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SELECTIVE BINDING OF CHICKEN PROGESTERONE RECEPTOR A SUBUNIT TO A DNA FRAGMENT CONTAINING OVALBUMIN GENE SEQUENCES

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SUMMARY: Progesterone receptor A protein, purified to near-homogeneity from laying hen oviducts, binds preferentially to a cloned fragment of the chicken ovalbumin gene containing a portion of transcribed gene sequences, including the transcriptional start site, as well as extensive sequences preceding the 5' end of the ovalbumin gene. As revealed using a nitrocellulose filter adsorption assay to isolate receptor-DNA complexes, the non-specific interaction of receptor A with DNA is characterized by selective binding to the largest DNA fragments present in mixtures of bacterial plasmid DNA. In contrast, the smaller ovalbumin gene fragment is preferentially bound from a mixture containing a larger plasmid DNA fragment. Furthermore, selectivity for the ovalbumin gene fragment is retained even in the presence of a six-fold molar excess of nonradioactive cloned chicken DNA containing sequences from the analogous region of the adult chicken β-globin gene. Direct competition studies using mixtures of ovalbumin and globin 5'-DNA sequences show at least a ten-fold preference by receptor for the hormone-responsive ovalbumin gene.

INTRODUCTION: A variety of steroid hormones have been shown to enhance the rate of transcription of specific target cell genes (1,2). These effects appear to be temporally associated with the accumulation of hormone-receptor protein complexes in the cell nucleus, suggesting that the change in transcriptional activity results from direct interaction between receptors and specific genomic sites (3,4). In situ, such an interaction has been observed for ecdysone binding to the Drosophila polytene chromosomes of larval salivary gland cells (5), while steroid receptors have been found to bind in vitro to DNA (6-8), and to chromatin (9,10). Recently, a sequence-specific interaction of glucocorticoid receptor with a cloned fragment of the mouse mammary tumor virus genome has been reported (11). Studies in our laboratory with purified progesterone receptor A subunit isolated from chicken oviducts have shown that the receptor can undergo a tight interaction with DNA, as assayed by nitrocellulose filter

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adsorption, by DNA-cellulose chromatography, and by sedimentation velocity studies (12). The high affinity of progesterone receptor A for any DNA tested in the above study led us to search more carefully for preferential interactions at specific high affinity sites near target gene DNA sequences. One of these target sites is the gene coding for ovalbumin. Using cloned DNA containing ovalbumin gene sequences, and highly purified progesterone receptor A protein, we now report preferential formation of receptor-DNA complexes with ovalbumin gene sequences, assayed by elution of [^{32}P]DNA-receptor complexes adsorbed to nitrocellulose.

MATERIALS AND METHODS:

Purification of receptor A: Receptor A was purified as previously described (13), except the final 1 ml heparin sepharose column was equilibrated in 10 mM Tris-Cl, pH 7.4, containing 1 mM Na_2EDTA and 12 mM 1-thioglycerol (Buffer A) and eluted with a 40 mL linear gradient of 0.1 M to 1.0 M NaCl in Buffer A. Receptor A eluted at 0.15-0.3 M NaCl. The purified preparations used in this study were > 80% receptor A protein as determined after SDS gel electrophoresis (14), and staining by the silver procedure of Wray et al. (15).

Labeling with [^{32}P]: Restriction endonuclease fragments were end-labeled on their 3' ends using the Klenow fragment of DNA polymerase I (Boehringer-Mannheim) and α-[^{32}P]dATP (2-3000 Ci/mmol, Amersham), or on their 5' ends with γ-[^{32}P]ATP (2-3000 Ci/mmol, Amersham) using T4 polynucleotide kinase (Boehringer) (12). After phenol extraction, the reaction mixtures were chromatographed in 10 mM Tris-Cl, pH 7.8, 0.1 mM EDTA (Buffer B) on Sephadex G50 to remove free label.

Nitrocellulose Filter Adsorption Assays: Filter binding reaction mixtures were prepared in 50 mM potassium phosphate buffer, pH 7.4, containing 10% glycerol and 0.1 mM EDTA. Solutions (200 µl) contained final concentrations of 25-50 mM NaCl, 0-3 µg/ml Receptor A, and 2-20x10^5 dpm [^{32}P]DNA (0.2-0.8 µg/ml). These mixtures were incubated at 37° for 90 min, rapidly cooled to 4° on ice, and then filtered as previously described (12). Washed filters were extracted twice with 0.5 M ammonium acetate and 0.5 M sodium acetate in 10 mM Tris-Cl, pH 7.4 buffer. Eluted DNA was precipitated and washed with 70% ethanol, dried, and dissolved in Buffer B. The receptor concentrations used in the experiments reported here resulted in retention of ≤ 10% of the input DNA to the filters. About 75% of the bound label from each filter could be subsequently eluted and analyzed.

