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DNA sequence preference of the progesterone receptor

(steroid receptors/protein–DNA interactions/gene regulation)

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ABSTRACT **Highly purified hen oviduct progesterone receptor A subunit was tested for binding to several chicken gene DNAs. Sequence preference detected by nitrocellulose filter adsorption of [^{32}P]DNA fragments obtained from recombinant plasmids revealed a marked retention of certain DNA fragments. About a 10-fold preference was seen for DNA fragments flanking the 5′ end of the steroid-regulated genes ovalbumin and gene Y. No preference was seen with analogous DNA fragments from chicken β-globin and α-actin genes. Restriction endonuclease mapping suggests the presence of multiple receptor interaction sites flanking the 5′ terminus of the ovalbumin gene. One of these preferential binding sites was localized between −135 and −247 base pairs upstream from the start of transcription. This region contains an 18-base-pair A+T-rich sequence, a likely candidate for the binding site itself, because earlier studies had shown receptor A to have marked preference for A+T-rich DNA.**

We have been studying the mechanism of steroid hormone regulation of gene expression in the chicken oviduct (for reviews see refs. 1 and 2). The progesterone receptor in this organ has two subunits, receptor A (M_r, 79,000) and receptor B (M_r, 108,000) (3, 4), but only receptor A contains strong DNA binding activity (5). This subunit was purified to apparent homogeneity from immature chick oviduct (6) and used in a series of DNA-binding studies (7). In the binding studies, nitrocellulose filter adsorption of [^{32}P]DNA showed high affinity of receptor A for heterologous DNA ($K_{diss} = 10^{-10}$ M) and a preference for A+T-rich DNA. Studies of binding at 0°C demonstrated several lines of evidence indicating that the protein was a helix-destabilizing protein. Using increased incubation temperatures, we recently demonstrated preferential interaction of receptor A with a 1.7-kilobase (kb) fragment flanking the 5′ end of the chicken ovalbumin gene (8). Using the same methods, Payvar *et al.* reported a binding preference for purified rat glucocorticoid receptor in the mouse mammary tumor virus genome (9). Concurrently, Mulvihill and co-workers have tested for progesterone receptor DNA preference with a DNA-cellulose competition assay using crude cytoplasmic receptor extracts (10). They reported a "consensus sequence" flanking the ovalbumin and related genes, which they identified as the most probable receptor-binding site. We have now carried out more detailed mapping of receptor-binding sites in the 5′ flanking region. We report here the identification of a preferential DNA sequence lying between −135 and −247 base pairs (bp) upstream from the ovalbumin gene.

MATERIALS AND METHODS

Chemicals. Radiolabeled nucleotides were obtained from Amersham at a specific activity of 2,000–3,000 Ci/mmol (1 Ci = 3.7 × 10^{10} becquerels). DNA polymerase I (Klenow fragment), T4 polynucleotide kinase, and bacterial alkaline phosphatase were purchased from Boehringer Mannheim. Restriction endonucleases and agarose were obtained from Bethesda Research Laboratories.

Preparation of DNA. Isolation of DNA from recombinant plasmids was as described for pOV1.7 (11), pCβG6.1 (12), and pY7.2 (13). Endonuclease digestions were performed in universal buffer (14) at temperatures for periods recommended by the supplier. The DNA fragment OV1.9 was produced by *Hpa* II digestion of pOV1.7 and isolated by preparative electrophoresis in 1% low-temperature-melting agarose (FMC). The fragment Y1.5 was purified from pY7.2 as described (13). DNA fragments were labeled at their 3′ termini by using ^{32}P-labeled deoxyribonucleotides and the Klenow fragment of DNA polymerase I or at the 5′ termini by using T4 polynucleotide kinase and were purified as described (8). Specific activities used were 2–10 × 10^6 dpm μg^{-1}.

