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Hamster estrogen receptor cDNA: cloning and mRNA expression

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Abstract

Estrogen-induced hamster kidney tumor model serves as a useful model to study the biochemical and molecular mechanisms of hormonal carcinogenesis. In this model, we have demonstrated an increased expression of estrogen receptor mRNA and protein in estrogen-treated kidneys and in estrogen-induced tumors. The sequence information for hamster estrogen receptor gene is not known and has been investigated in this study. A hamster uterus cDNA library was constructed and the 5′-region of the hamster estrogen receptor cDNA cloned from this library using polymerase chain reaction (PCR) methodology. Additionally, hamster kidney polyadenylated RNA was reverse transcribed and PCR amplified using primers that were designed based on maximum homology between mouse, rat and human estrogen receptor cDNAs. These PCR amplified fragments were cloned into plasmid vectors and clones with the expected size of the insert subjected to Southern blot analysis using human estrogen receptor cDNA as a probe. The positive clones on Southern blot analysis and the PCR amplified products from these clones were subjected to DNA sequence analysis. Using this strategy, a full length, 1978 bp hamster estrogen receptor cDNA has been cloned which shows 87% homology with human, 90% with rat and 91% with mouse estrogen receptor cDNA. The deduced amino acid shares 88% homology with human, and 93% with rat and mouse estrogen receptors. Hamster estrogen receptor domain C (DNA binding domain) shows a 100% homology with a similar domain from mouse, rat, human, pig, sheep, horse and chicken estrogen receptor (Genebank reference ID: AF 181077).

1. Introduction

Estrogens primarily elicit their responses by binding to their cognate receptors and then through the interaction of the receptor-ligand complex with the estrogen response elements on estrogen-responsive genes [1–4]. Estrogen receptor (ER) alpha has been shown to be involved in the regulation of the differentiation and maintenance of neural, skeletal, cardiovascular, and reproductive tissues [5,6]. ER alpha is important in the pathophysiology of the disease process and compounds that can modulate ER alpha transcriptional activity are currently being used to treat osteoporosis, cardiovascular disease, and breast cancer [7,8]. Although estrogenic hormones at plasma levels in the low picogram levels exert receptor-mediated biological effects, such as stimulation of uterine growth, at high doses estrogens may induce pathophysiological conditions including development of tumors. Prolonged use of estrogens has been associated with human cancers [9–14] and estrogens induce tumors in laboratory animals [15–17]. Chronic administration of the natural hormone, 17β-estradiol (E_2) or synthetic estrogen, diethylstilbestrol to Syrian hamsters induces kidney tumors which serves as a useful model to study the mechanism of hormonal carcinogenesis [15–18]. These estrogen-induced and -dependent tumors share several characteristics with human breast and uterine cancers, which point to a common mechanistic pathway [19]. Studies aimed at understanding the contribution of hormonal potency to the development of neoplasia in the hamster tumor model did not show any correlation

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between hormonal potency of various estrogens tested and their tumor incidence [20,21]. Since hormones like E_2 are essential in estrogen-induced and -dependent tumors, it is postulated that ER-mediated processes play an important role in the carcinogenic process.

The levels of estrogen receptor protein have been shown to increase in the renal corpuscle, renal arteries, interstitial cells and capillaries of estrogen-induced tumor-bearing kidneys compared to untreated controls [22]. Additionally, an intense immunoreactivity for ER protein has been observed in estrogen-dependent hamster kidney tumors [22]. ER mRNA levels have also been shown to increase in estrogen-induced kidney tumors of hamsters [22]. These data suggest that ER mRNA and protein levels increase as the tumor mass to kidney mass ratio increases which indicates that ER may be providing favorable conditions for cellular proliferation. The hamster ER gene has not been cloned yet. This sequence information is essential to understand the regulatory and carcinogenic role of ER in estrogen-induced carcinogenesis. Additionally, ER mRNA variants have been detected in several breast cancer patients and have been postulated to play a role in resistance to hormonal therapy [23,24]. In the hamster kidney tumor model, it is postulated that the free-radicals/reactive oxygen species generated by metabolic redox cycling of estrogen quinones, may provide favorable conditions for oxidative stress in certain cell types [19]. Oxidative stress may modulate the expression of certain genes or induce mutations [25–29]. Mutations in DNA polymerase beta and microsatellite DNA alterations have been reported in estrogen-induced hamster kidney tumors [30–32]. In order to study the modulation of ER expression or to identify if ER variants are produced during estrogen-induced hamster kidney tumorigenesis, the sequence information of the hamster ER cDNA is needed. Moreover, this information is needed for the design of specific primers for PCR and for the understanding of downstream regulatory mechanisms. In the present study, we report the cloning of hamster ER cDNA and its homology with other known ER cDNAs.

