Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 5424–5428, August 1986
Biochemistry

Cloning of the chicken progesterone receptor

(λgt11 expression screening/DNA binding domain/v-*erbA*/progesterone receptor forms A and B)

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Contributed by P. Chambon, April 10, 1986

ABSTRACT **Monospecific antibodies directed against the chicken progesterone receptor (PR) form B were used to screen a randomly primed phage λgt11 cDNA expression library prepared from size-fractionated chicken oviduct mRNA. Two independent immunoreactive clones, λcPR1 and λcPR2, were isolated. Antibodies selected from anti-PR form B antiserum on matrices of λcPR1 and λcPR2 fusion proteins detected two proteins on electrophoretic immunoblots of crude and purified PR preparations. These proteins had the same apparent molecular weights as did PR forms A and B crosslinked with the tritiated progestin R 5020. Thus, λcPR1 and λcPR2 fusion proteins contain epitopes present in both PR forms A and B. A cDNA clone, λcPR3, containing the inserts of both λcPR1 and λcPR2, was isolated from a randomly primed λgt10 oviduct cDNA library, indicating that both cDNA inserts were derived from the same oviduct mRNA. Additional evidence that these cDNAs correspond to PR mRNA was provided by sequencing the λcPR3 cDNA insert, since it was found to encode the sequence of three tryptic peptides prepared from purified PR form B. A fourth and a fifth cDNA clone, λcPR4 and λcPR5, were sequentially isolated from the same λgt10 cDNA library beginning with a probe derived from the 3′ end of the λcPR3 insert. Partial DNA sequencing of λcPR4 and λcPR5 revealed the presence of a sequence coding for a cysteine-rich domain that is strikingly homologous to the amino acid sequences present in the putative DNA-binding domain of the human and chicken estrogen receptors, human glucocorticoid receptor, and v-*erbA* gene product of the avian erythroblastosis virus.**

Steroid hormone receptors are target cell-specific mediators of the control of transcription by their respective hormones during development. It is assumed that the binding of the steroid to the receptor induces an allosteric transition of the structure of the receptor so that it becomes capable of controlling initiation of transcription of specific genes. It is also believed that hormone–receptor complexes act as transcription factors by directly binding to promoter elements of hormone-responsive genes (ref. 1 and references therein). Thus, a detailed knowledge of the structure–function relationship of steroid hormone receptor domains is a prerequisite to understanding hormone action at the molecular level. cDNAs corresponding to chicken (2) and human (3) estrogen and to rat (4) and human (5, 6) glucocorticoid receptors have been cloned recently. A comparison of the cDNA-deduced amino acid sequences of chicken (2) and human (7) estrogen and human glucocorticoid (8) receptors has revealed a high degree of conservation in the putative DNA-binding and hormone-binding domains (2). Moreover, sequences homologous to these two domains are present in the product of the v-*erbA* gene of the avian erythroblastosis virus (2, 7, 9).

The chicken (ref. 10 and references therein) and the human (ref. 11 and references therein) progesterone receptors (PR) exist in two forms, A and B, both of which bind progestins and DNA. In addition, a chicken PR form B antigen that does not bind the hormone has been described (12), and its cDNA has been partially cloned (13). Since there are contradictory reports concerning the relationship between these multiple forms of the PR (refs. 10 and 11 and references therein), there is an obvious need to determine the primary structure of each of them and, therefore, to clone the cDNAs of the hormone-binding forms A and B.

With the help of affinity-labeling techniques, we have isolated (14) the two hormone binding forms A and B of the chicken oviduct PR to apparent homogeneity and have raised antibodies against both of them. Using a chicken oviduct λgt11 cDNA expression library and anti-PR form B antibodies, we now have isolated cDNA clones that contain sequences encoding several peptides that were isolated after tryptic digestion of homogenous PR form B. Moreover, they also encode the putative DNA-binding domain that is highly conserved in the other steroid hormone receptors and in the product of v-*erbA*. However, we did not find any significant homology between amino acid sequences deduced from the present cDNA clones and those deduced from the cDNA corresponding to the nonhormone-binding receptor antigen. The present study paves the way toward the elucidation of the relationship between the different PR forms and structure–function studies of their various domains. In addition, the present clones may be useful for cloning PR genes from various species, including the human species where it is of obvious medical interest (15, 16).

