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Cloning of a novel estrogen receptor expressed in rat prostate and ovary

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ABSTRACT **We have cloned a novel member of the nuclear receptor superfamily. The cDNA of clone 29 was isolated from a rat prostate cDNA library and it encodes a protein of 485 amino acid residues with a calculated molecular weight of 54.2 kDa. Clone 29 protein is unique in that it is highly homologous to the rat estrogen receptor (ER) protein, particularly in the DNA-binding domain (95%) and in the C-terminal ligand-binding domain (55%). Expression of clone 29 in rat tissues was investigated by *in situ* hybridization and prominent expression was found in prostate and ovary. In the prostate clone 29 is expressed in the epithelial cells of the secretory alveoli, whereas in the ovary the granulosa cells in primary, secondary, and mature follicles showed expression of clone 29. Saturation ligand-binding analysis of *in vitro* synthesized clone 29 protein revealed a single binding component for 17β-estradiol (E2) with high affinity (K_d= 0.6 nM). In ligand-competition experiments the binding affinity decreased in the order E2 > diethylstilbestrol > estriol > estrone > 5α-androstane-3β,17β-diol ≫ testosterone = progesterone = corticosterone = 5α-androstane-3α,17β-diol. In cotransfection experiments of Chinese hamster ovary cells with a clone 29 expression vector and an estrogen-regulated reporter gene, maximal stimulation (about 3-fold) of reporter gene activity was found during incubation with 10 nM of E2. Neither progesterone, testosterone, dexamethasone, thyroid hormone, all-trans-retinoic acid, nor 5α-androstane-3α,17β-diol could stimulate reporter gene activity, whereas estrone and 5α-androstane-3β,17β-diol did. We conclude that clone 29 cDNA encodes a novel rat ER, which we suggest be named rat ERβ to distinguish it from the previously cloned ER (ERα) from rat uterus.**

Many aspects of vertebrate development, differentiation, and homeostasis are regulated by hormones and signaling molecules that control gene expression by binding to nuclear receptor proteins. Nuclear receptors are ligand-activated transcription factors, which regulate the expression of target genes by binding to specific response elements (1, 2). These receptors consist of a hypervariable N terminus that contributes to the transactivation function; a highly conserved DNA-binding domain responsible for specific DNA-binding and dimerization and a C-terminal domain involved in ligand-binding, nuclear localization, and ligand-dependent transactivation function (1, 2). In addition to these receptors for known ligands numerous so-called orphan receptors, which are putative receptors interacting with unknown ligands, have been found (3). It is highly probable that by nuclear orphan receptor research, novel ligands or activators with interesting biological properties will be found. An example is set by the orphan receptor PPAR (peroxisome proliferator activated receptor), a fatty acid-activated receptor (4) that regulates genes involved in fatty acid metabolism and adipocyte differentiation (5).

In an effort to clone and characterize novel nuclear receptors or unknown isoforms of existing receptors, we designed degenerate primers based upon conserved regions within the DNA- and ligand-binding domains (LBD) of nuclear receptors (6). These were used to PCR amplify rat prostate cDNA. Prostate was selected as an organ of interest given the high incidence of prostate cancer and benign prostatic hyperplasia. Initially prostate tumors are dependent on androgens for their maintenance and growth, but eventually nearly all tumors become independent of androgens and are thus beyond clinical control (7). The possibility exists that apart from androgens other (steroid) hormones or locally produced factors, interacting with nuclear receptors, are involved in cell proliferation, differentiation, and apoptosis of the normal prostate. This factor(s) could also be involved in growth stimulation of androgen independent prostate tumors. There is firm evidence that many biological processes of prostate epithelial tissue are controlled by androgens and estrogens through the production of stimulatory or inhibitory factors by the stroma, thereby indirectly influencing the adjacent epithelial tissue (8, 9). Human testicular receptors 2 and 4 (TR2 and TR4) and the estrogen receptor (ER)-related receptors (ERR1 and ERR2) are examples of orphan nuclear receptors expressed in the prostate (10, 11).

During this inventory of nuclear receptors expressed in the rat prostate we detected a novel ER, which is present in the secretory epithelial cells of the prostate and also in the granulosa cells of the ovary.

