Characterization of a Purified Chromatin Acceptor Protein (Receptor Binding Factor 1) for the Avian Oviduct Progesterone Receptor†

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ABSTRACT: The specific, high-affinity binding of the avian oviduct progesterone receptor (PR) with target-cell nuclei and chromatin has been shown to involve DNA complexed with specific chromatin acceptor proteins. One of these chromatin acceptor proteins has been partially purified and found to be a small hydrophobic protein with a broad p*I* of 5.0–6.0 [Goldberger, A., & Spelsberg, T. C., (1988) *Biochemistry 27*, 2103–2109]. This paper describes the final purification over 100 000-fold to apparent homogeneity of this candidate PR acceptor protein, termed the receptor binding factor 1 (RBF-1). When the avian genomic DNA is bound by RBF-1, saturable, high-affinity ($K_D \sim 2 \times 10^{-9}$ M) binding sites for PR are generated. RBF-1 has a unique, hydrophobic N-terminal sequence. The PR binding to the RBF-1–DNA complexes is shown to be dependent on an intact activated PR with which excess nonradiolabeled PR can compete. By use of a new, highly specific monoclonal antibody (mAb) to the RBF-1 with Western immunoblotting, RBF-1 was shown to be localized in the nucleus and to be tissue and species specific. Selective removal of the chromatin proteins containing RBF-1 results in the loss of the highest affinity class of PR binding sites. A second class of residual PR binding sites remains in the nucleoacidic protein (NAP), a complex of proteins more tightly bound to the DNA. This class of PR binding activity has been classified as the RBF-2. The RBF-1 is estimated to be 0.03% of the total chromatin protein with about 1.2×10^5 molecules/diploid cell.

The interaction of activated steroid hormone receptor complexes with nuclear acceptor sites is thought to be a critical step in steroid-specific alterations of gene expression. Many studies have described the properties of the interactions of purified steroid receptor complexes to steroid-responsive elements that neighbor steroid-responsive genes (Beato et al., 1989). However, less is known about the high-affinity binding of steroid receptor complexes to acceptor sites detected in isolated nuclei or chromatin. Studies in this laboratory on the chromatin acceptor sites for the avian oviduct progesterone receptor (PR)[1] have indicated that these sites consist of complexes of specific acceptor proteins and DNA, possibly involving specific DNA sequences (Rories & Spelsberg, 1989; Spelsberg et al., 1972, 1983, 1984, 1989; Hora et al., 1986a). By use of both cell-free and intact-cell binding assays as well as in vivo assays, the PR has been shown to bind the chromatin acceptor sites saturably and with high affinity (Pikler et al., 1976; Spelsberg et al., 1983, 1984, 1987). The binding to these sites is receptor dependent (Pikler et al., 1976), receptor specific (Kon & Spelsberg, 1982; Spelsberg et al., 1987), and mimics the patterns of nuclear binding observed in vivo (Boyd & Spelsberg, 1979; Boyd-Leinen et al., 1984). Many of these properties have been described for a variety of other chromatin acceptor sites in several other steroid target tissue systems (Spelsberg et al., 1983, 1989; Rories & Spelsberg, 1989). Specific chromatin acceptor sites have also been demonstrated for the receptors for retinoic acid (Liau et al., 1982; Crow et al., 1987) and thyroid hormone (Murray & Towle, 1989). Each steroid receptor species has been shown to have its own unique chromatin acceptor sites in a variety of biological systems (Hager et al., 1980; Higgens et al., 1973; Kon & Spelsberg, 1982; Spelsberg et al., 1987).

Both the estrogen receptor (ER) (Alexander et al., 1987) and the glucocorticoid receptor (GR) (Krisch et al., 1986; Kaufmann et al., 1986) have been localized in vitro to the nuclear matrix. The latter were shown to bind in vitro to the nuclear matrix of target cells in a high-affinity, saturable manner. Steroid receptors have also been shown to be tightly associated with the nuclear matrix in vivo (Berezney & Coffey, 1977; Barrack & Coffey, 1980). It should be noted that the steroid receptor binding properties of the chromatin acceptor sites described above and those in the nuclear matrix sites are very similar and have thus been speculated to be one and the same (Hora et al., 1986b; Rories & Spelsberg, 1989; Spelsberg et al., 1989).

Evidence that specific chromatin non-histone proteins play a role in these acceptor sites has been reported in many systems, e.g., PR (Spelsberg et al., 1983, 1984, 1989; Perry & Lopez, 1978; Lopez et al., 1985; Cobb & Leavitt, 1987; Edwards et al., 1989), ER (Ruh et al., 1981; Ross & Ruh, 1984; Singh et al., 1984; Ruh & Spelsberg, 1982), AR (Klyzsejko-Stefanowicz et al., 1976; Wang, 1978; Mainwaring et al., 1976; Davies et al., 1980; Foekens et al., 1985), GR (Simons et al., 1976), and finally thyroid receptors (Murray & Towle, 1989). The partial purification and characterization of a few specific acceptor proteins have been reported. Ruh and coworkers (Ross & Ruh, 1984; Singh et al., 1986; Ruh et al.,

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[1] Abbreviations: PR, progesterone receptor; ER, estrogen receptor; AR, androgen receptor; GR, glucocorticoid receptor; NAP, nucleoacidic protein; AP, acceptor protein; mAb, monoclonal antibody; TFA, trifluoroacetic acid; SDS, sodium dodecyl sulfate; RBF, receptor binding factor; Tris, tris(hydroxymethyl) aminomethane; Gdn HCl, guanidine hydrochloride; EDTA, ethylenediaminetetraacetate; HSETOH, β-mercaptoethanol; PAGE, polyacrylamide gel electrophoresis; EM, electron microscopy.

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1987) have identified several species of candidate acceptor proteins for the estrogen receptor in rabbit and cow uteri. A candidate "acceptor protein" for ER has been shown to be a 16-kDa hydrophobic protein resembling the acceptor protein for the avian oviduct PR described in this paper. Cheng, Bruchovsky, Foeken, and co-workers (Rennie et al., 1987; Foeken et al., 1985) have reported a 20-kDa candidate "acceptor protein" for AR that is bound in vivo in rat ventral prostate.