Purification of Progesterone Receptor A. Receptor A from laying hen oviducts was purified as previously described (15) except the final heparin-Sepharose column was eluted with a linear 0.1–1.0 M NaCl gradient in 10 mM Tris·HCl, pH 7.4, containing 1 mM Na_2EDTA and 12 mM 1-thioglycerol. Receptor A preparations were stored at 4°C, and each preparation was assayed for purity and concentration by sodium dodecyl sulfate/polyacrylamide gel electrophoresis.

DNA-Binding Reactions Using Receptor A. DNA binding reaction mixtures were prepared in 50 mM potassium phosphate buffer, pH 7.4, containing 10% (vol/vol) glycerol, 50 μg ml^{-1} bovine serum albumin and 0.1 mM EDTA. Solutions (200–400 μl) contained final concentrations of 10–50 mM NaCl, receptor A protein at 0–4 μg ml^{-1}, and 2–8 × 10^5 dpm of [^{32}P]DNA (0.2–0.8 μg ml^{-1}). The mixtures were typically incubated at 37°C, rapidly cooled to 4°C, and filtered immediately as described (7). DNA was extracted from the filters and analyzed by electrophoresis on agarose or polyacrylamide slab gels as detailed elsewhere (8).

RESULTS

Progesterone Receptor A Preparations. Fig. 1 shows an analysis by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (16) of six typical preparations of hen receptor A used in the binding studies. Receptor A preparations contained either homogeneous receptor A protein of 79,000 daltons (lane 3) or, in addition, small amounts of non-receptor A proteins (other lanes) generally at levels of a few percent relative to the amount of receptor A. These and other preparations showed no detectable variation in sequence-specific DNA binding assayed by nitrocellulose filter adsorption.

Preferential Binding of Progesterone Receptor A to DNA Containing Ovalbumin Gene and 5′ Flanking Sequences. We studied first the interaction of purified receptor A protein with

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Abbreviations: kb, kilobase(s); bp, base pair(s).

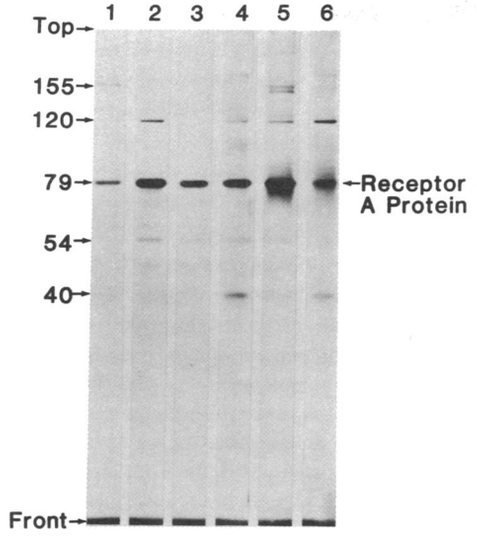

FIG. 1. Electrophoretic analysis of purified hen progesterone receptor A on 7.5% polyacrylamide gels under denaturing conditions. Samples from six different receptor A preparations (lanes 1–6) were analyzed separately on 1.5-mm slab gels according to the electrophoretic procedure of Laemmli (16). Protein bands have been visualized by the staining protocol of Wray *et al.* (17) using silver nitrate, which we estimate is capable of detecting 5 ng of protein. Apparent molecular weight $\times 10^{-3}$ is indicated on the left.

the 5′ flanking sequences adjacent to the ovalbumin gene, cloned in the recombinant plasmid pOV1.7 (11). Digestion of pOV1.7 with *Pst* I and *Eco*RI yields a 3.6-kb fragment of pBR322 DNA and the 1.7-kb chicken DNA insert as shown in Fig. 2*A*. Fig. 3*A* shows binding results obtained at 37°C with this digest. Although the two [^{32}P]DNA fragments were present in equal amounts initially (lane 1), the DNA bound at the lower concentrations of receptor A was enriched about 10-fold for the ovalbumin sequence relative to the larger plasmid fragment (lanes 3 and 4). This preferential adsorption of OV1.7 was obliterated at higher receptor A inputs (lanes 5 and 6), at which nonspecific binding to plasmid DNA became extensive. The influence of incubation temperature on preferential binding to OV1.7 is shown in Fig. 3*B*, with 0°C incubations. The preferential interaction observed at 37°C was absent at 0°C. This temperature dependence appears to be related to a transition at or below 15°C, because incubations at 15°C or 22°C gave readily detectable enrichment in OV1.7 binding (data not shown).

