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Estrogen regulates the expression of several different estrogen receptor mRNA isoforms in rat pituitary

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ABSTRACT **A 5.2-kb mRNA band that contains estrogen receptor (ER) sequence and exhibits sex- and tissue-specific expression has been identified in rat pituitary via Northern analysis; this band is composed of at least two distinctive ER mRNA isoforms. This mRNA is expressed in high levels in female pituitary but is absent in male pituitary and uterus, whereas the mRNA encoding the full-length receptor (6.2 kb) is expressed in all the aforementioned tissues. Estradiol treatment potently induces the expression of the 5.2-kb band in the male pituitary. Oligonucleotide hybridization and ribonuclease-protection experiments indicate that the pituitary ER variant is missing exons 1–4. Two corresponding cDNA clones, truncated estrogen receptor product 1 and 2 (TERP-1 and TERP-2), were isolated by using the anchored PCR. Both sequences contain a 31-bp segment of specific sequence upstream of exon 5; TERP-2, however, contains an additional 66 bp of specific sequence between the 31-bp segment and exon 5. On Northern analysis, probes complementary to the 31-bp segment of specific sequence hybridize only to the 5.2-kb band. Immunoblotting identified several proteins in rat pituitary that could represent the translation products of these or related transcripts. In summary, several ER isoforms have been identified that exhibit both tissue-specific expression and marked estrogen regulation and differ from full-length receptor by virtue of sequence upstream of the exon 4/5 boundary. Physiologically, the putative proteins encoded by these or similar isoforms might be important modulators of the tissue- and promoter-specific effects of estradiol.**

The estrogen receptor (ER) is an important mediator of growth and development in reproductive tissue. Like other members of the steroid-receptor family, it is a ligand-activated nuclear regulatory protein with discrete functional domains (1–3). Functioning primarily as a dimer, the ER has been shown to be capable of binding to highly conserved DNA sequences, collectively known as estrogen response elements, which are present in the regulatory regions of estrogen-responsive genes (4–8). It is through this sequence-specific binding that the ER influences transcription rates, although the presence of other facilitating transcription factors often appears to be required (9, 10).

Many ER variants have been identified in both human breast cancer cell lines and clinical breast cancer specimens. These variants, which frequently result from alternative mRNA splicing or single nucleotide alterations, include isoforms that have been shown to behave in either a dominant-negative or dominant-positive manner (11–15). Multiple ER mRNAs and proteins have also been identified in normal tissue from a variety of species, although the physiological significance of such heterogeneity has not yet been fully determined (16–20). Our laboratory previously reported the existence of a rat pituitary ER mRNA, identifiable on the basis of its more rapid migration on Northern analysis (21). Analysis of this band indicates that it consists of several ER mRNA isoforms with specific upstream sequence; expression of the predominant isoform is both tissue-specific and estrogen inducible.†

MATERIALS AND METHODS

RNA Isolation/Detection. Pituitary and uterine RNA was isolated via ultracentrifugation of tissue homogenates through a cesium gradient (22). Poly(A)$^+$ RNA was isolated on an oligo-(dT) column (Collaborative Research) (23). RNA was electrophoresed across a 1% agarose gel containing formaldehyde and then transferred to nitrocellulose (Schleicher & Schuell). A randomly primed cDNA complementary to the entire coding region of the ER was used as probe (23), as were oligonucleotides end-labeled with T4 kinase and [γ-^{32}P]ATP. RNase protection was done with a described protocol (23).

