Partial Purification and Preparation of Polyclonal Antibodies against Candidate Chromatin Acceptor Proteins for the Avian Oviduct Progesterone Receptor†

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ABSTRACT: Steroid hormones bind to specific receptors in target cells that in turn bind to chromatin acceptor sites to alter gene expression. These chromatin acceptor sites, for a variety of steroid receptors, appear to be composed of acceptor proteins tightly bound to the DNA. This paper describes the preparation of new polyclonal antibodies against the chromatin acceptor proteins of the avian oviduct progesterone receptor (PR) and their use in monitoring the purification of the acceptor proteins. This laboratory recently reported the preparation of monoclonal antibodies that do recognize the intact chromatin acceptor sites containing DNA-bound acceptor proteins but not the unbound acceptor protein for PR [Goldberger, A., Horton, M., Katzmann, J., & Spelsberg, T. C. (1987) *Biochemistry 26*, 5811–5816]. In order to obtain antibodies that recognize the unbound acceptor protein, polyclonal antibodies were prepared against a highly purified preparation of the acceptor protein(s). Analyses by ELISA indicate that the polyclonal antibodies recognize both the intact acceptor sites and the unbound (free) acceptor protein(s). Using these antibodies in Western immunoblots, two antigenic species of 10 and 6 kDa were detected in crude fractions of acceptor protein. These two protein species could be separated and further enriched while still retaining acceptor activity, i.e., the capacity to generate specific binding of the PR. Thus, the antigenic activity is closely associated with, if not identical with, the acceptor activity. Whether one or both species are used in vivo or whether the 6-kDa species is a proteolytic product of the 10-kDa species is unknown. The enrichment of the 10-kDa acceptor protein species has revealed that it is a small hydrophobic protein which focuses in the range of pH 5.0–6.0.

The mechanism of steroid regulation of gene transcription is not completely clear. The avian oviduct remains one of the best models to assess the regulation of gene expression by various steroids. Estrogen induces the growth and differentiation of the oviduct (O'Malley et al., 1969; Oka & Schimke, 1969). Two of the three epithelial cell types that evolve during this growth, goblet cells and tubular gland cells, are known to be steroid target cells (O'Malley et al., 1969; Kohler et al., 1969). The expression of egg-white protein genes in these cells is regulated by estrogens (Means et al., 1972; Liarakos et al., 1973; Palmiter et al., 1973, 1976, 1981), androgens (Palmiter et al., 1973, 1976; Tokarz et al., 1979; Compere et al., 1981), progestins (O'Malley et al., 1969; Chan et al., 1973; Palmiter et al., 1973, 1976, 1981; McKnight et al., 1975; Tuochimaa et al., 1976; Elo et al., 1980; Hora et al., 1986), and glucocorticoids (Hager et al., 1980). Progesterone appears to regulate the expression of many of the egg-white protein genes, especially the avidin gene in goblet cells (O'Malley et al., 1969; Tuohimaa et al., 1976; Elo et al., 1980; Hora et al., 1986). The steroids appear to regulate these genes primarily at the level of transcription.

The binding of steroid receptors to the nuclear acceptor sites in chromatin appears to be required for the regulation of gene transcription. One class of nuclear acceptor sites, termed the chromatin acceptor sites, has been identified in cell-free binding assay systems for all classes of steroids and in many different animal species (Spelsberg et al., 1971, 1976, 1979, 1983, 1984; Perry & Lopez, 1978; Tsai et al., 1980; Ross & Ruh, 1984; Ruh et al., 1986, 1987; Singh et al., 1986; Ogle, 1987; Shyamala et al., 1986; Cobb & Leavitt, 1987). The binding of steroid receptors to chromatin acceptor sites has been shown to be saturable, high affinity and receptor dependent, and therefore specific. These chromatin acceptors include specific acceptor protein(s) bound to genomic DNA (Spelsberg, 1971, 1976, 1979, 1983, 1984; Tsai et al., 1980; Ross & Ruh, 1984; Ruh et al., 1986; Singh et al., 1986; Cobb & Leavitt, 1987). The removal of these acceptor proteins from the chromatin DNA results in a loss of the specific binding to chromatin. The reannealing of these acceptor proteins to the genomic DNAs under certain conditions reconstitutes native-like acceptor sites (Spelsberg et al., 1979, 1983, 1984; Ross & Ruh, 1984; Singh et al., 1986).

Considerable effort in this laboratory has been devoted to the purification of these acceptor proteins from the chromatin acceptor sites of the avian oviduct progesterone receptor (PR). This class of nuclear binding sites has previously been shown to consist of specific protein bound to specific DNA sequences (Spelsberg et al., 1983; Toyoda et al., 1985). The high-affinity, saturable binding of PR to these sites is receptor dependent (Pikler et al., 1976) and receptor specific (Kon & Spelsberg, 1982) and mimics binding patterns seen in vivo (Boyd & Spelsberg, 1979; Spelsberg et al., 1983; Boyd-Leinen et al., 1984). The specific PR binding is lost once the protein is removed from the DNA (Spelsberg et al., 19799, 1983, 1984; Toyoda et al., 1985). Therefore, a method was devised by which the protein is reannealed to the DNA after each purification step to form a protein–DNA complex that can then be tested for PR binding in a cell-free assay (Spelsberg et al., 1984). These reconstituted nucleoproteins appear to be very similar to the native acceptor sites in exhibiting saturable,

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high-affinity PR binding with patterns of binding that mimic those observed in vivo (Spelsberg & Halberg, 1980; Spelsberg et al., 1984). However, use of this method to screen multiple purification steps is cumbersome since each fraction must be titrated at several protein to DNA ratios in order to accurately determine which fractions contain acceptor activity (Spelsberg et al., 1984).

