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Synergistic Functions of SII and p300 in Productive Activator-Dependent Transcription of Chromatin Templates

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SUMMARY

We have reconstituted a highly purified RNA polymerase II transcription system containing chromatin templates assembled with purified histones and assembly factors, the histone acetyltransferase p300, and components of the general transcription machinery that, by themselves, suffice for activated transcription (initiation and elongation) on DNA templates. We show that this system mediates activator-dependent initiation, but not productive elongation, on chromatin templates. We further report the purification of a chromatin transcription-enabling activity (CTEA) that, in a manner dependent upon p300 and acetyl-CoA, strongly potentiates transcription elongation through several contiguous nucleosomes as must occur in vivo. The transcription elongation factor SII is a major component of CTEA and strongly synergizes with p300 (histone acetylation) at a step subsequent to preinitiation complex formation. The purification of CTEA also identified HMGB2 as a coactivator that, while inactive on its own, enhances SII and p300 functions.

INTRODUCTION

In living cells, RNA polymerase II must efficiently transcribe DNA within a nucleosomal (chromatin) context, and several distinct transcriptional machineries have been implicated in this process. The general machinery that is necessary and sufficient for basal (core promoter-mediated) transcription from purified DNA templates consists of RNA polymerase II and the general initiation factors (GTFs) TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH (Orphanides et al., 1996; Roeder, 1996). Efficient activator-dependent transcription from DNA templates in purified systems requires, in addition, a circa 30 subunit Mediator complex that is viewed as the main conduit for communication between DNA bound regulatory proteins and the general transcription machinery (Malik and Roeder, 2000).

Subsequent to demonstrations that the nucleosomal packaging of DNA effectively blocks basal (activator-independent) transcription from DNA templates both in vitro (Knezetic and Luse, 1986; Lorch et al., 1987; Workman and Roeder, 1987) and in vivo (Han and Grunstein, 1988), a number of factors that facilitate activator-dependent transcription through chromatin modifications (both in vitro and in vivo) have been described. These cofactors include ATP-dependent chromatin remodeling factors (Neely and Workman, 2002) and factors that effect histone modifications such as acetylation, methylation, and ubiquitination (Vaquero et al., 2003). Whereas the intracellular functions of these factors in gene regulation have been demonstrated by various genetic and molecular biological assays, early in vitro studies using HeLa nuclear extracts as a source of the general transcriptional machineries and various cofactors showed that in vitro-reconstituted chromatin templates can be transcribed efficiently in a manner that is dependent on the presence of an activator, an ATP-dependent remodeling activity, and covalent histone modifications effected by histone acetyltransferases (Dilworth et al., 2000; Kraus and Kadonaga, 1998; Kundu et al., 2000; Mizuguchi et al., 2001; Neely et al., 1999; Utley et al., 1998). Together with other advances (below), the in vitro studies with purified DNA templates and purified factors and the in vitro studies with chromatin templates and crude transcription systems (nuclear extracts) set the stage for a more definitive biochemical analysis, through establishment of a fully defined system, for identification of other factors that might be involved in transcription of chromatin.

A notable advance in the studies of chromatin-templated transcription was the development (Ito et al., 1999) of methods to assemble chromatin with purified histones, recombinant assembly factors (ATP-dependent chromatin remodeling factors and histone chaperones), and DNA—thus eliminating possible contributions to transcription of unknown factors in the crude extracts commonly used to assemble chromatin (Becker and Wu, 1992).

Until now, however, most studies utilizing chromatin templates assembled with purified histones and recombinant assembly factors (NAP1 chaperone and ATP-dependent ACF complex [Ito et al., 1999]) have utilized unfractionated HeLa or *Drosophila* extracts as a source of transcription factors (An et al., 2002; Jiang et al., 2000; Levenstein and Kadonaga, 2002). Other studies using various sources of RNA polymerase II and GTF preparations (either purified or partially purified) in conjunction with various sources of chromatin templates (assembled either with crude *Drosophila* extract or with recombinant assembly factors) have led to the identification of several factors (such as FACT, RSF, PBAF, and TAF-I/SET/INHAT) that facilitate chromatin-templated transcription (Gamble et al., 2005; Lemon et al., 2001; LeRoy et al., 1998; Orphanides et al., 1998). FACT and TAF-I/SET/INHAT have histone chaperone activities, whereas PBAF is a member of the SWI/SNF family of ATP-dependent remodeling complexes and RSF is an ISWI-containing complex. SWI/SNF and ISWI-containing complexes have also been shown to play roles in chromatin transcription activation both in vivo and in vitro (Neely and Workman, 2002). Of the above-described factors, only FACT has been shown to work at the elongation step by overcoming a nucleosome-imposed transcription block that, somewhat surprisingly in view of later studies (below), was not affected by elongation factors TFIIF or TFIIS (SII) alone or in conjunction with FACT (Orphanides et al., 1998). Earlier in vitro studies using chromatin templates assembled with purified elongation complexes and oocyte extracts showed that SII and TFIIF help slightly in relieving this block (Izban and Luse, 1992). Consistent with the latter report, a recent study with an artificially reconstituted yeast RNA polymerase II elongation complex on a DNA fragment containing a single nucleosome did report SII-dependent disruption of the nucleosome (Kireeva et al., 2005). Notably, however, the in vitro SII study did not necessarily show a specific role for SII since it did not analyze the effect of other elongation factors in this artificial system. Furthermore, the SII study did not examine the more physiological situation of activator-dependent, chromatin remodeling-dependent, and histone modification-dependent transcription from nucleosomal array templates.

Because the previous biochemical studies of factors involved in chromatin transcription utilized reconstituted transcription systems of varying purity or systems that operated independently of histone modifications, they have led to the description of several different factors that act through different mechanisms. To define the minimal complement of factors necessary for this process, we have used chromatin templates assembled with pure histones and recombinant assembly factors, recombinant activators and coactivators, and a transcription assay system reconstituted with highly purified factors to identify, in HeLa nuclear extract, an activity (CTEA) that is dispensable for activator-dependent transcription from DNA templates but essential for productive activator-dependent transcription elongation from chromatin templates in conjunction with ATP-dependent chromatin remodeling and histone acetyltransferase activities. We have purified CTEA activity and reconstituted its major components.

RESULTS

Assembly of a Defined Chromatin Template and Transcription System

An initial objective of our study was to reconstitute a transcription assay system with highly purified factors shown previously to be necessary and sufficient for optimal activator-dependent transcription from DNA templates (Experimental Procedures). The TFIIA (p55 and p12 subunits), TFIIB, TFIIE (α and β subunits), and TFIIF (RAP30 and RAP74) components of the general transcription machinery, as well as the general coactivator PC4, were expressed in and purified from bacteria (Guermah et al., 2001). The multisubunit TFIID, TFIIH, and RNA polymerase II components of the general transcription machinery, as well as the TRAP/Mediator coactivator complex, were purified from cell lines expressing epitope-tagged subunits by combinations of conventional chromatography and affinity purification (anti-FLAG M2 agarose) methods (Experimental Procedures; Ge et al., 2002; Guermah et al., 2001). These procedures provided essentially homogenous preparations of these components as shown by SDS-PAGE with Coomassie blue and silver staining (Figures 1A and 1B, respectively).

