Evidence for specific DNA sequences in the nuclear acceptor sites of the avian oviduct progesterone receptor

(chromatin/acceptor protein/reconstituted deoxyribonucleoprotein/evolution)

HIROO TOYODA*, RALPH W. SEELEKE†, BRUCE A. LITTLEFIELD‡, AND THOMAS C. SPELSBERG§

Section of Biochemistry, Department of Cell Biology, Mayo Clinic and Graduate School of Medicine, Rochester, MN 55905

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ABSTRACT Recent studies have shown that saturable high-capacity nuclear binding sites (termed acceptor sites) for the avian oviduct progesterone receptor can be reconstituted by rehybridizing a specific oviduct chromatin protein fraction (CP-3) to pure hen DNA to generate a reconstituted nucleoacidic protein (NAP). Only a limited number of acceptor sites can be generated on hen DNA even at high protein/DNA ratios. This suggests the existence of a limited number of specific sequences in the avian genome that can participate in the acceptor sites. The studies presented in this paper show a specificity as to the source of DNA that can generate acceptor sites using hen oviduct CP-3 protein. The acceptor protein binds to all DNAs but generates acceptor sites only on DNAs from certain animals. The acceptor sites for the progesterone receptor, generated with heterologous mammalian DNAs and the avian oviduct CP-3 fraction, show saturation not only in number of acceptor sites generated on the DNAs but also in progesterone receptor binding. Binding to these sites is also receptor dependent. Using oviduct receptors from particular physiological states of the birds wherein the receptors do not bind to nuclear sites in vivo, it was found that the cell-free binding to these heterologous complexes of hen CP-3 protein and DNA from another species, termed heterologous NAP, is similarly absent. Thus, the cell-free binding to the native oviduct NAP and the heterologous NAP markedly resembles the nuclear binding in vivo. Interestingly, synthetic DNAs rich in adenine and thymine, but not those rich in guanine and cytosine, are capable of generating acceptor sites. Species-specific DNA sequences, as well as specific chromatin proteins, therefore, appear to be involved in the nuclear acceptor sites for the avian oviduct progesterone receptor. The DNA sequences appear to be conserved throughout most of the vertebrates but not among nonvertebrates as are the steroid hormones and their receptors. The exact numbers and distributions of these sequences in the avian genome are not known.

Nuclear acceptor sites (binding sites) for the avian oviduct progesterone receptor (PR) have been reported to involve both DNA and specific chromatin proteins in the tightly bound fraction termed CP-3 (1–6). These acceptor sites bind the PR with high affinity and saturability. Similarities between cell-free nuclear binding to these sites using isolated PR and nuclear binding in vivo have been reported (2, 3, 7–11). Nuclear acceptor sites for the avian oviduct estrogen receptor have also been reported to be composed of DNA and a tightly bound protein in the CP-3 fraction (12), although these sites appear to be distinct from the acceptor sites for PR (13). Similarly, a role for tightly bound proteins and DNA in the nuclear acceptor sites for androgens, estrogens, progesterones, and glucocorticoids in mammalian tissues has been reported (12, 14–17). It has recently been shown that the number of PR binding sites on hen DNA, generated by rehybridizing increasing quantities of acceptor protein to the hen DNA, is limited (6), suggesting that specific DNA sequences may be involved in the nuclear acceptor sites for PR. In this paper, we attempt to substantiate this possibility by determining whether or not DNA from other species can bind to the hen oviduct acceptor protein to generate nuclear acceptor sites for the PR.

MATERIALS AND METHODS

Isolation and Binding of the PR and Nuclear Components

The methods and materials used in these studies have been described (6). The PR binding assay used the streptomycin assay (18) with modification in the measurement of radioactivity. The method of isolating the nonfunctional PR, which can bind the steroid but cannot bind the nuclear acceptor sites in vivo or in vitro, is described elsewhere (7, 8, 10).

Nuclei and chromatin were isolated and purified from these homogenates by using modifications of previously described methods (7). All steps were performed at 0–4°C. Isolation of the native nucleoacidic protein (NAP) and pure hen DNA has been described (4, 6, 18). Chromatin, NAP, and DNA were suspended in 4 mM Tris-HCl/0.2 mM EDTA; pH 7.5, at 0.5–1.0 mg of DNA/ml for use in the PR binding assays. CP-3 protein was isolated from hen oviduct chromatin by using hen oviduct chromatin-hydroxylapatite chromatography as described (6). This chromatin-hydroxylapatite resin was treated with a stepwise gradient of increasing concentrations of guanidinium hydrochloride (Gdn-HCl)—0, 4, and 7 M—in 0.1 M sodium phosphate buffer, pH 6.0, at 4°C. The ratio of solvent to resin (ml/g of resin) was ~5 with a flow rate of 5.0 ml/min. The protein concentration in the eluting fractions was determined by the method of Bramhall et al. (19) substituting Coomassie blue stain or by the method of Bradford (20). In later studies, the method of Bradford was used for protein quantitation.

