Enrichment of a Second Class of Native Acceptor Sites for the Avian Oviduct Progesterone Receptor as Intact Chromatin Fragments†

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ABSTRACT: Several classes of specific progesterone receptor (PR) nuclear binding sites (acceptor sites) have previously been identified in avian oviduct chromatin on the basis of different binding affinities. Recently, two classes of acceptor proteins (AP) that are associated with these binding sites in the avian oviduct have been identified. These APs were termed receptor binding factors (RBF-1 and -2), and one (RBF-1) has been purified [Schuchard et al. (1991) *Biochemistry 30*, 4535–4542]. The RBF-1 is associated with the highest affinity class of sites in the intact chromatin, and the RBF-2 is associated with the second highest affinity class of sites. The PR binding sites and their associated RBF-2 protein remain with the residual chromatin fraction following extraction by 4 M Gdn-HCl. This Gdn-HCl-treated chromatin has been termed nucleoacidic protein (NAP). This paper describes the 200-fold enrichment of the native RBF-2 class of PR acceptor sites beginning with the DNase I digestion of NAP to obtain DNase-resistant fragment (NAP_f) containing ~150 bp of DNA. The PR binding sites are further enriched by high-performance or fast protein liquid chromatography and chromatofocusing. Anti-RBF-1/RBF-2 protein antibodies identify antigens that coelute with the PR binding activity. Hybridization analysis of the DNA_f from the enriched NAP_f demonstrates sequence homologies with the nuclear matrix DNA as well as with genomic sequences of the rapid steroid responding nuclear protooncogenes c-*myc* and c-*jun*. However, comparative analyses of the whole genomic DNA with the nuclear matrix DNA indicate that the RBF-2 (NAP_f) is largely nonnuclear matrix. These data show that discrete chromatin fragments containing the native nuclear acceptor sites for the avian oviduct PR can be isolated and used to analyze the structure and composition of these sites.

Previous studies from this laboratory have shown that the specific binding sites for the avian oviduct progesterone receptor (PR) in target cell nuclei consist of chromatin acceptor proteins bound to DNA (Pickler et al., 1976; Webster et al., 1976; Spelsberg et al., 1971, 1983, 1984). These sites reside in chromatin as complexes of acceptor protein tightly bound to potentially unique DNA sequences (Toyoda et al., 1985). In vivo and cell-free binding of PR to avian oviduct nuclei or chromatin acceptor sites have demonstrated a receptor-dependent, saturable, and high-affinity binding (Pikler et al., 1976; Spelsberg, 1983; Spelsberg et al., 1971, 1983, 1984, 1987a), which is receptor-specific (Kon & Spelsberg, 1982; Spelsberg et al., 1987b). The patterns of the cell-free PR binding to isolated nuclei or chromatin, but not to pure DNA, correlate with those measured in vivo with regard to receptor dependency, receptor specificity, and lack of binding by nonfunctional receptors in the avian oviduct (Boyd & Spelsberg, 1979; Spelsberg & Halberg, 1980; Spelsberg & Boyd-Leinen, 1980; Boyd-Leinen et al., 1984). Interestingly, early studies revealed the presence of two or more classes of high-affinity nuclear PR binding sites, each with a 3–10-fold difference in K_D but each requiring intact activated receptor. These sites were measured in vivo (Spelsberg, 1976; Spelsberg et al., 1976a) as well as in vitro (Spelsberg et al., 1977; Pickler et al., 1976; Hora et al., 1986). The exact nature and function of these distinct sites remains obscure.

Recently, this laboratory reported the identification and purification of a candidate acceptor protein, termed the receptor binding factor 1 (RBF-1),[1] which is associated with the highest affinity class of PR binding sites in whole oviduct chromatin (Goldberger & Spelsberg, 1988; Schuchard et al., 1991a). Characterization of this protein has shown RBF-1 to be a 10-kDa, hydrophobic protein that is dissociated from chromatin by 4 M Gdn-HCl concurrently with the loss of the highest affinity class of nuclear acceptor sites. However, the 4 M Gdn-HCl treated oviduct chromatin, termed the nucleoacidic protein (NAP), has been known for quite some time to contain significant levels of PR acceptor sites (Webster et al., 1976; Spelsberg et al., 1971, 1983, 1984; Hora et al., 1986; Goldberger & Spelsberg, 1988). These sites have recently been

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[1] Abbreviations: DNase I, deoxyribonuclease I; RNase A, ribonuclease A; ELISA, enzyme-linked immunosorbent assay; [^{3}H]PR, [^{3}H]progesterone-bound progesterone receptor from the avian oviduct; NAP, nucleoacidic protein (the residual DNA–protein complex following 4 M Gdn-HCl extraction of the avian oviduct chromatin consisting of genomic DNA tightly bound with about 20% of the total chromatin proteins); NAP_f, fragment of NAP remaining after extensive DNase I digestion of the whole NAP (it is a nuclear matrix like fragment); DNA_f, the DNA isolated from NAP_f; RBF-1 or -2, receptor binding factors 1 or 2; PAb, polyclonal antibodies; MAb, monoclonal antibodies; Gdn-HCl, guanidine hydrochloride; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; HPLC, high-performance liquid chromatography; FPLC, fast protein liquid chromatography; PBS, 0.15 M NaCl + 0.01 M NaH_2PO_4, pH 7.4; IgG, immunoglobulin G; p*I*, isoelectric point; M_r, relative molecular mass; AP, acceptor protein.

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classified as the RBF-2 class of sites (Schuchard et al., 1991a). The RBF-2 class of sites is distinguishable from the RBF-1 class of sites since the RBF-2 is not destroyed by 4 M Gdn-HCl treatment of chromatin and anti-RBF-1 MAb do not recognize any proteins in the RBF-2 protein fractions in the NAP (Schuchard et al., 1991a). Although the RBF-2 class of chromatin acceptor proteins have been shown to be an essential component of the specific PR binding sites on the NAP, the RBF-2 protein(s) itself has yet to be purified and characterized.

Interestingly, RBF-2-like classes of acceptor sites and chromatin acceptor proteins in the NAP preparations from the chromatins of other animal systems, and for other steroid hormones, have received extensive investigation. These NAP (RBF-2-like) acceptor sites, which are resistant to extraction by 4 M Gdn-HCl, have been identified for progesterone, androgen, glucocorticoid, and estrogen receptors in calf, rat, and rabbit uteri, rat placenta, mouse hypothalamus, and rat androgenic tissues (Klyzsejko-Stefanowicz et al., 1976; Perry & Lopez, 1978; Ruh et al., 1981; Ross & Ruh, 1984; Lopez et al., 1985; Ruh et al., 1985; Ogle, 1987).

