Identification of an isoform of the estrogen receptor messenger RNA lacking exon four and present in the brain

(orphan receptors)

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ABSTRACT   An isoform of the estrogen receptor messenger RNA (ER-mRNA) was identified in RNA from the brain of lizards and rats. Poly(A)$^+$ RNA from brain and uteri was reverse transcribed using gene-specific primer for the ER. The resulting complementary DNA was amplified in a polymerase chain reaction followed by cloning and sequencing of the amplified products. This isoform lacks exon four and is designated Δ4 ER-mRNA. Although several isoforms of the ER have been described from cancerous cells, to our knowledge, none has been identified previously in the brain. Furthermore, the Δ4 isoform is the only isoform detected in normal tissue. The Δ4 isoform appeared most abundant in RNA from brain tissue, whereas uterine RNA contained only trace amounts of the isoform. Apparently, tissue-specific alternative splicing accounts for these differences in abundance. Because exon four encodes a part of the steroid-binding domain, we predict that the corresponding protein encoded by the isoform will not bind estradiol and may therefore belong to a growing subclass of the steroid/thyroid/vitamin superfamily known as orphan receptors. We predict that the putative Δ4 protein may function as a ligand-independent transcription factor that acts on the same DNA response elements as the conventional ER. The abundance of this isoform in the brain relative to the uterus raises fundamental questions regarding the regulation of estrogen-responsive genes in different tissues.

The estrogen receptor (ER) is a member of the steroid/thyroid/vitamin superfamily of nuclear receptors. The most thoroughly studied members of this family function as ligand-activated transcription factors that bind to sequence-specific response elements in the DNA to modulate transcription of specific sets of genes (for reviews, see refs. 1–7). Molecular cloning of the cDNA for many of these steroid receptors has led to rapid advances in our knowledge of these proteins. Point mutation studies, deletion analysis, and domain swapping experiments have provided an understanding of structural and functional relationships. Discrete functional domains for DNA binding, ligand binding, and transcriptional activation have been identified. Nevertheless, many fundamental gaps and uncertainties remain. For example, the issues of whether or not steroid receptors exist as homodimers and bind ligand in a cooperative manner remain controversial (7). The mechanism whereby the receptor stimulates or represses gene transcription remains obscure.

There is growing evidence that some of the physiological effects of steroids may occur by binding to various membrane proteins (8). In addition, the vast majority of this superfamily may be comprised of orphan receptors, a term used to designate members of this family that have no known ligand (9). Although some orphan receptors may require as yet unidentified ligands to function, others may not bind or require any ligand to function. The latter subclass of orphan receptors would not be true receptors in the classical endocrine sense for they would not bind a hormone (9). Yet these proteins may retain their activity as transcription factors, perhaps being activated by other signaling pathways such as phosphorylation. Alternatively, such ligand-independent orphan receptors may modulate the activity of true receptors by protein–protein interactions.

The role of steroid hormones in regulating reproductive behavior in whiptail lizards (*Cnemidophorus inornatus*) is of long-standing interest in our laboratory. To advance these studies, in March 1991, we initiated experiments to map the brain nuclei containing ER-mRNA by *in situ* hybridization. To obtain the species-specific antisense probes required for such studies, we used reverse transcriptase (RT)-PCR to amplify segments of the ER complementary DNA (cDNA) from RNA extracts. DNA products of several sizes were produced. Mindful of the potential of PCR for amplification and detection of alternately spliced mRNAs, we subsequently cloned and sequenced these PCR products. The results indicated that the larger DNA fragment was derived from the conventional ER-mRNA, but the smaller DNA product lacked exon four and probably arises by alternative splicing of ER-mRNA. We designated this isoform Δ4 ER-mRNA. To extend these preliminary findings regarding Δ4 ER-mRNA, it was desirable to use a mammalian animal model that was commercially available, that provided greater amounts of tissue compared to our lizards, and for which the ER-cDNA had been cloned and sequenced. Only rat, human, and chicken ER had been sequenced in January of 1992 when these studies were initiated. Rats were chosen to continue these studies on Δ4 ER isoform, and it is the results of these studies that we report herein.

MATERIALS AND METHODS

Animals. Ovariectomized rats (Wistar strain) were purchased at 60 days of age (Harlan–Sprague–Dawley) and housed for 1 week before use. Tissues (uteri and brains) were excised within 2–4 min of CO_2 asphyxiation and immediately frozen in liquid nitrogen for subsequent storage at −80°C until use. For some animals, a block of tissue (about 1.5 cm^2 and 1 cm deep) containing the hypothalamus was dissected from the brains and frozen as described.

