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Tissue-dependent Expression of a Novel Splice Variant of the Human Oestrogen Receptor

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We have isolated a novel splice variant of ER mRNA from normal endometrial tissue using RT/PCR. The variant contains an unusual splice junction formed by splicing sequences within exons 4 and 7 together. The translated protein product would be predicted to lack part of exon 4, all of exons 5 and 6 and, due to a missense alignment at the new splice junction, the remaining sequence from exon 7 would be translated out of frame and terminate at the exon 7/8 splice junction. As a result, the protein would lack most of the hormone binding domain (HBD) and the major oestrogen-dependent transactivating region (AF-2), but still contain the DNA binding domain (DNA-BD) and N-terminal transactivating region (AF-1). In contrast to the exon 5 deleted variant of ER (Δ5), which was expressed in both normal endometrium and liver, this novel variant was present in endometrium but not in liver samples. These results confirm that some ER splice variants are expressed in normal, non-malignant oestrogen responsive tissues. In addition, they demonstrate the tissue specific expression of a novel and interesting splice variant of ER in these normal tissues.

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INTRODUCTION

Oestrogens play a pivotal role in the growth and development of the female reproductive system, with many tissues being responsive to them (including breast, endometrium, ovaries, liver, pituitary, cervix, vagina and bone). These hormones are also of importance pharmacologically [e.g. female oral contraceptives and hormone replacement therapy (HRT)] and pathologically (e.g. endometriosis, hyperplasia of the breast and endometrium, breast and endometrial carcinoma), when oestrogen deprivation is the treatment of choice [1].

It is generally accepted that the expression of oestrogen receptor (ER) protein is the major determinant of oestrogen-dependent events. The majority of oestrogen responsive tissues have been shown to express wild-type (WT) ER protein, although in the case of bone, the evidence is somewhat conflicting [1, 2]. Unliganded ER exists as a complex with a number of heat shock proteins, but upon binding to oestrogen the proteins dissociate, ER dimerises, binds to specific oestrogen responsive elements (EREs) upstream of oestrogen sensitive genes, and induces transcription [3, 4].

The human WT ER protein (66 kDa) consists of a number of functionally distinct regions, including a central DNA binding domain (DNA-BD) and a C-terminal hormone binding domain (HBD) [5]. There are two transcriptional activator regions: AF-1 which is located at the N-terminal region, and AF-2 which overlaps the HBD at the C-terminus. Activity of AF-2, the major transactivating region, is dependent on oestrogen binding, while AF-1 has been demonstrated to have a low level, cell type and promoter dependent constitutive activity [6].

The protein is translated from a 6.3 kb mRNA species consisting of 8 exons [7]. However, a number of splice variants exist in which exons have been accurately spliced out of the ER mRNA sequence [8–10], and others in which non-ER sequences have been precisely inserted at an exon/intron junction [11]. Some of these are predicted to result in protein products whose activity would differ from the WT ER protein. For example, the Δ5 ER variant has been shown to code for a protein which lacks AF-2, is constitutively active at the transcriptional level in the absence of oestrogens [12] and confers resistance

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in vitro to the anti-oestrogen tamoxifen [13]. The exon-7 deletion variant has been shown to inhibit the activity of WT [14]. It is clear that these variants may play a role in the differential sensitivity between cells and tissues to oestrogens and anti-oestrogens. For example, we have recently shown that the Δ5 ER variant may have a role in conferring resistance to tamoxifen in breast cancers which express the oestrogen-responsive proteins, progesterone receptor (PgR) or pS2 [15, 16].

The majority of the work on these receptor variants has been conducted in breast cancer tissues and cell lines, and some have also been demonstrated in malignant meningiomas [17] and hepatomas [18]. However, very little work has been performed on the expression of ER splice variants in normal oestrogen responsive tissues. The Δ5 ER variant has been detected in normal endometrium [12], and recently in a normal liver sample [18], indicating that this variant is expressed in non-malignant tissues. Tissue-specific or disease-specific expression of some of these variants may indicate an important role for them in physiological and/or pathological processes.

This paper describes the isolation and sequencing of a novel splice variant of ER. An unusual splice junction is formed from sequences within exons 4 and 7 of the ER. In addition, this variant was shown to be expressed in normal endometrium, but not in normal liver tissues.