2. Materials and methods

2.1. Isolation of RNA

Syrian hamsters (4–6 weeks of age; Harlan Sprague-Dawley, San Diego, CA) were housed in our animal facility with Purina Rodent chow and water available ad libitum. The hamsters were killed by decapitation, and kidneys of male hamsters and uteri of female hamsters were immediately excised, quick-frozen in liquid nitrogen and stored at −80°C. Polyadenylated (poly A^+) RNA was isolated from hamster kidneys and uteri using an OligotexTM Direct mRNA kit from Qiagen (Valencia, CA) according to the suppliers' recommendations.

2.2. Reverse transcription and polymerase chain reaction (PCR) amplification

Hamster poly A^+ RNA was reverse transcribed and subsequently PCR amplified using a GeneAmp RNA-PCR kit from Perkin Elmer Cetus (Norwalk, CT). For reverse transcription, 40 ng of poly A^+ RNA and downstream primer (250 ng) were added to a reaction buffer containing 5 mM $MgCl_2$, 1 mM each of deoxynucleotides, 1μl(40V/μl) RNase inhibitor and incubated at room temperature for 10 min. Then, 2.5 units of MMuLV reverse transcriptase was added and the reaction mixture incubated at 40°C for 90 min. After reverse transcription, the incubation mixture was treated with 2 units of RNAse H and incubated further at 37°C for 25 min. The reaction mixture was finally heated at 94°C for 10 min. Five microliters of this cDNA mixture was used for subsequent PCR amplification [33,34]. PCR amplifications were carried out in 50 μl of total volume containing 200 μM of each dNTP, 1.5 mM $MgCl_2$, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 100 ng of the appropriate primers and 2 units of Pwo DNA polymerase (Boehringer Mannheim, Indianapolis, IN) using a GeneAmp PCR system 2400 (Perkin–Elmer, Norwalk, CT). The amplification conditions were denaturation at 94°C for 1 min, annealing at 55°C for 1 min and polymerization at 72°C for 2 min. After 35 cycles, a 7 min extension at 72°C was performed.

2.3. Primers

Primers for reverse transcription and PCR amplification were designed based on maximum homology between rat, mouse and human ER cDNA sequence information [35–37]. The following primers were used for either reverse transcription or PCR amplification.

Primer name	Nucleotide sequence
HKER-1	5′-GGG CGC CGC CTA CGA GTT CAA-3′
HKER-2	5′-GCG CCA GAC CAG ACC AAT CA-3′
HKER-6	5′-CTC CAT GAT CAG GTC CAC CT-3′
HKER-7	5′-GGA GCC TGG GAG NTC TCA GAT-3′
HKER-9	5′-CAC TCG ATC ATT CGA GCA CA-3′
HKER-10	5′-CAC TCT GGC GTC GAT TAT CA-3′

2.4. Cloning

The PCR amplified products were size fractionated on a 1.5% agarose gel in Tris–acetate/EDTA electrophoresis buffer. The gel fragments were cut and DNA extracted using the Geneclean II kit (Bio 101 Inc. La Jolla, CA). The gel purified fragments were ligated into a pCRII vector from Invitrogen (San Diego, CA) using a TA cloning kit according to the suppliers' recommendations [38]. The ligated product was transformed into competent cells, single colonies were picked and plasmid DNA was amplified. The plasmid DNA was digested with EcoR1 and applied on to 1.5% agarose gels to screen for the presence of the cDNA insert.

