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Purification and Characterization of Chicken Ovalbumin Gene Upstream Promoter Transcription Factor from Homologous Oviduct Cells

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Previous studies established that the chicken ovalbumin gene upstream promoter (COUP) sequence, which lies between −70 and −90 base pairs upstream from the cap site, is essential for the efficient transcription of the ovalbumin gene. A transcription factor which binds to this sequence has been purified from the homologous chicken oviduct cells. The purification scheme starting from oviduct nuclear extract involved a combination of conventional column and sequence-specific DNA affinity chromatography steps. Using gel retardation and DNase I footprinting techniques to assay COUP-binding activity, we achieved extensive purification of this factor. Binding competition studies with the purified factor indicated that it bound specifically to the COUP sequence and that the binding could be competed for only by the promoter DNA fragments or synthetic oligonucleotides containing the COUP sequence. The purified protein preparation showed multiple polypeptide bands on polyacrylamide gel electrophoresis. Renaturation of separated polypeptides after extraction from the gel matrix was carried out. The majority of renatured polypeptides exhibited specific binding to the COUP sequence.

The chicken ovalbumin gene offers attractive features for studying the regulation of tissue-specific expression of eucaryotic genes. Synthesis of ovalbumin mRNA in chicken oviducts is controlled by steroid hormones at the level of transcription (22, 23, 29, 35). The molecular mechanism of this hormonal regulation of transcription is not clear at present. Although the steroid hormone receptor might play a major role as an activator of this steroid-controlled gene, it is probably not the only factor regulating this gene expression. Recent studies carried out in different laboratories indicate that multiple cellular factors interact specifically with distinct promoter elements of eucaryotic genes and regulate the synthesis of mRNA by RNA polymerase II. *trans*-Acting factors binding specifically to the GGGCGG (GC box) sequence motifs (2, 6–9, 12, 16–18) and the CAAT box sequences (13, 15, 17) have been isolated and characterized. In addition, an array of other *trans*-acting regulatory factors, e.g., the heat shock transcription factor (24, 25, 32, 36, 38–40), adenovirus major late transcription factor (3, 4, 20, 27, 28, 31), a factor binding to the octanucleotide sequence ATTTGCAT of immunoglobulin genes (33), and GAL4, GCN4, and HAP1 proteins binding specifically to upstream promoter elements of yeast genes (for a review, see reference 34), have recently been described.

An understanding of the molecular control of expression of the ovalbumin gene requires the isolation and biochemical characterization of the protein factors which bind specifically to discrete *cis*-regulatory sequences of this gene. We have demonstrated previously that an upstream promoter element of the ovalbumin gene called the chicken ovalbumin gene upstream promoter (COUP) box is located in the region from −70 to −90 base pairs (bp) and is essential for in vitro and in vivo transcription of the gene (10, 19, 26). We have also reported the isolation of a *trans*-acting factor from HeLa cells (30) which binds specifically to the COUP sequence and enhances in vitro transcription of this gene in a reconstituted transcription assay system. In this paper we describe the purification and characterization of the protein from homologous chicken oviduct tissue which binds to the same COUP box sequence.

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MATERIALS AND METHODS

Preparation of oviduct nuclear extract and purification of COUP-binding protein. The nuclear extract was prepared from diethylstilbestrol-stimulated chicken oviducts. About 250 g of oviduct tissue was minced and washed several times with ice-cold phosphate-buffered saline (20 mM potassium phosphate [pH 7.9], 130 mM NaCl, 5 mM KCl). All subsequent operations were carried out at 0 to 4°C. The tissue was minced in about 500 ml of buffer A (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.9], 10% sucrose, 4 mM dithiothreitol [DTT], 2 mM $MgCl_2$, 0.2 mM EDTA) and homogenized in a Tissuemizer for 2 min. The homogenate was centrifuged at 10,000 × *g* for 10 min; the pellet was collected and suspended in 500 ml of buffer B (10 mM HEPES, pH 7.9, 2 mM $MgCl_2$, 10 mM KCl, 4 mM DTT). The suspension was homogenized by several strokes of a Kontes pestle in a tissue grinder. The homogenate was centrifuged again at 10,000 × *g* for 10 min. The pellets were washed once more with buffer B (250 ml) and centrifuged. The next step involved the suspension of pellets in buffer C (20 mM HEPES, 2 mM $MgCl_2$, 0.2 mM EDTA, 0.6 M NaCl, 4 mM DTT, and 20% glycerol) to a final NaCl concentration of 0.5 M. After homogenization by a Kontes pestle, the homogenate was slowly stirred at 4°C for 30 min and was then spun at 10,000 × *g* for 30 min. The supernatant was collected, and the protein was precipitated by adding 0.43 g of ammonium sulfate per ml of extract. The resulting solution was stirred for an additional 20 min. This was followed by centrifugation, and the pellet was redissolved in a minimum volume of buffer D (20 mM HEPES, pH 7.9, 100 mM

KCl, 20% glycerol, 4 mM DTT, 0.2 mM EDTA) and dialyzed for 16 h against the same buffer with two changes. The dialyzed extract was finally clarified by centrifugation at 10,000 × *g* for 15 min.

