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Factors associated with the mammalian RNA polymerase II holoenzyme

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ABSTRACT

The RNA polymerase II (Pol II) holoenzyme in yeast is an essential transcriptional regulatory complex which has been defined by genetic and biochemical approaches. The mammalian counterpart to this complex, however, is less well defined. Experiments herein demonstrate that, along with Pol II and SRB proteins, proteins associated with transcriptional regulation as cofactors are associated with the Pol II holoenzyme. Earlier experiments have demonstrated that the breast cancer-associated tumor suppressor BRCA1 and the CREB binding protein (CBP) were associated with the holoenzyme complex. The protein related to CBP, the E1A-associated p300 protein, is shown in these experiments to be associated with the holoenzyme complex as well as the BRG1 subunit of the chromatin remodeling SWI/SNF complex. Importantly, the Pol II holoenzyme complex does not contain some factors previously reported as stoichiometric components of the holoenzyme complex, most notably the proteins which function in repair of damaged DNA, such as PCNA, RFC and RPA. The presence of the p300 coactivator and the chromatin-modifying BRG1 protein support a role for the Pol II holoenzyme as a key target for regulation by enhancer binding proteins.

INTRODUCTION

The biochemical characterization of factors required for transcription of mRNA in eukaryotic cells has revealed a requirement for RNA polymerase II (Pol II) plus the basal transcription factors TFIIB, -D, -E, -F and -H (1,2). Regulation of the transcription reaction additionally requires coactivator proteins which link these basal factors to the enhancer binding regulatory factors. Complementary genetic and biochemical approaches in yeast have shown that Pol II, basal factors and coactivators exist as a pre-assembled complex termed the Pol II holoenzyme, an entity which is highly responsive to regulation by DNA binding transcriptional activators (3–5). The holoenzyme was initially defined as a Pol II-containing complex associated with a class of proteins called SRB factors (suppressors of RNA polymerase B mutations). Nine SRB factors were originally identified in a yeast genetic screen as mutants which can reverse a Pol II mutation and which form a complex physically bound to the C-terminal domain (CTD) of Pol II (6). Pol II plus these SRB subunits are obligate components of the Pol II holoenzyme. Recruitment of the Pol II holoenzyme to a promoter is sufficient for activation of a test gene (7,8) and it has been shown that a temperature-sensitive mutation in SRB4 results in complete loss of transcription at the restrictive temperature (9), suggesting that the holoenzyme form of Pol II is responsible for virtually all mRNA transcription initiation in the yeast cell.

In mammalian cells the counterpart of the yeast holoenzyme has only recently been described. Mammalian homologs of three of the nine yeast SRB subunits have been identified based on sequence homology (10–13) and, similar to the yeast complex, are found stably associated with Pol II (10, 11). Additionally, the human SRB7 protein complemented a partial defect in yeast SRB7 (10), demonstrating conservation of function.

Multiple protocols for purification of the mammalian Pol II holoenzyme have been described. These include single step purification via an anti-cdk7 antibody from liver nuclear extract (14), a multistep purification of an SRB7-containing complex from calf thymus extracts (10), two chromatographic steps from a HeLa nuclear extract followed by an anti-TFIIF monoclonal antibody affinity matrix step (11,15), a single step purification from HeLa whole cell extract on a TFIIS affinity matrix (16) or a purification, developed by our laboratory, in which Pol II holoenzyme is purified from HeLa whole cell extract in two to three steps (17,18). This last preparation technique yields holoenzyme which is responsive to appropriate regulation *in vitro* by the phospho-CREB transcriptional activator (18).

These disparate purification protocols do not produce an identical complement of protein species co-purifying as the Pol II holoenzyme. In one study CBP failed to co-purify with the Pol II holoenzyme (16), while in another study CBP in the holoenzyme complex was the critical component for activation of

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transcription *in vitro* (18). In one report the factors which repair damaged DNA were identified as stable components of the Pol II holoenzyme and were present in the complex in amounts stoichiometric relative to the polymerase (11). In the experiments described here we demonstrate that the proteins which regulate repair of damaged DNA are not present in the holoenzyme complex, that the CBP homolog p300 is a component of the Pol II holoenzyme and that the BRG1 subunit of the SWI/SNF complex is in the holoenzyme complex.