A previous paper has described the purification of a 10-kDa candidate acceptor protein as a component of the chromatin acceptor sites for the avian oviduct progesterone (Goldberger & Spelsberg, 1988). This candidate acceptor protein generates a saturable, high-affinity binding of PR to avian genomic DNA. The present study outlines the properties of this candidate 10-kDa candidate acceptor protein. We have termed this protein receptor binding factor 1 (RBF-1) and demonstrate that it has a unique N-terminal sequence and that it generates a specific PR binding on avian genomic DNA. An abbreviated and improved method for purifying this candidate acceptor protein as well as the preparation of monoclonal antibodies to the RBF-1 is described. The mAb's are used to determine the nuclear localization and the tissue and species specificity of the RBF-1, as well as its dissociation from chromatin.

Materials and Methods

Buffers: NTE buffer, 10 mM Tris-HCl, 10 mM NaCl, 1 mM EDTA, pH 7.4; TKM buffer, 50 mM Tris-HCl, 25 mM KCl, 2 mM $MgCl_2$, pH 7.5; nuclei buffer 1, 0.5 M sucrose in TKM buffer; nuclei buffer 2, 2.0 M sucrose in TKM buffer; chromatin buffer 1, 2 mM Tris-HCl, 1 mM EDTA, pH 7.5; chromatin buffer 2, 20 mM Tris-HCl, pH 7.5; SDS solubilization buffer, 62 mM Tris-HCl, 2% SDS, 10% glycerol, 5% mercaptoethanol, pH 6.8.

Cell-Free Receptor Binding Assay. PR binding was measured according to the procedure of Spelsberg (1983) with modifications (Goldberger et al., 1987). Briefly, various amounts (0–170 μL) of the isolated [^{3}H]PR (57 Ci/mmol) were added to approximately 50 μg of chromatin DNA in a final assay volume of 500 μL. These reaction mixtures were incubated on ice for 45 min with frequent vortexing. Streptomycin [100 μL of 3% (w/w) streptomycin] was added when reconstituted RBF-1–DNA was being assayed, and the assay mixture was incubated for 15 min. The assay tubes were centrifuged at 4000*g* for 20 min, and the supernatant was aspirated. When whole nuclei or chromatin were being analyzed, no streptomycin was needed. The pellets were washed two times with 1 mL of 0.02% (w/w) streptomycin with 10 min of centrifugation. The pellets were extracted with 1 mL of 95% ethanol with shaking for 1 h, and the mixture was centrifuged at 4000 *g* for 10 min. The ethanol supernatants were removed and counted by liquid scintillation as a measure of the bound radioactivity. The DNA pellets were measured by the diphenylamine reaction (Burton, 1968).

Purification of RBF-1 with the Preparative SDS–PAGE Method. A modified procedure has been developed for purifying RBF-1 by utilization of preparative SDS–PAGE of fraction 3 from molecular sieve chromatography as described previously (Goldberger & Spelsberg, 1988). Fractions 11–17, i.e., the 4–20-kDa size range from the CL-Sepharose 6B column chromatography containing relatively concentrated amounts of the candidate RBF-1 antigen, were pooled as a pooled fraction 3 and separated on preparative SDS–PAGE. The gels were 1.5 mm thick and used one large preparative well. To remove oxidants, gels were preelectrophoresed for at least 3 h at 50 mA/gel with 50 μM glutathione solution in resolving gel buffer in the upper reservoir. A total of 8–10 mg of protein was resolved on each gel followed by electrophoretic transfer to four sheets of PVDF membrane. Initially, all sheets were analyzed by Western blotting. Routinely, only the fourth sheet was immunostained for RBF-1 and the RBF-1 band was cut from the first three membranes. The protein was extracted from the PVDF membrane with 70% (v/v) 2-propanol/5% (v/v) TFA overnight, dialyzed against 70% (v/v) 2-propanol, diluted 5-fold with distilled/deionized water, and lyophilized.

Analysis of the "Acceptor Activity" in the Protein Fractions from Preparative PAGE. Acceptor activity was analyzed in each of the isolated protein fractions by recombining the protein with the hen DNA to reconstitute the PR acceptor sites (Spelsberg et al., 1984; Goldberger & Spelsberg, 1988). The protein fractions at each stage of purification were first analyzed for those with the most intense staining of the 10-kDa protein antigen(s) with use of polyclonal antibodies (Goldberger & Spelsberg, 1988). These fractions were then recombined with hen DNA at several protein/DNA ratios to determine the ratio for generating optimal PR binding, as described previously (Spelsberg et al., 1984; Goldberger & Spelsberg, 1988). This ratio was then used for the analyses of the remaining fractions from each of the preparative SDS–PAGE gels. Specific PR binding to the reconstituted protein–DNA complexes was determined by subtracting the binding obtained with [^{3}H]PR in the presence of a 2–4-fold amount of nonradiolabeled PR (nonspecific binding) from the binding obtained with [^{3}H]PR alone (total binding). The addition of higher amounts of the nonradiolabeled, partially purified PR preparations was impossible due to the interference caused by excess protein in the binding assay. In certain instances, the residual PR binding to pure hen genomic DNA was also subtracted from the specific binding values.

Amino Acid Sequencing and Total Amino Acid Composition of RBF-1. The fractions from molecular sieve HPLC (Goldberger & Spelsberg, 1988) or from the preparative SDS–PAGE (described above) containing RBF-1 were applied to a gas-phase microsequencer for amino acid sequencing by the Edman degradation method. The analyses were performed with use of an Applied Biosystems 470A protein sequencer by the Molecular Analysis Core Facility of the Center for Reproductive Biology, Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, MN. Phenylthiohydantoin amino acids were identified by use of the Applied Biosystems 120A PTH analyzer. The Waters PICOTAG system was used to determine the amino acid composition.