The nuclear extract was fractionated on a DEAE Sephadex (A-25; Pharmacia) column as described previously (37). Approximately 200 ml of nuclear extract was loaded onto a 250-ml DEAE-Sephadex column preequilibrated with buffer D containing 50 mM $(NH_4)_2SO_4$. The flowthrough fraction (DE50) was dialyzed against buffer D containing 200 mM KCl and applied on a phosphocellulose column (30 ml) preequilibrated with the same buffer. The column was washed thoroughly with the same buffer, and the bound protein was eluted with a 240-ml linear gradient of 200 to 800 mM KCl in buffer D. The COUP-binding activity was assayed across the gradient fractions by the gel retardation assay. The active fractions were pooled and dialyzed against buffer D containing 0.2 M KCl. The dialyzed protein solution was then chromatographed on a 4-ml heparin-Sepharose column preequilibrated with buffer D containing 0.2 M KCl. The column was washed with the same buffer, and the protein retained on the column was eluted with a 40-ml linear gradient of 0.2 to 0.7 M KCl in buffer D. The COUP-binding activity was monitored as before, and the active fractions were pooled and adjusted to 0.3 M KCl. The protein solution was then subjected to DNA affinity chromatography.

DNA affinity column. The procedure for construction of the specific DNA affinity column was similar to that of Kadonaga et al. (18), except for a few modifications. The 23-mer synthetic oligonucleotide strands (see Fig. 4A) were purified separately on a 20% polyacrylamide gel. Equal amounts of these strands were then mixed together and allowed to anneal. The double-stranded oligonucleotide was further purified through a 15% acrylamide gel. About 500 μg of purified oligonucleotide was incubated in a 500-μl reaction mixture containing 50 mM Tris hydrochloride (pH 7.5), 10 mM $MgCl_2$, 4 mM DTT, 0.1 mM EDTA, 1 mM ATP, 0.1 μCi of [γ-^{32}P]ATP and T4 polynucleotide kinase at 37°C for 90 min. The reaction mixture was then chilled, 60 μl of 10 mM ATP and 200 U of T4 DNA ligase were added, and incubation was continued at 4°C for 16 h. A portion of the reaction mixture was tested to check concatemer formation. The mixture containing the DNA concatemer was then treated with phenol-chloroform (1:1), and the DNA was precipitated by ethanol in the presence of 1 M ammonium acetate. The precipitation step was repeated one more time, and the pelleted DNA was washed with 70% ethanol, dried, and redissolved in 20 mM potassium phosphate buffer, pH 8.0.

The coupling reaction was carried out essentially by the method of Arndt-Jovin et al. (1). About 300 mg of dry cyanogen bromide-activated Sepharose 4B (Pharmacia) was washed extensively with 1 mM HCl on a sintered glass funnel. The washed resin was then quickly washed with 20 mM potassium phosphate buffer (pH 8.0) and transferred immediately to a 15-ml polypropylene tube containing the concatemer DNA in 6 ml of 20 mM phosphate buffer (pH 8.0). The slurry was incubated overnight at room temperature with mild shaking. The slurry was then transferred on a sintered glass funnel and washed sequentially with 20 mM phosphate buffer, 1 M phosphate buffer, 1 M KCl, and water. Finally the resin was stored at 4°C in a buffer containing 10 mM Tris hydrochloride (pH 7.5), 100 mM KCl, 0.1 mM EDTA, and 0.02% sodium azide. The amount of DNA bound to the resin was determined by counting the radioactivity bound to a given amount of slurry. Typically, about 10 to 15% of the input DNA was coupled to the resin and about 5 μg of concatemer DNA was bound per 100 μl of Sepharose 4B.

For DNA affinity chromatography, about 200 μl of the affinity resin was incubated with a portion (3 ml) of the heparin-Sepharose-purified protein fractions in the presence of an excess (~100-fold) of poly(dI-dC) as nonspecific competitor. The final KCl concentration in the reaction mixture was 0.3 M, and the incubation was carried out for 2 h. The slurry was then packed into a column and washed extensively (30 column volumes) with buffer containing 0.3 M KCl. The column was then sequentially eluted with buffers containing 0.5, 0.6, 0.7, 0.8, 0.85, 0.9, and 1 M KCl. The eluates at different salt concentrations were checked for COUP-binding activity. Samples (50 μl) were taken from each of these fractions and precipitated by 10% trichloroacetic acid. The proteins were redissolved in gel loading buffer and analyzed on a 10% polyacrylamide gel. The gel was then silver stained by the method of Morrissey et al. (21). The protein concentrations of these fractions were estimated by comparing the intensities of the silver-stained protein bands in the sample lane with those of silver-stained standard protein bands of known concentration in adjacent lanes.

To carry out the second cycle of DNA affinity chromatography, the active fractions after the first cycle were pooled and adjusted to 0.3 M KCl. The procedure that was used for the first DNA column was repeated with only one difference. The concentration of the nonspecific competitor was much less (about 10-fold) to minimize the loss of the specific protein through nonspecific absorption. After assay of activity, the peak active fractions were pooled, divided into small vials, and stored frozen at −80°C. A sample (100 μl) of the purified protein was analyzed on a sodium dodecyl sulfate (SDS)-polyacrylamide gel as described above.

Gel retardation assays. Gel retardation assays were performed as described previously (11, 30) with two modifications. They were carried out in the absence of Mg^{2+} ions from the reaction mixture, and the incubations were done on ice. The gels were dried before being exposed to X-ray films. DNase I footprinting reactions were carried out as described previously (26, 30).

