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# Purification of Multiple Erythroid Cell Proteins That Bind the Promoter of the α-Globin Gene

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**Three erythroid cell factors that bind the murine α-globin promoter were enriched more than 1,000-fold by conventional and DNA sequence affinity chromatography. Visualization of enriched polypeptides revealed simple patterns suggesting that each binding activity was purified. Two of the purified proteins, α-CP1 and α-CP2, have been shown previously to interact with distinct binding sites that overlap in the α-globin CCAAT box. Affinity purification of α-CP1 revealed seven polypeptides with $M_r$s raging from 27,000 to 38,000. In contrast, purified α-CP2 was made up of a polypeptide doublet with $M_r$s of 64,000 and 66,000. The third purified binding activity, α-IRP, interacted with sequences that formed an inverted repeat (IR) between the α-globin CCAAT and TATAA boxes. Affinity-purified α-IRP was made up of a single polypeptide with an $M_r$ of 85,000. We confirmed that the purified polypeptides corresponded to α-CP1-, α-CP2-, and α-IRP-binding activities by UV cross-linking experiments (α-CP2 and α-IRP) or by renaturation of binding activity after elution of polypeptides from sodium dodecyl sulfate-polyacrylamide gels (α-CP1 and α-CP2). The apparent complexity of the polypeptides accounting for α-CP1 binding activity prompted a further physical characterization of this factor. Sedimentation of affinity-purified α-CP1 in glycerol gradients containing 100 mM KCl showed that all seven polypeptides migrated as a complex that cosedimented with α-CP1-binding activity. In contrast, when sedimented in glycerol gradients containing 500 mM KCl, α-CP1 dissociated into at least two components. Under these conditions, α-CP1-binding activity was reduced or lost. Activity was reconstituted, however, by combining fractions that were enriched in the two components. These results were confirmed by experiments in which we showed that α-CP1-binding activity can be recovered only by combining distinct sets of polypeptides that were isolated and renatured from sodium dodecyl sulfate-polyacrylamide gels. Our results suggest that the seven polypeptides visualized after affinity purification of α-CP1 interact to form a heterotypic complex (or set of complexes) required for α-CP1-binding activity.**

The purification and characterization of nuclear factors that interact with specific DNA sequences in eucaryotic promoters is an important step toward understanding the roles these proteins play in regulating differentiation-specific gene expression (for recent reviews, see references 23 and 25). To identify erythroid cell factors that might play a role in regulating globin gene expression, we have begun to characterize nuclear extracts of murine erythroleukemia (MEL) cells. This transformed erythroid precursor cell line can be readily induced to undergo erythroid differentiation in vitro (24).

We recently have described three factors that are present in MEL cell nuclear extracts that bind the α-globin promoter (3a, 10). Our results showed that these erythroid cells contain two distinct CCAAT-binding proteins, which we termed α-CP1 and α-CP2. Like other CCAAT factors (3), these two proteins have mutually exclusive binding sites that overlap. Both proteins interact selectively with the α-globin promoter, when compared with that of the β-globin promoter, and neither factor interacts with the adenovirus origin of replication (*ori*). This latter observation suggests that these two erythroid derived factors are distinguishable from the HeLa cell-derived CCAAT transcription factor CTF/NF-1 (19). In addition to the two CCAAT-binding proteins, we identified a third factor that interacts with two sequences that form a pair of inverted repeats (IRs) between the CCAAT and TATAA boxes. We termed this factor α-IRP.

As the next step in the characterization of these three erythroid factors, we report here the DNA sequence affinity purification of polypeptides that account for each binding activity. Our results showed that the CCAAT-binding protein, α-CP1, is made up of seven polypeptides with $M_r$s between 27,000 and 38,000. Sedimentation experiments and recovery of binding activity by renaturation of polypeptides eluted from sodium dodecyl sulfate (SDS)-polyacrylamide gels confirmed the identity of α-CP1 and suggested that these seven proteins are made up of two subgroups that interact to form a heterotypic complex that is capable of interacting with the α-globin promoter. The second CCAAT factor, α-CP2, is made up of a doublet of proteins with $M_r$s of 64,000 and 66,000. We confirmed that this polypeptide doublet is responsible for α-CP2-binding activity by performing UV cross-linking experiments in the presence or absence of specific oligonucleotide competitors and by reconstituting DNA-binding activity after proteins eluted from SDS-polyacrylamide gels were renatured. Lastly, our results show that a single polypeptide with an $M_r$ of 85,000 binds the α-globin IR box. The identification of α-IRP was confirmed by UV cross-linking experiments.

## MATERIALS AND METHODS

**Nuclear extracts.** MEL cells were maintained in roller bottles at densities between $1 \times 10^5$ and $2 \times 10^6$ cells per ml in α-medium (GIBCO Laboratories, Grand Island, N.Y.) containing 10% fetal bovine serum (10). For large-scale growth, cells were routinely expanded into 20-liter cultures (in roller bottles) in media containing 4% fetal bovine serum. Cells were grown to densities of $1 \times 10^6$ to $2 \times 10^6$ cells per

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ml, and extracts were prepared as described previously (10, 11).

**Protein chromatography.** Conventional chromatographic steps were performed as described previously (3a), and binding activities that were present in individual fractions were determined by electrophoretic shift assays (see below). Peak activities were pooled and dialyzed against buffer D (20 mM Tris [pH 7.9], 20% glycerol, 0.2 mM EDTA, 100 mM KCl, 0.5 mM dithiothreitol [DTT], and 0.5 mM phenylmethylsulfonyl fluoride). All extracts and chromatographic fractions were used immediately or frozen in liquid nitrogen and stored at −70°C. We note that in a previous study (10) we reported enrichment of the protein that we call α-CP1 in the present study. In the original enrichment scheme, binding activity flowed through both phosphocellulose and the cellulose-based weak anion exchanger DE52 (10). In our current fractionation protocol, we replaced DE52 with a slightly different anion exchanger, TSK-DEAE (EM Sciences). Using our current protocol, the protein described previously bound to the resin and was eluted at a low ionic strength (see below).

**DNA sequence affinity chromatography.** DNA affinity columns were constructed by modifying the procedures outlined by Kadonaga and Tjian (20) and Chodosh et al. (8). The following synthetic oligonucleotides were prepared on a DNA synthesizer (Applied Biosystems): α-CP1, 5′-GAT CGC ACA AAC CAG CCA ATG AGT AAC TGC TCC AAG-3′/5′-CTA GCT TGG AGC AGT TAC TCA TTG GCT GGT TTG TGC-3′ (positions −71 to −100 on the α-promoter [27]); α-CP2, 5′-CCC TAA CAA GTT TTA CTG GGT AGA GCA AGC ACA AAC CAG CCA ATG AG-3′/5′-TTA GGG CTC ATT GGC TGG TTT GTG CTT GCT CTA CCC AGT AAA ACT TG-3′ (positions −83 to −129 on the α-promoter [27]); α-IRP, 5′-GAT CTG GAG GAC AGC CCT TGG AGG GCA-3′/5′-AAT TTG CCC TCC AAG GGC TGT CCT CCA-3′ (positions −31 to −53 on the α-promoter [27]). These sequences corresponded to the DNase I footprint of each protein. Oligonucleotides were purified by gel electrophoresis, and complementary strands (100 μg of each strand) were annealed and phosphorylated (20). After organic extractions and ethanol precipitation, oligonucleotides were dissolved in 150 mM NaCl, 1 mM ATP, 10 mM DTT, 6.6 mM $MgCl_2$, 66 mM Tris (pH 7.4), and 10% polyethylene glycol (34) and incubated with T4 DNA ligase (4,000 U; New England BioLabs, Inc., Beverly, Mass.) at 37°C for 2 h and then at 16°C overnight. Under these conditions, 3-mers to 20-mers were produced. Ligated multimers were incubated with terminal transferase (45 U) in the presence of 4 mM TTP and dCTP, and biotin-11-dUTP (4 mM; Bethesda Research Laboratories, Inc., Gaithersburg, Md.) at 37°C overnight. After ethanol precipitation, biotinylated DNA was coupled to streptavidin agarose beads (Bethesda Research Laboratories) by mixing tailed DNA with 1 ml of beads overnight at 4°C. Fractions (500 μl) were poured into columns and washed extensively with the buffer Z described by Kadonaga and Tjian (20). In general, chromatographic fractions enriched in specific binding activities were passed over two to four 500-μl columns in parallel at 4°C. Columns were washed extensively with buffer Z containing 100 mM KCl and buffer Z containing 250 mM KCl. Sequence-specific proteins were eluted with buffer Z containing 1 M KCl. Active eluates were dialyzed against buffer D, incubated with 40 μg of poly(dI-dC) per ml, and then subjected to additional passes over the columns. Protein fractions were precipitated by the addition of deoxycholate and trichloroacetic acid (TCA) to 1 mM and 15%, respectively. Precipitates were washed with 1 ml of 95% acetone (−20°C), dried, and suspended in 20 μl of SDS gel sample buffer (21) containing 100 mM DTT in place of 2-mercaptoethanol.