Immunological methods were previously used to further characterize the intact acceptor sites for avian PR (Goldberger et al., 1986, 1987). Monoclonal antibodies were generated against the reconstituted acceptor sites composed of highly purified acceptor protein reannealed to hen DNA. These anti-acceptor site MAbs recognize the intact native acceptor sites in intact chromatin but not the unbound acceptor protein. Therefore, in an attempt to obtain a probe more suitable for rapid screening of acceptor protein fractions for use in the purification of these proteins, polyclonal antibodies were made against a highly purified preparation of the unbound acceptor protein. This paper describes methods to (1) enrich the hydrophobic chromatin acceptor protein(s) and (2) prepare polyclonal antibodies against this protein for use in the further purification and characterization of the protein.

Materials and Methods

General Methods. Chromatin and NAP[1] were isolated from hen oviduct, and DNA was isolated from hen spleen as described previously (Spelsberg et al., 1984). The preparation of chick oviduct [^{3}H]PR and the cell-free PR binding assay have been characterized in detail (Spelsberg, 1983). Binding of PR to reconstituted NAPs was corrected for PR binding to pure DNA. Total protein at each stage of acceptor purification was routinely determined by Coomassie dye binding (Bradford, 1976) or by a fluorescamine method sensitive down to 200 ng (Bohlen et al., 1973). Acceptor activity in each of the fractions was determined by reconsititution of acceptor sites by reannealing the protein onto hen DNA followed by PR binding in the cell-free assay (Spelsberg et al., 1984).

DNA Affinity Chromatography of Acceptor Protein. Isolation of a chromosomal protein fraction containing acceptor activity (CP-3) has been described in an earlier study (Spelsberg et al., 1984). Briefly, whole hen oviduct chromatin was adsorbed to hydroxylapatite (Bio-Gel HTP, Bio-Rad) through the phosphate groups of the DNA by using 0.1 M sodium phosphate buffer, pH 6.0. Histones (CP-1) were removed from the hydroxylapatite-bound chromatin with 3.0 M NaCl, pH 6.0. The CP-2 fraction was extracted with 4.0 M Gdn-HCl in 0.1 M sodium phosphate buffer, pH 6.0, and the CP-3 fraction was then removed from the DNA with 7.0 M Gdn-HCl in the same buffer. The procedure has been scaled up for bulk fractionation beginning with 6000 mg of chromatin DNA bound to 3000 g of hydroxylapatite.

Molecular Sieve Chromatography of Acceptor Activity. The CP-3 fraction was concentrated with DC-10 and DH-4 hollow fiber filter cartridges (Amicon) to about 500 mL (7–8 mg of protein/mL) and applied to a 10 × 92 cm column of Sepharose CL-6B equilibrated in 6 M Gdn-HCl, 2 mM sodium phosphate, pH 6.0, and 1 mM HSEtOH. Thirteen fractions of 500 mL each were collected and pooled on the basis of initial screening for acceptor activity. Fractions were dialyzed against deionized water and lyophilized.

Separation by pI Using Column Chromatofocusing. The PBE resin/Polybuffer system (Pharmacia) was used for fractionation of acceptor activity by p*I*. Due to the insolubility of the acceptor activity in aqueous buffers, 6.0 M urea was added to the system. Ten to twenty milligrams of fraction D from molecular sieve chromatography was resuspended in 7 mL of 6.0 M Gdn-HCl and dialyzed into start buffer (0.025 M Tris–acetate, pH 8.3, 6.0 M urea) overnight. After clarification by centrifugation at 10000 rpm for 10 min, the sample was applied to a 20-mL column of PBE-94 resin that had been equilibrated to pH 8.3 with start buffer. To ensure even sample entry, 1–2 cm of Sephadex G-25 coarse was placed on top of the column. The sample was then fractionated with an 8.0–5.0 pH gradient by using Polybuffer eluent (30% Polybuffer 96 and 70% Polybuffer 74, pH 5.0, with acetic acid and 6.0 M urea) at a flow rate of 0.5 mL/min. Protein distribution was monitored by absorbance at 280 nm. Fractions were pooled by half pH units, dialyzed against deionized water, and lyophilized.

Hydrophobic (HPLC) Chromatography. Acceptor activity in the p*I* 6.0–6.5 fraction from chromatofocusing was further fractionated by reversed-phase HPLC using an Altex Ultrapore RPSC C3 column (4.6 mm × 7.5 cm). All solutions for HPLC analyses were made with HPLC grade reagents in distilled H_2O purified on a Milli-Q unit (Millipore) and were filtered and degassed prior to use. The lyophilized protein (176 μg) was resuspended in 0.5 mL of 20% acetonitrile and 10 mM TFA in H_2O and filtered. The sample was injected and 20% acetonitrile and 10 mM TFA were continuously pumped for 10 min to allow binding to the column. A gradient of 20–100% acetonitrile and 10 mM TFA was then passed through the column over a 60-min period at 0.2 mL/min, and the absorbance was monitored at 280 nm. Fractions were pooled for easier screening. Aliquots either were taken immediately from these eluants for the reconstitution assay (analyses of PR binding) or were directly lyophilized without dialyses for later analyses.