In vitro transcription assays. For testing the transcriptional activity of the oviduct COUP factor, an in vitro-reconstituted transcription assay system was used as described previously (30). The transcripts were analysed by RNase A mapping (26). The fractions DE175, DE500, S300-IK, and S300-II were prepared from HeLa nuclear extract as described before (30). The DNA template pSVOG contained ovalbumin gene sequence from positions −221 to +41 and also sequences for simian virus 40 (SV40) early gene transcription (26, 30).

Renaturation of COUP-binding protein after SDS-PAGE. The method of Hager and Burgess (14) was followed with a few modifications. A portion (about 200 μl) of the affinity-purified protein was concentrated by acetone precipitation. The pellet obtained after centrifugation was dried and redissolved in SDS gel loading buffer and heated at 55°C for 15 min. The sample was applied then to a 10% SDS–polyacrylamide gel, and polyacrylamide gel electrophoresis (PAGE) was performed at 30 mA for 2 h. After electrophoresis the gel was stained briefly with ice-cold 0.25 M KCl until the standard molecular weight markers become visible. The gel slices were cut out along the sample lane, which did not show any visible KCl-stained band. The positions of these slices in comparison to the prestained (BioRad) and KCl-stained molecular weight markers in adjacent lanes were noted. Each gel slice was then placed into a small

dialysis bag containing SDS gel running buffer and 100 μg of bovine serum albumin (nuclease-free; BM Biochemicals). The bags were placed in a large horizontal gel trough filled with SDS gel running buffer, and electric current at 50 mA was applied for 3 h. The solutions containing the electro-eluted proteins were recovered from the bags and put into 15-ml siliconized corex tubes. The protein was precipitated by addition of 5 volumes of acetone (Gold-label; Aldrich Chemicals) at −20°C. The tubes were spun for 30 min at 8,000 rpm. The pellets were rinsed once with 80% acetone and dried. The pellets were finally dissolved in 100 μl of 6 M guanidine hydrochloride. The resulting solutions were dialyzed against buffer D for 16 h with one change. The dialyzed samples were assayed for COUP-binding activity.

RESULTS

Purification of COUP-binding protein from oviduct tissue. The COUP-binding activity was tracked throughout its purification by a gel retardation assay as described by Sagami et al. (30). A radiolabeled fragment of ovalbumin promoter (−269 to −44 bp) containing the COUP sequence was incubated with different protein fractions in the presence of an excess of a nonspecific competitor DNA, poly(dI-dC), to detect the formation of specific DNA-protein complexes on a native polyacrylamide gel. Another promoter fragment (−269 to −103 bp) lacking the COUP sequence was used as a control for these reactions. Due to the presence of high levels of contaminating nuclease activities in the earlier steps of purification, DNase I footprinting assays or in vitro transcription assays could not be carried out to monitor the COUP-binding activity. However, the gel retardation assay, modified by omitting magnesium ions from the reaction mixtures and carrying the incubations on ice, could be used successfully without significant interference from DNase activities. Consequently, the gel retardation assay was clearly the method of choice to monitor the purification of COUP-binding factor.

The crude oviduct nuclear extract was first applied to a DEAE-Sephadex column and fractionated into three fractions, DE50, DE175, and DE500, as described by Tsai et al. (37). By the gel retardation assay, the COUP-binding activity was found to be present in the DE50 fraction. This fraction was then applied to a phosphocellulose column preequilibrated with a buffer containing 0.2 M KCl. About 60% of the total protein loaded onto the column came out in the flowthrough fraction, which did not show any COUP-binding activity. The bound protein was eluted with a linear gradient of 0.2 to 0.8 M KCl in buffer. Individual fractions were assayed for COUP-binding activity. As shown in Fig. 1A, a distinct complex formation was observed across fractions 30 to 52 when the fragment −269 to −44 bp was used in the assay. When duplicate assays were performed with control fragment −269 to −103 bp, no such complex formation was observed (data not shown). The slightly lower molecular weight of the complexes formed when fractions 48 to 52 were used can be attributed to a protease-degraded form of COUP box-binding protein, which is probably due to the presence of a relatively high amount of protease contaminations in these fractions. The specific DNA-binding activity appeared approximately between 0.35 and 0.45 M KCl (Fig. 1B). The peak active fractions 32 to 44 were pooled and adjusted to 0.2 M KCl before being loaded on a heparin-Sepharose column.

The majority (about 70%) of the protein applied to the heparin-Sepharose column eluted in the unbound fraction