MATERIALS AND METHODS

RNA extraction

Frozen samples of normal endometrium and liver (from women) were kindly supplied by Professor E. Thomas (Princess Anne Hospital, Southampton, U.K.) and Dr A. Strain (Queen Elizabeth Hospital, Birmingham, U.K.). Frozen samples were pulverized in a dismembrator (Braun Biotech UK, Aylesbury, Buckinghamshire, U.K.). RNA was purified using RNAzol B (Biotecx Laboratory, Houston, TX, U.S.A.) as recommended by the manufacturers. Quantification was by spectrophotometry.

RT–PCR of endometrial RNA

2 μg RNA from 5 normal endometrial samples were reverse transcribed as described previously [16]. cDNA was subjected to 35 cycles of PCR in the presence of 0.25 μCi α[^{32}P]dCTP (per sample) and 500 nM concentrations of each primer [AAG GAG ACT CGC TAC TGT (sense; exon 2; 770–787 bp); ATA GAG GGG CAC CAC GTT (antisense; exon 8; 1824–1821 bp)] [19] using the conditions described previously [16].

Analysis and cloning of endometrial RNA RT–PCR products

PCR products of the ^{32}P-PCR (100 μl) were precipitated using ethanol (250 μl) and glycogen (2 μl of 20 mg/ml solution; Boehringer-Mannheim, Lewes, East Sussex, U.K.), for greater than 2 h at −20°C and centrifuged at 4°C for 20 min at 14,000 *g*. The pellet was washed with 100 μl of 70% ethanol, dried briefly and resuspended in 20 μl water.

5 μl of each sample was run on a 6% non-denaturing polyacrylamide gel in parallel with ^{32}P-labelled size markers, the gel was fixed for 5 min in 15% methanol, 5% acetic acid, dried and autoradiographed. Bands which were smaller than the expected 983 bp WT product were detected.

A band of 427 bp found to be expressed in the majority of endometrial samples was cloned into the pCRII vector and transfected into *E. coli* cells (TA cloning kit; Invitrogen, Abingdon, U.K.) as suggested by the manufacturers. Plasmid DNA was extracted using a plasmid purification kit (Qiagen Ltd., Dorking, Surrey, U.K.). A proportion was digested with EcoRI enzyme (2 h at 37°C; Boehringer Mannheim), electrophoresed on a 1% agarose gel, blotted and hybridized as described previously [16] using probes specific for exon 2/3 (AGA AGT ATT CAA GGA CAT; 863–880 bp), exon 4 (CCC TCC AGT GAA GCT TCG; 1229–1246 bp), exon 5 (ACC CTC CAT GAT GAG GTC; 1333–1350 bp), exon 6 (TGT GTA GAG GGC ATG GTG; 1481–1498 bp), exon 7 (TCC AGA GAC TTC AGG GTG; 1624–1641 bp) or exon 8 (GTA CAG ATG CTC CAT GCC; 1792–1808 bp).

The product was finally sequenced using a T7 or sp6-specific primer, [^{35}S]ATP and the Circumvent Thermal Cycling Kit (New England Biolabs) [15].

Amplification of the exon 5-deletion ER variant

RT, 25 cycles of PCR, and Southern blot hybridization were performed on the endometrial and liver RNA samples as described previously [16].

Amplification of the novel ER variant

Duplicate samples of endometrial or liver RNA were subjected to RT and 25 cycles of PCR. Novel splice variant (Δ4/7) and WT RNA (10 ng), produced by *in vitro* transcription (Ambion, Austin, TX, U.S.A.) were amplified in parallel with the sample RNA. After one cycle of PCR (95°C × 3 min; 61°C × 2 min, 72°C × 3 min) containing 500 nM sense primer (exon 3; GGC CTG CCG GCT CCG CAA ATG CTA CGA AGT GGG; 946–978 bp) one sample of each duplicate was restricted with StuI enzyme (Boehringer-Mannheim) which digests the DNA at the novel splice site while the other sample remained undigested (2 h at 37°C). The enzyme was denatured (65°C × 20 min), the variant-specific primer was added (500 nM), and the DNA was subjected to 9 cycles (95°C × 2 min; 61°C × 2 min, 72°C × 1 min) + 10 cycles (95°C × 2.5 min; 61°C × 2.5 min, 72°C × 1 min) + 5 cycles (95°C × 3 min; 61°C × 3 min, 72°C × 1 min), followed by 72°C for 5 min.

PCR products were precipitated as described above and run 4% on a nucsieve gel (Flowgen Instruments Ltd, Sittingbourne, Kent, U.K.). Blotting and hybridization were performed as described previously [16] using an exon 3/4 specific probe (CGG TCT TTT CGT ATC CCA CC; 989–1008 bp) for the novel variant Δ4/7.