The pBR322-derived recombinant plasmid pCβG6.1 (12), which contains the entire adult chicken β-globin gene sequence, was tested for receptor binding. Digestion of this plasmid with *Hin*dIII, *Bam*HI, and *Eco*RI yielded five DNA fragments (Fig. 2*B*). Receptor titration gave the results of Fig. 3*C*. The largest DNA fragments were bound at the lowest receptor concentrations, with successively higher protein concentrations required to bind the smaller pieces of DNA (lanes 1–4). Similar data (not shown) were obtained for the binding of receptor A to chicken α-actin gene sequences analyzed in a digest of the recombinant plasmid pAc3.2 (18) and for a digest of the parent plasmid pBR322 (8). Thus, receptor A appears to bind generally to these DNAs on the basis of fragment length. This preference for large DNA fragments contrasts with the preferential retention of OV1.7 (Fig. 3*A*) and is consistent with the known high affinity of receptor A for heterologous DNA (7).

Preferential Binding of Receptor A to Other Hormone Regulated Gene Sequences. Two other chicken genes regulated by steroids have also been tested. Genes *X* and *Y* are related to the ovalbumin gene and are located upstream 20–30 kb from the ovalbumin gene (19, 20). In two competition studies, we compared the interaction of progesterone receptor A with the analogous 5′ flanking DNA sequence from the *Y* and ovalbumin genes (Fig. 2*C*). The data in Fig. 3*D* were obtained from reaction mixtures containing a constant concentration of both pro-