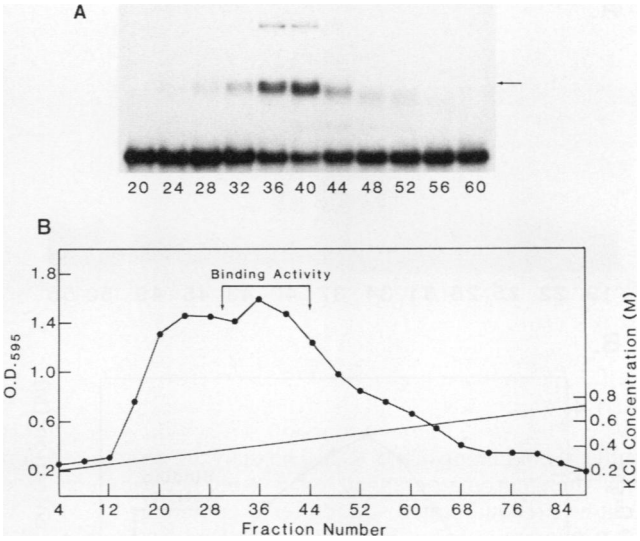

FIG. 1. Phosphocellulose column chromatography. DEAE-Sephadex column flowthrough fraction DE50 was applied to a phosphocellulose column and eluted with a linear gradient of potassium chloride as described under Materials and Methods. (A) Binding assay profile. A 1-μl amount of each of the indicated column fractions was incubated in a 10-μl reaction mixture containing 10 mM HEPES (pH 7.9), 100 mM KCl, 2 mM DTT, 0.1 mM EDTA, 10% glycerol, 0.5 ng of γ-^{32}P-labeled DNA fragment −269 to −44 bp, and 2 μg of poly(dI-dC) for 20 min at 0°C. The reaction mixtures were then loaded on a 5% native polyacrylamide gel. The electrophoresis was carried out in Tris-EDTA-borate buffer for about 2 h. The gel was then dried and exposed to X-ray films at −70°C. Arrow indicates the position of migration of the COUP factor-DNA complex in the gel. (B) Protein and salt gradient profiles. Salt concentration was determined by conductivity with calibrated standard solutions.

with no detectable COUP-binding activity. The protein retained on the column was eluted with a linear gradient of 0.2 to 0.7 M KCl. The results of the binding assay with the fragment −269 to −44 bp used as a probe indicate that a number of DNA-protein complexes of different molecular weights were formed across the gradient (Fig. 2). However, when the control fragment −269 to −103 bp was used, there was no detectable complex formation with fractions 40 to 49 (data not shown). This result indicated that fractions 40 to 49 from the heparin-Sepharose column contained a protein which did bind specifically to the COUP sequence. Recent experiments carried out in our laboratory suggest that the other complexes observed in the gradient profile between 0.3 to 0.45 M KCl (fractions 25 to 37) might be due to the binding of other specific *trans*-acting factors to regulatory sequences further upstream (5′) of the COUP sequence. As shown in Fig. 2, the COUP-binding activity appeared at the far shoulder of the peak of the protein profile at an approximate salt concentration of 0.45 to 0.55 M KCl. The peak active fractions were pooled and adjusted to 0.3 M KCl for the next chromatography step. Heparin-Sepharose column chromatography removed effectively most of the contaminating nuclease activities, and the protein was purified about 300-fold compared with the DE50 fraction (Table 1).

At this stage we tested the partially purified COUP-binding factor for transcriptional activity. For this purpose we used an in vitro-reconstituted transcription assay system as described previously (30). In this assay, the accurate transcription of the ovalbumin and SV40 early gene templates is carried out in the presence of a combination of

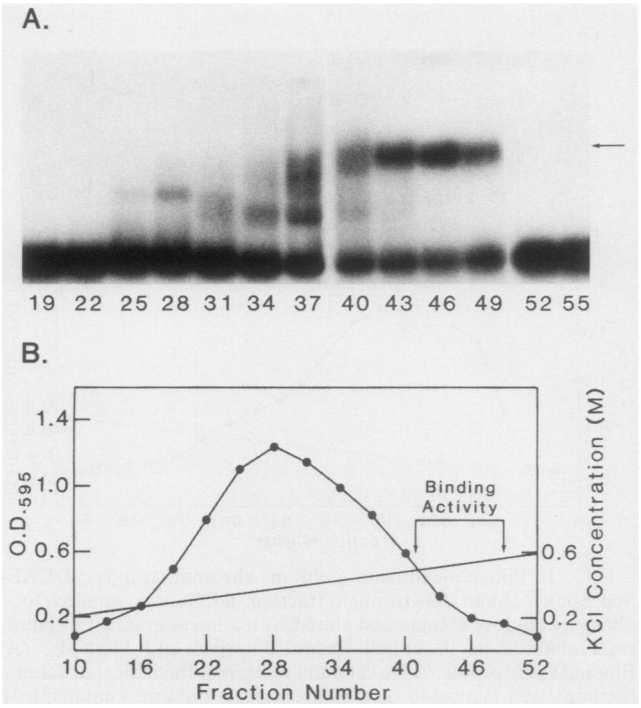

FIG. 2. Heparin-Sepharose column chromatography. Active fractions from the phosphocellulose column were pooled and applied to a heparin-Sepharose column and eluted with a linear KCl gradient as described under Materials and Methods. (A) Binding assay profile. Assays were carried out as described in the legend to Fig. 1. (B) Protein and salt gradient profiles.

fractions such as DE175, DE500, S300-I, and S300-II, which act as the source of transcription factors. These fractions were prepared by fractionation of HeLa nuclear extract (30). Our previous studies (30) established the requirement for fraction S300-I, which contained the COUP-binding transcription factor, for in vitro transcription of the ovalbumin gene. In the experiments described in Fig. 3, we replaced the HeLa S300-I fraction in the reconstituted system with different amounts of our heparin-Sepharose-purified oviduct COUP-binding protein. With increasing amounts of this factor, we observed a significant increase in the level of accurately initiated transcripts from the ovalbumin gene (Fig. 3A) compared with the control lane, which lacked any COUP factor. The transcription of the SV40 early genes, however, remained unaffected after addition of this factor (Fig. 3B). These results demonstrated that the oviduct COUP-binding factor possessed specific transcriptional activity and could substitute for the HeLa COUP transcription factor in the in vitro reconstitution assays.

