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Purification of transcription cofactor complex CRSP

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ABSTRACT **Transcription of protein coding genes in metazoans involves the concerted action of enhancer binding proteins and the RNA polymerase II apparatus. The cross talk between these two classes of transcription factors is mediated by an elaborate set of cofactor complexes. For the activation of transcription by the promoter specificity protein 1 (Sp1), TATA binding protein-associated factors in the TFIID complex originally were identified as necessary coactivators, but the identity of additional cofactors required for activated transcription was unknown. Recently, we have reported the isolation and properties of a cofactor complex, CRSP (cofactor required for Sp1), which functions in conjunction with the TATA binding protein-associated factors to promote efficient activation of transcription by Sp1. CRSP contains unique subunits as well as polypeptides that are shared with other cofactor complexes. Here, we report a detailed purification protocol for the isolation of CRSP from human HeLa cells. Our purification strategy takes advantage of the ability of CRSP to bind Ni^{2+}-nitrilotriacetic acid-agarose resin as well as other conventional chromatographic resins. We also describe a streamlined purification protocol that allows a more rapid and efficient means to isolate active CRSP.**

The mechanism by which enhancer binding proteins activate transcription has been studied extensively by using *in vitro* reconstituted transcription systems. Although general transcription factors suffice for basal levels of transcription *in vitro*, high levels of transcription in the presence of enhancer binding proteins occur only in the presence of additional accessory cofactors in mammalian cells (1). One class of cofactors, the TAF_{II} subunits of the TFIID complex, represents coactivators that function as direct targets for certain activators as well as promoter recognition factors (2–6). Their presence is required for enhancer-dependent transcription for most cellular genes in humans, *Drosophila,* and yeast (7). Another class of cofactors, USA (upstream stimulatory activity), has been identified as a complex chromatographic fraction that stimulates transcription by various activators. The USA fraction later was shown to include a single-stranded DNA binding protein (PC4), topoisomerase I (Dr2), a stimulatory fraction PC2, as well as several negative cofactors that repress basal transcription (8–11). *In vitro*, these proteins appear to influence both activator-dependent and basal levels of transcription. Lastly, a number of cofactors have been identified recently that appear to work in the context of specific activators. For example, the TRAP and DRIP complexes were reported to be specialized coactivators for ligand-dependent nuclear hormone receptor activation, whereas OCA-B/Bob1/OBF-1, mediate B-cell-specific transcription (12–16).

With the discovery of multiple cofactors that influence activated transcription, an important task was to delineate the relative roles of these factors. Does a single activator require multiple cofactors, and if so what are their respective contributions? To begin addressing these questions, we have focused our effort on specificity protein 1 (Sp1) as a model activator. Sp1 originally was identified and cloned as a factor that binds to the simian virus 40 early promoter (17). Since then, Sp1 has been implicated in the efficient transcription of many cellular and viral genes. The mechanism of activation by Sp1 has been well studied *in vitro*. These studies demonstrated that the $TAF_{II}110$ subunit of *Drosophila* TFIID or its human homologue, $TAF_{II}130$, could serve as a direct target and essential cofactor for Sp1 activation (18, 19). However, these early studies were conducted by using a partially purified transcription system and therefore did not address the potential requirement for additional cofactors necessary to mediate a high level of Sp1 activation.

To screen for additional cofactors required for transcription activation by Sp1, we have developed an *in vitro* transcription assay that includes the highly purified TFIID complex with a full complement of TAF_{II}s. By using this assay, we demonstrated in a separate report the existence of a cofactor distinct from TFIID and PC4, which we termed cofactor required for Sp1 or CRSP (20).

In this study, we present a purification strategy that allowed the identification of the polypeptides that comprise the CRSP complex. The nine-step purification protocol yields a homogeneous preparation of highly active CRSP complex, but it is labor intensive. To overcome this limitation, we recently have developed an alternative purification protocol that is simpler and more efficient.