Reconstitution of CP-3 to DNA to Obtain Reconstituted NAP Containing PR Binding Sites. The method for reconstituting the NAP that results in optimal recoveries of acceptor sites for PR when using the hen oviduct chromosomal protein fraction (CP-3 fraction) and hen DNA is described elsewhere (6). Briefly, lyophilized CP-3 proteins—e.g., those in the fraction extracted from the chromatin-hydroxylapatite column by 4–7 M Gdn-HCl—were resuspended in buffered 6.0 mM Tris-HCl.

Abbreviations: CP-3, chromosomal protein fraction 3; NAP, nucleoacidic protein or DNA–CP-3 complexes; PR, progesterone receptor.

*Present address: Department of Molecular Genetics, City of Hope Medical Center, Duarte, CA 91010.
†Present address: Department of Biological Sciences, George Washington University, Washington, DC 20052.
‡Present address: Department of Obstetrics and Gynecology, Yale University School of Medicine, New Haven, CT 06510.
§To whom reprint requests should be addressed.
M Gdn-HCl. Protein concentration ranged from 1 to 2 mg of protein/ml. The solution was homogenized with a Teflon pestle/glass homogenizer and incubated for 2-3 hr at 4°C. Pure DNA in 10 mM EDTA/1 mM Na2EDTA/H2O/0.5 mM phenylmethylsulfonyl fluoride/50 mM NaOAc, pH 6.0, was mixed with CP-3 protein to maintain a DNA concentration of 0.5 mg/ml and protein/DNA ratios (wt/wt) ranging from 0.0 to 1.2. The CP-3 protein and pure DNA were placed in dialysis bags in special chambers. The concentration of Gdn-HCl was reduced from 6.0 to 0 M over a 12-hr period and the reconstituted samples were prepared for PR binding.

Sources of the Various DNAs. DNAs from various species were isolated and suspended in 150 mM NaCl/15 mM Na3 citrate, pH 7.0, as described by Spelsberg et al. (6) and by Thrall and Spelsberg (21) except when obtained from other laboratories. The tissue sources from which the DNAs were isolated were as follows: human DNA, from human placental tissue; hen DNA, from hen spleens; cow DNA, from cow uteri (Pel-Freeze); dog DNA, from dog spleens; toad DNA, from Xenopus laevis larvae (a gift from Sharon Busby, Fred Hutchinson Cancer Research Center, Seattle, WA); hagfish DNA, from hagfish livers (a gift from Aubry Gorbman, Department of Zoology, University of Washington); wheat germ DNA was a gift of General Mills, Minneapolis, MN; insect DNA, from Drosophila viridis and Drosophila melanogaster larvae (gifts from Martin Blumenfeld, Department of Genetics and Cell Biology, University of Minnesota, and Ky Lowenhaupt, Department of Biology, Washington University, St. Louis, MO). Several DNAs were obtained already isolated and purified. These were as follows: salmon DNA from salmon testis DNA (type III) and Escherichia coli DNA (type VIII), from Sigma; virus DNA, from Charon 4A viral DNA (a gift from Mike Getz, Department of Cell Biology, Mayo Clinic and Graduate School of Medicine); poly(dA-T)poly(dA-T) and poly(dC-G)poly(dG-C), DNA, synthetic double-stranded DNA with precise alternating nucleosides in each strand [sedimentation (s20) = 6–9], from P-L Biochemicals; poly(dA)poly(dT) and poly(dG)poly(dC) DNA, synthetic double-stranded DNA with each strand consisting of homologous nucleosides, from P-L Biochemicals. All DNA preparations were assayed for RNA and protein contamination as described (6, 21).

RESULTS

Binding to Pure DNAs from Different Sources. Binding of the [3H]PR to the pure whole genomic DNAs from a variety of sources was examined first (Fig. 1). In all instances, cell-free binding of the [3H]PR to the purified DNAs from different species is linear over the receptor titration range used. In this range, binding of [3H]PR to native or reconstituted NAP (containing the hen oviduct acceptor protein bound to the hen DNA) displays saturation (3, 6). Further, DNAs from the different organisms (Fig. 1A) display similar levels of binding within the same receptor concentration range. The synthetic duplex homopolymers and copolymers (Fig. 1B) display marked differences in [3H]PR binding. The (A-T)-rich DNA DNAs bind the [3H]PR markedly higher than do the (G+C)-rich polymers or whole genomic DNAs.

