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Cloning and sequence analysis of rabbit progesterone-receptor complementary DNA

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ABSTRACT **Two λgt11 clones containing fragments of cDNA encoding the rabbit progesterone receptor were isolated with the aid of monoclonal and monospecific polyclonal anti-receptor antibodies. RNA gel blot analysis showed that the corresponding mRNA was ≈5900 nucleotides in size and present in the uterus, where its concentration was increased by estrogen treatment, and in the vagina. This mRNA was not detected in liver, in spleen, in intestine, and in kidney where the receptor protein is known to be absent or present in very small concentration. Cross-hybridizing clones were isolated from a λgt10 library. The DNA was sequenced, and the primary structure of the progesterone receptor was deduced. It consists of 930 amino acids and contains a basic, cysteine-rich region (residues 568–645) with extensive homology to the glucocorticoid and estrogen receptors and the v-erbA oncogene protein. This region is followed by a C-terminal domain that is similar in size to the corresponding domains of the other steroid receptors and v-erbA and shows striking amino acid homology with the glucocorticoid receptor and significant homology with the estrogen receptor. In contrast, the region extending from the cysteine-rich segment toward the N terminus differed in size and amino acid sequence from that of the other receptors and v-erbA. This region had a high proline content in the progesterone receptor.**

Steroid hormones act on target cells by binding to intracellular receptors. Steroid–receptor complexes in turn modulate the transcription of specific genes (reviewed in ref. 1). Elucidation of the structural and functional properties of steroid receptors will aid in the understanding of hormone action and of the mechanisms for the regulation of gene transcription. Several of these receptors have been purified, and antibodies against them, especially monoclonal antibodies, have been obtained (2–5). This allowed the cloning of cDNAs for the glucocorticoid receptor (GR) and the estrogen receptor (ER) (6–8).

We report here the preparation of λgt11 and λgt10 cDNA libraries from rabbit uterine mRNAs and the isolation and DNA sequence analysis of clones encoding progesterone receptor (PR) and the deduced primary structure of PR.

MATERIALS AND METHODS

Antibodies. The anti-PR monoclonal antibody Mi60-10 (3) has been extensively characterized (9–12).

The polyclonal anti-PR antibody was prepared in goat as described (13) except that extensively purified antigen was used. PR was purified to near homogeneity by immunoaffinity chromatography (9) and electrophoresed in NaDodSO$_4$/polyacrylamide gels. The 110-kDa band was excised and directly used to immunize a goat. The specificity of this antibody was also shown by immunoblot and immunocytochemical techniques (F.L., S. Brailly, and M. Applanat, unpublished observations).

Progesterone Receptor mRNA. Preparation of poly(A)$^+$ RNA, translation in reticulocyte lysates, and enrichment of receptor mRNA by sucrose-gradient ultracentrifugation have been described (10).

Preparation of the λgt11 Library. Single-stranded cDNAs were prepared by reverse transcription in the presence of random primers (14). The second strand was synthesized by reverse transcriptase and the Klenow fragment of DNA polymerase I (14) and then digested with S1 nuclease. The double-stranded cDNA was methylated by *Eco*RI methylase and repaired with the Klenow fragment of DNA polymerase, and phosphorylated *Eco*RI linkers were added using T4 DNA ligase (15). After digestion with *Eco*RI and chromatography on Bio-Gel A-50m, DNA fragments larger than 100 base pairs were ligated to *Eco*RI- and alkaline phosphatase-treated λgt11 arms. After *in vitro* packaging, the phages were used to infect *Escherichia coli* Y1090 (hsdR$^-$, hsdM$^+$) (Promega Biotec, Madison, WI). Screening of the cDNA library was essentially as described by Huynh *et al.* (15). The library was plated onto 24 × 24 cm plastic dishes, and two replicas were made onto isopropyl β-D-thiogalactoside-saturated nitrocellulose filters. The first filter was incubated with the polyclonal antibody (30 μg/ml) and, thereafter, with a second antibody raised in the rabbit against goat immunoglobulins (60 μg/ml). The second filter was incubated with the purified Mi60-10 monoclonal anti-PR antibody (10 μg/ml) and a rabbit anti-mouse immunoglobulin second antibody (10 μg/ml). Finally, the filters were incubated with ^{125}I-labeled protein A (10^6 cpm/ml), washed, dried, and autoradiographed. Two clones reacting with both antibodies were isolated by two additional rounds of screening. One contained a DNA insert of 300 base pairs (λrPR$_1$), and the second contained a 100-base-pair insert (λrPR$_2$).

