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Immunohistochemical Localization of the Avian Progesterone Receptor and Its Candidate Receptor Binding Factor (RBF-1)

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Abstract An avian oviduct nuclear matrix protein in the 6–10 kDa size range has been implicated to function in the cell-free nuclear binding of the avian oviduct progesterone receptor (PR). This protein, termed the receptor binding factor-1 (RBF-1), has been purified and partially characterized [Schuchard et al.: Biochemistry 30:4535–4542, 1991]. This paper describes the immunohistochemical co-localization of the RBF-1 and PR in the avian oviduct cell nuclei and rat reproductive cell nuclei using antibodies directed specifically against the RBF-1 and activated PR. In the undifferentiated oviduct, the immunoreactivities for both PR and RBF-1 were co-localized in the nuclei of only epithelial cells, but not the stromal cells or smooth muscle cells. In the partially differentiated oviduct of estrogen treated chicks, the immunoreactivity co-localized in the nuclei of not only epithelial but also glandular and stromal cells. Staining for the PR, but not RBF-1, was detected in the smooth muscle cells. The intensity of the PR but not the RBF-1 staining was markedly down-regulated in these cells at 2 and 6 h after treatment of the animals with progesterone (P). However, the band patterns for RBF-1 in the Western blots did show qualitative changes which may reflect P-induced post-translational modifications which alter the epitope on the RBF-1. Interestingly, immunohistochemical analysis of several reproductive tissues of the rat showed that certain cell types in the uterus, ovary, and prostate displayed strong positive nuclear staining for an RBF-1–like antigen(s). Western blot analyses of the rat reproductive tissues showed that the immunoreactive component was a 15 kDa antigenically related RBF-1 which was only present in the uterus. © 1993 Wiley-Liss, Inc.

Key words: epithelial cells, RBF-1, progesterone receptor, immunohistochemistry, estrogen

Advances in the last two decades have demonstrated that steroid hormone receptor-mediated gene transcription is an intricately complex process [for review see Carson-Jurica et al., 1990; Landers and Spelsberg, 1992]. While many studies have described the properties of the interaction between purified steroid receptor (SR) complex and steroid responsive DNA elements neighboring steroid responsive genes [for review see Beato, 1989; Martinez and Wahli, 1991], more recent studies have implicated other nuclear proteins, both soluble and chromatin-bound, to play a role in this process. For example, recent studies indicate that steroid receptors interact with the nuclear proto-oncoproteins, *Fos* and *Jun*, at AP-1 transcription factor binding elements (and perhaps with other proteins at other DNA elements) to regulate gene expression [see review by Landers and Spelsberg, 1992].

Soluble, receptor-specific factors, often termed accessory proteins, have been shown to enhance SR binding to the DNA. These include factors which augment receptor-DNA interactions with the thyroid [Murray and Towle, 1989; Burnside et al., 1990], glucocorticoid [Cavanaugh and Simons, 1990], vitamin D [Sone et al., 1991], and progesterone receptors [Edwards et al., 1989]. These proteins may differ from a second class of proteins, nonhistone chromatin proteins, which affect the interaction of SRs with DNA.

Although it has been accepted that the interaction of activated steroid hormone receptor complexes with endogenous nuclear acceptor sites (i.e., the nuclear binding sites) is a critical step in steroid-specific alterations of gene expression,

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little is known about the nature of these sites other than they appear to be associated with the nuclear matrix [see review by Rories and Spelsberg, 1989].

Early characterization of the chromatin acceptor sites has been reported for the progesterone (P) [Spelsberg et al., 1983, 1984, 1987a, 1989; Perry and Lopez, 1978; Lopez et al., 1985; Cobb and Leavitt, 1987], estrogen (E_2) [Ruh et al., 1981; Ross and Ruh, 1984; Singh et al., 1984], and androgen [Mainwaring et al., 1976; Klyzsejko-Stefanowicz et al., 1976; Wang, 1978; Rennie et al., 1987] receptors in a variety of animal systems. Chromatin acceptor sites for the avian oviduct progesterone receptor (PR) have been studied extensively and found to consist of complexes of specific acceptor proteins tightly bound to specific DNA sequences [Spelsberg et al., 1972, 1984, 1987a,b, 1988; Schuchard et al., 1991a,b; Pikler et al., 1976; Kon and Spelsberg, 1982; Hora et al., 1986; Goldberger et al., 1987; Goldberger and Spelsberg, 1988; Rejman et al., 1991]. The specificity of this interaction is highlighted by in vivo and in vitro studies which demonstrate that the binding of PR to avian oviduct chromatin is not only saturable and high affinity [Spelsberg et al., 1983, 1984, 1987a,b; Pikler et al., 1976], but also receptor dependent [Pikler et al., 1976] and receptor specific [Spelsberg et al., 1987a,b; Kon and Spelsberg, 1982]. Proteins involved in the acceptor site activity have been identified for other hormone receptors in a variety of target tissues systems in several animal species [Klyzsejko-Stefanowicz et al., 1976; Lian and Spelsberg, 1982; Ross and Ruh, 1984; Foekens et al., 1985; Ruh et al., 1986, 1987; Rennie et al., 1987; Murray and Towle, 1989]. Purification of one species of acceptor site proteins for avian PR has yielded a small (~ 6–10 kDa), chromatin-associated protein that is somewhat hydrophobic and has been localized to the nuclear matrix. This protein has been functionally termed the receptor binding factor-1 (RBF-1) [Schuchard et al., 1991a,b; Rejman et al., 1991]. Purification of an acceptor protein for the rabbit uterine estrogen receptor (ER) has yielded a similar but somewhat larger (17 kDa) protein [Ross and Ruh, 1984; Singh et al., 1984; Spelsberg et al., 1988]. A 20 kDa acceptor protein for the androgen receptor has also been reported [Rennie et al., 1987].