RNA Extraction and cDNA Synthesis. Total RNA was extracted from these tissues using the guanidinium pH 4 method of Chomczynski and Sacchi (10). Inclusion of an additional phenol/chloroform extraction was the only modification of this procedure. Poly(A)$^+$ RNA was separated from total RNA using the PolyATtract mRNA isolation

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system (Promega), according to the manufacturer's instructions. The purity and quantity of the RNA were assessed by spectrophotometric scanning from 300 to 220 nm. All preparations had A_{260}/A_{280} ratios of 1.8 or higher.

Maloney murine leukemia virus RT (GIBCO/BRL) was used to copy poly(A)$^+$ RNA (2 μg) into cDNA with a gene-specific primer, 7F, at 20 pmol per reaction (11). This gene-specific primer is an antisense primer complementary to a segment of the ER-mRNA in exon seven: nt 1673–1650 according to the numbering of the rat ER sequence reported by Koike et al. (12).

For the PCR, we used the nested primer strategy (13) to increase the specificity of the amplified products. An Eppendorf Microcycler E was used. Following an initial denaturation for hot start condition (95°C for 5 min), Taq DNA polymerase was added. Then, the thermocycler program cycled 30 times through the following steps: annealing at 57°C for 60 sec, primer extension at 72°C for 3 min, and denaturation at 95°C for 30 sec.

DNA products were cloned into pCR II plasmid (Invitrogen) using reagents supplied by this company. The resulting recombinant DNA was purified using Qiagen columns (Chatsworth, CA) using buffers and procedures described by the manufacturer.

DNA Sequencing. Double-stranded DNA sequencing was performed by the Sanger dideoxy method (14) using Sequenase T7 DNA polymerase and reagents as described by the supplier (United States Biochemical).

The following synthetic oligonucleotide primers were purchased from Midland Certified Reagent (Midland, TX): 1B (nt 611–630) 5'-CTACTACCTGGAGAACGAGC-3'; 3A (nt 868–888) 5'-GACATAATGACTACATGTGT-3'; 7F (nt 1650–1673) 5'-GTTGATCTTGTCCAGGACTCGGTG-3'; 5C (nt 1353–1374) 5'-AGGAGGCACACTCCAGAAGGTG-3'.

RESULTS

Poly(A)$^+$ RNA (2 μg) from hypothalamic-enriched brain tissue or from uteri was reverse transcribed into cDNA in a 20-μl reaction using an ER-gene-specific primer from exon seven (primer 7F). One-tenth of this cDNA reaction was then subjected to amplification by PCR using the nested primer strategy. For the first round amplification (30 cycles), we used primers 1B and 7F. For the second round of amplification, 2 μl of the first PCR reaction served as template with an internal set of primers, 3A and 5C. Analysis of the DNA products from the second round of PCR by agarose gel electrophoresis (Fig. 1) revealed the presence of two bands: one of 518 bp and the other of 180 bp. These bands have the exact lengths expected for the amplification of the normal and Δ4 ER isoform, respectively.

Each band was isolated from low-melt agarose gels and melted in 100 μl of water. A small fraction of this material (2–4 μl) was reamplified with the same set of internal primers. Analysis of the resulting products indicated selective reamplification of each individual DNA band (data not shown). These reamplified DNA molecules from each band were separately cloned into pCR II plasmid. The resulting recombinant DNAs were purified on Qiagen columns and subsequently sequenced. The DNA sequence data (Fig. 2) provided definitive evidence for the identity of these two bands. The sequence of the 518-bp band showed that its termini were bounded by the internal primers and that all three exons (three, four, and five) were continuously present in the sequence. Fig. 2 shows the exon boundaries for the 518-bp band. This band, therefore, was amplified from the conventionally spliced form of the ER-mRNA. The sequence of the smaller, 180-bp fragment showed that its termini were also defined by the internal primers. More importantly, exon 4
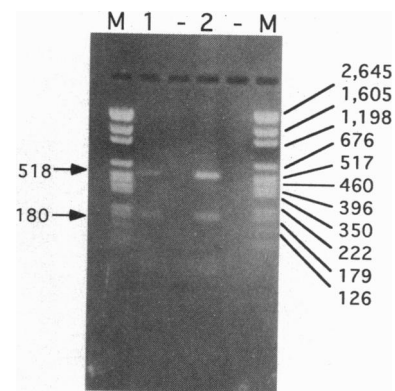

FIG. 1. Size analysis of PCR products by agarose gel electrophoresis. Either 10 μl (lane 1) or 20 μl (lane 2) of a second round PCR reaction (100 μl) was loaded onto a 1.0% agarose gel and subjected to electrophoresis at 44 V. Band sizes of products were determined from plots of the migration of size standards loaded in lanes marked M. The sizes of these standards are indicated in base pairs.

had been deleted as evidenced by the 3–5 junction sequence. Thus, the Δ4 isoform of the ER-mRNA is present in RNA from brain tissue enriched with hypothalamic cells.