**Glycerol gradients.** Peak activity fractions from the α-CP1 affinity column were pooled, dialyzed, concentrated (Centricon; Amicon Corp., Lexington, Mass.), and adjusted to 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.9), 10% glycerol, 0.1% Nonidet P-40, 1 mM DTT, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 0.1 or 0.5 M KCl (29, 30). Protein standards (2 μg each of bovine serum albumin [BSA], ovalbumin, and α-chymotrypsinogen) were prepared in the same buffer. Proteins were centrifuged through 15 to 35% glycerol gradients in a rotor (SW 55 Ti; Beckman Instruments, Inc., Fullerton, Calif.) at 48,000 rpm (218,300 × *g*) for 27 h at 4°C. Fractions (175 μl) were collected from the bottom of the tube and assayed for specific DNA-binding activity by electrophoretic shift and for protein content by precipitation with TCA (15%) and electrophoresis in SDS-polyacrylamide gels.

**Electrophoretic shift gels.** Mobility shift assays were performed as described previously (10, 14, 32). The probe used in all cases was a 120-base-pair fragment of the α-globin promoter extending from a *Hae*III site at position −130 to a *Pst*I site at position −10.

**SDS-polyacrylamide gels and renaturation of DNA-binding activity.** Electrophoresis in SDS-polyacrylamide gels was performed as described previously (21), except that 100 mM DTT replaced 2-mercaptoethanol in the sample buffer. Separated proteins were visualized by silver staining (ICN Pharmaceuticals Inc., Irvine, Calif.), and $M_r$s were determined by reference to commercially obtained molecular weight markers (Bethesda Research Laboratories). DNA-binding activity was renatured from SDS-polyacrylamide gels as described previously (15, 16) for α-CP2 and α-CP1, respectively.

**Quantifying protein enrichment.** Protein was determined by the method of Bradford (4) (Bio-Rad Laboratories, Richmond, Calif.), with BSA used as a standard, except for fractions from DNA affinity columns, in which the protein concentration was estimated from silver-stained gels by comparison with known amounts of stained markers (BSA and ovalbumin). Binding activity was quantified by a standard gel shift assay. In the standard assay, portions of the column fractions were mixed with 8.9 fmol of labeled DNA and 1 μg of poly(dI-dC) [except after affinity chromatography, when no poly(dI-dC) was added]. After electrophoresis, bound and free probe DNAs were visualized by autoradiography. Appropriate regions were cut out and quantified by liquid scintillation counting. The fraction of probe that bound was titrated with increasing amounts of protein, and only linear portions of the binding curves were used. One unit of activity is defined as the amount of protein required to shift 1 fmol of probe under standard conditions. We note that these measurements are subject to several errors because of changes in the amounts of competing proteins in the course of purification. This was particularly problematic during the early stages of factor purification and probably accounts for measured recoveries in excess of 100% at some early column steps.

**UV cross-linking.** For UV cross-linking experiments, a single-stranded template containing the α-globin promoter (from positions −130 to −10, cloned in M13mp8 and M13mp9) was extended in vitro with the Klenow fragment of DNA polymerase (from the M13 universal primer) in the presence of bromodeoxyuridine (100 μM) and α-$^{32}$P-labeled nucleotide triphosphates (20 μM of each triphosphate [8,

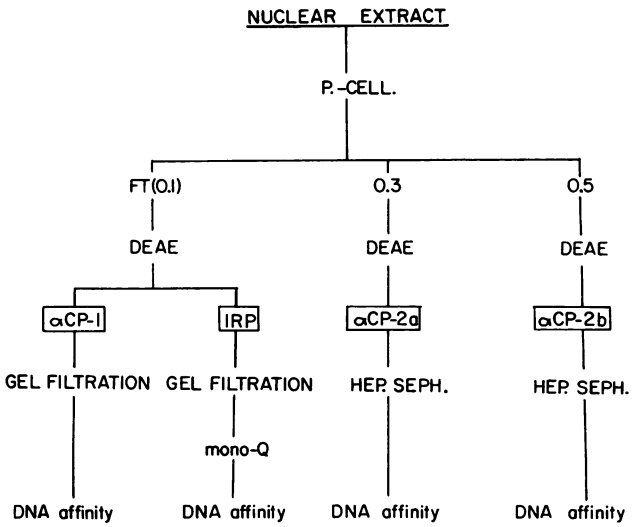


FIG. 1. Fractionation scheme for proteins that bind the α-globin promoter. Nuclear extracts were prepared and fractionated as described in the text. Extracts were applied to a phosphocellulose (P. Cell.) column, and a fraction that flowed through (FT) the column in 100 mM KCl was collected. The column was then eluted stepwise with a buffer containing 0.3 and 0.5 M KCl. Subsequent protein enrichment was via chromatography on DEAE, Ultrogel (GEL FILTRATION), heparin-Sepharose (HEP. SEPH.), and mono-Q columns. The last step in the purification of all proteins was DNA sequence affinity chromatography.

26]). After incubation with *Eco*RI and *Hin*dIII, the radiolabeled template was isolated by electrophoresis on a nondenaturing polyacrylamide gel, purified, and shown to bind enriched factors in electrophoretic shift experiments (data not shown). After incubation of purified protein with the radiolabeled probe ($5 \times 10^5$ cpm), samples were irradiated with a germicidal UV lamp (5,000 μW/cm$^2$) for 10 to 15 min (6, 17). $CaCl_2$ was added to 10 mM, and samples were digested with DNase I (2 μg) and micrococcal nuclease (20 U) for 30 min at 37°C. Samples were precipitated with TCA (15%), subjected to electrophoresis in SDS-polyacrylamide gels, and visualized by autoradiography. Labeled protein markers were obtained commercially (Bethesda Research Laboratories).

## RESULTS

**Three distinct α-globin-binding activities.** We have shown previously that fractionation of MEL cell extracts on phosphocellulose and DEAE columns resolves four activities that fall into three categories: two distinct α-globin CCAAT-binding proteins (α-CP1 and α-CP2) and a third factor (α-IRP) that interacted with a pair of IR elements that were located between the α-globin CCAAT and TATAA boxes (Fig. 1) (3a). Among the two CCAAT-binding proteins, one, α-CP2, was resolved during chromatography on phosphocellulose into two components, α-CP2a and α-CP2b. Apart from their behavior on phosphocellulose, α-CP2a and α-CP2b behaved similarly in all subsequent chromatographic steps, footprinted the same sequences on the α-globin promoter, and were made up of a nearly identical doublet of polypeptides (see below). Together, these results suggest that some α-CP2 activity remains on the phosphocellulose column after the first salt step and is eluted subsequently. α-CP2a and α-CP2b are, therefore, closely related, if not identical. We used the designations α-CP2a and α-CP2b to indicate their behavior on phosphocellulose under our conditions.

The scheme used to purify each binding activity is outlined in Fig. 1. As detailed in Tables 1 to 3, several conventional chromatographic steps enriched each binding activity less than 20-fold. To purify each activity further, we constructed three different DNA sequence affinity columns based on the DNase I footprint of each factor (3a, 10). Appropriate oligonucleotides for the affinity matrices were synthesized, hybridized, multimerized, tailed with biotinylated dUTP, and affixed to a streptavidin agarose matrix (see above).