The RBF-1 protein appears to play a role in the highest affinity class of nuclear binding sites for PR in the avian oviduct [Rejman et al., 1991; Schuchard et al., 1991a; Horton et al., 1991]. Selective removal of the chromatin protein fraction which contains RBF-1 results in the loss of the highest affinity class of PR binding sites. When RBF-1 is reconstituted to avian genomic DNA, the specific binding of PR is regenerated [Spelsberg et al., 1984, 1988; Schuchard et al., 1991a,b; Hora et al., 1986; Goldberger et al., 1987; Rejman et al., 1991]. The RBF-1 has been purified to homogeneity from oviduct nuclei and characterized. The N-terminal amino acid sequence is consistent with a protein that is hydrophobic, and the protein has an apparent M_r ~ 6–10 kDa as determined by SDS-PAGE [Schuchard et al., 1991a,b; Rejman et al., 1991]. Interestingly, using biochemical analysis and cell fractionation techniques, the RBF-1 has been localized to the nuclear matrix along with the nuclear bound PR [Rejman et al., 1991; Schuchard et al., 1991b]. This is the same intranuclear site where activated steroid receptors and much of the transcriptional machinery of the cell nucleus have been localized [see review by Barrack and Coffey, 1980].

As a means of further characterizing the RBF-1, an immunohistochemical approach has been utilized in this study to evaluate the subcellular localization of both the PR and the RBF-1 protein associated with the nuclear acceptor sites in two different animal species and a variety of tissues. Using polyclonal antibodies prepared against a highly purified preparation of RBF-1, the present study demonstrates a parallel nuclear co-localization of the RBF-1 and PR in specific cells of the avian oviduct that appears to be very cell-type specific. Moreover, a related RBF-1 antigen appears to be present in reproductive tissues of mammals that also appears to be cell-type specific.

METHODS

Animal and Treatments

For immunohistochemical studies, 21-day-old female White Leghorn chicks (n = 15) were injected in the right leg muscle with E_2 (17 β-estradiol) (Sigma, St. Louis, MO) at a dose of 1 mg/per chick/day for 1 week. This generates a partially differentiated oviduct. Five controls received propylene glycol only (100 μl/chick/day) and represent undifferentiated oviducts. After 1 week without any treatment, one group received a single injection of P (20 mg/kg; E. Merck,

Darmstadt, Germany) at 20 mg/ml in propylene glycol. The controls were injected with propylene glycol alone. The chicks (n = 5) were killed by cervical dislocation 0, 2, and 6 h after P or vehicle administration, and the oviducts were taken for immunohistochemical analysis of PR and RBF-1 and for Western blot of RBF-1. For Western blot analysis, chicks were injected with 0.2 ml estradiol benzoate (5 mg/ml in sesame oil) for 3 weeks with injections beginning 1 week after arrival. After a 4 day withdrawal, chicks were injected once daily with 0.2 ml of P (5 mg/ml in propylene glycol) for up to 6 days, sacrificed at the appropriate times, and the oviduct was removed. In a similar fashion, samples from the ovary, uterus, and prostate were taken from 3-month-old normal male and female Wistar rats and subsequently analyzed.

Antibodies

The antibodies used in this study were 1) anti-PR monoclonal antibodies PR13, PR6, and PR22 [Sullivan et al., 1986], 2) anti-PR polyclonal antibody (PAb) (PR60) which was made against human/chick PR C-terminal amino acids [$cPR_{772\text{-}786}$ = $hPR_{921\text{-}933}$ (L-A-G-M-V-K-P-L-F-H-K-K)] conjugated to thyroglobulin, and 3) anti–RBF-1 polyclonal (PAb) [Goldberger et al., 1986; Goldberger and Spelsberg, 1988; Schuchard et al., 1991a]. The latter was prepared against RBF-1 obtained from preparative PAGE purification procedure as described previously [Schuchard et al., 1991b]. The anti-PR polyclonal antibody was the IgG fraction precipitated with 40% ammonium sulfate. The precipitate was dissolved in water and dialyzed against PBS prior to use. Anti–RBF-1 polyclonal antibody was purified on protein A (Affigel). The anti–RBF-1 monoclonal antibody recognizes the free denatured protein but not the DNA-bound acceptor protein while the anti-RBF polyclonal antibody recognizes both the free and DNA-bound protein. The purification of RBF-1 and characterization of the monoclonal antibodies have been reported elsewhere [Rejman et al., 1991; Schuchard et al., 1991a,b].