DISCUSSION

Our first set of experiments was designed to test for the existence of the Δ4 isoform of the ER-mRNA isoform in RNA extracted from rat tissues. For this purpose, we used RT-PCR incorporating the nested primer strategy to achieve selective amplification of the target cDNA. With this second set of internal primers in the nested primer strategy, the PCR reaction should amplify across exon four and consequently produce only the normal form and the Δ4 isoform. This strategy excluded the possibility of amplifying other isoforms that might have been present and thereby simplified the analysis.

The results reported herein showed that this strategy did result in the amplification of only two DNA products that had sizes corresponding to those predicted for the target segments. Subsequent cloning and complete sequencing of these DNA products provided definitive evidence that the larger product (518-bp band) is the normal form, whereas the smaller product (180-bp band) is the Δ4 isoform (see Fig. 2). The smaller product has the same terminal sequences as the larger product as defined by internal primers, but had a 3–5 exon junction reflecting the complete and precise excision of exon four. We assume that the Δ4 isoform most likely arises by an alternative splicing mechanism even though we have not directly determined this in precursor product studies.

The identification of the Δ4 isoform in RNA from rat tissues extends our initial observations regarding the occurrence of this isoform in RNA from lizard brains (L.J.Y., unpublished work). These findings imply that the alternative splicing mechanisms for the production of the Δ4 isoform as well as the physiological function(s) of this isoform have been conserved in evolution from the common ancestors of reptiles and mammals.

Several laboratories have recently identified various mutations of the ER-mRNA that appear to be due to changes at the gene level (15, 16). Also, other isoforms of the ER-mRNA have been identified in cancerous cell lines or primary tumors (17–19). In these studies, isoforms lacking exons two, three, five, and seven have been identified. They apparently arise by aberrant splicing, perhaps due to unknown perturbations associated with the malignant state of these cells. These workers also used RT-PCR to detect these isoforms. However, their choice of primers located in exon four only
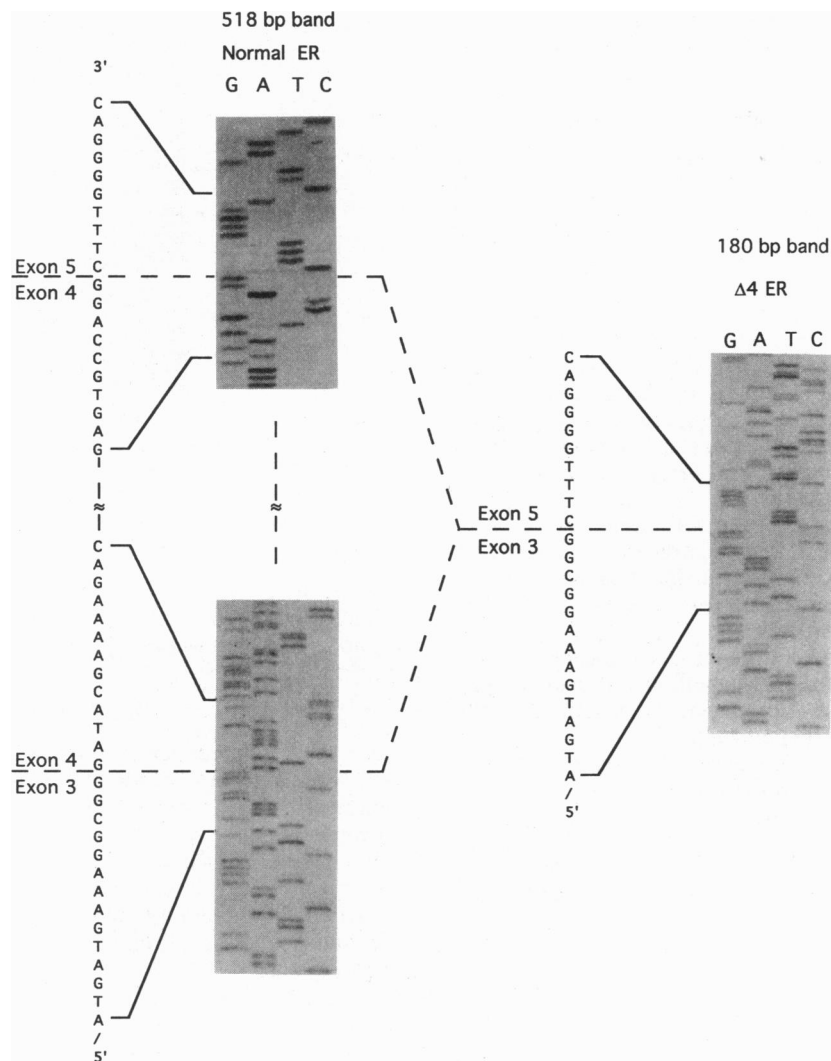
FIG. 2.   Nucleotide sequence of exon boundaries in the 518-bp clone and 180-bp clone. The two PCR products (518- and 180-bp bands) were cloned into pCR II vector and sequenced as described. Only the exon boundary sequences are shown for both clones.