MATERIALS AND METHODS

Purification of Pol II holoenzyme and basal transcription factors

HeLa S3 cells or HeLa cells carrying epitope-tagged TBP (19) were passaged in suspension culture using standard procedures. Whole cell extracts were prepared using the technique of Manley *et al.* (20), except that all buffers containing chloride were replaced with buffers containing acetate. Purification of this whole cell extract over Biorex70 ion exchange matrix, centrifugation of the 0.6 M KOAc protein fraction over sucrose gradients and chromatographic purification of the sucrose gradient peak over a Ni–NTA matrix have been described (17).

Nuclease treatment of samples for sucrose sedimentation analysis

For preparative sucrose gradients 6 ml Biorex70 0.6 M peak were applied to the sucrose gradients. For analysis of sensitivity to nucleases 0.6 ml Biorex70 0.6 M peak fraction were treated with 25 µg RNase A (DNase-free) at 20°C for 30 min and then layered directly onto the sucrose gradient without terminating the reaction. Alternatively, 10 µg DNase I were incubated with 0.6 ml Biorex70 0.6 M peak fraction in 10 mM $Mg(OAc)_2$ and 2 mM $CaCl_2$ at 20°C for 30 min and the reaction terminated by addition of EGTA to 2 mM final concentration and EDTA to 15 mM final concentration. The proteins were then layered onto the sucrose gradient as above. For ethidium bromide analysis all sucrose solutions and the sample had ethidium bromide added to a final concentration of 1 µg/ml and centrifuged as above.

Affinity purification of the Pol II holoenzyme with the CBP matrix

The glutathione S-transferase (GST) fusion protein encoding the holoenzyme binding domain of CBP (amino acid residues 1805–1890; a kind gift of T.Nakajima and M.Montminy; 18) was expressed in bacteria [BL21(DE3)]. One liter of induced bacterial culture was extracted using standard procedures and lysate was bound to 1 ml glutathione–agarose. This matrix was then washed extensively with 0.8 M KOAc in H buffer (20 mM Tris–OAc, 1 mM EDTA, 20% glycerol, 1 mM PMSF and 2 mM DTT). The concentration of the CBP polypeptide fragment on the matrix was 0.3 mg/ml matrix.

For analysis by immunoblot of Pol II holoenzyme polypeptides which bound to the CBP matrix 2 µg GST–CBP(1805–1890), equal amounts of GST fusion protein alone and GST–CBP(1–117) were bound to 25 µl glutathione–agarose beads and incubated with peak protein fraction from the Biorex70 0.6 M wash (25 µl, 75 µg total protein diluted in 0.5 ml buffer H with KOAc adjusted to 0.15 M, 0.5% NP-40 and BSA added to 0.2 mg/ml) at 4°C for 16 h. The beads were washed three times with 0.5 ml buffer H + 0.4 M KOAc. Bound proteins were eluted in buffer containing SDS and 2-mercaptoethanol for analysis by SDS–PAGE and immunoblotting. Immunoblotting was done as per standard procedures.

Affinity purification of the Pol II holoenzyme with the BRCA1 matrix

BRCA1 C-terminal amino acids 1560–1863 were fused to the biotin binding protein of the PinPoint Xa-3 vector (Promega) and in TG1 bacterial cells expressed a fusion protein of ~45 kDa. A BRCA1 domain identical to the protein described here except for a point mutation from methionine to glutamate at amino acid residue 1775 was also fused to the PinPoint vector. These fusion proteins and the PinPoint domain alone were purified on magnetic beads containing streptavidin (Dynal) and the amounts of fusion protein bound to the matrix were normalized to each other by analysis on SDS–PAGE, electrotransfer of the proteins to nitrocellulose and probing with HRP-conjugated streptavidin (Gibco-BRL). Equal amounts of immobilized fusion protein were incubated for 16 h at 4°C with 25 µl (75 µg) Biorex70 0.6 M peak fraction diluted to 0.5 ml in buffer H + 0.15 M KOAc, plus 0.2 mg/ml BSA, 0.1 mM DTT and 0.5% NP-40. The matrix was extensively washed in buffer H + 0.6 M KOAc, 0.2 mg/ml BSA, 1 mM DTT and 0.5% NP-40 and bound sample subjected to SDS–PAGE and immunoblotting by standard procedures.

RESULTS

Purification strategy

This purification strategy was similar to that used in the experiments which described the co-purification of CBP and of BRCA1 (17,18). For the purpose of identifying fractions the Pol II holoenzyme was operationally defined as containing both Pol II and the SRB10/11 homologs cdk8 and cycC. No other assumptions were made about composition of the Pol II holoenzyme. An advantage of the Biorex70 matrix is that most cellular proteins flow through (Neish *et al.*, submitted for publication), but, as will be shown below, many transcription factors bind. As can be seen in the Western blots depicted in Figure 1, about half of Pol II and nearly all of cycC eluted in the 0.6 M step elution from the Biorex70 column. These data were consistent with the co-elution profile of cdk8, BRCA1 and half of the Pol II reported earlier (17).