Immunohistochemistry

For RBF-1, the chick oviducts and rat tissues were frozen in liquid nitrogen immediately and stored at −70°C until sectioning. Frozen sections (10 μm thick) were cut in a cryostat (Leitz, cryostat 1720, Germany), thaw-mounted on poly-L-lysine coated glass slides, and fixed in Zamboni's fixative (2% (w/v) paraformaldehyde/15% (v/v) saturated picric acid) for 10 min at room temperature. Immunostaining for PR was performed as described previously [Ylikomi et al., 1985; Joensuu, 1990]. Briefly, after washing in PBS, non-specific binding was blocked with 10% (v/v) normal serum (normal horse serum for MAb and normal goat serum for PAb) for 15 min at room temperature. The sections were incubated with anti-PR and anti-RBF-1 antibodies overnight at 4°C. The antibody concentration was 5 μg/ml for the anti–RBF-1 antibodies. Then sections were incubated with biotinylated secondary antibody for 30 min at room temperature. Visualization was carried out using the avidin-biotin peroxidase complex (Vector Laboratories, Burlingame, CA) for 30 min, and 0.05% (v/v) diaminobenzine tetrahydrochloride (DAB) as chromogen with 0.01% (v/v) imidazole and H_2O_2 in 0.5 M Tris, pH 7.6, for 5 min. Primary antibodies were replaced by preimmune purified IgG (5 μg/ml) as the control.

For PR, immunohistochemical staining was carried out in frozen section with PAb PR60. For MAbs (PR13, PR6, and PR22), chick tissues were fixed in Baker's fixative (4% (v/v) paraformaldehyde, 1% $CaCl_2 \cdot 2H_2O$, pH 6.7) for 4 h at 0°C and embedded in paraffin. Immunohistochemistry was carried out according to the method described above. The working concentrations of PR13, PR6, and PR22 were 1 μg/ml and 5 μg/ml for PR60. No counterstaining was used.

Isolation of tissue nuclei. Tissue was weighed and minced into small pieces with scissors. A volume 3–4 times the weight of the tissue of nuclei solution #1 containing 0.5 M sucrose in 1 × TKM buffer (50 mM Tris, 25 mM KCl, and 2 mM $MgCl_2$, pH 7.5) was used to blend the tissue in a blender on high setting for 10 seconds followed by homogenization (5–8 strokes) in a Thomas homogenizer at medium setting. The homogenate was filtered through two layers of cheesecloth to remove debris. The filtrate was centrifuged at 17,000*g* for 5 min at 4°C and the supernatant discarded. The pellets were resuspended in a volume of nuclei solution #2 (2.0 M sucrose in 1 × TKM buffer, pH 7.5) equivalent to that used with nuclei solution #1 with a smooth pestle homogenizer. The concentration of sucrose was adjusted to 1.75 M by the addition of distilled water and the resultant

solution centrifuged at 27,000*g* for 10 min at 4°C. The resulting pellet represents clean nuclei.

Isolation of chromatin from nuclei. The nuclei pellets were resuspended in the same volume of chromatin solution #3 (2 mM Tris, 1 mM EDTA, pH 7.5) as used with nuclei solution #1 and #2. Using a smooth pestle homogenizer (by hand), the body of the homogenizer was briskly pulled away from the pestle six or seven times allowing the vacuum to encourage breaking the nuclei. The homogenates were centrifuged at 27,000*g* for 10 min at 4°C, and the pellets were resuspend in a volume of chromatin solution #4 (20 mM Tris, pH 7.5) equal to 1–1.5 times the starting tissue weight (w/v). The suspension was filtered through four layers of organza or fine mesh, and an aliquot was removed for diphenylamine assay to determine DNA concentration. The chromatin was stored at −80°C until used for Triton X-100 extraction. To obtain a Triton X-100 extract of the chromatin, the chromatin was thawed and homogenized, and the volume was adjusted to a salt concentration of 0.5 M using 2.0 M KCl. After 5 min on ice, the suspension was centrifuged at 17,000*g* for 10 min. The pellets were resuspended in the appropriate volume of solution #5 (1 × NTE) to obtain a a final DNA concentration of 0.2 mg/ml and centrifuged twice at 17,000*g* for 10 min.