Binding to Reconstituted Homologous NAPs Composed of Hen Oviduct CP-3 and DNAs from a Variety of Sources. Purified DNAs from different sources were reconstituted with increasing quantities of the CP-3 fraction isolated from hen oviduct chromatin. Typical results from multiple experiments are shown in Fig. 2. The binding values of the respective pure DNAs have been subtracted from those of the reconstituted NAPs. The binding patterns have been grouped according to whether or not the organisms are known to utilize progesterone. As shown in Fig. 2A, DNAs from organisms that are known to utilize progesterone can generate significant numbers of acceptor sites for oviduct PR. However, at lower protein/DNA ratios, only reconstituted NAPs containing human and hen DNA display marked binding by the [3H]PR, although at higher CP-3 protein/DNA ratios DNAs from many organisms are capable of generating acceptor sites. Several of these nonavian DNAs also displayed a saturation of sites. Fig. 2B shows that DNAs from organisms that are not known to utilize progesterone do not generate acceptor sites even at high CP-3 protein/DNA ratios. Wheat germ DNA is an exception.

Evolutionary Trend of DNA Specificity for the Hen Oviduct Accepter Protein. The data in Fig. 3 are summarized in Fig. 2 using as 100% PR binding to the reconstituted homologous NAP prepared with hen oviduct CP-3 and hen DNA. At low ratios (wt/wt) of CP-3 protein to DNA (e.g., 0.2–0.4), a marked specificity as to the source of the DNA that can generate acceptor sites for oviduct [3H]PR is observed; e.g., only human and hen DNA generate acceptor sites. This ratio represents the native situation, since the CP-3 protein/DNA ratio in native chromatin averages 0.25. In contrast, at higher protein/DNA ratios, DNAs from all mammals, as well as those from amphibians and bony fish, generate acceptor sites for the avian oviduct PR using the avian oviduct acceptor protein. DNAs from more evolutionarily distant organisms—e.g., insect and bacteria—fail to display any PR acceptor sites (wheat germ DNA being an exception). It should be mentioned that DNAs from different strains of the same species (e.g., insects or birds) generate equivalent binding values to each other (data not shown). In contrast, DNAs from some animals—e.g., human, dog, and cow—generate a greater number of acceptor sites than the homologous hen DNA. This increase is further enhanced on a per cell basis because these animals have more DNA per cell than chickens.

Receptor Titration and Receptor Dependency of [3H]PR Binding to the Reconstituted NAPs. Reconstituted NAPs made with hen, dog, and human DNAs display a high,
isolated NAPs from various DNA sources. NAPs were reconstituted using the isolated hen oviduct CP-3 fraction and pure DNA from a variety of sources as described (6). Reconstituted NAPs were suspended in 4 mM Tris/HCl/0.2 mM EDTA, pH 7.5, and binding experiments with the [3H]PR were carried out by the streptomycin method as described elsewhere (18). Results were corrected for DNA binding. The experimental design was the same as described for DNA binding (Fig. 1). Means of triplicate analyses of PR binding to the reconstituted NAPs in the same experiment are shown. Binding values for each NAP are corrected for PR binding to the respective pure DNA (the same pure DNA used in the reconstituted NAP). (A) NAPs containing DNA from organisms known to utilize the steroid progestrone. (B) DNAs from organisms that do not utilize progestrone. Sources of DNAs in the reconstituted NAPs were as follows: (A) X, human uteri; O, cow thymus ( calf); i, dog spleen; h, hen spleen; a, salmon sperm; d, frog liver. (B) Drosophila larvae; a, E. coli; o, Charon 4A virus; A, hagfish; m, wheat germ.

saturable binding of the steroid receptor similar to that of native hen oviduct NAP (data not shown). In the absence of receptor, the binding is markedly reduced and nonsaturable. In contrast, reconstituted heterologous NAP containing bacterial DNA was shown previously to display a low level, nonsaturable binding with the PR over this same range of receptor concentration (6). Thus, binding of the [3H]PR to the reconstituted heterologous NAPs appears to be receptor dependent and the binding to NAPs containing DNA from certain animals is saturable.