Oligonucleotides. The sequence and corresponding amino acid position (based on the published sequence of rat 6.2-kb mRNA) (24) of the synthetic oligonucleotide probes used in Northern blotting were as follows: ERR-1 [5′ untranslated region (UT)], 5′-AAGCGTCTTTCCAGAAATGTTCCATGGGTTTG-3′; ERR-2 (aa 4–14), 5′-CAAGGCCATTCCCGAGGCTTTGGTGTGAAGG-3′; ERR-3 (aa 183–193), 5′-ACACACAGCACAGTAGCGAGTCTCCTTGGCAGA-3′; ERR-4 (aa 203–214), 5′-AAGAAAGCCTTGCAGCCTTCACAGGACCAGAC-3′; ERR-5 (aa 274–284), 5′-TTCATTTCGGCCTTCCAAGTCATCTC-3′; ERR-6 (aa 391–400), 5′-GGAGCGCCAGACCAGACCAATCATCAGGAT-3′; ERR-7 (aa 508–517), 5′-GGAAAGGATGAGGAGGAGCTGGGCCAGACG-3′; ERR-8 (aa 526–536), 5′-CTTGCATTTCATGTTGTAGAGATGCTCCATGCC-3′; and truncated ER product 1 (TERP-1), 5′-GCCTGGTCGCTGTTCAACAAGCTCAAGAAATG-3′.

Anchored PCR/PCR. Anchored PCR was done by using the protocol outlined in the 5′ rapid analysis of cDNA end procedure (RACE) system (GIBCO/BRL). First-strand synthesis was done with 5′-GTTGTAGAGATGCACCATGCC-3′ (aa 530–536). RNA template was done with RNase H; homopolymeric tailing of the cDNA was done with terminal deoxynucleotidetransferase and dCTP. PCR was first done with the anchor primer included in the kit and 5′-AGGAGCAAACAGGAGCTTCCC-3′ (aa 405–412). A second PCR amplification was done with the kit's universal amplification primer and 5′-CAUCAUCAUCAUTGATCAGGATCTCCAACC-3′ (aa 388–394). The primers contained deoxyuridine to allow for cloning into the pAMP-1 vector with uracil DNA glycosylase.

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Abbreviations: E_2, 17β-estradiol; ER, estrogen receptor; UT, untranslated region; TERP, truncated ER product.

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†The sequences reported in this paper have been deposited in the GenBank data base (accession nos. L38930 and L38931).

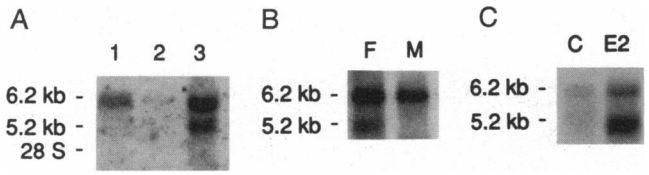

FIG. 1. Expression of the 5.2-kb ER mRNA is tissue- and sex-specific, estrogen inducible. (*A*) Northern blot of 20 μg of total uterine (lane 1), total female pituitary (lane 2), and poly(A)$^+$ female pituitary RNA (lane 3). (*B*) Northern blot of 20 μg of poly(A)$^+$ RNA isolated from female (lane F) and male (lane M) pituitary. (*C*) Northern blot of poly(A)$^+$ RNA from sham (lane C) and E_2-treated (lane E2) males. All blots were hybridized with a ^{32}P-labeled ER cDNA probe.

Full-length TERP-1 and TERP-2 clones were obtained by using 35 cycles of nested PCR with the following primer pairs: 5′-CCATTTCTTGACCTTGTTG-3′ and 5′-CGTTTCAGGGATTCGCAG-3′; 5′-CCATTTCTTGACCTTGTTGAACAGC-3′ and 5′-ATTCGCAGAACCTTGTGG-3′.

Immunoblots. Total cellular pituitary protein was isolated by directly homogenizing tissue in a 50 mM tris(hydroxymethyl)aminomethane solution/2% SDS. Proteins were denatured and separated via SDS/10% PAGE and transferred to nitrocellulose. Blots were blocked in a 5% nonfat dry milk (Carnation)/0.1% polyoxyethylene sorbitan monolaurate (Tween 20) phosphate-buffered solution (pH 7.6) for 60 min followed by primary and secondary antibody incubations for 90 min apiece and washed between each step as described (19). An enhanced chemiluminescence detection technique (ECL; Amersham) was used.

ER-21 (N terminus) (25) and ER-715 (hinge) (19) have been described. C542 (C terminus) is a monoclonal antibody generated by using a peptide sequence corresponding to aa 582–595 of the human ER. This antibody has been demonstrated to immunoprecipitate ^{3}H binding activity in both calf and rat uterus and results in a single immunoprecipitated silver-stained band from MCF-7 cells (D. P. Edwards, personal communication).