HPLC Molecular Sieve Chromatography. Two fractions from the C3 HPLC that contained acceptor activity were pooled and run on an Altex Spherogel TSK-2000 column (7.5 mm × 30 cm). The sample was resuspended in 20% acetonitrile and 5 mM TFA and chromatographed in the same buffer over a 30-min period at 0.5 mL/min. Protein was monitored by absorbance at 280 nm. These eluant fractions were handled as were those in the hydrophobic chromatography.

Antibody Production. Polyclonal antisera were produced by injection of a highly purified acceptor fraction, the p*I* 6.0–6.5 fraction from chromatofocusing. Two 5–7-lb male white New Zealand rabbits were given 44 and 22 μg, respectively, of acceptor protein emulsified in Freund's complete adjuvant at multiple intradermal sites. Pertussis toxin (50 μL) was also administered intramuscularly to further enhance the immune response. After 1 month, a booster containing half the original dose in Freund's incomplete adjuvant was given intramuscularly, and the animal was bled 1 week later. Boosting and bleeding was repeated at 1-month intervals as necessary. Serum was prepared and tested in an ELISA. The total IgG fraction was purified on protein A–Sepharose, quantitated in an ELISA, and stored at −70 °C. Preimmune sera were prepared in a similar manner.

[1] Abbreviations: Gdn-HCl, guanidine hydrochloride; NAP, nucleoacidic protein (a 4 M Gdn-HCl extract of chromatin); PR, progesterone receptor from chick oviduct; AP, acceptor protein; Ab, antibody; pAb, polyclonal antibody; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; PBS, 0.15 M NaCl and 0.01 M NaH_2PO_4, pH 7.2; kDa, kilodalton; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; p*I*, isoelectric point; HPLC, high-performance liquid chromatography; IgG, immunoglobulin G.

ELISA. Sera were screened for recognition of intact acceptor sites in NAP and for recognition of free acceptor protein fractions in an ELISA as described previously (Goldberger et al., 1986). Protein samples were allowed to solubilize in PBS or deuteriated H_2O for 2 h at 4 °C prior to plating, and some were homogenized to give an even suspension. Appropriate dilutions of serum were incubated in the wells for 1–2 h, after which time the wells were washed with 0.05% Tween 80 in PBS. The second antibody was alkaline phosphatase labeled goat anti-rabbit IgG (Sigma).

Western Analysis of Acceptor Protein Fractions. Lyophilized protein fractions were suspended in 0.125 M Tris-HCl, pH 6.8, 2% SDS, 5% 2-mercaptoethanol, and 10% glycerol and boiled for 30 min. Samples were electrophoresed into 0.75 mm wide, 17.5% SDS–polyacrylamide gels as described in Laemmli (1970). The proteins were then blotted to nitrocellulose in electrode buffer containing 20% methanol at 200 mA for 1 h (Towbin et al., 1979). Unbound sites on the nitrocellulose sheets were blocked with 5% BSA in PBS for 1 h at 37 °C. The sheets were then incubated in polyclonal antibodies diluted in 5% BSA overnight at 4 °C with agitation. After thorough washing with PBS or 0.05% Tween 80 in PBS, the blots were incubated in alkaline phosphatase labeled goat anti-rabbit IgG diluted 1:750 in 5% BSA for 1 h at 22 °C. The blots were again washed and then developed in 0.1 M NaCl and 0.1 M Tris-HCl, pH 9.6, containing 0.1 mg/mL nitroblue tetrazolium, 0.05 mg/mL 5-bromo-4-chloroindoxyl phosphate, and 0.004 M $MgCl_2$ (Blake et al., 1984).

Identical sets of column fractions containing 200 ng or less of protein in the same relative proportions as the immunoblots were silver-stained (Herskoven & Dernick, 1985) to determine protein distribution. Some blots were stained with India ink to detect protein. The sheets were blocked for 1 h at 37 °C in 1% BSA and 0.5% Tween 20 in PBS and then incubated in 0.1% India ink (Staedtler) in 0.1% ovalbumin and 0.05% Tween 20 in PBS.

Results

Specificity of Polyclonal Antibodies. The sera were initially tested in an ELISA for production of antibodies that bound the immunogen as well as the intact acceptor sites in NAP. The rabbit that had received 44 μg of immunogen was producing such antibodies after one boost, and the other rabbit was making antibodies after two boosts (data not shown). Immunoglobulin fractions were purified from the preimmune and second bleeding sera and screened for direct binding to partially purified acceptor protein fractions as well as recognition of intact acceptor sites in NAP and chromatin. Figure 1A shows that protein from two steps or purification was bound by the antibody, while preimmune IgG did not bind. These two fractions contained the acceptor activity when they were assayed for PR binding after reconstitution to DNA (see Figures 4A and 5A for reference). The fact that 2.5 μg of the chromatofocusing fraction gave about the same A_{410} value as 5 μg of the preceding molecular sieve fraction indicates the purification is occurring between the two steps. Figure 1B shows that the purified Ab also bound intact acceptor sites in NAP and chromatin, which were not recognized by the preimmune Ab. Neither the immune nor the preimmune IgG bound pure DNA. While the crude or partially enriched Abs also partially inhibited the specific PR binding to NAP in the cell-free binding assays, these studies were plagued by the fact that many control and preimmune sera caused a general inhibition of all PR binding. A negative effect of the sera on the receptor is speculated.

