© 1998 Oxford University Press
Nucleic Acids Research, 1998, Vol. 26, No. 15 **3505–3512**

Molecular cloning and characterization of human estrogen receptor βcx: a potential inhibitor of estrogen action in human

Sumito Ogawa[1,2], Satoshi Inoue[1,2], Toru Watanabe[1], , Akira Orimo[1], Takayuki Hosoi[2], Yasuyoshi Ouchi[2] and Masami Muramatsu[1,2,*]

[1]Department of Biochemistry, Saitama Medical School, 38 Morohongo, Moroyama-machi, Iruma-gun, Saitama 350-0495, Japan and [2]Department of Geriatric Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

Received April 20, 1998; Revised and Accepted May 13, 1998

DDBJ/EMBL/GenBank accession no. AB006589

ABSTRACT

We have identified and characterized a novel human estrogen receptor (ER) β isoform, ERβcx, which is truncated at the C-terminal region but has an extra 26 amino acids due to alternative splicing. The ERβcx transcript is expressed in testis, ovary, thymus and prostate as well as in human cultured cell lines such as HEC-1, HOS-TE85 and Saos-2 cells. ERβcx protein is also immunodetectable in these human cells. Biochemical analysis reveals that the average dissociation constants (K_d) of ERα and ERβ for 17β-estradiol (E_2) are 0.2 and 0.6 nM respectively, but ERβcx has no ligand binding ability. ERα and ERβ proteins bind to the estrogen response element, whereas ERβcx does not form any shifted complex in gel shift assays. In a transient expression assay, ERβcx shows no ligand-dependent transactivation ability of a basal promoter and also cannot interact with a cofactor, TIF1α, in the presence or absence of E_2. ERβcx preferentially forms a heterodimer with ERα rather than that with ERβ, inhibiting DNA binding by ERα. Interestingly, however, it shows a significant dominant negative activity only against ERα transactivation. Thus, this study indicates that ERβcx potentially inhibits ERα-mediated estrogen action and that alternative splicing of the C-terminal region and its inhibitory properties are characteristic of several members of nuclear receptor isoforms.

INTRODUCTION

Estrogen plays crucial roles in sexual development and the reproductive cycle (1). The estrogen receptor (ER), a member of the steroid/nuclear receptor superfamily, mediates this action by ligand-dependent binding to the estrogen response element (ERE), which exists in the enhancer region of target genes, regulating their transcription directly (2–4).

It has been shown that members of the nuclear receptor superfamily, such as RAR, RXR and TR, have multiple subtypes and isoforms (5–7). In the case of the ER, we and others have reported several isoforms that were mostly derived from alternative splicing (8,9). Most of these isoforms lost estrogen-dependent transactivation ability and some of them showed various effects on estrogen signaling, such as inhibitory effects on the wild-type ER (9,10).

In order to understand the mechanism of estrogen action and ER regulation of gene transcription, it is important to isolate and characterize novel subtypes and/or isoforms of the ER. Recently, another ER subtype, ERβ (11), has been identified from rat prostate and, therefore, the classical ER is renamed ERα. ERβ has been characterized by its distinct properties, including tissue localization and transactivation properties (12,13). The DNA binding domain (DBD) of ERβ is highly homologous with that of ERα, implying that both ERα and ERβ share the same DNA response element. Subsequently, human ERβ (14,15) and mouse ERβ (16) were isolated.

In this study, we screened human cDNA libraries with an ERα cDNA probe to identify novel ER subtypes and isoforms. We have obtained three independent ERβ-related clones, including a novel isoform, designated ERβcx.

In view of the structural and functional similarities among several nuclear receptor isoforms (17–19), some isoforms generated by alternative splicing of the C-terminal region may suppress ligand-dependent transactivation by canonical receptors. To test this possibility, we have investigated the effect of ERβcx on transcriptional regulation by wild-type ERα and ERβ using a transfection system. Biochemical properties of ERβcx are also described.

MATERIALS AND METHODS

Screening of human cDNA and genomic library

A human testis λZAPII cDNA library (5.0×10^5 plaques) constructed in pBK-CMV (Stratagene) was screened with a

*To whom correspondence should be addressed at: Department of Biochemistry, Saitama Medical School, 38 Morohongo, Moroyama-machi, Iruma-gun, Saitama 350-0495, Japan. Tel: +81 492 76 1490; Fax: +81 492 94 9751; Email: muramasa@saitama-med.ac.jp

^{32}P-labeled DBD fragment of rat ERα cDNA (20) encoding amino acids 177–281. The plaque-transferred filters were hybridized with the probe for 18 h at 63°C in 5× SSC, 0.5% (w/v) blocking agent (Amersham). The filters were washed for 15 min at room temperature in 2× SSC, 0.1% SDS twice and then exposed to X-ray film. Further screening was repeated until a single positive signal was obtained. The PCR-amplified DNA fragments specific for ERβcx (nt 2667–3229) and specific for ERβ (nt 2690–2918) (15) were used as probes to isolate the genomic clones from a human genomic DNA library (Japanese Cancer Research Resources Bank).

Plasmid construction

The ERβ cl 61-1 cDNA insert was cloned into the pCXN2 expression vector (21) and into pGEX4T-2 (Pharmacia) to construct pCXN2-hERβcx and GST-hERβcx respectively. pCXN2-hERα, pCXN2-hERβ, GST-hERα, GST-hERβ and ERE-GCAT were constructed as described (15,22). All constructs were verified by sequencing.

DNA sequence and analysis

The nucleotide sequences were determined by sequencing both strands of alkaline denatured plasmid DNA using the BcaBest sequencing kit (TaKaRa Co.). The obtained DNA sequence was compiled and analyzed using the DNASIS computer programs (Hitachi Co.).

Northern blot analysis of various human tissues

Human multiple tissue northern blots of poly(A)$^+$ RNA were purchased from Clontech. These blots were probed with ^{32}P-labeled DNA fragments of ERα (2.1 kb *Eco*RI-digested fragment of HEG0), ERβ (nt 2690–2918), ERβcx (nt 2667–3229) and glyceraldehyde 3-phosphate dehydrogenase (G3PDH), as an internal control, according to the manufacturer's instructions.

