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HUMAN PROGESTERONE A-RECEPTORS CAN BE SYNTHESIZED INTRACELLULARLY AND ARE BIOLOGICALLY FUNCTIONAL

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In order to investigate the origin and functional independence of the human progesterone receptor A binding protein, we have expressed a truncated human progesterone receptor cDNA in both gene transfer and *in vitro* translation assays. Proteins identical in size and antigenicity to the A-receptors found naturally in human progesterone target cells are synthesized from this cDNA that lacks the putative B receptor initiator methionine codon of the complete cDNA. The functional independence of A-receptors is suggested by their ability to bind hormone and to stimulate transcription from the progestin responsive mouse mammary tumor virus promoter.

Progesterone receptors (PR) are unique among steroid receptors because, in some species, there are two separate hormone-binding proteins which appear to be encoded by a single gene (1). In $T47D_{CO}$ human breast cancer cells, we have described two progestin binding forms: A-receptors of 94 kDa and B-receptors of 120 kDa (2). Chicken PR also have two hormone binding forms, the smaller A-protein and the larger B-protein (3,4), while rabbit receptors are composed of only the larger B-protein (5). The origin of the A form has been the subject of controversy. That A- and B-proteins are structurally similar has been demonstrated by peptide mapping of photolabeled receptors (6,7) and by immunoreactivity (8). This has led to suggestions that the larger B-protein is the authentic receptor and that A is a proteolytic artifact. However, investigators have been unable to generate A-receptors from B-receptors *in vitro* (9). In $T47D_{CO}$ cells, A- and B-proteins are equimolar in both the hormone-free untransformed state and the hormone-occupied transformed state (10). A and B form separate and independent 8S complexes on sucrose density gradients (11). Both proteins bind hormone and are able to interact with DNA (reviewed in 2). These and other data suggest that A is not a proteolytic artifact, but is a functional and independent natural receptor. This view has recently been bolstered by the studies of Conneely et al (12) and Gronemeyer et al (13), who have shown that A- and B-proteins can arise by alternate translation initiation of a single mRNA transcript. In this paper we demonstrate that human A-proteins function as independent receptors by expressing a human cDNA that codes for the A-protein to engineer a progestin-responsive cell.

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MATERIALS AND METHODS

Materials: hPR54 is a hPR cDNA whose 5' end is at position +259 bp relative to the putative first initiator methionine codon when compared to the published sequence (14). This cDNA was originally cloned into the pcD expression vector by the method of Okayama and Berg (15) and as a result of this cloning, it contains 45 bp of G-C residues at the 5' terminus. hPR50 lacking both putative initiation codons was used as a control and is described in detail elsewhere (16). pGEM 3, pGEM 4 and pGEM 7 Zf(+) were purchased from Promega (Madison, WI). AB-52 is a monoclonal antibody specific for the hPR whose production is described elsewhere (17). pHHCAT, is an expression vector which contains the mouse mammary tumor virus (MMTV) promoter (bp -223 to +109) linked to the chloramphenicol acetyltransferase (CAT) gene.

Cell culture: Thymidine kinase negative (tk-) mouse L cells were cultured in MEM (Gibco, Grand Island, NY) supplemented with 5% fetal bovine serum, 10 mM Hepes, 1% non-essential amino acids, 2 mM glutamine and 25 μg/ml gentamycin. Mouse L cells transfected with thymidine kinase were grown in the same medium with the additional supplement of 0.1mM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine (HAT medium). $T47D_{co}$ cells were cultured as previously described (16).

