Nuclear Matrix Localization and Specific Matrix DNA Binding by Receptor Binding Factor 1 of the Avian Oviduct Progesterone Receptor†

Mark Schuchard,‡ M. Subramaniam, Terry Ruesink, and Thomas C. Spelsberg*
Department of Biochemistry and Molecular Biology, Mayo Clinic and Mayo Foundation, Rochester, Minnesota 55905
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ABSTRACT: A chromatin acceptor protein for the avian oviduct progesterone receptor (PR), termed receptor binding factor 1 (RBF-1), has recently been shown to (1) be a component of the nuclear binding sites (acceptor sites) for PR and (2) generate high-affinity binding sites (termed the RBF-1 class of sites) on avian genomic DNA [Schuchard et al. (1991) *Biochemistry 30*, 4535–4542]. A second class of sites and its associated protein (termed RBF-2) were also identified. This paper demonstrates that RBF-1 and also the PR nuclear binding sites are localized in the oviduct nuclear matrix. RBF-1 is found in abundance in the nuclear matrix of liver but only in traces in the nuclear matrix of spleen. Extraction of the nuclear matrix with 4.0 M Gdn-HCl results in the complete removal of RBF-1 as occurs with whole chromatin. Interestingly, a second class of specific PR binding, termed RBF-2, remains on the nuclear matrix after the removal of all RBF-1. Southern blot analysis indicates that the nuclear matrix DNA contains sequences homologous with the 5′-flanking domains of the rapidly steroid regulated c-myc and c-jun protooncogenes and the β-actin gene, but not genomic sequences of the late sex steroid regulated gene, ovalbumin, or the α-actin gene. A specific, small region in the 5′-flanking domain of the c-myc gene appears to be associated with the nuclear matrix. Southwestern blot analysis using partially purified RBF-1 shows a marked affinity and specificity of the RBF-1 for the nuclear matrix DNA. These data support a direct action of progesterone on the rapidly regulated nuclear matrix protooncogenes. The possibility of an indirect action on late-regulated genes such as ovalbumin is discussed.

Steroid hormones are known to interact with their specific receptor proteins, and these complexes bind to specific nuclear sites (acceptor sites) to alter gene expression [for reviews, see Rories and Spelsberg (1989), Weinberger and Bradley (1990) and O'Malley (1990)]. Many laboratories have reported that steroid receptors and their nuclear binding sites are associated with the nuclear matrix (Barrack et al., 1977, 1979; Barrack & Coffey, 1980, 1982; Coffey & Barrack, 1980; Vollmer et al., 1982; Barrack, 1983, 1987; Buttyan et al., 1983; Rennie et al., 1983; Colvard & Wilson, 1984; Simmen et al., 1984; Hora et al., 1986; Alexander et al., 1987; Swaneck & Fishman, 1988; Metzger & Korach, 1990). Nuclear matrix has been described as the insoluble, skeletal framework of the nucleus. Many other functional properties have been found associated with the nuclear matrix including DNA loop attachment sites (Dijkwel et al., 1979; Berezeny & Buchholtz, 1981a; Razin et al., 1981), in vivo replicating DNA (Vogelstein et al., 1980: Berezney & Buchholtz, 1981b), DNA polymerase α (Smith & Berezney, 1980), DNA synthesis (Berezney & Smith, 1981; Smith & Berezney, 1982); and the avian oviduct ovalbumin and other transcriptionally active genes (Robinson et al., 1982; Ciejek et al., 1983).

This and other laboratories have previously reported that the nuclear acceptor sites for the avian oviduct progesterone receptor (PR)[1] consist both of genomic DNA and of specific chromatin proteins termed acceptor proteins (O'Malley et al., 1972; Webster et al., 1976; Hora et al., 1986; Goldberger & Spelsberg, 1988; Spelsberg et al., 1971, 1972, 1983, 1988, 1989; Rories & Spelsberg, 1989). Similar compositions for the nuclear acceptor sites of a variety of steroid target tissue systems have been reported. These chromatin acceptor proteins have been shown to be required for the receptor-specific, saturable, high-affinity binding of the steroid receptor complex to chromatin and DNA [for reviews, see Spelsberg et al. (1989) and Rories and Spelsberg (1989)]. Recently, a specific nuclear acceptor protein, termed receptor binding factor (RBF-1), for the avian oviduct PR has been purified and partially sequenced (Goldberger & Spelsberg, 1988; Schuchard et al., 1991). RBF-1 has an apparent molecular weight of 10K, a unique N-terminal sequence, and an acidic p*I* and is hydrophobic. When bound to hen genomic DNA, this protein generates high-affinity, saturable binding of isolated oviduct [^{3}H]PR to the RBF-1–DNA duplex (Goldberger et al., 1988; Schuchard et al., 1991). This protein can be dissociated from nuclei or chromatin by 4 M Gdn-HCl or by 0.2% (v/v) Triton X-100. The dissociation of all RBF-1 from chromatin/nuclei using Triton X-100 results in the corresponding loss in the higher affinity of the two classes of PR binding sites (Schuhard et al., 1991).

A second class of nuclear binding sites for PR in the avian oviduct chromatin has been identified which remains on the chromatin after the 4 M Gdn-HCl extraction. These sites are also dependent on protein(s), termed RBF-2, which are more tightly bound to the chromatin DNA than the RBF-1. This residual DNA–RBF-2 complex has been termed NAP (nucleoacidic protein) (O'Malley, et al., 1972; Spelsberg et al., 1983, 1988; Hora et al., 1986; Schuchard et al., 1991). The function of RBF-2 and its relationship to RBF-1 are unknown.