Cell transfection and whole cell extracts preparation

Cultured cells were maintained in Dulbecco's modified Eagle's medium (DMEM) without phenol red, supplemented with 10% dextran-coated charcoal-stripped fetal calf serum (23). Samples of 5×10^5 cells in 10 cm Petri dishes were transfected with a total of 20 µg plasmids using calcium phosphate (24). Cells were harvested 36 h after transfection and whole cell extracts were prepared by freeze-thawing and diluted in 100 µl TEG buffer (10 mM Tris, pH 7.5, 1.5 mM EDTA, 10% glycerol).

Antibody preparation and western blot analysis

To detect the specific ERβ and ERβcx proteins, rabbit polyclonal antibodies against synthesized peptides of the C-terminal region of ERβ (CSPAEDSKSKEGSQNPQSQ) and ERβcx (MKMETLL-PEATMEQ) respectively were prepared as described elsewhere (25) and purified on affinity columns bound with each synthetic peptide. Whole cell extracts were fractionated on SDS–7.5% polyacrylamide gels under reducing conditions. Twenty micrograms of protein were then subjected to western blot analysis using the anti-ERβ- and anti-ERβcx specific antibodies respectively as described, using the chemiluminescence-based ECL detection system (Amersham) according to the manufacturer's instructions.

Binding assay of estrogen to estrogen receptors

Ten microgram aliquots of whole cell extracts were incubated at 4°C for 16 h with various concentrations of [$^{2.4.6.7-3}H$]E_2 (91 Ci/mmol) (Amersham) in the presence and absence of a 150-fold excess of radioinert E_2. Free [$^{2.4.6.7-3}H$]E_2 was removed by dextran-coated charcoal adsorption and the bound form was quantified using a liquid scintillation counter. Specific binding was then calculated for each concentration of radiolabeled E_2 used. The data were analyzed by the method of Scatchard (26).

Gel shift assay

Double-stranded oligonucleotide (37 bp, 5′-GTCCAAAGT-CAGGTCACAGTGACCTGATCAAAGTT-3′, consensus ERE underlined) (27) was end-labeled with [^{32}P]dCTP using the Klenow fragment. An appropriate volume (4–20 µg protein) of cell extract was incubated with 2 µg poly(dI·dC) on ice for 15 min in a 15 µl reaction mix of 10 mM Tris–HCl (pH 7.5), 1 mM DTT, 10% (v/v) glycerol, 100 mM KCl. Radiolabeled probe (~10^4 c.p.m.) was added to the reaction mixture and incubated at room temperature for 15 min. ER–DNA complex and free probe were then separated on a 4% polyacrylamide gel run for 2 h at 4°C in 0.5× TBE buffer.

Chloramphenicol acetyltransferase (CAT) assay

The CAT assay was performed as described (28). Briefly, 5×10^5 COS-7 cells were transfected with a total of 15 µg DNA. Two micrograms of ERE-GCAT reporter plasmid were co-transfected with the indicated amounts of receptor expression vectors. All assays were performed in the presence of 2 µg PCH110 (Pharmacia), a β-galactosidase expression vector used as an internal control to normalize for variations in transfection efficiency. The total amounts of DNA and expression vectors for transfection were adjusted using pGEM3Zf (Promega) and pCXN2 respectively. After 12 h incubation with calcium phosphate-precipitated DNA, the cells were washed with fresh medium and incubated for an additional 24 h in the presence or absence of 10^{-7} M E_2. Whole cell extracts were prepared by freeze-thawing and assayed for CAT after normalization for β-galactosidase activity.

Glutathione S-transferase (GST) pull-down assay

ERβcx and TIF1α (29) proteins were synthesized *in vitro* using the TnT-coupled reticulocyte lysate system (Promega). GST, GST–hERα, GST–hERβ and GST–hERβcx proteins were induced, solubilized, estimated as of equal quality on Coomassie stained gels and bound to glutathione beads following the manufacturer's instructions (Pharmacia LKB). After binding to glutathione beads, GST fusion proteins were preincubated for 30 min in 500 µl NETN buffer minus NP-40 in the absence (–) or presence (+) of 10^{-7} M E_2. Then, 15 µl of the suspension were incubated with 1–2 µl appropriate ^{35}S-labeled, *in vitro* translated protein for 1 h in 500 µl NETN (20 mM Tris–HCl, pH 7.5, 100 mM KCl, 0.7 mM EDTA, 0.5% NP-40, 1 mM phenylmethylsulfonyl floride). Following incubation, the beads were washed three times with NETN. Bound proteins were eluted with 20 µl 1× SDS–PAGE buffer (50 mM Tris–HCl, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromo-phenol blue, 10% glycerol) and electrophoretically separated on a SDS–7.5% polyacrylamide gel.