2.5. Southern and northern blot analysis

The plasmid DNA containing the expected size insert, and the corresponding PCR amplified fragments were subjected to Southern analysis [39]. For Southern analysis, DNA from the agarose gels was transferred to nylon membranes by capillary transfer in the presence of 10 × SSC (1.5 M NaCl, 0.15 M sodium citrate, pH 7.0) buffer. The membrane was denatured for 5 min in 1.5 M NaCl, 0.5 M NaOH, neutralized for 5 min in 1.5 M NaCl, 0.5 M Tris–HCl, pH 8.0, rinsed in 2 × SSC buffer for 2 min, and the DNA on the membrane fixed by UV-Cross linker (Strategene, La Jolla, CA). For Northern blot analysis, total tissue RNA was prepared from the hamster uterus by the method of Chomczynski and Sacchi [40]. Total RNA (30 μg) in formamide (50%)-formaldehyde (2.2 M)-morpholino propanesulfonic acid (MOPS) buffer was heat denatured at 65°C for 10 min and separated by electrophoresis on a 1% agarose gel containing 20% formaldehyde after the addition of the loading dye. After destaining, RNA was transferred to Hybond-N+ nucleic acid transfer membrane (Amersham, Arlington Heights, IL) by capillary transfer in 10 × SSC. Membranes were UV-cross linked and prehybridized for 1 h at 68°C in a QuikHyb hybridization solution (Stratagene, La Jolla, CA). A ^{32}P-labeled estrogen receptor cDNA probe (provided by Dr. P. Chambon, France) was prepared using a High Prime DNA labeling system (Boehringer Mannheim, Indianapolis, IN) and [α-^{32}P]-dCTP (3000 Ci/mol) (ICN Radiochemicals, Irvine, CA) according to the suppliers' recommendations. Salmon sperm DNA (100 μl of 10 mg/ml) was added to the labeled probe and heat denatured by boiling for 5 min before addition to the hybridization mixture. The hybridization was carried out for 6 h at 68°C. After hybridization, blots were washed twice for 15 min each with a 2 × SSC buffer containing 0.1% (w/v) SDS at room temperature. Then, the blots were washed with a 0.1 × SSC buffer containing 0.1% (w/v) SDS for 30 min at 60°C.

2.6. cDNA library construction

A hamster uterus cDNA library was constructed using 5 μg of hamster uterus poly A$^+$ RNA, a Zap-cDNA synthesis kit and a Zap-cDNA Gigapack III Gold cloning kit (Strategene, La Jolla, CA) following suppliers' recommendations. Briefly, hamster uterus mRNA was reverse transcribed in the presence of a 50 base oligonucleotide primer containing an 18 base polydeoxythymidine, and an Xho1 restriction site and a GAGA sequence to protect the Xho1 restriction site. The second strand synthesis was performed in the presence of RNAse H, DNA polymerase 1, and polydeoxy ribonucleotides. The uneven termini of the double stranded cDNA were nibbled back or filled with Pfu DNA polymerase, and EcoR1 adaptors were ligated to the blunt ends. The blunt-ended and EcoR1 adaptor ligated cDNA was digested with Xho1 to release the EcoR1 adaptor and the residual linker primer. These two fragments were separated on a sepharose drip column, and the size-fractionated cDNA was then precipitated and ligated into the Uni-Zap XR vector. The lambda library was subsequently packaged into a high efficiency Gigapack III Gold packaging system and plated on the *Escherichia Coli* cell line XL1 Blue-MRF. The library was further amplified and stored at −80°C.

3. Results

A hamster uterus cDNA library was constructed and ER cDNA fragment amplified from this library. Several minor fragments and one major (~650 bp) fragment were amplified when a small aliquot (25 μl) of the hamster uterus cDNA library was PCR amplified (after heat denaturing at 95°C for 10 min) using primers HKER-9 and HKER-10 (data not shown). Southern blot analysis of the PCR amplified fragments using human ER cDNA as a probe showed that the 650 bp fragment cross hybridized with the human ER cDNA (data not shown). This 650 bp fragment was cloned into a pCR II vector from Invitrogen (San Diego, CA), transformed into *E. coli* cells, single colonies picked and plasmid DNA amplified as described in the Methods section. Plasmid DNAs digested with restriction enzyme EcoR1 showed the presence of the expected size insert (Fig. 1(A)).