**Purification of α-CP1.** After chromatography on phosphocellulose, DEAE, and gel filtration (Fig. 1), the binding activity corresponding to α-CP1 was enriched approximately 11-fold (Table 1). To purify this activity further, the enriched protein was incubated with poly(dI-dC) (40 μg/ml) and applied to a DNA sequence affinity column in a buffer containing 100 mM KCl (20) (see above). The column was washed with two to four column volumes of the same buffer containing 250 mM KCl, and binding activity was eluted with the same buffer containing 1 M KCl. Column fractions were collected and assayed for α-globin-binding activity. The results (Fig. 2) showed that the peak of α-CP1 activity was contained in the 1 M salt wash fractions. Active fractions were pooled, dialyzed to starting conditions, and reapplied to the affinity column. After three consecutive cycles of column binding and elution, proteins were precipitated with TCA (15%), separated by electrophoresis on SDS-polyacrylamide gels, and visualized by silver staining. A group of seven polypeptides with $M_r$s ranging from 27,000 to 38,000 were prominently enriched (Fig. 3A, lane 4). It should be noted that under the conditions illustrated in Fig. 3A, only five of these bands ($M_r$s from 33,000 to 38,000) stained well.

TABLE 1. Purification of α-CP1

| Fraction | Protein | | | Activity | | Sp act (U/mg) | Purification (fold) |
|---|---|---|---|---|---|---|---|
| | Vol (ml) | Concn (mg/ml)[a] | Total amt (mg [%]) | Total (U)[b] | Yield (%) | | |
| Nuclear extract[c] | 67 | 5.4 | 361.8 (100) | 294,800 | 100 | 814.8 | 1 |
| Phosphocellulose | 82.4 | 3.3 | 271.9 (75.2) | 367,504 | 124.66 | 1,351.6 | 1.7 |
| DEAE | 17 | 1.3 | 22.1 (6.11) | 55,760 | 18.9 | 2,523.0 | 3.1 |
| Ultrogel | 15 | 0.09 | 1.35 (0.037) | 12,600 | 4.3 | 9,333.3 | 11.5 |
| DNA affinity (3×) | 4.86 | 0.0009 | 0.00437 (0.0012) | 10,157 | 3.45 | 2,324,256 | 2,853 |

[a] Determined by a modified method described by Bradford (4) (Bio-Rad), with BSA used as a standard, except for the DNA affinity fraction, in which the protein concentration was estimated from silver-stained gels.
[b] One unit of activity is defined as the amount of protein required to bind 1 fmol of probe under standard gel shift conditions (see text).
[c] Prepared from $3.0 \times 10^{10}$ cells.

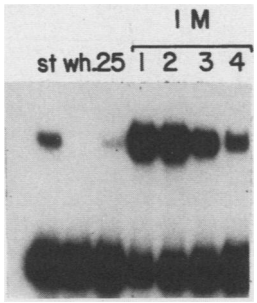


FIG. 2. Electrophoretic shift assays during affinity purification of α-CP1. α-CP1, which was enriched through the Ultrogel stage (st), was applied to an affinity column. Material in the column flow-through (wh) and 0.25 M KCl washes (.25) were tested for binding activity, as were four 500-μl fractions that were eluted from the column by application of a buffer containing 1 M KCl (1 M; lanes 1 through 4).

The two additional bands, with $M_r$s of 27,000 and 28,000, acquired a characteristic yellow stain that did not photograph well. Visual inspection of the original gels, however, suggested that the bands with $M_r$s of 27,000 and 28,000 were as abundant as the other five polypeptides. In addition, we subsequently found that when stained gels were washed and restained, the polypeptides with $M_r$s of 27,000 and 28,000 became readily visible (Fig. 3B). Affinity purification of α-CP1, therefore, results in the significant enrichment of seven polypeptides with $M_r$s ranging from 27,000 to 38,000.

It should be noted that we have previously described (10) an erythroid cell-derived CCAAT-binding protein that footprints a region of the α-promoter identical to that protected by α-CP1 (3a). These results suggest that the activity we reported earlier (10) and α-CP1 are caused by the same factor. To confirm this hypothesis, we applied material that was enriched by our previous protocol (10) to an α-CP1 affinity matrix (the fractionation of extracts by our previously published protocol [10] differed slightly from our current procedure; see above). After three applications to the affinity column, proteins were precipitated, subjected to electrophoresis on SDS-polyacrylamide gels, and visualized by silver staining. A comparison of the resolved proteins (Fig. 3A, compare lanes 4 and 5) confirmed that the factor that we characterized previously (10) is made up of the same seven polypeptides as α-CP1.

The pattern of polypeptides produced by affinity chromatography of α-CP1 was simple and did not change after additional affinity or conventional chromatographic steps. These results suggest that all seven polypeptides that were enriched by the affinity step contributed to α-CP1-binding activity. To obtain more insight into potential interactions between these polypeptides, we subjected affinity-purified α-CP1 to sedimentation through glycerol gradients in the presence of 100 or 500 mM KCl. During sedimentation in 100 mM KCl, all seven polypeptides comigrated with a sedimentation coefficient similar to that of BSA (4.2S; Fig. 4A, top). In addition, α-CP1-binding activity cosedimented with the seven polypeptides. In 500 mM KCl, on the other hand, the complex observed at 100 mM KCl dissociated into two major components. The first component, which we term α-CP1A, sedimented at 3.6 S (just ahead of an ovalbumin marker) and was made up of the three polypeptides with $M_r$s of 27,000, 28,000, and 35,000 (we note here that the polypeptide with an $M_r$ of 35,000 always stained more intensely than the polypeptides with $M_r$s of 27,000 and 28,000). The second component (α-CP1B) sedimented at approximately 2.8S and was

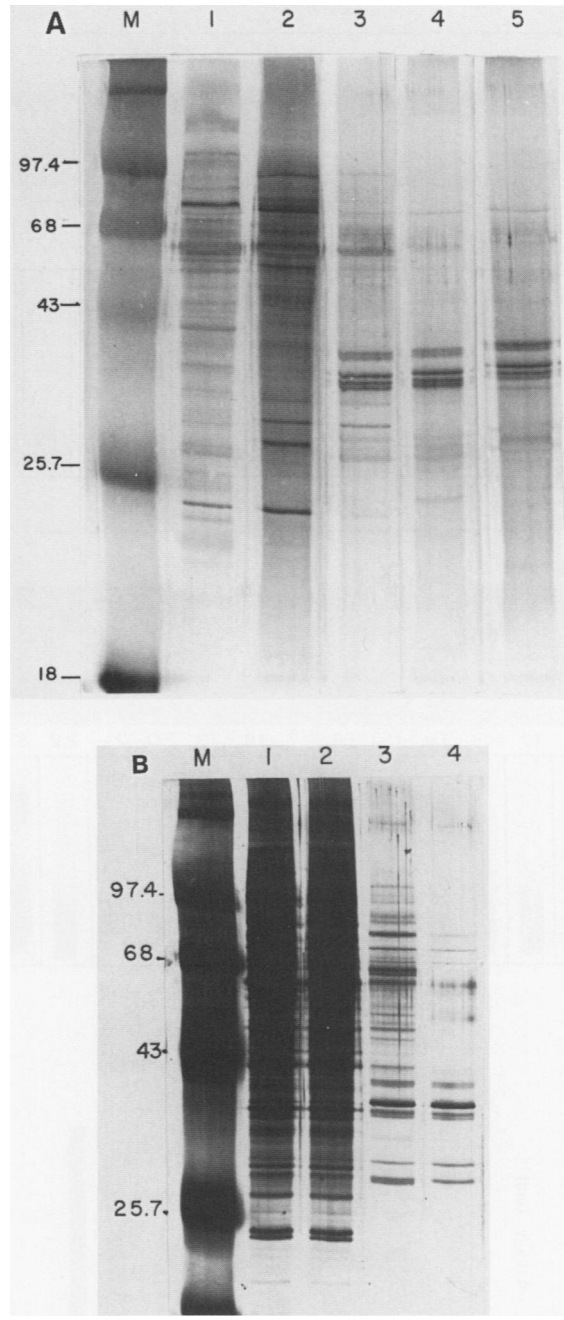


FIG. 3. SDS-polyacrylamide gel electrophoresis and visualization of affinity-purified α-CP1. (A) Marker proteins are indicated (lane M); molecular sizes (in thousands) are indicated on the left. Samples of protein from starting material (lane 1), the first affinity column flowthrough (lane 2), and the 1 M KCl elutions from the first and third affinity columns (lanes 3 and 4, respectively) were precipitated with TCA, subjected to electrophoresis on SDS-polyacrylamide gels (10%), and visualized by silver staining. Lane 5 shows the result of the visualization of a protein described previously (10) after affinity purification (see text). Five bands with $M_r$s of 33,000, 34,000, 35,000, 37,000, and 38,000 were easily detected. Two polypeptides with $M_r$s of 27,000 and 28,000, however, acquired a yellow color that did not photograph well. (B) Although the polypeptides with $M_r$s of 27,000 and 28,000 were poorly stained in panel A, they were readily visualized when gels were washed and restained. Lanes marked M and 1 through 4 are as described above for panel A (a separate purification is shown in panel B). The molecular size (in thousands) of marker proteins is also indicated on the left.

