Reconstituted transcription assays were performed as indicated above using Gal4-Sp1 on Ad-MLP, or native Sp1 on Ad-pIX promoter, which are indicated on the panel.

therefore analyzed whether the hSrb10-containing complex has a coactivator function. In contrast to the yeast Mediator complex, we found that the hSrb10-containing complex was devoid of such an activity to further potentiate transcription with GAL4-VP16 (data not shown). We extended these studies and investigated whether the complex affected activation of transcription in an assay containing the mammalian coactivators PC4 and TFIIA (Ma et al., 1996). The Srb10-containing complex was found to repress activated transcription (Figure 5a, lanes 6–9, and see below). The repressing effect on activated transcription was specific to the hSrb10-containing complex, as control beads were without effect (Figure 5a, lane 10). Repression was not due to squelching of the activator or coactivators, as the inhibition could not be overcome by the addition of excess activator (Figure 5b) or TFIIA and/or PC4 (data not shown, see below). Moreover, repression of activated transcription appears to be specific for activation of transcription since basal transcription was not decreased. More importantly, preincubation of the complex with anti-Med6 antibodies precluded the repressive effect of the complex in activated transcription without affecting the stimulation of basal transcription (Figure 5c). The effect was specific to the Med6 antibodies as the preimmune serum or unrelated antibodies were without effect (data not shown). The inhibition of activated transcription was not activator specific, as activation by Gal4-Sp1 was also repressed (Figure 5d), nor was the effect promoter specific, as inhibition was also observed using the natural adenovirus promoter encoding polypeptide IX with the natural activator Sp1 (Figure 5d). Because of its specific inhibitory effect on activated transcription, we refer to the complex as NAT for *n*egative regulator of *a*ctivated *t*ranscription.

Analysis of the Mechanism of Repression by NAT

To further understand the mechanism of repression of activated transcription by NAT, as well as the stimulation of basal transcription, we studied whether initiation of transcription was affected. Toward this end, the synthesis of the first phosphodiester bond was analyzed using an abortive initiation assay. Transcription reactions were reconstituted as above and were provided with the nucleotide precursors ATP and CTP with or without activator. Under these conditions, the activator stimulated the production of the ApC dinucleotide (Figure 6a). The addition of the NAT complex resulted in inhibition of activated transcription to levels similar to those produced in the absence of activator (Figure 6a, lanes 1–4). In contrast to the results observed under transcription conditions measuring initiation and elongation, the NAT complex did not stimulate initiation of basal transcription (Figure 6a, lanes 5–7). We conclude that inhibition of activated transcription by the NAT complex is mediated through, or prior to, the formation of the first phosphodiester bond, and that the positive effect on basal transcription is mediated through a step subsequent to the formation of the first phosphodiester bond.

We next analyzed whether repression of activated transcription could be bypassed by the formation of a transcription preinitiation complex. Factors required for activated transcription were incubated with the DNA prior to the addition of the NAT complex. Subsequently, NAT was added to reactions, and the extent of single-round transcription was compared to that of reactions

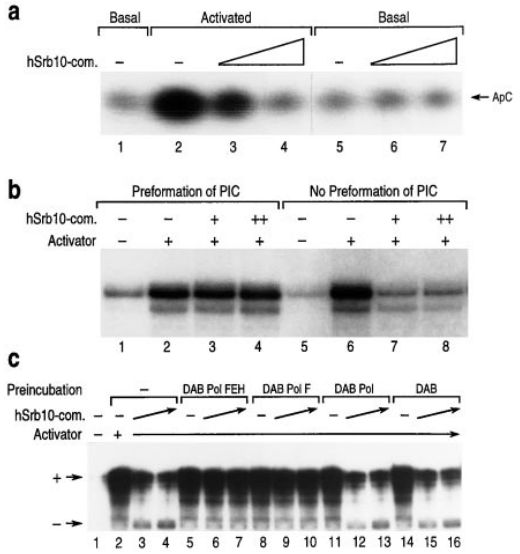

Figure 6. Transcription Repression by the NAT Complex

(a) The NAT complex represses transcription initiation. Abortive transcription reactions were performed as described in the Experimental Procedure. Addition of the NAT complex and activator/cofactors was as indicated.