Reverse transcription and PCR amplification of the poly A$^+$ RNA isolated from control hamster kidney in the presence of forward and reverse primers HKER-1 and HKER2 resulted in the amplification of ~1 kb

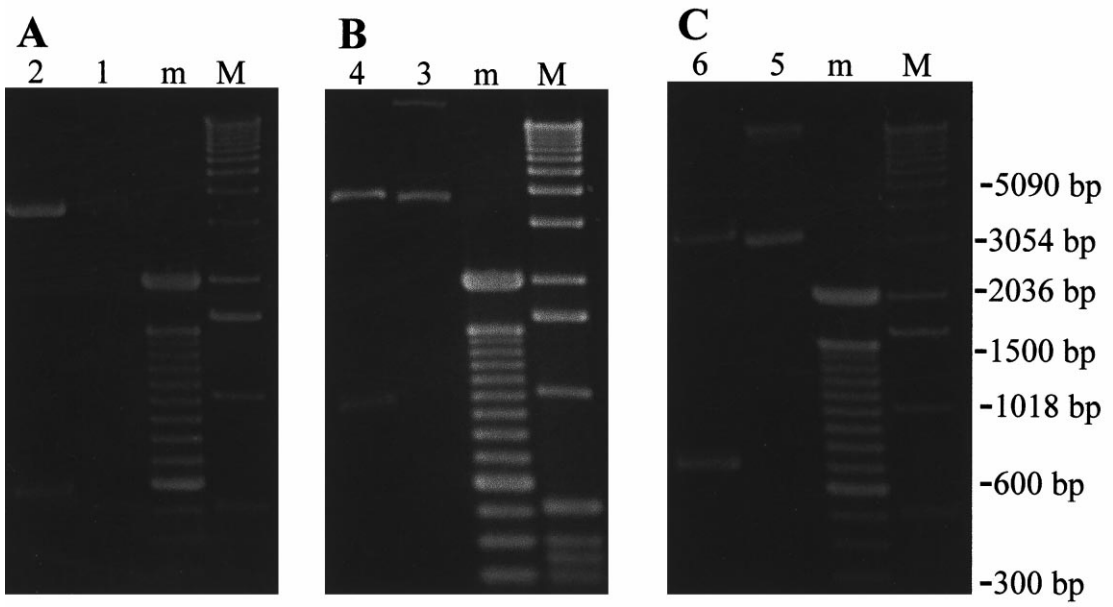

Fig. 1. Analysis of plasmid DNA for the presence of estrogen receptor inserts that were cloned into the plasmid cloning vectors. PCR amplified and purified fragments were cloned into pCR II vector (Invitrogen) as described in Section 2. The amplified plasmid DNA was then digested with restriction enzyme EcoR1 at 37°C for 1 h. The digested and undigested plasmid DNAs were size fractionated on a 1.5% agarose gel and stained with ethidium bromide. (A) Analysis of plasmid DNA for the cDNA insert corresponding to the 5′-end of hamster estrogen receptor; (B) analysis of plasmid DNA for the cDNA insert corresponding to the middle portion of hamster estrogen receptor; and (C) analysis of plasmid DNA for the cDNA insert corresponding to the 3′-end of hamster estrogen receptor. Lanes 1, 3 and 5 = undigested plasmid DNA; lanes 2, 4 and 6 = plasmid DNA digested with EcoR1; *m* = 100 bp DNA ladder; *M* = 1 kb DNA ladder.

fragment which was cloned into a pCRII vector using TOPO cloning kit (Invitrogen, San Diego, CA). The amplified plasmid DNA digested with EcoR1 showed the presence of the expected size insert (Fig. 1(B)). Additionally, hamster poly A^+ RNA was also reverse transcribed and PCR amplified in the presence of forward and reverse primers HKER-6 and HKER-7 in an attempt to amplify the 3′-end of hamster ER cDNA and this reaction resulted in the amplification of ~690 bp fragment which was also cloned into a pCR II plasmid vector from Invitrogen (Fig. 1(C)).