Preparation of Monoclonal Antibodies against RBF-1. New mAb's were prepared against the purified 10-kDa RBF-1 antigen. Purified RBF-1 (20 μg) was suspended in 6 mL of complete Freunds adjuvant, and 0.5 mL was injected (sc) into Balb/c mice. Three more injections of the same quantity of RBF-1 were given at 2-week intervals. Three days after the last injection, the mice were sacrificed and the spleen cells isolated and fused with NS-1 mouse myeloma cells as described previously (Foster et al., 1982). The media of the many cloned hybridomas were screened for anti-RBF-1 antibodies by use of Western immunoblotting of pure RBF-1. Several of these clones demonstrating the most specificity and sensitivity for the 10-kDa RBF-1 antigen were injected into Balb/c mice for bulk production as ascites growth as described previously (Goldberger et al., 1986).

Western Immunoblot Analysis. Western immunoblot analysis was carried out by SDS–PAGE according to the method of Schägger and von Jagow (1987). Briefly, 1.5-

mm-thick gels consisted of a 1-cm stacking gel [3.75% (w/w) acrylamide, 0.12% (w/w) bisacrylamide, 0.75 M Tris-HCl, 0.1% (w/w) SDS, pH 8.8] and a 12-cm resolving gel [10% (w/w) acrylamide, 0.31% bisacrylamide, 1 M Tris-HCl, 0.1% (w/v) SDS, pH 8.8]. The anode buffer contained 0.2 M Tris-HCl, pH 8.9, and the cathode buffer consisted of 0.1 M Tris, 0.1 M Tricine, and 0.1% (w/w) SDS. Protein samples were dissolved in SDS solubilization buffer and incubated at 100 °C for 10 min. The electrophoresis was run in the cathode to the anode direction at 50 V for 30 min and then increased to 150 V for 3.5 h.

The proteins in the gel were electrotransferred to a nitrocellulose sheet according to the method of Matsudaira (1987). The transfer was carried out in transfer buffer consisting of 10 mM CAPS and 10% (w/w) methanol, pH 11.0, with a Bio-Rad Trans-Blot apparatus at 80 V for 1 h. The nitrocellulose sheet was then washed with 0.05% Tween 80 in PBS (140 mM NaCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 2 mM KCl) followed by either protein staining according to the method of Hancock and Tsang (1983) or immunostaining RBF-1 as described below. RBF-1 was detected on the nitrocellulose blot with a specific monoclonal antibody by use of the method of Blake et al. (1984). The blot was blocked with blocking buffer [5% (w/v) nonfat dry milk in 0.05% (w/w) Tween 80 in PBS] at 37 °C for 1 h and incubated with the mAb (2 μg/mL in the blocking buffer) either overnight at 4 °C or for 2 h at room temperature. The blot was washed with PBS/Tween 80 and then incubated in 1 μg/mL goat anti-mouse IgG, alkaline phosphatase conjugate for 1 h at room temperature. The blot was then developed as per Blake et al. (1984).

Hen Tissue Nuclear Preparations for RBF-1 Quantitation. Nuclei were isolated by a modification of the method of Spelsberg et al. (1971). Typically, 70 g of frozen tissue was thawed and homogenized with 250 mL of nuclei buffer 1 in a small Waring blender for 10 s. The homogenate was further homogenized with several strokes in a Teflon pestle glass tissue grinder and filtered through cheesecloth to remove connective tissue. The solution was centrifuged at 17000*g* for 5 min. The nuclear pellet was resuspended in nuclei buffer 2 and homogenized with two strokes of a Potter-Elvehjem tissue grinder with use of a smooth pestle. The volume was adjusted with water to give a 1.75 M final sucrose concentration, and the mixture was centrifuged at 27000*g* for 10 min. The supernatant was decanted off, and the nuclei pellets were either stored at −80 °C or immediately carried to the next isolation step. To isolate chromatin, the nuclear pellets were resuspended in 250 mL of chromatin buffer 1 with a Teflon pestle glass homogenizer and the nuclei lysed by creating a vaccum in the glass homogenizer with the pestle. The solutions were then centrifuged at 27000*g* for 10 min. The chromatin pellets were resuspended in 100 mL of chromatin buffer 2 and either stored at −80 °C or used immediately.

Triton X-100 Extraction of RBF-1 from Nuclei or Chromatin. Aliquots of hen oviduct chromatin (4 mg of DNA) were precipitated in 0.2 M KCl and pelleted at 23000*g* for 10 min. The pellets were then resuspended in 4 mL of various concentrations of Triton X-100 [0–0.3% (v/v)] in 1× NTE buffer and incubated on ice for 2 h with frequent mixing. The extraction mixtures were centrifuged at 23000*g* for 10 min, and the supernatants were dialyzed and lyophilized for Western blot analysis for the RBF-1. The extracted chromatin pellets were resuspended in 20 mL of the same concentration of buffered Triton X-100 and recentrifuged, and the supernatants were discarded. The extracted chromatin pellets were then washed two times in NTE with centrifugation and used for measurement of residual RBF-1 and PR binding.

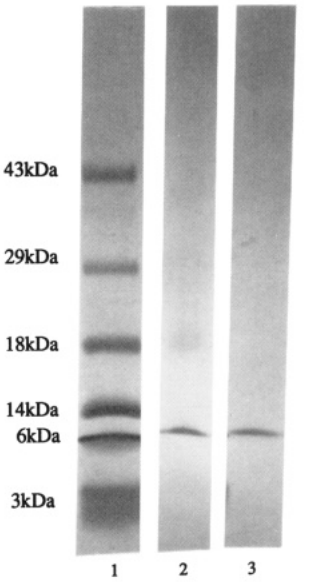

FIGURE 1: RBF-1 purified by the preparative SDS–PAGE: Western analysis and protein stain. RBF-1 (12 μg/lane) was run on a 10% polyacrylamide gel as indicated in Materials and Methods. Lane 1 (prestained molecular weight standards, Catalog No. 6040SA,BRL) and lane 3 (RBF-1) were Coomassie Blue stained. Lane 2 was immunostained as indicated in Materials and Methods.