Under these conditions Δ4/7 variant ER mRNA, but not the WT ER mRNA was amplified. A PCR product whose yield is decreased by StuI treatment is indicative of the expression of Δ4/7 mRNA in that sample.

RESULTS

Isolation and characterization of a novel ER splice variant

Following RT–PCR of endometrial RNA using exon 2- and exon 8-specific primers in the presence of [^{32}P]dCTP, a number of smaller bands were visible in addition to the WT ER band (983 bp). A 427 bp band was cloned into the pCRII vector, probed with sequences of DNA corresponding to different exons within the human ER sequence, and finally sequenced. The cloned product was shown to bind to exon 2/3 and exon 8 specific ER probes, but not to exon 4, exon 5, exon 6 or exon 7-specific ER probes (Fig. 1). Sequencing confirmed the product to be a novel splice variant of human ER which contained a new splice junction formed by sequences within exons 4 and 7 [Fig. 2(a)].

Sequence homology can be demonstrated between the novel splice sites within exons 4 and 7, and the consensus sequence of donor and acceptor sites for mRNA splicing [20] [Fig. 2(b)].

This variant mRNA therefore has part of exon 4, all of exons 5 and 6, plus part of exon 7 spliced out of the mRNA sequence (Fig. 3). Although exons 1 to the splice site within exon 4 should be translated identically to the WT ER sequence, the remaining sequence following the new splice site would be predicted to be translated out of frame until the protein terminates at a stop codon located at the exon 7/8 junction. Most of the hormone binding domain and the AF-2 region would therefore not be present in the Δ4/7 ER translated product, but would be replaced by a short unique amino acid sequence which is very high in Pro and Ala residues. Interestingly, the protein product would still contain AF-1, DNA-BD and the nuclear localization signal.

Tissue-specific expression of ER splice variants

The protein sequence of Δ4/7 ER is similar to that of the Δ5 ER splice variant previously published [12] in that the majority of the hormone binding domain and AF-2 region would be missing, whereas the remaining N-terminal region is identical to the WT ER sequence. We therefore compared the expression of Δ4/7 and Δ5 ER variant mRNA in two normal oestrogen-responsive tissues, endometrium and liver.

WT ER and Δ5 ER mRNA were detected in the normal endometrium and the liver samples (Fig. 4), although the relative levels of Δ5/WT ER PCR product were different in the two tissue types. The expression of Δ4/7 ER mRNA in these two tissues was then assayed using a variant-specific antisense primer which bound across the exon 4/7 splice site used in combination with an exon 4 specific sense primer in an RT–PCR. A variant-specific primer was used to confirm that the Δ4/7 ER variant product was not an artefact of the initial RT–PCR reaction. However, the variant-specific primer contained sequences which would bind to WT ER sequence under certain conditions. Amplification conditions were selected to minimize this but it remained important to ensure that WT ER was not amplified as this product would be indistinguishable from the variant PCR product. The novel splice junction itself is a StuI restriction enzyme site, and StuI would therefore digest the Δ4/7 ER, but not the WT ER cDNA sequence. A PCR product whose quantity is reduced by the StuI treatment would therefore be indicative of Δ4/7 ER expression in a sample. This was confirmed by the data shown in Fig. 5 (a and b). Using a relatively high annealing temperature (61°C), *in vitro*-transcribed Δ4/7 ER mRNA was clearly amplified, and was sensitive to StuI digestion prior to PCR cycling (at the first ds DNA product stage) [Fig. 5(a)] (see methods section for further details). However, *in vitro* transcribed WT ER mRNA was not amplified when processed in parallel using these conditions [Fig. 5(b)]. Interestingly, Δ4/7 ER could be detected in the 5 endometrial samples assayed, but not in the liver