PCR amplified fragments cloned into the plasmid vectors were analyzed by Southern blot analysis after digestion with restriction enzyme EcoR1. Plasmid clones containing the appropriate size inserts and showing cross hybridization with human ER cDNA upon Southern blot analysis were subjected to DNA sequence analysis, using M13 and T7 primers. To achieve complete complementarity of DNA bases, all the three cloned fragments were additionally analyzed with primers that were designed from the sequence information obtained on sequencing the clones with M13 and T7 primers (data not shown). All the three cloned fragments showed sequence homology with mouse, rat and human ER cDNA sequences reported in the literature. Based on the DNA sequence information of the three cloned fragments which had overlapping regions, a complete full length, 1978 bp ER cDNA sequence was derived which shows an ATG start site and a TGA termination signal (Fig. 2). The expression of the hamster ER was analyzed by Northern blotting of total RNA sample isolated from the hamster uterus. Hybridization of the RNA transferred to nylon membrane with cDNA fragment corresponding to the 5′-end of hamster ER cDNA resulted in the detection of a mRNA of ~6.4 kb in hamster uterus (data not shown). The same size fragment was detected when the same membrane was hybridized with a human ER cDNA probe (data not shown). Thus, hamster ER cDNA has been cloned using PCR meth-

Table 1
Percentage amino acid and cDNA homology between hamster estrogen receptor and some of the cloned estrogen receptors[a]

Species	Protein							cDNA
	Overall	A	B	C	D	E	F	Overall
Mouse	93	**95**	84	**100**	92	**98**	86	91
Rat	93	**95**	84	**100**	92	**98**	86	90
Human	88	**95**	84	**100**	74	**94**	63	87
Pig	88	**92**	85	**100**	79	**94**	60	87
Sheep	87	**92**	78	**100**	85	**93**	58	85
Horse	86	**86**	75	**100**	79	**92**	63	85
Chicken	79	**86**	61	**100**	49	**93**	51	81

[a] The three most highly conserved regions (A, C and E) are highlighted in bold.