Guanidine Extraction of RBF-1 from the Nuclei or Chromatin. Aliquots of hen oviduct chromatin (4 mg of DNA) were precipitated in 0.2 M KCl and pelleted at 23000*g* for 10 min. The pellets were then resuspended in 20 mL of various concentrations of Gdn-HCl (0–4 M) in 50 mM phosphate, pH 6.0, and incubated on ice for 2 h with frequent mixing. The extraction mixtures were centrifuged at 60000*g* for 48 h, and the supernatants were dialyzed and lyophilized for Western blot analysis for RBF-1. The extracted chromatin pellets were washed two times with 3 mL of NTE and centrifuged at 23000*g* for 10 min. The chromatins were used for the measurement of residual RBF-1 and PR binding.

DNA and Protein Quantitation. The method for Burton (1968) and Bradford (1976) was used to quantitate DNA and protein, respectively.

Results

Extensive effort was made to improve and shorten the isolation method for RBF-1 from that described previously (Goldberger & Spelsberg, 1988). An abbreviated purification step and method was developed with use of preparative SDS–PAGE. Pooled fraction 3 from the CL-Sepharose-6B chromatography (Goldberger & Spelsberg, 1988) was applied to preparative SDS–PAGE by use of a slab gel apparatus as described in Materials and Methods. The purified protein, isolated by this method, has the same amino acid sequence and PR acceptor activity as RBF-1, which was isolated by the longer method of Goldberger and Spelsberg (1988). The protein yield was increased 150-fold, and significant reduction in effort was achieved with this new approach. Figure 1 shows the Western blot of the RBF-1 purified by the short method and detected by immunostaining and protein staining. Both indicate an electrophoretically pure protein with an apparent molecular mass of around 10 kDa.

Two other analyses suggest that the protein is homogeneous. First, two-dimensional gel electrophoresis involving isoelectric focusing in the first dimension and molecular sizing in the second dimension, revealed a single-size protein with a rela-

Table I: N-Terminal Amino Acid Sequence of RBF-1 for the Avian Oviduct PR[a]

Met-Ile-Pro-Pro-Val-Gln-Val-Ser-Pro-Leu-Ile-Lys-Phe-Thr-X-Tyr-Ser-Ala-Leu-Leu-Val-Gly-Met-Ile-Tyr-Gly-(Lys)-(Lys)-X-Tyr-Asp-Tyr-Leu-Lys-Pro-Ile-Ala-Val

[a] The amino acid sequence analysis is described in Materials and Methods. The amino acids in parentheses represent tentative identification. The X represents unidentified or nondetectable amino acids. The double amino acid indicates the position where both amino acids have been identified. This sequence is representative of five additional analyses.

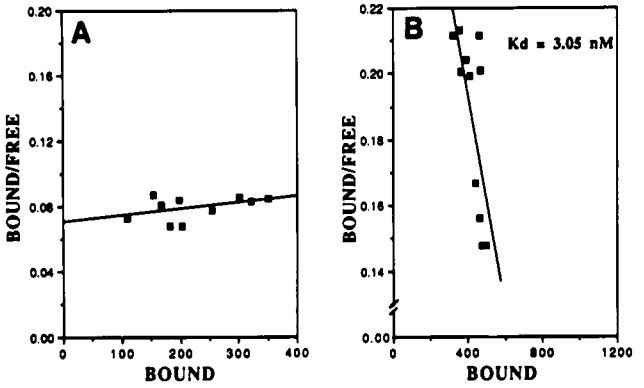

FIGURE 2: Scatchard analyses of PR binding to reconstituted sites composed of purified RBF-1 and avian DNA and to pure avain DNA alone. Pure hen DNA was isolated as previously described (Spelsberg et al., 1984). RBF-1 was purified as described in Materials and Methods. RBF-1 was reconstituted to pure hen DNA to form an RBF-1–DNA complex as described previously (Spelsberg et al., 1984), and pure hen DNA and RBF-1–DNA duplex were bound with [^{3}H]PR in the cell-free binding assay as described in Materials and Methods. The binding data from pure hen DNA (panel A) and RBF-1–DNA complex (panel B) were Scatchard analyzed.

tively broad p*I* (data not shown). Second, as shown in Table I, the N-terminal amino acid sequence analyses of this 10-kDa protein support the presence of a single protein. The first 37 amino acids of the N-terminal sequence have been obtained with five separate preparations of the 10-kDa protein. A search of the Protein Identification Resource database revealed no significant similarities with any other known proteins with a minor homology to the large T antigen of the SV-40 virus. The amino acid sequence in this N-terminal domain of RBF-1 is highly hydrophobic with a relatively high percentage of amino acids favoring a nonhelical extended sheet structure. Analysis of the total amino acid composition indicated that other parts of the molecule must contain an increased frequency of charged amino acids, but the protein overall still contains an excess (63%) of hydrophobic amino acids.

A series of studies were then performed to assess the specificity of the PR binding to the RBF-1–DNA duplexes. Figure 2 shows a Scatchard analysis of [^{3}H]PR binding to DNA alone (panel A) and to a reconstituted RBF-1–DNA duplex (panel B). The RBF-1–DNA complex displays a saturable, high-affinity binding ($K_D = 3 \times 10^{-9}$ M), whereas the hen DNA alone displays nonsaturable binding. To determine the receptor dependency of the steroid binding to the RBF-1–DNA duplex, the DNA binding activity of the receptor was inactivated by removal of reducing agents during the isolation and processing (Peleg et al., 1988). As shown in Figure 3, the inactivated PR fails to bind to the RBF-1–DNA, whereas the active PR does bind, indicating that the PR binding correlates with an increase in the amount of RBF-1 reconstituted to the DNA. Figure 4 shows that the preaddition (by 15 min) of a 2-fold excess of nonradiolabeled PR to the assays containing [^{3}H]PR results in a markedly reduced PR