MATERIALS AND METHODS

PCR-Amplification and Complementary DNA Cloning. A set of degenerate primers [DNA-binding domains (DBDs) 1, 2, 3 and WAK/FAK] was designed according to the most highly conserved sequences of the DNA-binding domain (P-box) and ligand-binding domain (Ti-stretch) of members of the nuclear receptor family. The sequences are as follows (5′ → 3′): DBD1, TGYGARGGXTGYAARGSXTTYTT; DBD2, TGYGGNWGXTGYAARGTXTTYTT; DBD3, TGYGARGGXTGYAARWGXTTYTT; WAK, RAANCCNGGXAXXXXYTTNGCCCA; FAK, RAANCCNGGXAXXXXYTTNGCYTT (X= deoxyinosine). Single-strand cDNA reverse transcribed from rat prostate total RNA was employed with the primers in PCR reactions as described (6). The amplifi-

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Abbreviations: ER, estrogen receptor; ERE, estrogen response element; E2, 17β-estradiol; LBD, ligand-binding domain; DBD, DNA-binding domain; CHO, Chinese hamster ovary.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession number U57439).

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cation products were separated on a 2% low melting agarose gel and DNA products between 400 and 700 bp were isolated from the gel and ligated to TA cloning vector (Invitrogen). As alternatives we also used the RP-1/RP-2 and DBD66–100/DBD210–238 primer sets in the DNA-binding domain of nuclear receptors exactly as described by Hirose *et al.* (12) and Chang *et al.* (10), respectively. Clone 29 (obtained with the DBD–WAK/FAK set), with a length of 462 bp, showed high homology (65%) with the rat ER cDNA. Two PCR primers directed to clone 29 (Fig. 1) were used to generate a probe of 204 bp, which was used to screen a rat prostate cDNA library (Clontech) under stringent conditions. The cDNA insert of a positive clone of 2.6 kb was subcloned into the *Eco*RI site of pBluescript (Stratagene). The complete DNA sequence of clone 29 was determined in the core facility (CyberGene, Huddinge, Sweden) by cycle sequencing.

Saturation Ligand-Binding Analysis and Ligand-Competition Studies. Clone 29 protein was synthesized *in vitro* using the TnT-coupled reticulocyte lysate system (Promega). Translation reaction mixtures were diluted five times with TEDGMo buffer (40 mM Tris/HCl, pH 7.4/1 mM EDTA/10% (vol/vol) glycerol/10 mM Na_2MoO_4/10 mM DTT) and 0.1 ml aliquots were incubated for 16 h at 8°C with 0.3–6.2 nM [2,4,6,7-^{3}H]-17β-estradiol (DuPont/NEN; specific radioactivity 85 Ci/mmol) in the presence or absence of a 200-fold excess of unlabeled E2.

For ligand-competition studies diluted reticulocyte lysate was incubated with 5 nM [2,4,6,7-^{3}H]-17β-estradiol in the presence of either 0, 5, 50, 500, or 5000 nM of various competitors as indicated for 16 h at 8°C. Bound and unbound steroids were separated with a dextran-coated charcoal assay (13).

***In Situ* Hybridization.** *In situ* hybridization was carried out as described (6, 14). Two oligonucleotide probes directed against nucleotides 994-1041 and 1981–2031 were used and labeled at the 3′ end with ^{33}P-dATP using terminal deoxynucleotidyltransferase (Amersham). Adult male and female Sprague–Dawley rats (age 2–3 months, $n = 10$) were used for this study.

Transactivation Analysis in Chinese Hamster Ovary (CHO) Cells. The expression vector pCMV29 was constructed by inserting the 2.6-kb clone 29 fragment in the *Eco*RI site of the expression vector pCMV5 (15). The pERE-ALP reporter construct contains a secreted form of the placental alkaline phosphatase gene (16) and the mouse mammary tumor virus–long terminal repeat in which the glucocorticoid response elements were replaced by a single vitellogenin promoter estrogen response element (ERE).

CHO-K1 cells were seeded in 12-well plates at approximately 1.7×10^5 cells per well in phenol-red free Ham F-12 medium with 5% fetal calf serum (dextran-coated charcoal treated) and 2 mM L-glutamine. After 24 h the cells were transfected with 250 ng pERE-ALP vector and 50 ng pCMV29 using lipofectamine (GIBCO) according to the manufacturer's instructions. After 5 hr of incubation the cells were washed and refed with 0.5 ml of phenol-red free Coon's F-12 medium containing 5% serum substitute (SRC 3000, Tissue Culture Services, Botolph Claydon, Buckingham, U.K.), 2 mM L-glutamine, and 50 μg/ml gentamicin plus hormones as indicated. After 48 hr the medium was assayed for alkaline phosphatase activity by a chemiluminescence assay.