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* To whom correspondence should be addressed.
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[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; Gdn-HCl, guanidine hydrochloride; RNase, ribonuclease A; DNase, deoxyribonuclease I; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; RBF, receptor binding factor; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PR, progesterone receptor.

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In this paper, RBF-1 is shown to be completely localized in the avian oviduct nuclear matrix. Further, all the nuclear binding sites for PR in the avian oviduct have, for the first time, been shown to be localized in this same nuclear structure. Interestingly, portions of the 5'-flanking regions of the c-myc and c-jun protooncogenes are also shown to be localized in this nuclear matrix. These genes are rapidly regulated by steroids (Fink et al., 1988; Lau et al., 1990, 1991; Rories et al., 1989). In addition, evidence is presented for a high-affinity, specific binding of RBF-1 to the nuclear matrix DNA using Southwestern blot analysis.

Materials and Methods

Buffers. NTE buffer, 10 mM Tris-HCl, 10 mM NaCl, and 1 mM EDTA, pH 7.4; *matrix buffer A*, 10 mM Tris-HCl, 0.2 $MgCl_2$, and 0.1 mM PMSF, pH 7.5; *matrix buffer B*, 10 mM Tris-HCl, 0.2 mM $MgCl_2$, 0.1 mM PMSF, and 2 M NaCl, pH 7.5; *TM buffer*, 50 mM Tris-HCl, 5 mM $MgCl_2$, and 0.25 M sucrose, pH 7.5; *DNase I stock solution*, 2 mg/mL DNase I dissolved in 0.15 M NaCl and 50% (v/v) glycerol; *RNase* stock solution, 27 mg/mL from Worthington Biochemical; *PBS*, 140 mM NaCl, 1.5 mM KH_2PO_4, 8 mM NaH_2PO_4, and 2.7 mM KCl; *transfer buffer*, 10 mM Tris-HCl, 50 mM NaCl, 2 mM EDTA, and 0.1 mM DTT, pH 7.0; *binding buffer*, 10 mM Tris-HCl, 1 mM EDTA, 0.02% (w/v) BSA, 0.02% (w/v) Ficol 400, and 0.02% (w/v) poly(vinylpyrolidone); *TE buffer*, 10 mM Tris-HCl and 1 mM EDTA, pH 7; *SDS solubilization buffer*, 62 mM Tris-HCl, 2% (w/v) SDS, 10% (v/v) glycerol, and 5% (v/v) mercaptoethanol, pH 6.8; *Denhardt's solution*, 0.02% (w/v) Ficoll, 0.02% (w/v) poly(vinylpyrrolidone), and 0.02% (w/v) BSA; *SSC buffer*, 150 mM NaCl and 15 mM sodium citrate, pH 7; *prehybridization solution*, 50% (v/v) formamide, 5× Denhardt's solution, 5× SSC buffer, 0.1% (w/v) SDS, 0.05 mg/mL salmon sperm DNA, and 0.01 mg/mL poly(A) DNA.

DNA and Protein Quantitation. The methods of Burton (1968) and Bradford (1976) were used to quantitate the DNA and protein, respectively. Aliquots of chromatin or matrix were adjusted to 0.5 mL with water, 0.05 mL of 3.3 N $HClO_4$ was added, and the mixture was vortexed. The tubes were incubated in a 90 °C waterbath for 1 h and then cooled on ice. The tubes were centrifuged at 5000 rpm for 5 min in a microfuge. Aliquots of the supernatant were adjusted to 0.3 mL with 0.3 M $HClO_4$ 0.8 mL of diphenylamine solution was added, and the mixture was stored in the dark at room temperature overnight. A sample (0.2 mL) of each was placed on a microtiter plate, and the absorption values at 600 and 410 nm were measured. The pellets were resuspended in 1 mL of 6 M Gdn-HCl and incubated for 30 min at room temperature and several aliquots were used for the protein assay.

Isolation of Nuclei and Chromatin. Nuclei and chromatin were isolated as described previously (Goldberger et al., 1987; Goldberger & Spelsberg, 1988; Schuchard et al., 1991). These methods are modifications of the method of Spelsberg et al. (1971). Nuclei were typically isolated from 70 g of hen oviduct and purified through 0.5 and 1.75 M sucrose. Chromatin was isolated by mechanically lysing the nuclei under hypotonic conditions. Triton X-100 was excluded in the nuclei chromatin preparations used in these studies since it dissociates RBF-1 from the chromatin (Schuchard et al., 1991).

Isolation of the Nuclear Matrix. A modification of the procedure of Berezney and Coffey (1977) was used for the isolation of the nuclear matrix. The method described here involves extensive DNase digestion resulting in very low residual genomic DNA associated with the nuclear matrix and small DNA fragments. A pellet of nuclei isolated from 8–10 g of tissue was gently resuspended in 25 mL of matrix buffer A, incubated on ice for 10 min, and centrifuged at 2000*g* for 15 min. The pellet was resuspended again in matrix buffer A and centrifuged. The pellet was gently resuspended in approximately 25–50 mL of matrix buffer B so that the DNA concentration (as determined from the starting amount of nuclei) was less than 1.5 mg of DNA/mL. This resuspension was incubated on ice for 10 min and centrifuged at 2000*g* for 30 min. The pellet was resuspended again in matrix buffer B and centrifuged. The pellet was then gently resuspended in 12.5 mL of TM buffer. DNase I stock solution was added to a final ratio of DNA/DNase I (mg/mg) of 40, RNase stock solution was added to a final concentration of 0.1 mg/mL. This digestion mixture was incubated at 34 °C for 30 min with occasional mixing, then cooled on ice, and centrifuged at 2000*g* for 15 min. The pellet was resuspended and centrifuged once in TM buffer and twice in NTE buffer. Triton X-100 was omitted from the nuclear matrix isolation procedure because it was previously shown to extract RBF-1 from the nuclei/chromatin (Schuchard et al., 1991).