Extraction and Characterization of Recombinant Fusion Proteins. *Escherichia coli* Y1089 (*hsdR*$^-$, *hsdM*$^+$) strain (Promega Biotec) was lysogenized with the λgt11 clones. Crude lysates were prepared as described (15). After lysis of bacteria, the fusion proteins (or β-galactosidase in control nonrecombinant λgt11) were precipitated by ammonium sulfate (at 35% of saturation at 4°C). Immunoblot experiments were performed as described (9).

Preparation of λgt10 cDNA Library. Double-stranded cDNAs were obtained by the RNase H method (16) and blunt-ended using T4 DNA polymerase. Methylation, addition of linkers, and size-fractionation were as described above. The first four drops eluting from the Bio-Gel A-50m column were used for cloning in λgt10 (15); 3 × 10^5 recombinants were screened (14) with the nick-translated insert of

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Abbreviations: PR, progesterone receptor; ER, estrogen receptor; GR, glucocorticoid receptor.

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λrPR1. Twenty-five positive clones were detected, and the inserts were subcloned into plasmid pBR325.

Sequencing. Four overlapping clones (λrPR3-6) were used to sequence the complete PR cDNA coding region on both strands by the M13-dideoxynucleotide chain-termination procedure (17).

RESULTS

Isolation of cDNA Clones. As steroid receptors are very rare proteins, it was necessary to greatly enrich the starting RNA for receptor mRNA molecules. For this reason, estrogen-treated rabbits were used, and uterine mRNAs were fractionated on a sucrose gradient. The cDNAs, synthesized using random primers, were inserted into the λgt11 vector, and the library was screened with both monoclonal and monospecific polyclonal antibodies. Two different types of antibodies, prepared in mice and goat, were used to decrease the probability of detection of nonspecific, nonreceptor clones. Of the 3×10^6 clones examined, two gave a reproducible signal with both antibodies.

Immunological Characterization of the cDNA Clones. Both antibodies (monoclonal and polyclonal) recognize a single protein—the 110-kDa receptor in uterine cytosol. A 79-kDa fragment is also detected when proteolysis of the receptor has occurred (9, 10). The fusion proteins (β-galactosidase fragment of receptor) encoded by the two recombinant clones were detected by monoclonal (Fig. 1) and polyclonal (data not shown) antibodies in immunoblot analysis. No protein was detected by these antibodies in *E. coli* cells infected with nonrecombinant λgt11 clones.

Competition experiments were used to show the immunological similarity between receptor and fragments of the fusion proteins. Highly purified PR was preincubated with monoclonal antibodies: this treatment suppressed the detection of receptor and also that of both fusion proteins (Fig. 1). Conversely, by using monoclonal antibodies, it was shown that preincubation with the fusion protein suppressed the detection either of purified receptor or of receptor in uterine cytosol (Fig. 1).

To clearly show that the uterine proteins that shared antigenic determinants with the fusion proteins were the steroid binding forms of receptor, the following experiment was performed. Partially purified, λrPR1 fusion protein was adsorbed onto a nitrocellulose filter that was then incubated with the polyclonal anti-receptor antiserum and washed, and the antibodies were eluted. A control experiment was performed with proteins from a nonrecombinant clone. Eluted antibodies were incubated with cytosol containing [^{3}H]progestin–receptor complexes and ultracentrifuged on a sucrose gradient (Fig. 2). Incubation with antibodies eluted from the filter bearing the recombinant fusion protein (Fig. 2*d*) showed two peaks of radioactivity corresponding clearly to the binding of either one (see Fig. 2*c*) or two molecules of antibody to the [^{3}H]progestin–receptor complexes. The proportion of the heavier peak increased with increasing concentration of eluted antibody (data not shown). There was no displacement of steroid–receptor complexes when incubated with the control antibody preparation (Fig. 2*e*).