For chromatin assembly and functional analyses, HeLa core histone octamers were purified from nuclear pellets, mouse NAP1 was expressed in and purified from bacteria, and recombinant Acf-1, ISWI, Topoisomerase 1 (Topo 1), and p300 were expressed in and purified from Sf9 cells (Figure 1C). Chromatin was then assembled from a relaxed $pG_5ML\Delta53$ DNA template and core histones using Acf-1, ISWI, NAP1, and Topo 1 as described (An et al., 2002; Ito et al., 1999). The $pG_5ML\Delta53$ plasmid contains the adenovirus (Ad) major late (ML) core promoter flanked by five GAL4 binding sites in the upstream end and a 390 nucleotide G-less cassette in the downstream side (Kundu et al., 2000). A DNA supercoiling assay showed extensive DNA supercoiling upon chromatin assembly (data not shown) and micrococcal nuclease (MNase) digestion revealed a pattern of at least 10–12 well-resolved and regularly spaced bands (Figure 1D), thus indicating complete assembly of chromatin with regularly spaced nucleosomes.

Identification, Purification, and Characterization of CTEA, an Activity that Supports Efficient Transcription of Chromatin Templates with the Purified System

An initial test of the suitability of the reconstituted template for further studies showed activator- and p300 plus acetyl-CoA-dependent transcription when HeLa nuclear extract was employed as a source of the general transcriptional machinery (Figure 2A). The activator (GAL4p65) used in this assay contains the activation domain of NF-κB p65

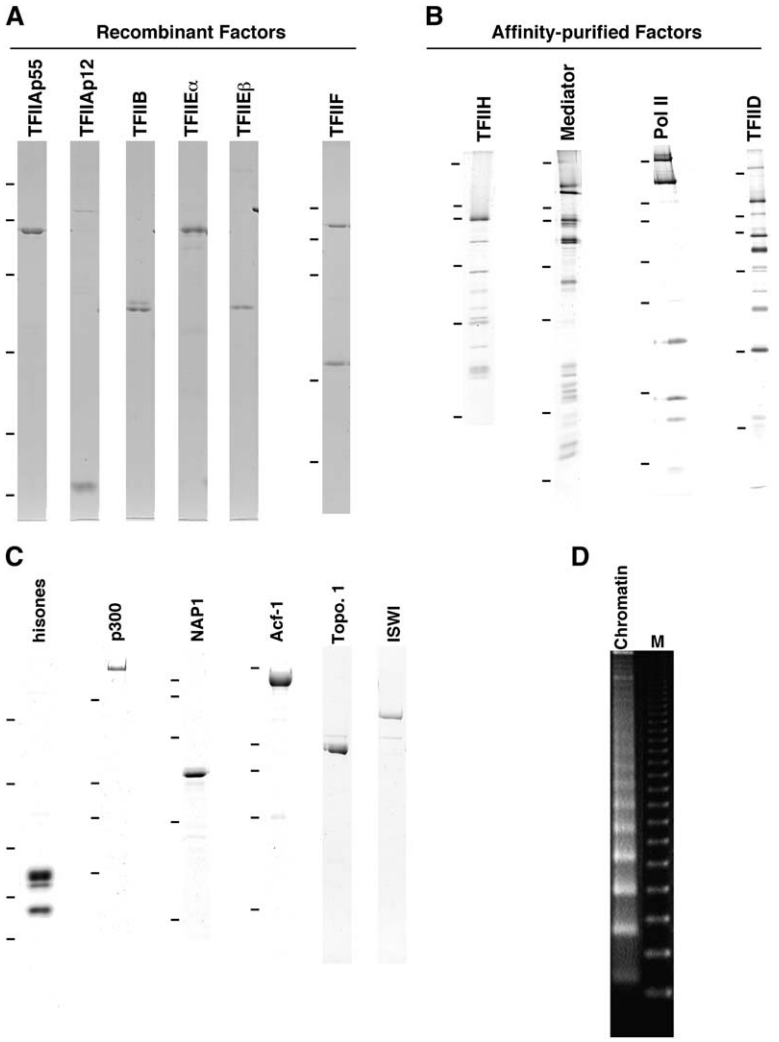

Figure 1. Analysis of Purified Recombinant GTFs, Affinity-Purified Complexes, Chromatin Assembly Factors, and In Vitro-Assembled Chromatin

(A) TFIIA (p55 and p12 subunits), TFIIB, TFIIE (α and β subunits), and TFIIF.

(B) TFIIH, Mediator, TFIID, and RNA polymerase II. Proteins were resolved on SDS-PAGE and visualized by Coomassie blue staining in (A) and silver staining in (B).

(C) HeLa histones, p300, NAP1, Acf1, ISWI, and Topo 1. Proteins were resolved on SDS-PAGE and visualized by Coomassie blue staining. In (A)–(C), relevant bars on the left indicate protein markers from to top to bottom, in kDa: 200, 116, 97, 66, 45, 31, 21, 14, and 8.

(D) MNase analysis of chromatin assembled with components in (C). Chromatin was digested and analyzed on 1.3% agarose gels with ethidium bromide staining. A 123 bp ladder was used as size marker (M).

fused to the GAL4 DNA binding domain. These results are consistent with earlier reports of activator- and p300 plus acetyl-CoA-dependent transcription from chromatin templates similarly assembled with purified histones and ACF and assayed in nuclear extracts (An et al., 2002; Kraus and Kadonaga, 1998; Kundu et al., 2000).

The same highly purified reconstituted transcription system described above (TFIIA, TFIIB, TFIID, TFIIE, TFIIF, TFIIH, RNA polymerase II, PC4, and Mediator) was previously shown to mediate robust activator-dependent transcription from histone-free DNA templates (Guermah et al., 2001). Importantly, and in contrast to nuclear extract, this system failed to support activator (GAL4p65)- and p300 plus acetyl-CoA-dependent transcription from the reconstituted chromatin templates when the 390 nucleotide transcript was monitored (Figure 2B, lane 4). However, addition of a nuclear extract-derived phosphocelulose (P11) 0.85 M KCl fraction, but not the P11 0.5 M KCl fraction, supported efficient activator- and p300 plus acetyl-CoA-dependent transcription of the chromatin template (Figure 2B, lane 3 versus lane 4 and lanes 6 and 7 versus lane 5). This potentially new activity in the P11 0.85 M fraction was tentatively designated *c*hromatin *t*ranscription-enabling *a*ctivity or CTEA.