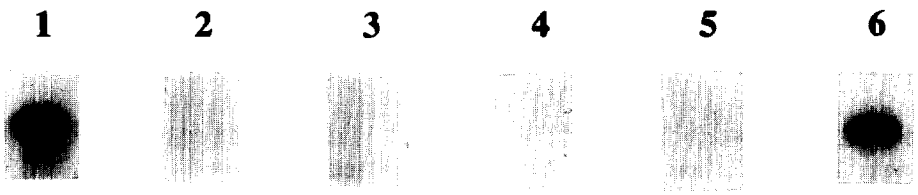
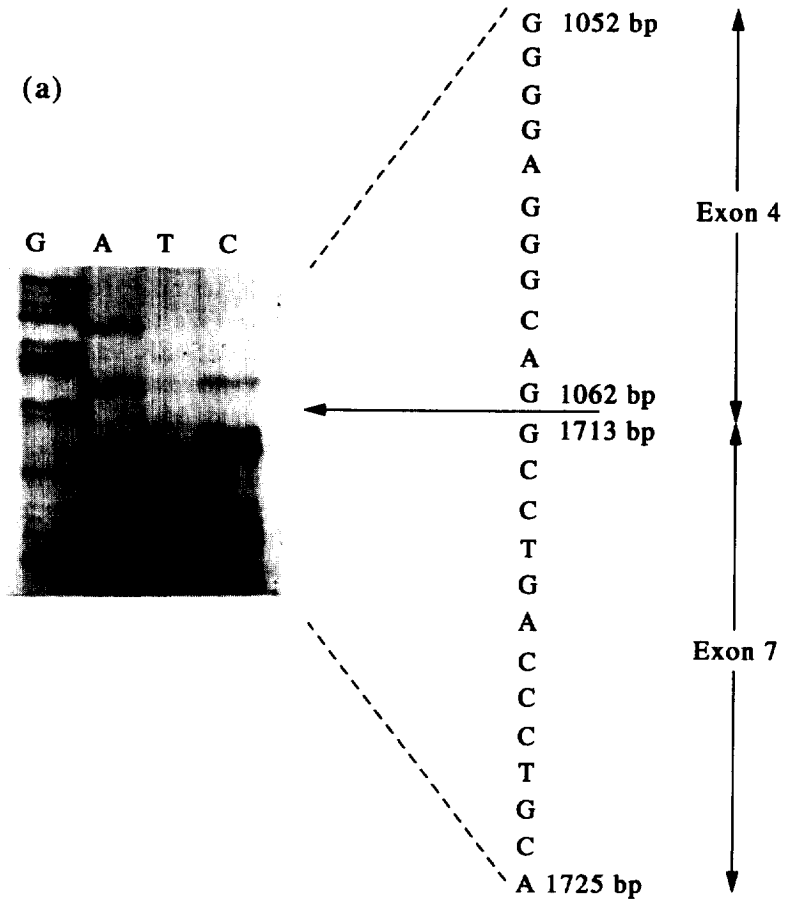


Fig. 1. Southern blot of an ER RT–PCR product amplified from normal endometrial RNA. The cloned 427 bp RT–PCR product (pCREδ) was digested with EcoRI, electrophoresed, blotted onto nylon and probed with ^{32}P-labelled DNA specific for exons 2/3 (lane 1), exon 4 (lane 2), exon 5 (lane 3), exon 6 (lane 4), exon 7 (lane 5) or exon 8 (lane 6) of the human ER sequence. Results were visualized by autoradiography, and demonstrated that exons 2, 3 and 8 of ER were present, but the sequences bound by the exon 4-, 5-, 6- and 7-specific probes were absent.