RBF-1 Isolation. RBF-1 was purified according to the preparative SDS–PAGE method of Schuchard et al. (1991). RBF-1, partially purified by hydroxylapatite–DNA and molecular sieve (CL-Sepharose 6B) chromatographies, was separated on a SDS–PAGE gel according to the method of Schägger and von Jagow (1987). The proteins in the gel were electrotransferred to several Immobilon membranes, the RBF-1 band was identified by immunostaining of one membrane, and the band was excised from the other membranes. RBF-1 was extracted from the excised membrane sections with a solution containing 70% (v/v) 2-propanol and 5% (v/v) TFA, and the extracts were dialyzed and lyophilized.

Western Immunoblot Analysis. SDS–PAGE (Schägger & von Jagow, 1987), electroblotting (Matsudaira, 1987), and immunostaining (Blake et al., 1984) were carried out as described in the references but with modifications (Schuchard et al., 1991). The antibody used was mouse anti-RBF-1 mAB 12 which was developed in this laboratory against SDS–PAGE-purified RBF-1.

Cell-Free Receptor Binding Assay. The interaction of progesterone receptor (PR) with chromatin, DNA, and nuclear matrix was measured by using the procedure described previously with modifications (Goldberger et al., 1987; Goldberger & Spelsberg, 1988) according to the method of Spelsberg (1983).

Southwestern Blot Analysis. Southwestern blot analysis was carried out with slight modification of the method of Bowen et al. (1980). Electrophoresis was carried out as described above. After electrophoresis, the gel was soaked in 300 mL of 4 M urea in transfer buffer for 1 h at room temperature. The gel was then sandwiched between two sheets of nitrocellulose in a Bio-Rad transblot apparatus. The apparatus was submerged in 2.5 mL of transfer buffer for 36–48 h with a buffer change after 20 h. The blot was washed and blocked in binding buffer at room temperature for 30 min. The dephosphorylated nuclear matrix DNA was 5' end labeled. The blot was probed with these ^{32}P-labeled DNA probes (3×10^5 dpm/mL of binding buffer) at room temperature overnight with gentle mixing. Excess probe was removed with binding buffer using various concentrations of NaCl or Gdn-HCl and autoradiographed.

Isolation of Nuclear Matrix DNA. Oviduct nuclear matrix was resuspended in NTE buffer, and SDS and proteinase K were added to 0.5% (w/v) and 50 mg/mL, respectively. The reaction mixture was incubated at 37 °C for 30 min with

occasional vortexing. The proteinase K treatment was repeated for 15 min. An equal volume of phenol was then added, and the mixture was vigorously vortexed and centrifuged at 44000*g* for 10 min. The aqueous phase was reextracted with chloroform. Sodium acetate (0.1 volume of 3 M) and 2 volumes of ethanol were added to the aqueous phases, and the mixture was stored overnight at −20 °C. The solution was again centrifuged at 44000*g* for 30 min to pellet the DNA. The supernatant was decanted and the pellet allowed to drain for 30 min at 4 °C. The pellet was resuspended in TE buffer. Electrophoretic analyses of this DNA in 1% (w/w) agarose gels with ethidium bromide staining revealed that the majority of this DNA averaged 100–200 base pairs in length. Approximately 8 μg of DNA was applied to each lane of the 1% (w/w) agarose gels and electrophoresis performed. The 100–200 bp region was cut from the gel, and the DNA was extracted from the gel slice by using a Costar Spin-X tube (Costar, Cambridge, MA). The DNA was then dephosphorylated, and the 5′ ends were labeled with ^{32}P.

DNA Slot Blot (Southern Blot) Analysis. DNA oligonucleotide sequences to be analyzed (0.25 or 5 μg) were diluted to 10 μL with TE buffer, incubated in boiling water for 5 min, and cooled on ice. SSC buffer (40 μL of a 20× stock) was added to each sample of DNA and then blotted to nylon fibers under vacuum using a Minifold II Slot-Blotter (Schleicher & Schuell, Keene, NH). The slots were rinsed with 10× SSC, and the blot was dried under vacuum at 80 °C for 2 h. The blot was blocked with prehybridization buffer for 2 h at 43 °C and hybridized overnight with 4×10^6 cpm/mL ^{32}P-labeled matrix DNA probe in prehybridization buffer. The blot was washed with 1× SSC and 0.1% (w/w) SDS at 43 °C for 15 min and autoradiographed.

Results

A typical nuclear matrix preparation represented about 1–2% (w/w) of the total DNA and approximately 15% (w/w) of the total protein in hen oviduct nuclei. This nuclear matrix isolation procedure was modified from previous reports by using more extensive DNase I digestion and by eliminating the TritonX-100 extraction step. Triton X-100 was eliminated because it was previously shown to extract RBF-1 from chromatin or nuclei (Schuchard et al., 1991). As mentioned under Materials and Methods, the size of the majority of the DNA fragments in the nuclear matrix ranged between 100 and 200 bp in length as determined by ethidium bromide staining of electrophoretic agarose gels (data not shown).