Binding of the [3H]PR to Reconstituted NAPs Composed of Hen Oviduct CP-3 and Synthetic DNAs. Synthetic homo- and copolymer duplexes of DNA were examined for their ability to generate PR binding sites when reconstituted with hen oviduct CP-3. Binding of the [3H]PR to pure synthetic DNAs is shown in Fig. 1 for comparison. It is noteworthy that (A+T)-rich synthetic DNAs show a higher nonsaturable binding of the PR than do native or (G+C)-rich synthetic DNAs. The binding values for the reconstituted NAPs (Fig. 4) were corrected for [3H]PR binding to sham-reconstituted pure synthetic DNAs alone. Interestingly, (A+T)-rich synthetic DNAs, especially the A+T copolymer, display a marked increase in PR binding over DNA alone, when reconstituted with hen oviduct CP-3 protein (Fig. 4). In contrast, (G+C)-rich synthetic DNA duplexes show little or no increase in [3H]PR binding when complexed with the avian CP-3 protein. The large number of reconstituted acceptor sites on (A+T)-rich synthetic DNA is receptor dependent (data not shown). It was found that the rate-limiting factor in the assays shown in Fig. 4 was the amount of [3H]PR in the assay. Addition of more [3H]PR caused a higher level of binding. Thus, saturation binding by the PR on poly[d(A-T)]-poly[d(T-A)] has not been achieved and may be much higher than that displayed in this figure. Further, it is possible that sufficient acceptor protein has not been rehybridized to the synthetic DNA to saturate all available PR binding sites on the DNA. In any event, (A+T)-rich synthetic DNAs appear to be capable of generating binding sites for the PR in contrast to (G+C)-rich synthetic DNA.

Binding of the Acceptor Protein to DNAs That Do Not Generate Acceptor Sites. Using hydroxylapatite to adsorb the reconstituted NAP, we redissociated the proteins from the reconstituted NAPs with increasing concentrations of Gdn-HCl and assayed for acceptor activity as described (6). Table 1 shows that bacterial DNA, which does not generate acceptor sites with the acceptor protein, does bind the acceptor protein (Fig. 2). The same results are found when the synthetic DNA poly[d(G-C)] is used (data not shown). Thus, equivalent amounts of acceptor activity are bound to the various DNA species, regardless of their capacity to generate acceptor sites. Preliminary studies have indicated that the acceptor protein may be less tightly bound (i.e., dissociated by 4.0 M Gdn-HCl) to DNAs that do not generate PR binding sites as compared with the protein bound to DNAs that do generate PR binding sites (i.e., dissociated only by 7.0 M Gdn-HCl).
Fig. 4. Binding of the isolated hen oviduct \[^{3}H\]PR to reconstituted heterologous NAPs composed of hen oviduct CP-3 protein and synthetic DNAs. The experiments were performed as described in the legend to Fig. 2 except that the NAPs were reconstituted using synthetic duplex DNAs and hen oviduct CP-3 protein. Sources of the DNAs in the reconstituted NAPs were as follows: \(\bullet\), poly(dA-T)poly(dA-T); \(\odot\), poly(dA)poly(dT); \(\bullet\), poly(dG-C)poly(dG-C); \(\circ\), poly(dG)poly(dC). Binding values were corrected for binding to DNA alone (i.e., by subtracting the values for \[^{3}H\]PR binding to the respective synthetic DNA duplexes alone from that to the reconstituted NAP) as described in the legend to Fig. 1.

DISCUSSION

A species specificity has also been found with regard to the sources of the DNAs used to generate PR acceptor sites with hen oviduct CP-3. Our results support a DNA sequence specificity for the avian oviduct acceptor protein for the PR. The following observations support the hypothesis that PR binding to the heterologous NAPs represents specific/biologically relevant acceptor sites: (i) the numbers of acceptor sites generated on heterologous DNAs (salmon and dog DNA) with hen CP-3 protein are saturable, (ii) receptor binding to these regenerated sites is saturable, and (iii) the binding is receptor dependent. Binding of the PR to the pure DNAs was nonsaturatable. Further, simple "binding" of the acceptor protein to an DNA was not found to be sufficient to generate acceptor sites since bacterial DNA, which does not generate acceptor sites, still bound these protein(s). Only the analysis of PR binding demonstrates a DNA sequence specificity. Thus, a more stringent criteria than DNA binding may be required to demonstrate a DNA specificity of eukaryote protein.