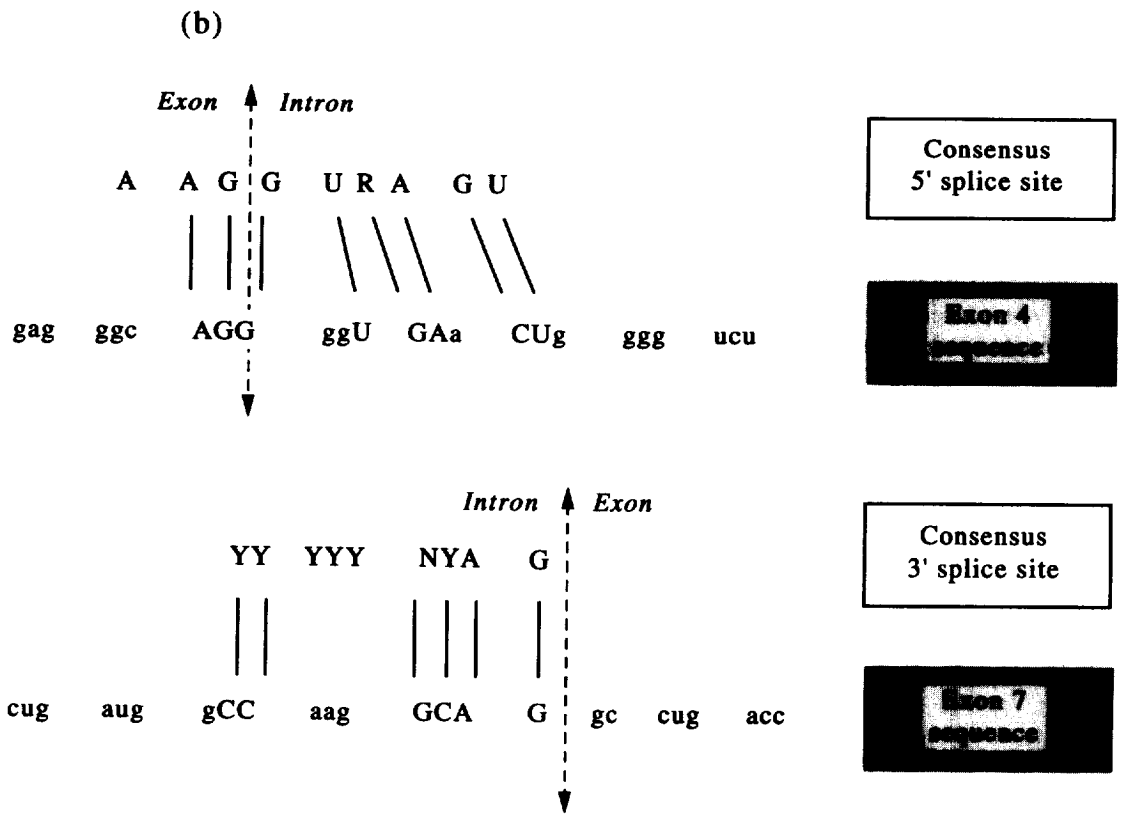


Fig. 2. Sequence of the novel splice junction of the ER variant. Sequences within exons 4 and 7 or ER are spliced together (a), and show homology to the consensus sequence for donor and acceptor splice sites (b) (Y, pyrimidine, R, any base).

samples (Fig. 4), demonstrating tissue specific expression of this novel variant.

DISCUSSION

The majority of work published thus far on ER variants has been concerned with their expression in malignant tissues, particularly of the breast [12], liver [18] and meninges [17]. Indeed, we ourselves have reported that the Δ5 ER mRNA is widely expressed in breast cancer, and seems to have a role in conferring the phenotype and possibly the sensitivity of certain breast cancers to anti-oestrogen therapy [16]. There are very few published reports on the expression of splice variants in non-malignant tissues. As far as we are aware this is limited to a brief mention of the Δ5 ER variant expressed in an endometrial sample [12] and in one normal liver sample [18]. In addition, we ourselves have been able to detect this variant in normal breast (unpublished data).

Using RT–PCR we have isolated and characterized a new splice variant of ER from normal endometrial tissue. This variant is not a RT–PCR artifact as it has been cloned and sequenced from two separate samples and detected in many samples using 2 different sets of primers, one which is specific for the variant form of ER. Southern blot hybridization initially indicated that some ER sequence was present, but that exons 4–7 were deleted from the mRNA sequence (Fig. 1). However, sequencing demonstrated that this variant was unusual in that the new splice junction was formed by sequences within exons 4 and 7 [Fig. 2(a)], and not at the usual exon/intron sites as occurs with the WT and other splice variants of ER.

Homology can clearly be demonstrated between the sequences of the consensus splice donor and acceptor splice sites [20] and the sequences in exon 4 and 7 which are spliced together to form this new variant [Fig. 2(b)]. Although we have predicted the position of the new splice site, it is difficult to be precise due to the homology between the sequences of exon 4 and 7 at this position. These new donor and acceptor splice sites have not previously been reported; in fact, this is the first report demonstrating that splicing of ER mRNA does not necessarily have to involve the splice sites predicted from the WT ER RNA sequence. Examples