MATERIALS AND METHODS

***In Vitro* Transcription and Preparation of Transcription Factors.** *In vitro* transcription assays were performed with a HeLa cell fractionated system. HeLa cell nuclear extract was prepared essentially as described (21). For the preparation of the phosphocellulose 1 M (P1M) fraction, the ammonium sulfate pellet of the nuclear extract was resuspended in buffer D [25 mM Hepes, pH 7.9/0.2 mM EDTA/20% (vol/vol) glycerol/2.5 mM $MgCl_2$,] containing 0.02 M KCl until the solution reached the conductivity equivalent to 0.3 M KCl. The resuspended solution was loaded on the P11 phosphocellulose resin (Whatman), washed with buffer D containing 0.5 M KCl, and eluted stepwise with buffer D containing 1 M KCl. For the preparation of RNA polymerase II basal factor fractions, the nuclear extract was completely dialyzed to 20 mM KCl in buffer D, applied to a P11 column at 0.1 M KCl, washed at 0.3 M KCl, and eluted at 0.5 M KCl. Routinely 1/10th of column volume eluates were collected and assayed for those fractions with no significant TFIID or CRSP contamination.

Sp1 was overexpressed in HeLa cells by using a vaccinia virus-expressing vector and purified to near homogeneity as described (18). Transcription assays were performed as described (22). Individual reactions were performed in 25-μl

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Abbreviations: Sp1, specificity protein 1; CRSP, cofactor required for Sp1; NTA, nitrilotriacetic acid; P1M, phosphocellulose 1 M; IP-TFIID, immunopurified TFIID.

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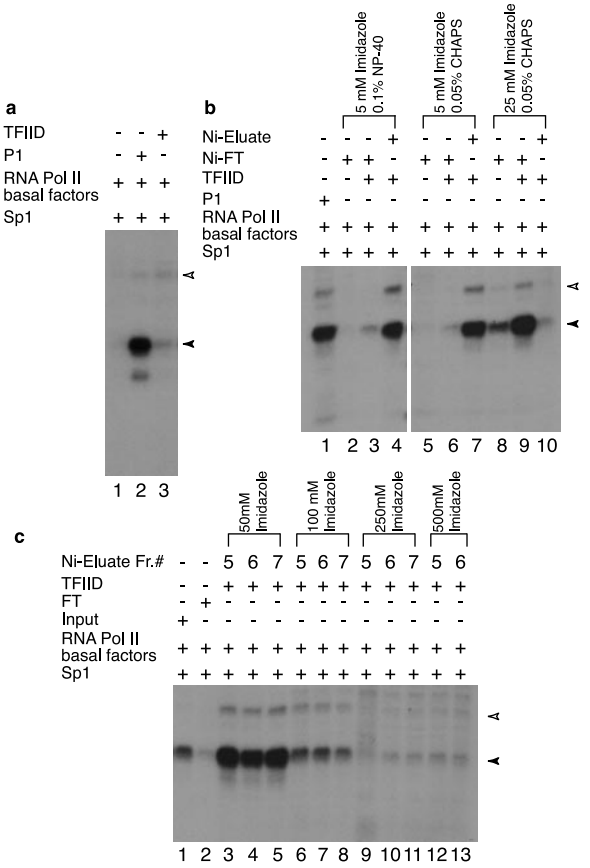

FIG. 1. CRSP binds to Ni^{2+}-NTA-agarose column. (*a*) Sp1 activation requires CRSP in the 1 M phosphocellulose fraction (P1M). Transcription reactions contain Sp1 (lanes 1–3), RNA polymerase II and basal transcription factors (lanes 1–3), the P1M fraction (lane 2), and immunopurified TFIID (IP-TFIID) (lane 3). Transcription from a GC-box template and a control DNA template lacking Sp1 binding sites is indicated by filled and open arrowheads, respectively. (*b*) CRSP binds to Ni^{2+}-NTA-agarose resin in the presence of competing imidazole. Transcription reactions were performed as described in *a*. The Ni^{2+}-NTA-agarose flow-through (FT) fraction and eluate fractions under various binding conditions are tested (lanes 2–10). All Ni eluates are eluted with 100 mM imidazole. (*c*) CRSP elution profile. Transcription reactions were performed as described in *a* in the presence of input fraction (lane 1), flow-through fraction (lane 2), or eluate fractions under varying concentration of imidazole (lanes 3–13).