During a typical nuclear matrix isolation procedure, equal fractions (based on the same proportion of the total preparation) of nuclei, nuclear matrix, and the nuclear extracts were each separated by SDS–PAGE. The proteins in the gel were electrotransferred to nitrocellulose and analyzed by Western blotting to compare the relative amount of the total RBF-1 that is associated with the nuclear matrix with the amount associated with whole nuclei and nuclear extracts. Figure 1 shows that the residual proteins remaining in the total nuclear extract (lane 1), in washed chromatin (lane 2), and in the nuclear matrix (lane 3), when applied in equivalent proportions, are quantitatively similar in their amounts of RBF-1. In addition, the proteins from the extracts during the matrix isolation by low salt (lane 4), 2 M NaCl (lane 5), and nuclease treatments (lane 6) display very little or no RBF-1. These data demonstrate that all of the nuclear RBF-1 appears to be associated with the nuclear matrix.

To determine the nuclear matrix localization and distribution of this protein among different tissues, the nuclear matrix was isolated from chick liver, hen oviduct, and hen spleen.

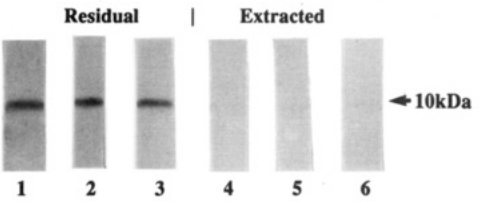

FIGURE 1: Nuclear localization of RBF-1: Western blot analysis. Nuclear matrix was isolated from a pellet of oviduct nuclei as described under Materials and Methods. Fractions (1/400) of the nuclear "extracts" were saved from the matrix buffer A (low-salt extraction) (lane 4), matrix buffer B (2 M NaCl extraction) (lane 5), and the nuclease digestion (lane 6). The "residual" RBF-1 associated with the whole nuclei (lane 1), washed chromatin control (lane 2), and the isolated nuclear matrix (lane 3) are shown. For the washed chromatin control (lane 2), one oviduct nuclei pellet was carried through the matrix isolation steps except that 2 M NaCl was removed from matrix buffer B and TM buffer was used without DNase I and RNase. All fractions were run on an SDS–PAGE gel, electroblotted, and immunostained for RBF-1 as described under Materials and Methods.

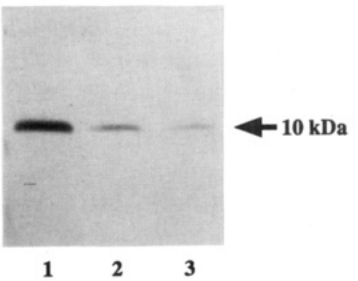

FIGURE 2: Tissue specificity of RBF-1 in the nuclear matrix of avian liver, oviduct and spleen: Western blot analysis. Nuclear matrix was isolated from the nuclei of chick liver (lane 1), hen oviduct (lane 2), and hen spleen (lane 3) as described under Materials and Methods. The fractions used for Western blot analysis were determined on a protein basis (50 μg of protein). These fractions were run on an SDS–PAGE gel, electroblotted, and immunostained for RBF-1 as described under Materials and Methods.

Equal fractions of the nuclear matrices (on a protein basis) were extracted with SDS–solubilization buffer at 100 °C for 10 min. The extracts were applied to SDS–PAGE [10% (w/v) acrylamide] and analyzed by Western blot analysis. Figure 2 demonstrates that there are higher concentrations of RBF-1 in the nuclear matrixes of both chick liver (lane 1) and hen oviduct (lane 2) compared to that from hen spleen (lane 3) with the greatest concentration in the liver.

It was of interest to determine the concentrations of Gdn-HCl and Triton X-100 required to dissociate RBF-1 from the nuclear matrix. Aliquots of hen oviduct nuclear matrix were extracted with solutions containing increasing concentrations of Gdn-HCl in 0.05 M phosphate (pH 6.0), and the extracts and residual proteins remaining on the matrix were separated on an SDS–PAGE gel and analyzed by Western blotting. Figure 3 shows that 4 M Gdn-HCl was required to completely extract RBF-1 from the nuclear matrix. Only a fraction of RBF-1 was extracted with 3 M Gdn-HCl. These results demonstrate that the RBF-1 is tightly bound to the nuclear matrix and is dissociated from the matrix at the same concentration of Gdn-HCl as previously shown in whole nuclei (Schuchard et al., 1991). Triton X-100 extracts from hen oviduct nuclear matrix were similarly analyzed by Western blot analysis (data not shown). Triton X-100, up to 0.5% (v/v), extracted only a small fraction of RBF-1 from nuclear matrix. Thus, RBF-1 in the nuclear matrix seems to be more resistant to Triton X-100 extraction than was previously shown for RBF-1 in whole chromatin but is similarly resistant to Gdn-HCl extractions (Schuchard et al., 1991).