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ggaattcCGGGGGAGCTGGCCCAGGGGGAGCGGCTGGTGCTGCCACTGGCATCCCTAGGC  60
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CTGGAGCACGGCCCCATCTACATCCCTTCCTCCTACGTAGACAACCGCCATGAGTATTCA 420
GCTATGACATTCTACAGTCCTGCTGTGATGAACTACAGTGTTCCCGGCAGCACCAGTAAC 480
   M  T  F  Y  S  P  A  V  M  N  Y  S  V  P  G  S  T  S  N
CTGGACGGTGGGCCTGTCCGACTGAGCACAAGCCCAAATGTGCTATGGCCAACTTCTGGG 540
L  D  G  G  P  V  R  L  S  T  S  P  N  V  L  W  P  T  S  G
CACCTGTCTCCTTTAGCGACCCATTGCCAATCATCGCTCCTCTATGCAGAACCTCAAAAG 600
H  L  S  P  L  A  T  H  C  Q  S  S  L  L  Y  A  E  P  Q  K
AGTCCTTGGTGTGAAGCAAGATCACTAGAGCACACCTTACCTGTAAACAGAGAGACACTG 660
S  P  W  C  E  A  R  S  L  E  H  T  L  P  V  N  R  E  T  L
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K  R  K  L  S  G  S  S  C  A  S  P  V  T  S  P  N  A  K  R
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S  R  F  R  E  L  K  L  Q  H  K  E  Y  L  C  V  K  A  M  I
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CAGAACCTACAGTCTCAGTGATGGCCAGGCCTGAGGCGGACAGACTACAGAGATGGTCAA 1920
Q  N  L  Q  S  Q  *
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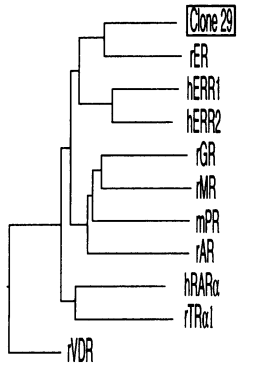
FIG. 1. Sequence of rat clone 29 cDNA and predicted amino acid sequence of clone 29 protein. Two potential translation start sites are indicated in boldface type. The predicted DNA-binding domain is double underlined and the PCR primers, used for generation of the probe for screening of a rat prostate cDNA library, are single underlined.

RESULTS

Cloning of a Novel Nuclear Receptor Protein. The PCR method, successfully used before to identify new members of the nuclear receptor family (6, 10, 12), was used to screen rat prostatic tissue. Use of the RP1/RP2 (12) and DBD66–100/DBD210–238 (10) primer sets resulted in the cloning of DNA sequences identical to several known members of the nuclear receptor family, whereas use of the DBD–WAK/FAK primer set resulted in the cloning of an unknown sequence (clone 29). Clone 29 DNA (462 bp) was found to be highly homologous to the cDNA of the rat ER (65%), which was previously cloned from rat uterus (17). The amino acid residues predicted by clone 29 DNA sequence suggested that this DNA fragment encoded part of the DNA-binding domain, hinge region, and the beginning of the LBD of a novel member of the nuclear receptor family. Two PCR primers were made (see Fig. 1) to generate a probe consisting of the hinge region of the novel receptor. This probe was used to screen a rat prostate cDNA library, resulting in four strongly positive clones with sizes of 0.9 kb, 1.8 kb, 2.5 kb, and 5–6 kb, respectively. The clone of 2.5 kb was sequenced and Fig. 1 shows the nucleotide sequence and deduced amino acid sequence of clone 29. Two in-frame ATG codons are located at nucleotide 424 and nucleotide 448, preceded by an in-frame stop codon at nucleotide 319, which suggests that they are possible start codons. The open reading frame encodes a protein of 485 amino acid residues with a calculated molecular weight of 54.2 kDa (counted from the first methionine). Analysis of the proteins synthesized by *in vitro*



Domain	A/B	C	D	E/F
rER	16.5	95.5	28.9	53.5
hERR1	16.5	69.7	15.6	34.2
hERR2	13.7	72.7	19.8	31.0
rAR	16.5	59.1	12.8	19.1
rGR	19.4	59.1	14.6	18.8
rMR	17.5	57.6	17.8	19.1
mPR	15.5	56.1	14.4	19.6
rTRα1	15.4	50.0	13.3	17.7
rRARα	26.1	59.1	14.8	17.3
rVDR	21.7	47.0	15.6	18.8

FIG. 2. Comparison of clone 29 protein with several representative members of the nuclear receptor family. Percentage amino acid identity in the domains A/B (N terminus), C (DNA-binding domain), D (hinge region), and E/F (ligand binding, dimerization, and ligand-dependent transactivation function) are depicted. For the alignment and phylogenetic tree Clustal analysis of the full-length receptor sequences using the MEGALIGN/DNASTAR software was used.

translation from the clone 29 cRNA in rabbit reticulocyte lysate revealed a doublet protein band migrating at approximately 61 kDa on SDS/PAGE gels (data not shown), confirming the open reading frame. The doublet protein band is probably caused by the use of both ATG codons for initiation of protein synthesis.