The chromatin remodeling SWI/SNF complex from yeast cells has been shown to be associated with the Pol II holoenzyme (21), but it is unknown whether the mammalian homologs of these factors are associated. CBP has been shown to associate with the Pol II holoenzyme (18), but it was unclear whether the related p300 protein (22) also associates significantly with the transcription complex. In the remaining panels of Figure 1, co-elution of these factors with the holoenzyme preparation was evaluated. As can be seen, both p300 and CBP eluted almost exclusively in the 0.6 M fraction. The BRG1 subunit of human SWI/SNF (23) eluted as did Pol II, half in the 0.6 M fraction and half in the 1.5 M fraction.

The proteins which repair damaged DNA, including RFC and RPA, were reported to be associated with the Pol II holoenzyme (11). In addition to these factors, purification of the PCNA replication and repair factor was evaluated. PCNA was not detected in any fraction containing Pol II, since PCNA did not bind to the Biorex70 matrix. RFC purification was tracked by

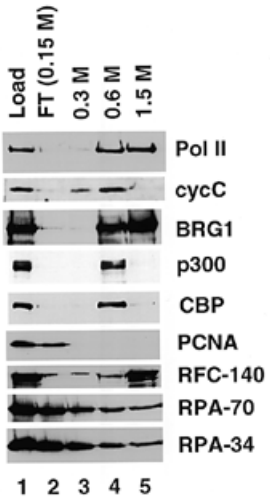

Figure 1. Analysis of protein composition of Biorex70 column chromatography fractions. Samples from the whole cell extract (Load, lane 1), the flow-through at 0.15 M KOAc (FT, lane 2), the protein peaks from the 0.3 M KOAc wash (lane 3), the 0.6 M KOAc wash (lane 4) and the 1.5 M KOAc wash (lane 5) were subjected to SDS–PAGE and immunoblotted against the indicated polypeptide. In each blot the indicated polypeptide was the predominantly staining species migrating at a position on the gel which correlated well with its published molecular mass.

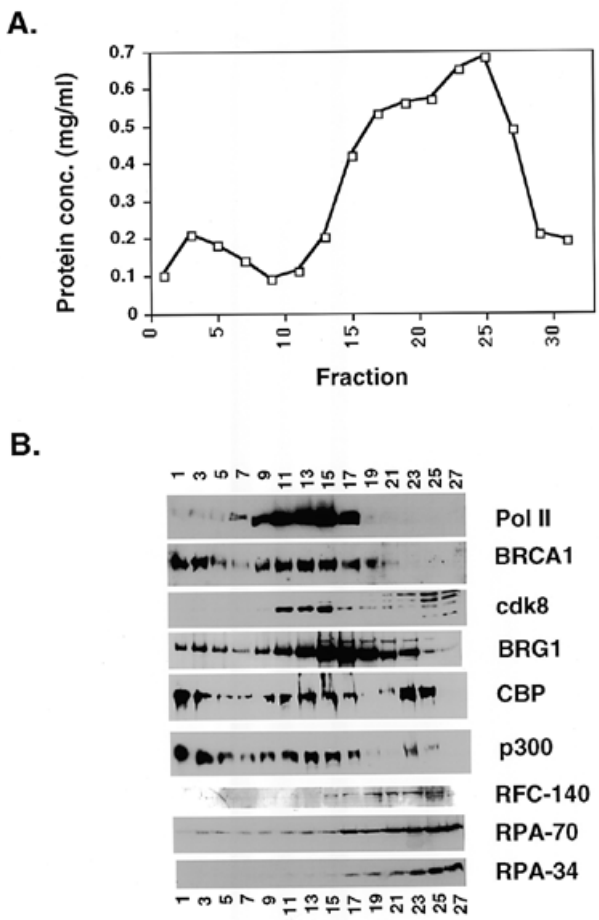

Figure 2. Analysis of the protein composition of sucrose gradient sample sedimentation from the Biorex70 0.6 M peak fraction. Protein fractions from the bottom of the gradient are in samples with low numbers and protein fractions with high numbers represent the low sedimentation rate and thus low molecular weight protein complexes. (**A**) Total protein concentrations across the sucrose gradient fractions. (**B**) Immunoblots of specific proteins, as in Figure 1, were performed across sucrose gradient samples.