MATERIALS AND METHODS

The purification of chicken oviduct PR forms A and B, their photoaffinity labeling with tritiated R 5020, and the generation of polyclonal anti-PR antibodies have been described (14). Tryptic digests from apparently homogenous PR form B were separated by using HPLC techniques, and peptides were sequenced at the sub-40-pmol level by using a gas-phase sequenator as will be described in detail elsewhere. Randomly primed phage λgt10 and λgt11 cDNA libraries were prepared from size-fractionated (above 4 kilobases) laying-hen oviduct poly(A)$^+$ mRNA as described (2, 3). Monospecific anti-PR form B antibodies were isolated by affinity chromatography of the crude anti-PR form B antiserum on matrices containing covalently bound homogenous PR form B as described for the glucocorticoid receptor (6). For

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Abbreviations: PR, progesterone receptor; bp, base pair(s).

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screening purposes and immunoblot analysis, crude as well as monospecific anti-PR form B antisera were subtracted from antibody populations that crossreacted with *Escherichia coli* proteins by successive filtration through two affinity resins containing covalently bound total *E. coli* proteins and purified *E. coli* β-galactosidase, respectively. The purification of fusion proteins from induced recombinant λgt11 lysogens and the selection of antibodies on fusion protein affinity adsorbents was performed as described (6). Screening of the libraries and immunoblot analyses were done by standard procedures (3, 17, 18). Sequencing of the cDNA clones was performed in phage M13 mp18 and mp19 by the Sanger dideoxy technique.

RESULTS

Cloning of cDNA Fragments Encoding PR Form A and B Epitopes Using the λgt11 Expression Vector. Antiserum directed against the PR form B was made monospecific by affinity chromatography on PR form B covalently coupled to Sepharose 4-B. A λgt11 cDNA expression library, prepared from size-fractionated laying-hen oviduct poly(A)$^+$ RNA (2), was screened (10^6 clones) with these antibodies; two clones, λcPR1 and λcPR2 (Fig. 1), were found to express epitopes recognized by monospecific anti-PR form B. The isopropyl-β-D-thiogalactopyranoside inducible β-galactosidase fusion proteins specific for these two clones are shown in Fig. 2*A* (compare lanes 1 and 2 for λcPR2 and lanes 3 and 4 for λcPR1). The apparent molecular masses were approximately 125 and 130 kDa for the λcPR1- and λcPR2-specific fusion proteins, respectively. Accordingly, inserts of approximately 250 base pairs (bp) (λcPR1) and 390 bp (λcPR2) were found in the two cDNA clones. Immunoblots of crude lysates from isopropyl-β-D-thiogalactopyranoside-induced recombinant phage lysogens (Fig. 2*A*, lanes 5 and 6) confirmed the immunological recognition of the expressed epitopes by the monospecific anti-PR form B antibodies (Fig. 2*A*, lanes 7 and 8 for λcPR1 and λcPR2, respectively). Purified *E. coli* β-galactosidase (114 kDa), included in the marker lane (M), did not give any immunoreaction, nor did preimmune serum (Fig. 2*A*, lanes 9 and 10).