TABLE 1. Purification of oviduct COUP-binding factor

Fraction	Total protein (mg)	Volume (ml)	Sp act (U/mg)[a]	Purification (fold)	Yield (%)
Nuclear extract	950	200	ND[b]	ND	ND
DEAE-Sephadex	700	340	24	1	100
Phosphocellulose	36	32	360	15	77
Heparin-Sepharose	1.5	8	7,000	290	63
DNA affinity 1	0.003	3	2.6×10^6	1.35×10^5	46
DNA affinity 2	0.001	1.25	4.6×10^6	1.90×10^5	27

[a] One COUP-binding unit = 1 fmol of DNA probe shifted in the gel retardation assay.
[b] ND, Not determined.

The results of DNase I footprinting analyses with the phosphocellulose and heparin-Sepharose column-purified fractions strongly suggested that the boundaries of the COUP factor-binding site on the ovalbumin promoter were between positions −70 and −90 bp (unpublished observation). Our previous studies with the COUP-binding protein isolated from HeLa cells also indicated that this protein footprinted between −70 and −90 bp upstream of the ovalbumin cap site. Using this information, we synthesized an oligomer (23 nucleotides) spanning this sequence (Fig. 4). A sequence-specific DNA affinity column was then constructed by ligating these synthetic oligomers to DNA concatemers and finally covalently linking the concatemers to a Sepharose resin. We estimated that about 5 μg of concatemer DNA was coupled per 100 μl of resin. The DNA-Sepharose resin was then incubated with the heparin-Sepharose-purified fractions in the presence of an excess of nonspecific poly(dI-dC). The resin was then packed into a column and washed extensively with buffer containing 0.3 M KCl. The bound protein was eluted in a stepwise manner with an increasing concentration of KCl (0.4 to 1 M) in buffer.

The flowthrough and the fractions eluted at different salt concentrations were tested for COUP-binding activity. There was no detectable activity in the flowthrough fraction. The COUP-binding activity eluted typically between 0.5 and 0.75 M salt (Fig. 4). Samples of each eluted fraction were precipitated by trichloroacetic acid, analyzed by SDS-PAGE, and silver stained. A similar analysis of the protein composition of a portion of the protein fraction applied to the column was done and revealed numerous polypeptide bands (Fig. 5A). The gel pattern of active fractions eluted from the affinity column exhibited at least four major polypeptides: M_r 44,000 (44K), 39K, 34K, and 27K polypeptides and

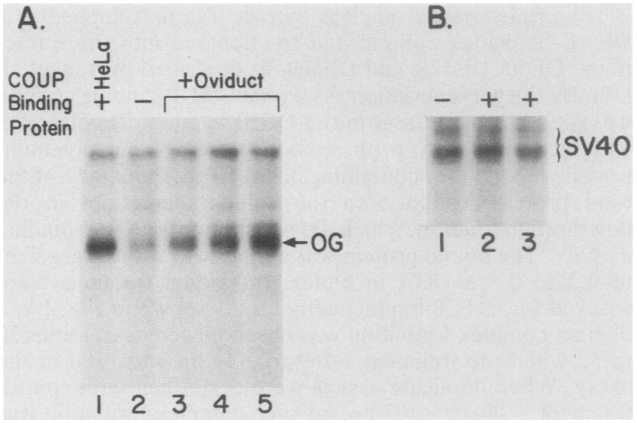

FIG. 3. Requirement of oviduct COUP-binding factor for ovalbumin gene transcription. The transcription of the ovalbumin gene and SV40 early genes was examined by using an in vitro-reconstituted system as described in the text. Fractions DE175, DE500, S300-I, and S300-II were obtained from HeLa nuclear extract. DE175 (3 μl), DE500 (5 μl), and S300-II (4 μl) were used in the absence (−) or presence (+) of HeLa S300-I (5 μl) or different amounts of oviduct COUP factor. pSVOG (1.05 μg) was used as the template. The arrow and the bracket indicate the positions of the correctly initiated transcripts. (A) Lanes: 1, with HeLa S300-I; 2, no COUP factor; 3 to 5, with 1, 2, and 3 μl of oviduct COUP factor, respectively. (B) Lanes: 1, no COUP factor; 2 and 3, 1 and 3 μl of oviduct COUP factor, respectively.