Protein sequence comparison (Fig. 2) showed that clone 29 protein is most related to the rat ER, cloned from uterus (17), with 95% identity in the DNA-binding domain (amino acid residues 103–167). A number of functional characteristics have been identified within the DNA-binding domain of nuclear receptors (2, 18). The clone 29 protein P-box and D-box sequences of EGCKA and PATNQ, respectively, are identical to the corresponding boxes in the ER (2, 17), thus predicting that clone 29 protein binds to ERE sequences.

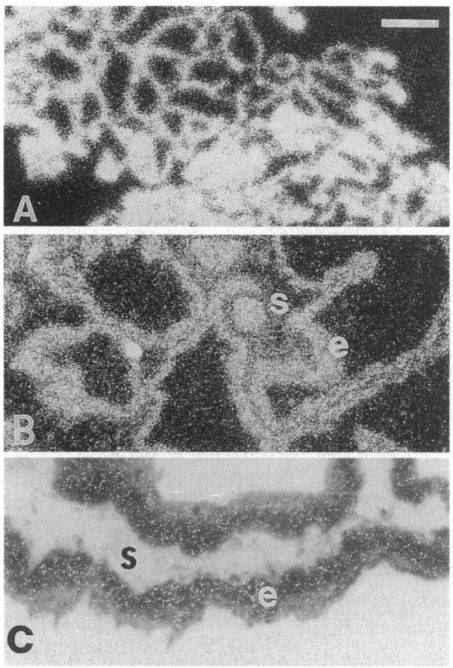


FIG. 3. *In situ* hybridization of clone 29 RNA in rat prostate. (*A*) Film autoradiography of prostate gland showing strong expression of clone 29. (*B*) Dark-field image showing prominent signal for clone 29 in epithelium (e) of prostatic alveoli. The stroma (s) exhibits weaker signal. (*C*) Epipolarization image of cresyl violet counterstained section showing silver grains over epithelium (e), whereas the stroma (s) contains fewer grains. Bar (in *A*) represents 0.7 mm for *A*, 200 μm for *B*, and 30 μm for *C*.

The putative LBD of clone 29 protein (amino acid residues 259–457) shows closest homology to the LBD of the rat ER (Fig. 2), whereas the homology with the human ERR1 and ERR2 proteins (11) is considerably less. With the human, mouse, and *Xenopus* ERs the homology in the LBD is also around 55%, whereas the homology with the LBD of other steroid receptors is not significant (Fig. 2). Several amino acid residues described to be close to or part of the ligand-binding pocket of the human ER-LBD (Cys 530, Asp 426, and Gly 521) are conserved in the LBD of clone 29 protein (Cys 436, Asp 333, and Gly 427) and in the LBD of ERs from various species (19–21). The ligand-dependent transactivation function TAF-2, identified in the ER (22), is almost completely conserved in clone 29 protein (amino acid residues 441–457). Steroid hormone receptors are phosphoproteins (23), and several phosphorylation sites identified in the N-terminal domain and LBD of the ER (24, 25) are conserved in clone 29 protein (Ser 30, Ser 42, and Tyr 443). Clone 29 protein consists of 485 amino acid residues, whereas the ERs from human, mouse, and rat consist of 590–600 amino acid residues. The main difference is a much shorter N-terminal domain in clone 29 protein, i.e., 103 amino acid residues as compared to 185–190 amino acid residues in the other receptor proteins. Also, the nonconserved so-called F-domain at the C-terminal end of the ERs is 15 amino acid residues shorter in clone 29 protein.

***In Situ* Hybridization of Clone 29 RNA in the Prostate and Ovary of the Rat.** Two antisense oligonucleotide probes were synthesized: one in the hinge region (nucleotide 994-1041) and