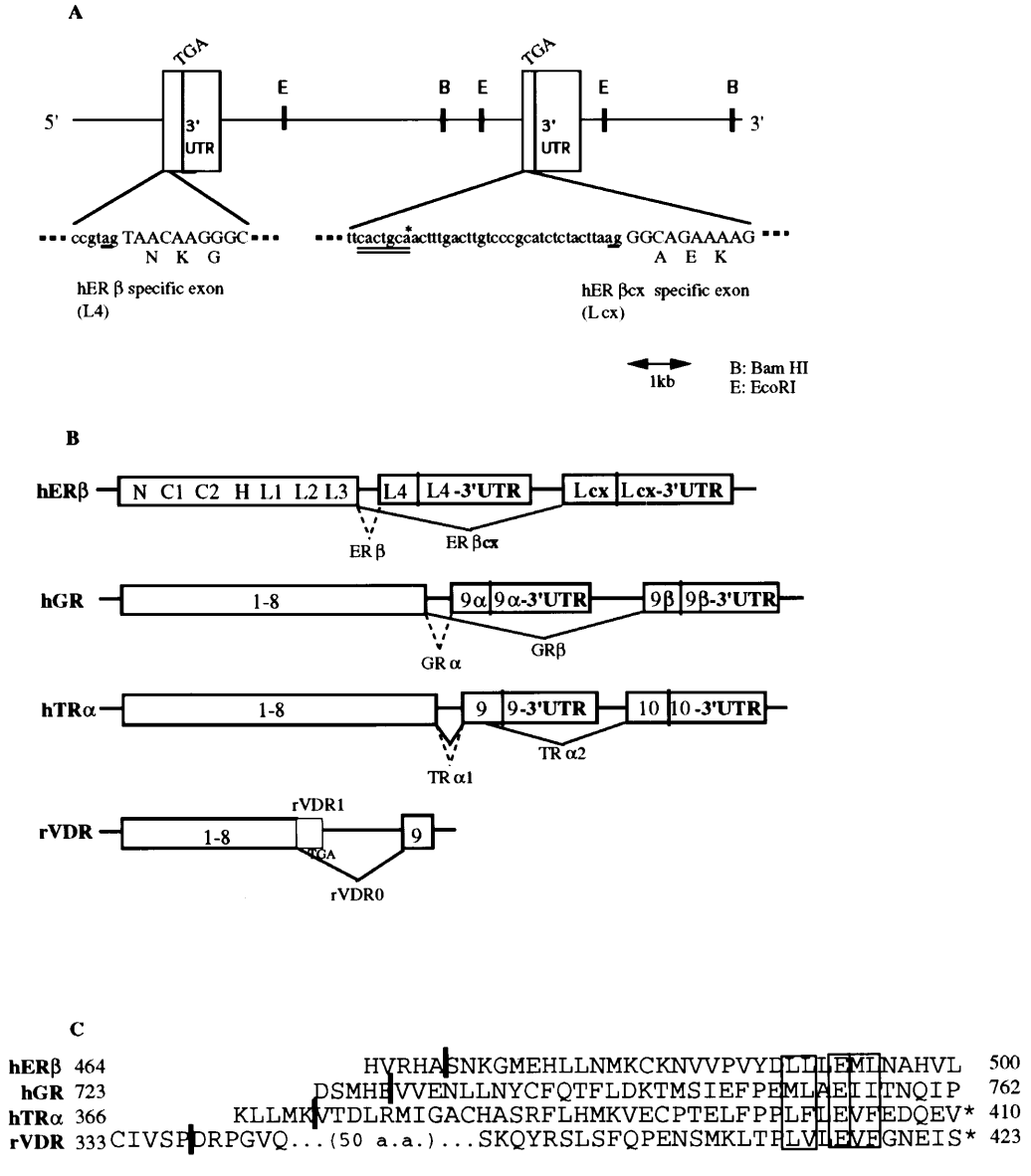

Figure 1. Genomic organization of ERβcx. (**A**) Restriction map and the sites of alternative exons for ERβ and ERβcx. The exons encoding the C-terminal-specific region of ERβ (L4) and ERβcx (Lcx) are schematically represented, with each intron–exon junction indicated below. The pyrimidine-rich branch site (CACTGCA) is double underlined and a putative conserved branch point sequence is indicated by an asterisk. The sites for restriction enzymes in the ERβ genomic region around the specific exons are abbreviated as follows: B, *Bam*HI; E, *Eco*RI. (**B**) Comparison of the alternative splicing of the 3′-end among nuclear receptor isoforms. Exons and introns are schematically represented by open boxes and solid lines respectively. The numbers and names of exons are in open boxes. (**C**) Sequence alignment of nuclear receptor proteins. The central conserved acidic amino acid and two pairs of hydrophobic amino acids in the AF-2 core region are boxed. Amino acid numbers are indicated and terminal amino acids are indicated by a star. The alternative splicing junctions are indicated by a solid bar.

RESULTS

Isolation of ERβcx cDNA

We screened the human testis cDNA library with a DNA probe generated from the coding sequence of the rat ERβ DBD and found 13 positive clones out of 200 000 plaques. Among them, three clones were ERβ-related and two were partial ERαs, as judged by sequence analysis. Restriction mapping and partial sequencing of the three ERβ-related clones indicated that all were derived from the same RNA. One of the three overlapping clones, cl 61-1, had the longest, and complete, open reading frame containing a poly(A)$^+$ tail. Its nucleotide sequence and deduced amino acid sequence were determined, designated ERβcx and deposited in the DNA Data Bank of Japan (accession no. AB006589). The predicted ERβcx protein consists of 495 amino acids, with a calculated relative molecular mass (M_r) of 55.5 kDa, counted from the ATG codon at nt 1276, preceded by an in-frame stop codon at nt 1210. This putative first ATG codon is identical to that of the ERβ cDNA (15). Computer-assisted analysis and database searches reveal that ERβcx encodes an A/B domain, DBD, hinge region and part of its ligand binding domain (LBD). It was found to be identical to the human ERβ cDNA except that the C-terminal 61 amino acids of ERβ were replaced by a unique

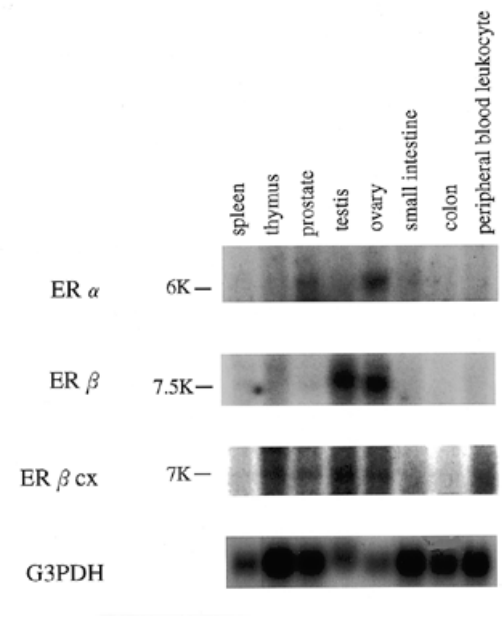

Figure 2. Expression of the ERα, ERβ and ERβcx transcripts in various human tissues. Northern blots with poly(A)$^+$ mRNA from various human tissues were hybridized with ^{32}P-labeled DNA probes specific for each ER. Each lane contained 2 µg poly(A)$^+$ mRNA and G3PDH was used as the internal control.

26 amino acid sequence in ERβcx. Thus, ERβcx is named for its C-terminal exchanged property.

The C-terminal ERβcx region is derived from a unique exon 3′-downstream of the human genomic ERβ

Six independent clones, ~20 kb insert size on average, were isolated from the human genomic DNA library probed with the C-terminal-specific region of ERβ (nt 2690–2918) or ERβcx (nt 2667–3229). The restriction map of the clones showed that these six genomic DNA fragments overlapped and were derived from the same region. Two DNA fragments digested with *Eco*RI, 5 and 1.8 kb in size, were hybridized with ERβ- and ERβcx-specific probes respectively. These *Eco*RI-digested fragments were subcloned and sequenced. The 5 kb fragment included the ERβ-specific sequence as a single exon and its intron–exon junction was CCGTAG (Fig. 1A). The 1.8 kb fragment included the ERβcx-specific sequence and its 3′-untranslated region (UTR) as a single exon (Lcx) and its intron–exon junction was CTTAAG. As shown in Figure 1A, the pyrimidine-rich branch site (CACTGCA) lies ~30 nt upstream of the ERβcx intron–exon junction and retains a conserved branch point sequence. The ERβcx-specific exon was located at 5.5 kb 3′-downstream of the ERβ-specific exon.