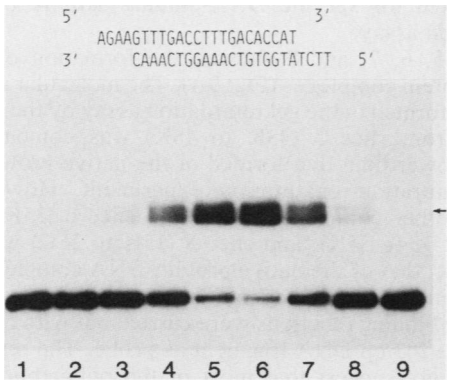

FIG. 4. Sequence-specific DNA affinity chromatography. (Top) Sequence of the 23-mer synthetic double-stranded oligonucleotide used for the construction of the sequence-specific DNA affinity column. The procedure for the affinity column construction is described in the text. (Bottom) Profile of COUP-binding activity eluted from the first DNA affinity column. The active fractions from the heparin-Sepharose column were pooled and incubated with the DNA affinity resin as described under Materials and Methods. Samples (2 μl) of each of the fractions eluted at different salt concentrations were assayed for binding activity. Lanes 1 to 9 indicate the binding activity with fractions eluted with buffer D containing 0.3, 0.4. 0.5, 0.6, 0.7, 0.8, 0.85, 0.9, and 1 M KCl, respectively. The arrow indicates the position of the COUP factor-DNA complex.

several minor high-molecular-weight polypeptides (Fig. 5B). To further purify the COUP protein, these active fractions were pooled and reapplied on the specific DNA affinity column. The procedure is similar to that described for the first DNA affinity chromatography step. The amount of nonspecific competitor DNA added in the second affinity column step was much less than in the first step. When the eluted COUP protein fraction from the second DNA column was analyzed on an SDS gel, the resulting gel pattern consisted of only four polypeptides again, 44K, 39K, 34K, and 27K (Fig. 6). The minor high-molecular-weight polypeptides were all removed after this step. As estimated from our binding assays, one cycle of specific DNA affinity column chromatography purified the protein an additional 500-fold compared with the heparin-Sepharose step. As indicated in Table 1, we achieved approximately 190,000-fold purification from the DE50 material with this protocol. We should, however, take into account the possibility of an overestimation of this fold purification. In early steps of purification, high amounts of nonspecific competitor DNA had to be added in the binding assays carried out with crude protein fractions. This created the possibility of loss of some specific binding units through nonspecific absorption to the competitor DNA. This might have led to underestimation of the purity of the cruder fractions and eventually to overestimation of the overall fold purification. In addition, the presence of inhibitors of binding (or function) in the crude preparations and their subsequent removal during purification may also have contributed to a similar overestimation. Nevertheless, in the absence of evidence to the contrary, the level of purification estimated in Table 1 most certainly is consistent with a rather low concentration of this protein in oviduct cells.

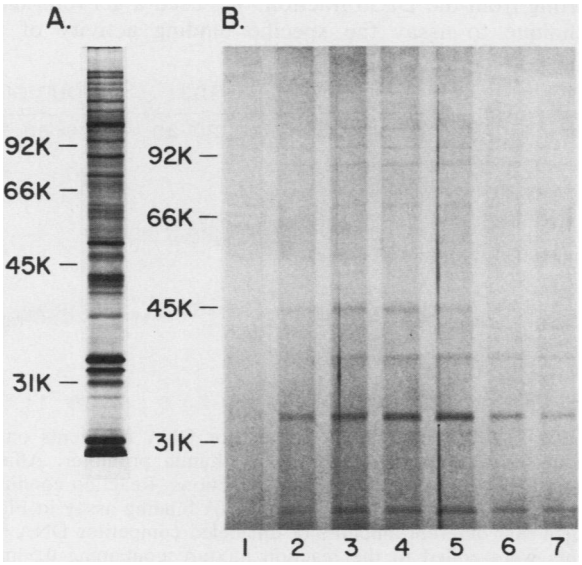

FIG. 5. SDS-PAGE analysis of fractions applied to and eluted from the first DNA affinity column. (A) A portion (20 μl) of the pooled heparin-Sepharose fractions was analyzed on an SDS-polyacrylamide gel as described under Materials and Methods. (B) Portions (50 μl) from each of the fractions eluted at different salt concentrations from the first DNA affinity column were analyzed similarly. Lanes 1 to 7 indicate the silver stain profile of the fractions eluted by 0.5, 0.6, 0.7, 0.8, 0.85, 0.9, and 1 M KCl, respectively.

Characteristics of oviduct COUP-binding factor. To demonstrate that the COUP factor purified by the specific DNA affinity chromatography had the same binding domain as the COUP factor isolated from HeLa cells, DNase I footprinting reactions were carried out with the purified oviduct factor. The results of this experiment (Fig. 7) show that the boundaries of the footprint by the oviduct COUP protein mapped between positions −70 and −90 bp of the ovalbumin promoter. The homologous oviduct factor footprinted at exactly the same region of the promoter as the HeLa factor.

To determine the specificity of binding of the purified COUP factor from oviduct, competition experiments were designed with unlabeled promoter DNA fragments with or without the COUP sequence, i.e., the −70 to −90 bp region (Fig. 8). An unlabeled DNA fragment (−753 to −44 bp) strongly competed with the binding of the labeled probe to the protein at a molar ratio of competitor to DNA fragment

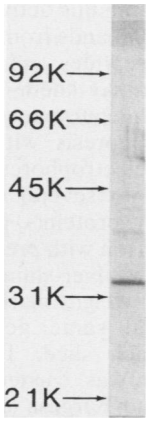

FIG. 6. SDS-PAGE analysis of the COUP-binding fraction after second cycle of DNA affinity chromatography. The purified protein (100 μl) was applied to an SDS gel and analyzed as described in the legend to Fig. 5.