Polyacrylamide gel electrophoresis and Western blot analysis of RBF-1. Samples were analyzed for the presence of RBF-1 by detection using SDS-polyacrylamide Tris-tricine gel electrophoresis system and Western immunoblotting as described previously [Schuchard et al., 1991b] with some minor modifications. Briefly, equal amounts of protein were blotted to nitrocellulose in electrode buffer containing 10% (v/v) methanol, 10 mM CAPS, pH 11, at 100 volts (constant current) for 1 h at 4°C. Unbound sites on the nitrocellulose sheets were blocked with 2% milk (w/v; dry stock nonfat) in PBS for 30 min at 37°C with agitation. The sheets were then incubated with 8 μg/ml polyclonal antibody in blocking solution for 1 h at 22°C with agitation. After thorough washing with 0.05% Tween 80 in PBS, the blot was incubated with biotin-conjugated anti-rabbit IgG in 2% milk for 1 h at 22°C followed by washing with 0.05% Tween 80 in PBS. Finally, the blot was incubated with alkaline phosphatase conjugated goat anti-biotin IgG diluted 1:750 in 2% (v/v) milk for 1 h at 22°C. The blots were again washed and then developed in 0.1 M NaCl and 0.1 M Tris-HCl, pH 9.6, containing 0.1 mg/mL nitroblue tetrazolium, 0.05 mg/mL 5-bromo-4-chloroindolyl phosphate, and 0.004 M $MgCl_2$. The polyclonal antibody detects 10 kDa and 6 kDa protein antigens which copurify as the major PR acceptor activity [Goldberger and Spelsberg, 1988]. Since a monoclonal antibody against the same purified RBF-1 preparation recognizes a protein in the 10 kDa region of the gel, the 10 kDa antigen, which also displays a major PR acceptor activity, is referred to as receptor binding factor-1 (RBF-1).

RESULTS

Immunohistochemical Localization

Chick oviduct

Progesterone receptor. As shown in Figure 1A–D, using anti-PR monoclonal antibody (MAb) PR13, PR was observed in the nuclei of epithelial, glandular, stromal, and smooth muscle cells of partially differentiated avian oviduct (1 week E_2 treated chicks) (Fig. 1B), while in undifferentiated oviducts (from non-E_2 treated chicks) only the epithelial cells showed positive staining (Fig. 1A). The staining was decreased in PR positive cells when tissues were obtained from animals treated with P for 2 h (Fig. 1C) and 6 h (Fig. 1D). Results using anti-PR MAb PR6 and PR22 were similar to those obtained with MAb PR13 (data not shown). Using the anti-PR polyclonal antibody PR60 (Fig. 1E), a similar nuclear location for PR was clearly evident in oviduct of E_2 pretreated chicks treated with P for 2 hours. However, the immunostaining with PR60 showed less reduction by P treatment compared to that obtained with PR13 (Fig. 1C).

Receptor binding factor (RBF-1). As shown in Figure 1F–I, the anti–RBF-1 PAb displayed a positive reactivity with the immunocytochemical methods employed in this study. The MAb to

Fig. 1. Immunohistochemistry of the progesterone receptor (PR) using monoclonal antibody PR13 (**A–D**) and polyclonal antibody PR60 (**E**) and of RBF-1 using polyclonal anti–RBF-1 antibody (**F–I**) in chick oviduct. These antibodies were used to probe the following oviduct tissues: A,F = undifferentiated oviducts (non-E_2 treated animals); B,**J** = partially differentiated oviducts (1 week of E_2 treated animals); C,H = oviducts from E_2 treated animals which were treated with P treatment for 2 h; D,I = oviducts from E_2 treated animals which were treated with P for 6 h; E = same as C or H but probed with antibody PR60; J (control) = same as C but the primary antibodies for PR and RBF-1 were replaced by PBS. E, epithelial; G, gland; M, mucosal layer of immature oviduct wall. The bars in the panels represent 25 μm distance.