The PR binding to the RBF-2 class of sites of avian oviduct NAP have been extensively studied (Webster et al., 1976; Spelsberg, 1982; Spelsberg et al., 1971, 1976a,b, 1983, 1984; Toyoda et al., 1985; Hora et al., 1986; Schuchard et al., 1991a). By use of a cell-free binding assay, the oviduct PR was shown to bind to the NAP saturably with high affinity and with the same patterns described above for intact nuclei and chromatin both in vivo (Spelsberg, 1976; Boyd & Spelsberg, 1979; Spelsberg & Halberg, 1980) and in vitro (Spelsberg et al., 1976a,b, 1983, 1984; Boyd & Spelsberg, 1979; Spelsberg & Halberg, 1980; Spelsberg & Boyd-Leinen, 1980; Spelsberg, 1982; Boyd-Leinen et al., 1984; Dani & Spelsberg, 1985; Goldberger et al., 1986, 1987; Goldberger & Spelsberg, 1988; Hora et al., 1986). The NAP itself appears to contain two classes of PR binding sites that are dependent on an intact, activated receptor and can be competed by nonradiolabeled PR (Hora et al., 1986). Therefore, three classes of PR acceptor sites have been resolved in the avian oviduct nuclei, one associated only with intact chromatin (RBF-1) and two with the NAP (RBF-2 and RBF-3) under conditions both in vivo (Spelsberg, 1976; Spelsberg et al., 1976a,b) and in vitro (Hora et al., 1986; Schuchard et al., 1991a).

It was previously reported that the specific PR acceptor sites in the avian oviduct NAP are resistant to the action of DNase I (Hora et al., 1986). This treatment generates protein-protected DNA fragments, termed NAP_f, which still retain specific, high-affinity PR binding activity at two classes of sites with K_Ds of $\sim 10^{-9}$ M and $\sim 10^{-8}$ M. The specific binding to the NAP_f is at least 4-fold greater on a per mass of DNA basis than that of undigested NAP due to the removal of much of the involved DNA by DNase. The NAP_f binding of PR is 20–25-fold greater than the binding to intact chromatin, probably not only due to the loss of extraneous DNA but possibly to the unmasking of many of the acceptor sites (Spelsberg et al., 1971, 1976a, 1979; Dani & Spelsberg, 1985; Hora et al., 1986). Electrophoresis of the DNA_f from NAP_f has shown that small DNA fragments (200 bp in length) were protected from DNase action. These small intact DNA fragments were not generated when pure DNA was treated with DNase, which resulted in monomers or small oligomers.

In this paper, a marked enrichment of the intact nucleoprotein fragments (DNA–protein complexes) that comprise RBF-2 chromatin acceptor sites for PR is described. The DNase I generated protein–DNA fragments (NAP_f) were resolved by molecular sieve HPLC and FPLC followed by chromatofocusing. These chromatographies result in an enrichment of the PR acceptor sites approximately 200-fold. Polyclonal and monoclonal antibodies directed against only partially purified RBF acceptor protein preparations and RBF protein–DNA complexes have been previously reported (Goldberger et al., 1986, 1987; Goldberger & Spelsberg, 1988). Interestingly, these antibodies identify antigens that coelute with the PR binding activity. However, some antigens do not coelute with the PR binding activity. Dot-blot hybridization analysis of the DNA from these enriched NAP_f fragments demonstrates some sequence homology with the nuclear matrix DNA as well as with genomic sequences of the c-*myc* and c-*jun* protooncogenes. Similar homologies as these genomic sequences have been identified in the nuclear matrix DNA (Schuchard et al., 1991b). These studies demonstrate that discrete chromatin fragments, containing the nativelike, specific nuclear binding sites for PR, can be isolated as intact chromatin fragments composed of complexes of DNA and protein. Further, the homology between the DNA_f and the nuclear matrix DNA supports the presence of the RBF-2 in the nuclear matrix as recently reported for the RBF-1 class of PR binding sites. However, evidence is presented for the presence of an even greater degree of homology with sequences in whole genomic DNA as opposed to those in the nuclear matrix, suggesting the presence of the RBF-2 class of PR acceptor sites outside the nuclear matrix.

Materials and Methods

Isolation of Chromatin, NAP, and DNA. Chromatin and its subcomponents were isolated and purified by methods described previously (Boyd-Leinen et al., 1984). Adult hens were sacrificed and oviducts were excised, cleaned, and homogenized in buffered sucrose solutions. Nuclei were partially purified by sedimentation through sucrose and disrupted in buffered saline–EDTA to release chromatin, which was further washed and resuspended in solution A [2 mM Tris-HCl + 0.1 mM EDTA (pH 7.5)]. The nucleoacidic protein (NAP) containing the RBF-2 class of acceptor proteins was isolated from avian oviduct chromatin as previously described (Spelsberg et al., 1971, 1983, 1984; Webster et al., 1976; Toyoda et al., 1985; Boyd-Leinen et al., 1984). The RBF-1 class of acceptor proteins has been removed with the 4 M Gdn-HCl extraction (Schuchard et al., 1990). The chromatin was sedimented from 4 M Gdn-HCl extracted chromatin by centrifugation at $10^5 g$ for 48 h and resuspended at 0.5–1.0 mg of DNA/mL in solution A. Pure avian genomic DNA was prepared from hen spleen nuclei and resuspended in solution B [10 mM Tris-HCl + 10 mM NaCl + 0.2 mM EDTA (pH 7.5)] as previously described (Webster et al., 1976; Spelsberg et al., 1984). Acceptable preparations of DNA contained less than 1.0% (w/w) protein or RNA per mass of DNA. The DNA was quantitated by the diphenylamine method of Burton (1956) and the protein by the method of Lowry et al. (1951).