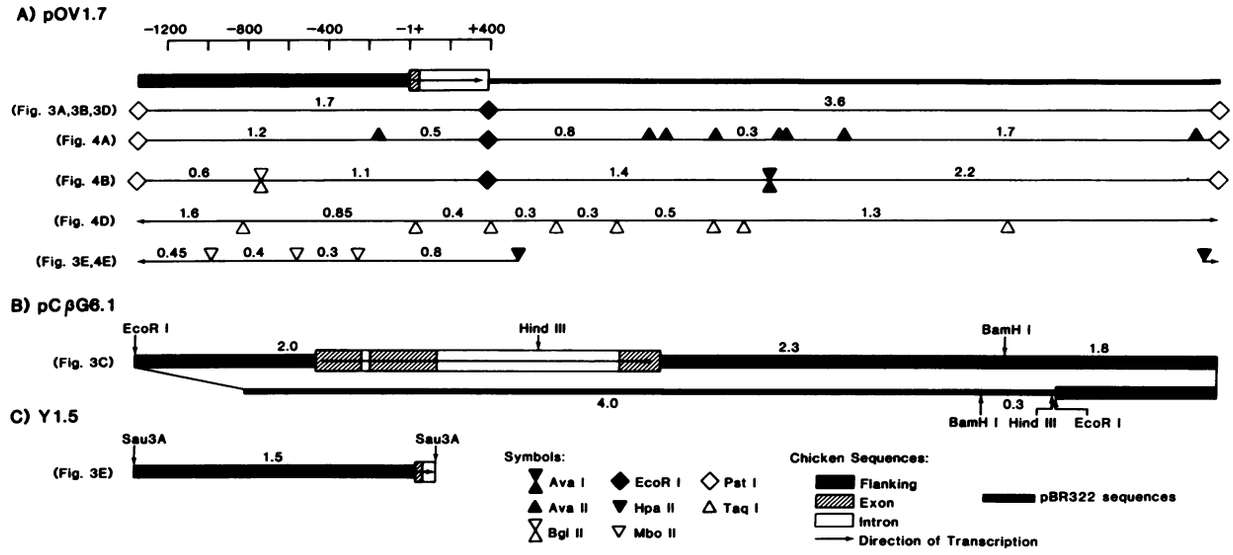

FIG. 2. Partial restriction maps of the DNAs utilized in receptor A-binding studies. DNA digests were prepared, end-labeled with ^{32}P, and used to obtain the results presented in the figures indicated at the left of each diagram. Sites of cleavage by restriction endonucleases and the sizes of the resulting DNA fragments are shown for the various digests. (*A*) The recombinant plasmid pOV1.7 (5.3 kb) containing 1,727 bp of chicken genomic sequence inserted into the *Pst* I and *Eco*RI sites of pBR322. The insert consists of 1,338 bp of sequence flanking the 5′ terminus of the ovalbumin gene and 389 bp of transcribed sequence including the first exon (47 bp) and part of the first intron. Also shown in addition to diagrams of four digests of this recombinant is a digest of OV1.9 containing the indicated portion of pOV1.7 including the entire 1.7-kb insert. (*B*) The recombinant plasmid pCβG6.1 (10.1 kb) contains the entire adult chicken β-globin gene (three exons and two introns) and the insert also includes 0.9 and 3.4 kb of 5′ and 3′ flanking sequence, respectively, all inserted into the *Eco*RI site of pBR322. (*C*) A 1.5-kb DNA fragment of the chicken *Y* gene isolated from a *Sau*3A digest of pY7.2 (13). This DNA contains 1.4 kb of 5′ flanking sequence and about 0.1 kb of the 5′ transcribed portion of the *Y* gene, including the first exon and part of the first intron.