To demonstrate that the epitopes expressed by λcPR1 and λcPR2 correspond to epitopes effectively present on PR forms A and B, we selected the corresponding antibodies from anti-PR form B antiserum by affinity chromatography on resins containing either one of the two covalently bound purified fusion proteins. These antibodies were used as immunoprobes on immunoblots of crude and partially purified PR. Fig. 2*B* shows the fluorograph of partially purified

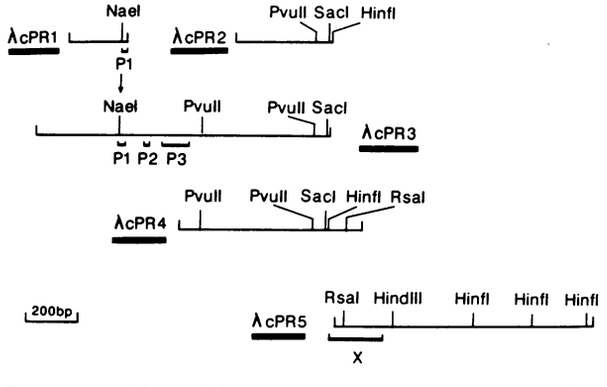

FIG. 1. Partial restriction enzyme maps of the chicken PR cDNA clones λcPR1 to λcPR5. P1, P2, and P3, location of the sequences encoding peptides A, B, and C (see Fig. 3), respectively. X, cDNA region encoding the amino acid sequence highly homologous to the putative DNA-binding domain of the estrogen and glucocorticoid receptors and the v-*erbA* gene product.

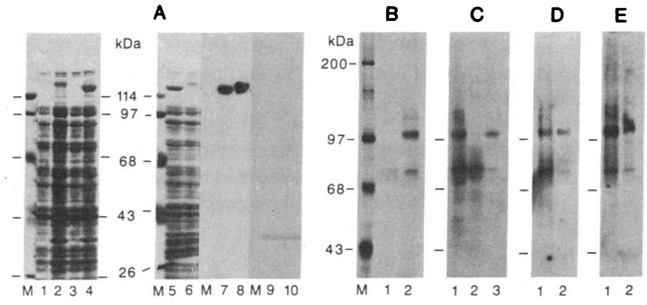

FIG. 2. λcPR1 and λcPR2 cDNA clones contain epitopes present in both PR forms A and B. (*A*) Coomassie blue-stained $NaDodSO_4$/7.5% polyacrylamide gel from a crude $NaDodSO_4$ lysate of non-induced (lanes 1 and 3) and induced (1 mM isopropyl-β-D-thiogalactopyranoside, lanes 2 and 4), recombinant phage lysogens specific for λcPR2 (lanes 1 and 2), and λcPR1 (lanes 3 and 4) in *E. coli* Y1089. Three immunoblots of crude lysates from isopropyl-β-D-thiogalactopyranoside-induced λcPR1 (lanes 5, 7, and 9) and λcPR2 (lanes 6, 8, and 10) were either stained with Coomassie blue (lanes 5 and 6) or probed with monospecific anti-PR form B (lanes 7 and 8) or preimmune serum (lanes 9 and 10). All sera were stripped of *E. coli* and β-galactosidase antibodies by filtration through corresponding affinity resins. Each immunoblot contained purified *E. coli* β-galactosidase in the marker lane (M) to control for crossreaction with β-galactosidase. (*B*) Fluorograph of $NaDodSO_4$/7.5% polyacrylamide gel of photoaffinity-labeled partially purified PR form A (DNA-cellulose II fraction; see ref. 14) (lane 1) and partially purified mixture of PR forms A and B (phosphocellulose II fraction, see ref. 14) (lane 2). (*C*) Immunoblot of a crude PR preparation (DEAE-Sepharose fraction; see ref. 14) (lane 1) and of the same partially purified PR form A and mixture of PR forms A and B as in *B*, probed with crude anti-PR form B antiserum. (*D* and *E*) Immunoblots of the same PR preparations as shown in *B* and *C*, probed with λcPR2 (*D*) and λcPR1 (*E*) fusion protein-selected antibodies derived from crude anti-PR form B antiserum. Lanes: 1, crude mixture of PR forms A and B; 2, partially purified preparation of forms A and B. The immunocomplexes in *A*, *C*, *D*, and *E* were visualized by autoradiography after incubating the blots with ^{125}I-labeled *Staphylococcus aureus* protein A. (*C–E*) $NaDodSO_4$/7.5% polyacrylamide gels. The position of each marker (lanes M) is indicated by bars on the left side of the panels.