The purified Abs were also tested on Western blots of AP

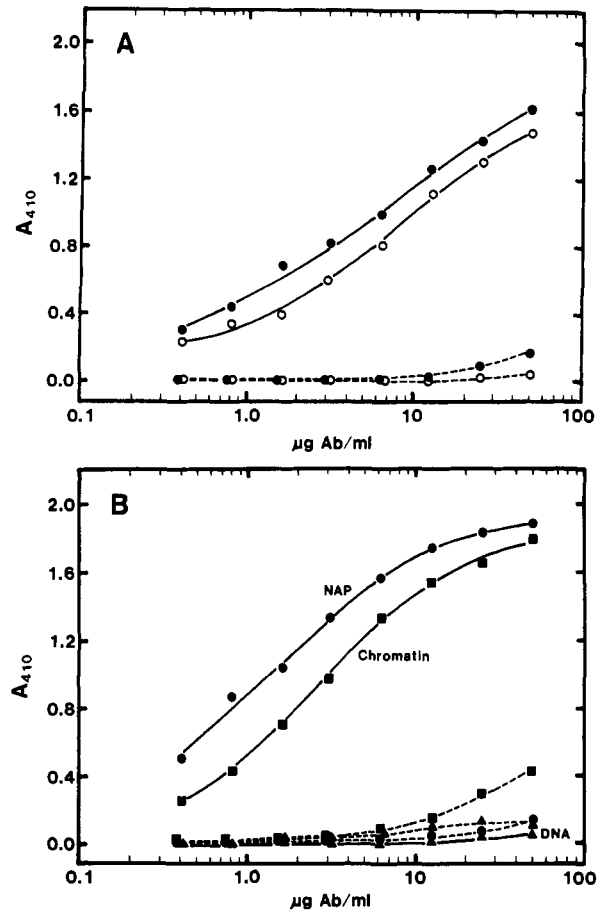

FIGURE 1: Polyclonal antibody binding to AP and intact acceptor sites in an ELISA. (Panel A) Five micrograms of fraction D from Sepharose CL-6B chromatography (MS-D, ●) and 2.5 μg of p*I* 6.0–5.5 fraction from chromatofocusing (CF-4, ○) were bound to microtiter plates and assayed for binding of pAb as described under Materials and Methods. (Panel B) Five micrograms of DNA as NAP (●), chromatin (■), or pure DNA (▲) were plated and assayed as described. (Both panels) Preimmune IgG (---); immune IgG (—).

fractions that had been separated by SDS–polyacrylamide gel electrophoresis for the ability to detect specific antigens. Earlier studies utilizing molecular sieve chromatography in 6 M Gdn-HCl had shown that the acceptor activity had a molecular weight of about 10000–20000 (Spelsberg et al., 1983). Three bands of M_r 12000, 10000, and 6000 were localized in fraction D with immune Ab from both rabbits (Figure 3, lanes 3 and 5). This fraction represents the pool or individual fraction from molecular sieve chromatography previously shown to contain PR binding activity when reconstituted to hen DNA (Spelsberg et al., 1983). However, the reaction with the 12-kDa band appeared to be nonspecific as it was also detected by preimmune Ab (Figure 2, lanes 2 and 4). India ink staining of a similar strip to show total protein distribution indicated that the 12-kDa protein was a major component of this fraction, while the 10- and 6-kDa bands were minor components (Figure 2, lane 1).

Correlation of the Presence of the 6- and 10-kDa Antigens with Acceptor Activity during Purification. Fractions from the various steps of AP purification were screened with the pAb by using both Western immunoblots and an ELISA. As both pAbs gave the same staining pattern (Figure 2), pAb 2 was employed for all subsequent studies since it had a higher titer. The PR acceptor activity was found in the CP-3 fraction but not in the CP-1 or CP-2 fractions (Figure 3A; Spelsberg et al., 1984). Figure 3B shows that the two antigens were faintly detected in the CP-3 fraction obtained from DNA

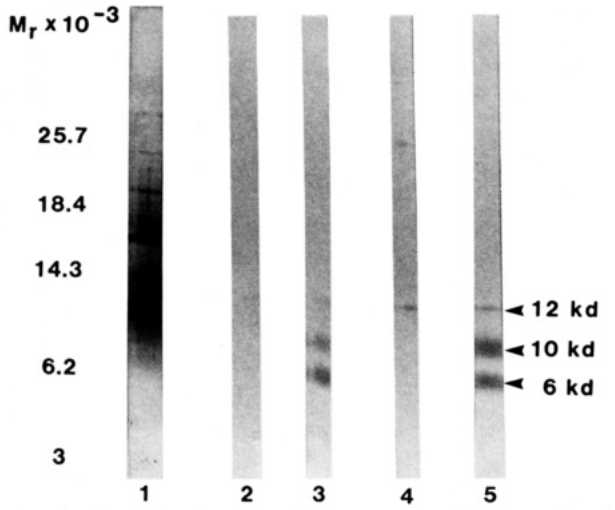

FIGURE 2: Detection of antigens in acceptor protein preparations by Western immunoblot using pAbs. Each strip contains 5 μg of fraction D from molecular sieve chromatography that was separated on 17.5% SDS–polyacrylamide gels and blotted to nitrocellulose as described under Materials and Methods. Strips were stained with pAbs diluted to 10 μg/mL. (Lane 1) India ink staining for protein distribution; (lane 2) preimmune IgG 1; (lane 3) immune IgG 1; (lane 4) preimmune IgG 2; (lane 5) immune IgG 2.