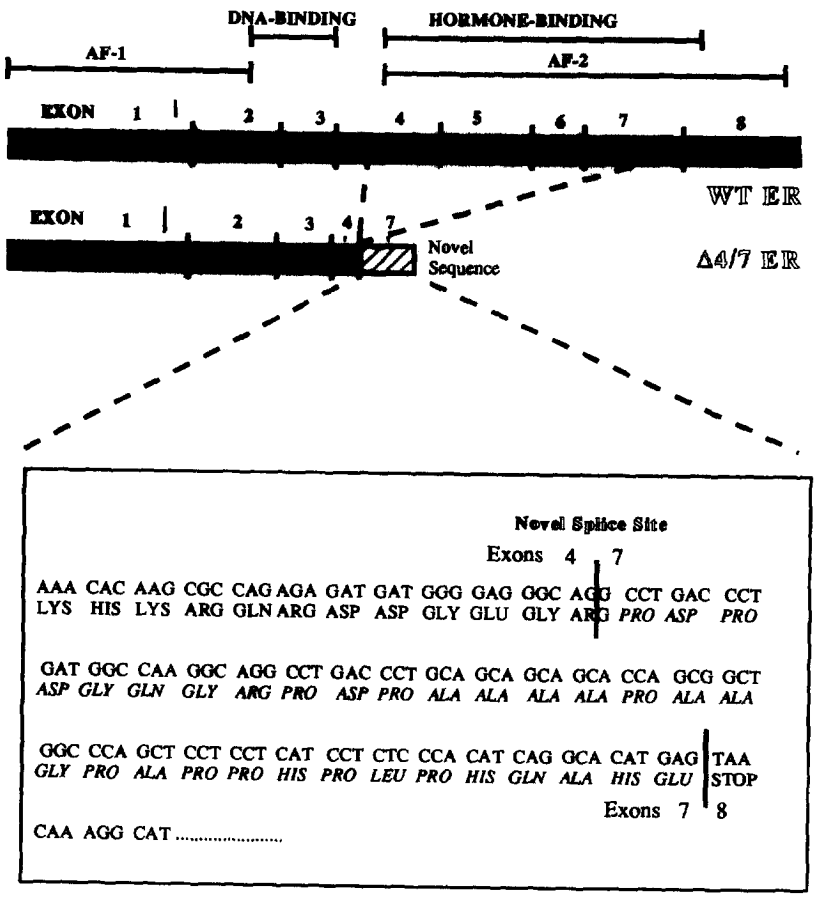


Fig. 3. A comparison of the protein sequence of WT and that predicted for Δ4/7 ER variant ER, showing the position of the transactivation regions (AF-1 & AF-2), DNA binding- and hormone-binding domains. The codons downstream from the new splice junction of the Δ4/7 ER variant would be translated out of frame resulting in a novel sequence of 32 amino acids and termination of the protein at the junction of exon 7 and 8.

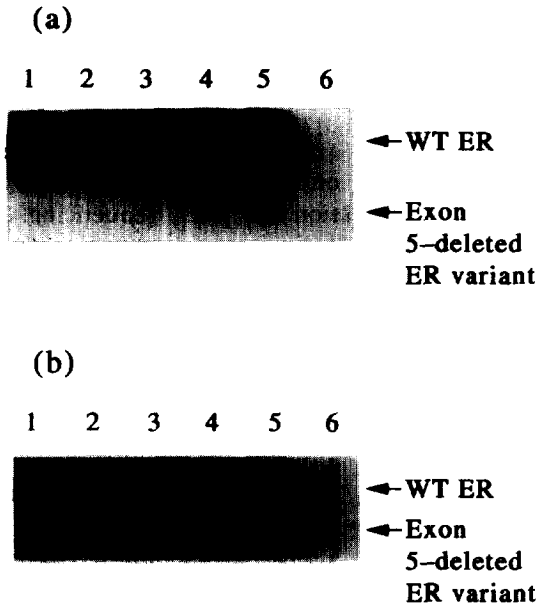


Fig. 4. Expression of WT and Δ5 ER mRNA in normal human endometrium and liver samples. Total RNA was extracted from 5 normal endometrial (a) and 5 normal liver (b) samples. 2 μg RNA (samples 1–5) and a negative control (no RNA; sample 6) were subjected to RT–PCR (25 cycles) using primers which span exon 5 of the human ER sequence. After agarose gel electrophoresis the DNA products were denatured, neutralized, blotted onto, and probed with an exon 4 specific probe [15].

of similar forms of alternative splicing of WT sequences which alter the property of the final translated product can be demonstrated to exist for a number of different genes. For example, one of the c-*Ha-ras* proto-oncogene mRNA transcripts has been shown to contain an extra exon between exons 3 and 4 [21]. This variant c-*Ha-ras* mRNA species has been shown to be unstable, and the extra exon to contain a stop codon which results in premature termination of translation with the resultant protein being inactive. Similarity exists between the predicted new ER variant and the Δ5 ER variant protein sequence, which has previously been shown to be transcriptionally active [12]. Upon translation, the HBD and AF-2 regions of the Δ4/7 protein would be absent, although the DNA-BD, AF-1 and nuclear localization signal regions would still be present (Fig. 3). Laboratory mutations in which the HBD and AF-2 sequences have been deleted can be shown to be transcriptionally active in the absence of oestrogens. However, whether these mutants are constitutively active or inactive seems to be cell- and promoter-dependent [22]. The novel splice junction produces a frame shift resulting in the remaining sequences of exons 7 and 8 being translated out of frame. Indeed, translation from the exon 4/7 splice site would result in a short region of 32 novel amino acids and termination at a stop codon occuring precisely at the exon 7/8 junction (Fig. 3). Of these, 9 would be Pro and 8 would be Ala residues.

