Receptors for glucocorticosteroid and progesterone recognize distinct features of a DNA regulatory element

(chicken lysozyme gene/hormone regulatory element/DNase I "footprint"/methylation protection/DNA computer graphics)

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ABSTRACT The chicken lysozyme gene can be induced in oviduct cells by four classes of steroid hormones, including glucocorticosteroids and progestins. The glucocorticosteroid receptor of rat liver and the progesterone receptor of rabbit uterus both bind, although with different relative affinities, to two sites in the promoter region of the chicken lysozyme gene located, respectively, between 50 and 80 and between 160 and 200 base pairs upstream of the transcription start point. Now we show that the purified progesterone binding site of the chicken oviduct progesterone receptor (Mf, 110,000, or so-called B subunit) generates a DNase I protection pattern ("footprint") in the promoter-distal site that is longer than the footprint generated by the glucocorticosteroid receptor. Methylation protection studies within the promoter-distal binding site identify four contact points for the chicken progesterone receptor and three contact points for the glucocorticosteroid receptor, of which only one is shared by both receptors. Computer graphics models allow one to envisage a different interaction of each receptor with the B form of the DNA double helix.

Gene regulation by steroid hormones is mediated by an interaction of the hormone receptor with DNA elements around the regulated promoters. Such elements have been identified at variable distances from the promoter in the genes for mouse mammary tumor virus, human metallothionein IIa, chicken lysozyme, rabbit uteroglobin, and human growth hormone (for a review see ref. 1). The expression of some of these genes, as for instance the chicken lysozyme gene, can be regulated by several steroid hormones, including glucocorticosteroids and progesterone (2, 3). The question, thus, arises of whether the action of individual hormones is mediated by the same or by separate DNA regulatory elements.

In the promoter region of the chicken lysozyme gene two receptor binding sites have been found, of which the promoter-proximal one (located around position −60) exhibits high affinity for the glucocorticosteroid receptor and low affinity for the progesterone receptor, whereas the promoter-distal site (around position −180) has low affinity for the glucocorticosteroid and high affinity for the progesterone receptor (4, 5). No functional data are available concerning the promoter-proximal binding site, but deletions destroying the promoter-distal site result in parallel loss of inducibility by both glucocorticosteroid and progesterone, suggesting that binding of the receptor to this site is relevant for induction by both hormones (4).

In exonuclease III protection experiments with the promoter-distal site we found that the region covered by the glucocorticosteroid receptor is shorter than the region protected by the rabbit uterus progesterone receptor (5), indicating that subtle differences may exist between the interaction of each receptor with the regulatory sequences. To analyze this question in more detail we decided to use the homologous progesterone receptor purified from chicken oviduct in DNase I "footprint" and methylation protection experiments. Here, we show that the 110-kDa form of the chicken progesterone receptor binds to the promoter-distal site in the lysozyme gene and yields a footprint that is 9–13 base pairs longer than that generated by the glucocorticosteroid receptor. Within the promoter-distal site the contact points between each receptor and the DNA double helix differ in six out of seven positions, suggesting different recognition mechanisms for each receptor protein.

MATERIALS AND METHODS

Receptor Purification. The 94-kDa form of the rat liver glucocorticosteroid receptor was purified as previously published (6); it is free of progesterone receptor activity as demonstrated by binding of [3H]R 5020 [17,21-dimethyl-pregnen-4,9(10)diene-3,20-dione].

The so-called B subunit (7) of the chicken oviduct progesterone receptor was purified by affinity chromatography (8). After elution from the affinity column by [3H]R 5020 and subsequent DEAE-Sephacel chromatography, the pooled radioactive peak eluting at 0.3 M KCl during the linear salt gradient was further concentrated by another DEAE-Sephacel chromatography, in which it was eluted by 0.5 M KCl.

Identification of the receptor bands was performed by photoaffinity labeling with radioactive ligand followed by fluorography (9, 10).