Nuclease Digestion of NAP. This method has been described in detail previously (Hora et al., 1986). Briefly, lyophilized preparations of NAP were resuspended in solution C [100 mM Tris-HCl + 10 mM $MgCl_2$ (pH 7.4)] at a concentration of 1.0 mg of DNA/mL. The solution of NAP was incubated at 4 °C for 2 h and then digested for the designated times with 300 units of DNase I/mg of DNA. Digestion was terminated by the addition of EDTA to 20 mM, and aliquots were taken for DNA measurement. The extent of digestion of the DNA in chromatin was estimated by the solubility in 0.5 N perchloric acid of the A_{260}-absorbing material (Hora

et al., 1986). This NAP_f containing ~2% of the total cellular DNA was sedimented by centrifugation at 225000*g* for 45 min. Sedimented NAP_f was washed once and either (1) resuspended by homogenization in solution B for quantitation and PR binding analysis or (2) resuspended in solution D [5 M urea, 10% (v/v) glycerol, 0.2 M KCl, 1 mM EDTA, 0.5 mM PMSF, and 0.01 M Tris-HCl (pH 7.5)] for chromatography by either molecular sieve HPLC or, in more recent studies, molecular sieve FPLC as described below.

Isolation of the DNA_f from the Nuclease-Digested NAP (NAP_f). For the isolation of the DNA fragments (DNA_f) from the digested NAP, the NAP_f was resuspended in solution E [50 mM Tris-HCl, 1 mM EDTA (pH 7.5)] at 1.0 mg of DNA/mL and treated for 30 min at 37 °C with 100 μg/mL RNase A, followed by a similar treatment with 100 μg/mL pronase. The RNase (Worthington Biochemicals, Freehold, NJ 07728) was heated to 90 °C for 1 h and the pronase (Calbiochem, 10933 North Torrey Pines Rd., La Jolla, CA 92037) self-digested for 2 h at 37 °C before use. Following phenol extraction, the DNA_f was recovered by ethanol precipitation.

Molecular Sieve Chromatography of NAP_f Using HPLC or FPLC. Freshly prepared digests of NAP_f were resuspended in solution D by homogenization in a glass/Teflon homogenizer, and the suspension was clarified by centrifugation at 5000*g* for 5 min. Two-milliliter aliquots of the supernatant fraction were injected onto a preparative TSK 4000 SW column and pumped at 100 psi with a flow rate of 2 mL/min by a Waters HPLC system. Eluents were monitored by absorbance at 260 nm and then collected, and the fractions were pooled into 20-mL fractions. An equal volume of double-distilled, deionized, microfiltered water was added to each pooled fraction, and the fragments were sedimented by centrifugation at 180000*g* for 36 h. The pellets of the HPLC-purified NAP_f were washed and homogenized in solution C for analysis by PR binding and ELISA or for further purification by chromatofocusing. In later studies the molecular sieve FPLC separation of NAP_f was used in place of the HPLC. With this instrument an aliquot of NAP_f (500 μL) containing 230 μg of DNA was diluted to a final volume of 2.0 mL with solution E and left to stand for 30 min at 4 °C. During this time, a preparative Superose 6 molecular sieve column (Pharmacia, 800 Centennial Ave., Piscataway, NJ 08854) was equilibrated with solution E by using a Pharmacia FPLC system. This was accomplished by washing the column with a minimum of 3 bed volumes (150 mL) of buffer at a slow flow rate (0.25 mL/min). Gel-filtration separation of the entire 2.0-mL sample was carried out with a flow rate of 0.5 mL/min (back pressure of 0.4 MPa) and absorbance measured at both 280 and 260 nm.

Chromatofocusing of NAP_f. The pooled fractions from molecular sieve HPLC or FPLC that displayed maximal PR binding and immunologic activity were further enriched and characterized by chromatofocusing. These fractions were dialyzed against solution D without KCl, followed by the addition of solid imidazole to a concentration of 25 mM. The pH was adjusted to 7.4 and the soluble NAP_f was applied to a column of PBE 94 exchange resin (Pharmacia) that had been equilibrated with the modified solution D containing 25 mM imidazole. The NAP_f was eluted from the column with solution F [1:8 dilution of polybuffer PB 74 (Pharmacia) in 5 M urea, 10% (v/v) glycerol, and 0.5 mM PMSF (pH 4.0)]. Eluents were monitored by absorbance at 280 nm and the pH was determined for each fraction. Fractions were pooled according to half-unit pH values and then dialyzed and lyophilized. The lyophilized fractions were resuspended in solution C, and the NAP_f fragments were sedimented by centrifugation in a Beckman TL-100 ultracentrifuge at 350000*g* for 24 h. The pellets were gently resuspended with solution C and analyzed for PR binding and antigenicity.

Isolation of Partially Purified Avian Oviduct Progesterone Receptor. The procedures for the isolation, labeling, and partial purification of PR from 5-week estrogen-stimulated chicken oviduct are modifications of methods described previously (Spelsberg et al., 1984; Boyd-Leinen et al., 1984). Briefly, cytosol preparations of chicken oviducts homogenized in solution G [10 mM Tris-HCl, 1 mM EDTA, and 12 mM monothioglycerol (pH 7.4)] were labeled with 20 mM [^{3}H]-progesterone and then precipitated with saturated ammonium sulfate to a final concentration of 35% saturation. The [^{3}H]PR was sedimented and the resulting pellets were frozen and stored at −80 °C for future use in binding assays.

Cell-Free PR Binding Assay. The streptomycin binding assay of steroid receptors, used in previous studies from this laboratory (Spelsberg, 1983), was modified to accommodate the small size of the NAP_f and DNA_f. Pellets of freshly prepared NAP_f were resuspended in 110 μL of solution B at 4 °C with gentle homogenization. All binding assays were performed in siliconized, 1.0-mL microfuge tubes in a total volume of 0.3 mL with 0.1–10 μg of DNA (as NAP, NAP_f, or DNA_f) per assay. Binding was initiated by the addition of the [^{3}H]PR and was allowed to proceed for 60 min at 4 °C. The PR-DNA_f and/or PR-NAP_f complexes were precipitated by the addition of streptomycin sulfate at 0.2% (w/v) followed by a 15-min incubation at 4 °C. The precipitated complexes were sedimented by centrifugation (5000*g* for 5 min) and washed three times with 0.02% (w/w) streptomycin sulfate in solution C followed by centrifugation after each wash. The radioactivity in the pellets was extracted with 1 mL of 95% (v/v) ethanol and counted in 10 mL of a 2:1 (v/v) mixture of phase combining system (Amersham Corp., 2636 S. Clearbrook Dr., Arlington Heights, IL 60005) and PCS xylene. The DNA in the pellets was determined by a micromodification of the standard diphenylamine reaction (Burton, 1956). Streptomycin pellets from duplicate PR binding assays of the chromatofocused NAP_f were pooled to allow more accurate quantitation of the DNA. Recoveries of input DNA in the PR binding assays were consistently between 70% and 80%.