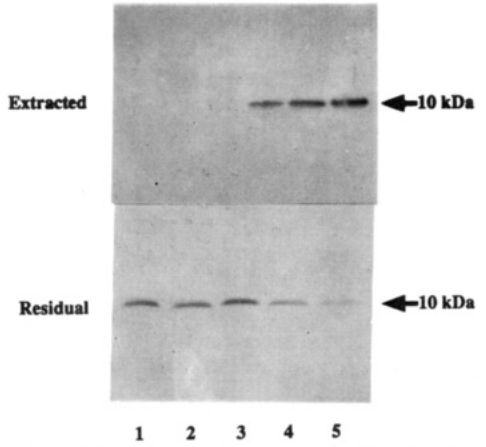

FIGURE 3: Guanidine hydrochloride extraction of RBF-1 from oviduct nuclear matrix: Western blot analysis. Oviduct nuclear matrix was extracted on ice for 2 h with various concentrations of guanidine hydrochloride in 50 mM sodium phosphate, pH 6.0: lane 1, 0 M; lane 2, 1 M; lane 3, 2 M; lane 4, 3 M; lane 5, 4 M Gdn-HCl. The extraction mixtures were microfuged for 20 min at 12000 rpm. The *extracts* (supernatants) were dialyzed against water for 2 h and against SDS solubilization buffer overnight. The *residual* matrix pellets were washed with NTE, microfuged for 10 min, and extracted with SDS solubilization buffer. The *extracts* and *residuals* were run on SDS–PAGE, electroblotted, and immunostained for RBF-1 as described under Materials and Methods.

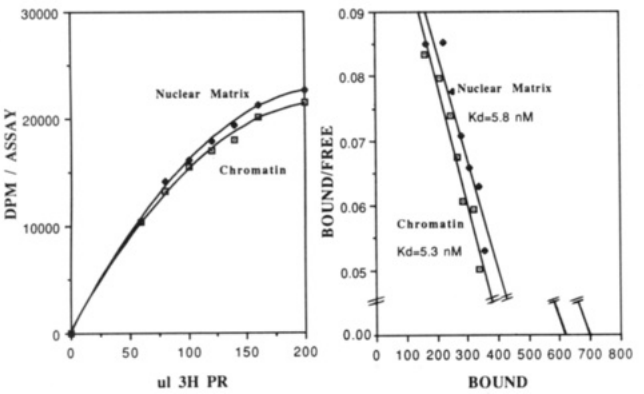

FIGURE 4: Comparison of [^{3}H]PR binding to oviduct chromatin and nuclear matrix. Chromatin and nuclear matrix were isolated from two identical oviduct nuclei pellets as described under Materials and Methods. The chromatin and nuclear matrix pellets were resuspended in equal volumes of NTE buffer by Potte–Elvehjem homogenization and PolyTron homogenization, respectively. The [^{3}H]PR binding to each was assayed by cell-free receptor binding as described under Materials and Methods. [^{3}H]PR titration (left panel) and a Scatchard analysis of the data (right panel) of oviduct chromation and nuclear matrix are shown. "Bound" on the absissa of the right panel is picomolar concentrations of [^{3}H]PR bound in the assay reaction mixture. The specific activity of [^{3}H]progesterone was 57 Ci/mmol, and the dpm of 100 μL of [^{3}H]PR was 224000.

To determine whether the nuclear [^{3}H]PR binding sites are localized in the nuclear matrix, whole chromatin and nuclear matrix were quantitatively isolated from the same hen oviduct nuclei and the preparations assayed by the cell-free [^{3}H]PR binding assay. Equivalent amounts of either chromatin or nuclear matrix were added to each assay. Figure 4 displays the titration curve and Scatchard analysis of these binding studies. Both the nuclear matrix and whole chromatin display similar high-affinity, saturable binding as indicated by the Scatchard plot analysis of the data with K_D values of 5.8 and 5.3 nM, respectively. Further, the similar number of sites as shown by the similar intercepts on the abscissa demonstrates that all the PR binding sites in the oviduct nuclei appear to be localized in the isolated nuclear matrix. The approximate

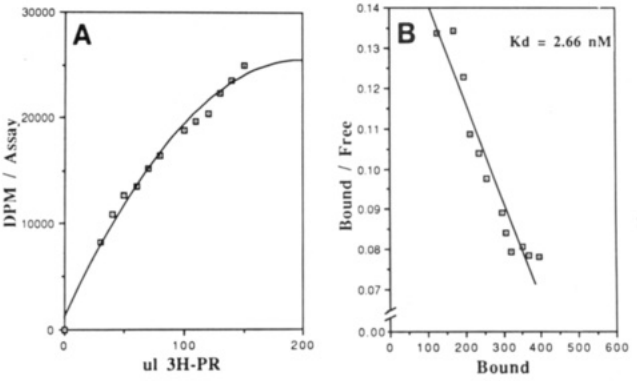

FIGURE 5: [^{3}H]PR binding to 4 M guanidine hydrochloride extracted oviduct nuclear matrix. Oviduct nuclear matrix (0.135 mg of DNA, 15.1 mg of protein) was washed with NTE buffer and extracted with 10 mL of 4 M guanidine hydrochloride and 50 mM phosphate, pH 6, on ice for 1 h. The nuclear matrix was centrifuged at 17000*g* for 30 min, and the pellet was washed 2 times with NTE buffer. The pellet was resuspended in 8.5 mL of NTE buffer with a Polytron homogenizer for two 15-s bursts. A 165-μL aliquot was used for the cell-free receptor binding assay as described under Materials and Methods. Panel A is the saturation plot, and panel B is the Scatchard plot with "Bound" representing picomolar concentration. The specific activity of [^{3}H]progesterone was 57 Ci/mmol, and the dpm of 100 L of [^{3}H]PR was 224000.

number of PR binding sites per nucleus from chromatin and the nuclear matrix is 15700 and 18000, respectively. These numbers were calculated on the basis of 30 μg of chromatin DNA added per 0.5 mL of assay mix and assuming 2.5 pg of DNA per chick somatic cell (Sober, 1970). This laboratory has previously shown that there are 6000–10000 sites per nucleus (Pickler et al., 1976). The slight differences in the number of sites in chromatin and the previously published number of sites in nuclei may be due to the loss of DNA during chromatin isolation from nuclei.