RNA Gel Blot Experiments (18). Initial experiments verified that probes from both recombinant λgt11 clones detected the same messenger on RNA gel blots of uterine RNAs (data not shown). Thereafter, the concentration of this 5900-nucleotide mRNA was studied in the uterus both before and after estrogen treatment and in various other organs (Fig. 3). As expected, the concentration of PR mRNA was markedly increased by estrogen. It was also present in the vagina but

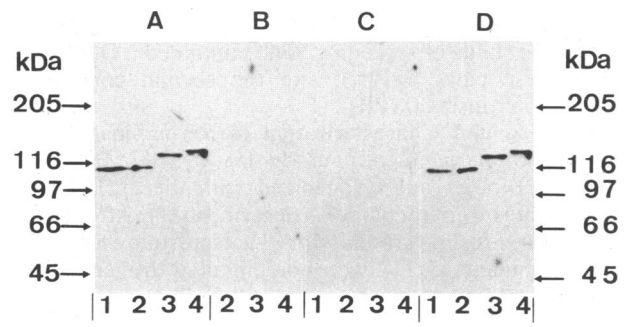

FIG. 1. PR and recombinant fusion proteins compete for the same antibody binding sites. (*A*) Immunoblot with Mi60-10 anti-PR monoclonal antibody. (*B*, *C*, and *D*) Immunoblot with Mi60-10 antibody preincubated with purified receptor (*B*), with fusion protein from clone λrPR1 (*C*), and with protein from a nonrecombinant λgt11 clone (*D*). Lanes 1, uterine cytosol (100 μg of protein containing 0.3 pmol of receptor). Lanes 2, purified receptor (1 pmol). Lanes 3, fusion protein from clone λrPR1 (total protein, 3 μg; fusion protein, 0.15 μg or 1 pmol). Lanes 4, fusion protein from clone λrPR2 (total protein, 6 μg; fusion protein, 0.15 μg or 1 pmol). The antibody (0.2 μg) was preincubated with 200 pmol of purified receptor (*B*), fusion protein (*C*), or control proteins from a nonrecombinant clone (*D*).

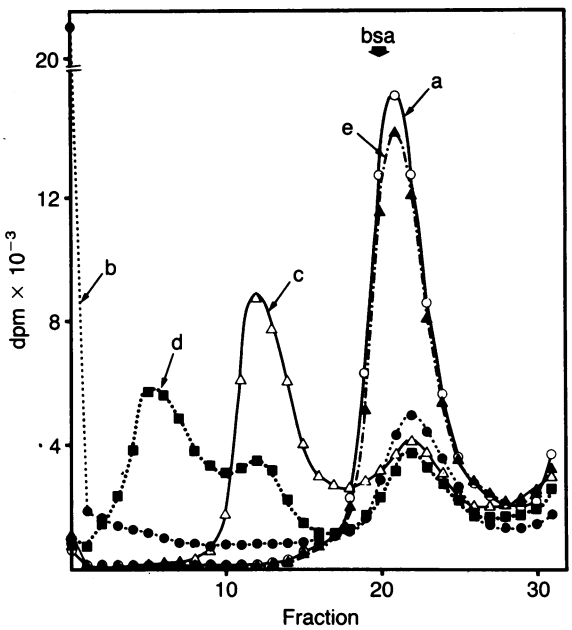

FIG. 2. The recombinant fusion proteins bind antibodies that interact with [^{3}H]progestin–receptor complexes in uterine cytosol. Uterine cytosol was incubated for 2 hr at 0°C with 30 nM 16α-ethyl-21-hydroxy-19-nor[6,7-^{3}H]pregn-4-en-3,20-dione (specific activity, 47 Ci/mmol; Amersham). Aliquots (30 μl) of cytosol were incubated for 4 hr at 0°C with the following: curve a, goat preimmune immunoglobulins (500 μg); curve b, goat anti-PR immunoglobulins (500 μg); curve c, Mi60-10 monoclonal anti-PR antibody (20 μg); curve d, immunoglobulins adsorbed to λrPR1 fusion protein (80 μl); curve e, immunoglobulins adsorbed to proteins from nonrecombinant λgt11 (80 μl). The complexes were ultracentrifuged for 20 hr in a SW50 rotor at 2°C at 49,000 rpm in a 5–20% (wt/vol) sucrose/0.3 M KCl gradient. Immunoglobulins binding to proteins either from λrPR1 or from control λgt11 were prepared as follows. Proteins extracted from bacteria and precipitated by ammonium sulfate were adsorbed onto 8.5 × 8.5 cm nitrocellulose filters (1 ml of extract containing 2 mg of protein). After washing and saturation with 10% (wt/vol) bovine serum albumin, the filters were incubated for 2 hr at 20°C with polyclonal anti-receptor immunoglobulins (10 ml per filter of a solution of 1 mg of protein per ml). After washing the antibodies were eluted from the filters with 10 mM glycine·HCl, pH 2.3/150 mM NaCl (two elutions of 5 ml each). The antibodies were concentrated by ultrafiltration, dialyzed, and clarified by ultracentrifugation. bsa, Bovine serum albumin.