ELISA. Monoclonal antibodies (MAb) were prepared against complexes of partially purified RBF-2 reannealed with hen genomic DNA as described elsewhere (Goldberger et al., 1986) and used in the ELISA. These MAb preparations (raised against the protein–DNA complex) were originally screened for their ability to inhibit PR binding to whole NAP (i.e., the RBF-2 class of acceptor sites). In addition, polyclonal antibodies (PAb) were prepared against a highly purified preparation containing both the RBF-1 and RBF-2 classes of proteins (Goldberger et al., 1987). The antigenicity of the NAP_f fragments was determined by ELISA. The NAP_f fragments were adsorbed to microtiter plates, and unbound sites were blocked with 9% (w/w) BSA in PBS. Samples were then incubated with appropriate dilutions of antibodies in 9% (w/w) BSA for 1 h, followed by a wash with 0.05% (v/v) Tween 20 in PBS. Each experiment included both positive and negative (control) antibodies. For the PAb, preimmune serum was used. For the MAb, specific MAb to other proteins were used. Goat anti-mouse IgG, linked to alkaline phosphatase, was then added to the microtiter wells, followed by washing. The color development was carried out for 20–30

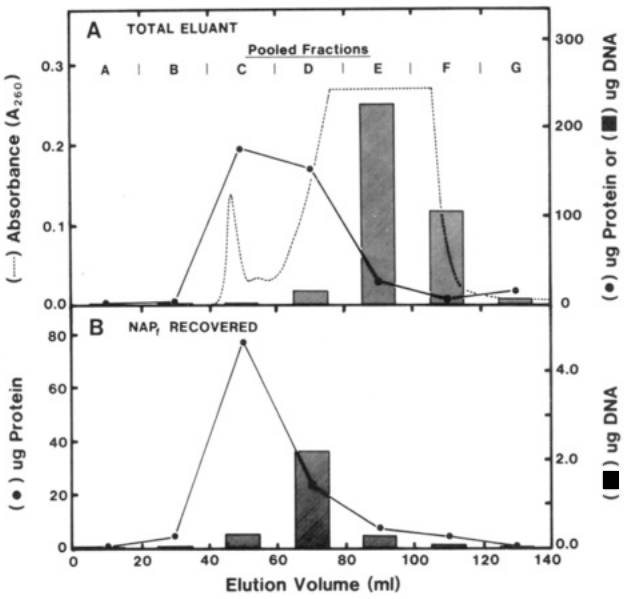

FIGURE 1: HPLC molecular sieve chromatography of the NAP$_f$. Fragments from 5-min digests were solubilized in solution E and clarified by centrifugation. Two-milliliter aliquots were injected and passed through a TSK 4000 SW column under conditions described under Materials and Methods. Twenty-eight 5-mL fractions were collected, which were subsequently pooled into 7 fractions. In panel A, the elution profile of a typical chromatographic run, monitored at A_{260} (---), is shown along with protein (●) and DNA (■) content in each pooled fraction. Panel B shows the protein (●) and DNA (■) recovered from the pooled fractions after centrifugation at 45 000 rpm for 36 h.

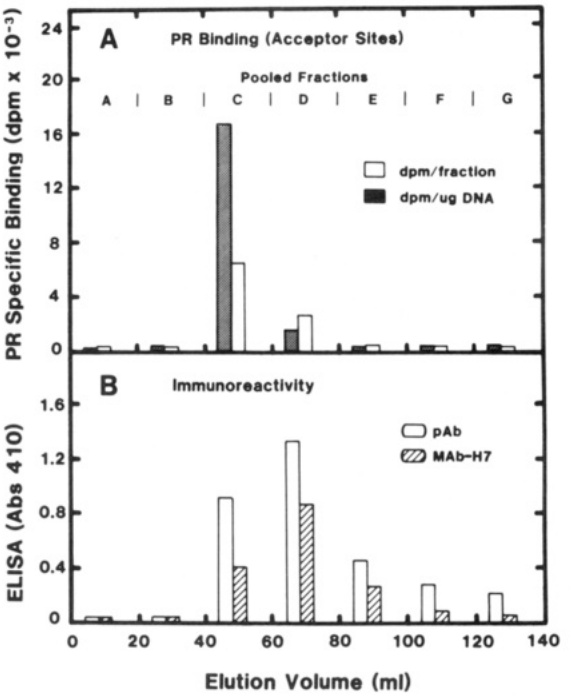

FIGURE 2: Characterization of molecular sieve HPLC fractions. The seven pooled fractions from the molecular sieve chromatography were centrifuged and the sedimented NAP$_f$ was resuspended in solution C for analysis of specific binding with progesterone receptor (panel A) and immunoreactivity by ELISA (panel B). Binding assays were conducted according to procedures described under Materials and Methods. The data represents the average of values from three binding assays with NAP$_f$ obtained from separate chromatographic runs and are plotted as dpm/fractions (open bars) and dpm/μg of DNA (hatched bars). Determinations of immunoreactivity were made by ELISA as described under Materials and Methods, with polyclonal (pAb) antibodies (open bars) and monoclonal (mAb-H7) antibodies (hatched bars). The data represent values from single representative assays in which 5-μL aliquots of chromatographic fractions were tested.

min by addition of substrate containing 1 mg/mL *p*-nitrophenyl phosphate in diethanolamine buffer (pH 9.8). Colorimetric determinations were made at 410 nM on a Dynatech plate reader, and all values were corrected for the nonspecific contribution of a second (nonspecific) antibody binding to the antigen.

Slot Blot Analysis of DNA$_f$ Fragments. Denatured DNA samples were absorbed to nylon membranes as described by Davis et al. (1986). The DNA samples analyzed were the vectors Bluescript SK (Stratagene, La Jolla, CA), pBR322, and pUC13, genomic chicken ovalbumin pOv12 gene (a 12-kb genomic DNA including all exons and introns plus 3 kb of 5′ flanking region and 1 kb of 3′ flanking region cloned in pBR322), chicken genomic c-*myc*, which contains all three exons of the chicken c-*myc* gene plus approximately 1.0 kb of flanking DNA at either end, the matrix DNA prepared from chicken oviduct nuclei as described previously (Schuchard et al., 1991), and finally, whole genomic avian DNA.