In vitro translation: hPR54 and hPR50 were subcloned into pGEM 3 and pGEM 4 respectively. *In vitro* transcriptions were carried out using the SP6 riboprobe system (Promega) according to the manufacturer's instructions to produce the sense strand RNA. The resultant RNA was translated in a rabbit reticulocyte lysate system (Bethesda Research Laboratories, Gaithersburg, MD) according to the manufacturer's instructions in the presence or absence of [^{35}S]methionine (~1100 Ci/mmole, New England Nuclear, Boston, MA). Immediately following translation, 80 nM unlabeled R5020 and protease inhibitors (0.1 mM leupeptin, 77 μg/ml aprotinin, 1 μg/ml pepstatin A, and 100 μg/ml bacitracin) were added to stabilize protein products. Synthesized receptors were immunoprecipitated with 20 μg/ml of the antibody AB-52. T47D cytosol containing 25 pmoles of unlabeled hPR protein was used as a source of carrier protein only when *in vitro* synthesized receptors were labeled with [^{35}S]methionine. Immunoprecipitation was as described previously (16). The immunoprecipitate was then mixed with 150 μl of sample buffer (7% SDS, 15% β-mercaptoethanol and .02% bromophenol blue in 50% glycerol), boiled 5 min, centrifuged (3000 x g, 10 min), and the supernatants were subjected to SDS-polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose and exposed to X-ray film for autoradiography (receptors labeled with [^{35}S]-methionine) or immunoblotted (unlabeled receptors).

Immunoblotting: Following electrophoresis, proteins were transferred to nitrocellulose filters (Schleicher and Schuell, 0.45 μm) using a Polyblot transfer apparatus (American Bionetics, Inc., Emeryville, CA) operating at 2.5 mA/cm^2 for 30 min. After transfer, the nitrocellulose was treated as described previously (18). For the [^{35}S]methionine labeled receptors, the filter was incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Cappel, Malvern, PA). Unbound antibody was removed by 1 wash with wash buffer (10 mM Hepes, pH7.4, 1 mM EDTA, 30 mM NaCl, 0.5% Triton X-100, 0.25% gelatin), followed by 3 washes with substrate buffer (50 mM Hepes, pH 7.4, 150 mM NaCl) and immunoreactive bands were visualized as described previously (18). The nitrocellulose was then sprayed with Enhance (New England Nuclear) and exposed to Kodak X-AR5 film using 2 Dupont Cronex Lightning-Plus intensifying screens. For unlabeled receptors, the filter was incubated with [^{125}I]labeled anti-mouse $F(ab')_2$ (Amersham, Arlington Hts., IL) at a concentration of 0.33 μCi/ml in dilution buffer (wash buffer containing 1% BSA) for 6 hrs at 4°C. The nitrocellulose was then washed extensively with wash buffer, air dried and exposed to X-ray film as described above.

Hormone Binding: The hydroxylapatite method (19) was used with the following modifications. A final concentration of 20 nM [^{3}H]R5020 was used to label the receptors and a concentration of 2 μM R5020 was used to determine non-specific binding. The absorbed proteins were eluted with 1.0 ml of ethanol and radioactivity assayed by liquid scintillation counting using 3a70 scintillation cocktail (Research Product International, Mount Prospect, IL).

Transfections: Stable: tk- mouse L cells were cotransfected with Herpes Simplex virus thymidine kinase gene in pBR322 and pHHCAT (1:20 ratio) using the calcium phosphate co-precipitation method of Wigler et al (20). Transfectants were selected with HAT medium. Individual HAT resistant colonies were subcloned and the presence of regulated reporter gene

was confirmed by glucocorticoid induction of CAT activity. **Transient**: Mouse L cells with stably integrated MMTV-CAT reporter genes were plated at 1 x 10^6 cells/35 mm dish. After 24 hrs the growth medium was replaced with a transfection solution of HAT medium containing 200 μg/ml DEAE-dextran, 5 μg/ml plasmid DNA and 0.1 mM chloroquine. The cells were incubated for 2 hrs at 37°C, then washed with phosphate buffered saline. The cells were shocked for exactly 1 min with HTB (21 mM Hepes, pH 7.1, with 137 mM NaCl, 5 mM Na_2HPO_4 and 6 mM dextrose) containing 15% glycerol. Cells were washed with phosphate buffered saline, HAT medium was added, and incubated at 37°C for 48 hrs. Cells were then hormone-treated for an additional 24 hrs.

CAT Assays: Tritiated acetate was used as the substrate in a soluble enzymatic CAT assay as described in detail by Nordeen et al (21). Protein in the cell lysates was measured by the method of Bradford (22).