reaction volume, which contained the following: 50 ng of $(GC)_3$BCAT (3), which contains three Sp1 sites upstream of the E1B TATA box; 50 ng of control template same as $C(_TC)_3$BCAT but lacks Sp1 binding sites and contains a 15-bp insertion producing a longer transcript; 50 ng of Sp1; 300 ng of RNA polymerase II basal factor fraction; 5–25 ng of immunopurified TFIID; and varying amounts of CRSP fraction. The reactions were incubated at 30°C for 30 min before ribonucleotides were added to the final concentration of 0.5 mM. The final reaction products were detected by primer extension.

Purification of CRSP. The P1M fraction (see above) was used as starting material. For a P1M fraction derived from 100 liters of HeLa cells, routinely 10 ml of Ni^{2+}-nitrilotriacetic acid (NTA)-agarose (Qiagen) was used. The resin first was extensively equilibrated with Ni^{2+}-binding buffer [25 mM Hepes/0.7 M KCl/5 mM imidazole/0.1% NP-40 (vol/vol)/12.5 mM $MgCl_2$/10 mM β–mercaptoethanol (β–ME)/10% glycerol (vol/vol)]. Before loading the P1M fraction onto the Ni^{2+}-NTA-resin, the fraction was supplemented with imidazole, NP40, and β–ME to achieve a final concentration of 5 mM imidazole, 0.1% NP-40 (vol/vol), and 10 mM β–ME. This solution was incubated with the Ni^{2+}-NTA-agarose resin for

Table 1. Purification table for CRSP starting with 100 liters of HeLa cells

Chromatographic procedures	Total protein amount	Approximate fold purification
Nuclear extract	2 g	
Ammonium sulfate ppt.	1 g	2
Phosphocellulose	40 mg	40
Ni-NTA-agarose	3 mg	1,000
Blue sepharose	1 mg	2,400
Poros heparin	0.5 mg	3,000
Glycerol gradient	0.05 mg	9,000
MonoS	.004 mg	45,000

8–12 hr. After binding, the resin was packed into a gravity column, and the unbound P1M solution was slowly passed over this column 2–3 times at a flow rate of no more than 20 ml/hr. The slow flow rate in this step is important to ensure maximum