RESULTS: Previous studies have demonstrated a high affinity (K_{diss} = 10^{-10}M) of receptor A for heterologous, double-stranded DNA (12). In the experiments reported here, we have isolated receptor-DNA complexes using the nitrocellulose filter technique (16). The filters adsorb protein but not double-stranded DNA. After filtration, DNA molecules retained as receptor-[^{32}P]DNA complexes were eluted and analyzed by electrophoresis on agarose or polyacrylamide gels and subsequent autoradiography. Because the presence of high affinity binding of receptor A protein to specific DNA would be reflected by an enrichment in

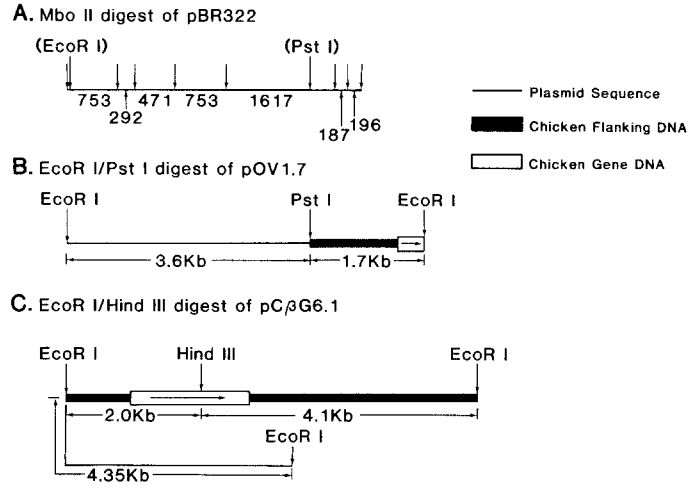

Figure 1. Restriction endonuclease maps of plasmids used in the experiments. Panel A, map of plasmid pBR322, showing the observed cleavage sites (↓) and lengths of the seven fragments found after digestion with MboII. The EcoRI and PstI sites are shown for orientation. The MboII digest was end-labeled as described in Methods. Panel B, map of recombinant plasmid pOV1.7, a cloned fragment of the 5'-end of the chicken ovalbumin gene constructed by replacing about 700 bp in pBR322 with a 1.7 Kb fragment from a partial clone of the ovalbumin gene, OV4.5 (22). An EcoRI, PstI double digest yields two fragments, a 3.6 Kb plasmid fragment, and a 1.7 Kb chicken DNA fragment (OV1.7), beginning 1338 bp upstream from the transcription start site and extending 389 bp into the gene. The black bar shows 5'-flanking DNA; the open bar shows the location of the start of the ovalbumin gene primary transcript in vivo. Arrow in the sequence shows the direction of transcription. The digest was end-labeled as in Methods. Panel C, map of recombinant plasmid pCβG6.1 (23), a cloned fragment of the 5'-end of the adult chicken β-globin gene. A HindIII digest yielded the fragments shown. The 2.0 Kb fragment (βG2.0) contained 5'-flanking sequence extending from 980 bp upstream of the start of transcription to a point 1020 bp into the gene. This DNA was isolated by preparative agarose gel electrophoresis, eluted and either end-labeled or used directly for competition as described in Methods and in Figures 2 and 3. Symbols on the Panel are the same as in Panel B.

the complexes bound to the filters, this method makes it possible to analyze binding solutions containing mixtures of DNA molecules and to detect preferential binding of certain sequences. Figure 1 shows restriction maps of the three plasmids used as the source of DNA for these experiments. Fragments were prepared and labeled as described in the Figure legends and in Methods.