Comparison of the alternative splicing ends between ERβcx and other nuclear receptor isoforms and their splicing junctions are shown in Figure 1B and C respectively. Besides ERβcx, human glucocorticoid receptor β (GRβ) (17) and thyroid hormone receptor α2 (TRα2) (18) are derived by alternative splicing of the last exon and a rat vitamin D receptor isoform (rVDR1) (19) is an alternative splicing variant retaining intron 8 of rVDR (Fig. 1B). The splicing junctions are located in the more N-terminal portion than the activation function 2 (AF-2) core region (29–31; Fig. 1C) and, therefore, all these splicing variants show substitution of part of the last exon, including the AF-2 core region, by unique sequences.

Expression of ERβcx in various human tissues

Northern blot analysis was performed using DNA fragments of specific regions of ERs as probe. It revealed that an ~7 kb ERβcx transcript was detected in testis, ovary, prostate and thymus. In contrast, ERα transcript was expressed in ovary and prostate and ERβ transcript was distributed in testis and ovary (Fig. 2).

Immunoblot of human cultured cells

Western blot analysis using the ERβ- and ERβcx-specific polyclonal antibodies against their C-terminal amino acid residues respectively demonstrated the ERβ protein as 60 and 57 kDa

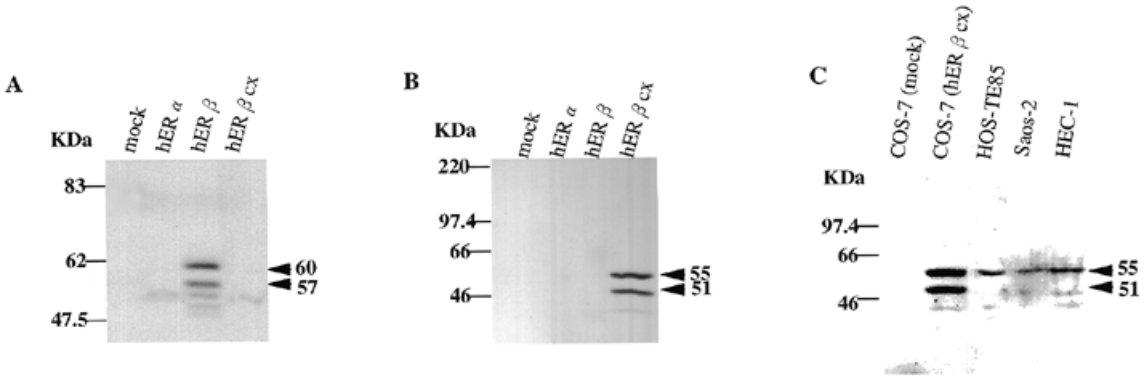

Figure 3. Immunoblot analysis of ERβ and ERβcx. (**A**) Determination of ERβ by immunoblot analysis of transfected cells. Twenty micrograms of transfected COS-7 cell extracts were resolved by SDS–PAGE and transferred to nitrocellulose. Blots were probed with the ERβ-specific polyclonal antibody (1:500). (**B**) Immunoblot analysis of transfected cells with the anti-ERβcx antibody (1:500). The same volumes of the cell extracts were used as shown in (A). (**C**) Immunoblot analysis with the anti-ERβcx antibody (1:500) in human cultured cells. COS-7 cells transfected with either 20 µg pCXN2 or 20 µg pCXN2-hERβcx, HOS-TE85 (derived from human osteosarcoma), Saos-2 (derived from human osteosarcoma) and HEC-1 (derived from human endometrial carcinoma) cells were cultured and harvested. Twenty micrograms of whole cell extracts were used for the analysis.

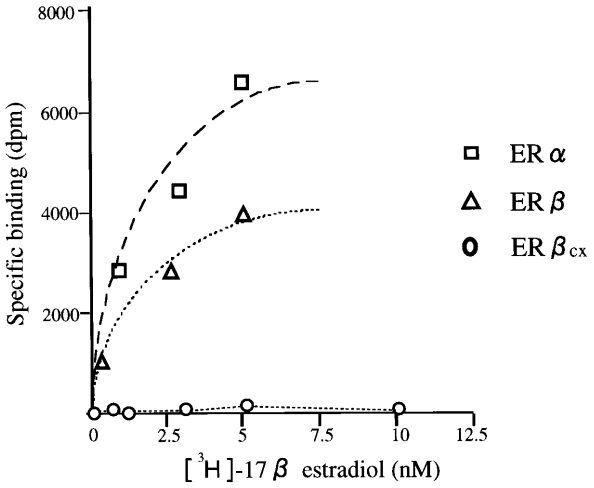

Figure 4. Comparison of E_2 binding activities among ERα, ERβ and ERβcx. Whole cell extract of COS-7 cells transfected with ERα, ERβ or ERβcx was incubated for 16 h with 0.1–1 nM [$^{2,4,6,7-3}H$]E_2 in the presence or absence of various concentrations of unlabeled E_2. Unbound E_2 was removed by centrifugation and the receptor-bound radioactivity was measured with a liquid scintillation counter. Only one representative result is shown from the three independent experiments showing similar results.