CTEA was then purified through the chromatographic scheme depicted in Figure 2C, using the purified transcription system in conjunction with GAL4p65 and p300 plus acetyl-CoA to monitor activity. The CTEA transcriptional activity profiles for gradient elution from Mono S and gel filtration on Superose 6 are shown in Figures 2D and 2E, respectively. Both columns reveal relatively uniform elution profiles with the peak fractions (39 and 41) on Superose 6 corresponding to a molecular size of about 100 kDa.

Silver staining of the Superose 6 fractions revealed that the CTEA peak activity in fractions 39 and 41 strongly correlates with two protein bands of 38 kDa (p38) and 50 kDa (p50) (Figure 2F). Microsequencing of these two bands by mass spectrometry identified p38 as human transcription elongation factor SII and p50 as human vaccinia-related kinase 1 (VRK1). These data are potentially significant for both SII and VRK1. Thus, SII has been shown to be

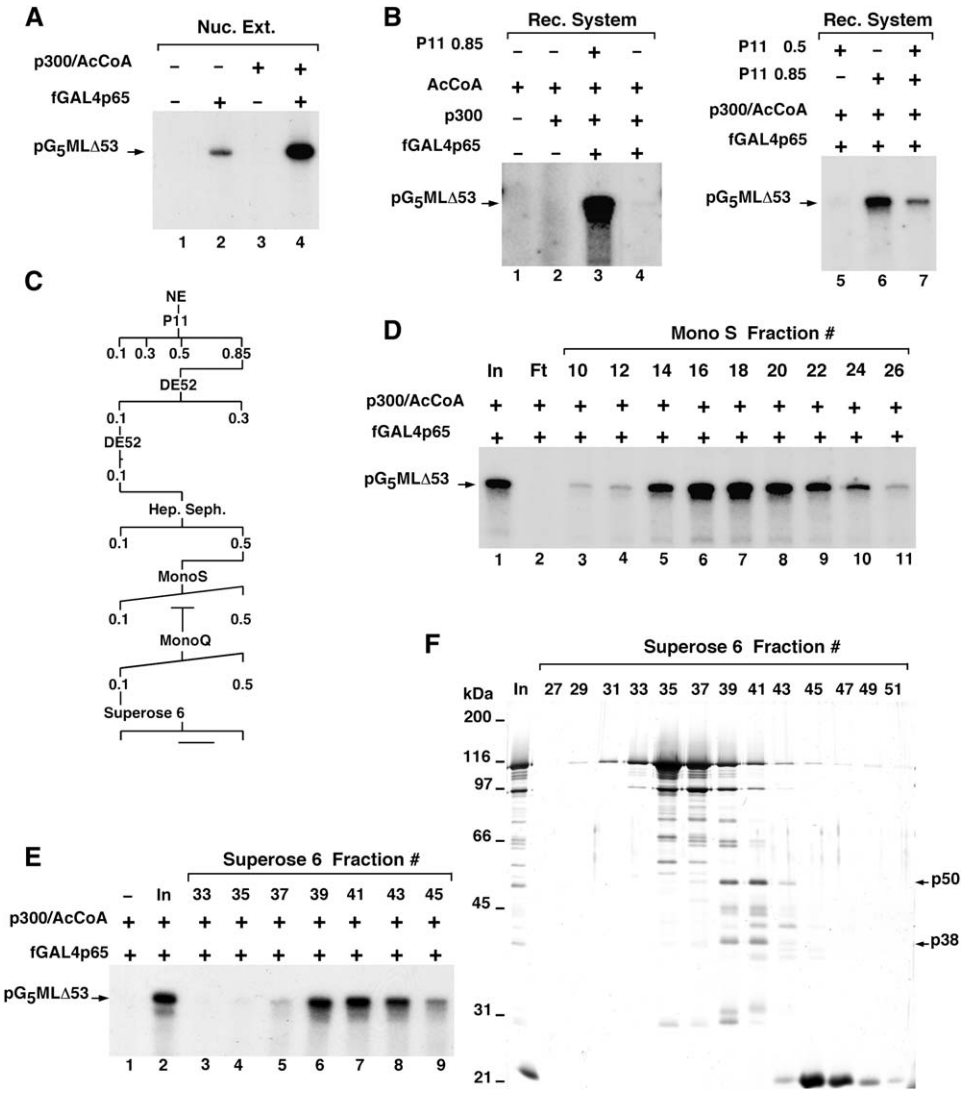

Figure 2. Identification, Characterization, and Purification of CTEA Activity

(A) Chromatin transcription in HeLa nuclear extract.

(B) Chromatin transcription in the reconstituted system in the presence of a novel activity (CTEA) in the P11 0.85 M fraction.

(C) Chromatographic scheme for purification of CTEA.

(D and E) Chromatography of CTEA on Mono S (D) and Superose 6 (E). Input (In) and indicated alternate fractions were assayed for transcriptional activity in the reconstituted system.

(F) Polypeptide composition of the Superose 6 column fractions and the Mono Q flowthrough input (In) analyzed by SDS-PAGE and silver staining. Transcription assays in (A), (B), (D), and (E) contained the $pG_5MLΔ53$ chromatin template and factor additions as indicated.

involved in the reversal of arrested RNA polymerase II molecules on DNA templates (Reines et al., 1989) and to facilitate transit of RNA polymerase II in an artificially formed elongation complex through a single positioned nucleosome (Introduction), although there has been no previous report of a role for SII in overcoming nucleosome repression of activator-dependent or chromatin remodeling/modification-dependent transcription. VRK1 is related to the vaccinia virus kinase B1 (vvB1), which is essential for the replication of the vaccinia virus genome (200 kbp; Rempel et al., 1990).