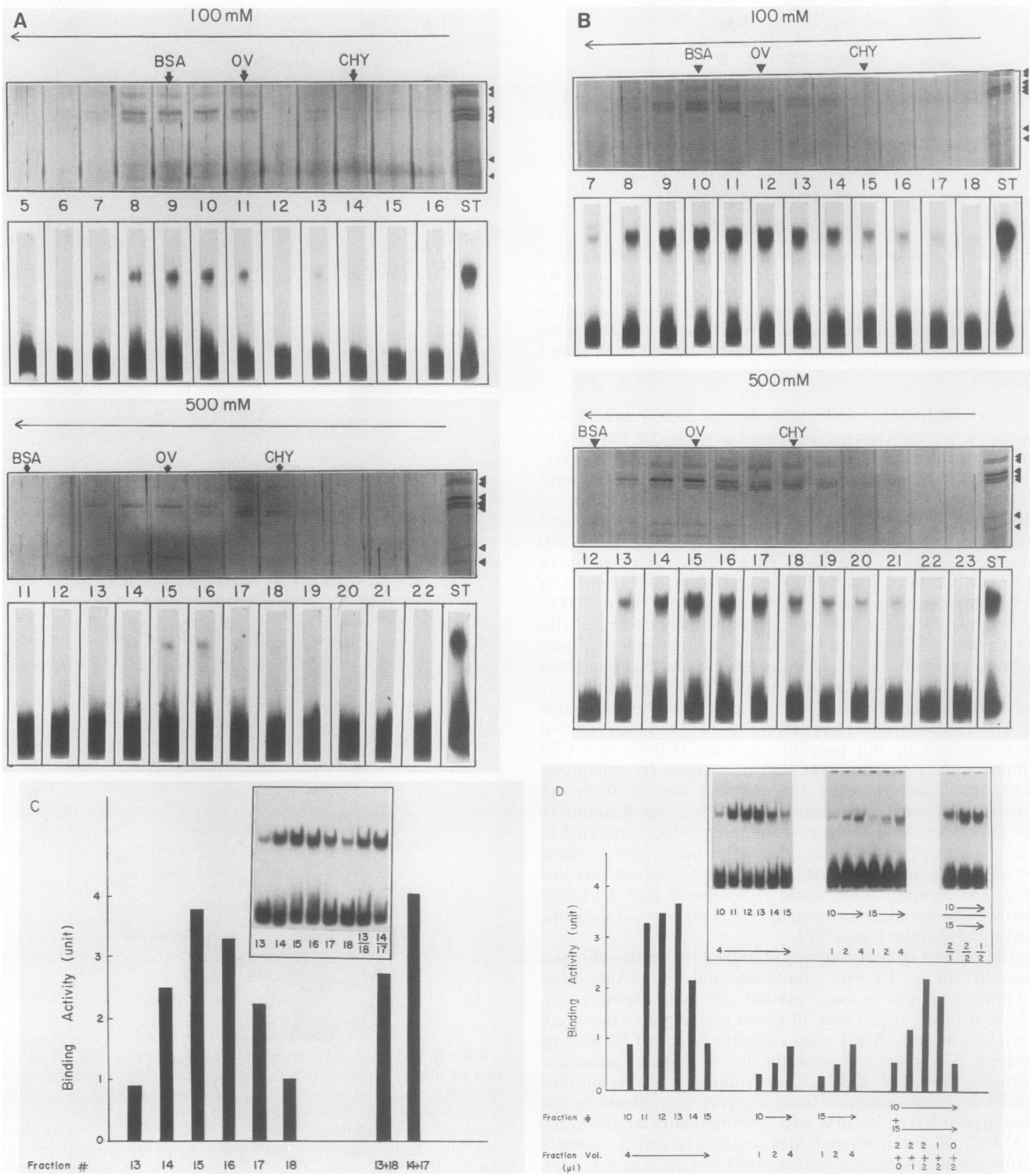


FIG. 4. Glycerol gradient sedimentation of affinity-purified α-CP1. (A) Affinity purified α-CP1 (ST) was subjected to sedimentation in glycerol gradients containing 100 (top) or 500 (bottom) mM KCl, as indicated. Fractions were collected and analyzed for their protein content by electrophoresis in SDS-polyacrylamide gels (shown at the top of each gradient) and for DNA-binding activity by electrophoretic shift (shown below each SDS-polyacrylamide gel). The peak positions of marker proteins (run in separate tubes) are indicated at the top of each SDS-polyacrylamide gel (OV, ovalbumin; CHY, α-chymotrypsinogen). Arrowheads at the right of the SDS-polyacrylamide gels indicate the seven polypeptides that correspond to affinity-purified α-CP1. Individual fraction numbers are also indicated, as is starting material (ST). (B) A second gradient was run for the purpose of quantifying α-CP1 activity in individual fractions and after fractions were combined. The protein and electrophoretic shift gels are labeled as described above for panel A. (C) The DNA that was shifted by adding 4 µl of each fraction (as indicated below the bar graphs) across the 500 mM gradient shown in panel B was quantified by liquid scintillation counting. Binding activity (in standard units; see text) was plotted for each fraction. Two microliters from fractions 13 and 18 and fractions 14 and 17 were then

made up of the four polypeptides with $M_r$s of 33,000, 34,000, 37,000, and 38,000 (Fig. 4A, bottom). After sedimentation in the higher salt concentration, α-CP1-binding activity was reduced (Fig. 4A, bottom) and the residual activity was maximal in the region where the two sets of polypeptides overlapped (note that all binding experiments were performed at the same, optimal salt concentration, 80 mM KCl). We tested whether fractions enriched in α-CP1A and α-CP1B had complementary activities. Under these circumstances, we anticipated that the components of α-CP1, which were enriched at the boundaries of the sedimentation profile, might be able to interact to form a complex similar to that detected during sedimentation in 100 mM KCl. The interaction might produce complexes with substantially greater binding activities than that expected from the summation of activities found in the individual fractions. To test this possibility, affinity-purified α-CP1 was again separated on glycerol gradients containing 100 or 500 mM KCl (Fig. 4B and C). Again, all seven polypeptides migrated together in 100 mM KCl (Fig. 4B, top), whereas sedimentation in 500 mM KCl produced a distinct shift in the sedimentation profile of the polypeptides (Fig. 4B, bottom). A total of 4 μl of each fraction from the 500 mM salt gradient was assayed, and binding activity was quantified. Peak activity (in fractions 15 and 16) migrated between the ovalbumin and α-chymotrypsinogen markers (Fig. 4C). Comparison with the protein pattern (Fig. 4B, bottom) showed that the seven polypeptides overlapped to the greatest extent in this region. In contrast, while fraction 13 was enriched in the α-CP1A polypeptides, 4 μl of this fraction contained only 23% of the peak binding activity. Similarly, 4 μl of fraction 18 was enriched in the α-CP1B set of polypeptides, but it contained only 28% of peak binding activity. When we combined 2 μl of fraction 13 and 2 μl of fraction 18, however, the two fractions complemented and 70% of peak activity was restored (Fig. 4C). To ensure that the electrophoretic shift assay was responding linearly in this binding activity range, a third experiment was performed (Fig. 4D). Again, α-CP1 was separated on glycerol gradients containing 500 mM KCl. Binding activity was quantified across the gradient, and two fractions at the margins of the activity peak were titrated in the electrophoretic shift assay. The measured binding activity increased linearly with the addition of increasing amounts of each fraction (Fig. 4D). Combinations of these two fractions, however, resulted in a 156% increase in the activity expected from the contribution of each fraction alone (Fig. 4D). These results suggest that α-CP1 is made up of at least two distinctive sets of polypeptides, which we term α-CP1A and α-CP1B. The results suggest further that these polypeptides can interact to form a heterotypic complex with a high affinity for the α promoter.