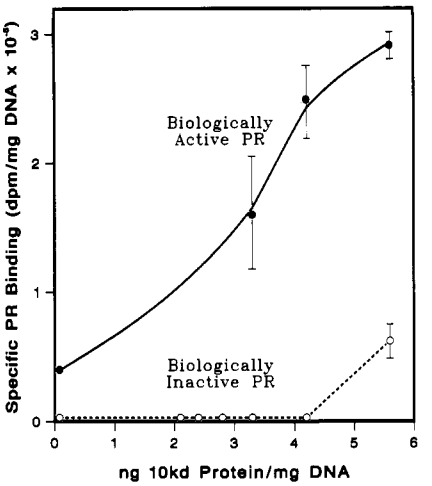

FIGURE 3: Dependence on a biologically active progesterone receptor for the binding to the reconstituted PR acceptor sites using RBF-1 acceptor protein. Biologically active (PR) (●) or inactive PR (○) were isolated as previously described (Peleg et al., 1988), and each were bound to the 10-kDa RBF-1–DNA duplex and reconstituted at various ratios of RBF-1 to the hen genomic DNA. Each of these reconstituted assays were then assessed for specific PR binding in the cell-free assays as described in the Materials and Methods section. The mean ± standard deviation of the replicate assays in one experiment is shown.

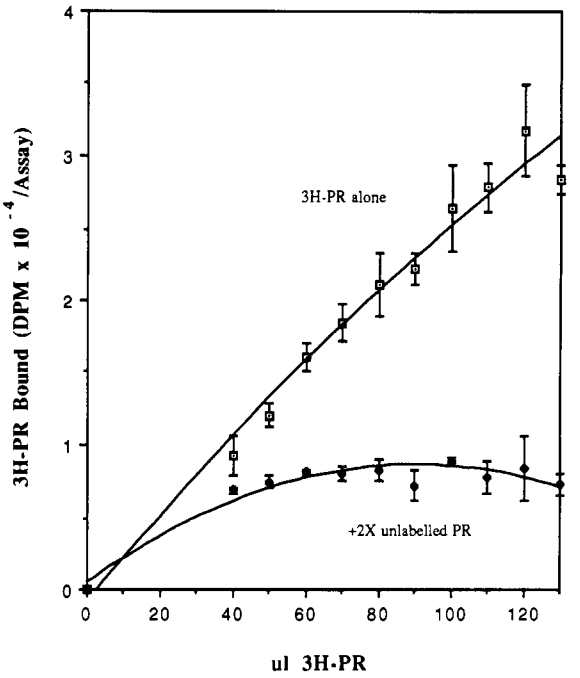

FIGURE 4: Competition of nonradiolabeled PR for [3H]progesterone receptor binding to the reconstituted RBF-1–DNA duplex. RBF-1–DNA duplex was bound with [^{3}H]PR alone (□) or with the addition of a 2-fold excess of nonradiolabeled PR (♦). The complex was prepared by reconstituting 10 ng of protein/1 mg of hen genomic DNA. The means ± standard deviations of three replicate analyses at each point from one experiment are shown.

binding to the reconstituted RBF-1–DNA duplex. This data gives evidence that the PR binding to the RBF-1–DNA duplex is saturable.

To determine the conditions required to dissociate RBF-1 from chromatin, extractions of oviduct chromatin with increasing concentrations of Gdn HCl and Triton X-100 were performed. Figure 5 shows Western immunoblots of the extracted (panels A and C) and the residual protein still bound to nuclear chromatin (panels B and D). The data demonstrates that RBF-1 is wholly dissociated from the chromatin either

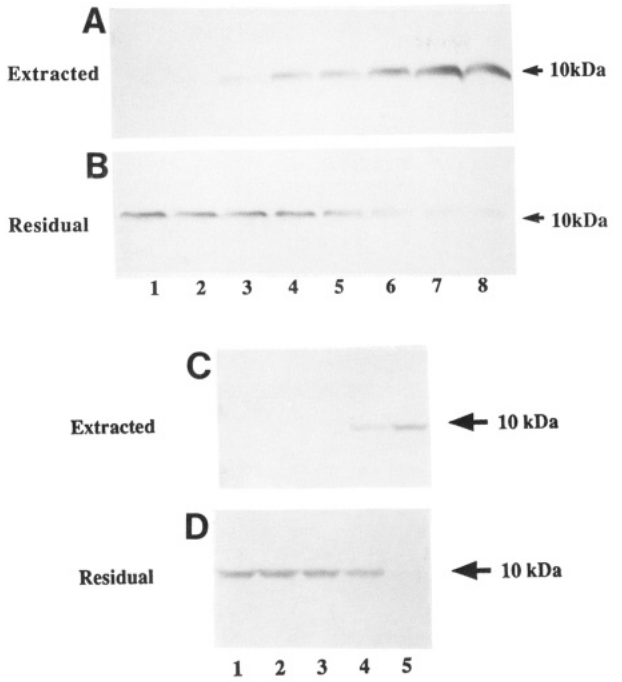

FIGURE 5: Western blot analysis of RBF-1 in Triton X-100 and Gdn HCl extracts from oviduct chromatin. Avian oviduct chromatin was extracted with Triton X-100 or guanidine hydrochloride as described in the Materials and Methods Section. Panels A and B are the Western blots of Triton X-100 extracted proteins (40 μg of DNA) and the residual chromatin proteins, respectively: lane 1, 0%; lane 2, 0.02%; lane 3, 0.04%; lane 4, 0.06%; lane 5, 0.08%; lane 6, 0.1%; lane 7, 0.2%; and lane 8, 0.3% Triton X-100. Panels C and D are the Western blots of the Gdn HCl extracts from chromatin (40 μg of DNA) and the residual (DNA-bound) proteins, respectively: lane 1, 0 M; lane 2, 1 M; lane 3, 2 M; lane 4, 3 M; and lane 5, 4 M guanidine hydrochloride.

by 0.2% (v/v) Triton X-100 (panels A and B) or by 4.0 M Gdn HCl (panels C and D). Interestingly, Figure 6 shows that untreated chromatin containing RBF-1 contains two high-affinity classes of sites (panel B), while the 0.2% (v/v) Triton X-100 treated chromatin, devoid of RBF-1 (panel D), shows a loss of the higher affinity class of sites. The number of [^{3}H]PR binding sites per cell was calculated from the Scatchard plots to be approximately 19 500 for the higher affinity class of sites and approximately 38 100 for the lower affinity class of sites. These values were determined assuming 2.5×10^{-12} g of DNA/avian oviduct diploid cell. Further studies demonstrated that the gradual removal of the RBF-1 by increasing Triton X-100 concentrations in the extractions correlated with the gradual loss of the higher affinity PR binding sites (data not shown).