It is difficult to predict the effect of replacing most of the HBD with this short stretch of Pro- and Ala-rich amino acids would have on the properties of the variant protein with respect to its DNA binding and transcriptional activities. However, the similarity between the structure of this novel variant protein and the Δ5 ER variant is striking. The Δ5 ER protein has been shown to be transcriptionally active in the absence of oestrogens in a yeast reporter system [12]. In addition to its constitutive activity upon transfection of Δ5 ER into the MCF-7 breast cancer cell, the variant was found to confer resistance to the antioestrogenic activity of the antioestrogen tamoxifen [13]. As with other splice variants, the effects of Δ4/7 ER may prove to be promoter and/or cell type specific.

The normal endometrial and liver samples were shown to express both WT and Δ5 ER mRNA (Fig. 4). This has confirmed the finding of Fuqua *et al.* [12] who were able to detect this variant in endometrial tissue. Although present in hepatic carcinomas, Δ5 ER mRNA was reported to be expressed in only 1 of 5 normal liver samples assayed [18]. This is in contrast to our finding the variant in all 5 of our normal liver samples. In fact, using the ratio of Δ5 to WT ER RT–PCR product as a measure of the relative expression of variant ER mRNA in the samples (for validation see [15]), Δ5 ER mRNA was more abundant relative to WT ER mRNA in the liver samples than in the endometrial samples. This discrepancy may be due to differences in the sensitivity of the RT–PCR techniques in the different laboratories. However, it is interesting to note that the normal liver samples in the report by Villa *et al.* [18] were from male patients whereas our samples were all

from females aged between 11 and 47 years of age suggesting that the expression of Δ5 ER mRNA in the normal liver may be sex-dependent.

In contrast to the Δ5 ER variant, Δ4/7 mRNA was expressed in a tissue specific manner. The methodology used specifically amplified and detected the Δ4/7 ER variant message and not the WT ER sequence [Fig. 5(a and b)]. This was achieved by designing a primer which contained more of the exon 7 than the exon 4-specific sequence of the 4/7 splice junction and using a relatively high annealing temperature (61°C). In addition, the novel splice region forms a new restriction site for the StuI enzyme. Therefore, by digesting the sample with StuI after one round of PCR (after RNA has been reverse transcribed to cDNA and then replicated to dsDNA), Δ4/7 ER but not WT product would be digested at the novel splice site. The StuI treatment would reduce the amount of variant PCR product compared to a duplicate sample in which the restriction enzyme was not added. A reduction in the amount of RT–PCR product was clearly demonstrated following StuI treatment during amplification of *in vitro* transcribed Δ4/7 ER mRNA [Fig. 5(a)]. This reduction was unlikely to be as extensive when the DNA has been synthesized from cellular RNA, as this would contain many more competing StuI sites. A RT–PCR product, the quantity of which was reduced by StuI treatment, was therefore indicative of the expression of Δ4/7 ER mRNA.

Using this methodology Δ4/7 ER mRNA was found to be expressed in normal endometrial and breast cancer samples, but not in normal liver tissues [Fig. 5(c and d)]. The quantity of variant RT–PCR products demonstrated in the endometrial tissue cannot be taken as an indication of the relative amounts of variant message in the samples, but is rather an indication of the RT–PCR efficiencies for that tissue sample. The unclear bands of RT–PCR product seen in a couple of the lanes in the liver tissue samples are not Δ4/7 ER, and are probably due to a low level of amplification of WT ER mRNA in these samples. Other examples of tissue specific alternative splicing of mRNA have been previously reported in mammalian systems. For example, the β-tropomysin gene mRNA transcript has