Interaction of receptor A with the Mbo II fragments from pBR322 (see Figure 1A) was studied as shown in Figure 2A. A constant amount of [^{32}P]DNA was incubated with purified receptor A at receptor concentrations varying from 0 to 33 nM. Figure 2A shows the autoradiograms resulting following electrophoresis of the [^{32}P]DNA retained on the filters. The [^{32}P]DNA fragments appear to be labeled to equal specific activity (lane 1), and when filtered

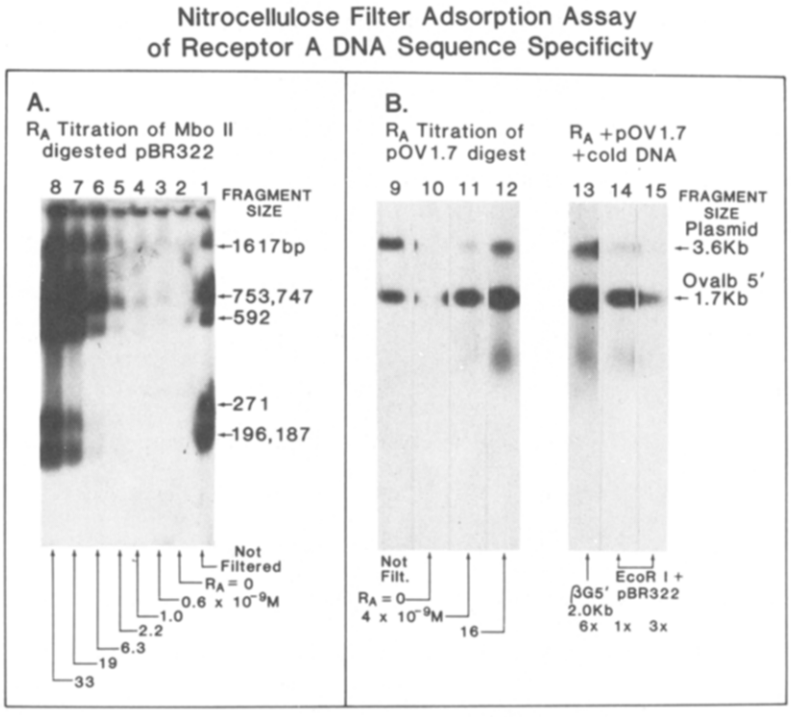

Figure 2: Selective binding of DNA fragments to receptor A (R_A). [^{32}P]DNA bound to receptor A on nitrocellulose filters was eluted and analyzed by electrophoresis on slab gels in Buffer B containing 50 mM sodium acetate followed by autoradiography of the gels. Receptor A concentrations were estimated (± 50%) from the intensity of staining of the 79,000 dalton band after electrophoresis in SDS. Panel A: Analysis on 4% polyacrylamide gels of binding to MboII-digested pBR322 labeled at both 5' ends with [^{32}P]. Binding solutions contained about 1.5×10^6 cpm [^{32}P]DNA at 2 µg/mL and the indicated amounts of receptor A. Panel B: Analysis on 0.7% agarose gels of binding to EcoRI, PstI digested pOV1.7 3' end-labeled with [^{32}P] at the EcoRI ends. Binding mixtures contained 5×10^5 cpm [^{32}P]DNA at 0.6 µg/mL and receptor A at 4×10^{-9}M (lane 11) or 16×10^{-9}M (lanes 12-15). The competition experiment with chicken β-globin DNA in lane 13 also contained 1.5 µg/mL unlabeled βG2.0, while lanes 14 and 15 contained 0.6 and 1.8 µg/mL, respectively of unlabeled pBR322 linearized with EcoRI. This represents the indicated excess of competitor relative to [^{32}P]OV1.7 fragment.

in the absence of added protein gave essentially no retention of DNA (lane 2). At low receptor concentrations not all the DNA fragments were bound by receptor (lanes 3 and 4). Instead, the binding was mainly to the large DNA fragments, compared to the initial mixture of DNA (lane 1). In the presence of increasing receptor A, progressively smaller DNA fragments were bound, while even the shortest DNA fragments became bound at the highest receptor concentrations (lanes 7 and 8). This result is consistent with a random, sequence-independent binding mode in which the probability of a given molecule of DNA being bound to the receptor depends solely on the number of non-specific binding sites it contains (a number proportional to the length of the DNA fragment).

Figure 1B shows a restriction endonuclease map of the plasmid pOV1.7. Digestion of this DNA with EcoRI and PstI yields two DNA fragments, a 1.7 Kb piece (OV1.7) containing only chicken ovalbumin gene sequences and a 3.6 Kb plasmid DNA fragment. These two fragments were used in the receptor-binding experiment shown in Figure 2B. Both fragments were equivalently labeled (lane 9). Virtually no DNA was retained on the filters in the absence of receptor (lane 10). Analysis of the DNA bound at low receptor A concentration reveals marked preferential binding to the 1.7 Kb ovalbumin gene DNA (lane 11). The relative enrichment of ovalbumin sequences over plasmid sequences was about ten-fold when the band intensities of the autoradiogram were compared by scanning densitometry. Increased binding to the 3.6 Kb fragment was observed upon the addition of a four-fold higher concentration of receptor A protein (lane 12). Lanes 14 and 15 show the effect of including an equal or three-fold greater molarity of unlabeled EcoRI-cut pBR322 in the binding mixtures. Preferential binding to OV1.7 is still observed, although the amount of binding is reduced.