CTEA Works Mainly at the Elongation Step of Transcription and Allows RNA Polymerase II to Overcome Blocks from Several Contiguous Nucleosomes

The CTEA-dependent transcription assays described above monitored the synthesis of a 390 nucleotide transcript that requires both accurate initiation and productive transcription elongation events through at least two nucleosomes. We then used alternative assays to gain insights into CTEA function during early steps of transcription (initiation and early elongation events resulting in

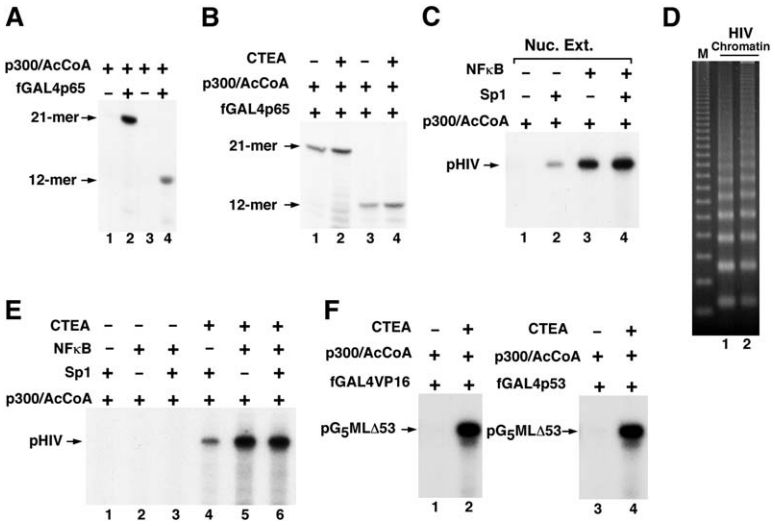

Figure 3. Characterization of CTEA Function

(A) Activator-dependent production of 12 and 21 nucleotide transcripts from the pG_5HML-based chromatin templates in the reconstituted system.

(B) Minimal CTEA effects on the production of 12 and 21 nucleotide transcripts from chromatin templates assayed as in (A).

(C) CTEA-dependent synthesis of long transcripts from the pHIV chromatin template in nuclear extract.

(D) MNase analysis of pHIV chromatin templates; lane 1 (0.8 mU) and lane 2 (0.4 mU).

(E) CTEA-dependent transcription of the pHIV chromatin template in the reconstituted system.

(F) CTEA-dependent transcription of the $pG_5MLΔ53$ chromatin template by fGAL4VP16 and fGAL4p53 in the reconstituted system.

formation of 12 and 21 nucleotide transcripts). To analyze the effect of CTEA (Superose 6) on the synthesis of short (12 and 21 nucleotide) transcripts, chromatin templates containing pG_5HM plasmids with 12 and 21 nucleotide G-less cassettes were assayed in the reconstituted system. First, and in the absence of CTEA, there is a strong activator-dependent production of both 12 and 21 nucleotide transcripts (Figure 3A, lane 2 versus lane 1 and lane 4 versus lane 3). Furthermore, and as shown in Figure 3B, CTEA had only a modest (<2-fold) effect on the synthesis of these transcripts in the presence of the activator (Figure 3B, lanes 2 and 4 versus lanes 1 and 2, respectively). For the initiation step, we used an abortive initiation assay to analyze synthesis of the first phosphodiester bond. Consistent with only modest effects of CTEA on the synthesis of 12 and 21 nucleotide transcripts, CTEA similarly had only a modest, but reproducible, effect on the initiation step (data not shown). The much greater magnitude of the CTEA effect on synthesis of the 390 nucleotide transcript relative to 3, 12, and 21 nucleotide products indicates that CTEA works mainly at the elongation step of transcription to overcome the nucleosome barrier.

The transcription assays (described above) used to identify and purify CTEA produce a transcript of 390 nucleotides, which corresponds to an occupancy of about two nucleosomes. We next wanted to examine whether CTEA can support transcription through several contiguous nucleosomes as must occur in vivo. To this end, we used a pHIV template that contains the HIV promoter and a 400 nucleotide G-less cassette 955 bp downstream of the transcription initiation site (Zhou and Sharp, 1995). To first establish activator (NF-κB p50/p65 and Sp1)- and coactivator (p300)-dependent transcription on the chromatinized HIV template (Figure 3D), we used exogenous activators and p300 in conjunction with HeLa nuclear extract as a source of general transcription factors. Indeed, and as expected (Pazin et al., 1996), Sp1 and NF-κB activate transcription in this assay (Figure 3C, lanes 2 and 3 versus lane 1), with the levels of activated transcription obtained with NF-κB alone or a combination of NF-κB/Sp1 being much higher than those obtained with Sp1 alone (Figure 3C, lanes 3 and 4 versus lane 2). We next analyzed the requirement of CTEA (Superose 6 fraction) for transcription of the pHIV chromatin template in the purified reconstituted system described above. Results show that there is a strong dependence on CTEA activity for transcription of the HIV promoter in the presence of Sp1, NF-κB, or NF-κB plus Sp1 (Figure 3E, lanes 4, 5, and 6 versus lanes 1, 2, and 3, respectively). This, therefore, shows that CTEA can support transcription by RNA polymerase II through several contiguous nucleosomes in a manner that resembles in vivo situation.

Although CTEA was shown to function with GAL4p65, NF-κB (p50/p65), and Sp1, we wished to determine if it mediated activation by a broader range of activators. For this purpose, we tested GAL4 fusion proteins containing the activation domains of VP16 (fGAL4VP16) and p53 (fGAL4p53) in the purified reconstituted transcription system containing p300 and acetyl-CoA. Indeed, CTEA strongly potentiates the transcriptional activity of both fGAL4VP16 and fGAL4p53 from the $pG_5MLΔ53$ chromatin templates (Figure 3F, lanes 2 and 4 versus lanes 1 and 3, respectively). These data further establish CTEA activity as a general elongation activity that is required for activated transcription of chromatin templates.

Recombinant SII (rSII) Can Substitute for Purified CTEA in Chromatin-Templated Transcription

To investigate the relevance to CTEA of the major components (SII and VRK1) that copurify with CTEA activity, we expressed and purified these proteins both from bacteria (Figure 4A and data not shown) and from HeLa cells that stably express epitope-tagged proteins (data not shown). As shown in Figure 4B, using equimolar amounts of CTEA (based on endogenous SII) and recombinant SII (rSII) from

bacteria, rSII can substantially substitute for CTEA activity (Superose 6) in supporting activator- and p300 plus acetyl-CoA-dependent transcription of chromatin templates (Figure 4B, lane 3 versus lanes 1 and 2). Further experiments have shown that rSII derived from a stable HeLa cell line also can substitute for the purified CTEA fraction (data not shown). Additional experiments using purified recombinant VRK1 from bacteria and HeLa cells have failed, thus far, to show a role for VRK1 individually or in combination with SII in the current assay (data not shown). It remains possible, however, that a role for VRK1 in transcription could become evident when other (noncore histone) chromatin-associated proteins and/or chromatin-interacting proteins are present in the assay.

Finally, additional characterization of SII protein by Western blot analysis revealed that, in agreement with the functional data for CTEA activity, SII is restricted to the 0.85 M KCl fraction on P11 and to the 0.1 M KCl fraction on DE52 (Figure 4C). Of potential significance, SII protein in the P11 0.85 M fraction runs, on SDS-PAGE, as two protein bands, with only the fast-migrating form present in the purified CTEA fraction (Figure 4C).