RESULTS AND DISCUSSION

Recent cloning of steroid receptor cDNAs has helped to elucidate their protein structure (23). As shown in Figure 1, the structure of steroid receptors consists of a carboxy terminal hormone binding region, a cysteine-rich DNA binding domain and a more variable amino terminus. The sequence of hPR mRNA reveals two in-frame AUGs codons 492 bp apart that could potentially code for the B- and A-proteins through alternative initiation of translation at these sites (24). Both Conneely et al (12) and Gronemeyer et al (13) have shown this to be possible for chicken PR. We have used a truncated human cDNA, hPR54, that contains the downstream ATG (ATG_A), but does not contain the upstream ATG (Fig. 1). This cDNA should encode the A-protein but not the B-protein.

To determine if hPR54 is capable of expressing the A-receptor, we transcribed and translated this cDNA *in vitro* as described in the methods. The products of [^{35}S]methionine incorporation were either fractionated directly on an SDS- polyacrylamide gel, or immunoprecipitated with AB-52 and then fractionated on a polyacrylamide gel. A shorter

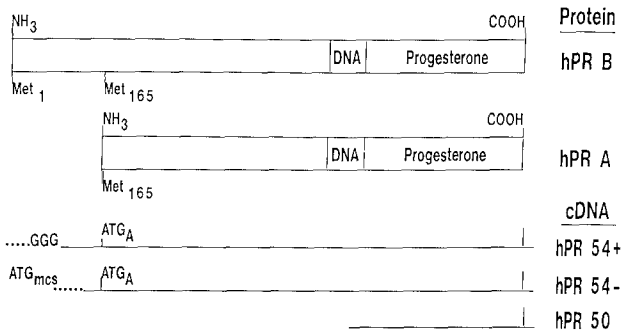

FIGURE 1: **Schematic representation of hPR protein structure and of hPR cDNAs.** Alternate translation initiation from two in-frame methionine codons could lead to synthesis of two homologous PRs: full length B-receptors and A-receptors truncated ~165 amino acids from the amino terminus. hPR54+ is a cDNA which contains enough information to code for A- but not B-receptors. The G-C residues were removed from the 5' end of hPR54+ to produce hPR54- and the resultant cDNA was subcloned into pGEM7. ATGmcs refers to the in frame ATG present in the multiple cloning site (mcs) of pGEM7. hPR50 is a cDNA that does not contain a complete DNA binding domain and is used as a control.

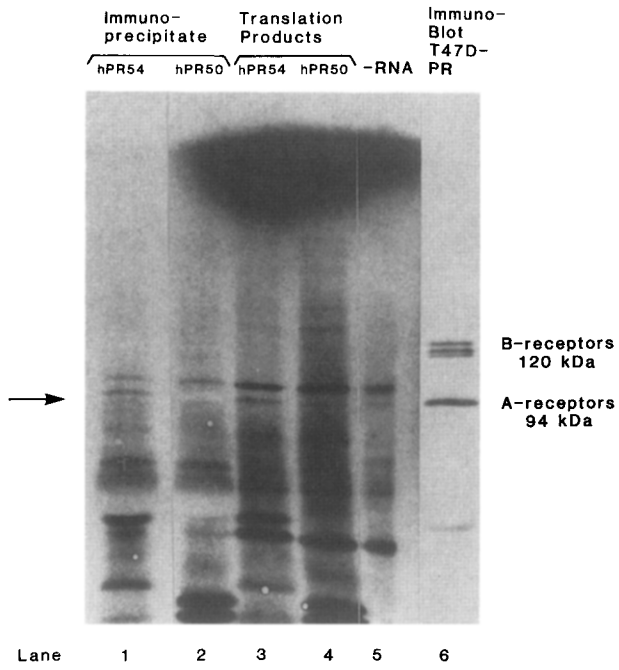

FIGURE 2: **Analysis of** in vitro **translation products from hPR cDNA constructs.** hPR54 and hPR50 cDNAs were transcribed and translated *in vitro* in the presence of [^{35}S]methionine. The products were immunoprecipitated with AB-52 and separated by electrophoresis in lanes 1 and 2. The translation products were directly separated by electrophoresis in lanes 3 and 4. Lane 5 is a minus RNA control of the translation system. Lane 6 is immunoprecipitated PR from T47D cell cytosol.

cDNA hPR50 (see Fig. 1), lacking both ATG_A and the first DNA binding finger (16), served as a control. The results are shown in Figure 2.