In order to test whether the observed selectivity in DNA binding represented simply preferential affinity for eukaryotic DNA in general, a competition experiment was performed using an analogous 5' sequence of a chicken gene not under hormonal control in vivo, specifically a 2.0 Kb DNA fragment (βG2.0) containing sequences from the chicken adult β-globin gene (Figure 1C). As seen in lane 13 of Figure 2B, the presence of a six-fold molar excess of unlabeled β-globin gene fragment over ovalbumin gene molecules had no apparent effect on the selective binding seen in the absence of competition (lane 12). This result was confirmed by performing a similar experiment in which both the ovalbumin and β-globin DNAs were end-labeled (Figure 3). Lanes 1-5 show the retention by receptor of [^{32}P]βG2.0 in the absence and presence of receptor. In Figure 3B several concentrations of [^{32}P]βG2.0 (specific activity 19×10^6 cpm/pmol) were added to mixtures containing a constant receptor concentration and a constant concentration of [^{32}P] labeled digested pOV1.7 (specific activity = 4.5×10^6 cpm per pmol of 1.7 Kb ovalbumin DNA).

Receptor A DNA Sequence Specificity Competition Study

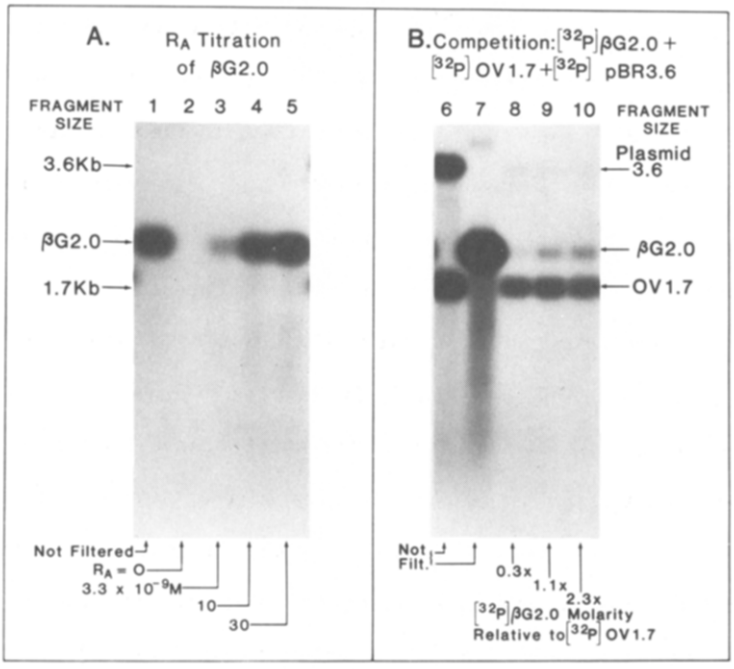

Figure 3: Effect of competition with [^{32}P]-labeled chicken β-globin DNA (βG2.0) on the selective binding of receptor A to EcoRI, PstI digested pOV1.7. Autoradiographs are shown of 0.7% agarose gels following electrophoresis of DNA eluted from receptor A-DNA complexes adsorbed to nitrocellulose filters. Panel A: Analysis of [^{32}P]DNA starting material (lane 1) and binding solutions (lanes 2-5) containing 3×10^5 cpm of 5' end-labeled [^{32}P]βG2.0 at 0.1 μg/mL. Panel B: Analysis of [^{32}P]DNA binding by 10×10^{-9}M R_A in mixtures containing a constant amount of end-labeled EcoRI, PstI double digested pOV1.7 (0.5 μg/ml, specific activity = 4.5×10^6 cpm per pmol of OV1.7 fragment) together with increasing amounts of [^{32}P]βG2.0 (specific activity = 19×10^6 cpm per pmol βG2.0). DNA alone in lanes 1 and 2. Lanes 8-10 contained 0.06, 0.22, and 0.46 μg/mL [^{32}P]βG2.0 representing molarities of 0.3, 1.1, and 2.3 relative to the molarity of ovalbumin gene sequences, OV1.7 (1.5×10^{-10}M).