antibody specific for the 140 kDa subunit and it was primarily found in the 1.5 M fraction, although as much as 10% of total RFC co-eluted on this first column with the SRB proteins and Pol II. RPA elution was followed by immunoblotting for two of the subunits, RPA-70 and -34. The RPA polypeptides eluted similarly in many fractions. Perhaps as much as 10% co-eluted with the holoenzyme subunits in the 0.6 M step on this first column, although the predominant pool of RPA was found in the flow-through fraction. A DNA recombination protein, hRAD51, was evaluated in the same purification scheme and was found only in the flow-through of the Biorex70 column (17). Using the purification protocol described herein very little, if any, of these repair proteins could be associated with the Pol II holoenzyme.

The second step in purification exploits the size of the Pol II holoenzyme in order to separate it from many contaminating proteins. A 10–60% sucrose gradient was empirically determined to be optimal for separation of large complexes, such as the Pol II holoenzyme was predicted to be, while lower molecular mass complexes would not sediment as rapidly and would thus remain at the top of the gradient. Protein (usually ~20 mg in 6 ml) from the Biorex70 0.6 M fraction was layered onto such a sucrose gradient, centrifuged and fractions were obtained by dripping sample from a hole punctured in the bottom of the tube. Assaying for total protein revealed the bulk of the protein near the top of the gradient (high number fractions; Fig. 2A). As was seen before with similar preparations (17,18), immunoblot analysis of fractions showed that the Pol II subunit sedimented in fractions in the middle of the 10–60% gradient (Fig. 2B). BRCA1 was observed in two pools, in fractions 1–3 at the very bottom of the gradient and in a pool that co-sedimented with Pol II (fractions 9–17). The SRB10/11 homologs cdk8 and cycC co-sedimented with Pol II and BRCA1 (Figs 2B and 4A).

As can be seen in Figure 2B, BRG1 sedimented in a broad peak which overlapped those of Pol II and cdk8. The observation of BRG1 polypeptide sedimentation extending into fractions 19–23 suggested that some of the BRG1 was in a complex independent of the holoenzyme, but it was possible that a subset of the BRG1 in this 0.6 M fraction was associated with the holoenzyme.

p300 eluted in fractions similar in pattern to BRCA1. The fractions with the highest amounts of p300 were 11–15, consistent with the Pol II holoenzyme, and 1–3. A small amount of p300 was observed in fraction 23. In contrast to p300, CBP consistently sedimented with the Pol II holoenzyme, peaking in fractions 11–15 as well as fractions 1–3, but there was significant CBP in fractions 23–25 at the very top of the gradient. Interestingly, sedimentation of the 270 kDa CBP molecule in fractions 23–25 suggested that this polypeptide did not enter the gradient except when associated with other polypeptides in a large complex. It has been shown that if similar sucrose gradient samples were immunoprecipitated using antibody specific for CBP, Pol II would be bound only in fractions which also contained cdk8 (18). Thus CBP in fractions 11–15 was associated with the Pol II holoenzyme. The antibodies used in these immunoblots for p300 and CBP were specific for each of these two related proteins. This

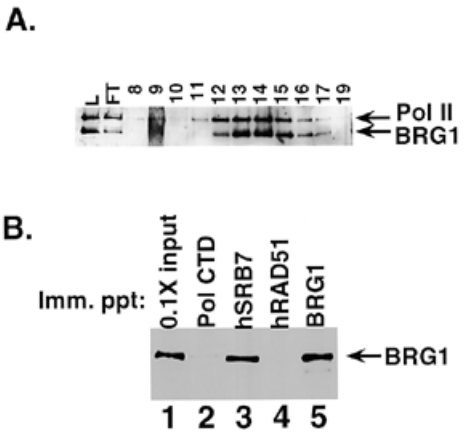

Figure 3. BRG1 is a component of the Pol II holoenzyme. (**A**) Analysis of metal chelate chromatography of Pol II holoenzyme-containing samples. The Pol II holoenzyme peak from the sucrose gradient was chromatographed on a Ni–NTA matrix and eluted by washing with a linear gradient of imidazole. The elution profiles of Pol II and the BRG1 subunit of the mammalian SWI/SNF complex were analyzed by immunoblotting. (**B**) Immunoprecipitation of the BRG1 polypeptide via the holoenzyme-specific anti-hSRB7 antibody. Immunoprecipitations were performed with anti-Pol II CTD antibody 8WG16 (lane 2), affinity-purified anti-hSRB7 (lane 3), affinity-purified anti-hRAD51 (lane 4), anti-BRG1 (lane 5) and immunoblotted and stained with antibody specific for BRG1. The resulting 190 kDa band is indicated.

was evidenced by the distinct presence of CBP in fractions 23–25 in the same gradient as was used for p300 and also by the differences in migration on protein gels, with the p300 polypeptide having a slightly slower migration than did CBP (data not shown).