affinity chromatography of chromatin proteins, while they were not present in the CP-2 fraction. Previous studies have shown that the PR binding activity was found primarily in the CP-3 fraction (Spelsberg et al., 1984). The faint localization of the antigens in the CP-3 fraction indicates that the antigens are a minor component at this early stage of purification.

Pooled fractions from molecular sieve chromatography of CP-3 were next screened for the 10- and 6-kDa antigens. The acceptor activity elutes as a low molecular weight protein, and these fractions were pooled as fraction D (Figure 4A). As shown in Figure 4A, fraction A (no. 1–3) and fraction B (no. 4 and 5) contained much of the total protein, while fraction C (no. 6–8) contained a small amount of acceptor activity as well as a large amount of protein. Western immunoblot analysis indicated that the majority of the two antigens was detected in fraction D with a small amount in fraction C (Figure 4C). The intensity of staining reflected the relative distribution of acceptor activity between the two pools as the staining of fraction D was more intense than that of fraction C. Fraction D was also enriched in the lower molecular weight band, as would be expected of a molecular sieve fractionation.

Fraction D was then further purified on the basis of p*I* values by column chromatofocusing. An ELISA performed on the resulting fractions showed that Ab was bound by protein not retained by the column (above p*I* 7.0) and was also bound by fraction 2 (p*I* 6.5–7.0) and fraction 4 (p*I* 5.5–6.0) (Figure 5A). Reconstitution to hen DNA and PR binding of these fractions indicated that acceptor activity eluted in fractions 2 and 4 (Figure 5A). Antibody staining of a Western blot agreed with the ELISA but also revealed that the 6- and 10-kDa antigens were separated by this method (Figure 5B). The 6-kDa antigen was the more basic, eluting in fraction 2, while the 10-kDa antigen eluted primarily in fraction 4.

Hydrophobic chromatography with C3 reversed-phase HPLC was used to further purify the p*I* 5.5–6.0 fraction from the chromatofocusing step. An ELISA of pooled fractions indicated that both antigenic and acceptor activity eluted in fractions 4–6 (Figure 6). The peak of PR binding activity eluting in fraction 1 apparently resulted from aggregation, as it also eluted in fractions 4–6 when it was rechromatographed (data not shown).

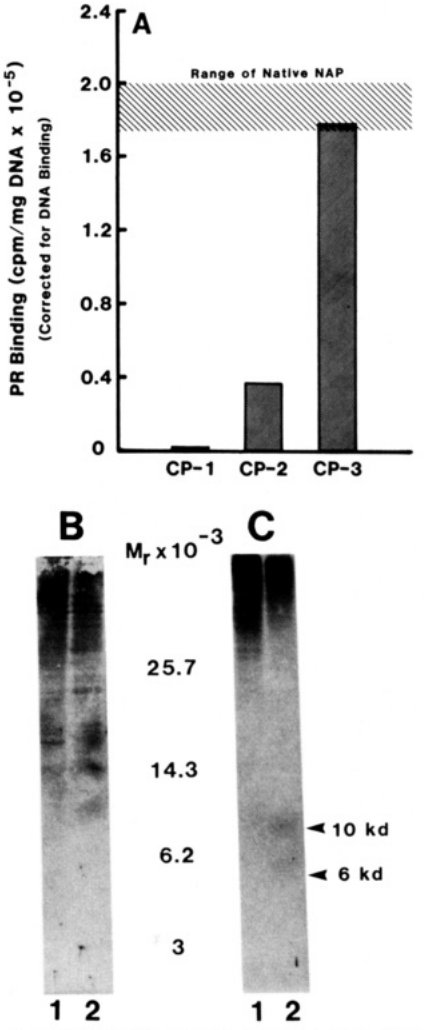

FIGURE 3: Acceptor activity in the fraction from the chromatin–hydroxylapatite (DNA affinity) chromatography. (Panel A) Acceptor activity in chromatin protein fractions that were reannealed to hen DNA at a ratio of 0.4 mg of protein/mg of DNA (w/w). (Panels B and C) Five micrograms each of CP-2 (lane 1) and CP-3 (lane 2) were electrophoresed and blotted onto nitrocellulose. (Panel B) India ink staining for total protein distribution; (panel C) immunostaining with 10 μg/mL pAb.

Fractions 4 and 5 from the reversed-phase chromatography were then subjected to HPLC molecular sieve chromatography on a SWTSK 2000 column. This second separation by size was performed because the large Sepharose CL-6B column gives relatively poor resolution in the low molecular weight range. The resulting fractions were pooled, and portions of these pools were combined for ELISA and for analysis of acceptor activity. Again the antigenic activity was found in the same fractions as the PR acceptor activity that eluted as low molecular weight species (Figure 7). It should be noted that the Western immunoblots were too faint for reproduction when portions of the HPLC reversed-phase and HPLC molecular sieve fractions were screened (data not shown). There may not have been enough antigen present for visualization by this method.