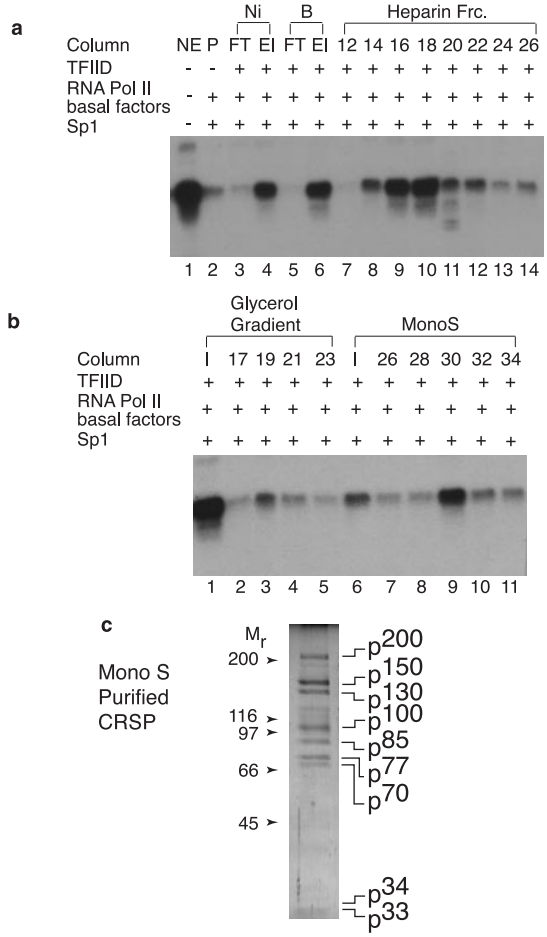

FIG. 2. Purification of CRSP and identification of CRSP polypeptides. (*a*) Reconstituted transcription reactions with the GC box template, Sp1 supplemented with nuclear extract (NE) (lane 1), or the P1M and RNA polymerase II and basal transcription factor fraction (lane 2). The rest of the reactions contained GC box template, Sp1, RNA polymerase II and basal transcription factors, and IP-TFIID (lanes 3–14), supplemented with Ni^{2+}-NTA-agarose flow-through (FT) and eluate (El) (lanes 3 and 4), Cibacron Blue Sepharose (B) FT and El (lanes 5 and 6), and various fractions derived from Poros Heparin (lanes 7–14). (*b*) Reconstituted transcription reactions with GC box template, Sp1, RNA polymerase II and basal factor fraction, and IP-TFIID (lanes 1–11) supplemented with input and fractions derived from glycerol gradient centrifugation (lanes 1–5) or input and fractions derived from MonoS chromatography (lanes 6–11). (*c*) Silver-stained SDS/PAGE gel of the MonoS purified CRSP. Molecular size standards are shown on the left, and the apparent molecular weight of each polypeptide is listed on the right.

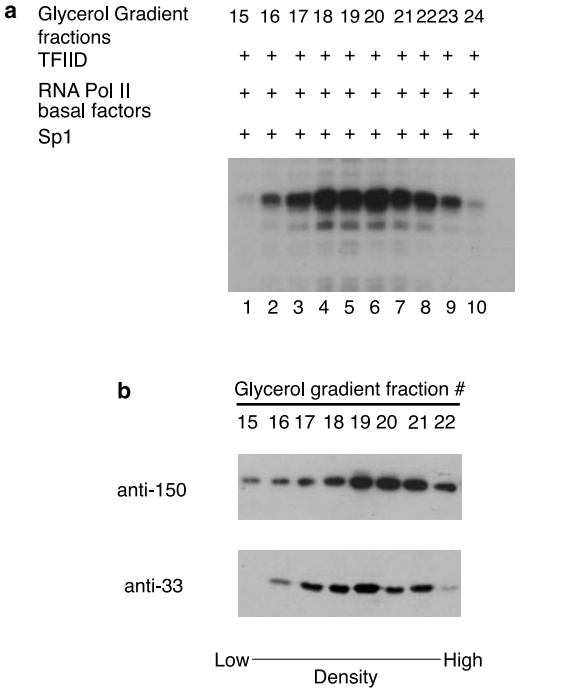

FIG. 3. The presence of CRSP subunits correlates with the CRSP transcription activity. (*a*) Reconstituted transcription reactions with GC box template, Sp1, RNA polymerase II and basal factor fraction, and IP-TFIID (lanes 1–10) supplemented with various fractions derived from glycerol gradient centrifugation. (*b*) Western blots of the glycerol gradient fractions probed with anti-CRSP150 or anti-CRSP33 antibodies.