It was of interest to determine the intracellular localization of RBF-1. The soluble cytosol protein, insoluble protein (cell membranes and organelles), and the isolated nuclei, purified through heavy sucrose from the avian hen oviduct, were analyzed by Western immunoblots for the RBF-1. Figure 7 shows that the highest concentration of immunoreactive RBF-1 was associated with the purified nuclei. The amount used for this experiment was determined by assuming a 1/5 volume ratio between the nuclei and cytosol by use of the diameters of the nucleus and the whole cell as determined from EM photos of oviduct cells. Figure 8 shows the tissue specificity of RBF-1, by use of Western blots (panel A). The liver nuclei contain significantly higher quantities of RBF-1 than oviduct nuclei while spleen nuclei contain very little RBF-1. While the reason for this particular tissue distribution is unclear, a general correlation was shown between the amount of RBF-1 per tissue and the capacity of the chromatin to bind PR (panel

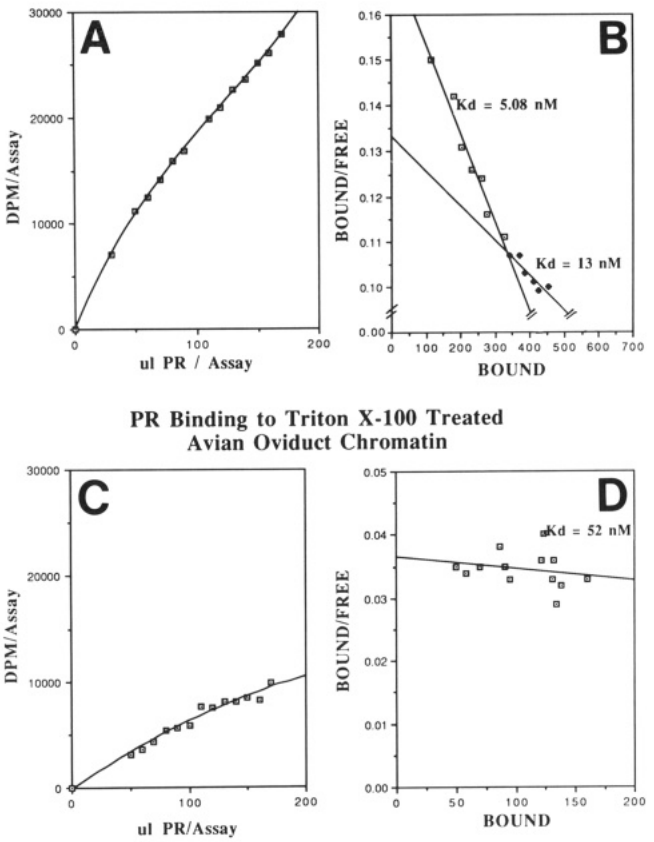

FIGURE 6: [^{3}H]PR binding to untreated and 0.2% Triton X-100 extracted chromatin. Chromatin isolated from hen oviduct was assayed for cell-free [^{3}H]PR binding following either no treatment (panels A and B) or 0.2% Triton X-100 extraction (panels C and D) to remove RBF-1. Panels B and D represent Scatchard analyses wherein the abscissa represents picomolar (10^{-12} M) [^{3}H]PR bound to the chromatin.

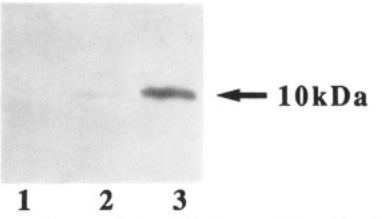

FIGURE 7: Western blot analysis of the intracellular density distribution of RBF-1 in the avian oviduct. Oviduct tissue was homogenized as described in the Hen Oviduct Nuclei Isolation section in Materials and Methods. Aliquots from the low sucrose treatment (cystosol) (lane 1) and the high sucrose treatment (membrane and organelles) (lane 2) during oviduct nuclei isolation were separated by SDS–PAGE and immunostained for RBF-1 as described in Materials and Methods. In lanes 1 and 2, the fraction taken of the total extract was one-fifth of that used from the isolated nuclei (40 μg of DNA) (lane 3).

B). Preliminary analysis of the animal species specificity showed that this protein as an antigen was not detected in human endometrium (data not shown). A small amount of the RBF-1 antigen was found in the nuclei of a fibroblast cell line of QT6 quail cells (a gift from Dr. Nita Maihle). In any case, a tissue and species specificity of the RBF-1 is indicated. Since liver chromatin displays a significantly higher number (5-fold) of RBF-1 than does spleen chromatin, it was further analyzed for specific [^{3}H]PR binding (Figure 9, panel A) and Scatchard analyzed (Figure 9, panel B). As shown in panel B of Figure 9, the liver chromatin contains a high-affinity, saturable binding of the oviduct PR with an affinity (K_D = 4.56×10^{-9} M) similar to and a level higher than that of oviduct chromatin.