(b) Preformation of transcription initiation complex precludes NAT-mediated repression. Single-round transcription conditions were described in the Experimental Procedures. In lanes 3 and 4, the NAT complex was added to the reactions after the formation of the preinitiation complex that was accomplished during a 40 min preincubation at 30°C. In lanes 7 and 8, the NAT complex was added together with the other general transcription factors.

(c) Formation of the DABPolF complex precludes NAT-mediated repression. Different preinitiation complex intermediates, including the activator and cofactors, were incubated for 60 min with the DNA as indicated at the top of the panel. The NAT complex together with the remaining factors were added, and reactions were performed under single-round transcription conditions, as described above. In lanes 3 and 4, the NAT complex was added together with the other general transcription factors.

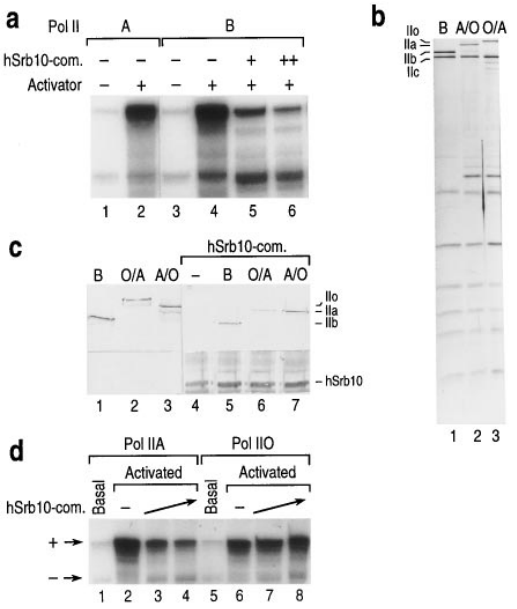

Figure 7. Interaction of the NAT Complex with RNAPII

(a) The NAT complex inhibits activated transcription mediated by the CTD-less form of RNAPII. Transcription reactions were performed as described above. Lanes 1 and 2 were reconstituted using RNAPIIA; lanes 3–6 were reconstituted using the CTD-less form of RNA polymerase II (IIB). The NAT complex was added as indicated.

(b) Silver staining analysis of the RNAPII preparation IIB, IIA/IIO, and IIO/IIA used in the experiments described above and below.

(c) The NAT complex interacts with RNA polymerase II. Different forms of RNA polymerase II, shown in (b), were mixed with purified NAT complex as indicated in the panel. Immunoprecipitation was performed using anti-hSrb10 antibody as described in the Experimental Procedures. Lanes 1–3 represent the RNAPII used in the analysis. Lane 4 is the beads with the NAT complex in the absence of RNAPII. Lanes 5–7 are the beads after incubation with RNA polymerase IIB, IIO/IIA, and IIA/IIO, respectively.

(d) The NAT complex cannot inhibit activated transcription mediated by the phosphorylated form of RNAPII. Transcription reactions were performed as described above. Lanes 1–4 were reconstituted using RNAPIIA; lanes 5–8 were reconstituted using the phosphorylated form of RNA polymerase II. The NAT complex was added as indicated.

performed with no preincubation. We found that the formation of a preinitiation complex precluded inhibition of activated transcription (Figure 6b). This finding provided us with an experimental approach to study the factor(s) targeted by the NAT complex.

The effect of NAT on different preinitiation complex intermediates was analyzed. Specifically, we asked which complex rendered the reaction resistant to inhibition by the NAT complex. The formation of the DABPolF preinitiation complex intermediate precluded NAT-mediated inhibition (Figure 6c, lanes 8–10). However, the formation of the DAB preinitiation complex intermediate (lanes 14–16) or the omission of TFIIF from the DABPolF complex (lanes 11–13) rendered the reaction susceptible to inhibition by the NAT complex. Since the activator and cofactors necessary for activation (PC4 and TFIIA) were preincubated with the different preinitiation complex intermediates, we concluded that it is the stable association of RNAPII and factors (PC4, TFIIA, TFIIB, TFIID, TFIIF) with the template that renders the reaction resistant to inhibition by the NAT complex. However, resistance to the NAT complex does not require the presence of TFIIE and TFIIH.