To confirm our interpretation of the identity and structure of α-CP1, we reconstituted α-CP1-binding activity by eluting and renaturing selected polypeptides from SDS-polyacrylamide gels (Fig. 5). After electrophoresis of purified binding activity, five regions of an SDS-polyacrylamide gel were excised and subjected to an elution and renaturation protocol (16). The five selected regions included polypeptides with $M_r$s above 97,000 (region A), 43,000 to 97,000 (region B; we note here that purified α-CP1 is sometimes contaminated by a protein that migrates in this region), 30,000 to 43,000 (region C; this region contains one α-CP1A polypeptide and the polypeptides making up α-CP1B), 25,000 to 30,000 (region D; this region includes the remainder of α-CP1A), and less than 25,000 (region E) (Fig. 5A). After elution and renaturation of the polypeptides contained in each region, samples were tested individually, or in various combinations, for their ability to reconstitute α-CP1-binding activity. The results (Fig. 5B) showed that regions A, B, and E, either alone or in combination, were devoid of any DNA-binding activity. In contrast, both regions C and D contained weak amounts of DNA-binding activity. Neither of the electrophoretic shifts produced by the polypeptides that were renatured from these two regions, however, were identical to that

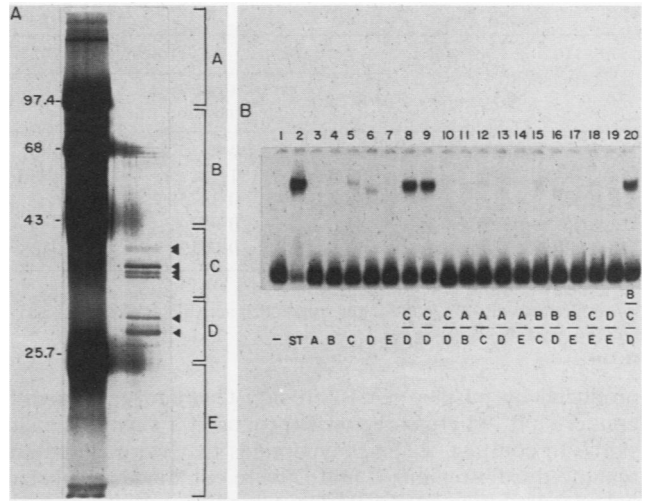


FIG. 5. Reconstitution of α-CP1-binding activity from SDS-polyacrylamide gels. (A) Affinity-purified α-CP1 was applied to an SDS-polyacrylamide gel, and a portion of the gel was stained with silver. $M_r$s (in thousands) of marker proteins are indicated on the left. Five regions that were excised from the remainder of the gel are also indicated on the right of panel A by the letters A through E. (B) The regions of the gel indicated alphabetically in panel A were subjected to an elution and reconstitution protocol and were then tested individually, or in combination, for their ability to reconstitute α-CP1-binding activity (see text) in an electrophoretic shift assay (all assays were performed with 80 mM KCl). Probe DNA was incubated without (lane 1) or with (lane 2) 2 μl of purified protein. Lanes 3 through 7 show the results that were obtained by incubating 2 μl of reconstituted protein from regions A through E, respectively. Lane 8, Protein (1 μl) eluted and renatured from region C plus 1 μl of protein from region D; lane 9, same as lane 8, but reactions contained 100 ng of poly(dI-dC); lane 10, same as lane 8, but reactions contained 100 ng of an oligonucleotide containing the α-CP1-binding site (see text); lanes 11 through 20 contained 1 μl of protein reconstituted from each of the regions indicated below each lane.

combined, and their binding activities were plotted (indicated on the rightmost part of the bar graph). The inset shows the electrophoretic shift gel corresponding to the plotted data. (D) To ensure that electrophoretic shift gels responded linearly, a third experiment was performed. α-CP1 was sedimented on a glycerol gradient containing 500 mM KCl. Individual fractions were tested for binding activity, and the results are plotted in the bar graphs on the left. The fractions tested and volumes used were as indicated above for panel C. In the middle two sets of bar graphs, fractions 10 and 15, at the periphery of the binding peak, were tested for their linear response in the shift gel assay. Fractions 10 and 15 were then combined and retested for binding activities, as illustrated in the bar graphs on the right. The inset shows the electrophoretic shift data plotted in the bar graphs.

TABLE 2. Purification of α-CP2a

| Fraction | Protein | | | Activity | | Sp act (units/mg) | Purification (fold) |
|---|---|---|---|---|---|---|---|
| | Vol (ml) | Concn (mg/ml) | Total amt (mg [%]) | Total (U) | Yield (%) | | |
| Nuclear extract[a] | 96 | 4.0 | 384 (100) | 206,496[b] | 100 | 537.75 | 1 |
| Phosphocellulose | 33.3 | 1.58 | 52.61 (13.7) | 129,870 | 62.9 | 2,468.5 | 4.59 |
| DEAE | 39.6 | 0.3375 | 13.36 (3.48) | 89,676 | 43.43 | 6,712.3 | 12.5 |
| Heparin Sepharose | 81.3 | 0.07 | 5.69 (1.48) | 51,056 | 24.73 | 8,973.0 | 16.7 |
| DNA affinity (3×) | 3.9 | 0.0012 | 0.00468 (0.0012) | 3,368 | 1.82 | 805,128.2 | 1,497.2 |

[a] Prepared from $3.94 \times 10^{10}$ cells.
[b] Approximately 10% contributed by α-CP2b activity.

produced by purified α-CP1 (region C produced a slightly greater shift, whereas region D produced a slightly smaller shift). In contrast, when polypeptides that were eluted and renatured from regions C and D were combined, a substantial reconstitution of α-CP1 DNA-binding activity was observed (Fig. 5B, lane 8). Additional tests showed that only mixtures containing polypeptides renatured from regions C and D (that is, only combinations including all polypeptides corresponding to α-CP1A and α-CP1B) reconstituted α-CP1-binding activity. These results confirm our identification of α-CP1-binding activity. They also confirm the results of the glycerol gradient experiments and strongly suggest that α-CP1-binding activity is made up of heterotypic protein subunits.

We quantified the enrichment and recovery of α-CP1-binding activity during purification. Significant enrichments were achieved on the DNA sequence affinity columns (Table 1). We estimate that conventional chromatographic steps followed by three successive applications of α-CP1 to the affinity column enriched activity nearly 3,000-fold, with an overall yield of slightly more than 3% (Table 1).