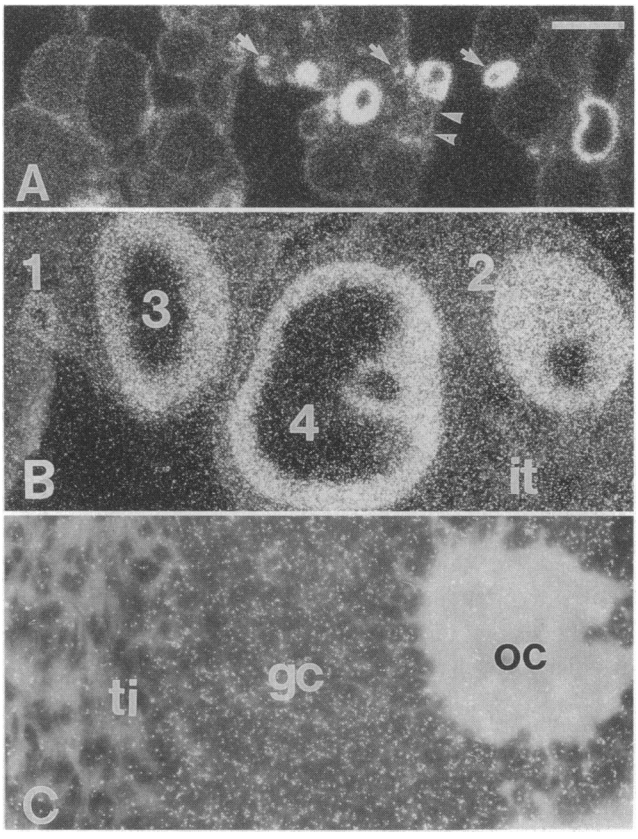


FIG. 4. *In situ* hybridization of clone 29 RNA in rat ovary. (*A*) Film autoradiography of ovary showing strong expression of clone 29 in follicles at different developmental stages (some are indicated by arrows). The interstitial tissue (arrowheads) shows low signal. (*B*) Dark-field image showing high expression of clone 29 in granulosa cells of primary (1), secondary (2), tertiary (3), and mature (4) follicles. Low signal is present in interstitial tissue (it). (*C*) Epipolarization image of ovary showing strong signal in granulosa cells (gc), whereas the oocyte (oc) and the theca interna (ti) are devoid of clear signal. Bar (in *A*) represents 0.9 mm for *A*, 140 μm for *B*, and 50 μm for *C*.

the other in the 3′-untranslated region (nucleotide 1981–2031). Clear expression of clone 29 was observed in the reproductive tract of both male and female rats, whereas in all other rat tissues the expression was very low or below the level of detection with *in situ* hybridization (not shown). In male reproductive organs high expression was seen in the prostate gland (Fig. 3), whereas very low expression was observed in testis, epididymis, and vesicula seminalis (not shown). In dipped sections, expression was clearly visible in prostate epithelial cells (secreting alveoli), whereas the expression in smooth muscle cells and fibroblasts in the stroma was low (Fig. 3). In female reproductive organs expression was seen in the ovary (Fig. 4), whereas expression in the uterus and vagina were negative (not shown). In dipped sections high expression was seen in the granulosa cell layer of primary, secondary, and mature follicles (Fig. 4), whereas primordial follicles, oocytes, and corpora lutea appeared completely negative. Low expression was seen in the interstitial cells of the ovary. Both anti-sense oligonucleotide probes used produced similar results. Addition of a 100-fold excess of the respective unlabeled oligonucleotide probes during the hybridization reactions abolished all signals.

Ligand-Binding Characteristics and Transactivation Function of Clone 29 Protein. On the basis of the described high homology between clone 29 protein and the rat ER in the DBD and LBD we hypothesized that clone 29 protein might encode a novel ER. Furthermore, biologic effects of estrogens on rat prostate and ovary, which show high expression of clone 29 RNA, are well known (7, 26, 27). To analyze the steroid binding properties clone 29 protein was synthesized *in vitro* and incubated with [^{3}H]E2. Linear transformation of saturation data revealed a single population of binding sites for E2 with a K_d (dissociation constant) of 0.6 nM (Fig. 5). Competition curves are indicative of an ER in that only estrogens competed efficiently with [^{3}H]E2 for binding (Fig. 5). Fifty percent inhibition of specific binding occurred at a 0.6-fold excess of unlabeled E2; diethylstilbestrol, estriol, estrone, and 5α-androstane-3β,17β-diol were 5, 15, 50, and 150 times, respectively, less effective as competitors. Neither testosterone, progesterone, corticosterone, nor 5α-androstane-3α,17β-diol were efficient competitors, even at the highest concentrations used (1000-fold excess). The K_d and the steroid binding specificities measured are in agreement with data previously reported for ERs in rat and human prostate, rat granulosa