NAT Interacts with RNAPII, and Its Repressive Function Is Independent of the CTD, but Precluded by Phosphorylation of the CTD

Since the *SRB* genes were isolated as suppressors of CTD truncations (Nonet and Young, 1989), and NAT contains at least three Srbs, we asked whether the function of NAT requires the CTD of RNAPII. A CTD-less form of RNAPII (IIB form) was purified from calf thymus (Figure 7b). Transcription reactions were reconstituted with approximately equivalent amounts of CTD-less or CTD-containing RNAPII, estimated by quantitative Western blots using antibodies against the Rpb7 subunit (data not shown). We first analyzed whether the CTD-less RNAPII responded to Gal4-VP16 in vitro. To our surprise, we observed that transcription reactions reconstituted with the nonphosphorylated or CTD-less form of RNAPII responded to activators to similar levels (Figure 7a). Interestingly, the NAT complex inhibited activated transcription mediated by the CTD-less form of RNAPII (Figure 7a).

Since the results presented above demonstrated that stable association of RNAPII with template was necessary to bypass NAT-mediated repression, we analyzed whether the NAT complex interacts with RNAPII, and if so, whether the phosphorylation of the CTD is important for interaction. Highly purified forms of RNAPII (phosphorylated IIO, nonphosphorylated IIA, and CTD-less IIB) (Figure 7b) were incubated with the NAT complex that was attached to beads through the anti-CDK8 antibodies. After incubation, the beads were washed extensively. We then used Western blots with antibodies against the largest subunit of RNAPII to determine whether the different forms of RNAPII were coimmunoprecipitated with the NAT complex. This analysis revealed that the NAT complex interacts with RNAPII, yet the interaction was specific for the nonphosphorylated or CTD-less form of RNAPII. The phosphorylated form of RNAPII was unable to interact with the NAT complex (Figure 7c, lanes 4–7). These results are in full agreement with the functional studies demonstrating that the NAT complex is not able to inhibit activated transcription mediated by the phosphorylated form of RNAPII (Figure 7d). We suggest that the phosphorylation of CTD induces a conformational change in the polymerase that precludes its interaction with the NAT complex.

Discussion

We have isolated a complex, NAT, that represses activation of transcription by RNAPII. The NAT complex is composed of approximately 20 polypeptides and includes a subset of the Srb polypeptides, hSrb7, hSrb10, and hSrb11, as well as hRgr1 and hMed6. The presence of hSrb10, hSrb11, and hRgr1 in a complex that functions to down-regulate transcription is consistent with studies in yeast demonstrating that Srb8–Srb11, as well as Rgr1, function as negative regulators of transcription in vivo. We suggest that the NAT complex is a subcomplex of the RNAPII holoenzyme and its function and regulation is through the RNAPII holoenzyme. The presence of the human homologs of the yeast holoenzyme components Rgr1, Srbs, and Med6 in the complex is in agreement with this hypothesis.

Yeast Med6 was isolated as a subunit of the Mediator complex, which mediates the response to transcriptional activators (Kim et al., 1994; Lee et al., 1997). Moreover, yeast Med6 was found to be important for the yeast Mediator function, and to be required for the expression of a subset of genes in yeast. In our studies, we found that hMed6 has no function on its own, and is present in an RNAPII subcomplex with dual functions: the complex represses the response to transcriptional activators, yet stimulates basal transcription. These two functions are likely due to different activities in the subcomplex, as the repressing activity is mediated through RNAPII and prior to its stable association with template DNA. On the other hand, the stimulation of basal transcription occurs after initiation of transcription.