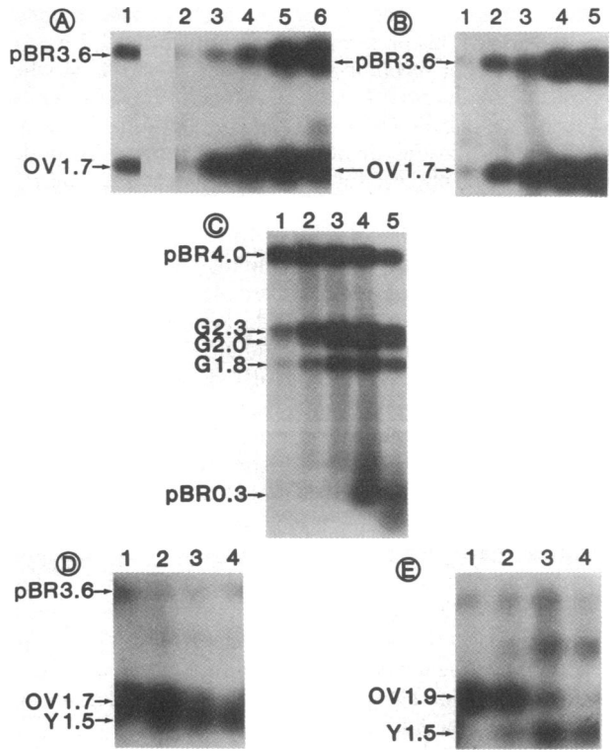

FIG. 3. Preferential interaction of progesterone receptor A with DNA fragments derived from the target genes ovalbumin and *Y*, and the nontarget β-globin gene. [^{32}P]DNA bound to receptor A was eluted from nitrocellulose filters and analyzed by autoradiography after electrophoresis in 0.7% agarose. Symbols to the left of each autoradiograph identify the bands of DNA as to their origin and size (in kb) as indicated in Fig. 2. (*A*) Receptor A binding to *Eco*RI/*Pst* I-digested pOV1.7 at 37°C. The DNA was labeled at the *Eco*RI termini with ^{32}P (lane 1). Reaction mixtures contained [^{32}P]DNA at 0.7 μg ml^{-1} and 0, 3, 9, 27, or 81 nM receptor A (lanes 2–6, respectively). (*B*) Titration with receptor A at 0°C. Lanes 1–5 correspond to the same solutions described for *A*. (*C*) Receptor A titration of binding to a *Hin*dIII/*Pst* I/*Bam*HI digest of pCβG6.1 labeled at the *Hin*dIII termini. Binding mixtures for lanes 1–4 contained 3, 9, 27, and 81 nM receptor A, respectively, and a constant amount of [^{32}P]DNA (0.3 μg ml^{-1}). (*D*) Competition by ^{32}P-labeled Y1.5 for binding of receptor A to *Eco*RI/*Pst* I-digested pOV1.7. Solutions contained 4.5 nM receptor A and *Eco*RI/*Pst* I-digested pOV1.7 (labeled with ^{32}P at the *Eco*RI termini) at 0.8 μg ml^{-1} in the absence (lane 1) and presence of a 0.24-, 0.54-, and 1.6-fold molar ratio of ^{32}P-labeled Y1.5 to ^{32}P-labeled OV1.7 (lanes 2–4, respectively). These molar ratios correspond to mass ratios of 0.2-, 0.47-, and 1.4-fold, respectively. The specific activity of each DNA fragment was about 3.5 × 10^6 dpm pmol^{-1}. (*E*) Competition experiment analogous to that in *D*, showing the DNA bound from mixtures containing 4.5 nM receptor A, ^{32}P-labeled OV1.9 at 0.13 μg ml^{-1}, and 0, 0.2, 0.5, or 1.6 molar ratio of ^{32}P-labeled Y1.5 to ^{32}P-labeled OV1.9. The mass ratios are 0.16-, 0.4-, and 1.3-fold, respectively. The fragments had equivalent specific activities. Two contaminant DNA bands, not removed by preparative electrophoresis, were a contaminant in OV1.9 (the largest band) and one present in the Y1.5 preparation (the next largest band).

tein and digested ^{32}P-labeled pOV1.7, and increasing amounts of ^{32}P-labeled Y1.5. The Y1.5 DNA effectively competed with OV1.7 for receptor binding and both DNAs were bound preferentially compared to pBR3.6. This competition is clearly evident in the data shown in Fig. 3*E* from a similar competition study between ^{32}P-labeled Y1.5 and a 1.9-kb fragment containing all of the OV1.7 sequence isolated from pOV1.7 (see Fig. 2*A*). These results show that receptor A interacts selectively with DNA from the 5′ ends of both the *Y* and ovalbumin genes. Similar results were obtained with the analogous 5′ region of the *X* gene (data not shown).

Receptor A Interacts Preferentially at Several Sites Within the OV1.7 Sequence. Studies were performed to map receptor A-binding sites within OV1.7. Digestion of pOV1.7 by the nucleases *Ava* II, *Eco*RI, and *Pst* I divides OV1.7 into two fragments (Fig. 2*A*). As seen in Fig. 4*A*, preferential binding to this digest was to the 1.2-kb DNA fragment containing the sequence to the left of the *Ava* II site. Histones (lane 8) adsorbed the [^{32}P]DNA fragments proportional to their labeling intensities (lane 1).

Another digest, with the enzymes *Bgl* II, *Ava* I, *Eco*RI, and *Pst* I (Fig. 2*A*), was used in the receptor titration experiment shown in Fig. 4*B*. Again, histones bound all the DNA fragments equally (compare lanes 1 and 8). When receptor was added over a 16-fold concentration range (lanes 3–7), the two fragments OV1.1 and OV0.6 containing ovalbumin gene sequences were both preferentially retained relative to the plasmid fragments. Thus at least two sites of preferential interaction with receptor A are present on the full length of OV1.7. A displacement assay with excess unlabeled DNA was performed to discriminate between these two OV1.7 fragments, as shown in Fig. 4*C*. Suf-