The ~10% of the DNA repair factors RPA-70 and -34 and RFC-140 which were in the 0.6 M fraction were further analyzed by sucrose gradient sedimentation. These were only detected in fractions at the top of the gradient, suggesting that they were uncomplexed or in complexes insufficiently large to co-sediment with the Pol II holoenzyme. A low amount of RPA-70 was observed in the more rapidly sedimenting fractions (lower number fractions), but subsequent analysis on a CBP affinity matrix suggested that RPA-70 was not associated with the holoenzyme (see Fig. 5B). PCNA was not detectable in any of the sucrose gradient fractions in this analysis (data not shown).

The pattern of sedimentation of BRG1 in the sucrose gradient step was consistent with either a subset of SWI/SNF being in the holoenzyme complex or, alternatively, with the SWI/SNF complex merely having a broad peak which overlapped the narrower holoenzyme peak. The pooled sucrose gradient peak fractions containing holoenzyme components Pol II, cdk8, cycC, p300 and BRCA1 were chromatographed on a Ni–NTA column as reported previously (17). The sucrose gradient pool was bound to a Ni–NTA column in the presence of 5 mM imidazole and then washed with a linear gradient of 5–130 mM imidazole. In this case we asked specifically whether the BRG1 subunit of the SWI/SNF complex co-purified on this third affinity chromatography step. About half of the Pol II in the input sample bound to the Ni–NTA matrix and eluted as a peak in fractions 12–15. Nearly all of the BRG1 polypeptide bound to the Ni–NTA matrix and eluted in fractions 13–15, coincident with the Pol II elution profile (Fig. 3A). These data supported the hypothesis that the SWI/SNF which co-sedimented with the Pol II holoenzyme was indeed bound to the holoenzyme complex.

It was further confirmed that a subfraction of BRG1 was indeed a component of the Pol II holoenzyme by immunoprecipitation of BRG1 from the Biorex70 0.6 M fraction using a holoenzyme-specific antibody which binds the hSRB7 subunit (Fig. 3B). Affinity-purified anti-hSRB7 purified the BRG1 polypeptide, while affinity-purified anti-hRAD51 antibody did not. The monoclonal antibody specific for the Pol II CTD has been shown to disrupt the holoenzyme complex and purify the polymerase containing only its core subunits (5,17) and thus this antibody does not purify the BRG1 subunit, supporting the hypothesis that presence of the BRG1 polypeptide in the hSRB7 immunoprecipitate was due to its presence in the holoenzyme complex.

Co-sedimentation of holoenzyme components was not dependent upon nucleic acids

It was possible that the entity we defined as the Pol II holoenzyme complex was in fact composed of proteins which were not physically associated, but rather linked via DNA or RNA molecules. For example, a Pol II molecule elongating along a DNA template and synthesizing a nascent mRNA might appear to bind to other factors via the DNA or RNA molecules. This possibility was tested by treating the input sample for the sucrose gradient with RNase A or DNase I or by running the entire sucrose gradient in ethidium bromide (Fig. 4 and data not shown). If a polypeptide was associated with the Pol II holoenzyme via a DNA or RNA tether then one of these treatments would cause it to sediment at the top of the gradient and no longer in the holoenzyme peak fractions. Aliquots of the same input Biorex70 fraction were treated with RNase or DNase and then run simultaneously in four identical sucrose gradients in order to improve comparability of results. As can be seen in Figure 4A, the control/untreated gradient was similar to that in Figure 2. Pol II peaked in fractions 9–17 in this centrifugation run. The p300 protein did not have a significant pool in the low sedimentation samples but was reproducibly seen in pools accumulating in fractions 1–3 and 11–17. Both cdk8 and cycC co-sedimented in fractions 9–13 and were present in lower concentrations in fraction 15. These results were typical of many repeated centrifugation runs in which cdk8 and cycC were in higher concentration in the more rapidly sedimenting portion of the holoenzyme peak. BRCA1 protein was seen in two main pools, one coincident with holoenzyme fractions 9–15 and one pool in fractions 1–3. The SWI/SNF subunit BRG1 was in multiple fractions, including a pool in fractions 1–3 and a broad peak from 9–19 overlapping with the holoenzyme peak fractions.