DNase I Footprinting. The recombinant plasmid pUCLysA-208, containing the chicken lysozyme promoter region (4), was digested with HindIII and labeled either at the 5' end with [γ-32P]ATP and T4 polynucleotide kinase to a specific activity of 1 × 106 dpm/pmol or at the 3' end with [α-32P]dATP and the Klenow fragment of DNA polymerase I to a specific activity of 3 × 106 dpm/pmol. After digestion with EcoRI a 208-base-pair labeled fragment was isolated by electrophoresis in low-melting agarose gels. Aliquots of about 30 fmol of DNA fragments were used for each point. Incubation with the receptor at 25°C for 45 min in 0.1 M KCl, digestion with

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DNase I, and electrophoresis on denaturing gels were as previously described (11).

**Methylation Protection Experiments.** The same restriction fragment and end-labeling protocol as described for DNase I footprinting were used for the methylation protection with the promoter-distal site. For the experiments with the promoter-proximal site the plasmid pUC18-Δ-208 (4) was digested with EcoRI and labeled either at the 3' end or at the 5' end, prior to secondary digestion with HindIII and isolation through electrophoresis in agarose gels (specific activities 4.2 × 10⁶ dpm/pmol and 0.9 × 10⁶ dpm/pmol, respectively). Aliquots (2–2.5 × 10⁶ dpm) of the labeled fragment were methylated by dimethyl sulfate in the presence or absence of the corresponding receptor and were analyzed in sequencing gels as previously described (11, 12).

**RESULTS**

A polyacrylamide gel analysis of a typical preparation of the B form of the chicken oviduct progesterone receptor used for these studies is shown in Fig. 1. A densitometric evaluation of the gel indicated that the purity is higher than 80%. The 110-kDa band, identified as the receptor by photoaffinity labeling with 5020 (8, 10), has been purified by affinity chromatography on a receptor column and therefore the final preparation contains almost exclusively steroid binding species of the receptor. The specific activity of the purified progesterone receptor is 9.1 nmol of bound steroid per mg of protein, and the specific activity of a pure receptor should be about 87% pure. This preparation did not contain measurable amounts of glucocorticosteroid receptor as measured by specific binding of [³H]dexamethasone (9a-fluoro-11β, 17,21,-trihydroxy-16a-methylpregn-1,4-diene-3,20-dione).

DNase I footprint analysis confirms previous exonuclease protection experiments with respect to the promoter-distal binding site. In the sense ("upper") strand the oviduct progesterone receptor yields a footprint extending from nucleotide −202 to −163, whereas the footprint of the glucocorticosteroid receptor extends from −202 to −174 (Fig. 2A). In the antisense ("lower") strand of the DNA the progesterone receptor generates a footprint between −197 and −160, interrupted by a series of receptor unhindered cutting sites centered at position −189 (Fig. 2B, lanes 4 and 5). With the glucocorticosteroid receptor a DNase I footprint is found between −199 and −173 that is also interrupted by a series of accessible cutting sites centered at −192 (Fig. 2B, lanes 1 and 2) and located within an A+T stretch of DNA (position −195 to −187, Fig. 4A).

In the presence of the glucocorticosteroid receptor, but not with the oviduct progesterone receptor, an additional promoter-proximal DNase I footprint is found. In the upper strand this footprint extends between −76 and −49 and in the

![Fig. 1. Affinity labeling of pure chicken oviduct progesterone receptor. The B subunit of the chicken oviduct progesterone receptor was purified as described in ref. 8 and analyzed in 7.5–15% gradient polyacrylamide gel electrophoresis. Lane 1, silver staining of 1 μg of pure chicken oviduct progesterone receptor. Lane 2, the same amount of receptor photoaffinity labeled with 10 pmol of [³H]HR 5020 (87 Ci/mmol; 1 Ci = 37 GBq) by UV irradiation. The fluorograph reveals that the progesterone was specifically bound to the chicken oviduct progesterone receptor, since no signal was obtained when the UV irradiation of an identical sample was performed after exchange with 2 μM unlabeled progesterone (24 hr at 4°C), lane 3.](image-url)