Analysis of the DNA bound in mixtures containing molar ratios of β-globin fragment to ovalbumin fragment of 0.3, 1.1, and 2.3 (lanes 8-10 respectively) reveals that the presence of β-globin gene sequences had no detectable effect on the selective binding of receptor to the 1.7 Kb ovalbumin DNA piece. Had equal binding by both of these DNAs been observed, the βG2.0 band would have been about four times as intense as that of the OV1.7 band, due to the higher specific radioactivity of the βG2.0 fragment. Thus, the preference for ovalbumin sequence is at least ten-fold greater than for globin sequence under these conditions.

DISCUSSION: In these studies, our experimental approach focuses on the hypothesis that hormone-receptor complexes specifically recognize and bind to DNA sequences at or near the affected genes. Our initial study, utilizing both nitrocellulose filters and sedimentation failed to demonstrate significant differences in the affinity of purified receptor A for a variety of test DNAs, including phage, bacterial, and ovalbumin gene sequences (K_D about 10^{-10}M in all cases) (12). We reasoned then that the presence of large numbers of non-specific binding sites might overwhelm the sensitivity of these methods to detect specific binding, especially if binding to these sites differed by only a factor of 100 or less. The preferential adsorption assay used here, although semi-quantitative in nature, has the virtue that smaller differences of affinity can be detected. Furthermore, the presence of suspected non-specific binding sites can be manipulated by selecting the size and nature of the DNA fragments present. Finally, binding to various DNAs occurs in the same reaction mixture, providing a sensitive, internally controlled comparison of the relative strength of the binding interactions.

Receptor A purified from hen oviducts by our procedure results in preparations containing stoichiometric amounts of progesterone (17,18). However, since we do not know the extent of dissociation of progesterone from the receptor, the exact role of the steroid in the observed preferential binding is unclear. In addition, while it seems unlikely that the effects seen in these experiments could be attributed to one of the minor non-receptor proteins present in these preparations, further studies are being pursued to address this question.

The preferential binding obtained with purified receptor A to the 1.7 Kb ovalbumin gene fragment cannot be attributed to a general, non-specific interaction such as that observed to MboII-cut pBR322 DNA, where preferential adsorption of plasmid fragments varied directly with their length (Figure 2A). If this phenomenon were acting with the pOV1.7 digest experiments, the 3.6 Kb plasmid sequence would have been adsorbed preferentially. Figure 2B shows that this was not the case. The degree of specificity may not be much greater

than 10-fold, however, since excess nonradioactive EcoRI-cut pBR322 DNA did decrease retention of the labeled OV1.7 fragment (Figure 2B). This result is consistent with our earlier studies (12).

The results in Figure 2B and Figure 3 demonstrate that the sequence preference of the receptor is not merely for elements present in all eucaryotic DNA's, since the ovalbumin gene sequence was adsorbed about 10 times as well as the analogous 5'-region of the chicken globin gene. Since the globin gene is not under steroid hormonal control in vivo, it will be interesting to compare more exactly the structural or sequence features of these two DNA's which account for these differences.

At present it is not possible to deduce a function in vivo for this DNA sequence preference of the receptor. Considering the size of the eucaryotic genome, the interaction would appear to be insufficient to account for selective gene regulation. However, the DNA sequences available for interaction with receptors may be limited by chromatin structure, as are the sequences susceptible to nuclease attack, for example (19). Thus, the degree of specificity reported here may be sufficient to account for gene induction in vivo, especially if the receptor A is localized to regions near active genes by virtue of its companion chromatin-binding subunit, receptor B. From other considerations, we have speculated in the past that this may be the case (20,21).

Since the OV1.7 fragment is so large, the results here do not directly address the question of the proximity of the specific site or sites to the start of the ovalbumin gene transcript. In results to be presented elsewhere, we have observed several higher-affinity sites within OV 1.7; all are associated with markedly A-T rich regions of the DNA. Our earlier study (12) had shown a marked preference of receptor A for binding to A-T-rich synthetic DNA. Thus, we hypothesize that receptor A may bind preferentially to DNA regions rich in A-T. Finer mapping necessary to test this idea is in progress.

A recent study by Payvar et al., (11) mapped the binding of rat liver glucocorticoid receptor to a restriction fragment of the mouse mammary tumor virus genome. Those workers found a pronounced preference for an internal DNA

sequence far downstream of the start of transcription. A similar site has now been identified at or near the transcription initiation (K. Yamamoto, personal communication). It will be of interest to compare the features of the virus system with that reported here for a highly inducible steroid target gene.

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