When the input sample was treated with RNase A prior to centrifugation no effect was observed in the holoenzyme pool in fractions 9–15 (Fig. 4B). All of the tested factors were again present in these fractions. The Pol II and BRG1 polypeptides did extend into more slowly sedimenting fractions, as observed in the control gradient. Clearly, the complexes in fractions 9–15 were unaffected by the treatment. Strikingly, the pool of material in fractions 1–3 containing p300, BRCA1 and BRG1 disappeared, suggesting that the high sedimentation rate complex was assembled on an RNA molecule.

The DNase I and ethidium bromide treatments yielded results which were largely comparable with the control sample (data not shown). We concluded from these analyses that although BRCA1, BRG1 and p300 could be associated with RNA, co-purification of these proteins with Pol II and the SRB factors

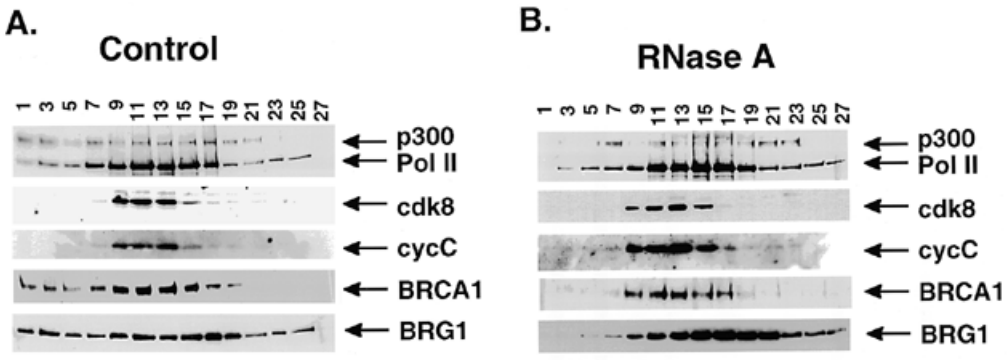

Figure 4. Effect of nucleic acids on sedimentation rates of putative holoenzyme components. Matched sucrose gradient centrifugation analyses were performed with the Biorex70 0.6 M protein peak fraction which was untreated (**A**) or treated with RNase A (**B**). Samples from each were analyzed by immunoblotting.

as a complex in the middle of the gradient was not a consequence of their interaction with DNA or RNA.

CBP affinity matrix

It had been noted that a Pol II complex physically interacted with a CBP domain spanning amino acid residues 1805–1890 (18). The CBP(1805–1890) protein was tested as an affinity matrix to determine whether it could purify the other putative holoenzyme subunits along with Pol II. The CBP matrix specifically bound to Pol II, as previously reported, and also retained p300, CBP and BRCA1 (Fig. 5B). GST or GST–CBP(1–117) control matrices did not bind any of these proteins. Purification of holoenzyme containing p300 and CBP on the CBP affinity matrix suggested that multiple binding sites for CBP/p300 exist in the holoenzyme complex. Since the CBP matrix could interact with the holoenzyme, some of these binding sites were unoccupied.

Identical results were obtained whether the input protein source was the Biorex70 0.6 M fraction or Pol II holoenzyme fractions from the sucrose gradient (data not shown). Thus BRCA1, Pol II and CBP/p300 exist as a size-selected complex which was bound to the CBP affinity matrix. Affinity purification of Pol II on CBP matrix was unaffected by RNase digestion (data not shown).

The RPA proteins, which did not co-purify with the holoenzyme on conventional chromatography, did not bind to the CBP affinity matrix (Fig. 5B). The Biorex70 column fraction input contained both the Pol II holoenzyme and the RPA proteins, but the RPA proteins did not bind to this affinity matrix. These results supported the conclusion that the proteins involved in repair of damaged DNA were not components of the Pol II holoenzyme complex being purified in this study.

The SWI/SNF subunit BRG1, a fraction of which co-purified with the holoenzyme (Figs 1–3) and which was in a complex containing hSRB7 (Fig. 3B), was not purified significantly on the CBP matrix. This result suggests that the SWI/SNF subunit is only in a small subpopulation of the Pol II holoenzyme. As will be shown below, a similar matrix containing BRCA1 specifically purifies BRG1, suggesting that this SWI/SNF component may be associated with certain subpopulations of the Pol II holoenzyme.