preparations of PR form A (79 kDa) (lane 1) and of a mixture of PR forms A and B (109 kDa) (lane 2) crosslinked with tritiated progestin R 5020. Fig. 1*C* demonstrates, in agreement with our previous report (14), the crossreactivity of anti-PR form B antiserum with PR form A. A crude preparation of a mixture of PR forms A and B (Fig. 2*C*, lane 1), a partially purified PR form A (lane 2), and a partially purified mixture of PR forms A and B (lane 3) were separated on 7.5% $NaDodSO_4$/polyacrylamide gels and analyzed by immunoblotting. The major bands that were visualized with a crude anti-PR form B antiserum have clearly the same apparent molecular masses as those revealed by fluorography in Fig. 2*B*. The same preparations of crude and partially purified mixtures of PR forms A and B as those used in Fig. 2*C* were then analyzed with the two populations of fusion protein-selected antibodies. Fig. 2 *D* and *E* corresponds to immunoblots with λcPR2 and λcPR1 fusion protein-selected antibodies, respectively. Again, two bands were revealed with the same apparent molecular masses as the PR forms A and B visualized in Fig. 2*B*. Thus, we conclude that λcPR1 and λcPR2 fusion proteins contain epitopes that are present in both PR form A and form B.

Cloning of a cDNA Fragment Encoding Three Peptides Contained in the PR Form B. To demonstrate that the cDNA inserts present in λcPR1 and λcPR2 were derived from the same mRNA, both inserts were used to screen a randomly primed λgt10 library prepared from fractionated laying-hen oviduct poly(A)$^+$ RNA by described methods (2, 3). A 1.1-kilobase cDNA clone, λcPR3, containing both the inserts

of λcPR1 and λcPR2, was isolated (Fig. 1). Sequencing of the λcPR3 cDNA between the *Nae* I and *Pvu* II sites revealed that three deduced amino acid sequences encoded by the cDNA segments P1, P2, and P3 (Fig. 3) are identical to the sequences of three peptides, A, B, and C, which were isolated from tryptic digests of apparently homogenous PR form B (R.J.S., B. Grego, M. V. Govindan, and H.G., unpublished results). We conclude from these data that the cDNA insert present in λcPR3 encodes a segment of PR form B.

The Cysteine-Rich Putative DNA-Binding Domain of Estrogen and Glucocorticoid Receptors Is Encoded in Progesterone cDNA Clones. Comparison of the sequence of the human and chicken estrogen receptors and of the human glucocorticoid receptor has revealed the presence of a highly conserved cysteine-rich region that is also present in the product of the avian erythroblastosis virus v-*erbA* gene (see Fig. 4 and refs. 2, 7, and 9). Studies from this laboratory have shown that this region of homology is required for tight nuclear binding of the estrogen receptor and, therefore, may correspond to the DNA binding domain of steroid hormone receptors (V. Kumar, S. Green, and P.C., unpublished results).

Using the λcPR3 cDNA insert as a probe, we isolated λcPR4, which was then used to isolate λcPR5 from the same chicken oviduct λgt10 library as above (see Fig. 1). Interestingly, sequencing the 3′ end of λcPR4 cDNA and the 5′ end of λcPR5 cDNA revealed the presence of a cysteine-rich region "X" (Figs. 1 and 3), which is highly homologous to the putative DNA-binding domain of estrogen and glucocorticoid receptors (Fig. 4). The highest homology was found with the human glucocorticoid receptor as indicated by crosses along the top of the PR sequence. There are only six amino acid differences between the putative DNA-binding domains of the chicken PR and the human glucocorticoid receptor. These striking homologies strongly support the conclusion that the cDNA clones that have been isolated here correspond to the PR.