Factors that positively and negatively regulate transcription coexist in a large RNAPII holoenzyme complex. Young and coworkers isolated a complex containing the entire set of Srbs as well as Gal11, Sin4, and other factors. This complex was purified using antibodies against different Srb polypeptides in Western blot analysis and irrespective of specific function. On the other hand, Kornberg and colleagues isolated the Mediator complex scoring for an activity that mediates the response to transcriptional activators. The Mediator is free of RNAPII and Srb8–Srb11, but contains other Srbs, Rox3 (Gustafsson et al., 1997), Gal11, Sin4, Rgr1 (Li et al., 1995), and additional polypeptides referred to as Meds (Lee et al., 1997; Myers et al., 1998). However, the negative regulators within the Mediator complex, Sin4, Rgr1, in addition to Gal11 and Med3, can be dissociated from the Mediator complex without an effect on Mediator function. The kinase-cyclin pair CDK8/cyclin C are the human homologs of the yeast Srb10/Srb11 complex (Liao et al., 1995). Previous studies have demonstrated the existence of different CDK8/cyclin C complexes in human cells (Rickert et al., 1996). In agreement with these previous observations, we have chromatographically separated three different hSrb10-containing complexes. One complex was isolated by conventional chromatography, as well as by affinity chromatography on columns containing monoclonal antibodies against RAP74, the large subunit of TFIIF. TFIIF was found to be present in stoichiometric amounts within human and yeast RNAPII complexes (Kim et al., 1994; Koleske and Young, 1994; Maldonado et al., 1996; Pan et al., 1997). Interestingly, the human complex, containing TFIIF, and other GTFs (TFIIE and limiting amounts of TFIIH), includes factors, in addition to hSrb10 and hSrb11, that mediate the response to transcriptional activators (Maldonado et al., 1996; Cho et al., 1997). Two other hSrb10(CDK8)-containing complexes have been isolated using antibodies against CDK8. One complex is described in the present study and functions as a negative modulator of activated transcription and is devoid of GTFs, RNAPII, Srb2, and Srb4–Srb6. The other complex includes polypeptides similar to those present in the NAT complex, but additionally contains the chromatin remodeling Swi/Snf complex as well as the histone acetylases p300/CBP and PCAF (Cho et al., 1998). The NAT complex, like the yeast Mediator, probably represents one of the subcomplexes of a larger RNAPII holoenzyme complex. The subcomplexes likely dissociate from the RNAPII holoenzyme either because of the assay conditions and/or because of the procedures used to purify the complex.

In light of previous results, and the findings presented in the current study, we suggest that the RNAPII holoenzyme is a transcriptional control panel, capable of responding to specific stimuli. Different forms of RNAPII complexes and subcomplexes are likely generated from different purification schemes and functional assays applied in different laboratories. Regardless of the differences in the polypeptide composition, it appears that the RNAPII holoenzyme is capable of integrating and responding to specific signals leading to activation or repression of gene expression. The important questions that remain to be answered are how this specificity is achieved, and how the RNAPII complex is regulated.

Three important regulatory modes, likely to be involved in the regulation of the complex, are (1) targeting of the complex to specific genes, (2) induced-conformational changes upon interaction of the complex with

gene-specific regulators, and (3) posttranslational modification of polypeptides within the complex. The presence of polypeptides with enzymatic activity within the complex is well established (Maldonado et al., 1996; Nakajima et al., 1997a, 1997b; Pan et al., 1997; Cho et al., 1998). At least two acetyltransferases (p300/CBP and PCAF) and different protein kinases are components of the RNAPII complex. In previous studies, Young and coworkers described the Srb10/Srb11 kinase complex in the yeast RNAPII complex. The studies of Carlson and coworkers demonstrated a negative role for this kinase complex in gene regulation in vivo. In the present studies, we isolated a subcomplex from HeLa cells containing the hSrb10/hSrb11 kinase complex and biochemically demonstrated that the complex functions as a negative modulator of transcription using a reconstituted system. This complex also has a positive effect on basal transcription, which is likely mediated through a step subsequent to the formation of the first phosphodiester bond, since it does not affect abortive transcription initiation. We found that the NAT complex phosphorylates the CTD of RNAPII with specificity different from that of the TFIIH-associated CTD kinase. It is possible that phosphorylation at residues other than serine 5 in the CTD, the residue phosphorylated by TFIIH, is important for the function of hSRB10 complex. Our studies also revealed that the CTD of RNAPII is not the only target for transcriptional repression by NAT, as we observed that transcription mediated by the CTD-less form of RNAPII was also subjected to negative regulation by the NAT complex. This finding is in agreement with the genetic studies demonstrating that, in addition to the extragenic suppressors isolated in response to CTD truncations (the Srbs), intragenic mutations were also isolated as suppressors of CTD truncations (Nonet and Young, 1989). Our studies establish that the NAT complex is directed to RNAPII by a direct interaction between components of the NAT complex and RNAPII; however, this interaction is independent of the CTD of RNAPII. Importantly, however, we observed that the CTD was important in modulating the interaction between the NAT complex and RNAPII, as its phosphorylation precludes the interaction between the NAT complex and RNAPII. In light of our findings, it is likely that the NAT complex targets the CTD as well as other components of the transcription machinery involved in establishing a complex between RNAPII and promoter sequences.