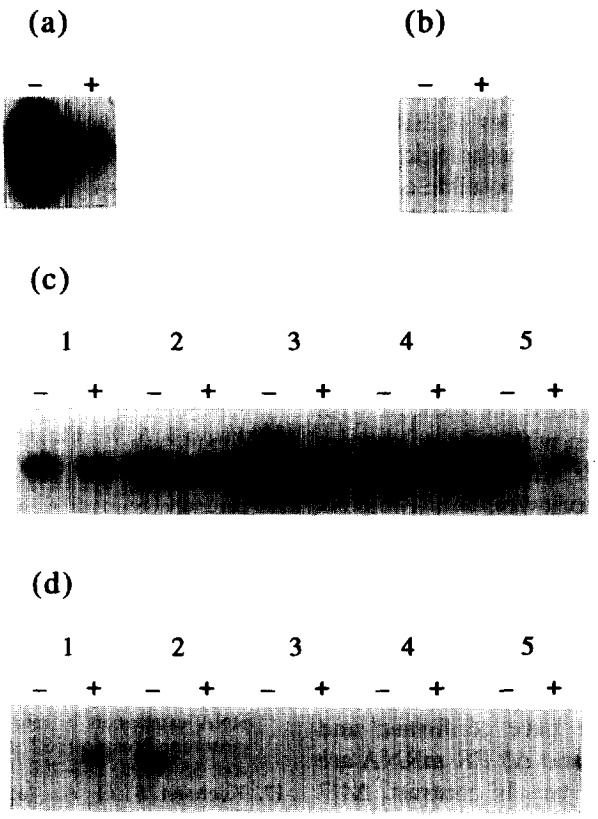


Fig. 5. Tissue specific expression of Δ4/7 ER mRNA. 1 ng of (a) variant or (b) WT ER RNA, produced by *in vitro* transcription, were amplified in parallel with total RNA was extracted from 5 normal endometrial (c) and 5 normal liver samples (d). 2 μg RNA (in duplicate) were subjected to RT–PCR (25 cycles) using variant specific primers which span exon 4 to the novel splice site. After RT and one round of PCR, one sample of each duplicate was restricted with StuI which digests DNA at the novel splice site (+) while the other sample remained undigested (–). After agarose gel eletrophoresis the DNA products were denatured, neutralized and blotted onto nylon membrane. The membrane was then probed with an internal exon 4 specific probe. These results indicated that variant (a), but not WT ER (b) mRNA was amplified by this technique, and that the amount of variant product was reduced by the StuI treatment described. It therefore follows that the novel variant is expressed in normal endometrium (c), but not in normal liver (d).

been shown to be cell type specific in rats [23]. That is, exon 5 splices to exon 6, and exon 6 splices to exon 8 in smooth muscle and fibroblast cells, while exon 5 splices to exon 7 which then splices to exon 8 in skeletal muscle cells. The tissue-specific expression of the ER variant indicates a possible role for this variant in some oestrogen responsive tissues, but not others. With respect to the Δ4/7 ER variant, many tissues respond differently to oestrogens and anti-oestrogens with respect to proliferation, and this variant and/or other variants of ER may be involved in conferring this tissue specificity. In addition, tissues express different oestrogen-responsive genes. For example, oestrogen stimulation of endometrium and breast results in an increased synthesis of progesterone receptor [24, 25] and decreased expression of ER [26]. In contrast, liver responds to oestrogens by increased synthesis of sex hormone binding globulin (SHBG) [27], and increased production of high density lipoprotein cholesterol (HDL-C) with concomitant decrease in low density lipoprotein cholesterols (LDL-C) [28], and increased expression of ER [26]. One may speculate that the variant ER mRNA products may demonstrate gene or promoter-specific DNA-binding or transcription and therefore be responsible in part for the control of such specific genes within these tissues. Thus the finding of this new variant and others previously reported may have exciting implications for the roles of ER splice variant in normal physiology and pathological conditions particularly as, in addition to possible cell- and promoter- specific activities, we have shown tissue type specific expression of certain ER splice variants also occurs.

However, an effect by ER variants in the oestrogen-responsive tissues is clearly dependent on their being translated into protein. Identification of translated variant protein has previously proved to be difficult with respect to the Δ5 variant protein with only one report published to date [29]. Further work on the protein products of these variants is a priority.

In summary, we have detected a novel splice variant of ER formed when regions within exons 4 and 7 are spliced together to form an unusual splice junction. The protein sequence of the Δ4/7 variant of ER would be relatively similar to that of the Δ5 variant in that the HBD and AF-2 regions would be missing, but AF-1 and DNA-BD still intact. We have confirmed and extended the findings that WT and Δ5 ER mRNA are expressed in endometrium and liver. In contrast, Δ4/7 ER mRNA was expressed in normal endometrial and in breast cancer samples, but could not be detected in the liver.

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