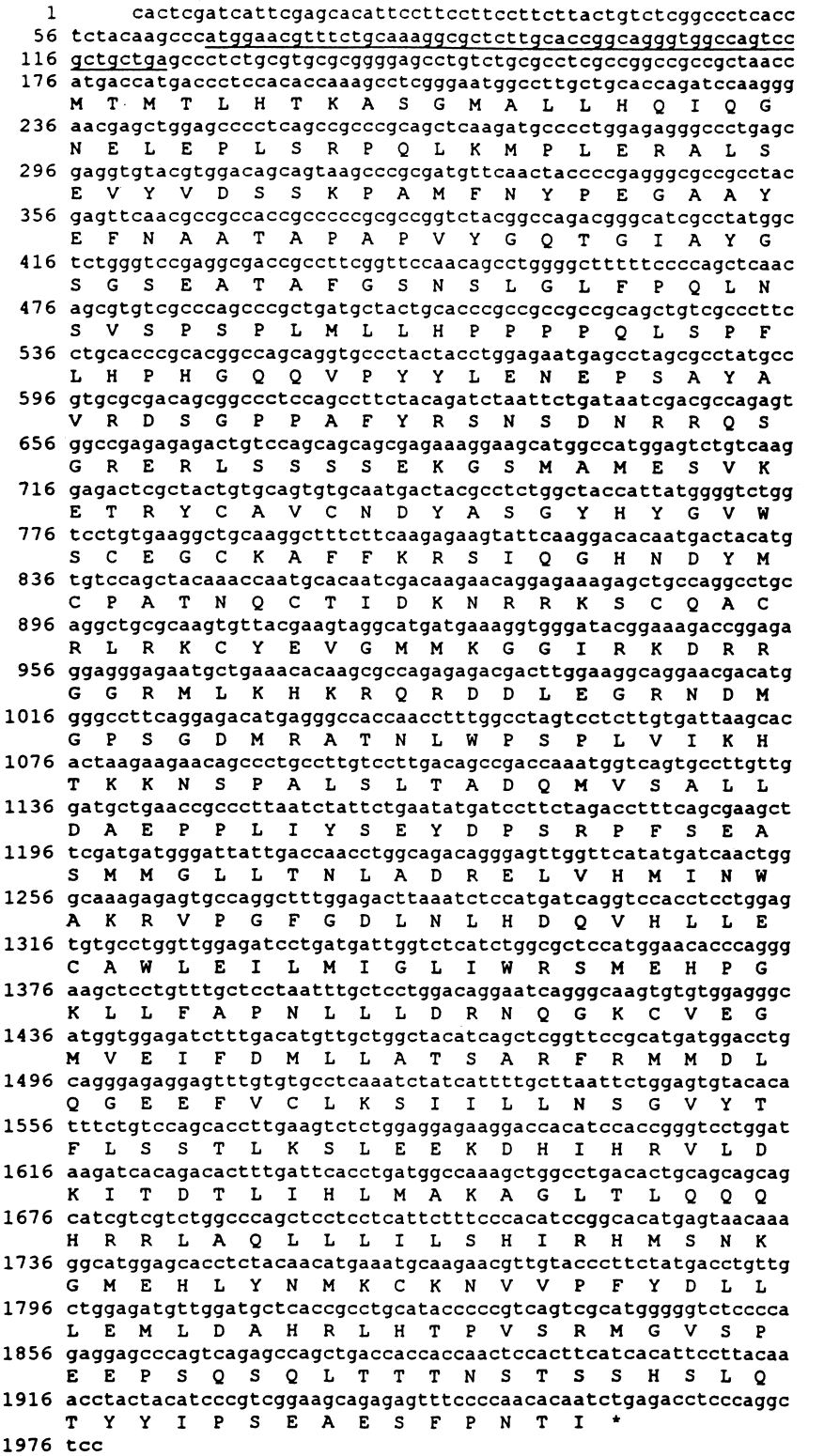

Fig. 2. Nucleotide sequence analysis and deduced amino acid sequence of hamster estrogen receptor. The untranslated open reading frame in the 5′-end of the receptor is underlined.

odology which has extensive homology with mouse, rat and human ER cDNA and protein (Table 1).

4. Discussion

In the present study, we report the cloning of hamster ER alpha cDNA. This 1978 bp cDNA contains a 595 amino acid long open reading frame and shares high nucleotide sequence homology with that of the mouse, rat, human, pig, sheep, horse and chicken ER cDNAs (Table 1). The nucleotide sequence organization of hamster ER is similar to that of mouse ER cDNA [36] and shows a small open reading frame of 57 bp (Fig. 2, underlined region) in the 5′-end which may code for a 19 amino acid peptide. There is a TGA termination signal 52 nucleotides upstream of the longest open reading frame. The longest open reading frame starts with a TAG initiation codon at nucleotide 176.

ER gene is a member of a large family of steroid/thyroid hormone receptors which acts as a transcription factor when bound to specific ligands [3,4]. ER has three principal domains, a variable N-terminal domain, a highly conserved DNA binding domain, and a moderately conserved C-terminal domain [41,42]. The human ER gene is greater than 10 kb in length and is split into eight exons [42]. The DNA binding domain enables the receptor to bind to its cognate target site consisting of two inverted repeats of two half-sites with consensus motif AGGTCA (or closely related sequence) spaced by 3 bp and referred to as an estrogen response element [43]. The A/B region of the ER gene is important for stimulating transcription from certain estrogen-responsive promoters. The poorly conserved C-terminal region F can be deleted without affecting hormone binding or transcription. The ligand binding-domain also harbors a nuclear localization signal as well as sequences necessary for dimerization and transcriptional activation (AF2). A second activation function, AF1, is present in the amino-terminal domain of the receptor [44].

The hamster ER gene has a structural organization similar to ERs cloned from other species. The deduced amino acid shares 93% homology with rat [35] and mouse ERs [36], and 88% homology with human [37] and pig ERs [45] (Table 1). A comparison of the amino acid sequences of different domains of hamster ER with those reported for other species show extensive homologies in certain domains (Table 1). Hamster ER domain C (DNA binding domain) shows 100% homology with a similar domain from mouse, rat, human, pig, sheep, horse and chicken ER [35–37,41,45–47] (Table 1). Domain E (hormone binding domain) shows 98% homology with domain E of mouse and rat ER [35,36], 94% homology with human and pig [37,45], 93% homology with sheep and chicken [41,46], and 92% homology with horse ER [47] (Table 1). Domain A of hamster ER shows 95% homology with mouse, rat and human ERs [35–37], 92% homology with pig and sheep [45,46], and 86% homology with horse and chicken ERs [41,47]. The least conserved domain is domain F. Northern blot analysis shows a 6.4 kb transcript in the hamster uterus. A similar size transcript has been reported for the mouse ER mRNA in the uterus [36]. This suggests that there must be a large 3′-untranslated region for hamster ER as reported for many other species [35–37]. Thus, the cloned hamster ER cDNA is closely related in nucleotide sequence and peptide domain homology to mouse, rat and human ER gene.

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