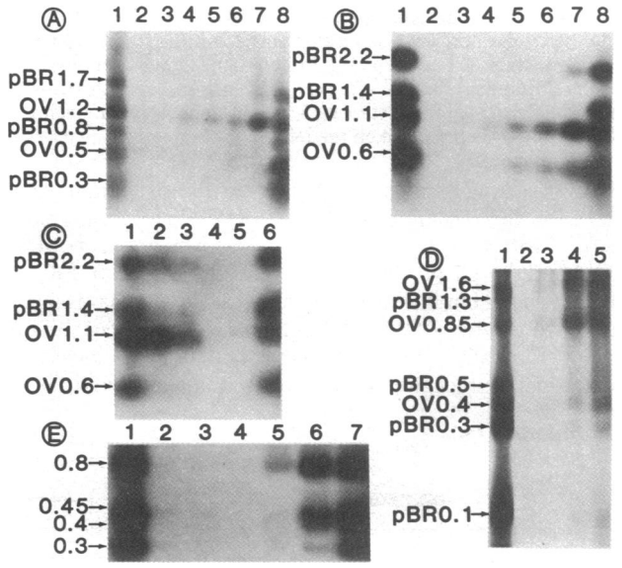

FIG. 4. Mapping of preferential interactions of receptor A within the OV1.7 sequence. DNA bands are labeled to the left of each autoradiograph according to their origin and size (in kb) as indicated in Fig. 2*A* for various digests of pOV1.7 and OV1.9. Electrophoresis was in 0.7% agarose (*A–C*) or in 4% (*D*) or 6% (*E*) polyacrylamide. (*A*) Results of an analysis of binding to *Ava* II/*Eco*RI/*Pst* I-digested pOV1.7 (labeled at the *Ava* II termini) at 0.5 μg ml^{-1}. The [^{32}P]DNA mixture is shown in lane 1 and that bound to filters in the presence of 0, 1.5, 3, 12, and 24 nM receptor A or histones at 4 μg ml^{-1} is shown in lanes 2–8, respectively. (*B*) Analysis of binding to an *Ava* I/*Bgl* II/*Eco*RI/*Pst* I digest of pOV1.7 with the *Ava* I and *Bgl* II termini labeled (lane 1). Lanes 2–7 show DNA bound from solutions containing [^{32}P]DNA at 0.9 μg ml^{-1} and protein as described for *A*. (*C*) Relative stability of the preferential interaction of receptor A with the 0.6- and 1.1-kb fragments derived from *Ava* I/*Bgl* II/*Eco*RI/*Pst* I-digested pOV1.7. Receptor A (12 nM) was incubated with [^{32}P]DNA at 0.9 μg ml^{-1} at 37°C and an aliquot was assayed for bound DNA (lane 1). A 15-fold mass excess of unlabeled pBR322 DNA was added and samples were analyzed after 3, 7, 11, and 20 min of competition (lanes 2–5, respectively). Lane 6 shows the distribution of ^{32}P on the labeled digest of pOV1.7. (*D*) Receptor A titration of binding to *Taq* I-digested pOV1.7 (lane 1). Lanes 2–5 show the [^{32}P]DNA bound from mixtures containing DNA at 0.7 μg ml^{-1} and 0, 8, 24, or 50 nM receptor *A*. (*E*) Analysis of receptor A binding to *Mbo* II-digested OV1.9. Lane 1 contained the labeled DNA digest and lanes 2–7 show the DNA bound to filters from solutions containing [^{32}P]DNA at 0.3 μg ml^{-1} and 0, 0.3, 0.9, 2.7, 8.1 or 24 nM receptor A, respectively.

ficient receptor A was present so that all four fragments were bound to the filters (lane 1). After addition of a 15-fold mass excess of pBR322 DNA, aliquots of the binding mixture were periodically removed and filtered, and the retained DNA was analyzed (lanes 2–5). The time-dependent decrease in binding of the 1.1-kb fragment was significantly slower than that observed for the other three fragments, thus assigning a stronger interaction of receptor with the 1.1-kb chicken DNA.