**Purification of α-CP2a and α-CP2b.** The chromatographic steps used to purify α-CP2a and α-CP2b are also illustrated in Fig. 1. After gradient elutions of DEAE and heparin-Sepharose columns, α-CP2a was enriched by less than 20-fold (Table 2). Fractions containing α-CP2 activity were applied to a DNA sequence affinity column, and the column was developed as described above. After three to four successive passes of α-CP2a over the affinity matrix, binding activity was significantly enriched, as determined by electrophoretic shift assays (Fig. 6). Visualization of the enriched proteins after separation on SDS-polyacrylamide gels (Fig. 7, lane 5) showed that α-CP2a-binding activity was made up of a polypeptide doublet with $M_r$s of 64,000 and 66,000. (In some experiments, the polypeptide with an $M_r$ of 64,000, itself, appeared to be made up of a nearly comigrating doublet.) Affinity purification of α-CP2b also enriched polypeptides with $M_r$s of 64,000 and 68,000 (Fig. 7, lane 6). Purified α-CP2a and α-CP2b appeared to be very similar when their one-dimensional protein patterns were compared. We note, however, that the ratio of the $M_r$-64,000 to the $M_r$-66,000 polypeptides was less in α-CP2b when compared with that in α-CP2a. As determined by densitometric

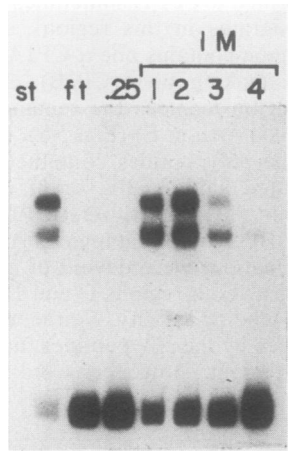


FIG. 6. Electrophoretic shift assays during affinity purification of α-CP2. α-CP2a (see text), which was enriched to the heparin-Sepharose step (st), was applied to an affinity column. Material in the column flowthrough (ft), 0.25 M KCl salt washes (.25), and four 500-μl fractions eluted from the column with a buffer containing 1 M KCl (1 M; lanes 1 through 4) were tested for their binding activities by electrophoretic shift.

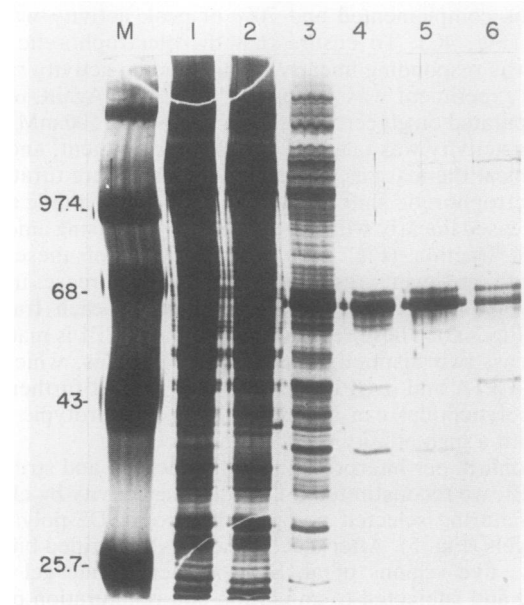


FIG. 7. SDS-polyacrylamide gel electrophoresis and visualization of affinity-purified α-CP2. Proteins were prepared and visualized as described in the legend to Fig. 3. Marker proteins (M) are indicated. Samples of protein from starting material (lane 1), the first affinity column flowthrough (lane 2), the first affinity column 1 M eluate (lane 3), and the 1 M eluate from the second and third affinity columns (lanes 4 and 5, respectively) are illustrated. Lane 6 contains the 1 M KCl eluate of the third affinity column of α-CP2b (see text). The molecular size (in thousands) of marker proteins is indicated on the left.

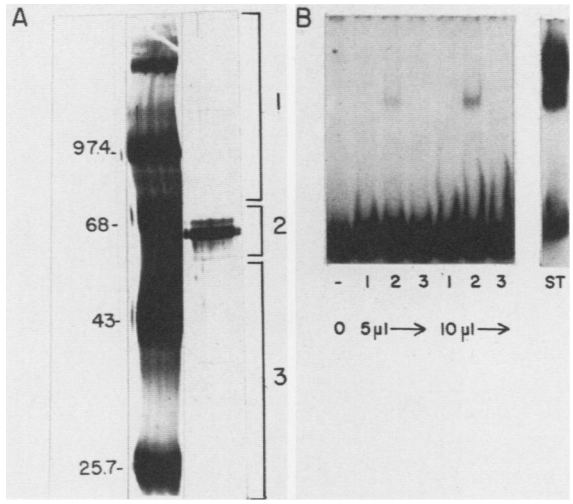


FIG. 8. Reconstitution of α-CP2-binding activity from SDS-polyacrylamide gels. (A) Affinity-purified α-CP2 was run on an SDS-polyacrylamide gel, and a portion of the gel was stained with silver. Marker proteins are also indicated on the left (molecular sizes are in thousands). Three regions excised from the remainder of the gel are indicated on the right. (B) Regions 1 to 3, indicated in panel A, were subjected to an elution and reconstitution protocol and tested for their ability to reconstitute α-CP2-binding activity (see text). Probe DNA was incubated without (indicated as 0) or with 5 or 10 μl of reconstituted proteins that eluted from the regions indicated at the bottom of the panel. Only region 2 reconstituted binding activity. The binding activity in the starting material is also indicated on the right (ST).

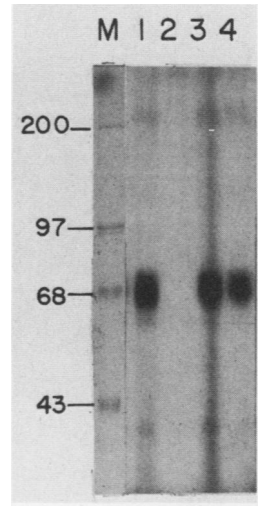


FIG. 9. UV-cross-linking of α-CP2-binding activity to the α-globin promoter. Affinity-purified α-CP2 was incubated with a uniformly radiolabeled fragment of the α-globin promoter in the presence or absence of specific nonradioactive competitors (see below), irradiated with UV, and prepared for electrophoresis in SDS-polyacrylamide gels as described in the text. Samples were incubated in the presence of 100 ng of poly(dI-dC) (lane 1) or with 80 ng of poly(dI-dC) plus 20 ng of the double-stranded oligonucleotide that was used to affinity purify α-CP2 (lane 2), 80 ng of poly(dI-dC) and 20 ng of a double-stranded oligonucleotide that was used to affinity purify α-IRP (lane 3), or with 80 ng of poly(dI-dC) and 20 ng of single-stranded oligonucleotide that was used to affinity purify α-CP2 (lane 4; see text). An autoradiograph of the dried SDS-polyacrylamide gel is shown, and the mobilities and molecular sizes (in thousands) of marker proteins (M) are indicated on the left.

scans of photographic negatives, these ratios were 5:1 for α-CP2a and 2:1 for α-CP2b.

We confirmed that the polypeptides with $M_r$s of 64,000 and 66,000 accounted for α-CP2-binding activity by two independent means. First, after electrophoresis of purified protein, three regions of an SDS-polyacrylamide gel were excised and subjected to an elution and renaturation protocol (15). A single region that contained the polypeptides with $M_r$s 64,000 and 66,000 was capable of reconstituting α-CP2-binding activity (Fig. 8). As an additional means of confirming that the polypeptides with $M_r$s of 64,000 to 66,000 accounted for α-CP2-binding activity, we used UV cross-linking to transfer isotope from a radiolabeled nucleic acid probe to a specifically bound protein. For these experiments, one strand of an α-globin promoter template was uniformly radiolabeled by primer extension and incubated with proteins that were enriched by the affinity columns. Incubations were performed in the presence or absence of oligonucleotides that are known to compete specifically for the binding of α-CP2a to the α-globin promoter (see above). After UV irradiation, proteins that interacted tightly with the labeled nucleic acid were covalently attached to the probe. Residual label and unlinked nucleic acid were removed by digestion with micrococcal nuclease and DNase I. Samples were precipitated with TCA, separated on an SDS-polyacrylamide gel, and subjected to autoradiography (see above). The results of this experiment (Fig. 9, lane 1) showed that polypeptides with $M_r$s of 64,000 to 66,000 are specifically labeled by UV cross-linking to the α-globin promoter. As expected, labeling was completely abolished by the addition of an oligonucleotide containing the α-CP2-binding site (Fig. 9, lane 2). In contrast, coincubation with an oligonucleotide containing either the α-globin IR box or a single-stranded oligonucleotide containing only one strand of the α-globin CCAAT box had no effect on protein labeling by UV cross-linking (Fig. 9, lanes 3 and 4). Results of these experiments strongly suggest that the polypeptides with $M_r$s of 64,000 to 66,000 account for the binding behavior of α-CP2a.