binding of CRSP to the resin. The column was washed with 10 bed vol of Ni^{2+}-wash buffer [25 mM Hepes/0.7 M KCl/10 mM imidazole/0.1% NP-40 (vol/vol)/12.5 mM $MgCl_2$/10 mM β–ME/10% (vol/vol) glycerol] and with 10 bed vol of the same buffer but with 0.15 M KCl and no NP-40. The column was eluted with Ni^{2+}-elution buffer [25 mM Hepes/0.15 M KCl/12.5 mM $MgCl_2$/100 mM imidazole/10% (vol/vol) glycerol]. The Ni^{2+}-NTA fractions containing CRSP activity were directly loaded onto a 10-ml Cibacron Blue Sepharose column (Amersham Pharmacia) with a slow flow of approximately 15 ml/hr. The flow-through fraction was reloaded on the same column multiple times. The column was washed with 10 bed vol of HEMG buffer [25 mM Hepes/0.2 mM EDTA/12.5 mM $MgCl_2$/10% (vol/vol) glycerol] containing 0.2 M KCl and eluted with HEMG/0.5 M KCl. The CRSP protein peak was pooled, dialyzed to HEMG 0.2 M KCl, and 5–10 ml of the eluate was applied to a 600 μl Poros Heparin column (PerSeptive Biosystems, Framingham, MA). The column was eluted with a linear salt gradient starting at 0.2 M KCl to 1 M KCl over 10 column vol. Approximately 130-μl fractions were collected, and those heparin fractions containing CRSP activity (CRSP elutes around 0.5 M) were pooled and applied to 15% (vol/vol) to 30% (vol/vol) glycerol gradient (in HEMG/0.2 M KCl) and centrifuged at 50,000 rpm for 7 hr in a TLS55 rotor (Beckman). The CRSP-containing fractions were pooled and applied to a MonoS PC 1.6/5 column (Amersham Pharmacia), and proteins were eluted with a linear salt gradient from 0.2 M to 1 M KCl in HEMG buffer. CRSP elutes around 0.3 M KCl. Before testing in transcription reactions, Ni eluates with 100 mM imidazole were dialyzed to HEMG/0.1 M.

Shorter Purification Protocol for CRSP. The P1M fraction was loaded on Ni^{2+}-NTA-agarose column and washed as described above. Small eluate fractions (1/20th column bed vol) were collected and kept separated until further analysis. Each fraction was analyzed by silver stain and transcription. Early Ni^{2+} eluate fractions that contained CRSP activity and eluted before most of the protein were pooled. These fractions were applied to MonoS PC 1.6/5 and eluted as described above.

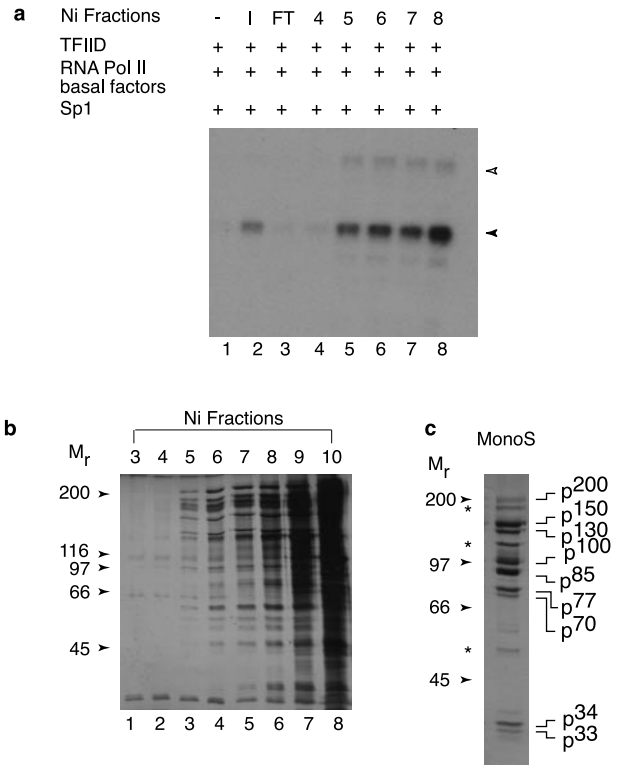

FIG. 4. CRSP elutes before the protein peak from the Ni^{2+}-NTA-agarose. (*a*) Reconstituted transcription reactions with GC box template, Sp1, RNA polymerase II and basal factor fractions, and IP-TFIID (lanes 1–8) supplemented with input fraction (lane 2), flow-through fraction (FT) (lane 3), or various elution fractions from Ni^{2+}-NTA-agarose chromatography (lanes 4–8). Transcription from a GC box template and a control DNA template lacking Sp1 binding sites is indicated by filled and open arrowheads (*b*) Silver-stained SDS/PAGE gel of various Ni^{2+} eluate fractions. Molecular size standards are shown on the left. (*c*) Silver-stained SDS/PAGE gel of CRSP fraction derived from MonoS chromatography directly after the Ni^{2+}-NTA-agarose step. Molecular size standards are shown on the left, and the apparent molecular weight of the CRSP subunits are listed on the right. * indicate contaminating polypeptides present in this preparation that do not represent CRSP subunits.