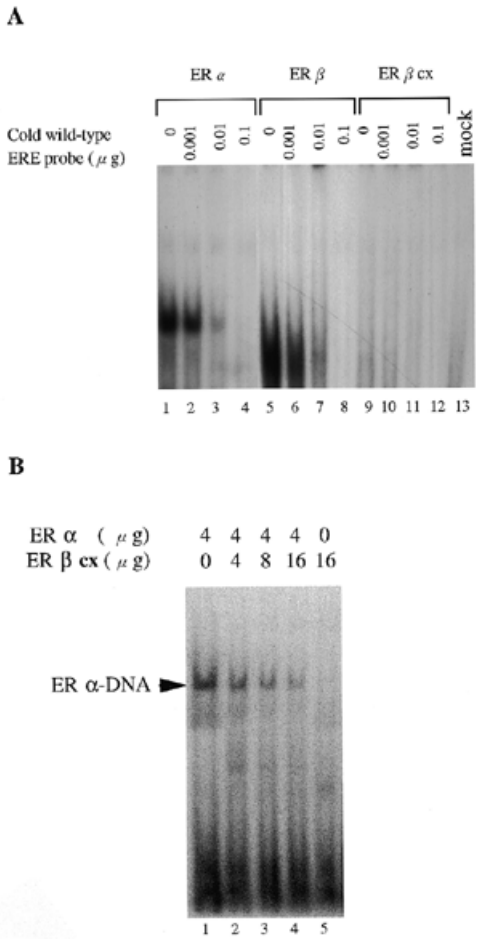

Figure 5. DNA binding abilities of ERα, ERβ and ERβcx. (**A**) Whole cell extracts of COS-7 cells transfected with the indicated ER expression vectors were analyzed for their ability to bind the ^{32}P-labeled ERE. ERα and ERβ bind to the ERE and competition is shown by adding an increasing amount of cold ERE (lanes 1–8). ERβcx–DNA complexes were not detected (lanes 9–12). (**B**) DNA binding of ERα is inhibited in the presence of ERβcx. A constant amount (4 μg) of whole cell extracts of COS-7 cells transfected with pCXN2-hERα was applied to lanes 1–4, with increasing amounts (0, 4, 8 or 16 μg) of whole cell extracts transfected with pCXN2-hERβcx. Sixteen micrograms of this extract alone were run in lane 5. The amount in each lane was equalized with untransfected cell extracts.

bands (Fig. 3A) and the ERβcx protein as 55 and 51 kDa bands (Fig. 3B) in transfected COS-7 cells. The signal intensities of the bands corresponding to the ERβ and ERβcx proteins respectively were almost equivalent under the conditions used here, suggesting that both proteins are expressed at similar efficiency. Since the ERβcx transcript was detected endogenously in some human cultured cells, such as HEC-1, HOS-TE85 and Saos-2 cells, by reverse transcription PCR (RT–PCR) (data not shown), immunoblotting of these cells was carried out. An immunoreactive band was detected in HEC-1, HOS-TE85 and Saos-2 cells at ~55 kDa, corresponding to the heavier band in COS-7 cells transfected with pCXN2-hERβcx (Fig. 3C). No detectable band was detected in mock-transfected COS-7 cells.

Estrogen binding activities of ERα, ERβ and ERβcx

[^{3}H]E_2 radioligand binding assays were performed with whole cell extracts of transfected COS-7 cells. A representative result of the saturation test is shown in Figure 4, which demonstrates that the average dissociation constants (K_d) of ERα and ERβ are 0.2 and 0.6 nM respectively, as determined by Scatchard analysis (data not shown). In contrast, ERβcx showed little binding to E_2.

DNA binding ability of ERα, ERβ and ERβcx

Whole cell extracts were prepared from COS-7 cells transfected with ER expression vectors and used for gel shift assay. The ERα–DNA complexes, which were blocked by cold ERE probe, were detected as a distinct band (Fig. 5A, lanes 1–4). The ERβ–DNA complexes were also observed at a higher mobility than ERα–DNA complexes, which were also blocked by cold ERE (Fig. 5A, lanes 5–8). However, we could hardly detect any shifted band when whole cell extracts of COS-7 cells transfected with pCXN2-hERβcx were used (Fig. 5A, lanes 9–12). The effect of ERβcx on the ERα–DNA and ERβ–DNA complexes was also examined. As shown in Figure 5B, DNA binding by ERα was inhibited in the presence of ERβcx (a reduction of ~40% at 16 μg whole cell extract transfected with pCXN2-hERβcx), in contrast to the lack of an influence on ERβ–DNA complexes (data not shown).

Lack of transcriptional activity of ERβcx and lack of interaction of ERβcx with the cofactor TIF1α

Upon stimulation of cells transfected with ERα or ERβ expression vectors with 10^{-7} M E_2, CAT activity from the ERE-GCAT construct increased 25- and 9-fold respectively, whereas it did not change in the case of ERβcx (Fig. 6A). *In vitro*-translated TIF1α was pulled down ligand-dependently by GST–hERα and GST–hERβ (Fig. 6B, lanes 2–5) and, at the same time, it was not pulled down by either GST–hERβcx (Fig. 6B,

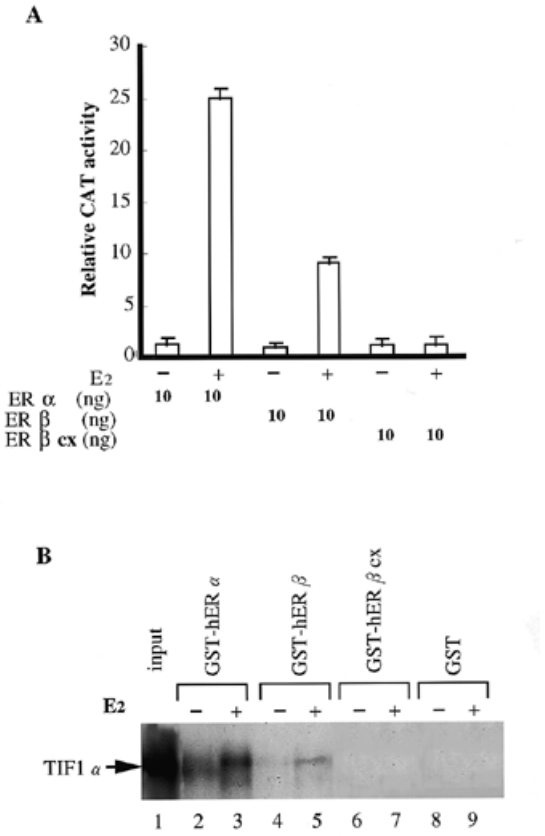

Figure 6. Transcriptional activity of ERβcx and its inability to interact with a cofactor, TIF1α. (**A**) Transactivation of the ERE-GCAT reporter by ERα, ERβ and ERβcx. COS-7 cells were transfected with 2 µg ERE-GCAT reporter plasmid and 10 ng wild-type ER expression vectors respectively. The transfected cells were incubated for 24 h in the presence (+) or absence (–) of 10^{-7} M E_2 and CAT activities were normalized relative to β-galactosidase activity expressed from the PCH110 internal control vector and are reported as means ± SD, calculated from three independent experiments. CAT activities are indicated as the amounts of fold induction. (**B**) Lack of interaction between ERβcx and TIF1α. The GST pull-down assay was performed as indicated except that GST fusion proteins, each estimated as of equal quality on Coomassie stained gels (data not shown), were preincubated for 30 min in 500 µl NETN buffer minus NP-40 in the presence (+) or absence (–) of 10^{-7} M E_2; 20% of the input is shown in lane 1.