Experimental Animals. CD-1 rats, weighing between 200 and 225 g, were obtained from Charles River Breeding Laboratories in accordance with the guidelines established by the University of Virginia. Where indicated, animals received 17β-estradiol (E_2) at 20 μg/100 g of body weight in sesame oil.

RESULTS

Expression of 5.2-kb ER mRNA. ER mRNA is less abundant in pituitary than in the uterus (Fig. 1*A*), and previous studies with total RNA required prolonged exposure times for visualization of the 5.2-kb band (previously called 5.5 kb) (21). Scanning densitometric measurements indicate that the 5.2-kb mRNA represents 20–50% of the total ER mRNA in the multiple samples of pituitary poly(A)$^+$ RNA pooled from females at various stages during the estrous cycle. Uterine poly(A)$^+$ RNA contained little, if any, of the 5.2-kb mRNA (data not shown), as described (21).

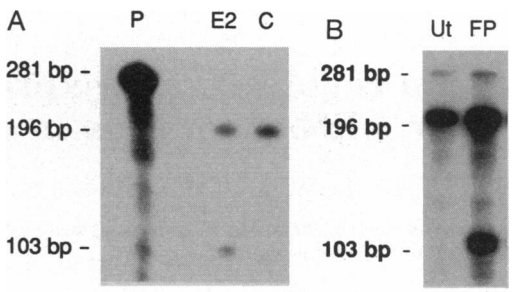

FIG. 3. The structural divergence between the full-length ER and the pituitary isoform(s) maps to within a few bases of the exon 4/5 splice site. (*A*) RNase protection of male control (lane C) and E_2-treated (lane E2) male pituitary RNA. Lane P, probe alone. (*B*) Uterine (lane Ut) and female pituitary (lane FP) RNA. The complementary portion of the probe extends from the *Xba I* site in exon 4 to the *Sma I* site in exon 5. Probe alone migrates at 281 bases (includes 85 bases of vector sequence), full-length-protected product migrates at 196 bases, and a pituitary isoform migrates at 103 bases. Each lane contains 25 μg of total RNA, except for the uterus lane, which contains 12.5 μg.

As demonstrated in Fig. 1*B*, the 5.2-kb mRNA was quite abundant in female pituitary RNA but was not detectable in mature male pituitary poly(A)$^+$ RNA. When males were treated with E_2 for 3 days (pooled serum E_2 level at 1168 pmol/liter), the expression of the 5.2-kb mRNA was markedly induced, increasing from an undetectable level to approximately twice that of the full-length receptor (Fig. 1*C*).

Structural Analysis of 5.2-kb ER mRNA. *Oligonucleotide mapping.* An experiment using an oligonucleotide complementary to the 3′ end of the coding region (which hybridized to both ER mRNA forms on Northern blots) and ribonuclease H, thus removing the large 3′ UT, indicated that the size difference could be localized to 5′ UT and coding regions of the molecule (data not shown). More specific structural analysis was done by using synthetic oligonucleotide probes complementary to different areas of the 5′ UT and coding regions of the 6.2-kb message. Poly(A)$^+$ pituitary RNA from mature females was probed with eight separate synthetic oligonucleotides. As expected (Fig. 2), all oligonucleotides hybridized to the larger mRNA. However, only the oligonucleotides complementary to the hormone-binding domain (ERR-6 to -8) hybridized to the 5.2-kb message; the DNA-binding region (C), A/B region, and 5′ UT oligonucleotides (ERR1-4) did not hybridize to the 5.2-kb mRNA. The hinge region (ERR-5) oligonucleotide hybridized poorly to the 5.2-kb band, suggesting that most mRNA in this band did not contain this sequence; the weak signal, however, suggests that there may be some heterogeneity in these transcripts. These experiments provided gross structural evidence that the 5.2-kb mRNA