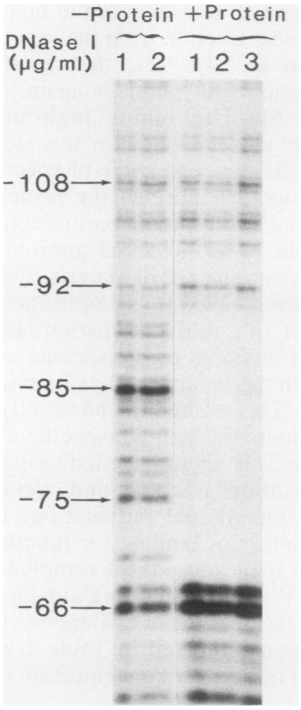

FIG. 7. DNase I footprinting analysis of DNA affinity column-purified COUP-binding protein. Portions (5 μl) of affinity-purified protein were added to the lanes indicated. The numbers on the left indicate positions on the ovalbumin upstream sequence (in base pairs) determined by Maxam-Gilbert sequencing.

of 10. A control fragment from −753 to −103 bp, which had the COUP sequence deleted, failed to compete even at a higher molar ratio of 30. When we used the synthetic 23-mer oligonucleotide as a competitor, we found it able to compete for binding with the labeled fragment completely at a molar ratio of 20. The oligomer was found to be an effective competitor even when a lower molar ratio of 10 was tested (data not shown). These studies indicated clearly that the oviduct COUP factor possessed the same binding specificity as the HeLa COUP transcription factor.

Renaturation and identification of oviduct COUP binding activity after SDS-PAGE. To identify the polypeptide(s) responsible for COUP-binding activity, we decided to separately excise the protein bands from an SDS-polyacrylamide gel, recover the polypeptides and renature them after removal of SDS, and assay them individually for specific binding activity. For this purpose we carried out a preparative SDS gel electrophoresis with a sample of affinity-purified protein. After electrophoresis the gel lane containing the sample was cut into several slices. The approximate molecular weights of the protein(s) present in each slice were determined by comparison with prestained molecular weight markers as well as the silver-stained patterns of a sample protein and molecular weight marker proteins which were run alongside in the same gel. The proteins were electroeluted from each slice. The renaturation of the electroeluted proteins was carried out by following the procedure of Hager and Burgess (14). In essence, the SDS was at first removed with acetone, and the proteins were completely unfolded by treatment with 6 M guanidine hydrochloride and then allowed to renature slowly be removal of the denaturing agent by dialysis. The renatured proteins were tested for specific DNA-binding activity by the gel retardation assay.

Slices 5, 6, 7, and 8 showed the formation of discrete DNA-protein complexes (Fig. 9A). The molecular size of the complex formed in the gel retardation assay by the renatured protein from slice 5 (43K to 48K) was comparable but slightly lower than that formed of the native protein before the denaturation-renaturation experiment. However, the sizes of other complexes formed by slice 6 (35K to 43K), slice 7 (25K to 35K), and slice 8 (15K to 25K) were much lower than that of the native protein-DNA complex. To test the specificity of binding of the proteins in these slices, duplicate binding reactions were carried out with the control −269 to −103 bp DNA fragment as probe. The results (Fig. 9B) strongly suggest that most of the polypeptides eluted from these slices bound specifically to the DNA test fragment containing the COUP sequence.

DISCUSSION

The hormonal regulation and expression of the ovalbumin gene is highly tissue specific. One approach toward understanding the molecular regulation of this gene would be to purify the *trans*-acting factors interacting with the promoter element of the ovalbumin gene and then to create a homologous reconstituted system to study the regulation of its expression. This type of approach was initiated earlier by Tsai et al. (37), who demonstrated that a chicken oviduct cell extract can be fractionated into multiple components which can functionally replace similar components in a reconstituted HeLa cell-free transcription system. In this paper we describe the purification of a homologous *trans*-acting factor which binds specifically to the COUP box promoter element of the ovalbumin gene. We fractionated the oviduct nuclear extract by DEAE-Sephadex, phosphocellulose, and heparin-Sepharose column chromatography. By using specific DNA affinity chromatography in the final step of purification, we achieved extensive purification of the COUP-binding protein starting from the DE50 fraction. We used a gel retardation technique to assay the specific binding activity of this

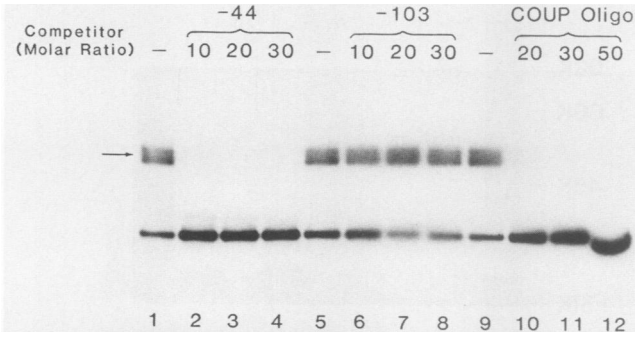

FIG. 8. Effect of different competitor DNA fragments on the binding of COUP protein to the ovalbumin promoter. Affinity-purified protein was used for these reactions. Reaction conditions were the same as described for the DNA binding assay in Fig. 2, except that different amounts of unlabeled competitor DNA fragments were added to the reaction mixture containing 0.5 ng of labeled DNA fragment before the protein fraction was added. The molar ratios of the competitor to the labeled DNA fragment are indicated on the top of each lane. Lanes 1, 5, and 9, no competitor DNA fragment; lanes 2 to 4, fragment −753 to −44 bp as competitor; lanes 6 to 8, fragment −753 to −103 bp as competitor; lanes 10 to 12, synthetic double-stranded COUP oligomer −90 to −70 bp as competitor. The arrow indicates the position of the COUP factor-DNA complex.

protein. The assay was both simple and rapid, and with only a little modification we successfully avoided any interference from contaminating nuclease activities during assays of the early purification steps.