DISCUSSION

A method to enrich an acceptor protein for PR from avian oviduct chromatin has been developed. Special conditions had to be employed to keep these hydrophobic proteins soluble during their purification, including use of chaotropic agents/solvents in the mobile phases of the various chromatographies. The purification steps and solubilizing reagents

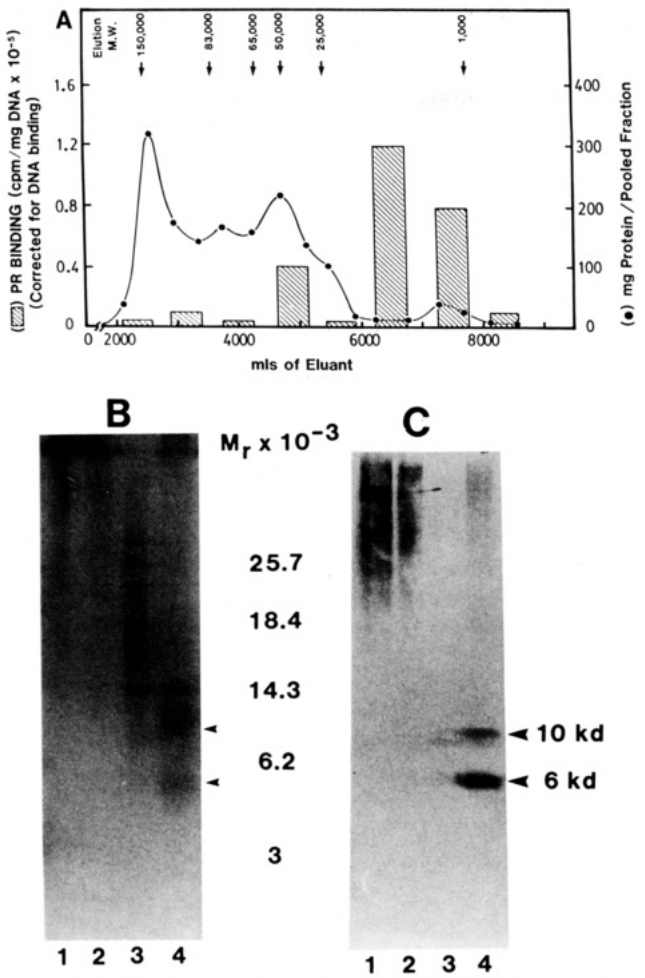

FIGURE 4: Molecular sieve chromatography of the CP-3 fraction from the DNA affinity chromatography. (Panel A) CP-3 fraction separated on a 10 × 100 cm column of Sepharose CL-6B run with 6 M Gdn-HCl. Fractions (500 mL each) were assayed for protein (●) and acceptor activity (▨). (Panel B) Silver staining of pooled fractions separated by SDS–polyacrylamide gel electrophoresis. (Pool 1) Fractions 1–3; (pool 2) fractions 4–6; (pool 3) fractions 7–10; (pool 4) fractions 11–15. Each lane contains 200 ng of protein. (Panel C) Immunoblot analysis of the same fractions stained with pAb at 10 μg/mL. Each lane contains 5 μg of protein.

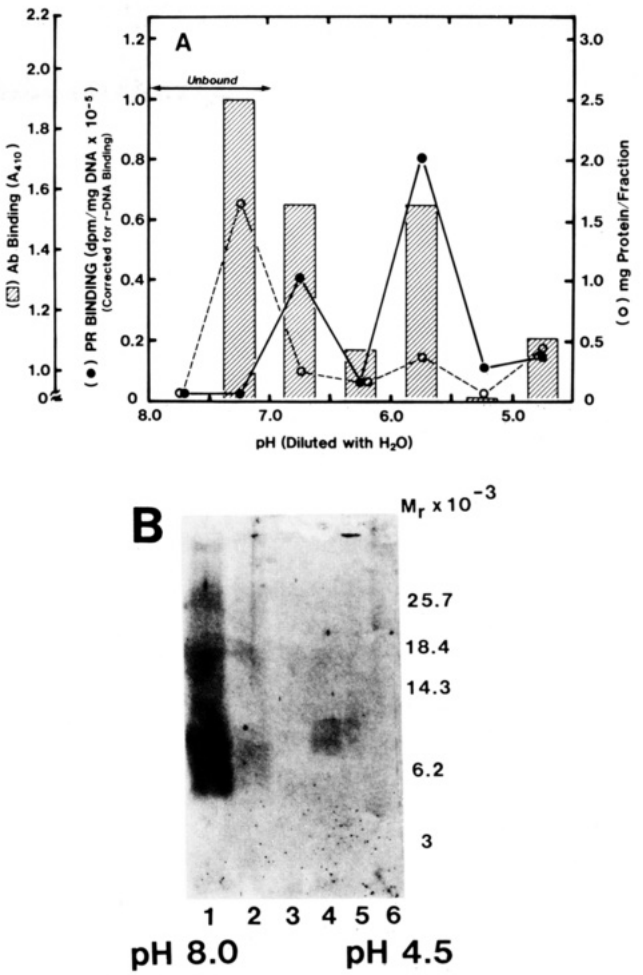

FIGURE 5: Chromatofocusing of acceptor protein in pooled fraction 4 as per the legend of Figure 4. (Panel A) Fraction D from molecular sieve chromatography was further purified by column chromatofocusing using a 8.0–4.5 pH gradient and pooled by half pH units. The fractions were assayed for protein (○), acceptor activity (●), and pAb binding by ELISA (▨). (Panel B) Immunoblot of 5 μg of each fraction reacted with pAb at 10 μg/mL.