BRCA1 affinity matrix

The C-terminus of BRCA1 (residues 1560–1863) has been shown to function as a transcriptional activator when expressed in eukaryotic cells as a fusion protein with the GAL4 DNA binding domain (24). When the methionine at codon 1775 was mutated to glutamate this transcriptional activation was lost (24). The wild-type BRCA1 fragment and the M1775E mutant were fused to the biotin binding PinPoint protein and used as affinity matrices. When these matrices were incubated with the Biorex70 0.6 M fraction the wild-type BRCA1 matrix purified Pol II, while the M1775E mutation failed to purify the polymerase (Fig. 6B). Washing of the matrix in these assays was quite stringent (0.6 M KOAc and 0.5% NP-40 in buffer H). The stringency of the wash protocol and the failure of a protein containing a single point mutation to purify the holoenzyme suggested that this assay was quite specific for holoenzyme components. These results suggest that the failure of this M1775E mutant BRCA1 protein to activate transcription in a transfection assay (24) might be explained by a failure to bind to the Pol II holoenzyme.

It was found that the wild-type BRCA1 matrix purified the BRG1 subunit of the SWI/SNF complex (Fig. 6B, bottom panel). It had been shown that a significant amount of BRG1 was in a complex with the Pol II holoenzyme (Figs 1–3) but that very little bound to the CBP affinity matrix. BRG1 was purified by the wild-type BRCA1 matrix but not the M1775E matrix, indicating that the interaction with this SWI/SNF subunit was specific.

DISCUSSION

Purification of the Pol II holoenzyme in these experiments revealed that p300 and BRG1, but not DNA repair factors, are components of the holoenzyme complex. The specificity of the purification protocol is demonstrated by these latter proteins, which are abundant in the nucleus and which do not co-purify with the holoenzyme complex.

The protein composition of the Pol II holoenzyme preparation was tested by several rigorous criteria. Pol II holoenzyme sedimentation characteristics were not affected by treatments which would perturb protein–nucleic acid interactions. Strikingly, RNase A treatment of the sucrose gradient input material did not affect sedimentation of holoenzyme peak fractions but did cause disappearance of BRCA1, p300 and BRG1 from fractions with very high sedimentation rates (fractions 1–3; Fig. 4). This pool of BRCA1 had been the second major pool of BRCA1 in the cell extract (17) and this result with RNase A treatment suggests that virtually all of the BRCA1 in the cell extract is associated with the holoenzyme. It is possible that this RNase-sensitive complex

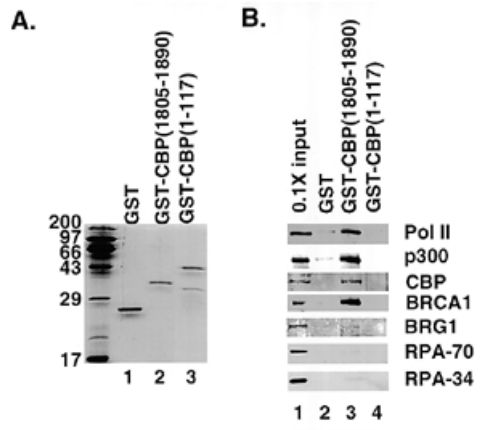

Figure 5. Affinity chromatography using the CBP affinity matrix. (**A**) Composition of affinity matrices. The protein composition of the bound bacterial protein was evaluated by SDS–PAGE and stained with Coomassie brilliant blue. Proteins were: GST alone (lane 1); GST–CBP(1805–1890) (lane 2); GST–CBP(1–117) (lane 3). (**B**) Affinity purification of Pol II holoenzyme components on CBP(1805–1890). Affinity purification of specific polypeptides was analyzed by immunoblotting bound proteins after incubation of the CBP matrix with the Biorex70 0.6 M peak fraction. Samples were a tenth of the input (lane 1) or material bound by GST alone (lane 2), GST–CBP(1805–1890) (lane 3) and GST–CBP(1–117) (lane 4).