![Fig. 2. DNase I footprint experiments. (A) Sense strand. Lane 1, G sequence reaction (12). Lanes 2, 3, 6, and 7, digestion with DNase I in the absence of added receptor. Lanes 4 and 5, DNase I digestion after incubation at 25°C with 1.5 and 3.0 μg, respectively, of rat liver glucocorticosteroid receptor (rLIGR). Lanes 8 and 9, DNase I digestion after incubation with 0.75 and 1.5 μg, respectively, of chicken oviduct progesterone receptor B (cOvPRB). (B) Antisense strand. Lanes 1 and 4, DNase I digestion in the absence of added receptor. Lane 2, DNase I digestion in the presence of 2.7 μg of glucocorticosteroid receptor. Lane 5, DNase I digestion in the presence of 1.5 μg of chicken oviduct progesterone receptor B. Lanes 3 and 6, G-sequence reaction (12). The numbers on the left refer to the distance from the transcription initiation site. The DNase I-protected regions are indicated by dotted lines. The arrows point to positions that are hypersensitive to DNase I in the presence of receptor.](image-url)
lower strand between $-80$ and $-53$ (Fig. 2A and B). We have previously shown that the rabbit uterus progesterone receptor binds, although with low affinity, to this promoter-proximal site (5). Thus, there are differences in the properties of the preparations of progesterone receptors from rabbit uterus and chicken oviduct. Note that in Fig. 2B, lanes 2 and 5 are darker throughout, giving the wrong impression of enhanced DNase I digestion in the presence of receptor. A densitometric evaluation of the relative intensity of each band showed that this is not the case for most of the positions. Interestingly, in the presence of either the progesterone or the glucocorticosteroid receptor, a very evident DNase I cutting site is detected at $-45$ in the lower strand, in a position where there is no DNase I cut in the absence of the receptors (Fig. 2B, arrows). This could reflect the vicinity of bound receptor molecules (in the case of the glucocorticosteroid receptor) but could also result from indirect effects of bound receptor molecules upon the DNA conformation. This seems probable for the progesterone receptor, as the binding site is located 120 base pairs upstream.

An analysis of the contact points between the receptors and the promoter-distal binding site was carried out by methylation-protection experiments (13). A densitometric analysis of the relative intensity of the individual bands of the autoradiograms in Fig. 3 allows the following conclusions. In the lower strand both receptors protect the N-7 position of the G at $-185$, whereas only the glucocorticosteroid receptor protects the G at $-176$, which is clearly methylated in the presence of the progesterone receptor (Fig. 3B, lanes 1–4). This latter receptor protects the G at $-170$ and to a lesser extent the G at $-171$, whereas these positions are accessible to methylation in the presence of the glucocorticosteroid receptor. In the upper strand of the DNA no common contact point for both receptors is detected. The glucocorticosteroid receptor protects only the G at $-180$, whereas the progesterone receptor protects the guanines at $-177$ and $-166$ (Fig. 3A, lanes 1–7).

In the promoter-proximal binding site, no contacts for the oviduct progesterone receptor were found, and the methylation protection pattern detected with the glucocorticosteroid receptor (Fig. 4A) is similar to that found in other glucocorticosteroid regulatory elements (16, 17). The guanines at $-74$ and $-64$ in the upper strand (Fig. 3A, lanes 8–11) and those at $-72$ and $-61$ in the lower strand (Fig. 3B, lanes 5 and 6) were protected by the receptor. A summary of these data is shown in Fig. 4A. It is clear that although the binding sites for the glucocorticosteroid and the progesterone receptor do partly overlap, each receptor recognizes different features in the major groove of the DNA.

A computer graphic representation of the binding sites, compared to the glucocorticosteroid regulatory element of mouse mammary tumor virus (11) is shown in Fig. 4B. The van der Waals areas of the N-7 atoms of the relevant G residues are shown in black for the glucocorticosteroid receptor and in stippling for the oviduct progesterone receptor. Three of the contact points for the glucocorticosteroid receptor are similar in mammary tumor virus and in the two binding sites of the chicken lysozyme promoter, whereas the progesterone receptor exhibits an additional set of contact points in the major groove of a subsequent turn of the DNA double helix. The axial projection of the DNA helices (Fig. 4C) shows clearly that the glucocorticosteroid receptor contact points are located within a small sector that overlaps but differs from that covered by the progesterone receptor, suggesting that the two receptors approach the DNA from different angles.