Labeling of the DNA$_f$ Fragments. DNA$_f$ fragments were labeled with ^{32}P by random hexanucleotide primer extension using the multiprime DNA labeling system from Amersham (Arlington Heights, IL). [^{32}P]dCTP with a specific activity of approximately 3000 Ci/mmol (New England Nuclear Research Products, Boston, MA) was used to label the DNA fragments. The hybridization and washes of slot-blotted DNA were performed as described previously (Schuchard et al., 1991b).

RESULTS

As described in the introductory section, NAP represents native protein–DNA complex isolated by the 4 M Gdn-HCl extraction of chromatin. NAP contains the RBF-2 class, but not the RBF-1 class, of PR acceptor sites (Schuchard et al., 1991a,b). NAP$_f$ (i.e., the nuclease-digested NAP) represents the residual DNA–protein complexes following DNase I digestion of NAP, which still contains the RBF-2 class of PR acceptor sites (Hora et al, 1986). The first step used to enrich the intact PR acceptor sites from the original "crude" NAP$_f$ preparation was molecular sieve HPLC or FPLC. Figure 1, panel A, shows a representative profile of the molecular sieve chromatography of the NAP$_f$ on HPLC. The fractions were monitored via UV absorption at 260 nm and then pooled as described in the legend and indicated in Figure 1. The first absorbance peak (fraction C) eluted at a position consistent with a molecular weight slightly larger than thyroglobulin (M_r = 330 000). This was followed by a second broad absorbance peak in the lower molecular weight regions. Profiles similar to those obtained with HPLC were observed by using FPLC. The concentration of eluting protein was greatest in fractions C and D (panel A). In contrast, the highest concentration of DNA was found primarily in fractions E and F, representing very small fragments of DNA including nucleotides. Figure 1, panel B, shows the actual recovery of NAP$_f$ obtained after the pooled fractions were sedimented by centrifugation. Under these conditions the supernatants would contain free nucleotides and small oligonucleotides as well as proteins. The sedimented fractions containing the protein–DNA complexes were localized in Fraction D with some in fractions C and E. Again, the patterns for the elution of DNA and protein, and the absorbance were very similar with molecular sieve FPLC.

Figure 2 (panel A) shows the elution of the recovered acceptor site activity (i.e., PR binding) from the molecular sieve HPLC. The levels of PR binding activity were greatest in fraction C with lesser amounts in fraction D. Figure 2 (panel

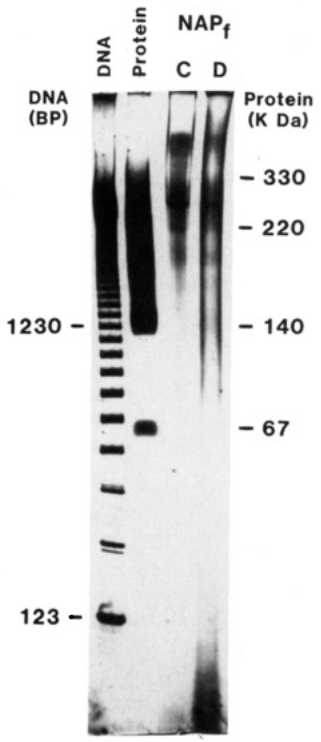

FIGURE 3: Nondenaturing electrophoresis of intact NAP_f. NAP_f samples from size-exclusion chromatography were resuspended in solution K containing 10% glycerol and 0.5 mM PMSF and electrophoresed in a 3–10% (w/w) polyacrylamide gradient gel by techniques described under Materials and Methods and elsewhere (Goodwin & Sahlberg, 1982). Samples analyzed by electrophoresis included NAP_f-C (30 μg of protein) and NAP_f-D (24 μg of protein). Size markers represent a set of high M_r proteins including thyroglobulin, ferritin, catalase, lactate dehydrogenase, and BSA and a 123 base pair DNA.

B) identifies the antigenic activity in the same chromatographic fractions (C and D) by ELISA. Both the anti-acceptor protein polyclonal antibodies (PAb) prepared against highly purified preparations of RBF-1 and RBF-2 acceptor protein (Goldberger et al., 1988) and the anti-acceptor site monoclonal antibodies (MAb-H7) prepared against reconstituted acceptor protein–DNA complexes containing similar preparations of RBF-1 and RBF-2 acceptor proteins (Goldberger et al., 1987) showed similar activity in each of the fractions. In the case of both classes of antibodies, the majority of the immunoreactivity was found in the same fractions (C and D) that contain the PR acceptor sites and the recovered NAP_f (Figure 1). The other fractions displayed lesser antigenic activities.

Figure 3 shows the denaturing polyacrylamide gel electrophoretic analysis of the intact NAP_f fractionated by molecular sieve HPLC, which revealed four diffuse, high molecular weight bands of NAP_f in both fractions C and D by silver staining. These four species of NAP_f migrated with an approximate charge/mass ratio equivalent to the protein standard (thyroglobulin subunit), with an M_r ~300 000. Interestingly, the DNA_f isolated from the NAP_f fraction D, resolved in a 4% (w/w) Nu-sieve agarose gel, migrated in a broad region at an average of ~200 bp (data not shown). This size of DNA_f is consistent with previous size estimations of DNA_f from the crude (nonchromatographed) NAP_f (Hora et al., 1986).

Figure 4A shows a Southern blot using the ^{32}P-labeled DNA_f from the enriched NAP_f obtained from FPLC molecular sieve chromatography as a probe for nuclear matrix DNA, the genomic sequences of the c-*myc*, c-*jun*, and ovalbumin genes, and the plasmid vectors. Interestingly, the enriched