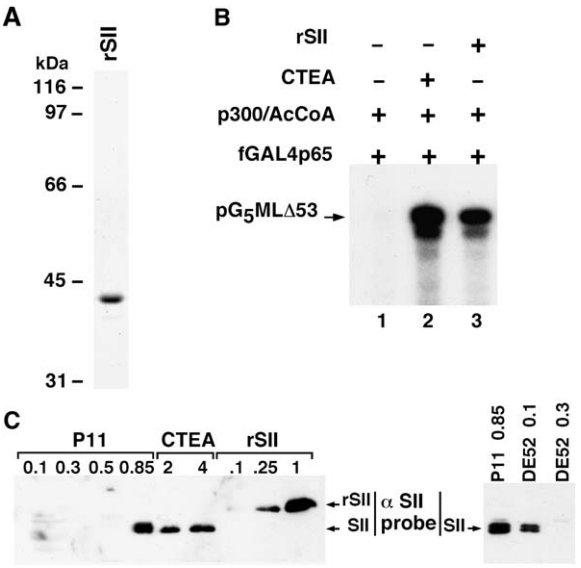

Figure 4. Recombinant SII (rSII) Substitution for the CTEA Fraction in Chromatin-Templated Transcription

(A) Analysis of purified recombinant SII (rSII) by SDS-PAGE and Coomassie blue staining.

(B) rSII substitution for CTEA in transcription from the $pG_5ML\Delta53$ chromatin template in the reconstituted system.

(C) Western blot analysis of SII protein in P11 fractions, the purified CTEA fraction and purified rSII (left panel) and in DE52 fractions derived from the P11 0.85 M fraction (right panel). Different samples were resolved by SDS-PAGE and analyzed by Western blotting with SII antibodies.

Collective Requirement for Activator, SII, p300, and Acetyl-CoA for Optimal Transcription of a Chromatin Template in the Purified Reconstituted System and Synergy of SII and p300 plus Acetyl-CoA at a Step after Preinitiation Complex Assembly

Although an earlier experiment (Figure 2) showed a requirement for p300 plus acetyl-CoA in conjunction with the GAL4p65 activator and CTEA in chromatin transcription, we wanted to analyze in a more complete fashion the contribution of each of these components. These assays utilized recombinant SII in place of CTEA, as well as chromatin assembled with recombinant histones that, unlike HeLa histones, are free of covalent modifications. As shown in Figure 5A, there is a collective requirement of activator (lane 6 versus lane 7), SII (lane 6 versus lane 5), p300 (lane 6 versus lane 13), and acetyl-CoA (lane 6 versus lane 12) for optimal transcription of the recombinant chromatin template. This coordinate dependency resembles the in vivo situation where there is a requirement for the concerted action of activators, coactivators, and histone modifiers to achieve optimal activated transcription. The residual activity observed with activator and SII (Figure 5A, lane 11) in the absence of p300 and acetyl-CoA could reflect either an incomplete nucleosome assembly on a small proportion of the chromatin templates or an intrinsic activity that might be repressed in a more physiological chromatin state with chromatin-associated proteins (including H1) other than nucleosomal core histones.

As a control for the experiment shown in Figure 5A and in order to show that the effects of SII, p300, and acetyl-CoA are specific to transcription of chromatin templates, we performed the same experiments with equimolar amounts of the histone-free DNA template (Figure 5B). In stark contrast to what was observed with the chromatin template, activator-dependent transcription from the DNA template (Figure 5B) showed no requirement for or effect of either SII (lane 6 versus lane 5) or p300 plus acetyl-CoA (lane 6 versus lane 11). Of note, in the absence of acetyl-CoA, there was a significant inhibitory effect of p300 on activated transcription that is independent of SII (Figure 5B, lane 4 versus lane 2 and lane 12 versus lane 11). Importantly, there was a reversal of this effect by acetyl-CoA (Figure 5B, lane 5 versus lane 4 and lane 6 versus lane 12), which suggests that p300, without its substrate, may interfere with the action of some component in the assay. Although the basis for this reversal by acetyl-CoA is unknown, it may conceivably reflect an acetyl-CoA-dependent p300 conformational change and/or a p300-mediated acetylation event that negates the inhibitory effect.

The data described above (Figure 5A) showing the collective requirement for SII, activator, p300 plus acetyl-CoA for productive transcription do not preclude the possibility of an exclusive effect of p300 plus acetyl-CoA (and histone acetylation) at the step of preinitiation complex formation with no effect on elongation. To address this critical issue, we first assembled a complete preinitiation complex with DNA, RNA polymerase II, GTFs, PC4, Mediator, and the activator. This was followed by chromatin assembly, activator-dependent histone modifications, and transcription in the reconstituted system. Our results show that p300

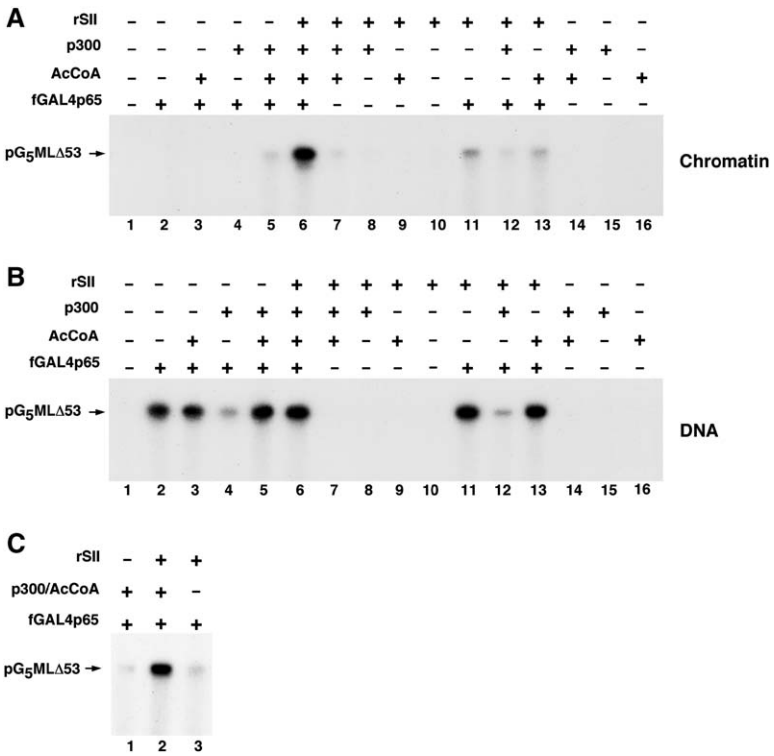

Figure 5. Collective Requirement for Activator, rSII, p300, and Acetyl-CoA (AcCoA) in Chromatin-Templated Transcription

(A) Transcription from the $pG_5ML\Delta53$ chromatin template assembled with recombinant histones.

(B) Transcription from a histone-free DNA $pG_5ML\Delta53$ template (control for [A]).