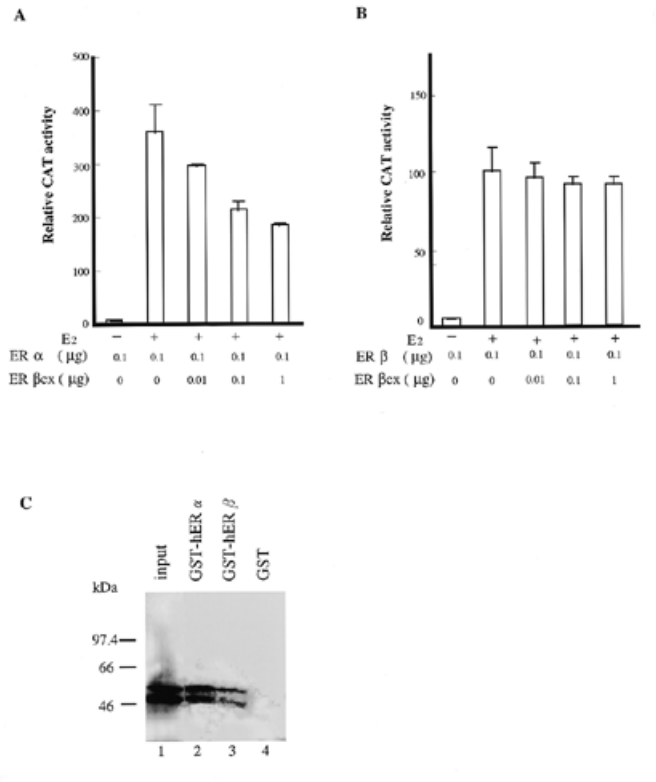

Figure 7. Dominant negative activity of ERβcx in the ERα signaling pathway. (**A**) Dose-dependent inhibition by ERβcx of ERα transactivation. The cells were transfected with 0.1 µg ERα expression vector and ERβcx expression vector at the indicated amounts (0.01–1 µg) in the presence (+) or absence (–) of 10^{-7} M E_2. CAT activities are reported as means ± SD, calculated from three independent experiments. (**B**) ERβcx did not influence transactivation by ERβ. The experimental conditions were as in (A) except that the cells were transfected with 0.1 µg ERβ expression vector instead of ERα expression vector. (**C**) GST pull-down assay. ERβcx protein was synthesized *in vitro* using the TnT-coupled reticulocyte lysate system (Promega); 20% of the input is shown in lane 1. After binding of GST–hERα, GST–hERβ and GST proteins, each estimated as of equal quality on Coomassie stained gels (data not shown), to glutathione beads, 15 µl suspension were incubated with 1–2 µl of the appropriate ^{35}S-labeled, *in vitro* translated protein for 1 h in 500 µl NETN buffer. Bound proteins were eluted and electrophoretically separated on a SDS–7.5% polyacrylamide gel.

lanes 6 and 7) or GST alone (Fig. 6B, lanes 8 and 9) in the presence or absence of E_2.

Transcriptional activity of ERβcx in the presence of ERα or ERβ

When ERα or ERβ was co-transfected with ERβcx, ERβcx rather inhibited ligand-induced transactivation by ERα (Fig. 7A), but did not influence transactivation by ERβ (Fig. 7B), which was also confirmed when we used 293T cells (data not shown). The inhibitory activity of ERβcx against ERα was more pronounced as increased amounts of the ERβcx expression vector were added in the presence of ERα (0.1 µg expression vector), a 10× co-expression ratio leading to 52% inhibition of transactivation of ERα (Fig. 7A). *In vitro* translated products of ERβcx consisted of two proteins of 55 and 51 kDa, both of which were preferentially pulled down using GST–hERα and GST–hERβ (Fig. 7C, lanes 2 and 3), although they were not pulled down by GST protein alone (Fig. 7C, lane 4).

DISCUSSION

In this study we have identified an endogenous variant of ERβ and named it ERβcx. Among three in-frame ATG codons of ERβcx, located at nt 1276, 1412 and 1435, preceded by an in-frame stop codon at nt 1210, the first and second ATG codons conform to the Kozak consensus sequence (32). This first ATG codon is identical to that of ERβ, recently reported (15), encoding the additional 53 amino acids in its A/B domain compared with the previously published ERβ sequence (14). The open reading frame of ERβcx encodes a protein of 495 amino acid residues with a calculated molecular weight of 55.5 kDa (calculated from the first methionine).

ERβcx is identical to ERβ for the first 469 amino acids, but differs from ERβ in replacement of the last 61 amino acids of the

latter by a unique 26 amino acid sequence. The 61 amino acids of ERβ include the AF-2 core, which is essential for ligand-dependent transcriptional activation, including cofactor binding (29–31), and their substitution suggests that ERβcx may lose its transactivation capacity (see below).

We have shown that alternative splicing of the human ERβ primary transcript produces a novel isoform, ERβcx, differing at the last exon. Both the ERβ- and ERβcx-specific intron–exon junctions obeyed the splice consensus sequence (33); AG nucleotides at the end of each intron followed by the exon sequences. Moreover, the branch site is also conserved at ~30 nt upstream of the splice site of the ERβcx-specific exon (Lcx), retaining the target A nucleotide (34). Although distinct splicing mechanisms between Lcx and the ERβ-specific exon (L4) (35) have yet to be elucidated, we have also shown that ERβcx inhibits E_2-dependent transactivation by wild-type ERα. Similar cases exist for other nuclear receptors, such as hGRβ (17), hTRα2 (18) and rVDR1 (19). These are also alternative splicing variants substituting part of the last exon, including the AF-2 core, by unique sequences (see Fig. 1B and C). As a result, GRβ, TRα2 and rVDR1 expressed endogenously act as dominant negative isoforms against cognate wild-type receptors. This type of regulation may be conserved among several members of the nuclear receptor superfamily.