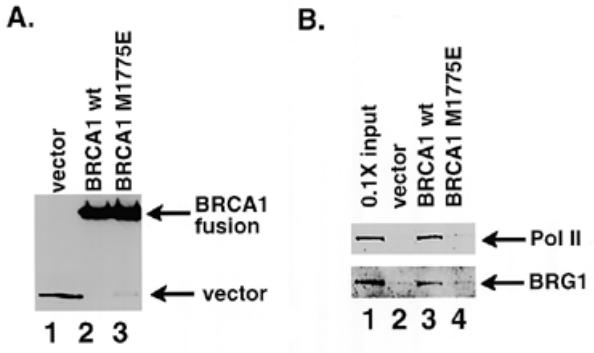

Figure 6. Affinity purification of the Pol II holoenzyme with BRCA1. (**A**) BRCA1 wild-type and point mutant C-termini (1560–1863) were fused to the PinPoint vector and expressed in bacteria. The amount of fusion protein expressed and retained on a streptavidin matrix was determined by staining a blot of the protein gel with HRP-conjugated streptavidin. The PinPoint vector alone produces a 10 kDa polypeptide and the BRCA1 wild-type and BRCA1 point mutant fusion proteins migrated at ~45 kDa. (**B**) Affinity purification of Pol II and BRG1 with the BRCA1 matrix. The Biorex70 0.6 M protein fraction was incubated with matrix containing the BRCA1 fusion proteins and washed with buffer H + 0.6 M KOAc. The presence of bound proteins was evaluated by immunoblotting for Pol II (top) and BRG1 (bottom).

represents the Pol II holoenzyme with attached nascent transcript and this possibility is currently being tested.

Only proteins directly associated with the gene expression process were identified in the Pol II holoenzyme. Proteins involved in DNA repair did not co-purify. Also of significance, from this limited sampling, proteins which bind to DNA with sequence specificity do not co-purify with the Pol II holoenzyme (17). These findings support a role for the Pol II holoenzyme as a complex containing some of the basal transcription factors and coactivators which may bridge enhancer binding regulatory proteins to the basal machinery.

An advantage of the described purification is that the Pol II holoenzyme is already enriched prior to the affinity purification step. It is possible that the affinity matrix step biases the purification of the Pol II holoenzyme. For example, the CBP matrix will select for holoenzyme complexes containing coactivators. Our affinity purification experiments suggest that the holoenzyme may bind coactivator species (p300 or CBP) with a greater than 1:1 stoichiometry relative to Pol II, while basal factors are present at substoichiometric concentrations (for example, compare input and bound p300 or BRCA1 in Fig. 5B). While these results may reflect physiological conditions, it is also possible that our observations reflect our choice of affinity matrices. A CBP matrix would be expected to bind tightly to a putative docking protein which would link CBP to Pol II and select for complexes enriched in these factors, while basal factors may be gradually depleted over the purification steps. Conversely, purification via TFIIF (11) may, for example, select for holoenzyme complexes containing stoichiometric amounts of TFIIF relative to Pol II as well as factors involved in elongation of transcription, since TFIIF also functions in the elongation phase of mRNA synthesis (25,26). This may explain how the proteins which repair damaged DNA may co-purify with the holoenzyme when purified with TFIIF monoclonal antibody (11), since the repair factors may be linked with transcription elongation.

The SWI/SNF component BRG1 was found to co-purify with the Pol II holoenzyme on Biorex70, sucrose gradient sedimentation, metal chelate and BRCA1 affinity chromatography. It was shown to be a component of the same complex by immunoprecipitation with a holoenzyme-specific antibody. The amount of BRG1 associated with the Pol II holoenzyme, relative to the total BRG1 in the extract, was at most ~20% (estimated from the Western blots of Figs 1–3). The BRCA1 affinity matrix purified substoichiometric amounts of BRG1 relative to the Pol II purified on these matrices, suggesting that BRG1 was only present in a small subset of the holoenzyme complexes. It is possible that BRG1 has similar affinity for the holoenzyme complex as do the basal transcription factors, which appear to be specifically associated but are depleted upon purification (10).

The CBP present in the Pol II holoenzyme has demonstrated functional importance *in vitro* (18). The related p300 was found associated with the holoenzyme in this study. Use of CBP and BRCA1 affinity matrices revealed interesting properties of the Pol II holoenzyme. As shown in Figure 5B, binding by CBP, p300 and BRCA1 were not mutually exclusive of each other, since the CBP matrix could specifically purify all three proteins. This implies that factors which dock CBP to the holoenzyme must have multiple sites available. While it is expected that CBP and p300 have the same docking site, it would be anticipated that BRCA1 would have a different binding site. We have recently identified the docking factor for CBP as the RNA helicase A factor, which appears to bridge CBP(1805–1890) directly to the Pol II core (27). The suggestion that there exist in the Pol II holoenzyme unoccupied docking sites could explain how expression of high levels of CBP in cells which already have endogenous CBP can drive transcription of a specific reporter even higher (28). Overexpression of either CBP or p300 would raise the stoichiometry of these factors in the Pol II holoenzyme and thus increase the responsiveness to upstream activators such as CREB.

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