were selected from a variety of conditions examined, many of which were eliminated because of handling difficulties or poor yield after the purification steps. The analysis of the biological activity of the acceptor proteins, i.e., acceptor activity, involves the reannealing of protein fractions to whole genomic hen DNA followed by the assessment of PR binding. However, this approach is cumbersome for the screening of multiple fractions from purification steps. Polyclonal antibodies were made against a partially purified acceptor protein preparation to obtain a good probe for rapid screening for these acceptor protein(s) during their purification. The Abs described here are able to recognize both the bound acceptor protein in intact acceptor sites in NAP and chromatin and the free acceptor protein. Two antigenic bands of 6 and 10 kDa were detected on Western blots of a partially purified AP fraction with the immuno Ab but not with the preimmune Ab. Subsequently, other fractions from the other steps of the purification procedure were screened by immunoblotting to determine whether these two antigens would correlate with the acceptor activity as assayed by PR binding. An ELISA was used with the final HPLC steps in which quantities were limited.

The recognition of antigens by the Ab on Western blots or in the ELISA correlated well with the presence of acceptor

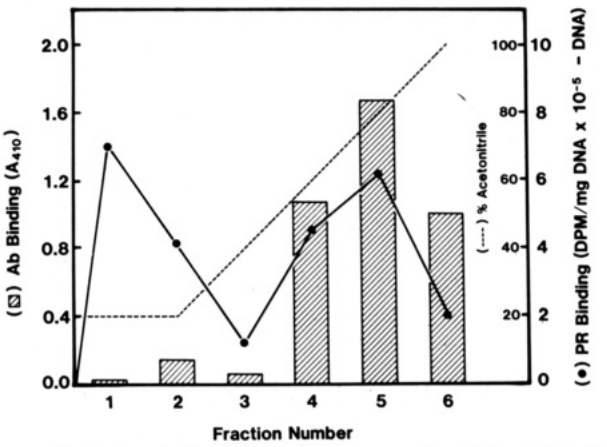

FIGURE 6: Hydrophobic HPLC chromatography of the p*I* 6.0–5.5 fraction of Figure 5. The p*I* 6.0–5.5 fraction from chromatofocusing was separated by C3 reversed-phase HPLC using a linear 20–100% acetonitrile gradient in 0.01 M TFA. Fractions were pooled for easier handling and screened for acceptor activity (●) and pAb binding by ELISA (▨). Antibody concentration in the ELISA was 15 μg/mL. An equal proportion of the total protein in each pool was adsorbed to the wells. (Fraction 1) 0.425 μg; (fraction 2) 1 μg; (fraction 3) 0.35 μg; (fraction 4) 0.625 μg; (fraction 5) 0.5 μg; (fraction 6) 0.5 μg.

activity in all fractions studied. The sizes of the two antigenic proteins on SDS–polyacrylamide gels, 10 and 6 kDa, are within

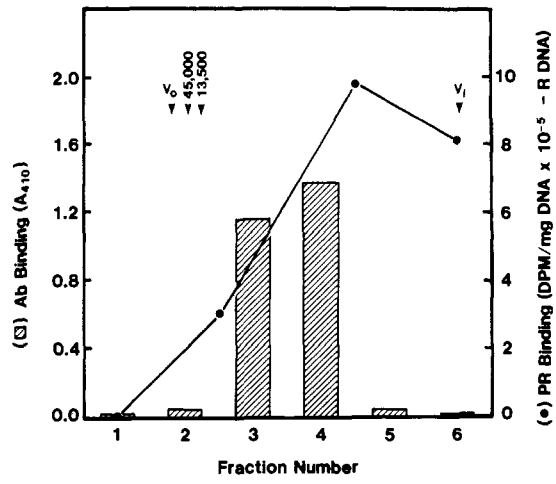

FIGURE 7: Molecular sieve HPLC chromatography of the combined fractions 4 and 5 from Figure 6. Pools 4 and 5 from C3 HPLC were run separately on a molecular sieve SWTSK 2000 column in 40% (v/v) acetonitrile in 0.01 M TFA. The resulting fractions were pooled and assayed for acceptor activity (●) and pAb binding by ELISA (▨). Antibody concentration was 15 μg/mL. An equal proportion of the total protein of each fraction was applied to the wells. (Fraction 1) 0.56 μg; (fraction 2) 0.49 μg; (fraction 3) ≥2.5 μg; (fraction 4) ≥2.5 μg; (fraction 5) 1.63 μg; (fraction 6) 0.33 μg.

the range of elution of acceptor activity from the Sepharose CL-6B and HPLC molecular sieve columns. Some PR acceptor activity was also seen in fraction C from molecular sieve chromatography, which contains more protein. In fact, previous experiments had shown that the acceptor activity might be somewhat larger (13–25 kDa) Spelsberg et al., 1984). However, the immunoblot indicated that the immunoreactive bands in fraction C were the same size as those in fraction D. The activity eluting in fraction C may have been an aggregate or multimers of the smaller protein species. This is plausible since the acceptor activity is extremely hydrophobic, as illustrated by its general insolubility in aqueous solutions, its tight binding to hydrophobic resin (C3), and the fact that it must be boiled for 15–30 min in 2% SDS to be completely solubilized for polyacrylamide gel electrophoresis.