(C) Synergistic functions of SII and p300 plus AcCoA in effecting productive transcription from the $pG_5ML\Delta53$ chromatin template at a step after preinitiation complex assembly. All assays were in the reconstituted system with additions as indicated.

plus acetyl-CoA and SII are both required at a step after preinitiation complex assembly for optimal transcription of chromatin templates (Figure 5C, lane 2 versus lanes 1 and 3). This is a very significant finding since it establishes a strong synergy between SII and histone modifications at a postinitiation step.

HMGB2 Enhances rSII- and p300-Dependent Chromatin Transcription from the pHIV Template Mainly at the Level of Elongation

Although significant CTEA activity was recovered during purification on Mono Q (Figure 2C), there was a significant loss of overall activity relative to the input (data not shown), and concentration of the CTEA-containing flow-through fraction was required for activity in the transcription assays. This could reflect, at least in part, separation (from SII and VRK1) of other factors that potentiate SII function. In addition, a parallel purification of CTEA activity that was eluted from heparin-Sepharose with 0.3 M KCl and further purified by Mono S and Superose 6, but not Mono Q, chromatography revealed a potent CTEA activity that correlates with SII, VRK1, and two other proteins (data not shown). Mass spectrometric analysis identified the latter two proteins as HMGB2 and PC4.

We then complemented transcription assays containing HIV chromatin templates (as described in Figure 3E) with SII, recombinant PC4, and different Mono Q gradient fractions. We choose the HIV template on the assumption that the requirement for transcription through a longer array of nucleosomes (about seven) might impose a greater requirement for factors that enhance SII function. Indeed, our analysis revealed an activity, which eluted from Mono Q at 330–350 mM KCl, that enhances SII-dependent chromatin transcription. One fraction (44) that correlated with this activity was analyzed by SDS-PAGE and silver stain (Figure 6A) and contained a highly purified 29 kDa protein (p29) that was determined by mass spectrometry to be HMGB2. A further analysis revealed that p29/HMGB2 can markedly (3-fold) enhance SII-dependent transcription of the pHIV chromatin template (Figure 6B, lanes 6 and 7 versus lanes 2 and 3, respectively). Importantly, despite its stimulatory effect in the presence of SII, p29/HMGB2 alone is unable to substitute for SII function in chromatin transcription (Figure 6C, lane 3 versus lanes 2 and 1).

The HMGB2 effect was observed for productive transcription elongation events through at least seven nucleosomes. We then used assays described above to assess a possible HMGB2 effect on early steps of transcription. To analyze the effect of HMGB2 on the synthesis of short (12 nucleotide) transcripts, chromatin templates containing pG_5HM plasmids with 12 nucleotide G-less cassettes were employed. First, and in the absence of HMGB2, there was a strong activator- and p300 plus acetyl-CoA-dependent production of 12 nucleotide transcripts (Figure 6D, lane 2 versus lane 1). Furthermore, and as shown in Figure 3B for CTEA, rSII had no effect on the synthesis of these transcripts in the presence of the activator and p300 plus acetyl-CoA (Figure 6D, lane 3 versus lane 2). The further addition of HMGB2 also had no effect on the production of 12-nucleotide transcripts (Figure 6D, lane 4 versus lane 3). Therefore, HMGB2 effects SII- and p300

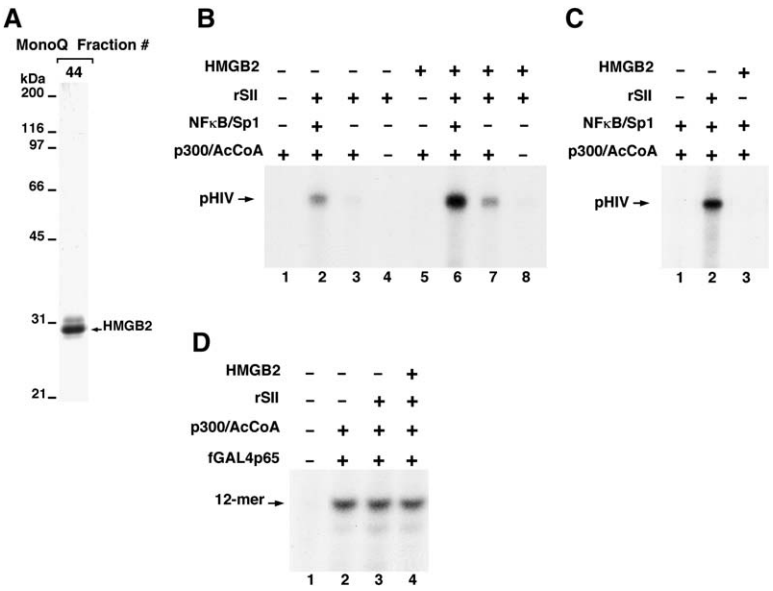

Figure 6. HMGB2 Enhances rSII and p300 Plus Acetyl-CoA-Dependent Transcription from the pHIV Chromatin Template Mainly at the Level of Elongation

(A) SDS-PAGE and silver staining analysis of a Mono Q Fraction (number 44, Figure 2C). The 29 kDa band was identified as HMGB2.

(B) HMGB2 (Mono Q fraction 44) enhancement of rSII-dependent transcription of the pHIV chromatin template.

(C) Failure of HMGB2 alone to substitute for rSII in mediating transcription of the pHIV chromatin template.

(D) Lack of an HMGB2 effect on the synthesis of 12-nucleotide transcripts from a pG_5HML-based chromatin template. All assays were in the reconstituted system with additions as indicated.

plus acetyl-CoA-dependent chromatin transcription mainly at the level of elongation.

DISCUSSION

In vivo transcription by RNA polymerase II takes place in the context of chromatin. However, as demonstrated here, a purified, reconstituted RNA polymerase II system that suffices for activator-dependent transcription on DNA templates is incapable of transcribing chromatin templates even in the presence of factors (the ATP-dependent chromatin remodeler ACF and the histone acetyltransferase p300) that effect transcription of chromatin in less-purified assay systems. Using an unbiased complementation and nuclear extract fractionation scheme, we have identified and purified an activity, CTEA, that allows for transcription through chromatin templates in a manner that is both activator and p300 plus acetyl-CoA dependent. CTEA acts primarily at the elongation step and enables the RNA polymerase II machinery to transcribe efficiently through several contiguously positioned nucleosomes. The major functional component of CTEA is transcription elongation factor SII; and while SII function is essential for productive transcription elongation, its function at this step is critically dependent upon p300-dependent acetylation. These highly synergistic transcriptional elongation activities on chromatin templates are potentiated by HMGB2.