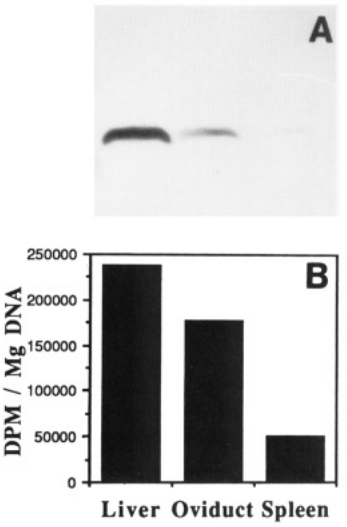

FIGURE 8: Tissue specificity of RBF-1 in the avian system by use of Western blot analysis and [^{3}H]PR binding. Chromatin was isolated from liver, oviduct, and spleen as described in Materials and Methods. Aliquots of each from 40 μg of DNA were separated by SDS–PAGE and immunostained from RBF-1 as described in Materials and Methods (panel A). Panel B shows the [^{3}H]PR binding to each chromatin with 150 μl of [^{3}H]PR.

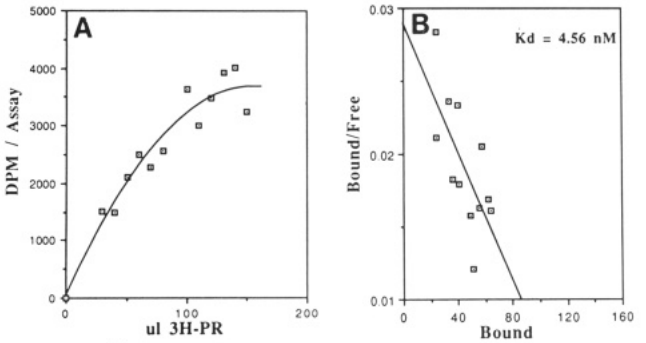

FIGURE 9: [^{3}H]PR binding to avian liver chromatin. Chromatin was isolated from chick liver and bound with [^{3}H]PR in a cell-free assay as described in Materials and Methods. The chromatin was titrated with increasing amounts of [^{3}H]PR (panel A) and Scatchard analyzed (panel B).

The relative amount of RBF-1 in oviduct chromatin was quantitated by Western blot analysis (Figure 10). Chromatin DNA (5 and 25 μg) was compared to known RBF-1 standards. The amount of the chromatin-associated protein was 11.1 and 55.7 μg, respectively. Approximately 0.02 μg of RBF-1 was calculated to exist in 55.7 μg of chromatin protein (i.e., 25 μg of chromatin DNA). By use of these data, there are an estimated 1.2×10^5 molecules of RBF-1/avian cell. Therefore, to purify RBF-1 to homogeneity, a 2785-fold purification from whole chromatin, or a 27 000-fold purification from whole tissue, is calculated from these data and the assumption that each avian cell contains 2.5×10^{-12} g of DNA/cell and the molecular weight of RBF-1 = 10 000 g/mol.

Discussion

This paper describes the final purification of a candidate PR acceptor protein, termed Receptor Binding Factor (RBF-1). A new, improved method of purification of RBF-1 compared to the previous method (Goldberger & Spelsberg, 1988) is described that uses preparative SDS–PAGE. RBF-1 isolated by this new abbreviated method displays the same PR binding activity and N-terminal sequence as the long method (Goldberger & Spelsberg, 1988) and gives a 150-fold greater yield. RBF-1 appears pure by its single N-terminal amino acid sequence and its single band on 2-D PAGE (data not shown).

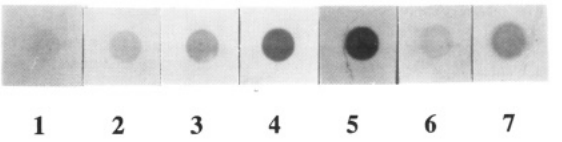

FIGURE 10: Western blot analysis of the quantitation of RBF-1 in oviduct chromatin. Various amounts of purified RBF-1 and oviduct chromatin were solubilized in 4 M guanidine/50 mM phosphate, pH 6, and vacuum blotted to nitrocellulose membrane. The membrane was then immunostained for RBF-1 as described in Materials and Methods: lane 1, 0.01 μg; lane 2, 0.02 μg; lane 3, 0.05 μg; lane 4, 0.1 μg; lane 5, 0.2 μg of purified RBF-1 protein. Lanes 6 and 7 contain 5 μg and 25 μg of chromatin DNA respectively.

When RBF-1 is recombined with hen genomic DNA, a specific binding site for the avian oviduct PR is generated.

The results presented here support that the PR binding to sites generated on avian DNA by RBF-1 require an intact, activated receptor. The [^{3}H]PR binding that nonradiolabeled PR can compete with displays a saturable, high-affinity binding ($K_D \sim 10^{-9}$ M) via Scatchard analyses. In contrast, the binding of [^{3}H]PR to native avian genomic DNA alone or to the reconstituted genomic DNA alone (without the RBF-1) shows a nonsaturable binding. Since RBF-1 generates specific PR binding sites when bound to avian genomic DNA, its role as a candidate acceptor protein for PR in the avian oviduct chromatin is supported. With use of a new highly specific mAb to RBF-1, the factor is shown to be, in part, tissue specific and species specific. Interestingly, there is a direct correlation between the extent of PR binding and the amount of RBF-1 in the various tissue chromatins. Furthermore, RBF-1 density is low in the cytosol and in the membrane/cytoplasmic organelles of the oviduct and appears to be highly localized in the nucleus. The removal of RBF-1 from oviduct chromatin by Triton X-100 without disrupting chromatin structure results in the selective loss of the highest affinity class of PR binding sites.

These studies raise an interesting dilemma on the basis of information in a previous paper (Goldberger & Spelsberg, 1988). The dissociation of RBF-1 from the hydroxylapatite–chromatin resin in the previous paper is achieved by 7.0 M Gdn HCl. We found that RBF-1 does dissociate from the chromatin in 4 M Gdn HCl but then rebinds to the hydroxylapatite resin. It can then be dissociated from the resin by 7.0 M Gdn HCl. Second, RBF-1 required $>10^5$-fold purification in an earlier paper (Goldberger & Spelsberg, 1988) but only 27 000-fold in these studies. The more extensive purification procedure described earlier gives lower yields and probably a greater loss of activity, which may have increased the estimated required purification. Lastly, the use of the mAb to directly measure the quantity of RBF-1 in oviduct chromatin as described in this paper is probably more accurate.