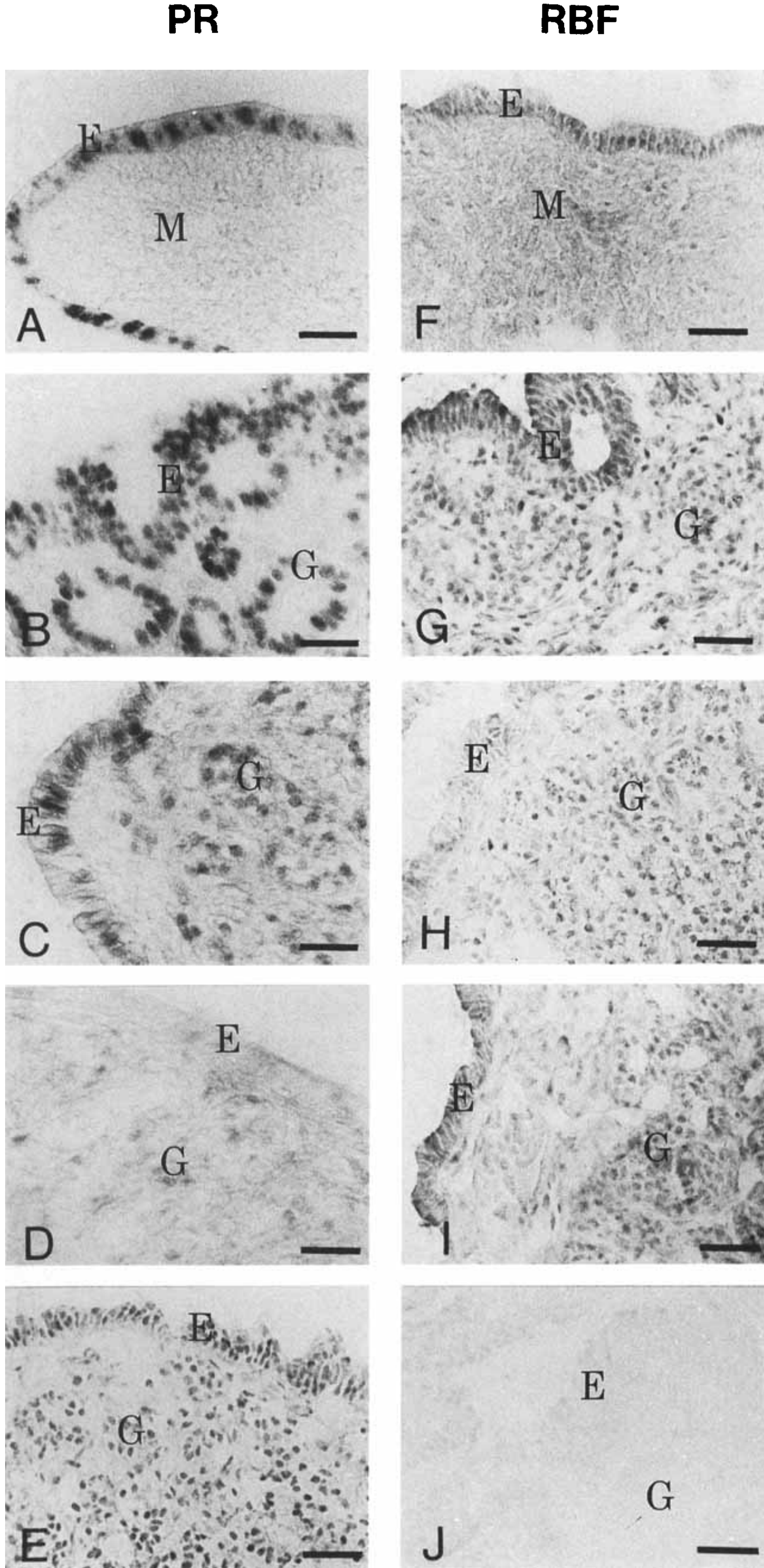

Figure 1.

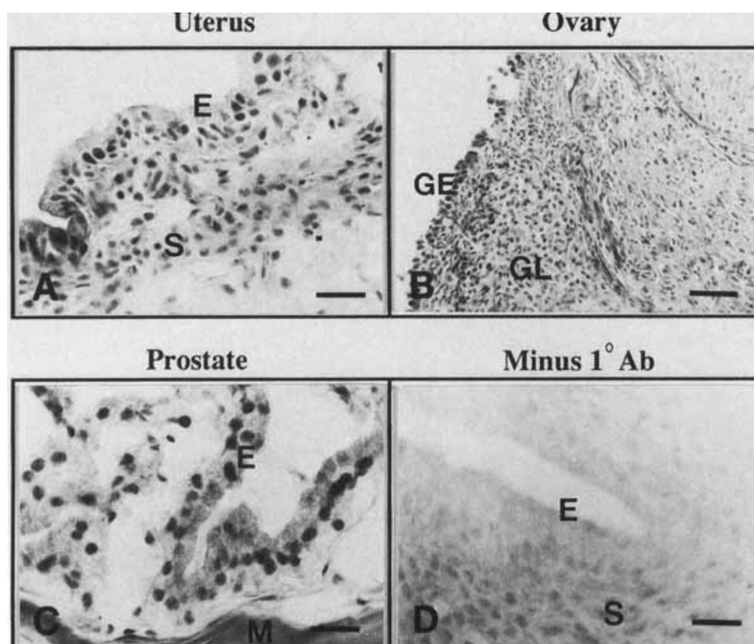

Fig. 2. Immunohistochemistry of RBF-1 in rat reproductive tissues using a polyclonal antibody. **A:** Uterus. **B:** Ovary. **C:** Prostate. **D:** Control staining of the rat uterus with preimmune IgG wherein the primary antibody was replaced by preimmune IgG. E, epithelial; GE, germinal epithelium; GL, granulosa luteum; M, smooth muscle; S, stromal. The bars in the panels represent 25 μm distances.

RBF-1 did not show any immunoreactivity in these tissues (data not shown). Immunoreactivity with the anti–RBF-1 polyclonal antibody was localized in the nuclei of the oviduct cells. Similar to the PR localization (Fig. 1A), the undifferentiated oviducts displayed a definite immunopositive reaction in the nuclei of only the epithelial cells with no immunoreactivity in stromal or smooth muscle cells (Fig. 1F). Also, similar to PR localization (Fig. 1B), immunostaining for RBF-1 was now observed in the nuclei of epithelial, glandular, and stromal cells of the partially differentiated oviducts (i.e., 1 week of E_2 treatment) with no detectable staining in the smooth muscle cells (Fig. 1G). The immunoreactivity was markedly stronger in the epithelial cells in the partially differentiated oviducts compared to the epithelial cells in the undifferentiated oviducts. In the partially differentiated oviducts, the glandular and stromal cells show less immunostaining than the epithelial cells. Unlike the localization with PR, the staining intensity of RBF-1 did not change markedly over 2 and 6 h following P treatment (Fig. 1G–I). The specificity of the immunostaining is shown by the lack of staining in Figure 1J where preimmune antibodies were substituted for the primary anti–RBF-1 PAb.