DISCUSSION

Three lines of evidence support the conclusion that the cDNA clones λcPR1 and λcPR2, which have been isolated here from a chicken oviduct λgt11 library, correspond to the chicken PR. First, antibodies raised previously (14) against the purified chicken PR form B and selected on matrices of the fusion proteins prepared from either λcPR1 or λcPR2 reveal selectively two proteins with molecular masses of PR forms A and B on immunoblots of crude and partially purified PR preparations. Second, a cDNA clone that was isolated from a chicken oviduct λgt10 library and contains sequences of both λcPR1 and λcPR2 encodes the amino acid sequences of three peptides obtained from an apparently homogenous preparation of PR form B. Third, two additional λgt10 cDNA clones, λcPR4 and λcPR5, contain sequences encoding the highly conserved putative DNA-binding domain that is present in the human and chicken estrogen receptors and in the human glucocorticoid receptor. Moreover, further sequencing of the λcPR5 cDNA insert (data not shown) indicated that it contains sequences homologous to the putative steroid hormone-binding domain E of the estrogen and glucocorticoid receptors (2).

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P1      ...AAGGCTGTGGACGCCGGCCCGGGGGCTCCCGGTCCCTCGCAGCCGCGACCCGGGGCTCCG...
        ...LysAlaValAspAlaGlyProGlyAlaProGlyProSerGlnProArgProGlyAlaPro...

Peptide A     AlaValAspAlaGlyProGlyAlaProGlyProSerGlnProArgProGlyAla

P2                  ...GTGAAAGCGCGTCCGGGACCCGAGGACGCCAGC...
                    ...ValLysAlaArgProGlyProGluAspAlaSer...

Peptide B                   AlaArgProGlyProGluAsp

P3      ...CCGGAGCGGGACGCGGGGCCCGGAGAAGGCGGACTGGCGCCGGCCGCGGCTGCTTCCCCG
        ...ProGluArgAspAlaGlyProGlyGluGlyGlyLeuAlaProAlaAlaAlaAlaAlaSerPro

Peptide C           AspAlaGlyProGlyGluGlyGlyLeuAlaProAlaAlaAlaAlaAlaSerPro

P3          GCGGCCGTGGAGCCGGGCGCGGGGCAGGACTACCTGCACGTGCCCATCCTGCCGCTCAAC...
            AlaAlaValGluProGlyAlaGlyGlnAspTyrLeuHisValProIleLeuProLeuAsn...

Peptide C   AlaAlaValGluProGlyAlaGlyGlnAspTyrLeuHisValProIleLeu

X       ...CCCCAGAAGATTTGTCTCATCTGTGGTGATGAGGCTTCTGGTTGCCACTACGGAGTACTC
        ...ProGlnLysIleCysLeuIleCysGlyAspGluAlaSerGlyCysHisTyrGlyValLeu

X           ACCTGTGGAAGCTGTAAAGTCTTCTTTAAAAGGGCAATGGAAGGGCAGCACAACTATTTA
            ThrCysGlySerCysLysValPhePheLysArgAlaMetGluGlyGlnHisAsnTyrLeu

X           TGTGCTGGAAGAAATGACTGCATAGTTGATAAAATTCGTAGGAAGAACTGTCCAGCGTGT
            CysAlaGlyArgAsnAspCysIleValAspLysIleArgArgLysAsnCysProAlaCys

X           CGCTTGAGGAAGTGCTGTCAAGCTGGAATGGTCCTGGGAGGT...
            ArgLeuArgLysCysCysGlnAlaGlyMetValLeuGlyGly...
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FIG. 3. Selected sequences of λcPR3, λcPR4, and λcPR5 cDNA inserts and the deduced amino acid sequences of regions P1, P2, P3, and X. The sequences of peptides A, B, and C obtained from tryptic digests of homogenous PR form B are aligned below the corresponding sequences of P1, P2, and P3 (see text).