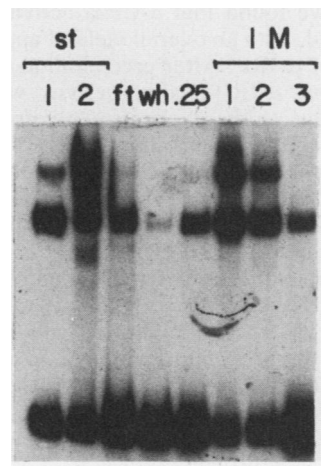


FIG. 10. Electrophoretic shift assays during affinity purification of α-IRP. α-IRP was enriched to the mono-Q step. Starting material (st) incubated without (lane 2) or with (lane 1) 100 ng of poly(dI-dC) is illustrated. α-IRP was applied to an affinity column. Material in the column flowthrough (ft), 0.1 M KCl salt washes (wh), 0.25 M KCl salt washes (.25), and three 500-μl fractions eluted from the column by a buffer containing 1 M KCl (1M; lanes 1 through 3) were tested for their binding activities by electrophoretic shift.

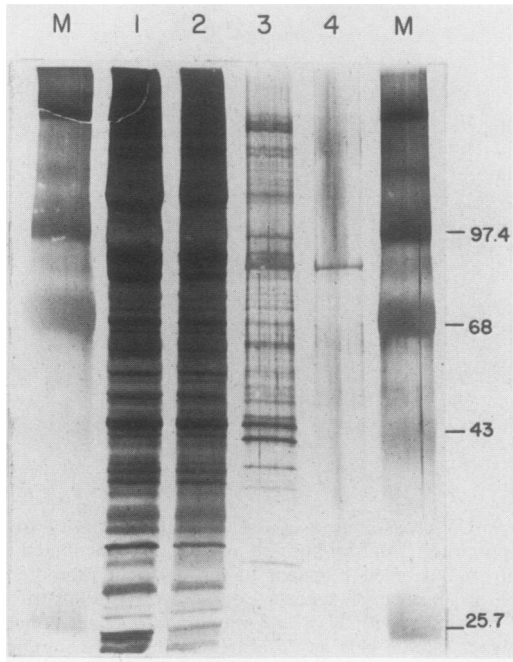


FIG. 11. SDS-polyacrylamide gel electrophoresis and visualization of affinity-purified α-IRP. Proteins were prepared and visualized as described in the legend to Fig. 3. Marker proteins (M) are indicated. Samples of protein from starting material (lane 1), the first affinity column flowthrough (lane 2), the first affinity column 1 M eluate (lane 3), and the 1 M eluate from a second affinity column (lane 4) are illustrated. The molecular sizes (in thousands) of marker proteins are also indicated on the right.

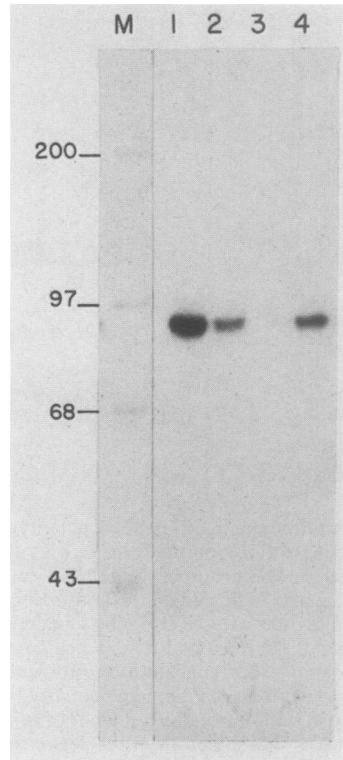


FIG. 12. UV-cross-linking of α-IRP-binding activity to the α-globin promoter. Affinity-purified α-IRP was incubated with a uniformly radiolabeled fragment of the α-globin promoter in the presence or absence of specific nonradioactive competitors (see below), irradiated with UV, and prepared for electrophoresis on SDS-polyacrylamide gels as described in the text. Samples were incubated with 100 ng of poly(dI-dC) (lane 1) or with 80 ng of poly(dI-dC) plus 20 ng of a double-stranded oligonucleotide that was used to affinity purify α-CP2 (lane 2) and α-IRP (lane 3; see text) or with 80 ng of poly(dI-dC) and 20 ng of a single-stranded oligonucleotide that was used to purify α-IRP (lane 4). An autoradiograph of the dried SDS-polyacrylamide gel is shown, and the mobilities of marker proteins (M) are indicated on the left (molecular sizes are indicated in thousands).

Results of a similar experiment confirmed that the polypeptides with $M_r$s of 64,000 and 66,000 that make up α-CP2b are also specifically radiolabeled by UV cross-linking (data not shown).

Table 2 quantifies α-CP2 purification through the affinity steps. After conventional chromatography followed by three affinity steps, we found that α-CP2a activity was enriched nearly 1,500-fold, with an overall yield of approximately 2%.

**Purification of α-IRP.** After precipitation with $(NH_4)_2SO_4$ and gel filtration, α-IRP-binding activity was applied to a high-performance anion exchange column (mono-Q) and recovered by salt gradient elution (Fig. 1). Peak activity fractions from the mono-Q column were then applied to a DNA affinity column. α-IRP-binding activity was significantly enriched after the DNA sequence affinity step (Fig. 10). The efficiency of the α-IRP affinity column, however, was not as great as those of the columns used to purify α-CP1- and α-CP2-binding activities. Thus, a substantial fraction (30%) of α-IRP-binding activity is found in the flowthrough of the first affinity column. In addition, a small fraction (10%) of the material bound to the column eluted with 0.25 M KCl (Fig. 10). The cause of this low binding efficiency is not known. The activity that eluted in the affinity column flowthrough and the 0.25 M salt cuts, however, were recovered and recycled onto the column. Following this protocol, most (80%) of the starting activity was eventually retained by the column and was eluted with 1 M salt.

After two such passes of α-IRP over the affinity column, a silver-stained SDS-polyacrylamide gel of the enriched polypeptides showed a single polypeptide species with an $M_r$ of 85,000 (Fig. 11, lane 4). To ensure that this polypeptide corresponded to α-IRP-binding activity, we again performed a UV cross-linking experiment. The results obtained by UV cross-linking in the presence (Fig. 12, lane 3) or absence (Fig. 12, lanes 1, 2, and 4) of specific oligonucleotide competitors confirmed that the protein with an $M_r$ of 85,000 coincides with α-IRP-binding activity.

Table 3 shows a quantification of the purification of α-IRP. After conventional chromatography and two passes over an affinity column, activity was enriched approximately 4,800-fold, with a total recovery of about 0.5%.

## DISCUSSION

We have previously described the enrichment of three factors that are present in MEL cell nuclear extracts that interact strongly with the promoter of the α-globin gene (3a). Two distinct CCAAT-binding proteins (α-CP1 and α-CP2) were described. These two factors have mutually exclusive binding sites and interact selectively with the α-globin promoter. Neither factor interacts with the adenovirus origin of replication (*ori*), suggesting that neither factor is related to the HeLa cell-derived CCAAT transcription factor CTF/

TABLE 3. Purification of α-IRP

| Fraction | Protein | | | Activity | | Sp act (units/mg) | Purification (fold) |
|---|---|---|---|---|---|---|---|
| | Vol (ml) | Concn (mg/ml)[a] | Total amt (mg [%]) | Total (U) | Yield (%) | | |
| Nuclear extract[a] | 67 | 5.4 | 361.8 (100) | 85,492 | 100 | 236.3 | 1 |
| Phosphocellulose | 82.4 | 3.3 | 271.9 (75.2) | 52,736 | 61.7 | 194.4 | 0.82 |
| DEAE | 17 | 1.8 | 30.6 (8.46) | 25,330 | 29.6 | 827.8 | 3.5 |
| Ultrogel | 17 | 0.335 | 5.7 (1.58) | 7,480 | 8.75 | 1,312.3 | 5.6 |
| Mono-Q | 3.05 | 0.18 | 0.549 (0.15) | 939.5 | 2.3 | 3,532.88 | 15 |
| DNA affinity (2×) | 3.71 | 0.0001 | 0.00037 (0.0001) | 422.5 | 0.49 | 1,142,000 | 4,833 |

[a] Prepared from $3.0 \times 10^{10}$ cells.