The most significant specific for the generation of acceptor activity was found at low CP-3 protein/DNA ratios similar to levels found in native chromatin. Interestingly, even at high protein/DNA ratios, \[^{3}H\]PR binding values followed a grouping of organisms based roughly on evolutionary divergence. The fact that wheat germ DNA can generate sites is currently inexplicable. Overall, the quantitative binding follows a general evolutionary pattern with the division occurring somewhere during the divergence of fish and amphibians 350 to 450 million years ago (22). The evolutionary sequence follows the general path of bacteria, fungi, plants, insects, fishes (first hagfish, then sharks, and finally bony fishes), amphibians, reptiles, birds, and lastly mammals. The utilization of mammalian steroid hormones appears to be restricted to animals under the subphylum Craniata, phylum Chordata (for reviews, see refs. 23–26). This includes the classes of lampreys, sharks, bony fish, amphibians, reptiles, birds, and mammals. Among the various classes of animals responding to mammalian steroids, there is considerable variation in the exact steroid species synthesized and circulating in the plasma (23). Complete information on the action of steroids in most of the classes of the subphylum Craniata is lacking. Knowledge of the presence of specific steroid receptors in these species is even more scarce. The actual induction of specific mRNA and proteins by common mammalian steroids has been identified only in fish (27), amphibians (24, 28–30), birds (31–35), reptiles (24, 34, 36), and mammals (37, 38). The identification of steroid receptors (primarily estrogen receptors) is limited to amphibians (30, 39), reptiles (40), birds (31, 41, 42), and a number of mammals (37, 38). Studies specifically with the progesterone receptor have been limited largely to birds and mammals (5, 38, 43). Alternatively, it is possible that rather than an evolutionary trend, the results observed in these studies reflect only a complexity of the genome. In other words, organisms such as plants, which do not contain steroid receptors but do contain high amounts of DNA, have a greater probability of having sufficient quantities of sequences needed to generate acceptor sites.

Concerning the nature of the specific DNA sequences involved, the high-affinity binding sites generated with (A+T)-rich synthetic DNAs suggest that such sequences may play a role in acceptor sites in the animal genomes. Obviously, many of the DNAs that fail to generate acceptor sites contain significant numbers of A+T bases. However, it may be the lengths and sequences of the (A+T)-rich regions that are important, not just the A+T composition of the genome. These data and previous data from our and other laboratories (1–17) appear to conflict with another model of acceptor sites wherein specific DNA sequences are recognized directly by steroid receptors (44–48). The existence of two classes of sites, one containing both DNA and protein and one containing just DNA, has been suggested (49–51). It should be mentioned that, based on the data shown in Fig. 1, any DNA containing regions rich in adenine and thymine would display an enhanced, but nonsaturable, binding of a steroid receptor. This phenomenon might explain the reports of enhanced binding of steroid receptors to pure cloned DNA sequences that were reported to be enriched in A+T sequences and have nonsaturable binding.

In conclusion, we propose that the initial acceptor sites (nuclear binding sites) for avian oviduct PR are composed of

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Table 1. Analysis of the amount of acceptor activity (protein) in various reconstituted NAPs with and without PR binding

<table>
<thead>
<tr>
<th>Source of DNA reconstituted with hen oviduct CP-3 protein</th>
<th>Total protein recovered, mg</th>
<th>Total acceptor activity, (^{10^{-2}}/\text{mg DNA})</th>
<th>Total acceptor activity, (^{10^{-2}}/\text{mg DNA})</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAP containing hen DNA</td>
<td>4.8</td>
<td>3.8</td>
<td>3.8</td>
</tr>
<tr>
<td>NAP containing bacterial DNA</td>
<td>1.2</td>
<td>3.9</td>
<td>3.9</td>
</tr>
<tr>
<td>NAP containing poly(dA-T)poly(dT-A)</td>
<td>1.1</td>
<td>2.8</td>
<td>2.8</td>
</tr>
</tbody>
</table>

Reconstituted heterologous NAPs containing hen DNA, bacterial DNA, or the synthetic DNA poly(dA-T)poly(dA-T) were prepared using 10 mg of DNA and a CP-3 protein/DNA ratio of 0.6. Complexes were adsorbed to hydroxyapatite, and protein was eluted with a stepwise gradient of Gdn-HCl (0–7.0 M) in low-phosphate buffer (as described in the Methods and Materials for isolating CP-3 protein) to keep the DNA adsorbed to the resin. Eluted proteins and the acceptor activities were quantitated as described (6). Means of two replicate analyses of protein are presented.

\(^{1}\)Total acceptor activities recovered in the DNA-bound proteins eluted from the hydroxyapatite column are reported. Means of four replicate analyses of acceptor activity performed in the same experiment are presented. \[^{3}H\]PR binding values of the NAPs are corrected by subtraction of the corresponding DNA binding values.
specific acceptor proteins bound to specific DNA sequences. These sequences appear to be highly conserved among vertebrates, as is the case with the steroid hormones and their receptors (31–53). We further speculate that these sequences are repetitive in nature, generating 9000 to 18,000 identical nuclear binding sites per cell for the [3H]PR in the avian oviduct (2–5, 54).

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