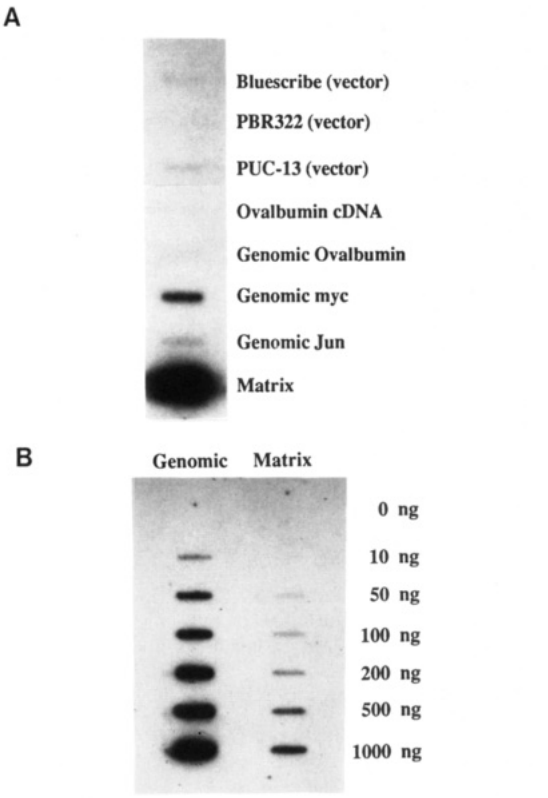

FIGURE 4: Southern blot analyses of DNA_f. Panel A shows DNA slot-blot analysis of various DNAs to show the specificity of different genes of NAP_f fragments enriched by FPLC molecular sieve chromatography. One microgram of matrix DNA and 5 μg of the remaining DNA preparations were denatured and slot-blotted as described under Materials and Methods. The DNA slot-blotted membrane was probed with ^{32}P-labeled NAP_f fragments. The signal in each slot shows the specificity of the DNA to NAP_f fragments. Panel B shows different concentrations of the chicken genomic and matrix DNA were slot-blotted onto a nylon membrane and the blot was hybridized with ^{32}P-labeled NAP_f fragments. The concentrations of the blotted DNAs are shown at the side of the figure.

DNA_f displays marked homology with the nuclear matrix DNA. Furthermore, the genomic sequences of the rapid steroid responding nuclear protooncogenes, c-*myc* and to a lesser extent c-*jun*, are found in the DNA_f. In contrast, the genomic sequences in the whole ovalbumin gene (pOV-12) and the pUC13 vectors were not observed even with longer exposures and appear to have no homology with the DNA_f. This sequence distribution is the same as that measured for the oviduct nuclear matrix DNA (Schuchard et al., 1991b). Figure 4B shows the slot-blot analysis comparing the homology of DNA_f to chicken nuclear matrix DNA and chicken genomic DNA. When compared to matrix DNA, the DNA from the NAP_f fragments hybridized more strongly to the genomic DNA. These results reflect that there are multiple copies of NAP_f throughout the genomic DNA, possibly with greater frequency outside the nuclear matrix.

The two NAP_f species from the molecular sieve chromatography (fractions C and D) were further purified via chromatofocusing on a Pharmacia ion-exchange resin–polybuffer system. As shown in Figures 5 and 6, the 280-nm absorbance profile for material eluting (panel A) and the recovered protein and DNA (panel B) from the chromatofocusing column indicates a further fractionation and enrichment of the protein–DNA complexes of the molecular sieve fractions C and D, respectively. Figures 7 and 8 show the results of PR binding analyses in these fractions. Fractions 5 and 6 of both the NAP_f-C and -D fragments from the molecular sieve chromatography, which eluted from the chromatofocusing runs

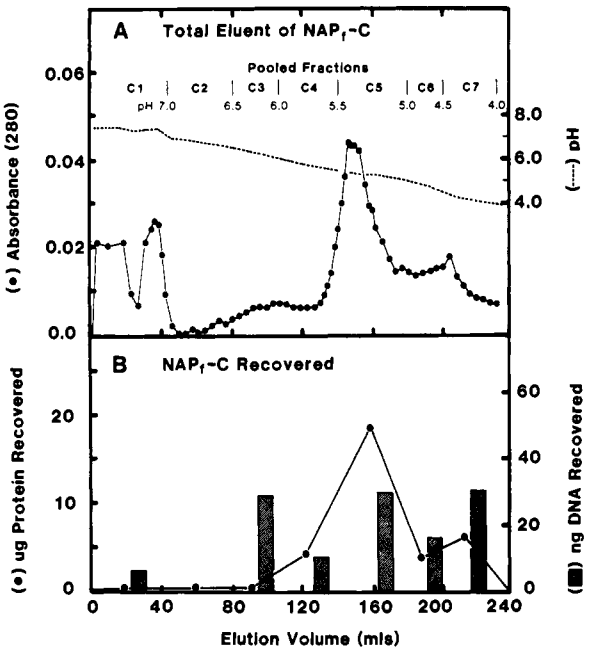

FIGURE 5: Chromatofocusing of HPLC molecular sieve fraction NAP_f-C. NAP_f-C, freshly collected from size-exclusion chromatography, was adjusted to 25 mM imidazole and dialyzed against modified solution D without KCl and containing 25 mM imidazole as described under Materials and Methods. Panel A shows the elution profile from chromatofocusing in which 12.6 mL of NAP_f-C was applied at 4 °C and exchanged with a 1:8 dilution of polybuffer in urea and glycerol. UV absorbance (●) was read immediately, and the pH gradient (---) was determined at 4 °C. Panel B shows protein and DNA values, determined on streptomycin-precipitated pellets pooled from PR binding assays from two runs of chromatofocusing. Values represent protein (●) and DNA (hatched bars) recovered per fraction of one example chromatographic run.

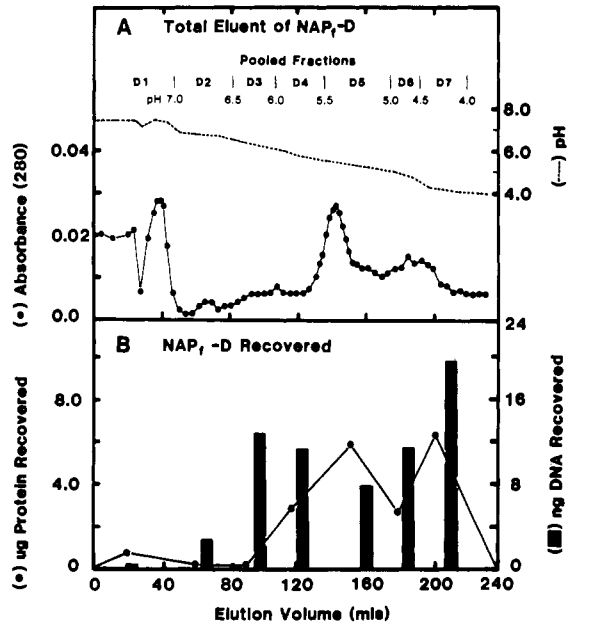

FIGURE 6: Chromatofocusing of HPLC molecular sieve fraction NAP_f-D. Procedures for treatment of samples and chromatofocusing are described under Materials and Methods and in the legend to Figure 4. NAP_f-D (13.4 mL) was applied to the column. Panel A shows the elution profile from chromatofocusing for absorbance (●) and pH profile (---). Panel B shows protein (●) and DNA (solid bars) values, determined on streptomycin-precipitated pellets as described in the legend to Figure 4.