precluded the possibility of detecting a Δ4 isoform. Preliminary data from Koehorst *et al.* (20) noted that a PCR product similar in size to Δ4, but no sequence data were provided to confirm this. We have not detected any of these isoforms in normal tissues under conditions where Δ4 is readily apparent (J.K.S., unpublished results). After this paper was submitted, however, Pfeffer *et al.* (21) reported convincing evidence for the existence of the Δ4 isoform in mammary carcinoma cells.

In contrast to most of the isoforms that have been identified in malignant cells, the Δ4 isoform of the ER-mRNA reported here occurs in normal tissues of rats and lizards. It apparently arises by tissue-specific alternative splicing with the highest abundance in the hypothalamus as judged by the amount of amplified DNA. The relative intensities of the normal and Δ4 isoform products generated by PCR and visualized by ethidium bromide staining in agarose gels would suggest that this isoform comprises 40–60% of the amplified cDNA forms present in hypothalamic cDNA, whereas only trace amounts of the Δ4 isoform are amplified in uterine RNA. Although these apparent levels were very reproducible under the PCR conditions described, we nevertheless consider such estimates to be very crude at best. Preferential amplification of smaller bands may occur in PCR, and if this occurs in an early cycle of an exponential amplification, then the magnitude of this preferential amplification would be greatly exaggerated.

If these estimates are confirmed by Northern analysis or ribonuclease protection assays, they imply that the Δ4 isoform may have a prominent role in hypothalamic nuclei and that these cells may be the proper source of material to search for the corresponding protein.

Comparison of the nucleotide sequence of the normal ER-mRNA and the Δ4 isoform shows that a frameshift mutation would not be introduced by excision of exon four. Thus, an open reading frame is retained in the Δ4 isoform. Although it is generally assumed that levels of a poly(A)$^+$ RNA correspond to the levels of the corresponding translational product, the proteins for this isoform or any of the other isoforms have yet been identified in cell extracts.

Blaustein (22) has detected immunohistochemical staining ER in the cytoplasm of neurons. Whether this represents synthesis of new ER on polyribosomes or shuttling of the ERs between nucleus and cytoplasm remains unclear. Blaustein has suggested on the basis of results obtained with three different antibodies that there may be fundamental differences in the function of some ERs in the brain compared to peripheral reproductive tissues. Also, he suggested that different neurons may have either different classes of ERs or at least ERs with different functions. Our finding lends support to these ideas. Immunohistochemical studies using H222 antibody, which recognizes the C-terminal portion of the steroid-binding domain, would have detected both forms.

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Similarly, when probes that are complementary to common sequences in both isoforms are used in *in situ* hybridization studies to locate estrogen-sensitive cells, both isoforms would be detected. Thus, quantification based on such probes may overestimate the normal form of ER.

The identification of this isoform of the ER-mRNA in a normal hypothalamic tissue is inherently interesting even if it is not translated into protein since it would imply the use of splicing mechanisms to regulate normal ER-mRNA levels. Its presence in the hypothalamus raises many new questions. Is Δ4 ER-mRNA translated into the corresponding protein and, if so, what is its function? Because exon four encodes a portion of the steroid-binding domain, we assume that the corresponding protein of this isoform of the RNA will be translated into an orphan receptor. If so, its presence may demand some modifications in current models for the mechanism of estrogen action, especially in brain tissues. Currently, one model of estrogen action is assumed to be generally applicable to all tissues. Among the possible functions for the putative Δ4 ER protein is that it may act as a ligand-independent transcription factor to effect a degree of constitutive (estrogen-independent) regulation of estrogen-responsive genes, whereas the conventional ER may further modulate the expression of the same set of target genes in the cells. Several estrogen-responsive genes in the ovariectomized rat uterus are expressed at a basal level and estrogen substantially enhances the expression of these genes (23). This model would predict that reproductive and neural cells may display differing degrees of sensitivity to estrogen activation of gene expression, depending on the relative abundance of these two receptor forms.

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