Rat tissues

Receptor binding factor (RBF-1). With the same polyclonal antibody to the avian RBF-1 as described above, immunohistochemical staining was found in all rat reproductive tissues but not in all cell types (Fig. 2). In all instances the staining with the anti–RBF-1 polyclonal antibody was localized in the cell nuclei. In the uterus, the strongest staining was observed in the endometrium and uterine glands (Fig. 2A), with strong staining also in the myometrium and stromal cells. In the ovary, the granulosa luteal cells and major components of the ovarian parenchyma, including follicles, stromal, and interstitial cells, were positive for RBF-1 immunostaining (Fig. 2B). Interestingly, the smooth muscle cells in blood vessels were negative. In the prostate, positive staining was found predominantly in epithelial cells with a weaker immunoreaction in stromal cells. Again, no staining was found in the smooth muscle cells around the prostate glands (Fig. 2C). Specific binding of the PAb was corroborated in Figure 2D whereby the immunohistochemical staining with preim-

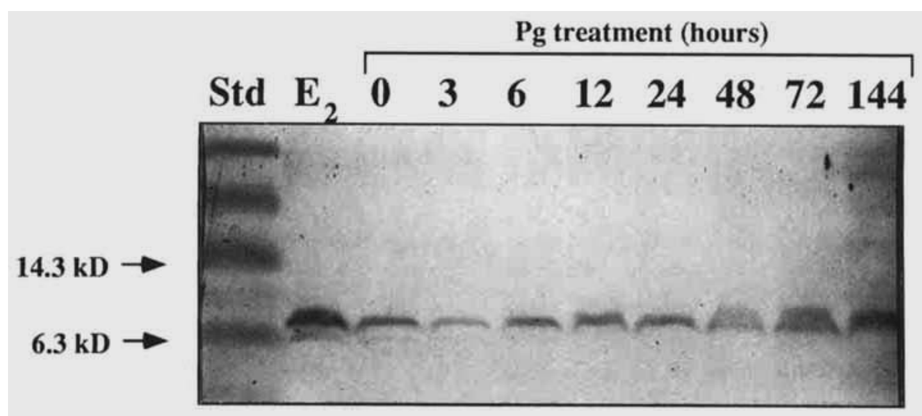

Fig. 3. Effect of in vivo progesterone treatment on the levels of avian oviduct chromatin RBF-1 using Western blot immunostaining with polyclonal antibody for RBF-1. This procedure is described in Material and Methods. Each lane contains the oviduct chromatin protein associated with 100 μg of DNA. Chromatin was isolated from chickens pretreated with E_2, withdrawn for 4 days, and then treated with P for 0 h, 3 h, 6 h, 12 h, 24 h, 48 h, 72 h, and 144 h. These values represent time of sacrifice after progesterone treatment.

mune IgG substituted for the primary antibodies showed little or no staining in the uterus.

Western Blot Analysis of Chick and Rat Tissue Chromatin

Western blot analyses were performed to further characterize the immunoreactivity of the extracted proteins containing RBF-1 from the oviduct chromatins (Fig. 3). The gel lanes were loaded on a per μg of DNA basis (100 μg DNA/lane) with chromatin isolated from each of the tissues. We have found this to be a more reliable loading parameter than the per mg protein basis. The Western blot analyses of the avian oviduct chromatin protein from the undifferentiated oviducts showed that the anti–RBF-1 PAb recognized two closely migrating bands in the 6–10 kDa region of the gel. Termination of the E_2 treatment results in a decrease in the level of RBF-1 detected by the polyclonal antibody (lane E_2 vs. 0 h). Subsequent P treatment of these E_2 treated animals did not lead to any significant changes in the RBF-1 concentration over the first 24 h after treatment as determined by scanning reflective densitometry. However, the lower band disappears by 6 h and at 48 and 72 h post-injection of P, a band having a higher molecular weight appears, thus creating a new doublet. These same pattern changes were observed in a repeat experiment. There was no immunostaining associated with these bands when Western blot analysis was carried out in the absence of the 1° antibody (data not shown).

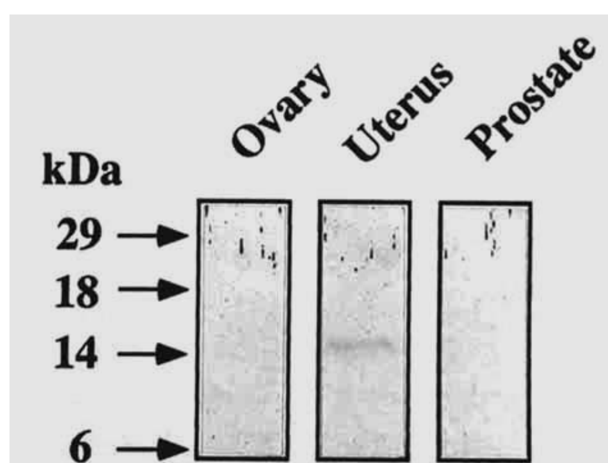

Fig. 4. Western blot anti–RBF-1 polyclonal antibody detection of RBF-1–like proteins in rat tissues. Each lane contains the protein associated with 200 μg DNA of chromatin isolated from the indicated rat tissues.