Several protein bands (seen in the minus RNA sample, Lane 5) are lysate derived and nonspecific. However, there are three major translation products of 94 kDa, 42 kDa and 34 kDa that are unique to RNA synthesized from hPR54 (Lanes 1 and 3) and are not present in hPR50 (Lanes 2 and 4). The 42 kDa and 34 kDa proteins do not contain a complete DNA binding domain as mapped by the positions of their presumed AUG start codons at amino acids 595 and 632; the DNA binding domain spans amino acids 567 to 644 (14). Further downstream are 2 additional methionine codons that are found in both constructs, but are preferentially used only in the shorter cDNA. They are not expressed in hPR54 because upstream AUG codons are preferentially utilized. The smaller *in vitro* products are not detectable *in vivo* (11).

The arrow (Fig. 2) points to the A-receptors, encoded by hPR54, but not by hPR50. Their position is marked by an immunoblot of $T47D_{CO}$ cytosol from a parallel lane (Lane 6). Thus hPR54 codes for a protein identical in size to 94 kDa A-receptors. Hormone binding activity was weak in this lysate due to inefficient translation.

To improve the translation efficiency of hPR54, we removed a 45 base pair stretch of G-C residues at the 5' end of the cDNA which results from the G-C tailing used in the cloning procedure. The G-C minus hPR54 was then inserted into the multiple cloning site of pGEM 7.

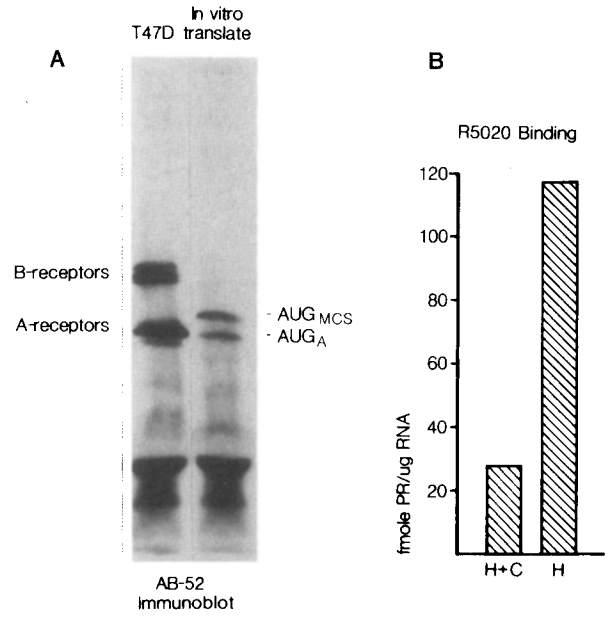

FIGURE 3: **Immunoreactivity and hormone binding of** in vitro **synthesized hPR.** G-C minus hPR54 subcloned into pGEM 7 was transcribed and translated *in vitro* using unlabeled methionine. The translated proteins were immunoprecipitated and then immunoblotted (Panel A) with T47D cytosol run separately as a molecular weight marker of A-receptors. The same translated material was used in a hormone binding assay (Panel B). [^{3}H]R5020 binding was measured in the absence (H) or presence (H + C) of excess unlabeled R5020. Data represent the average of triplicate determinations.