The foregoing results establish limits for the location of the strongest receptor A binding site within OV1.7. It must lie to the right of the *Bgl* II site at −732 bp and to the left of the *Ava* II site at −135 bp, upstream from the gene. This finding was confirmed by results for a *Taq* I digest of pOV1.7 (Fig. 2*A*) as shown in Fig. 4*D*. Lanes 3 and 4 show preferential retention of the 0.85-kb band, containing the proximal 5′ flanking DNA upstream from the gene, including the *Bgl* II/*Ava* II sequence. Fig. 4*D* also shows retention of the 1.6-kb fragment containing the upstream flanking DNA, similar to the results of Fig. 4*B*. Another experiment was performed with the 1.9-kb *Hpa* II fragment, OV1.9, which was digested with *Mbo* II into four fragments (see Fig. 2*A*). The receptor titration results shown in Fig. 4*E* show preferential retention of the 0.8-kb fragment (lanes 2–8). This interaction is most evident in lane 5; slight alterations in input receptor level (e.g., lanes 4 and 6) change the intensities dramatically. Lanes 5 and 6 show that this fragment is preferentially retained relative to the next adjacent upstream 0.3-kb *Mbo* II fragment. The latter fragment contains the consensus sequence proposed by Mulvihill *et al.* (10) for receptor interaction. Because this difference in intensity is greater than 10-fold, it cannot be ascribed merely to the 2.5-fold difference in fragment size. Preferential retention of the 0.45- and 0.4-kb fragments relative to the 0.3-kb sequence further substantiate the conclusion that multiple weaker receptor A sites exist within OV1.7. Thus, the *Mbo* II digest data of Fig. 4*E* suggest that the strongest receptor interaction lies to the right of the *Mbo* II site at −247 bp.

DISCUSSION

In order to detect DNA sequence-specific binding of the progesterone receptor A subunit, we have isolated the population of DNA fragments adsorbed to nitrocellulose filters by receptor A and compared their relative abundance with the composition of the initial DNA mixtures. The property of receptor A to bind preferentially to the largest fragments in a mixture made it necessary to choose carefully the distribution of genomic and bacterial sequences among different size DNAs. The absence of artifactual enrichment in a particular DNA fragment arising from differential elution of the filters was confirmed by data for histone binding. Analysis of the extracted DNA showed the relative distributions bound to the filters by histones to be indistinguishable from the distribution in the initial mixtures of DNA fragments. The same result was obtained in the presence of an excess of receptor A itself.

Nearly homogeneous progesterone receptor A (about 10,000-fold purified) was used in the assays to minimize contributions from non-receptor DNA-binding proteins. No variation was observed in any DNA-binding property reported in this study, which used about 50 different receptor A preparations containing a variety of minor contaminants. No nuclease or phosphatase activity was observed in any of the preparations. All preparations contained nearly stoichiometric amounts of progesterone as determined by radioimmunoassay. Any role of progesterone in determining the nature or extent of the sequence-specific binding of receptor remains to be elucidated.

The binding studies indicate the presence of several receptor A–DNA interactions within OV1.7 that are stronger than the corresponding interaction with plasmid DNA fragments of equivalent length. By comparing the binding to OV1.7-derived fragments, a region of strongest receptor A interaction can be deduced, as shown diagrammatically in Fig. 5. The data of Fig. 4 *A–C* place limits for this binding between the *Bgl* II site (−732 bp) and *Ava* II site (−135 bp). This placement is substantiated in Fig. 4*D* by binding to the sequence between the *Taq* I sites at −807 bp and +41 bp. Within this region it is probable that several interactions occur, as shown by the binding to *Mbo* II-derived fragments from both pOV1.7 (data not shown) and OV1.9 (Fig. 4*E*). Quantitative considerations suggest the strongest interaction lies downstream from the *Mbo* II site at −247 bp. By this exclusion argument, the region containing the strongest receptor A binding site lies within the 114-bp region between −247 and −135 bp upstream from the start of ovalbumin gene transcription.