Western blot analyses of the rat reproductive tissues was carried out with the hope of characterizing the antigens observed with immunohistochemistry. Interestingly, the rat uterus contained a distinct immunoreactive band with a $M_r \approx 15$ kDa, while rat prostate and ovary showed no bands attributed to the specific PAb to RBF-1 (Fig. 4). As with the immunohistochemistry studies, the uterine nuclear extracts showed no immunoreactivity with the anti–RBF-1 monoclonal antibody (data not shown). Also, there was no immunostaining associated with these bands when Western blot analysis was carried out in the absence of the 1° antibody.

DISCUSSION

While the composition of the nuclear acceptor sites which are involved in the binding of steroid receptors to chromatin has been controversial, the requirement of transcription factors along with activated receptors for the transcriptional activation of steroid responsive genes has created a renewed interest in these proteins [for a review see Landers and Spelsberg, 1992]. For the avian PR, a small hydrophobic protein, localized in the nuclear matrix of oviduct nuclei, has been implicated in playing a role in the interaction of the activated PR with DNA. The exact biological function of the RBF-1 protein has not been determined. The RBF-1 protein has been purified to apparent homogeneity and partially characterized [Schuchard et al., 1991a,b; Rejman et al., 1991]. Several hormone acceptor sites for several steroid-receptor systems have been partially characterized, and in some instances, the "acceptor" proteins identified [Spelsberg et al., 1983, 1984, 1989; Schuchard et al., 1991a,b; Goldberger et al., 1987; Ruh et al., 1986, 1987; Klyzsejko-Stefanowicz et al., 1976; Lian and Spelsberg, 1982; Ross and Ruh, 1984; Foekens et al., 1985; Hora et al., 1986; Goldberger and Spelsberg, 1988; Murray and Towle, 1989; Rejman et al., 1991]. All of these systems, in particular the one associated with the avian PR, have been studied exclusively in cell-free systems. The present study describes the first in situ visualization of candidate nuclear acceptor sites for a steroid receptor using immunohistochemical methods. The immunohistochemical localization of RBF-1 confirms other studies using biochemical methods which have shown this protein to be primarily located in the nucleus [Spelsberg et al., 1984; Hora et al., 1986; Schuchard et al., 1991a,b]. Perhaps more importantly, this nuclear localization of both RBF-1 and PR in avian oviduct cells has been further delineated to the nuclear matrix [Barrack, 1987; Rejman et al., 1991; Schuchard et al., 1991b]. This is an important observation in the light of the growing realization that the nuclear matrix is an important site for gene regulation [see review by Barrack and Coffey, 1980].

The present study shows 1) the localization of RBF-1 in the nuclei of only select cells by in situ immunohistochemistry, 2) the co-localization of the RBF-1 and PR in the nuclei of the same avian oviduct cells, 3) that not all cells in the oviduct contain the RBF-1 or the PR, 4) that the banding patterns in Western blots of oviduct chromatin show qualitative changes in response to steroid treatment, and 5) that the rat uterus, but not other rat tissues, contains a cell-specific, nuclear-localized RBF-1–like protein of similar size as the avian RBF-1. In the undifferentiated oviduct, positive nuclear immunostaining for both PR and RBF-1 was observed primarily in the epithelial cells lining the oviduct lumen. In the partially differentiated oviduct, both the PR and the RBF-1 were found in the nuclei of epithelial, glandular, and stromal cells, but the RBF-1 was not found in smooth muscle cells where PR immunostaining was strong. Western blot analysis of RBF-1 in purified form or in crude chromatin preparation have previously shown that the polyclonal antibody recognizes a doublet of $M_r \approx 6{,}000$ and 10,000 while monoclonal antibody recognizes a single protein band or doublet proteins of $M_r \approx 10{,}000$ [Rejman et al., 1991; Schuchard et al., 1991a]. Interestingly, the immunohistochemical studies presented in this paper demonstrate that the polyclonal antibody against RBF-1 but not the MAb were reactive in situ. The negative result with the MAb was not unexpected since the anti–RBF-1 PAb was previously shown to recognize both the DNA-bound and unbound (free of DNA) RBF-1 while the MAb only recognized the unbound RBF-1 (data not shown). The anti–RBF-1 PAb but not the anti–RBF-1 MAb was also found to inhibit PR binding to avian oviduct chromatin but not PR binding to pure hen DNA.