The 4 M Gdn-HCl-extracted nuclear matrix, devoid of all RBF-1, was found to contain specific PR binding sites. Figure 5 shows that the RBF-1 deficient nuclear matrix still contains a significant number of specific [^{3}H]PR binding sites. These 4 M Gdn-HCl-resistant non-RBF-1 class of sites have been reported in intact chromatin (O'Malley et al., 1972, 1979, 1984; Webster et al., 1976; Goldberger et al., 1986, 1987; Spelsberg et al., 1971, 1972, 1976, 1979, 1983, 1984, 1986, 1987) and recently termed the RBF-2 class of sites composed of DNA and the RBF-2 protein(s) (Schuchard et al., 1991). These results suggest that this second class of sites resides, at least in part, in the nuclear matrix as do the RBF-1 class of sites.

To determine whether RBF-1 binds to DNA of the nuclear matrix, partially purified RBF-1 was applied to SDS–PAGE as described under Materials and Methods and subjected to Southwestern blot analysis. The protein was transferred to nitrocellulose, renatured and then bound with ^{32}P-labeled nuclear matrix DNA. The Southwestern blot analysis (Figure 6) reveals that certain ^{32}P-labeled sequences of the matrix DNA are specifically bound by the RBF-1 as well as by many other chromatin proteins in this preparation. A portion of this binding remains even after a 1 M Gdn-HCl wash, indicating high-affinity binding of the RBF-1 with matrix DNA. Interestingly, RBF-1 dissociates from the DNA with 4 M Gdn-HCl similar to its dissociation from intact nuclear matrix and whole chromatin.

To ascertain whether certain steroid-regulated genes have homology with the sequences in the nuclear matrix DNA, Southern blot analysis was performed. As shown in panel A of Figure 7, the genomic sequence of c-myc and c-jun and

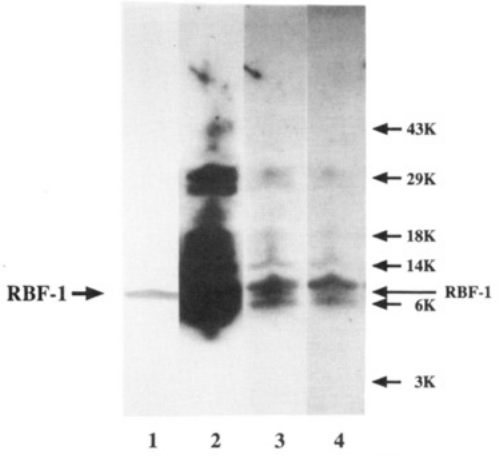

FIGURE 6: Partially purified RBF-1 probed with ^{32}P-labeled nuclear matrix DNA: Southwestern analysis. Partially purified RBF-1 [fraction C from the CL-Sepharose column (Rejman et al., 1990)] (500 μg) was run on SDS–PAGE and transferred to nitrocellulose membrane as described under Southwestern Blot Analysis under Materials and Methods. Lane 1 was immunostained for RBF-1 as described under Materials and Methods. The remaining lanes were probed with ^{32}P-labeled nuclear matrix DNA and washed. Lanes 2–4 are autoradiographs of the blot after the following washes: lane 2, 0.05 mM NaCl; lane 3, 1.0 M Gdn-HCl; lane 4, 4.0 M Gdn-HCl.

β-actin hybridized with matrix DNA. In contrast, neither the ovalbumin nor the α-actin genomic sequences, including its 5′-flanking domain, nor several cloning vectors hybridized to the nuclear matrix DNA. The subfragments of the c-myc gene were further analyzed, as shown in panel B of Figure 7. The subdomains of c-myc are divided into sequences A, B, C, and E as described in the restriction map shown in Figure 8. The *Pst*I 5′-flanking region (sequence A) and the *Sma*I 1021 bp fragment (sequence E) of the c-myc gene hybridized to the ^{32}P-labeled nuclear matrix DNA. Interestingly, no hybridization was observed with the *Pst*I 883 bp (sequence B) fragment, with *Pst*I 1000 bp fragment (5′ to fragment A), with the *Pst*I 3′-flanking region of the c-myc genome (sequence C), with the progesterone response element, or with the estrogen response element (Bagchi et al., 1988).

DISCUSSION

RBF-1 has been recently shown to be concentrated in the nuclei of hen oviduct cells (Schuchard et al., 1991). In this paper, RBF-1 is shown to be associated with the nuclear matrix. The requirement of 4 M Gdn-HCl for the dissociation of the RBF-1 from whole nuclei and chromatin, as well as the nuclear matrix, supports the concept that the RBF-1 is not relocating to the matrix after the disruption of the nuclei and DNase I digestion. The nuclear matrix isolation method utilized in this paper involves extensive digestion with DNase I and results in shorter lengths of DNA in the matrix preparations than has been isolated in other labs (Robinson et al., 1982; Ciejek et al., 1983). Interestingly, the specific, high-affinity nuclear binding sites for the avian oviduct PR are also shown for the first time to be localized in the nuclear matrix which has also been found to be the case for many steroid receptors in different target tissue systems (see the introduction for references). The localization of both RBF-1 and [^{3}H]PR acceptor (binding) sites to the nuclear matrix provides further evidence of the connection of the nuclear acceptor sites for PR with the RBF-1.