The DNA sequence preference we have observed presents several interesting questions that remain to be resolved. One question concerns the apparent multiplicity of receptor binding sites flanking the same gene. All of the OV1.7-derived fragments are preferentially retained compared to plasmid-derived sequences, although receptor A shows the highest preference for a short region in OV1.7. Thus it is not possible, at present, to ascribe the best site identified here as being the site utilized

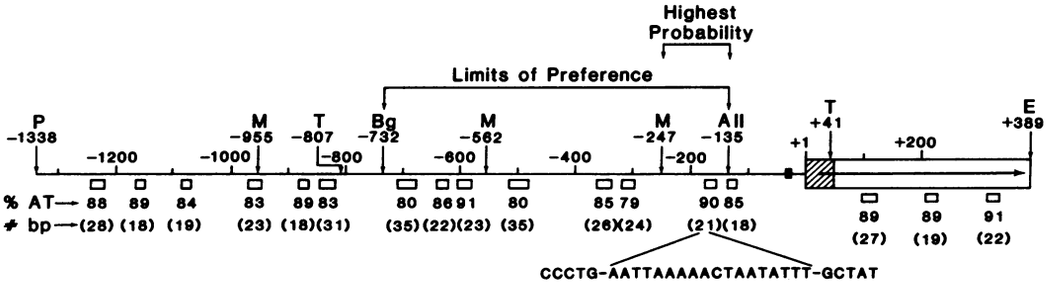

FIG. 5. Summary diagram showing the organization of pOV1.7 and the location of a receptor A-binding site inferred from the preferential adsorption experiments. The transcribed 5′-terminal sequence of the ovalbumin gene is indicated by the bar at the right and contains the first exon (shaded bar) and part of the first intron (open bar). The 5′-flanking sequence is represented by the narrow line and the Hogness sequence is shown as the dark block at −30 bp. Also indicated are the positions of cleavages by *Ava* II (AII), *Bgl* II (Bg), *Eco*RI (E), *Mbo* II (M), *Pst* I (P), and *Taq* I (T). The open bars below the map show the locations of A+T-rich sequences (≥79%) extending 18 bp or more. The length and percent of A+T composition are shown below each box. One bracket identifies the *Bgl* II/*Ava* II region having the tightest interaction with receptor A and the other delimits the highest probability sequence inferred to contain a receptor A-binding site. The sequence of the very A+T-rich portion in the latter region is shown.

in vivo. The second question concerns the exact nature of the DNA sequence involved in the preferential binding of receptor A. One interesting feature of the sequence between −135 and −247 bp is the presence of a highly A+T-rich (90%) 18-bp region (Fig. 5). Similar regions are present elsewhere in OV1.7, whereas very few are found in pBR322. Also, the 5′ flanking region of the β-globin gene with which receptor A does not preferentially interact (Fig. 3*C* and ref. 8), is high in G+C content (70%; see ref. 12).

A number of the DNA-binding properties of receptor A are consistent with A+T-rich sequences playing a role in receptor–DNA interaction. These include preferential binding to poly(dA-dT) compared with poly(dG-dC) and to single-stranded DNA compared with double-stranded DNA (7), and enhanced specificity in the presence of DNA helix-destabilizing agents (glycerol or dimethyl sulfoxide) or at increased temperatures (>15°C). However, while helix destabilization may be involved in receptor A binding, an A+T-rich region cannot be solely responsible for preferred binding because A+T-rich regions are also present on DNA fragments not showing preferential binding to receptor A (e.g., OV0.5 in Fig. 4*A*). One possibility is that the receptor binds through recognition of a DNA sequence within the highest-affinity region identified here but exerts its action as a helix-destabilizer at a proximal A+T-rich region.

One final consideration is whether the 10-fold sequence preference of receptor A binding reported here reflects the *in vivo* situation. Possibly the degree of sequence preference we observe may be underestimated due to alterations in receptor structure during purification. The observed sequence preference may also be a function of the DNA fragment size. Because the exact length of a receptor site remains unknown, nonspecific DNA sites present on a fragment may tend to reduce the apparent degree of specificity. Finally, selective binding *in vitro* to naked linear DNA may differ substantially from that to the DNA in chromatin *in situ*.

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