NF-1. In addition to the CCAAT-binding factors, a third factor (α-IRP) was enriched from MEL cells that interacted with a IR region that was located between the α-globin CCAAT and TATAA boxes. The affinity purification of all three proteins described here allowed us to begin to characterize the physical nature of each protein in more detail.

Our results suggest that α-CP1 is made up of seven polypeptides with $M_r$s that range from 27,000 to 38,000 (Fig. 3). This complex polypeptide pattern is not unusual when compared with those of other affinity-purified DNA-binding proteins (1, 2, 19, 22, 30, 31, 33). When we characterized the sedimentation behavior of the purified factor, we found that, in 100 mM KCl, all seven polypeptides migrated as a single complex that sedimented near BSA (4.2S). In contrast, during sedimentation in the presence of 500 mM KCl, the α-CP1 complex was at least partially disrupted. After sedimentation in the higher salt concentration, two distinct species of polypeptides were observed. These ensembles migrated at 3.6S (α-CP1A; made up of polypeptides with $M_r$s of 27,000, 28,000, and 35,000) and 2.8S (α-CP1B; made up of polypeptides with $M_r$s of 33,000, 34,000, 37,000, and 38,000). Under these conditions, α-globin-binding activity was distributed in the region of the gradient in which these two components overlapped. In addition, when fractions enriched in α-CP1A were combined with those enriched in α-CP1B, binding activity was greatly enhanced. Thus, the two components complemented one another to produce the observed binding activity (Fig. 4). These results were confirmed by experiments designed to reconstitute α-CP1-binding activity by eluting and renaturing specific polypeptides from SDS-polyacrylamide gels (Fig. 5). The results of these experiments confirmed that polypeptides with $M_r$s from 27,000 to 38,000 are required for α-CP1-binding activity. In addition, they showed that α-CP1-binding activity can be fully reconstituted only when all polypeptides corresponding to α-CP1A and α-CP1B are combined. Taken together, results of these experiments strongly suggest that α-CP1-binding activity is made up of a heterotypic protein complex. We emphasize, however, that a consideration of the observed sedimentation coefficients and $M_r$s of the individual proteins that make up α-CP1 indicate that additional information is required before a more specific model for this factor can be suggested.

The finding that α-CP1 is a heterotypic protein complex is consistent with results of several recent studies that suggest that heterologous complexes might be typical of eucaryotic CCAAT-binding proteins (7, 9, 16). The yeast transcriptional activators HAP2 and HAP3, for example, act in concert to bind a sequence (TGATTGGT) that is homologous to the CCAAT sequence found in promoters of higher eucaryotes (13, 28). In addition, in a recent series of reports, Chodosh et al. (7, 9) have shown that a CCAAT protein isolated from HeLa cells (and termed CP-1) is made up of heterologous subunits and that the yeast factors HAP2 and HAP3 are functionally interchangeable with the HeLa subunits. In this regard, we note that we have previously published the dimethyl sulfate protection pattern of α-CP1 bound to the α-CCAAT box (10). Comparison of this pattern of protection with the methylation interference pattern of the protein termed CP-1 by Chodosh et al. (7) or to the ubiquitously distributed CCAAT-binding protein termed NF-Y (12, 18) suggests that all these proteins interact with similar bases in the CCAAT box. In addition, α-CP1, NF-Y, and the HeLa cell-derived protein termed CP-1 all bind poorly to the adenovirus origin of replication. Together, these results suggest that these factors are related or are identical. It will be interesting to see whether affinity-purified NF-Y and HeLa CP-1 are made up of the same set of polypeptides that we observed for α-CP1. In addition, the functional similarity of HeLa CP-1 to HAP2 and HAP3 (9) raises the possibility that α-CP1, or its components, might also be related functionally to the yeast factors.

The finding that α-CP1 is a heterotypic complex suggests novel ways for regulating the binding of this factor to DNA. In this regard, we note that in a previous report (3a) we demonstrated that α-CP1 activity decreases three- to fivefold during MEL cell differentiation. The findings reported here suggest that this decrease might be mediated by mechanisms that do not require any reduction in the intracellular concentration of the components of α-CP1. During differentiation, for example, other cellular proteins (not yet identified) might form complexes with the components of α-CP1, thereby preventing their interaction and decreasing α-CP1-binding activity. While many other mechanisms might be envisaged, it is clear that the production of antisera against purified α-CP1 is required to test any mechanism that is postulated to play a role in regulating the level of α-CP1 activity during MEL cell differentiation.

The second factor we purified, α-CP2, bound largely upstream of, but overlapped with, the α-globin CCAAT box (3a). Affinity purification of this activity revealed a polypeptide doublet with $M_r$s of 64,000 and 66,000 (Fig. 7). Both UV cross-linking and renaturation of activity from SDS-polyacrylamide gels confirmed that these polypeptides are responsible for α-CP2-binding activity (Fig. 8 and 9). The affinity purification of α-CP2 should expedite further physical characterization of these polypeptides. We are interested in determining, for example, whether the polypeptides with $M_r$s of 64,000 and 66,000 share any peptides. If they do, it becomes of interest to determine what accounts for the difference in mobilities of the two proteins in SDS-polyacrylamide gels. In this regard, we note that in preliminary experiments we were unable to effect any changes in α-CP2-binding activity by treating the purified protein with phos-

phatases. We also note that the binding of α-CP2 to the α-globin promoter produces a characteristic doublet of shifted bands. Based on the results of renaturation and UV cross-linking experiments, however, we think it is highly unlikely that some other protein, which we have not visualized, could account for any of the observed band shifts. This suggests either that there are two α-CP2-binding sites in the target sequence or that protein-protein interactions are involved in the binding of α-CP2 to the α promoter. Experiments to distinguish between these possibilities are currently in progress. Lastly, it is of interest to determine the relationships between α-CP2 and other CCAAT-binding proteins. In this regard we note that Chodosh et al. (7) have characterized a second CCAAT-binding factor from HeLa cells which they also termed CP-2. Results of preliminary experiments in which the published methylation interference data of Chodosh et al. (7) were compared with the methylation protection pattern of α-CP2, however, suggest that these factors are distinct species (data not shown).

In addition to the two CCAAT-binding proteins, we purified a third factor (α-IRP) that interacted with two sequences that formed a pair of IRs between the CCAAT and TATAA boxes (Fig. 10 and 11). α-IRP is made up of a single polypeptide with an $M_r$ of 85,000, and UV cross-linking experiments confirmed that this polypeptide accounts for α-IRP-binding activity (Fig. 12). We note that the two binding sites for α-IRP on the α-promoter probably account for the doublet of shifted bands observed on the addition of concentrated α-IRP. We also note that because the α-globin IR boxes contained GC-rich sequences reminiscent of SP1-binding sites, we have previously tested the ability of α-IRP to bind the GC boxes in the 21-base-pair repeats of the simian virus 40 early promoter. We found that α-IRP binds as well to the α-promoter as it does to the simian virus 40 early promoter, suggesting that this erythroid factor might be related to the HeLa cell-derived transcription factor SP1 (3a). In addition to many chromatographic differences, however, the observed mobility of purified α-IRP in SDS-polyacrylamide gels is distinct from that of purified SP1 (5). Results of preliminary experiments also suggest that α-IRP footprints the simian virus 40 early promoter in a manner distinctive from that of SP1 (K. M. Barnhart and M. Sheffery, unpublished data). While there may be a family of SP1-like polypeptides, the simplest interpretation of our data is that α-IRP is distinct from SP1.

While we purified three polypeptides that interacted strongly with the α-globin promoter, the function that these proteins play in regulating globin gene expression during MEL cell differentiation is unknown. Both in vivo footprinting experiments and the development of an erythroid-derived in vitro transcription system should help to clarify the role that these proteins play in mediating globin gene expression. Lastly, we note that while we purified three factors that interacted strongly with the α-globin promoter, we observed additional erythroid factors that also bound the α-promoter. We are in the process of characterizing and purifying these factors.

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