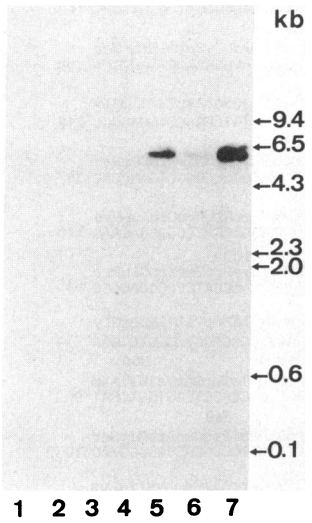

FIG. 3. Progesterone receptor mRNA in different tissues. RNA gel blot analysis (18) was performed with poly(A)$^+$ RNAs (35 μg per lane) prepared from the following organs of estrogen-treated rabbits (10): lane 1, kidney; lane 2, spleen; lane 3, intestine; lane 4, liver; lane 5, vagina; lane 7, uterus. Lane 6, uterine mRNAs from nontreated rabbits. The radioactive probe (10^6 cpm/ml) was prepared by nick-translation (14) of the insert from λrPR1. kb, Kilobases.

absent (or at a concentration below the detection limit) from liver, spleen, kidney, or intestinal tissues. Thus, variations in the concentration of the receptor mRNA closely paralleled the variations in the abundance of the receptor protein itself (11, 19).

Cloning in λgt10 and Sequencing of Receptor cDNA. The λgt10 library contained large cDNA inserts due to the use of the RNase H method to synthesize the double-stranded cDNAs and of size selection by chromatography. Among the 25 clones that cross-hybridized with λrPR1, 4 were used for sequencing experiments (Fig. 4). Their overlapping sequences corresponded to the same mRNA that contained an open reading frame of 930 codons (Fig. 5). The nucleotides surrounding the putative initiation codon closely match the consensus sequence of Kozak (20). The calculated molecular weight of the receptor is 98,554. A search for possible sites of post-translational modification of the protein revealed a putative N-linked glycosylation site (21) (at amino acids 665–667) and the absence of conspicuous consensus sites for cAMP-dependent phosphorylation (22) or ATP binding (23). Several tyrosines (amino acids 328, 376, and 602) have the characteristics that have been described for substrates of phosphorylation (24). Putative sites for casein kinase type phosphorylation (25) are also present (amino acids 42–44, 225–229, 551–553, and 788–790). Amino acid sequences have been described that are involved in nuclear localization of some proteins. The sequence present in simian virus 40 large tumor antigen (26) is not found in the receptor, whereas the sequence observed in MATα_2 protein (27) is homologous to that existing at positions 184–188 and 237–241 of the PR.

The amino acid sequence of PR was compared to those of the proteins present in the National Biomedical Research Foundation bank† and to GenBank.‡ The only significant homologies were with GR, ER, and v-erbA.

Comparison with ER (28–30), GR (31), and v-erbA (32) Sequences. Comparison of these sequences shows a highly homologous central region (83% homology with human GR, 56% homology with human ER, and 44% homology with v-erbA) characterized by its high content of cysteines and basic amino acids (Fig. 6). Many of the cysteines and basic amino acids are in conserved positions in the three receptors and also in v-erbA (Fig. 6*B*). Starting from this region and extending towards the C terminus is a sequence whose length is conserved in the four proteins (varying between 279 and 345 amino acids) that is homologous in PR, GR, and ER (Fig. 6 *A* and *C*). No significant homology with v-erbA was observed. In contrast, the segment extending from the cysteine-rich region toward the N terminus is very different in the four proteins. Its size varies from 567 amino acids (PR) to 36 amino acids (v-erbA). No significant homology between the amino acid sequences could be observed. In PR this region is characterized by an unusually high proportion of proline residues (15.17% of the residues). For comparison it should be noted that in the C-terminal region (amino acids 646–930) this proportion is only 5.21% and thus similar to that observed in other proteins (33). The N-terminal regions (upstream from the cysteine-rich sequence) of ER also contain relatively high amounts of proline (11.76%) that are markedly lower than in PR. On the contrary, the N-terminal regions of the GR and v-erbA are not very proline rich (7.62 and 2.77%, respectively).