SII, the Major Active Component of CTEA, Acts to Overcome the Nucleosome Block to Activator/Coactivator-Dependent Transcription by RNA Polymerase II

Our finding that SII is the major active component of CTEA has strong support from earlier biochemical and genetic analyzes. In vitro studies have shown that SII effects transcription elongation by inducing RNA polymerase II-mediated cleavage of nascent RNA, a step required to overcome transcription arrest caused by some DNA sequences (Fish and Kane, 2002; Wind and Reines, 2000). Structural studies show that SII remodels the active site of RNA polymerase II through interactions of domains II and III, respectively, with the RPB1/RPB9 jaw and the pore and active site of RNA polymerase II (Kettenberger et al., 2003). The antiarrest function of SII was identified during in vitro transcription analyses of DNA templates that contain natural gene sequences implicated in RNA polymerase II arrest (Fish and Kane, 2002; Wind and Reines, 2000). It also has been shown that both SII and the RNA polymerase subunit RPB9 are required for RNA polymerase II to overcome transcription arrest (Awrey et al., 1997). In addition, studies in yeast have shown that, under certain conditions such as growth in 6-azauracil (which depletes the GTP or UTP pool) or mutations in other factors, the absence of SII is often associated with defects in transcription elongation. As further evidence of SII involvement in RNA polymerase II transcription in vivo, other studies have indicated genetic interactions between SII and components of the ATP-dependent chromatin remodeling complex SWI-SNF (Davie and Kane, 2000), as well as other elongation factors such as Spt4-Spt5, the Paf1 complex, Elongator, and FACT (Arndt and Kane, 2003; Sims et al., 2004). Perhaps more important are observations that there is a strong genetic interaction between SII and the RNA polymerase II subunits and that SII-induced transcript cleavage is critical for survival of yeast in the context of RPB mutants (Hartzog et al., 1998; Lennon et al., 1998; Malagon et al., 2004; Ubukata et al., 2003; Wery et al., 2004). Further indicative of an SII role in elongation is its specific association with elongating RNA polymerase II in open reading frames, but not at promoters, of induced genes (Adelman et al., 2005; Pokholok et al., 2002).

With respect to an SII effect on chromatin-templated transcription, an earlier study using a crude chromatin assembly system and a purified elongation complex showed a modest stimulatory effect of SII in overcoming a nucleosome-imposed block to elongating RNA polymerase (Izban and Luse, 1992). A later study from the same group failed to reproduce this effect, but this may have reflected the use of chromatin assembled with purified histones by salt dialysis and the absence of any ATP-dependent chromatin-remodeling factor (Chang and Luse, 1997). Nonetheless, and while this manuscript was in preparation, Kireeva et al. (2005) reported that a "minimal" yeast RNA polymerase II elongation complex, artificially constructed with a short RNA primer-annealed DNA and ligated to a single nucleosome, is incapable of overcoming a single nucleosome block during transcription and that RNA polymerase II arrest is followed by a backtracking process that can be relieved by the addition of recombinant SII. This study is relevant since it shows that backtracking also occurs when RNA polymerase II encounters a nucleosome and that SII, or agents that prevent backtracking, can overcome the block (Kireeva et al., 2005).

In contrast to the work of Kireeva et al., the present study describes an unbiased isolation, from transcription-competent HeLa nuclear extract, of major components that facilitate activator- and coactivator-dependent transcription by RNA polymerase II through several nucleosomes on a chromatin template. One of the isolated components is transcription elongation factor SII, a major active component of CTEA activity that is able to potentiate activator-dependent transcription from a chromatin template in a highly purified transcription system that is also dependent on both p300 and acetyl-CoA. Most importantly, and in the context of this more physiological assay system, we have shown (1) that SII function is required during the productive transcription elongation step, but not during transcription initiation or promoter clearance, and (2) that SII and p300 (via histone acetylation) act in a highly synergistic fashion at a step subsequent to preinitiation complex assembly. This analysis also provides the first demonstration that the broadly used coactivator p300 (and histone acetylation) necessarily acts at a step (elongation) subsequent to PIC formation, as opposed to having a selective role in PIC formation. The previous demonstration (An et al., 2002; Ito et al., 2000) that p300-dependent acetylation of histones in ACF-assembled chromatin depends upon ACF makes it likely that ATP-dependent chromatin remodeling (by ACF) is also required in the present study. Importantly, in the same purified system, SII is not required for activator-dependent transcription from DNA templates, indicative of an important and specific SII role in transcription from natural chromatin templates. Also indicative of an apparent general role of SII in activator-dependent chromatin transcription, SII has been shown to act with different activators that include NF-κB (p50/p65), Sp1, GAL4p65, GAL4VP16, and GAL4p53. Consistent with its previously demonstrated function as an elongation factor in model (activator-independent) systems, the present study shows that SII and p300 plus acetyl-CoA also act at the elongation step to allow RNA polymerase II to transcribe through several contiguous nucleosomes, in response to activators.

Finally, we have found that HMGB2, while showing no coactivator activity on its own, can potentiate the activity of the major active component, SII, of CTEA together with p300 plus acetyl-CoA and that it acts mainly at the level of elongation. This is an important finding in light of the fact that HMGB2, like its closely related paralog HMGB1, is a prominent nonhistone chromatin-associated protein (Bustin, 2001). Several roles have been proposed for HMGB1 and HMGB2 proteins, including architectural and chaperone roles (Thomas, 2001). Some HMGB2 coactivator function on a histone-free DNA template in a semipurified transcription system has been reported earlier (Shykind et al., 1995). However, our demonstration that HMGB2 potentiates chromatin transcription is likely to be of greater significance since this is a bona fide chromatin-associated protein. The close structural similarity between the ubiquitous HMGB1 and the tissue-restricted HMGB2 suggests that both will have a similar function; and whereas SII has a potent activity on its own in the unfolded chromatin template employed here, the HMGB2 stimulatory effect observed here could assume greater significance in vivo on more physiological (e.g., constrained) templates and at potentially limiting concentrations of SII.

Relationship of SII to Other Elongation Factors that Facilitate Chromatin Transcription

Using biochemical and genetic approaches, several factors in addition to SII have been shown to play a role in transcription elongation. They include, but are not restricted to, TFIIF, ELL, Spt6, Spt4-Spt5 (DSIF), NELF, the Paf1 complex, Elongator, and FACT (Shilatifard et al., 2003; Sims et al., 2004). Among these factors, FACT has been shown to facilitate transcription through nucleosomes in vitro (Orphanides et al., 1998) and is composed of Spt16 and SSRP1 proteins (Orphanides et al., 1999). Genetic studies in yeast have implicated Spt16 in transcription regulation (Belotserkovskaya et al., 2004), whereas biochemical studies have shown that mammalian FACT has a histone chaperone activity and acts by destabilizing nucleosome structure during passage by RNA polymerase II (Belotserkovskaya et al., 2003).