A rapid steroid regulation of the c-myc and c-jun protooncogenes has been reported by this laboratory (Fink et al., 1988; Rories et al., 1989; Lau et al., 1990, 1991). Interestingly, we have now found that genomic sequences of the c-myc and

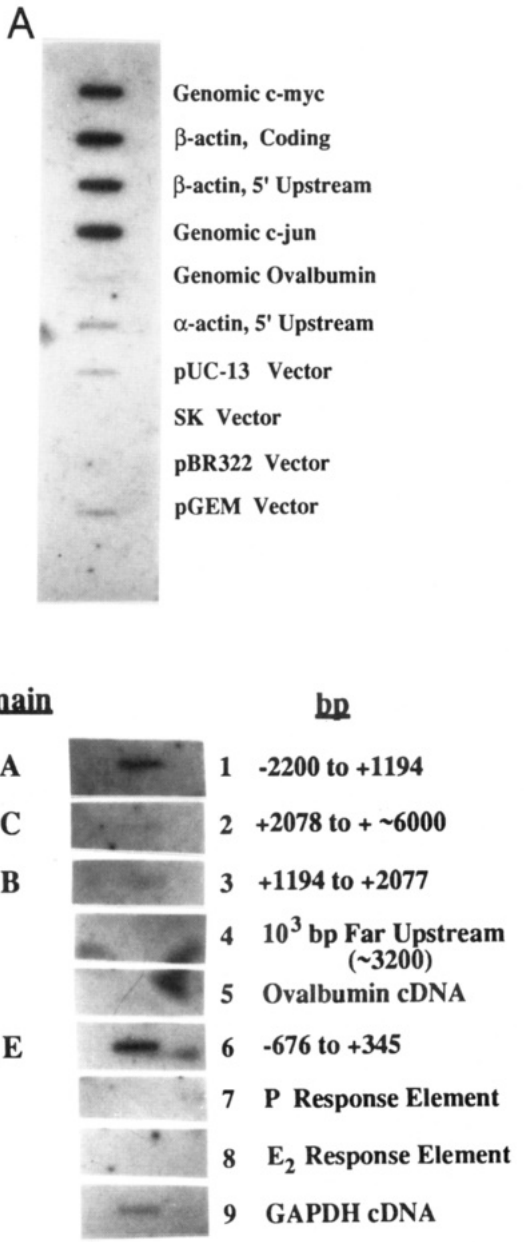

FIGURE 7: Gene sequence specificity in hen oviduct nuclear matrix DNA: Southern blot analyses. Various cloned genes and oligonucleotide sequences were vacuum slot-blotted to nylon membranes, probed with ^{32}P-labeled hen oviduct nuclear matrix DNA, and autoradiographed. Five micrograms of each DNA was slotted unless otherwise indicated. (Panel A) Slot 1, genomic c-myc in pBR-322 vector; slot 2, β-actin coding region in pGEM vector; slot 3, 4.5-kb 5′-upstream region of β-actin in pGEM vector; slot 4, genomic c-jun in pUC-V3 vector; slot 5, genomic ovalbumin in pBR-322 vector; slot 6, α-actin including 1070 bp upstream from the transcription start site; slot 7, pUC-13 vector; slot 8, Blue Scribe SK vector; slot 9, pBR-322 vector; slot 10, pGEM vector. The four vectors (pUC-13, SK, pBR-322, and pGEM) were used as negative control DNA. (Panel B) Slot 1, sequence A; slot 2, sequence C; slot 3, sequence B; slot 4 (0.25 μg), 1000 bp 5′ to sequence A; slot 5, ovalbumin cDNA (xx b 1.2); slot 6, 1021 bp sequence E; slot 7 (0.25 μg), progesterone response element; slot 8 (0.25 μg), estrogen response element; slot 9, GAPDH cDNA. For identification of sequences A, B, C, and E, see Figure 8 (restriction map of c-myc gene).

c-jun protooncogene and a non-steroid-regulated gene (β-actin) are associated with these matrix preparations whereas the genomic sequences of the late steroid regulated ovalbumin gene and some other non-steroid-regulated genes (GAPDH and α-actin) are not. While other laboratories have shown the ovalbumin gene to be associated with nuclear matrix, the

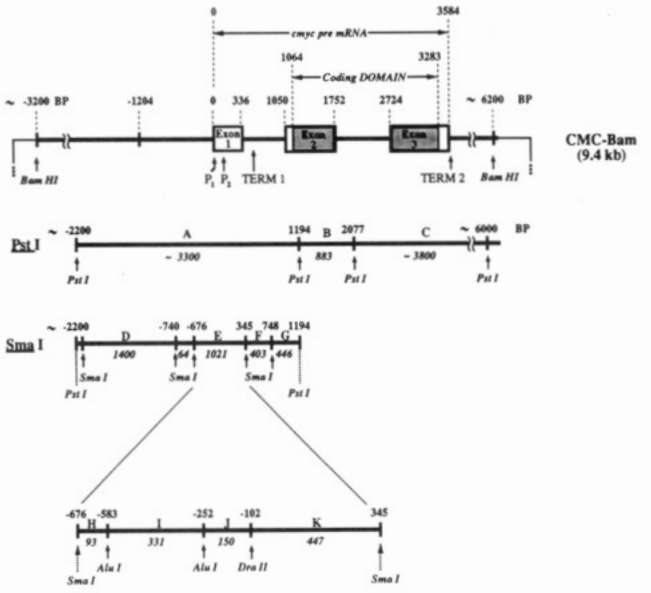

FIGURE 8: Restriction map of the avian c-myc gene (9.4 kb) cloned into a *Bam*HI site of pBR-322 (4.3 kb). CMC-Bam, American Type Culture Collection (Rockville, MD).