RESULTS

CRSP Binds to Ni^{2+}-NTA Resin. To identify potentially novel Sp1 cofactors we first developed an *in vitro* transcription assay that consisted of purified recombinant Sp1, recombinant TFIIA, and partially purified fraction containing RNA polymerase II and general transcription factors (GTFs) TFIIB, E, F, and H. The addition of a phosphocellulose fraction (P1M) containing endogenous TFIID and other cofactors (including USA) to this system resulted in the strong stimulation of transcription from template bearing three GC-box Sp1 binding sites but not from a control template lacking Sp1-binding sites (Fig. 1*a*, lane 2). In contrast, the addition of antibody affinity-purified TFIID resulted in little apparent Sp1-directed activation, thus suggesting the presence of a cofactor required for Sp1 (CRSP) in the P1M fraction that is distinct from TFIID (Fig. 1*a*, lane 3). Furthermore, the CRSP activity present in this P1M fraction was shown to be distinct from the USA cofactor PC4 and therefore may represent a novel activity (20). Importantly, although TFIID alone is insufficient, both TFIID and CRSP are required for Sp1 activation (20). Therefore, we have used transcription reactions containing Sp1, RNA polymerase II, GTFs, and IP-TFIID to purify CRSP.

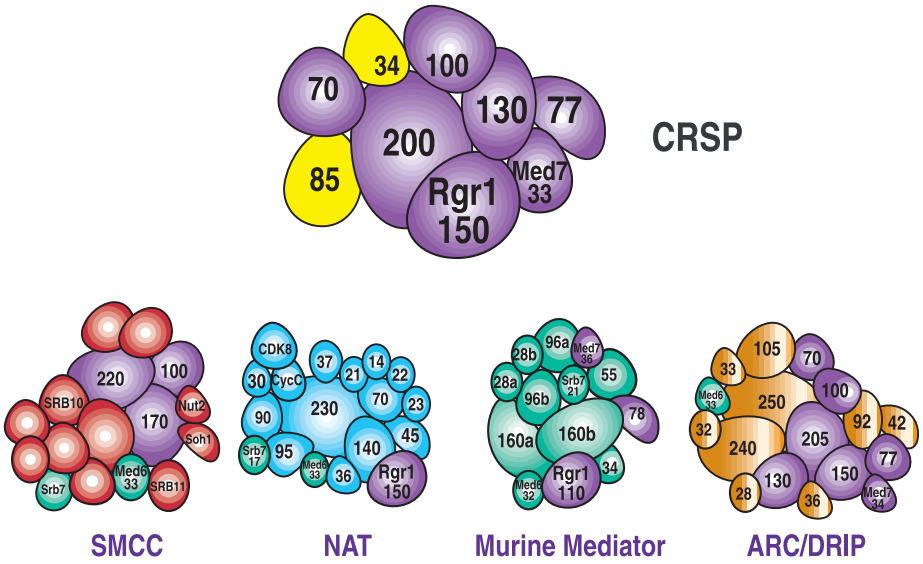

FIG. 5. Preliminary schematic comparison of mammalian cofactor complexes that share common subunits. The common subunits are represented by the same color in different complexes.

As a first step, we incubated the P1M fraction with various chromatographic resins and assayed both the flow-through and eluted fractions for CRSP activity. Because the P1M fraction contains endogenous TFIID and CRSP, the flow-through was tested with or without additional TFIID. Because TFIID is required for Sp1 activation, this strategy allowed us to determine the presence of CRSP alone or of CRSP and TFIID in the flow-through fraction. Among the resins tested, the Ni^{2+}-NTA-agarose resin was found to be most effective in separating CRSP. When the P1M fraction was incubated with Ni^{2+}-NTA-agarose resin very little CRSP was found to be present in the flow-through even in the presence of low levels of competing imidazole (Fig. 1*b*, lanes 2, 3, 5, and 6). In contrast, most of the other proteins, including TFIID, which is present in the P1M fraction, do not efficiently bind to Ni^{2+}-NTA-agarose at 5 mM imidazole concentration (data not shown). At higher imidazole concentrations (25 mM), CRSP failed to bind Ni^{2+}-NTA-agarose (Fig. 1*b*, lanes 8 and 9). After CRSP binds to the Ni^{2+}-NTA-agarose resin most of the activity can be eluted with 50 mM imidazole, while a small amount of CRSP remains bound up to 100 mM imidazole (Fig. 1*c*).