DISCUSSION

PR cDNA was inserted into the expression vector λgt11, and the identity of the isolated clones was established in several ways. The bacterial clones and the fusion proteins, which they contained, reacted with both monoclonal and polyclonal antibodies that have been shown to recognize only receptor in the uterine cells by immunochemical and immunocytochemical methods. Moreover, highly purified receptor preparations inhibited antibody binding to the fusion proteins. The latter in turn inhibited monoclonal antibody binding to receptor. Finally, the antibodies bound to the fusion protein were shown to interact with [^{3}H]progestin–receptor complexes formed in uterine cytosol. Among the several different antibodies present in the polyclonal antiserum, two bound to the fusion protein.

The mRNA detected by RNA gel blot using both cloned cDNAs as probes, also displayed the characteristics expect-

†National Biomedical Research Foundation (1986) Protein Sequence Database, Protein Identification Resource (Natl. Biomed. Res. Found., Washington, DC 20007), Release 9.0.

‡National Institutes of Health (1986) Genetic Sequence Databank: GenBank (Research Systems Div., Bolt, Beranek, and Newman, Inc., 10 Moulton Street, Cambridge, MA 02238), Tape Release 42.

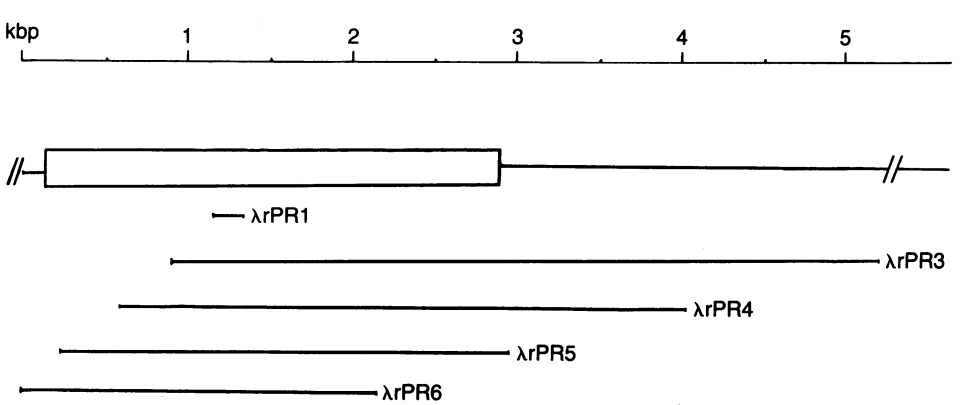

FIG. 4. cDNA clones used for sequencing of the complete progesterone receptor coding region. The open reading frame is boxed. kbp, Kilobase pairs.