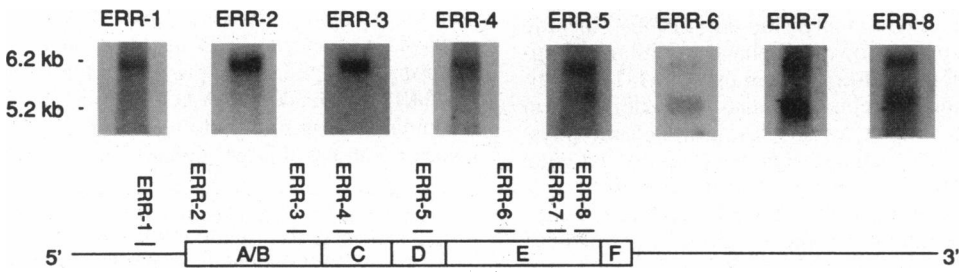

FIG. 2. Oligonucleotides complementary to the area encoding the N terminus of the ER do not hybridize to the 5.2-kb mRNA. Eight oligonucleotides were hybridized to separate Northern blots containing 20 μg of female pituitary poly(A)$^+$ RNA. The region of ER mRNA to which each oligonucleotide corresponds is shown below.

TERP-1 CCATTTCTTGAGCTTGTTGAACAGCGACCAGGCTTTGGGACTTGAATCTGGA

TERP-2 CCATTTCTTGAGCTTGTTGAACAGCGACCAGATGGGAGATGCTTTCAGAGAT
CCCAAGAGTAAGCAGAGCAGCGAGCCAGGTCCACTTGACTTTTCGGCTTTGG

FIG. 4. Anchored PCR isolation of the pituitary ER isoforms (TERP-1 and -2). The 31-base sequence common to both TERP-1 and -2 is contained in the open box; exon 5 sequence is double underlined.

contained a deletion of the area encoding the N-terminal end of the receptor.

RNase protection. On the basis of the oligonucleotide hybridization results, a series of probes were made for RNase protection. These probes included the following portions of the full-length receptor, all crossing the putative exon 4/5 splice site (aa 370): (*i*) HGA-1 (aa 270)–SMA-1 (aa 404), (*ii*) MBO-2 (aa 311)–SMA-1 (aa 404), and (*iii*) XBA-1 (aa 339)–SMA-1 (aa 404). All probes protected full-length mRNA in addition to a 103-base band in tissues where the 5.2-kb mRNA was observed; examples with the XBA-1–SMA-1 probe are depicted in Fig. 3. These experiments demonstrated that the smaller mRNA lacked all sequence upstream of the area roughly corresponding to the exon 4/5 boundary. Expression of the mRNA corresponded to that seen in Northern blotting: absence in uterus and sham-treated male pituitary, high levels in female and E_2-treated male pituitary. A faint band at 99 bases, corresponding to the length of exon 5 included in these probes and most likely representing the exon 4 deletion or a related transcript (20), was seen in male, male E_2-treated, and female pituitary specimens.

Anchored PCR. Anchored PCR was done to determine whether the 5.2-kb mRNA contained specific upstream sequence; cDNA clones TERP-1 and TERP-2, shown in Fig. 4, were obtained. Both were identical to the full-length receptor downstream of the exon 4/5 splice site. TERP-1 possessed 31 bp of specific sequence upstream of the exon 4/5 splice site, whereas TERP-2 had an additional 66 bp located between the 31-bp segment and exon 5. By using RNase protection, TERP-1 was shown to be present in female pituitary but absent from male pituitary or uterus (Fig. 5*A*). No TERP-1 sequence was detectable in female hypothalamus, ovary, or liver (data not shown).