The present immunological data support fully our previous conclusions (14, 21) that the chicken PR forms A and B are structurally related and share common epitopes. The cDNA insert of λcPR3, which contains epitopes shared by both PR forms and encodes amino acid sequences present in PR form B, may correspond to a unique chicken gene that encodes both PR forms. Alternatively, the two PR forms may be encoded by two closely related genes that have evolved by gene duplication. At the present time, we favor the first possibility because we have isolated a chicken genomic DNA cosmid clone that contains the sequence present in the λcPR3 cDNA insert, and we did not find any evidence supporting the possible presence of additional genomic sequences closely related to the DNA inserted in this cosmid clone (unpublished results). More studies are needed to show whether alternative splicing or specific proteolytic cleavage are responsible for the existence of the two PR forms. None of the DNA sequences that are present in the various λcPR clones isolated here share any significant homology with the DNA sequences of the cDNA corresponding to the so-called "chicken progesterone receptor B antigen" (13), whose cDNA-deduced amino acid sequence is itself closely related to that of a yeast heat shock protein previously described (22) (J.M.J., F.J., and P.C., unpublished results).

The finding that the PR cDNA clones λcPR4 and λcPR5 encode an amino acid sequence that is highly homologous to the putative DNA-binding domain found in both the estrogen and glucocorticoid receptors and in the product of the avian erythroblastosis virus v-*erbA* gene (Fig. 4) supports our previous suggestion that all steroid hormone receptors belong to a multigene family of transcriptional regulatory factors and that c-*erbA* gene product should be a receptor for a ligand closely related to steroid hormones (2). The present study excludes the possibility that the c-*erbA* gene product could be the PR. Therefore, it may be the receptor for another steroid—e.g., vitamin D_3. The striking homology (60 amino acids of 66) between the putative DNA-binding domains of the chicken PR and human glucocorticoid receptor is in agreement with a recent report (23) showing that the chicken lysozyme gene and mouse mammary tumor virus promoter elements that are recognized *in vitro* by the rat glucocorticoid receptor and rabbit PR are closely related. However, since not all of the genes that are induced by progestins are induced by glucocorticoids and vice versa, this striking homology suggests that additional domains of the receptors and/or additional factors may be involved in the specific recognition of hormone-responsive DNA elements by these receptors *in vivo*.

The resemblance between the cysteine-rich putative DNA-binding domain of steroid hormone receptors and the presumptive DNA-binding domains of the TFIIIA transcription factor of the *Xenopus 5S* gene (19, 24), the protein products of the *Drosophila* developmental genes Krüppel (25) and