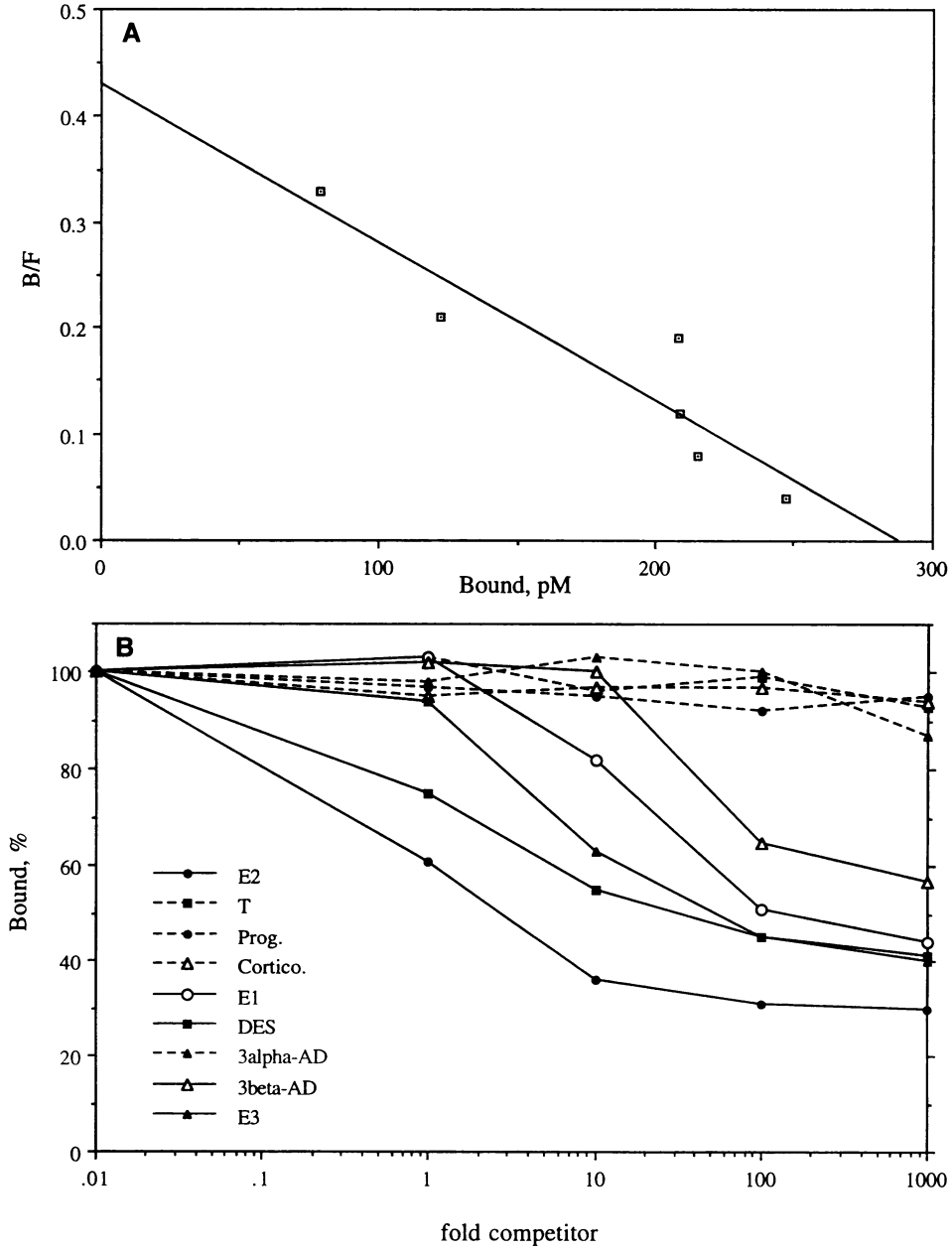


FIG. 5. E2 binding of clone 29 protein. (*A*) Saturation ligand-binding analysis of clone 29 protein. Reticulocyte lysate containing clone 29 protein was incubated with [^{3}H]E2 as described in *Materials and Methods*. The K_d (0.6 nM) was calculated from the slope of the line in the Scatchard plot shown ($r = -0.93$), and the number of binding sites was extrapolated from the intercept on the abscissa (Bmax. = 2100 fmol/ml undiluted translation reaction mixture or 280 fmol/ml in the binding assay mixture). (*B*) Specificity of ligand binding by clone 29 protein. Reticulocyte lysate containing clone 29 protein was equilibrated for 16 hr with 5 nM [^{3}H]E2 and the indicated fold excess of competitors. Data represent [^{3}H]E2 bound in the presence of unlabeled E2, testosterone (T), progesterone (Prog.), corticosterone (Cortico.), estrone (E1), diethylstilbestrol (DES), 5α-androstane-3α,17β-diol (3alpha-AD), 5α-androstane-3β,17β-diol (3beta-AD), and estriol (E3). [^{3}H]E2 binding in the absence of competitor was set at 100%.