RNase protection with TERP-2 demonstrated that full-length TERP-2 transcript is also estrogen inducible. However, a slightly shorter transcript, consistent in size with an mRNA containing the 66-base addition, but not containing the 31-base segment, appears to be inversely regulated by estrogen (Fig. 5*B*). The relative abundance of these two transcripts is 5- to 10-fold less than that of TERP-1 at its peak expression levels. Northern blotting with an oligonucleotide complementary to the 31-bp segment (Fig. 6) confirmed that this sequence was present in the 5.2-kb but not the 6.2-kb band. Thus, the 5.2-kb mRNA appears to consist of at least two ER isoforms, TERP-1 and TERP-2, and arises as a separate transcript from that previously identified as full-length ER mRNA. Full-length TERP-1 and TERP-2 clones, spanning the sequence from the specific upstream portion of the clones to the 3′ UT of the full-length receptor (exons 5–8), were obtained from female pituitary RNA by using the primer pairs detailed in *Materials and Methods* by reverse transcription–PCR (data not shown).

Immunoblotting. Immunoblotting was done with three different ER antibodies to determine whether these or related isoforms were being translated *in vivo* (Fig. 7). Immunoblots using ER-21 (N terminus) and ER-715 (hinge) detected a dominant product at ≈67,000 M_r, which is consistent with the previously reported value for the full-length receptor. However, antibody C542 (C terminus) bound equally well to a number of smaller proteins, several of which appeared to be estrogen-regulated. Bands at ≈59,200 M_r and 36,800 M_r decrease with E_2 treatment, whereas bands at ≈20,500 and 19,000 M_r increase. Although the absolute identity of each band is unknown, the sizes of the smaller two bands are comparable to what might be produced from the translation of TERP-1 and TERP-2.

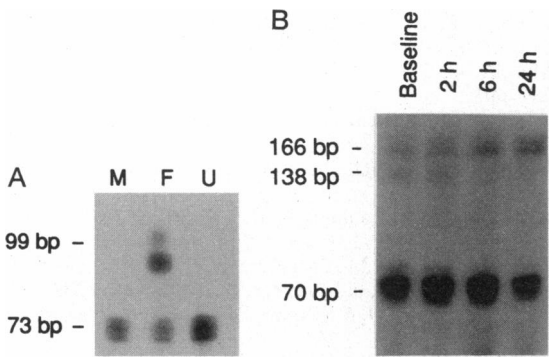

FIG. 5. RNase protection with anchored PCR products TERP-1 and -2. (*A*) TERP-1 is present in female pituitary (lane F) but not in male pituitary (lane M) or uterine (lane U) RNA. The probe shares 73 bases of homologous sequence with the full-length receptor and contains 27 bp of specific sequence. Therefore, transcripts from this clone would be expected to migrate at 100 bases. Each lane contains 25 μg of total RNA, except for the uterus, which has 12.5 μg. The relative positions of 99- and 73-base size markers are shown. (*B*) TERP-2 is estrogen-inducible in male pituitary. TERP-2 RNA protects a band at 166 bases; full-length receptor protects a band at 69 bases.

DISCUSSION

We have identified two estrogen-inducible isoforms of ER mRNA that are present in rat pituitary. The predominant form, TERP-1, exhibits both tissue- and sex-specific expression. In comparison with the full-length receptor, both TERP-1 and TERP-2 lack the A/B, DNA-binding, and hinge regions, as well as a small portion of the hormone-binding domain. In addition, both isoforms contain specific upstream sequence: a 31-base segment common to both TERP-1 and TERP-2 and a 66-base segment specific to TERP-2. The 66-base portion of TERP-2 may represent the intron sequence immediately upstream of exon 5. This sequence (intron–CTTTTCGGCTTTG–exon) has high homology to the previously cloned human sequence (intron–GTTTTCAGGCTTTG–exon) (26). The 31-base segment appears to be a separate exon.

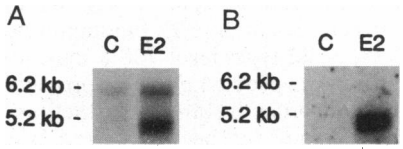

FIG. 6. An oligonucleotide complementary to the specific sequence in the anchored-PCR clone hybridizes only to the 5.2-kb band. (*A*) Northern blot of poly(A)$^+$ RNA from sham (lane C) and E_2-treated (lane E2) males hybridized with an ER cDNA probe. (*B*) Same blot hybridized with TERP-specific oligonucleotide.