The silver-stained SDS gel profile of the affinity-purified COUP protein revealed four polypeptides of 44K, 39K, 34K, and 27K. When these polypeptides were individually extracted from the gel and renatured, the majority of them exhibited specific COUP-binding activity. As shown in Fig. 9, there was also a distinct difference in the molecular sizes of the protein-DNA complexes formed by using these polypeptides. The mechanism by which multiple COUP-binding polypeptides of various molecular sizes are generated from the native protein and detected during the denaturation-renaturation experiment is not totally clear at present. In experiments with the purified HeLa COUP protein, we found that more than one polypeptide in the 45- to 50-kilodalton size range exhibited specific COUP-binding activity after renaturation (unpublished observation). Recently, Jones et al. (15) reported that the purified CAAT box-binding protein from HeLa nuclear extracts consisted of a heterogeneous population of structurally related polypeptides. Two structurally related polypeptides, 105K and 95K, which bound specifically to the GC boxes were described by Briggs et al. (2).

It is possible that the multiple forms of these *trans*-acting proteins were generated by proteolysis during isolation. In the case of the oviduct COUP protein, the protein-DNA complex band often migrated as a closely spaced doublet in the gel retardation assay. Moreover, when some of the COUP protein samples were subjected to repeated freeze-thaw cycles, a smaller complex appeared in the gel pattern during binding assays. From all of these observations we favor the following interpretation of our results. During isolation from the oviduct tissue, the COUP protein suffered some proteolytic nicking. The fragments of the nicked protein remained together in the native form, perhaps due to sulfhydryl linkages or protein-protein interactions. After complete denaturation and PAGE separation, these proteolytic fragments appeared on the gel as discrete polypeptides of various molecular sizes; those bands containing peptide sequences which exhibit COUP-binding activity after extraction from the gel and renaturation were detected in our assay.

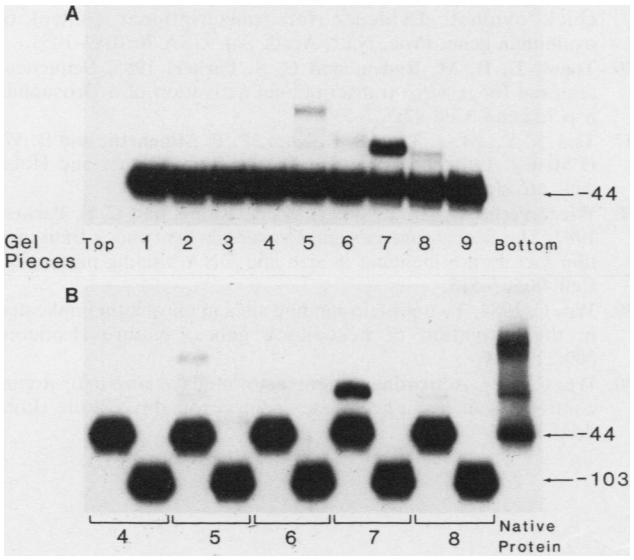

FIG. 9. Renaturation of COUP-binding activity after SDS gel electrophoresis. The renaturation experiment was carried out as described under Materials and Methods. (A) Portions (20 μl) of renatured fractions were assayed for binding activity with the promoter fragment −269 to −44 bp as probe. Lanes 1 to 9 indicate the binding activities after renaturation of the proteins eluted from slices 1 to 9, respectively, cut from the SDS gel (top to bottom). (B) Fragments −269 to −44 bp and −269 to −103 bp were used as probes to assay renatured fractions 4 to 8. The far right lane indicates the binding pattern of 0.1 μg of native heparin-Sepharose-purified COUP protein.

With a similar protocol which also includes DNA affinity column chromatography, the COUP box transcription factor was also purified from HeLa cells (Wang et al., manuscript in preparation). DNase I footprinting results show that both of these proteins have exactly the same footprint on the ovalbumin promoter, i.e., −70 to −90 bp (26, 30). Both exhibit high specificity in promoter binding. The binding of both the oviduct and HeLa factors to the COUP sequence can be competed for by the COUP oligonucleotide but not by the same oligonucleotide containing a single point mutation. These results suggest a high degree of evolutionary conservation of the COUP-binding protein. Nevertheless, we are aware of the fact that there might be subtle differences in the molecular properties among COUP-binding proteins prepared from different species. A transcription factor which binds specifically to the CCAAT sequence of the β-globin and herpes simplex virus thymidine kinase gene promoters has been well described in the literature (13, 15, 17). Based on the binding competition studies by Sagami et al. (30), our present understanding is that the COUP and CCAAT sequence-binding factors are distinct molecules.

A future aim of this work is to obtain the amino acid sequence of and antibodies to COUP peptides and to clone the gene for the COUP-binding protein. At that point we should have sufficient information to make more definitive statements about the structure-function relationships and evolutionary conservation of this interesting and important gene-regulatory factor.

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