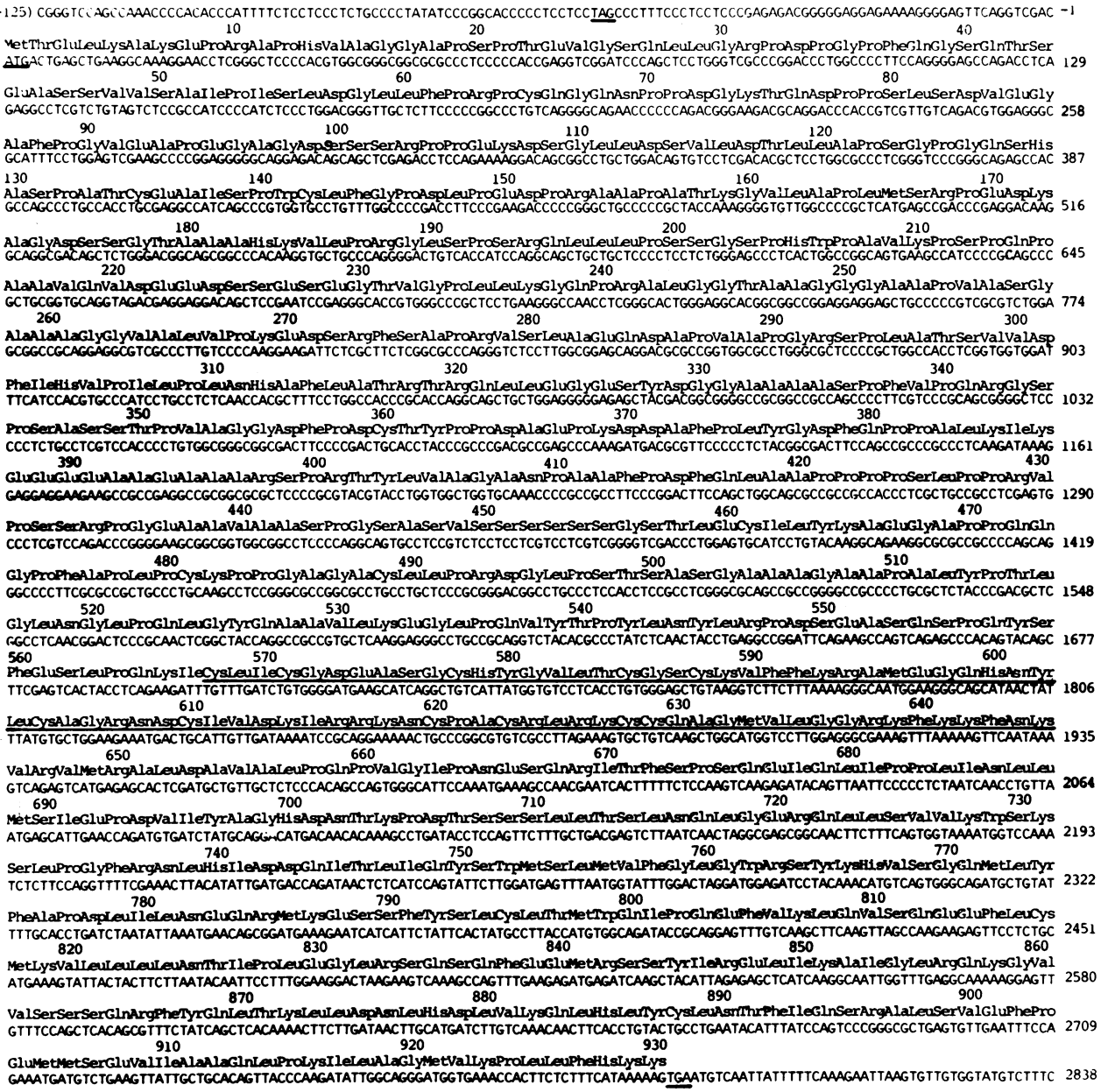

FIG. 5. Nucleotide sequence of the complete coding region of progesterone receptor cDNA and predicted amino acid sequence of the deduced protein. The cysteine-rich basic region is underlined. Stop codons and initiator codon defining the beginning and the end of the open reading frame are also underlined.

ed of the PR mRNA. It was induced by estrogen in the uterus to an extent similar to that observed for the protein itself. Its distribution in target and nontarget organs agreed with that of the protein as determined by biochemical or histoimmunochemical studies (11, 19).

A report has been published on the cloning of a cDNA possibly related to the chicken PR (34), but results indicate that this gene actually encodes a heat shock protein (35).

Comparison of the amino acid sequence of the PR with the GR, ER, and v-erbA shows the presence of a highly conserved basic cysteine-rich central region that may be involved in the DNA binding function of the receptor (30, 36). Identification of this region enabled us to delineate two other segments of the proteins. The C-terminal domain is similar in size in the three receptors and v-erbA and also shows significant sequence homology especially between PR and GR. This observation is not unexpected as there is evidence showing that this segment is involved in steroid binding (30–36) and since there is a high similarity between ligands of GR and PR. On the contrary, the segment of the protein that extends towards the N terminus from the cysteine-rich region is extremely variable in the three receptors and v-erbA. Differences in its length account entirely for the marked differences in the size of the receptors (930 amino acids in the PR, 595 amino acids in the ER). No significant homology is found between the amino acid sequence of the three receptors and v-erbA in this region. The function of this segment of the receptors is not understood, and the significance of its high proline content is also unclear. Steroid receptor organization resembles somewhat that of another DNA binding protein, the *fushi taragu* locus gene product in *Drosophila* (37). This protein contains a basic central region involved in DNA binding and showing homology with corresponding fragments of several other homeotic genes; this region is encompassed by two proline-rich segments. These comparative data will have to be substantiated, however, by direct experimental evidence including *in vitro* mutagenesis, before the function of the various regions of PR is fully understood.