**FIG. 3.** Methylation protection experiments. (A) Sense strand. Lanes 1, 2, 5, 6, 8, and 9, methylation in the absence of receptor. Lanes 3 and 4, methylation in the presence of 0.75 and 1.5 μg, respectively, of chicken oviduct progesterone receptor B (cOvRB). Lanes 7, 10, and 11, methylation in the presence of 2.9, 2.0, and 3.0 μg, respectively, of rat liver glucocorticosteroid receptor (rLiGR). (B) Antisense strand. Lanes 1, 3, and 5, methylation in the absence of receptor. Lane 2, methylation in the presence of 1.5 μg of chicken oviduct progesterone receptor B. Lanes 4, 6, and 7, methylation in the presence of 3.0, 1.5, and 3.0 μg, respectively, of glucocorticosteroid receptor. The numbers refer to distance from the transcription initiation site. * and o mark positions protected against methylation in the presence of chicken oviduct progesterone receptor B and glucocorticosteroid receptor, respectively.
Proteolytic degradation have the chicken oviduct position by the glucocorticosteroid both within binding "subunit" of the glucocorticosteroid receptor are indicated by dotted lines, and those generated by chicken oviduct progesterone receptor B are marked by the continuous lines. The triangles and rhombuses point to G residues that are protected against methylation in the presence of the chicken oviduct progesterone receptor B or the glucocorticosteroid receptor, respectively. The thick vertical arrow shows the approximate position of a DNase I cutting site that is seen only in the presence of receptor. The horizontal arrow indicates a decanucleotide that is found within both footprints (4). (B) Computer graphic models of the B-DNA helix within the footprint. The models were generated by using an Evans and Sutherland Colour Multipicture System hosted by a Digital Equipment VAX-11/780 computer, and the UCSP MIDAS molecular modeling software (14, 15). The DNA segments are from mouse mammary tumor virus (MMTV) and chicken lysozyme promoter regions II and I (Lys-II and Lys-I). The van der Waals spheres of the N-7 atoms of the relevant guanine residues are shown in solid black for the sites protected by the glucocorticosteroid receptor, and in stippling for those protected by the chicken oviduct progesterone receptor B. The numbers refer to the distance from the transcription initiation site. (C) Axial view of the three helices shown in B. Only the linked phosphorus atoms and the van der Waals spheres of the guanine N-7 atoms are shown. The arrows indicate the different approach angles of the two receptors.

**DISCUSSION**

It is not clear to us why several previous reports have failed to detect the DNA binding activity of the B component (M, 110,000) of the chicken oviduct progesterone receptor and have concentrated on the A protein (M, 79,000) as the DNA binding "subunit" of the receptor (for a review see ref. 7). Evidence is accumulating that the intact hormone binding species of the progesterone receptor is the so-called B "subunit," and that the A protein, which is recognized by antibodies to the B protein (18, 19), could represent a proteolytic degradation product (19, 20). This could explain why no convincing DNase I footprints have been obtained with the purified A protein (21). That the B form of the receptor and not a contaminating protein in the preparation binds to DNA has recently been shown. As measured by steroid binding, the partially purified salt-activated B form of the chicken oviduct progesterone receptor binds to calf thymus DNA-cellulose even at 100 mM NaCl (19).

It is possible that the DNA binding ability of the progesterone receptor from different species may be slightly different or may be influenced by the presence of receptor-associated (10) or other proteins. It is otherwise difficult to explain the fact that the progesterone receptors of chicken oviduct and of rabbit uterus show considerable differences in affinity for the promoter-proximal binding site in the chicken lysozyme gene (5). The binding of both receptors to the promoter-distal sites, however, shows identical exonuclease footprints (D.v.d.A., unpublished data), indicating that in this functionally relevant region both progesterone receptors recognize the same regulatory information.

As we know that the promoter-distal binding site mediates the inducibility of the lysozyme gene by both glucocorticosteroids and progestins, the observation that the receptors for the two hormones recognize different features of a DNA regulatory segment offers the possibility of dissociating the regulation by the two hormones by means of selective point mutations. Physiologically, overlapping but distinct interac-
tions of different hormone receptors with DNA also may help to explain the particular kinetics and quantitative features of the increased transcription of the same genes by different hormones (2, 3).

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