Given a demonstrated requirement of FACT in promoting activator (GAL4VP16)-dependent transcription by RNA polymerase II through 2 nucleosomes (390 nucleotide transcript) in a well-defined reconstituted system (Orphanides et al., 1998), with no apparent requirement for (or effect of) SII, it is important to consider why SII functions in the absence of FACT in the present study and in that of Kireeva et al. (2005). Several points are relevant. First, the assay system utilized by the investigators (LeRoy et al., 1998; Orphanides et al., 1998) contained S190-assembled/purified chromatin, purified RNA polymerase II and GTFs, and PC4. Notably, it did not contain Mediator and a histone modifying coactivator such as p300, which we found to

be essential for high levels of activator-dependent transcription from DNA and chromatin templates, respectively. Hence, the activity observed by Orphanides et al. (1998) may be a minor component of that in the present study. It is also possible that the S190-assembled chromatin contained associated S190-derived SII. Second, FACT is well characterized as a chaperone that facilitates removal of the H2A:H2B dimer during transcription-induced nucleosome disassembly and (through SSRP1) may also interact with the $(H3:H4)_2$ tetramer (Belotserkovskaya et al., 2003). However, while our system does not contain (or require) FACT, it retains the ACF and NAP1 components that were used initially for chromatin assembly. Hence, given the well-documented role of NAP1 as an H2A:H2B chaperone (Ito et al., 2000) and indications that it interacts with $(H3:H4)_2$ as well (reviewed in McBryant et al. [2003]), it is probable that NAP1 can also serve as an effective chaperone during the transcription reaction. In support of this view, others have shown NAP1-enhanced release of H2A:H2B from nucleosomes during transcription (Levchenko et al., 2005; Levchenko and Jackson, 2004) and, especially relevant to our p300-dependent transcription system, that p300-mediated acetylation facilitates transfer of H2A:H2B dimers from nucleosomal templates to NAP1 (Ito et al., 2000). These observations support the view that NAP1 may bypass any requirement for FACT in our assay system but do not argue against a normal role for FACT in the absence of other H2A:H2B chaperones or in a more physiological setting. Our results nonetheless demonstrate a clear SII requirement for activator-, ACF/NAP1-, and p300/acetyl-CoA-dependent transcription from chromatin.

Finally, It remains to be determined if some of these components such as SII, CTEA, FACT, and/or histone-modifying enzymes can work in a redundant, synergistic, and/or selective manner to promote transcription of chromatin templates in vitro. In this regard, it is interesting to note that, unlike SII, which thus far has specific functions in transcription elongation, FACT has been implicated in both transcription and replication and possibly in DNA-damage repair and recombination as well (Belotserkovskaya et al., 2004).

EXPERIMENTAL PROCEDURES

Chromatin Assembly

HeLa core histones were purified from HeLa nuclear pellet as described (Kundu et al., 2000). Recombinant histone octamers were prepared as described (An et al., 2002). FLAG-Acf1, FLAG-ISWI, FLAG-Topo 1, and FLAG-p300 were expressed in Sf9 cells and purified using M2 agarose (Guermah et al., 2001). Histidine-tagged NAP1 (Kundu et al., 2000) was expressed in bacteria and purified on Ni-NTA and Q Sepharose columns. Chromatin assembly was on $pG_5ML\Delta53$, pG_5HML, and pHIV plasmids essentially as described (Ito et al., 1999). Assembled chromatin was characterized by MNase analysis as in Figures 1D and 3D.

Purification of CTEA Activity

CTEA was purified from HeLa nuclear as outlined in Figure 3C. Following P11 chromatography, CTEA activity was found in the BC100 (100 mM KCl) eluate from DE52 and in the 0.5 M KCl eluate from Heparin Sepharose. CTEA activity was further purified on Mono S and Mono Q (FPLC) columns. The 0.1 M Mono Q flowthrough, containing CTEA activity, was concentrated, equilibrated to 0.2 M KCl/0.02% NP-40, and loaded on a Superose 6 (FPLC) column that was run in BC200/0.02% NP-40.

Mass Spectrometry

Mass spectrometry was used to identify the p38 and p50 proteins in the purified CTEA activity (Superose 6, concentrated fractions 40 and 41) and the p29 protein in the SII-enhancing activity (Mono Q fraction 44) essentially as described (Krutchinsky et al., 2001; Tackett et al., 2005).

Purification of Recombinant SII (rSII)

Recombinant mammalian SII was expressed in bacteria and purified on Ni-NTA and a subsequent HiTrap SP column (Szentirmay and Sawadogo, 1993).

In Vitro RNA Polymerase II Transcription Assays

Nuclear extracts (NE) were prepared as described (Dignam et al., 1983). TFIID, TFIIH, and the Mediator complex were purified from cell lines expressing FLAG-TBP, FLAG-ERCC3, and FLAG-TRAP220/Med1 (AB1), respectively, on phosphocellulose (Whatman P11), DEAE-cellulose (DE52), and anti-FLAG M2 agarose antibody columns (Ge et al., 2002; Guermah et al., 2001). RNA polymerase II was purified from nuclear pellet of a HeLa cell line expressing the FLAG-RPB9 subunit, essentially as described (Guermah et al., 2001) but with addition of an M2 agarose affinity step at the end.

TFIIA subunits (p55 and p12), TFIIB, and TFIIE subunits (α and β) were expressed as FLAG- tagged proteins in *E. coli* and purified on M2 agarose. TFIIF subunits (RAP30 and RAP74) were expressed as histidine-tagged proteins in *E. coli* and purified on Ni-NTA. TFIIA and TFIIF were reconstituted from individually purified components following denaturation and renaturation (Guermah et al., 2001). Native TFIIA, purified through several columns (Guermah et al., 1998), was used in experiment described in Figure 3E.

FLAG-GAL4p65 (fGAL4p65), FLAG-GAL4VP16 (fGAL4VP16), and FLAG-GAL4p53 (fGAL4p53) were purified as described (Guermah et al., 1998). FLAG-Sp1 and NF-κB (p65/p50) were purified from Sf9 cells as described (Guermah et al., 1998), except that a FLAG-p65 subunit was used instead of a histidine-tagged p65 subunit.

Transcription assays with the reconstituted system (GTFs, RNA polymerase II, PC4, and Mediator) were performed as described (Guermah et al., 2001; Kundu et al., 2000), except that PC4 was omitted from the assays used during the purification of CTEA. Reconstituted chromatin templates (35 ng DNA) or equivalent amounts of histone-free DNA were incubated with activators for binding, followed by a p300 acetylation step in the presence of 2 μM acetyl-CoA (AcCoA). An active CTEA fraction or recombinant SII then was added, followed by the general transcription machinery described above. Transcription assays for 12- and 21-nucleotide transcripts used pG_5HML plasmids with corresponding G-less cassettes (Fukuda et al., 2002).

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