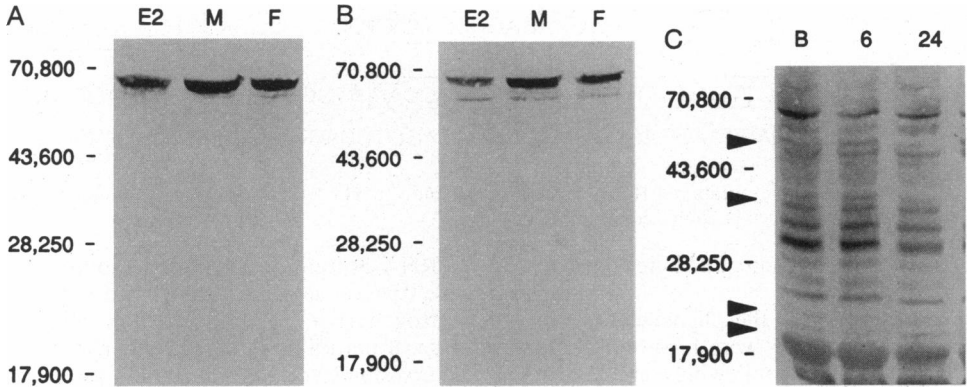

FIG. 7. Immunoblotting. Each lane contains 150 μg of total cellular protein. Positions of size markers are shown for each blot. (*A*) ER-21 (N terminus). Protein preparations from E_2-treated males (lane E2), control males (lane M), and females at various stages of the estrous cycle (lane F) are shown. (*B*) ER-715 (hinge). (*C*) C542 (C terminus). Protein preparations from untreated males (lane B) and males 6 (lane 6) and 24 hr (lane 24) after receiving E_2 injections are shown. Arrowheads show relative positions of bands that appear to either increase or decrease with E_2 treatment.

The specific portion of TERP-2 contains two initiation codons, neither of which is in phase with the intact portion of the ER, raising the possibility that TERP-2 might encode an entirely different protein. Alternatively, TERP-2 may simply be an unprocessed RNA transcript in which the 66-base intron is spliced out to form TERP-1. Also there could be other exons further upstream of some of these transcripts that are not yet identified. The 138-bp fragment seen in Fig. 5*B* is one such candidate transcript because it has not yet been cloned via anchored PCR and additional sequence would not be protected with a TERP-2 probe.

No initiation codons (ATG) were present in the specific portion of TERP-1, although there are six in-phase methionine codons within the first 300 nt of exons 5 and 6. Several of these codons are consensus sites (A/GNNATGG) for initiation, suggesting that translation from these sites is possible (27). Previous *in vitro* translation studies with a mutant ER lacking the first 281 aa residues of the wild type yielded multiple proteins, ranging in size from ≈10,000 to 35,000 M_r, clearly indicating that downstream areas could serve as translation initiation sites (28). Several synthetically generated ER variants with N-terminal truncations have been demonstrated to be stable proteins in yeast and mammalian expression systems; variants containing intact hormone-binding domains could bind E_2 (29).

Immunoblot experiments identified a number of smaller proteins that could be the translation products of these or related transcripts. Although these studies have not definitively identified the TERP-1 and TERP-2 translation products, they are consistent with the concept that there may be several ER isoforms that lack N-terminal residues. Clearly, further analysis with immunoprecipitation and/or direct protein sequencing is required to provide more definitive information.

The ER isoforms we have identified may arise from transcription from an alternate promoter, as has been suggested to occur in the human ER gene (17, 18), or they may be the product of a separate gene. Insertional disruption of the mouse ER gene resulted in E_2 binding in uterine tissue of ≈5% of the wild-type level (30), indicating that an alternate gene or gene product might be present. Analysis of ER mRNA expression or E_2 binding in the pituitary in this mouse model has not yet been reported. Regardless of whether or not these isoforms are transcribed from an alternate promoter or a separate gene, it is clear—at least in the case of the predominant form TERP-1–that both sex- and tissue-specific expressions are present; this expression is closely associated with estrogen exposure, suggesting that this isoform may be an important mediator of the effects of estrogen in the pituitary.

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