discrepancy with the data presented here can probably be explained by the much more extensive DNase digestion used in the studies described in this paper which resulted in the smaller DNA fragments and the reduced levels of DNA in the nuclear matrix preparations. It is interesting to note that the PRE was not found to be associated with the nuclear matrix DNA. One possible explanation is that the RBF-1 binding site on the nuclear matrix DNA may be located upstream of the PRE site. Interestingly, exon 3 at the 3′ end of the c-myc gene has recently been found associated with the nuclear matrix in the human leukemia HL-60 cell line (Chou et al., 1990).

It was previously shown that there is tissue specificity of RBF-1 in hen chromatin (Schuchard et al., 1991). The largest concentrations are found in hen liver and oviduct with very little found in spleen. Similarly, a tissue specificity of the RBF-1 (at least a quantitative one) is demonstrated in the nuclear matrix. The high levels of RBF-1 in avian liver found in the whole nuclei and nuclear matrix are inexplicable. Recent studies have shown that liver chromatin significantly binds the oviduct PR in vitro (Schuchard et al., 1991). Possibly RBF-1 serves a function for both PR and glucocorticoid receptors, as is the case for the cross-reactivity of these two steroids between their respective receptor species as well as the common steroid response elements (Strahle et al., 1987; vonDerahe et al., 1985).

RBF-1 has been previously shown to be tightly bound to chromatin, requiring 4 M Gdn-HCl or 0.2% (v/v) Triton X-100 for dissociation (Schuchard et al., 1991). The binding affinity of RBF-1 to nuclear matrix is similar since 4 M Gdn-HCl was also required to extract the protein. The fact that RBF-1 was readily extracted from nuclei and whole chromatin with 0.2% (v/v) Triton X-100 but not from the nuclear matrix is unclear, but it may be caused by a trapping of RBF-1 in the aggregate nuclear matrix preparations.

One additional more tightly bound RBF has been identified by PR binding. Two high-affinity classes of sites were originally demonstrated in whole nuclei for PR (Spelsberg, 1976; Spelsberg et al., 1977). RBF-1 is associated with the higher affinity of these two classes of sites (Schuchard et al., 1991). The lower affinity, second class of sites remains after the RBF-1 has been removed by 4 M Gdn-HCl (Schuchard et al., 1991). This factor residing in the 4 M Gdn-HCl-extracted chromatin has been classified as RBF-2. This activity has been characterized in the past as a complex of tightly bound protein and DNA and termed NAP for nucleoacidic protein (O'-Malley et al., 1972; Spelsberg et al., 1972, 1977, 1979, 1983, 1984, 1989; Webster et al., 1976). The removal of protein containing RBF-2 from the DNA in NAP results in the loss of specific PR binding sites. The studies presented here support that the PR binding sites associated with RBF-2 are also localized in the nuclear matrix. Futher characterization of RBF-2 is planned.

In any case, these data support the cascade model of steroid action on the transcription of genes. This model was first proposed for the ecdysone–insect steroid system to explain the early and late puffs (Cleaver, 1963). This laboratory applied that model to early regulated and late-regulated genes in mammalian systems for sex steroids (Spelsberg et al., 1983, 1987). This theory was proposed because of an extensive lag period following the nuclear binding of the steroid receptor complex before the transcriptional responses of structural genes to sex hormones occurred. The model proposes that sex steroid receptor complexes would bind to the primary steroid response elements of a rapidly protooncogene (regulatory gene). Transcription and translation of these early regulated genes (i.e., "regulatory genes") occur, and their protein products in turn regulate the expression of structural genes at later periods. The protein products could represent transcription factors.

The rapid regulation of the c-myc and c-jun protooncogenes by steroid hormones in the avian oviduct supports a role of these genes as early "regulatory genes" in this model which are under direct control by steroid receptors. The fact that upstream regions of c-myc have been found to be associated with the nuclear matrix as are the nuclear acceptor sites and acceptor proteins (RBF-1) for PR, and the fact that the RBF-1 binds with high specificity to the nuclear matrix DNA, support this model. In this instance, the primary event in steroid hormone action would be the interaction of steroid receptor complexes with the nuclear matrix via RBF-1 and RBF-2 at sequences which neighbor the rapidly regulated protooncogenes and other regulatory genes whose protein products would regulate the expression of the late structural genes. This model does not necessarily rule out that steroid receptors also work on the late responding genes as well. In fact, recent studies have shown that this might be the case with the c-jun/c-fos proteins interacting with the receptors at AP-1 sites to regulate the expression of many "late" genes. The c-jun and c-fos oncoproteins have been shown to bind to the ERE of the late-regulated ovalbumin gene (Gaub et al., 1990). Transfection studies demonstrated that c-jun, c-fos, and ER coactivated the ovalbumin promoter. Further experiments are needed to identify the locale and DNA sequence to which RBF-1 binds.

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