Experimental Procedures

Affinity Purification of CDK8

Affinity purification of human CDK8-containing complexes was performed using antibodies against CDK8. Approximately 150 μg of affinity-purified anti-CDK8 antibodies were covalently immobilized onto 0.8 ml of protein A agarose beads (Repligen). The control column was made from anti-CDK8-depleted serum. Following equilibration in buffer C (20 mM Tris–HCl [pH 7.8], 0.2 mM EDTA, 10 mM β-Mercaptoethanol, 10% (v/v) glycerol, and 0.2 mM PMSF) containing 0.1 M KCl and 0.1% NP-40, beads were incubated with ≈10 mg of input, the DEAE-cellulose flowthrough (derived from 0.5 M phosphocellulose fraction of HeLa nuclear extract) fraction at 4°C with rotation. After 4 hr of incubation, the beads were washed extensively with buffer C containing 0.7 M KCl, and 0.1% NP-40, and further washed with buffer C containing 0.1 M KCl. Protein complexes were eluted from the beads with 100 mM glycine (pH 2.6). The beads were neutralized with 0.1 M Tris–HCl (pH 8.0) and equilibrated with the above buffer prior to being reused. In the functional assays, the complex was not eluted and used directly on the beads.

Western Blot Analysis

Blots were incubated with 3% nonfat dry milk for 1 hr at room temperature with shaking. Following three washes with TTBS [10 mM Tris–HCl buffer (pH 7.5), 0.2 M NaCl, and 0.05% (v/v) Tween 20], blots were incubated with primary antibodies in 0.1% BSA containing TTBS for 2 hr at room temperature with gentle agitation. Blots were washed again with TTBS and incubated with secondary antibodies conjugated to alkaline phosphatase (Promega), for 30 min at room temperature. Blots were washed with TTBS and developed according to the manufacturer's protocol (Bio-Rad).

Peptide Sequencing

Eluates derived from the immunoaffinity column steps were separated on a polyacrylamide–SDS gel after they were concentrated by TCA precipitation. The gel was stained with Coomassie brilliant blue R-250 and destained extensively. The polypeptide bands were excised and subjected to in gel reduction, carboxyamidomethylation, and tryptic digestion (Promega), and a single 10% aliquot from each was analyzed as follows. Sequence information was determined by capillary (180 μm × 15 cm column, LC Packings, Amsterdam) reverse-phase HPLC coupled to the electrospray ionization (ESI) source of a quadruple ion trap mass spectrometer (Finnigan LCQ, San Jose CA). The instrument was programmed to acquire successive sets of three scan modes consisting of full scan MS over the m/z range 395–1118 amu, followed by two data-dependent scans on the most abundant ion in that full scan. These data-dependent scans allowed the automatic acquisition of high resolution (zoom scan) spectra to determine charge state and exact mass, and MS/MS spectra for peptide sequence information. Interpretation of the resulting MS/MS spectra of the peptides was facilitated by searching the NCBI nr and dbest data bases with the algorithm Sequest (Eng et al., 1994). MS/MS spectra with significant correlation to data-base entries were then confirmed by manual interpretation.

cDNA Cloning of Human Med6

A human EST clone (#231995) was obtained from Research Genetics and sequenced. Two oligonucleotides (5′-CATTCGGAAGCAACAGC GGC-3′ and 5′-TAGATCACTCCAGCAATG-3′) located in the N-terminal coding region were designed to generate a probe to be used for screening. The probe (∼80 nucleotides long) was labeled by random priming (Boehringer Mannheim). A HeLa cell cDNA library was used (Stratagene). After three rounds of screening, eight positive clones were selected from about 0.5×10^6 phage plaques. Two of these clones contained the full open reading frame. The full-length human Med6 cDNA cloned was inserted into pET21a(+) vector by PCR. Recombinant hMed6 was expressed in bacteria and purified first by Ni-NTA column chromatography. hMed6 was further purified to homogeneity by chromatography on a phosphocellulose column.