The linear, nonsaturable binding of PR to avian genomic DNA is not a unique observation. We have reported nonspecific PR interaction with avian genomic DNA via seasonally active receptors (Boyd & Spelsberg, 1979; Boyd-Leinen et al., 1984), Scatchard analyses (Goldberger & Spelsberg, 1988; Webster et al., 1976; Spelsberg et al., 1983, 1988), and noncompetitive binding by nonradiolabeled PR (Goldberger & Spelsberg, 1988). As outlined in several reviews and papers, the binding of a variety of steroid receptors to steroid-response elements (SREs), displays a nonsaturable, linear binding not requiring the steroid ligand, activation, or possibly even native structure (Rories & Spelsberg, 1989; Spelsberg et al., 1989). Furthermore, not all steroid-regulated genes contain SREs. However, these reviews also describe a marked preference of the steroid receptor binding to SREs, a critical role of receptor

structure for binding SREs, and the essential role of SREs in the steroid regulation of gene transcription. Interestingly, the requirements for single-strand DNA structure, i.e., coding strand (Lannigan & Notides, 1989), as well as a role of other proteins (e.g., transcription factors and other chromatin proteins) (Miller et al., 1984; Simons et al., 1976; Edwards et al., 1989; Jost et al., 1984; Bagchi et al., 1988; Cordingly et al., 1987; Diamond et al., 1990) in generating specific saturable, high-affinity binding sites for steroid receptors on SREs have been reported. The RBF-1 may bind to the SREs or their flanking domains to generate specific PR binding sites.

RBF-1 shows little similarity in N-terminal sequence to other proteins by a computer search with the Protein Identification Resource database. The amino acid composition reveals the presence of two histidines and several basic amino acids, indicating that one DNA-binding "finger" domain could exist in the molecule (Vincent, 1986). Interestingly, a proline-rich, extended (β sheet) domain in the N-terminal domain of this RBF-1 has a similar counterpart in the myc and myb nuclear oncogene proteins (Weiss et al., 1985). A positively charged, hydrophobic cluster in the N terminus of the retinoblastoma protein, a protein reported to be a DNA-binding protein, has also been demonstrated (Lee et al., 1987). The hydrophobic amino acid composition and the overall acidic p*I* of this 10-kDa protein calculated from its total amino acid composition agrees with the known physical characteristics of this protein determined empirically during its purification.

Specific chromatin acceptor proteins for several other steroid receptor systems have been identified. These studies include the PR in the avian oviduct (Bagchi et al., 1988) and in other tissues (Edwards et al., 1989) as well as glucocorticoid receptors in rat liver (Okamoto et al., 1988). It should be mentioned that by use of similar methods, a related family of "acceptor proteins" have been identified for the estrogen receptors in the rabbit and cow uteri by Ruh and co-workers (Ross & Ruh, 1984; Singh et al., 1986; Spelsberg et al., 1988). One of these, a 16-kDa protein, has recently been purified and found to be hydrophobic and tightly bound to the DNA. This protein closely resembles but is different in size from the avian oviduct RBF-1 described here. The anti-RBF-1 mAb described here does not recognize this 16-kDa protein (data not shown). Lastly, a 20-kDa acceptor protein bound by the rat prostate androgen receptor in vivo was identified by cross-linking the receptor that was bound to its nuclear/chromatin acceptor sites (Rennie et al., 1987). About 80% of the AR is reportedly bound to this protein while 20% of the AR is bound to DNA in this system (Foekens et al., 1985). As stated earlier, an acceptor-like protein has been identified for binding the thyroid hormone receptors to the genomic DNA (Murray & Towle, 1989).

Whether or not the sites generated by RBF-1 in the avian oviduct system reflect the true (native) PR acceptor sites in chromatin remains to be determined. However, the properties of RBF-1 including its ability to generate a high-affinity binding of PR on hen genomic DNA support RBF-1 as a candidate acceptor protein for PR in the avian tissues.

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Wheat Germ Initiation Factors 4F and (iso)4F Interact Differently with Oligoribonucleotide Analogues of Rabbit α-Globin mRNA†

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ABSTRACT: The binding of capped oligoribonucleotide analogues of the 5′ terminus of rabbit α-globin mRNA to wheat germ protein synthesis initiation factors eIF-4F and eIF-(iso)4F was measured by direct fluorescence techniques. An analysis of the equilibrium association constants (K_{eq}) indicates that both eIF-4F and eIF-(iso)4F recognize primarily the m^7G cap structure but differ in the recognition of other structural features. eIF-4F is sensitive to the position and sequence of hairpin structures within the oligoribonucleotide, while eIF-(iso)4F shows a preference for linear sequences. These differences suggest that wheat germ eIF-4F and eIF-(iso)4F may have discriminatory activity for mRNA recognition.

The cap-binding protein eIF-4E,[1] a 25-kDa polypeptide, has been shown to bind directly to the m^7G cap of mRNA (Tahara et al., 1981; Sonenberg, 1981; Sonenberg et al., 1981; Hellmann et al., 1982; Webb et al., 1984; Carberry et al., 1989, 1990; Goss et al., 1990). However, a recent study on the interaction of human eIF-4E with oligoribonucleotide analogues of rabbit α-globin mRNA has shown that eIF-4E is also capable of interacting with other structural features of the globin mRNA (Carberry et al., 1991b).

Two cap-binding proteins have also been isolated from wheat germ (Lax et al., 1986a,b): eIF-(iso)4F (previously designated eIF-4B) which consists of a 28- and a 82-kDa polypeptide in

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[1] Abbreviations: m^7G, 7-methylguanosine; eIF, eukaryotic initiation factor; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; DTT, dithiothreitol; kDa, kilodalton.

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