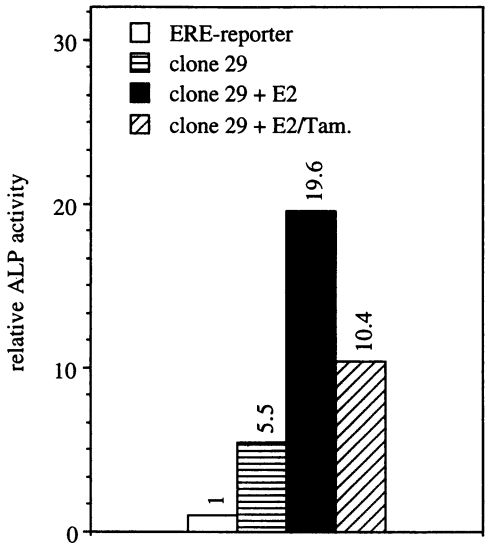


FIG. 6. Activation of transcription by clone 29 protein. CHO cells were transiently transfected with ERE-reporter plasmid alone (ERE-reporter) or together with a clone 29 protein expression plasmid. Cells were incubated in the absence of E2 (clone 29) or in the presence of 100 nM E2 (clone 29 + E2) or in the presence of 100 nM E2 and 1 μM tamoxifen (clone 29 + E2/Tam.). Alkaline phosphatase activity (ALP) was measured as described and the result of a representative experiment is shown. The ALP activity of ERE reporter alone was set at 1.

cells, rat antral follicles, and whole rat ovarian tissue (13, 28–30). When clone 29 protein was labeled with a saturating dose of [^{3}H]E2 and analyzed on sucrose density gradients, a single peak of specifically bound radioactivity was observed. The sedimentation coefficient of this complex was about 7S, and it shifted to 4S in the presence of 0.4 M NaCl (not shown). In control experiments the K_d of the human ER was 0.7 nM, and the order of affinity for the described competitors was the same (not shown). To investigate the transcriptional regulatory properties of clone 29 protein, we performed cotransfection experiments in which CHO cells were transfected with a clone 29 protein expression vector and an estrogen-responsive reporter gene construct. In the absence of exogenously added E2, clone 29 protein showed considerable transcriptional activity, which could be further increased by the addition of 100 nM E2 (Fig. 6). Simultaneous addition of a 10-fold excess of the antiestrogen tamoxifen partially suppressed the E2 stimulated activity (Fig. 6). The constitutive transcriptional activity of clone 29 protein could also be suppressed by tamoxifen (not shown). Although we have done everything possible to exclude estrogenic compounds from the medium (see *Materials and Methods*) we cannot exclude the presence of very low amounts of estrogenic compounds. It has been shown previously that the wild-type mouse and human ERs stimulate transcription in the absence of added E2 and that the transcriptional activity can be stimulated further by the addition of E2 (31, 32). In dose-response experiments, clone 29 protein began to respond at 0.1 nM E2 and maximal stimulation was observed between 1 nM and 10 nM E2 (Fig. 7). The maximal stimulation factor was 2.6 ± 0.5-fold (mean ± SD, n = 9) as compared to incubation in the absence of E2. Apart from E2, estrone and 5α-androstane-3β,17β-diol could also stimulate transcriptional activity, albeit at higher concentrations (Fig. 7). Dexamethasone, testosterone, progesterone, 5α-androstane-3α,17β-diol, thyroid hormone, and all-trans-retinoic acid could not stimulate transcriptional activity of clone 29 protein, even at the highest concentration (1000 nM) tested (not shown). In control experiments, the wild-type human ER also showed transcriptional activity in the absence of E2, which could be increased by the addition of E2 (not shown).

DISCUSSION

In this study a novel member of the steroid receptor family was identified. Clone 29 protein displays high-affinity binding of estrogens and in a transactivation assay system activation of the expression of an ERE-containing reporter gene construct by clone 29 protein in the presence of estrogens could be measured. We suggest that this clone be named rat ERβ, to differentiate it from the previously cloned ER (ERα) from rat uterus (17).

High expression of ERβ was detected in the granulosa cells of rat ovary by *in situ* hybridization. Autoradiographic studies have demonstrated the localization of [^{3}H]E2 over granulosa cells in the rat ovary and suggested therefore the granulosa cells as an estrogen target site (26, 33). This was confirmed by the identification of specific ERs in rat granulosa cells (29). Estrogens synergize with follicle stimulating hormone in ovarian weight augmentation, which is associated with a pronounced proliferation of granulosa cells, and the growth of small- and medium-sized follicles (26, 33).