Purification Scheme for CRSP. Combining the Ni^{2+}-NTA-agarose affinity purification with other chromatographic steps we devised a complete purification protocol for CRSP (Table 1 and Fig. 2). After the preparation of nuclear extract by using a previously defined protocol (21), we performed an ammonium sulfate precipitation step at 55% saturation, which effectively precipitates virtually all of the CRSP activity (data not shown). We next used phosphocellulose chromatography to separate CRSP and TFIID from the rest of the RNA polymerase II general transcription factors followed by the Ni^{2+}-NTA-agarose step. Because CRSP bound to Ni^{2+}-NTA-agarose at various salt concentrations, the P1M fraction can be directly loaded to Ni^{2+}-NTA-agarose without a need for dialysis. By testing various resins, we discovered that CRSP also selectively bound to Cibacron Blue-Sepharose, Poros Heparin, and MonoS columns (Fig. 2 *a* and *b*). The CRSP activity was eluted from Cibacron Blue-Sepharose in a stepwise fashion and further purified on Poros Heparin with a linear salt gradient. Subsequently, the fractions that contained the peak of CRSP activity were subjected to glycerol gradient sedimentation centrifugation. Active fractions from glycerol gradient centrifugation were purified further by MonoS chromatography. A set of polypeptides that consistently correlated with the transcription activity includes species of 33 K, 34 K, 70 K, 77 K, 85 K, 100 K, 130 K, 150 K, and 200 K (Fig. 2*c*). Proteolytic peptides obtained from these CRSP subunits were subjected to microsequence peptide analysis, and the sequences obtained were used to generate probes and/or to identify expressed sequence tag sequences in the genome database. Several probes obtained by this strategy were used to screen human cDNA libraries for sequences encoding the putative CRSP subunits. Partial cDNA clones were expressed and used for antibody generation (20).

By using these antibodies, we tested for the presence of CRSP subunits in transcriptionally active fractions by using Western blots to further confirm that the polypeptides isolated indeed correspond to the CRSP activity. On glycerol gradient, CRSP activity sediments near the bottom of the gradient indicating a large molecular weight species (Fig. 3*a*). Western blot analysis confirmed that the peak of CRSP transcription activity coincides with the peak of CRSP150, CRSP130, and CRSP33 protein (Fig. 3*b* and data not shown). More importantly, we have performed an immuno-depletion experiment to show that removal of CRSP polypeptides reduces the cofactor activity, suggesting that these polypeptides do indeed confer CRSP activity (20).

Alternative Purification Scheme for CRSP. Although the above purification strategy was critical for identifying the polypeptides that consistently correlated with CRSP activity, it is both time consuming and labor intensive. Therefore we sought to develop a purification strategy that will provide a more convenient means to prepare CRSP. Because the Ni^{2+}-NTA-agarose step represents our most effective step in CRSP purification, we tested whether it could be optimized. To better understand CRSP's behavior on this resin, we first analyzed in greater detail CRSP's elution properties. Small fraction volumes (1/20th of column volume) were collected and analyzed both for transcription activity and protein pattern (Fig. 4 *a* and *b*). We found that fractions 6, 7, and 8 contained high transcription activity but relatively low protein concentration. Thus, by taking small fractions we could separate CRSP from the peak of the protein, which eluted in fraction 9 and 10. When we applied this Ni^{2+}-eluate directly to a MonoS column, we found that a nearly homogeneous CRSP preparation could be obtained with few additional contaminating polypeptides (Fig. 4*c*). Thus, this strategy provides a useful alternative purification protocol for routine CRSP preparations.