In contrast, all MAbs to the PR (PR13, PR6, and PR22) showed positive reaction in the oviduct tissues. The monoclonal antibodies PR13 and PR22 are specific for the A and B forms of PR while PR6 was specific for only the B form (activated and unactivated) [Sullivan et al., 1986]. The PAb PR60 gives nuclear staining similar to that obtained with the other monoclonal antibodies. PR60 is specific for the C-terminus of the PR and the antibody has been shown to be occluded when PR has bound progesterone [Vegeto et al., 1992; Weizel et al., 1992] and, hence, may be the cause of the reduced P effect. The specific nuclear localization of PR in the tissues evaluated in this study is consistent with the results of previous studies [for review of this topic see Beato, 1989; Rories and Spelsberg, 1989; Carson-Jurica et al., 1990; Martinez and Wahli, 1991; Landers and Spelsberg, 1992]. Other studies have shown unoccupied PR to be

located mainly on the transcriptionally inactive heterochromatin in avian oviduct. Following treatment with P, PR is found primarily on the transcriptionally active euchromatin, with a corresponding decrease in the level of PR in the heterochromatin [Isola, 1987; Tuohimaa et al., 1989; Pekki, 1991]. A similar change in the localization of androgen receptor (AR) after dihydrotestosterone (DHT) have also been reported in rat prostate [Zhuang et al., 1992]. The observed down-regulation of PR is consistent with the observation that PR is induced by E_2 and down-regulated by P [Joensuu, 1990]. Similar changes in PR staining were detected immunohistochemically with MAb PR13 and to a lesser degree with PAb PR60.

Although the RBF-1 appears to show a modest quantitative reduction in the Western blots after E_2 withdrawal and P treatment of the animals, no apparent change in the immunohistochemistry was observed after this steroid treatment or after P treatment. The changing patterns on the Western blots induced by E_2 withdrawal and P treatment, however, may be indicative of steroid regulation of a post-translational modification of the RBF-1 such as a phosphorylation of RBF-1. The phosphorylation of the PR which is regulated by P has been documented [Logeat et al., 1985]. Support for this type of modification on RBF-1 arises by the fact that the 72 h RBF-1 doublet is reversed to a single band with acid phosphatase treatment (data not shown). Overall, the Western blot data provide the first intimation that steroids may regulate the level or modification of RBF-1.

The presence of RBF-1–like antigen(s) in rat tissues by immunohistochemistry is interesting and may suggest that RBF-1 is evolutionarily conserved, at least in part, among different animal species. In rat ovary, PR did not appear in the granulosa luteal cells where RBF-1 immunostaining was strong. In contrast, PR was present and RBF-1 absent in smooth muscle cells; the functional significance of this is not clear. It is interesting that the Western blot analyses only detected an immunopositive 15 kDa RBF-1–like protein from the rat uterine nuclei and not the other tissues which were positive by histochemistry. This may be the result of tissue-specific differences in the antigens (e.g., stability to denaturation or degradation due to released proteases). Whether or not this 15 kDa rat uterine protein is functionally related to the 10 kDa avian oviduct RBF-1 is presently unknown but is being examined by amino acid sequence analyses. Interestingly, this 15 kDa RBF-1 protein isolated from the rat uterine nuclei is in a similar molecular weight range as a 17 kDa candidate acceptor protein identified for the ER in the nuclei of rabbit and cow uteri [Ross and Ruh, 1984], as well as a 20 kDa nuclear protein reported for the AR acceptor system [Goldberger et al., 1986; Rennie et al., 1987].

Whether or not the RBF-1 plays a role in the mechanism of action of steroids has yet to be absolutely proven. Although a function involving the specific guiding of PR and other steroid receptors to their nuclear acceptor sites has been suggested [Spelsberg et al., 1983; Rories and Spelsberg, 1989; Landers and Spelsberg, 1992], the steroid receptor specificity of RBF-1 effects on PR binding to DNA is not known unequivocally. Some preliminary evidence exists for the presence of RBF-1 in several glucocorticoid-responsive but progesterone non-responsive tissues [Landers et al., in preparation]. This presents the possibility that RBF-1 may serve acceptor functions for both the progesterone and glucocorticoid receptors, especially in light of the similarity of the glucocorticoid and progesterone receptor systems (including the similarity of the steroid responsive DNA elements).

In summary, several properties implicate RBF-1 as playing a role in P action: enhancing the nuclear binding of PR to DNA, the nuclear localization of the cellular RBF-1, the co-localization of PR and RBF-1 in the same chicken oviduct cells and their co-localization in the nuclear matrices, the role of RBF-1 in the cell-free binding of PR to DNA, the presence of RBF-1 in only the rat uterus which is a target tissue for P, and the possible E_2 and P regulation of RBF-1 levels. The nuclear localization of RBF-1 also supports the previous cell-fractionation studies by Schuchard et al. [1991b] wherein RBF-1 was localized to the nuclear matrix of the oviduct. The localization of a protein antigen in rat uterine nuclei and the discovery of a 15 kDa antigen with anti–RBF-1 PAb in the size range of the mammalian nuclear acceptor proteins for estrogen [Ross and Ruh, 1984] and dihydroxytestosterone [Goldberger et al., 1986; Rennie et al., 1987] raises the possibility of a conserved family of protein factors in the same manner as found with the steroid receptor family [Cobb and Leavitt, 1987; Carson-Jurica et al., 1990; Landers and Spelsberg, 1992].

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