In addition to the ATG_A of hPR54, this construct contains an in-frame ATG in the multiple cloning site (mcs) at the SphI restriction site (GCATGC). Sense strand RNA from this new construct was synthesized using SP6 RNA polymerase and found to be both transcriptionally and translationally efficient. The G-C minus RNA actively directs synthesis of 94 kDa A-receptors as seen in the direct immunoblot of the lysate proteins immunoprecipitated with the antibody AB-52 (Fig. 3, panel A). A second protein ~6,000 daltons larger than A-receptors initiates in the mcs and could conceivably function as a progesterone receptor. The *in vitro* translation lysate had considerable R5020 binding activity which was competable by excess unlabeled hormone (Fig. 3, panel B). Thus, hPR54 codes for a protein of the appropriate size, which has the hormone binding activity and immunoreactivity of A-receptors. This protein can only be demonstrated in *in vitro* translations with an antibody that reacts with both A- and B-receptors (17); it was not detected by Misrahi et al (25) using a B specific antibody.

To test whether these A-receptors are functional, we used hPR54 cloned into the pcD expression vector for transfection studies. The only intact DNA binding protein this construct can encode is the A-receptor (Fig. 1). We first constructed a progestin responsive system by stably transfecting PR negative mouse L cells, which contain endogenous glucocorticoid receptors (GR) with pHHCAT, a construct containing 332 bp of the long terminal repeat (LTR) of the mouse mammary tumor virus linked to the CAT reporter gene. This LTR has response elements for both GR and PR (26). A subclone of these permanently modified cells was then

TABLE I
Progestin and glucocorticoid regulation of MMTV-CAT in mouse L cells transiently expressing PR

	hPR50	hPR54
Control	100 ± 30	100 ± 10
Dexamethasone	6366 ± 1623	4724 ± 1592
R5020	79 ± 11	474 ± 101
RU 486	320 ± 46	218 ± 37
Dexamethasone + RU 486	315 ± 95	294 ± 33
R5020 + RU 486	282 ± 56	197 ± 12

GR positive and PR negative mouse L cells with stably integrated MMTV-CAT reporter genes were used to transiently express hPR. The cells were transiently transfected with either a human PR cDNA containing ATG_A (hPR54) or a control cDNA (hPR50) as described in the Methods. Transfected cells were treated with hormone for 24 hrs at the following concentrations: 0.1 μM Dexamethasone; 0.1 μM R5020; and 1 μM RU 486. The numbers represent the average of duplicate determinations from two experiments, showing radioactive [^{3}H]acetyl chloramphenicol formed/μg lysate protein ± the S.E.M. (21).

transiently transfected with control hPR50 or with hPR54. CAT activities corrected for background expression, from 2 representative experiments, were averaged and are shown in Table 1.

In cells expressing proteins synthesized from hPR50, the glucocorticoid dexamethasone, acting through the endogenous GR, markedly stimulated CAT levels. The progestin R5020, had no effect. This confirms the lack of PR in these cells and the inability of R5020 to have any glucocorticoid-like action in this system. In contrast, the antiglucocorticoid/antiprogestin RU 486 stimulated CAT 3- to 4-fold above controls. This confirms that RU 486 is a weak glucocorticoid agonist. Therefore, it was able to suppress dexamethasone only to the partially stimulated level, and not to the basal state. Its agonist-like properties are unaffected by R5020.

Cells transfected with hPR54 remain glucocorticoid sensitive, but now acquire progestin sensitivity, responding to R5020 with a 4- to 5-fold induction of CAT. This stimulation was seen in 7 of 7 experiments. The induction of CAT by R5020 does not reach the same level as the induction by dexamethasone, since transient transfection introduces PR into only a fraction of the cells. RU 486 suppresses both the R5020 and the dexamethasone inductions to its weak agonist levels.

We conclude that the second methionine codon, present 492 bp downstream from the initiator methionine in human PR cDNAs, is competent to serve as a translation start site. Its use would lead to synthesis of a protein identical to the A-receptors found naturally in human progesterone target cells. This provides a mechanism for the natural intracellular synthesis of A-receptors; a mechanism that does not invoke proteolysis. Since A-receptors bind hormone and interact with the progesterone response elements of MMTV, they appear to be functionally independent of the B-receptors. We have previously shown that A- and B-receptors are structurally independent (11). We can now ask whether the genes targeted by A-receptors

differ from those regulated by B-receptors, and whether differential expression of these two proteins may correlate with the hormone responsiveness of breast cancers.