DISCUSSION

Given the multitude of coregulators of transcription that have been recently discovered, we believe that defining specific cofactor requirements for a single activator in the context of a simplified promoter provides a useful step toward understanding the role of these proteins. To this end, we report here a purification strategy for transcriptionally active cofactor CRSP that mediates activation by human Sp1. Because most, if not all, of the human general transcription factors required for RNA polymerase II transcription are now available as either recombinant or immunopurified proteins, the identification of CRSP allows us to test whether a complete reconstitution of Sp1 transcription activation can be achieved. A complete reconstitution for Sp1 activation with purified components also will provide a system whereby the contribution of individual cofactors can be compared and dissected. As expected of a Sp1 cofactor, CRSP appears to be ubiquitously expressed in multiple tissue types (unpublished data). In future studies, it will be important to determine whether CRSP can mediate the activation properties of other enhancer binding proteins. It will also be important to determine which step in transcription is mediated by CRSP.

The purification of the CRSP polypeptides reported here revealed that CRSP represents a unique cofactor complex that shares some subunits with other cofactors but also contains unique subunits (20). For example, CRSP33 and CRSP150 share homology with components of the yeast mediator complex that has been shown to stimulate transcription *in vitro*. More specifically, CRSP33 shares significant extended sequence similarity with Med7, while CRSP150 shares limited similarity to a domain of Rgr1 (20, 23). Furthermore, a murine complex that contains some yeast mediator homologues also includes polypeptides related to CRSP77 and CRSP150 (24). Another CRSP subunit found in other coactivator complex is CRSP200 which is identical to TRIP2/PBP/RB18A/DRIP230/TRAP220, a subunit of a large coactivator complex implicated in the inducible activation by various nuclear hormone receptors (12, 13, 25–27). In contrast, other CRSP subunits represent previously uncharacterized gene products. For example, CRSP 70 shares homology with the N-terminal domain of transcription-elongation factor SII (28), whereas CRSP 34, 77, and 130 represent polypeptides with no apparent homology to previously identified proteins.

Recently, other human cofactor complexes have been identified that affect activated transcription and share some of their subunits with CRSP. Most striking among these complexes is ARC/DRIP, a multisubunit coactivator that binds directly to certain activators such as SREBP and nuclear hormone receptors and is responsible for mediating transcription activation with a chromatin template *in vitro* (29, 30). Surprisingly, ARC/DRIP represents a larger complex than CRSP that contains seven subunits in common with CRSP (Fig. 5). Two other human complexes that share subunits with CRSP are NAT, a polymerase II-interacting complex that represses activated transcription and SMCC, a SRB/Med-containing cofactor complex that acts synergistically with PC4 to enhance activator-dependent transcription (31, 32). Although these cofactor complexes contain shared subunits, they appear to constitute distinct activities and the relationship between these complexes and their mode of action remains to be determined.

The apparent mixing and matching of CRSP subunits with other transcription cofactors suggests a model whereby these proteins may be involved in regulating a diverse set of genes by their ability to form structurally related, but functionally distinct, complexes. Given the accumulation of evidence for a combinatorial mechanism to generate diverse sets of cofactors and coactivators that mediate transcription activation, it has become increasingly evident that a significant degree of regulation may take place during the course of preinitiation complex formation at the core promoter. This level of control may be superimposed on the more classical mechanism directed by gene-specific enhancers and repressors. An important goal for future research will be to determine how the functional differentiation of these complexes may be achieved. Does each complex harbor unique subunits that alter the specificity and function of the complex? If so, that would suggest that the shared subunits in these complexes might provide a more general or structural role common to multiple cofactor complexes. Alternatively, do unique arrangements of the subunits and their resulting conformation contribute to their specialized functions? To address these questions, it is critical to obtain pure sources of each of these putative cofactor complexes to characterize their mechanism. The protocol for purification of CRSP, reported in this manuscript, contributes an important step toward the more general goal of determining the mechanism of action of these important cofactor complexes.

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