In the case of ERα, although the corresponding C-terminal spliced isoform remains to be identified, several other isoforms have been reported. The ER ΔE3 isoform, which harbors a deletion of exon 3 encoding the second zinc-finger of the DBD, especially inhibits E_2-dependent transcription activation in a dominant negative fashion when it is co-transfected with the wild-type ER and a reporter plasmid (10). We previously reported that the ERα Δexon 4/5 isoform also inhibits wild-type ERα (9). Recently, ERβ isoforms other than ERβcx have been reported, although they were not characterized for their transactivation properties (36,37).

Northern blot analysis showed that in some human tissues, such as ovary and testis, expression of ERα, ERβ and ERβcx overlapped. Co-localization of these ERs may play some role in reproduction, at least in ovary and testis. It was reported that both ERα and ERβ mRNAs are co-expressed in some human breast tumors, suggesting their involvement in malignancy (38). Although expression of ERβ was not confirmed specifically in human prostate, ERβcx was still detectable where expression of ERα and ERβcx overlapped. In these organs, ERα/ERβcx heterodimer formation and their transcriptional suppression may be of physiological importance.

The specificity of the anti-ERβ and anti-ERβcx antibodies was verified by immunoblotting of whole cell extracts of COS-7 cells transfected with each ER expression vector (Fig. 3A and B), along with the result that ERβ and ERβcx proteins were expressed with similar efficiency. The sizes of the ERβ and ERβcx proteins in transfected COS-7 cells were 60 and 57 kDa for the former and 55 and 51 kDa for the latter, as determined by mobility on the gel, suggesting their translational initiation from both the first ATG and the second downstream ATG. We also demonstrated that the 55 kDa ERβcx protein was detectable in HEC-1, HOS-TE85 and Saos-2 cells. Taken together, these results suggest that ERβcx is translated from the first ATG *in vivo*. The precise distribution of these ERs at the tissue and cellular levels in the human will be required for analysis of the detailed mechanisms of regulation.

Unlike ERα and ERβ, ERβcx lacks ligand binding ability and does not have or has only very low ERE binding ability, resulting in the loss of ligand-dependent transactivation ability. In the case of ERα, the 521–528 amino acid region of exon 8 is essential for ligand binding (39). The E_2 binding inability of ERβcx must be due to substitution of the corresponding L4 exon by the Lcx exon. However, we cannot exclude the possibility that ERβcx has a much reduced ERE binding activity, because the gel shift assay may require a threshold level of affinity for gel shifted protein–DNA complexes (40). Furthermore, ERβcx was incapable of interacting with TIF1α. It was suggested that TIF1α induces chromatin remodeling, thereby allowing nuclear receptors and other transactivators to bind to their cognate response elements (41). The lack of transcriptional activity of ERβcx, as well as its much reduced ERE binding ability, might be partly due to its inability to bind cofactors, such as TIF1α.

We have shown that the dominant negative activity of ERβcx was especially against ERα transactivation, rather than that of ERβ. DNA binding by ERα was inhibited by ERβcx, possibly due to formation of ERα/ERβcx heterodimers. We also showed a preference for forming ERα/ERβcx heterodimers rather than ERβ/ERβcx heterodimers. Recently, Cowley *et al.* (42) reported that ERα homodimers and ERα/ERβ heterodimers bound to the ERE better than ERβ homodimers (~4-fold greater K_d), suggesting that the former are more functional than the latter under physiological conditions *in vivo*. Taken together, we assume that selective suppression of the ERα signaling pathway by ERβcx may be due to the easier formation of transcriptionally inactive ERα/ERβcx heterodimers that have no or much reduced DNA binding activity than that of ERβ/ERβcx heterodimers. Considering the results of the GST pull-down assay, indicating ERβ/ERβcx heterodimers, we cannot exclude the possibility that ERβcx inhibits transactivation by ERβ beyond the experimental conditions described here.

There are other possibilities for the inhibitory mechanism: (i) ERβcx competes with ERα for ERE binding; (ii) specific transcriptional silencing, such as titering out cofactors and basal transcription factors essential for ERα transactivation (40,43–45). Based on our results that ERβcx was hardly capable of binding to the ERE, competition for ERE binding is unlikely to be responsible for the dominant negative properties of ERβcx against ERα. In addition, transcriptional squelching may be negligible in the experimental ranges, since suppression of ligand-induced transactivation was not observed even when 1 μg ERα or ERβ expression vector was added (data not shown).

Our studies do not rule out the possibility that ERβcx may act through a novel, as yet unknown estrogen-responsive pathway, through specific response elements, specific phosphorylation, novel ligand binding and so on. Further characterization of the physiological function of ERβcx will provide more insights into the diverse effects of estrogens *in vivo*.

ACKNOWLEDGEMENTS

We thank Dr Chambon for the gift of the TIF1α plasmid and Drs H.Toyoshima and S.Kato for helpful discussion. S.O. was supported by a JSPS Research Fellowship for Young Scientists. This work was supported in part by research grants from the Ministry of Education, Science and Culture of Japan and in part by CREST (Core Research for Evolutional Science and Technology) of the Japan Science and Technology Corporation.