with isoelectric points between 5.0 and 5.5, contained the majority of the specific PR binding activity. Fraction 4 of the

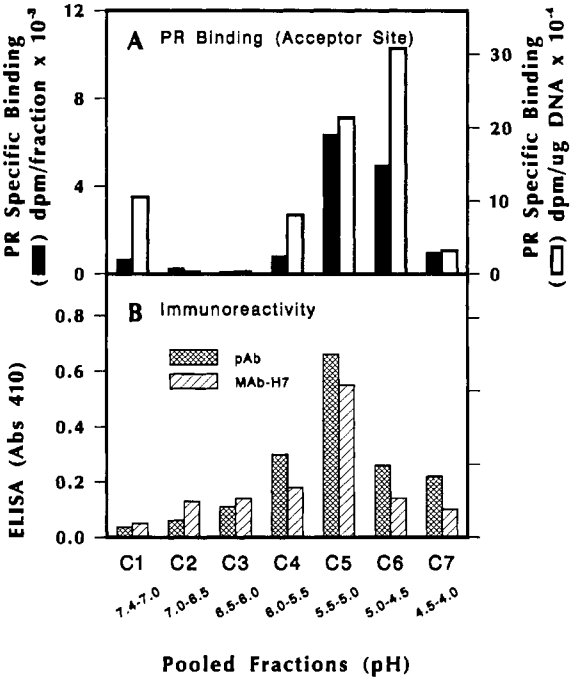

FIGURE 7: PR binding and immunoreactivity of fractions of NAP_f-C from chromatofocusing. After chromatofocusing, eluent fractions of NAP_f-C were pooled by 0.5 pH units and dialyzed to remove the urea, glycerol, and polybuffers. The dialyzed fractions were lyophilized and resuspended by homogenization in solution C. NAP_f was pelleted from the latter resuspensions by centrifugation at 100 000 rpm for 24 h in a Beckman TL-100 microultracentrifuge. Pellets were resuspended in 120 mL of solution C for binding assay and ELISA. PR binding to the fractions (panel A) was performed by procedures described under Materials and Methods using partially purified PR. The PR binding per fraction (solid bars) and per microgram of DNA (open bars) are shown. Data represent values from one representative binding assay. DNA values for calculation of specific binding activity were determined in pooled streptomycin-precipitated pellets of two bindings and then divided proportionately between assays. Panel B: ELISA of chromatofocused NAP_f-C fractions was carried out with anti-acceptor protein antibodies (pAb) (crosshatched bars) and anti-reconstituted acceptor site monoclonal antibodies (MAb-H7) (hatched bars) by techniques described under Materials and Methods. Data represent values from single experiments.

NAP_f-C chromatofocusing (Figure 7, panel A) also displayed some PR binding. Interestingly, as shown in panels B of Figures 7 and 8, the immunoreactivity of the eluted fractions from the chromatofocusing of both the molecular sieve fractions C and D was greatest in fraction 5 accompanied by diminished antigenicity in the neighboring fractions. It should be stated that in these subsequent chromatographies, part of the antigenic activity recognized by the PAb and MAb did not coelute with the PR binding activity.

Figure 9 summarizes and compares the specific PR binding on a per mass of DNA basis of the most active DNA–protein fractions resolved on the chromatographies. Panel A shows that the enrichment of the acceptor sites in chromatofocusing fraction C-6 of the molecular sieve C eluent was 112-fold greater than with intact NAP and 19 times that of NAP_f. In panel B of Figure 9, chromatofocusing fraction D-5 of the molecular sieve D fraction shows the greatest PR binding (acceptor sites) with a 200-fold increase in binding in comparison with that to intact NAP or chromatin/nuclei and 34-fold increase over that which occurs with NAP_f.

Discussion

As described in the introductory section, several distinct classes of high-affinity PR binding sites in the avian oviduct chromatin (i.e., acceptor sites) have been reported (Spelsberg

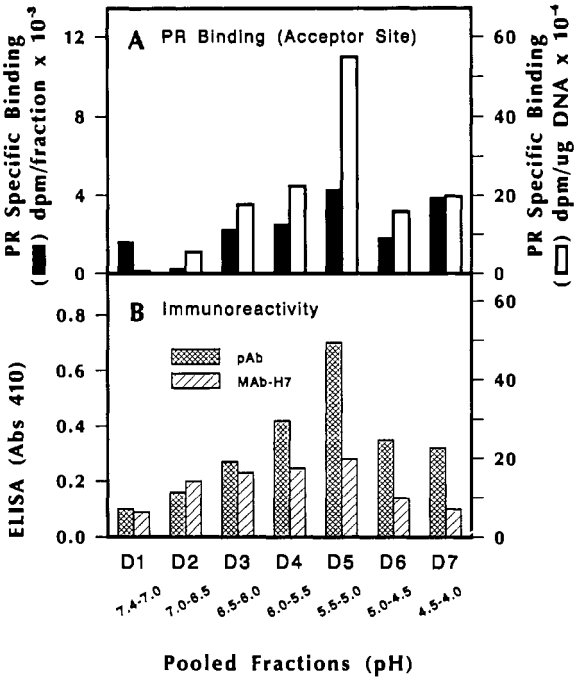

FIGURE 8: PR binding and immunoreactivity of fractions of NAP_f-D from chromatofocusing. Eluent fractions from chromatofocusing were treated as outlined for Figure 7 and under Materials and Methods. Binding assays (panel A) showing PR binding per fraction (solid bars) and per microgram of DNA (open bars) and ELISA (panel B), showing use of PAb (crosshatched bars) and MAb (hatched bars), were likewise performed as described for Figure 7.