Recently, a mutant mouse line without a functional ERα was created and assessed for estrogen responsiveness (34). Female

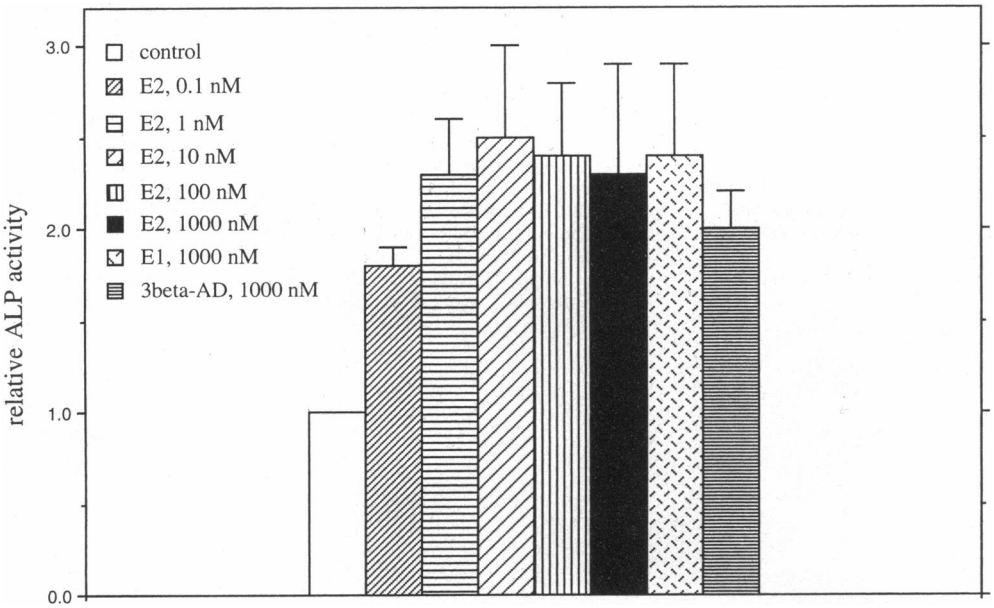


FIG. 7. Estrogen-stimulated activation of transcription by clone 29 protein. CHO cells were transiently transfected with the ERE-reporter plasmid and the clone 29 protein expression plasmid. Cells were incubated with increasing concentrations of E2 (0.1–1000 nM), estrone (E1, 1000 nM), 5α-androstane-3β,17β-diol (3beta-AD, 1000 nM), or with no ligand added. Alkaline phosphatase activity (ALP) was measured as described, and the activity in the absence of ligand (control) was set at 1. The figure shows relative ALP activities (± SD) from three independent experiments.

mice were infertile and showed hypoplastic uteri and hyperemic ovaries with no detectable corpora lutea (34). Although we have no information yet on the amount of ERα and ERβ protein expressed in granulosa cells of mice and rat, the presence of ERβ in the ovaries is in apparent contrast with the lack of estrogen action in the knock-out mice ovary. Maybe ERα regulates the expression of ERβ, or ERα and ERβ are expressed during different stages of follicle development. In some tissues from the ERα knock-out mice residual [^{3}H]E2 binding (5–10% of the wild-type level) with a K_d of 0.7 nM could be measured (34, 35). The authors ascribed this to a possible "splicing over" event, resulting in the production of a smaller mutant ERα protein that could be the source of the residual [^{3}H]E2 binding (35). In retrospect it could also be explained by the—perhaps compensatory—expression of ERβ. The presence of estrogen receptors in preimplantation mouse embryos (36) and the apparent absence of reported human ER mutations was interpreted as an indication for an essential role of estrogens during embryonic development (34, 36). This view seemed to be challenged by the recent discovery of a male with estrogen resistance caused by a mutation in the estrogen receptor gene (37). With the identification of a second ER, the unexpected existence of the ERα knock-out human could also be explained by complementation through ERβ. The human homologue of rat ERβ has recently been identified in our laboratory (E.E., unpublished data).

ERs are present in human and rat prostate, as evidenced by ligand-binding studies (13, 28, 38). In contrast with androgen receptors, the major part of the ERs is localized in the stroma of the rat prostate (38), although the epithelial cells of the secreting alveoli contain ER. Estrogens are, besides androgens, implicated in the growth of the prostate (7), and consequently estrogens have been implicated in the pathogenesis of benign prostatic hyperplasia (27). Neonatal estrogen treatment of rats was shown to down-regulate androgen receptor expression in the epithelial cells of all the three prostate lobes, thus leading to an overall prostate growth retardation (9).

The biological significance of the existence of two different ERs is at this moment unclear. Differences in the ligand-binding properties and/or transactivation function on certain target genes may exist.

An alignment of the LBD of ERα (rat, mouse, and human) and ERβ (rat) revealed various regions of conservation, whereas other segments are nonconserved (not shown). Comparative studies of the LBD of ERα and ERβ by protease mapping (39, 40) and crystal structure determination could provide more detailed information on the structural requirements for E2 binding.

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