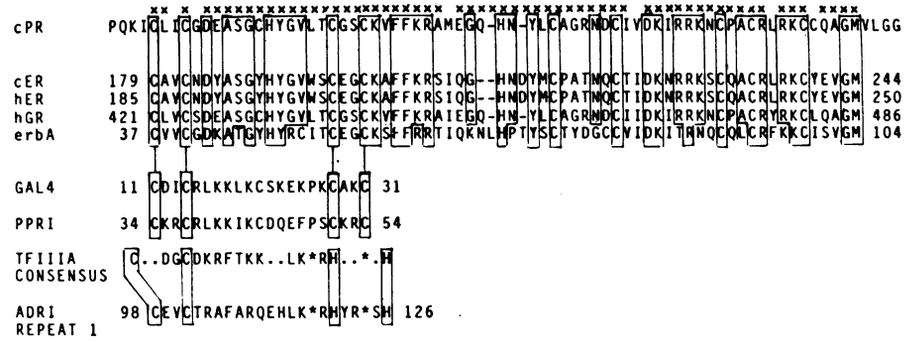

FIG. 4. Alignment of the putative DNA-binding domains of the chicken (cER) and human (hER) estrogen receptors, the human glucocorticoid receptor (hGR) and the v-*erbA* gene product (see ref. 2) and comparison with the corresponding sequence of the chicken PR (cPR). Boxed areas indicate complete homology of all steroid hormone receptors, and dashes indicate amino acid gaps for optimal alignment. Crosses above the cPR sequence indicate residues conserved in cPR and hGR. The Cys-$(Xaa)_2$-Cys-$(Xaa)_{13}$-Cys-$(Xaa)_2$-Cys motif present in the putative DNA binding domain of the GAL4 and PPR1 yeast regulatory protein is aligned with the corresponding motif of the steroid hormone receptors and v-*erbA* gene products. The consensus motif of the *Xenopus* transcription factor TFIIIA and the corresponding repeat 1 of the yeast regulatory protein ADR1 are also shown (see text). The pairs of cysteine and histidine residues dictating the postulated "finger" structures are boxed. Stars indicate positions where amino acid insertions may occur, and dots in the consensus sequence indicate variable residues (see refs. 19 and 20). Numbers indicate the position of amino acid residues in each sequence. Arrows below the C-terminal half of the putative DNA binding domains of the receptors indicate the cysteine and histidine residues that may possibly be involved in the generation of additional loop structures (see text).

Serendipity (26), and the yeast regulatory protein ADR1 has already been stressed (refs. 2, 9, 20 and 27; see Fig. 4). It has been proposed (19) that the repeating unit of TFIIIA folds into DNA-binding loops (fingers), each centered on invariant pairs of cysteine and histidine residues (boxed in Fig. 4) bound to Zn^{2+} in a tetrahedral arrangement. Although the pairs of histidine residues are not present in the putative DNA binding domains of steroid hormone receptors and v-*erbA* gene product, it is striking that the N-terminal half of these domains contains two pairs of cysteine residues spaced by 13 amino acids, similar to the TFIIIA and ADR1 arrangements (Fig. 4). Thus, it is possible that a finger of similar size could be formed with four cysteine residues binding a Zn^{2+} instead of two cysteine and histidine pairs, as it was proposed recently (20) for two yeast regulatory proteins GAL4 (28) and PPR1 (29, 30), which contain such cysteine pairs in their presumptive DNA-binding domains (see Fig. 4). Although the fingers that can be formed will have different lengths, similar loop structures involving cysteine and histidine residues also may be formed in the C-terminal half of the putative DNA-binding domain of steroid hormone receptors (Fig. 4). Thus, it is possible that all of these cysteine- and histidine-rich domains have evolved by duplication of a primordial unit that was present in primitive eukaryotic cells. However, the structure of the receptor cysteine-rich domain clearly does not represent a repeating unit, which suggests that its two halves may have diverged very early in evolution. In this respect, it is worth mentioning that, in fact, they are encoded in the chicken PR by two neighbor exons separated by an intron (unpublished results), which is reminiscent of the organization of the TFIIIA gene, where most of the exonic sequences coding for the fingers are separated by introns (31). Further studies on steroid hormone receptors and related genes will show whether this organization is common to all of them.

J.M.J. and Z.K. should be considered as equal first authors. We thank A. Staub for synthesis of oligonucleotides and T. Lerouge for some of the DNA sequencing, Dr. B. Grego for peptide separation, Dr. A. Ulman for a gift of β-galactosidase, C. Werlé and B. Boulay for the preparation of figures, the secretarial staff for typing the manuscript, and Drs. S. Green and M. V. Govindan for useful discussions. Z.K. was a recipient of a fellowship from the National Health and Medical Research Council of Australia. This research was supported by the Institut National de la Santé et de la Recherche Médicale, the Centre National de la Recherche Scientifique, the Ministère de la Recherche et de la Technologie (84-V-0803), the Fondation pour la Recherche Médicale, and the Association pour le Developpement de la Recherche sur le Cancer.

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