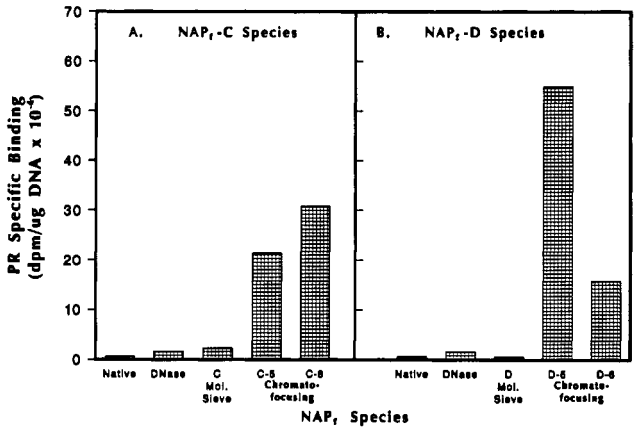

FIGURE 9: Enrichment of native chromatin acceptor sites for PR. Data were taken from experiments that are representative of typical binding values for (panels A and B) chromatin fractions as native NAP, NAP_f (DNase), (panel A) NAP_f-C (C molecular sieve), NAP_f-C_5, NAP_f-C_6, and (panel B) NAP_f-D (D molecular sieve), NAP_f-D_5 and NAP_f-D_6. All binding assays were performed with partially purified PR isolated as described under Materials and Methods. Absolute binding values were determined by using values of DNA recovered in streptomycin-precipitated pellets.

et al., 1976, 1977; Pikler et al., 1976; Spelsberg, 1976). The RBF-1 has recently been shown to be associated with the highest affinity class of these multiple classes of PR binding sites in the avian oviduct chromatin and has been localized to the nuclear matrix in the avian oviduct nuclei (Schuchard et al., 1991a,b). Similar to the RBF-2 class of sites, the RBF-1 class of sites are DNase I resistant. The RBF-1, however, is dissociated from the chromatin by 4 M Gdn-HCl, corresponding to the loss of the highest affinity PR binding sites (Schuchard et al., 1991b). In contrast, the RBF-2 protein remains with the NAP after chromatin extraction by 4 M Gdn-HCl and represents the second highest affinity class of PR binding sites (RBF-2 class) detected in whole nuclei/chromatin (Schuchard et al., 1991a,b).

The intact PR acceptor sites are shown to be enriched by HPLC and FPLC molecular sieve chromatography as one (and possibly two) groups of high molecular weight nucleoprotein complexes (NAP_f-C and NAP_f-D). Up to four species of deoxyribonucleoproteins are distinguished by nondenaturing gel electrophoresis (data not shown). Studies with antibodies against the partially purified RBF-2 activity alone and reconstituted RBF-2–DNA complexes show that the fractions that contain the majority of the PR binding activity are also recognized by these antibodies. Therefore, these two distinct preparations of antibodies appear to have some common epitopes in the chromatographic fractions. The fractions 5 from chromatofocusing of both NAP_f-C and NAP_f-D were the most immunologically reactive as well as the most active in PR binding among all the fractions. The chromatofocusing fraction C-6, however, also displayed significant PR binding. It should be mentioned that the MAb were prepared against acceptor protein–DNA complexes reconstituted with highly purified nuclear preparations containing both RBF-1 and RBF-2 acceptor proteins (Goldberger et al., 1986, 1987). These MAb were screened for inhibition of PR binding to NAP. In contrast, the PAb were prepared against the same nuclear protein fraction alone, i.e., uncomplexed to DNA. The PAb have been shown to recognize the 10-kDa RBF-1 candidate acceptor protein as well as other proteins that may or may not represent the RBF-2 class of acceptor sites (Goldberger & Spelsberg, 1988).

We examined several different types of chromatographic methods for the enrichment of the NAP_f and selected the HPLC molecular sieve and chromatofocusing on the basis of enrichment of the PR binding activity and recovery of the DNA. Additional steps failed to enhance the enrichment of the sites. Overall, the enrichment of PR acceptor sites on a per milligram of DNA basis is substantial since the specific PR binding activity is increased 112-fold in NAP_f-C6 and 200-fold in NAP_f-D5 over that of undigested, nonchromatographed NAP or over that of chromatin/nuclei. Moreover, the elution of this enhanced specific PR binding activity among the many apparent species of NAP_f further supports that nativelike chromatin acceptor sites for PR are being enriched.

The NAP_f described in this paper appears to be somewhat related to the nuclear matrix. The latter is a nuclear entity that is also resistant to high salt extraction, contains small amounts of DNA protected from DNase I activity, specifically binds steroid receptor complexes (Barrack & Coffey, 1980, 1982), contains steroid receptor binding sites resistant to DNase action, and contains about 1% of the total cellular DNA. These are also properties of the NAP_f (Hora et al., 1986; Schuchard et al., 1991b). Furthermore, as shown in this paper, the DNA_f contains some sequences homologous to those in the nuclear matrix DNA. In addition, the c-*myc* and c-*jun* genomic sequences are found in the DNA_f with a conspicuous absence of the ovalbumin gene, a similar finding with the nuclear matrix (Schuchard et al., 1991b). These data support the concept that both the RBF-1 and the RBF-2 classes of PR acceptor sites reside in the nuclear matrix. However, the extensive homology of the DNA_f sequences with the whole avian genomic DNA indicates that a high proportion of the RBF-2 sites reside also outside the nuclear matrix.

It is interesting that the same nuclear protooncogenes that rapidly respond to steroids (within minutes) in the avian oviduct are localized in the same chromatin fragments that bind the PR (i.e., the NAP_f and nuclear matrix). These data fit the cascade model of steroid action (Spelsberg et al., 1983,

1987a; Rories & Spelsberg, 1989; Landers & Spelsberg, 1991). In this model the nuclear protooncogenes would serve as "early"-responding regulatory genes whose protein products in turn regulate the expression of "late" structural genes. In support of this model, steroids have been shown to rapidly regulate the nuclear protooncogenes in the avian oviduct system (Fink et al., 1988; Rories et al., 1989; Lau et al., 1990, 1991; Spelsberg et al., 1989; Rories & Spelsberg, 1989). Steroid hormones also regulate these same protooncogenes in other steroid target tissue systems (Eastman-Reks & Vedeckis, 1986; Murphy et al., 1987; Travers & Knowler, 1987; Forstheofel & Thompson, 1987). Unexpectedly, ovalbumin, a late-responding gene, does not appear to be localized in this structure. Although previous studies suggest that the ovalbumin gene is associated with the nuclear matrix (Ceijek et al., 1983), the studies presented in this and previous reports indicate that ovalbumin sequences are absent. This may be the result of more extensive DNase digestion of our preparations yielding shorter lengths of DNA (Schuchard et al., 1991b).

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