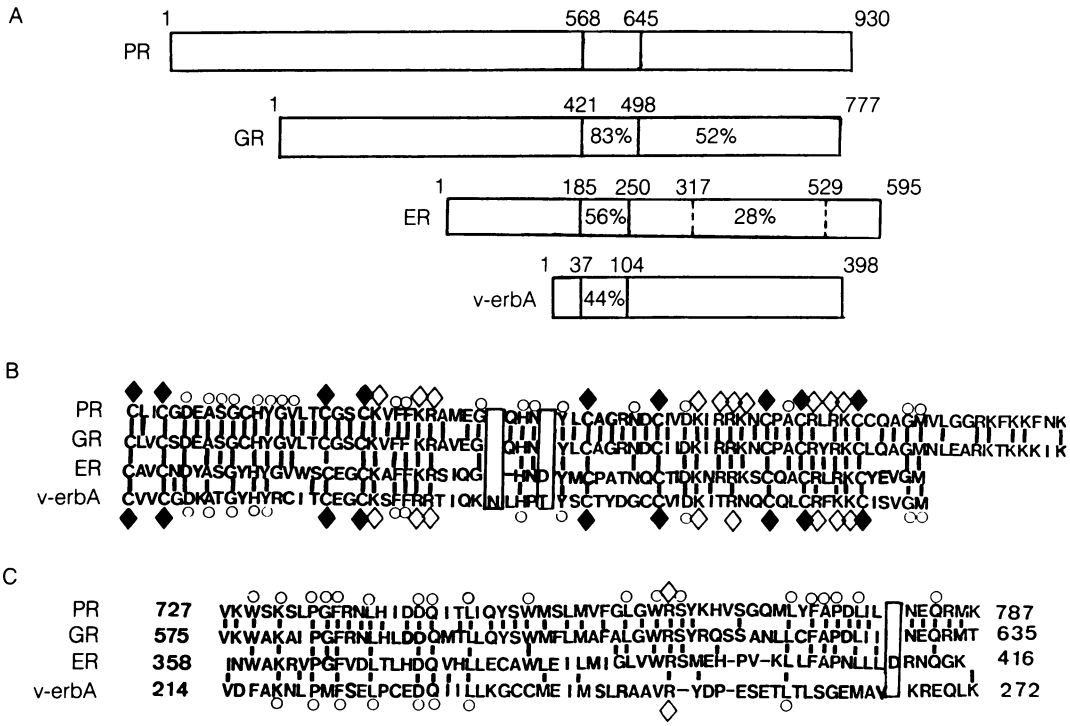

FIG. 6. Comparison between PR, GR, ER, and v-erbA. (*A*) Receptor and v-erbA sequences were aligned via their cysteine-rich basic region. The following significant homologies with equivalent regions in PR are shown: 83% homology between PR amino acids 568–645 and GR amino acids 421–498; 56% homology between PR amino acids 568–633 and ER amino acids 185–250; 44% homology between PR amino acids 568–633 and v-erbA amino acids 37–104; 52% homology between PR amino acids 645–930 and GR amino acids 498–777; 28% homology between PR amino acids 687–923 and ER amino acids 317–529. (*B*) Detailed sequence comparison in the cysteine-rich basic region. (PR amino acids 568–645; GR amino acids 421–498; ER amino acids 185–250; and v-erbA amino acids 37–104.) Symbols displayed above the sequences show homologies among the three receptors. Symbols displayed underneath the sequences show homologies among the four proteins (the three receptors and v-erbA). ◆, Homologous cysteines; ◇, homologous basic amino acids (lysines or arginines); ○, homology in other amino acids. (*C*) Detailed sequence comparison in the C-terminal region. (PR amino acids 727–787; GR amino acids 575–635; ER amino acids 358–416; and v-erbA amino acids 214–272). Symbols are as in *B*.

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