Transcription Factors and RNA Polymerase

Affinity-purified epitope-tagged TFIID was purified as described (Maldonado et al., 1996a). Expression and purification of recombinant TFIIA was done as described (Sun et al., 1994). Expression and purification of recombinant TFIIB, TFIIE, and TFIIF was as previously described (Maldonado et al., 1996a). Human RNA polymerase IIA and IIO forms were purified from HeLa cell nuclear pellets as described by Lu et al. (1991). The CTD-less form of RNAPII (IIB form) was purified as described (Kim and Dahmus, 1988). The IIB form of RNAPII was further purified by affinity chromatography using an anti-CTD monoclonal antibody column. RNAPIIB was recovered from the flowthrough of the column. Human TFIIH was purified from HeLa cells as described (Flores et al., 1992). Additionally highly purified TFIIH, used in the functional transcription assays described in Figures 5, 6, and 7, was obtained by affinity purification according to the method of LeRoy et al. (1998). Recombinant PC4 was expressed

and purified as described (Ge and Roeder, 1994). Human recombinant Sp1 was purchased from Promega.

Reconstituted Transcription Assays

Transcription assays (20 μl reactions) were performed as previously described (Ma et al., 1996) using two DNA templates (100 ng each) as indicated in the figures. Briefly, transcription factors used in these assays were: epitope-tagged holo-TFIID (eTFIID, 5 ng of TBP determined using quantitative Western blots, Zhou et al., 1992), rTFIIA (α-β and γ subunits, 75 ng), rTFIIB (20 ng), RNAPII (DEAE-5PW fraction, 40 ng), rTFIIF (20 ng), rTFIIE (15 ng), and TFIIH (S200 fraction, 65 ng or affinity purified as described by LeRoy et al., 1998). Transcription activation reactions additionally contained GAL4-VP16 (50 ng) and rPC4 (60 ng). Factors and templates were preincubated for 30 min at 30°C in the presence of 0.5 mM ATP, nucleotides were added, and reactions incubated for another 45 min at 30°C. The hSrb10-containing complex on the beads was added as indicated in the figure legends. The RNA products were separated by electrophoresis on a 6% denaturing polyacrylamide-urea gel. Transcription from the Ad-pIX promoter was as described above, except that 100 ng of pIX promoter DNA was used (Olave et al., 1998).

Single-round transcription conditions were established using a pulse-chase protocol and nucleotide starvation. Briefly, after preincubation of the factors with the template DNA as described above, transcription reactions were pulsed by the addition of ATP (0.5 mM) and CTP (0.5 mM) and [α-^{32}P]-UTP (5 μCi). After 4 min of incubation, the reactions were chased for 30 min by the addition of cold UTP (0.5 mM), and the products were separated as above.

Kinase Reactions

Kinase assays were performed in standard kinase buffer containing 50 mM HEPES–KOH (pH 7.4), 10 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM DTT. Substrate and the amount of ATP are indicated in the figure legends. After incubation at 37°C for 60 min, the reactions were spanned and the supernatants of the reactions were separated on a 6% polyacrylamide–SDS gel with the addition of the Laemmli buffer.

Abortive Transcription Initiation Reactions

Transcription reactions measuring abortive initiation were performed as described (Akoulitchev et al., 1995), and ribonucleotides ATP (1 mM), CTP (1 μM), and 10 μCi of [α-^{32}P]-CTP were added. Reactions were incubated at 30°C for 60 min. Products were treated with calf-intestine phosphatase (1 U, Boehringer) for 60 min at 37°C, and resolved by electrophoresis on a 15% polyacrylamide-urea gel.

Coimmunoprecipitation

10 μl of beads of anti-CDK8 affinity-purified complex was incubated with RNAPII (0.5 μg) at 4°C for 8 hr. The beads were washed six times with buffer C containing 150 mM KCl, and 0.1% NP-40. Beads were resuspended in Laemmli buffer and subjected to polyacrylamide–SDS gel electrophoresis followed by Western blot analysis.

Other Proteins and Antibodies

Three subunits of CAK were coexpressed in insect Sf9 cells, and rCAK was purified by Ni-NTA column. hSrb10/hSrb11 heterodimer was affinity purified from Sf9 cell lysate using 12CA5 antibody, after Sf9 cells were coinfected with baculovirus containing HA-CDK8, and cyclin C. Monoclonal antibodies against phosphorylated Ser-2 and Ser-5 were purchased from BAbCO (Berkeley Antibody Company).

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GenBank Accession Number

The accession number for the sequence of the human Med6 polypeptide is AF074723.