The antigen distribution after the chromatofocusing step was particular interest since one of those fractions was used as the immunogen. ELISA analysis showed that the flow-through fraction with p*I* above 7.0 was not able to reconstitute PR binding activity. The immunoblot of this fraction showed a large smear that appeared to contain the 6-kDa band. Possbily some irreversible modification of the antigens has occurred that prevents proper reconstitution of acceptor sites but does not alter the antigenic recognition by the Ab. The immunoblot also showed that the 10- and 6-kDa antigens were separated by the chromatofocusing method, with the 6-kDa antigen being more basic. As the p*I* 5.5–6.0 fraction containing the 10-kDa band served as the immunogen, the two bands would appear to be antigenically related. Whether or not the 6-kDa antigen represents a proteolytic product of the 10-kDa antigen is presently unknown.

ELISA analysis of the two final purification steps, HPLC hydrophobic and molecular sieve methods, indicated that the antigenic activity continued to copurify with the acceptor activity. This observation was not due to the fact that there was more protein in some ELISA wells than others, since no antibody bound to some of the wells to which more protein was applied. A peak of acceptor activity that was eluted from the C3 column in the void volume and was not antigenic appeared to be an aggregate as it eluted in the later acceptor fractions when it was rechromatographed (data not shown). Similarly, fraction 6 from HPLC molecular sieve chromatography contained acceptor activity that failed to react with the pAb. The reason this fraction did not bind Ab but could reconstitute PR binding is unknown but may indicate the presence of nonantigenic acceptor proteins, which are different protein species or are the same protein with modified immunodeterminants. Although there was not sufficient antigen in these highly purified fractions for adequate detection on Western blots, the antigen appeared to run true as predicted from chromatofocusing. When a large batch of the less pure p*I* 5.5–6.0 fraction containing the 10-kDa antigen was run directly on HPLC molecular sieve, only the 10-kDa band was detected where the acceptor activity eluted.

We have produced polyclonal antibodies that detect two antigens of 10 and 6 kDa which copurify with acceptor activity through all steps of acceptor purification. These proteins appear to be the acceptor protein(s) or a closely related species. This idea is further supported by the fact that these Abs recognize intact acceptor sites in chromatin and NAP. The small size of the acceptor protein explains the ability to denature and renature its biological activity in the reconstitution assays. The pAb can now be used to optimize the final purification of both these antigens, which can then be reexamined for acceptor activity by reconstitution and PR binding to definitely identify them as acceptor proteins. The purification will be performed on a bulk scale to obtain enough purified acceptor protein for analyses of primary and secondary structure of the proteins.

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Deposition of Newly Synthesized Histones: Hybrid Nucleosomes Are Not Tandemly Arranged on Daughter DNA Strands†

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ABSTRACT: Density labeling procedures have been utilized to study the dynamics of histone–histone interactions in vivo. Cells were labeled for 60 min with dense amino acids, and the label was chased for up to 22 h (two replication events for these cells). Nuclei were isolated and treated with formaldehyde to stabilize the histone–histone interactions with a covalent cross-link that produces an octameric complex of two each of H3, H2B, H2A, and H4. This complex was then extracted from the DNA and analyzed on density gradients. The results indicate that new H3,H4 deposits as a tetramer and does not dissociate in the subsequent chases. New H2A,H2B deposited as a dimer and also does not dissociate in subsequent chases. These new histones form hybrid octamers with old histones. On the basis of the new:old ratio in the hybrid octamers, we propose that additional old H2A,H2B from elsewhere in the genome interacts with tetramers of new H3,H4 to form the newly synthesized nucleosomes. It is also observed that 5% of the cross-linked complexes produced by formaldehyde are octamer–octamer (dioctamer). Upon analysis of the density of the dioctamer, the hybrid octamers were found adjacent to octamers that were homogeneous with respect to containing normal density histones. Control experiments are presented to demonstrate that the octamer–octamer cross-links are a product of intrastrand and not interstrand interactions between nucleosomes. These same control experiments also indicate that these procedures do not induce histone exchange during the preparative procedure prior to density gradient analysis. The significance of these results with regard to the dynamics of histone–histone interactions at the replication fork and the potential role in the maintenance of differentiation is discussed.

The basic repeat unit in chromatin is the nucleosome. Each nucleosome is composed of two each of histones H3, H2A, H2B, and H4 [see review of McGhee and Felsenfeld (1980)] organized in an octameric structure upon which two supercoils of DNA [approximately 200 base pairs (bp)] are associated. Histone H1 is thought to bridge one nucleosome to another in the repeat structure. There is increasing evidence that active genes have a more diffuse periodic structure than repressed genes. The mechanisms involved in establishing this more open

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