REFERENCES

1 George,F.W. and Wilson,J.D. (1988) In Knobil,E. (ed.), *The Physiology of Reproduction*. Raven Press, New York, NY, pp. 2–27.
2 Jensen,E.V. and Jacobson,H.I. (1962) *Recent Prog. Hormone Res.*, **18**, 387–414.
3 Evans,R.M. (1988) *Science*, **240**, 889–895.
4 Green,S. and Chambon,P. (1988) *Trends Genet.*, **4**, 309–314.
5 Kastner,P., Krust,A., Mendelsohn,C., Garnier,J.M., Zelent,A., Leroy,P., Staub,A. and Chambon,P. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 2700–2704.
6 Leroy,P., Krust,A., Zelent,A., Mendelsohn,C., Garnier,J.M., Kastner,P., Dierich,A. and Chambon,P. (1991) *EMBO J.*, **10**, 59–69.
7 Zelent,A., Mendelsohn,C., Kastner,P., Krust,A., Garnier,J.M., Ruffenach,F., Leroy,P. and Chambon,P. (1991) *EMBO J.*, **10**, 71–81.
8 Zhang,Q.X., Borg,A. and Fuqua,S.A. (1993) *Cancer Res.*, **53**, 5882–5884.
9 Inoue,S., Hoshino,S., Miyoshi,H., Akishita,M., Hosoi,T., Orimo,H. and Ouchi,Y. (1996) *Biochem. Biophys. Res. Commun.*, **219**, 766–772.
10 Wang,Y. and Miksicek,R.J. (1991) *Mol. Endocrinol.*, **5**, 1707–1715.
11 Kuiper,G.G., Enmark,E., Pelto-Huikko,M., Nilsson,S. and Gustafsson,J.Å. (1996) *Proc. Natl. Acad. Sci. USA*, **93**, 5925–5930.
12 Kuiper,G.G., Carlsson,B., Grandien,K., Enmark,E., Haggblad,J., Nilsson,S. and Gustafsson,J.Å. (1997) *Endocrinology*, **138**, 863–870.
13 Byers,M., Kuiper,G.G.J.M., Gustafsson,J.Å. and Park-Sarge,O.K. (1997) *Mol. Endocrinol.*, **11**, 172–182.
14 Messelman,S., Polman,J. and Dijkema,R. (1996) *FEBS Lett.*, **392**, 49–53.
15 Ogawa,S., Inoue,S., Watanabe,T., Hiroi,H., Orimo,A., Hosoi,T., Ouchi,Y. and Muramatsu,M. (1998) *Biochem. Biophys. Res. Commun.*, **243**, 122–126.
16 Tremblay,G.B., Tremblay,A., Copeland,N.G., Gilbert,D.J., Jenkins,N.A., Labrie,F. and Giguere,V. (1997) *Mol. Endocrinol.*, **11**, 353–365.
17 Oakley,R.H., Sar,M. and Cidlowski,J.A. (1996) *J. Biol. Chem.*, **271**, 9550–9559.
18 Koenig,R.J., Lazar,M.A., Hodin,R.A., Brent,G.A., Larsen,P.R., Chin,W.W. and Moore,D.D. (1989) *Nature*, **337**, 659–661.
19 Ebihara,K., Masuhiro,Y., Kitamoto,T., Suzawa,M., Uematsu,Y., Yoshizawa,T., Ono,T., Harada,H., Matsuda,K., Hasegawa,T., Masushige,S. and Kato,S. (1996) *Mol. Cell. Biol.*, **16**, 3393–3400.
20 Koike,S., Sakai,M. and Muramatsu,M. (1987) *Nucleic Acids Res.*, **15**, 2499–2513.
21 Niwa,H., Yamamura,K. and Miyazaki,J. (1991) *Gene*, **108**, 193–199.
22 Watanabe,T., Inoue,S., Ogawa,S., Ishii,Y., Hiroi,H., Ikeda,K., Orimo,A. and Muramatsu,M. (1997) *Biochem. Biophys. Res. Commun.*, **236**, 140–145.
23 Inoue,S., Kondo,S., Hashimoto,M., Kondo,T. and Muramatsu,M. (1991) *Nucleic Acids Res.*, **19**, 4091–4096.
24 Graham,F.L., and van der Eb,A.J. (1973) *Virology*, **52**, 456–467.
25 Orimo,A., Inoue,S., Ikeda,K., Noji,S. and Muramatsu,M. (1995) *J. Biol. Chem.*, **270**, 24406–24413.
26 Scatchard,G. (1949) *Ann. N.Y. Acad. Sci.*, **51**, 660–672.
27 Klein-Hitpass,L., Schorpp,M., Wagner,U. and Ryffel,G.U. (1986) *Cell*, **46**, 1053–1061.
28 Inoue,S., Orimo,A., Hosoi,T., Kondo,S., Toyoshima,H., Kondo,T., Ikegami,A., Ouchi,Y., Orimo,H. and Muramatsu,M. (1993) *Proc. Natl. Acad. Sci. USA*, **93**, 11117–11121.
29 LeDouarin,B., Zechel,C., Garnier,J.M., Lutz,Y., Tora,L., Pierrat,P., Heery,D., Gronemeyer,H., Chambon,P. and Losson,R. (1995) *EMBO J.*, **14**, 2020–2033.
30 Danielian,P.S., White,R., Lees,J.A. and Parker,M.G. (1992) *EMBO J.*, **11**, 1025–1033.
31 Durand,B., Saunders,M., Gaudon,C., Roy,B., Losson,R. and Chambon,P. (1994) *EMBO J.*, **13**, 5370–5382.
32 Kozak,M. (1989) *J. Cell Biol.*, **108**, 229–241.
33 Breathnach,R. and Chambon,P. (1981) *Annu. Rev. Biochem.*, **50**, 349–383.
34 Sharp,P.A. (1987) *Science*, **235**, 766–771.
35 Enmark,E., Pelto-Huikko,M., Grandien,K., Lagercrantz,S., Lagercrantz,J., Fried,G., Nordenskjold,M., and Gustafsson,J.Å. (1997) *J. Clin. Endocrinol. Metab.*, **82**, 4258–4265.
36 Chu,S., and Fuller,P.J. (1997) *Mol. Cell. Endocrinol.*, **132**, 195–199.
37 Vladusic,E.A., Hornby,A.E., Guerra-Vladusic,F.K. and Lupu,R. (1998) *Cancer Res.*, **58**, 210–214.
38 Dotzlaw,H., Leygue,E., Watson,P.H. and Murphy,L.C. (1997) *J. Clin. Endocrinol. Metab.*, **82**, 2371–2374.
39 Ekena,K., Weis,K.E., Katzenellenbogen,J.A. and Katzenellenbogen,B.S. (1996) *J. Biol. Chem.*, **271**, 20053–20059.
40 Ince,B.A., Zhuang,Y., Wrenn,C.K., Shapiro,D.J. and Katzenellenbogen,B.S. (1993) *J. Biol. Chem.*, **268**, 14026–14032.
41 Le Douarin,B., Nielsen,A.L., Garnier,J.M., Ichinose,H., Jeanmougin,F., Losson,R. and Chambon,P. (1996) *EMBO J.*, **15**, 6701–6715.
42 Cowley,S.M., Hoare,S., Mosselman,S. and Parker,M.G. (1997) *J. Biol. Chem.*, **272**, 19858–19862.
43 Meyer,M.E., Gronemeyer,H., Turcotte,B., Bocquel,M.T., Tasset,D. and Chambon,P. (1989) *Cell*, **57**, 433–442.
44 Tasset,D., Tora,L., Fromental,C., Scheer,E. and Chambon,P. (1990) *Cell*, **62**, 1177–1187.
45 Schodin,D.J., Zhuang,Y., Shapiro,D.J. and Katzenellenbogen,B.S. (1995) *J. Biol. Chem.*, **270**, 31163–31771.