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REFERENCES

1. Huckaby, C.S., Conneely, O.M., Beattie, W.G., Dobson, A.D.W., Tsai, M-J., and O'Malley, B.W. (1987) Proc. Natl. Acad. Sci. USA 84, 8380-8384.

2. Horwitz, K.B., Wei, L.L., Sedlacek, S.M., and D'Arville, C.N. (1985) Recent Prog. Horm. Res. 41, 249-316.

3. Schrader, W.T., and O'Malley, B.W. (1972) J. Biol. Chem. 247, 51-59.

4. Schrader, W.T., Toft, D.O., and O'Malley, B.W. (1972) J. Biol. Chem. 247, 2401-2407.

5. Logeat, F., Pamphile, R., Loosfelt, H., Jolivet, A., Fournier, A., and Milgrom, E. (1985) Biochemistry 24, 1030-1035.

6. Gronemeyer, H., Harry, P., and Chambon, P. (1983) FEBS Lett 156, 287-292.

7. Birnbaumer, M., Schrader, W.T., and O'Malley, B.W. (1983) J. Biol. Chem. 258, 1637-1644.

8. Gronemeyer, H., Govindan, M.V., and Chambon, P. (1985) J. Biol. Chem. 260, 6916-6925.

9. Horwitz, K.B., Francis, M.D., and Wei, L.L. (1985) DNA 4, 451-460.

10. Horwitz, K.B. and Alexander, P.S. (1983) Endocrinology 113, 2195-2201.

11. Wei, L.L., Sheridan, P.L., Krett, N.L., Francis, M.D., Toft, D.O., Edwards, D.P., and Horwitz, K.B. (1987) Biochemistry 26, 6262-6272.

12. Conneely, O.M., Maxwell, B.L., Toft, D.O., Schrader, W.T., and O'Malley, B.W. (1987) Biochem. Biophys. Res. Commun. 149, 493-501.

13. Gronemeyer, H., Turcotle B., Quirin-Stricker, C., Bocquel, M.T., Meyer, M.E., Krozowski, Z., Jeltsch, J.M., Lerough, T., Garnier, J.M., and Chambon, P. (1987) EMBO 6, 3985-3994.

14. Misrahi, M., Atger, M., d'Auriol, L., Loosfelt, H., Meriel, C., Fridlansky, F., Guiochon-Mantel, A., Galibert, F., and Milgrom, F. (1987) Biochem. Biophys. Res. Commun. 143, 740-748.

15. Okayama, H., and Berg, P. (1982) Mol. Cell. Biol. 2, 161-170.

16. Wei, L.L., Krett, N.L., Francis, M.D., Gordon, D.F., Wood, W.M., O'Malley, B.W., and Horwitz, K.B. (1988) Mol. Endo. 2, 62-72.

17. Estes, P., Suba, E., Lawler-Heavner, J., El-Ashry, D., Wei, L.L., Toft, D.O., Sullivan, W., Horwitz, K.B., and Edwards, D.P. (1987) Biochemistry 26, 6250-6262.

18. Sheridan, P.L., Krett, N.L., Gordon, J.A., and Horwitz, K.B. (1988) Mol. Endo. 2, in press.

19. Garola, R.E., and McGuire, W.L. (1978) Cancer Res. 38, 2216-2220.

20. Wigler, M., Pellicer, A., Silverstein, S., Axel, R., Urlaub, G., and Chasin, L. (1979) Proc. Natl. Acad. Sci. USA 76, 1373-1379.

21. Nordeen, S.K., Green, P.P., and Fowlkes, D.M. (1987) DNA 6, 173-178.

22. Bradford, M. (1976) Anal. Biochem. 72, 248-254.

23. Evans, R.M. (1988) Science 240, 889-895.

24. Kozak, M. (1986) Cell 47, 481-483.

25. Misrahi, M., Loosfelt, H., Atger, M., Meriel, C., Zerah, V., Dessen, P., and Milgrom, E. (1988) Nucleic Acids Res. 16, 5459-5472.

6. Cato, A.C.B., Miksicek, R., Schutz, G., Arnemann, J., and Beato, M. (1986) EMBO 5, 2237-2240.