Tissue-specific expression of multiple mRNA variants of the mouse estrogen receptor α gene[1]

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Abstract The isolation of five new mouse estrogen receptor α mRNA variants (mERα A, B, F1, F2 and H) is described. All of these transcripts are generated by alternative splicing and all encode the 66 kDa ERα protein that the previously identified mRNA C variant generates. However, these transcripts differ in their 5′ untranslated regions. RT-PCR and S1 nuclease protection assays revealed a tissue- and sex-specific expression pattern of all variants. The C and F mRNA variants are the predominantly expressed mERα variants in mouse. The expression of mERα H mRNA is restricted to liver, although female mice produce around a five fold higher level of this transcript than males. Our results show that the mERα gene is a complex genomic unit in mice that exhibits alternative splicing which is regulated in a tissue-specific manner.

Key words: Estrogen receptor alpha; Alternative splicing; Gene expression

1. Introduction

Estrogens play a pivotal role in sexual development and in reproduction [1], as well as in physiological processes in various tissues such as mammary gland, pituitary, bone, liver and the cardio-vascular system. Estrogens are also involved in various pathological processes such as breast and endometrial cancer [2] and osteoporosis [3]. The physiological effects resulting from estrogen action on target tissues often induce changes in the expression patterns of specific target genes. These are mediated by intracellular receptors, which bind estrogen [4,5]. To date, two nuclear estrogen receptors ERα-NR3A1 and ERβ-NR3A2 (Nuclear Receptors Nomenclature Committee, 1999 [33]) have been identified [6,7]. Both receptors belong to the superfamily of nuclear receptors and steroid receptors family that act as ligand-inducible transcription factors [8–10]. These factors share a common overall structure, consisting of six domains (A–F) as demonstrated by homology comparison [11,12]. Two transactivation functions, known as AF-1 and AF-2 are located in the A/B and E domains, respectively. Domain C mediates binding to target DNA through a zinc finger motif. Domain D has a nuclear localisation signal and domain E interacts with ligand, thereby conferring ligand dependency on the AF-2 domain. Finally Domain F has been shown to modulate the magnitude of target gene activation [13].

ERα and ERβ are expressed in various cell types and tissues and the expression levels of their respective mRNAs appear to be regulated in a tissue-specific and temporal manner. Furthermore, the ER mRNA levels are influenced by estrogen itself [14–16] or by other hormones [17–19]. A more complete understanding of the mechanism(s) that regulate(s) the tissue-specific and temporal expression of ERs is essential towards understanding how the pleiotropic effects of estrogens are achieved in complex organisms.

During the late 1980s the cDNAs encoding human [6], chicken [11], mouse [20] and rat [21] ERα were cloned. Surprisingly, the first exon of mouse and rat had low homology to the human first exon [20,21]. However, the discovery of a second promoter and exon located approximately 1.9 kbp upstream of the initially reported transcription start site [6] in the human estrogen receptor gene [22,23] showed high homology to the first exon of the previously described mouse estrogen receptor α (mERα) gene [20]. Subsequently, other upstream exons in the ERα gene of human [24] and rat [25,26] were identified. Additionally, we have recently shown that the estrogen receptor α gene is transcribed from at least four promoters in chicken and from six promoters in human [27,28].

Although the mouse is extensively used as a model organism to study ERα regulation in vivo, the complete structure of the mERα gene has not, to date, been elucidated. Thus, the aim of this study has been to investigate if multiple mERα mRNA variants are expressed in mouse as has been described in humans and in chickens. Using the rapid amplification of cDNA ends (RACE) and RT-PCR techniques we have identified five novel mRNA variants generated by the mERα. These transcripts are generated in a tissue-specific manner by differential promoter usage and through alternative splicing of upstream exons. Moreover, sequence alignment of the new upstream exons showed high homology to human and rat ERα genes. This study indicates that the estrogen receptor gene α is a complex transcription unit with tissue-specific promoter usage and alternative splicing that has been conserved between various species during evolution.

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[1] The nucleotide sequences presented in this paper have been submitted to the EMBL/GenBank database under the following accession numbers: A – AJ272161; B – AJ272162; B2 – AJ272163; F1 – AJ272164; F2 – AJ272165; H – AJ272166; promoter region – AJ276597.

Abbreviations: mERα, mouse estrogen receptor α; RACE, rapid amplification of cDNA ends

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2. Materials and methods

2.1. Note to the terminology used in this study

All upstream untranslated exons are named with a single capital letter starting with the letter 'A'. All exons and mRNA variants homologous to human ERα (A, B, C, F) bear a name based on that given in Flouriot et al. [28]. The mRNA originally cloned by White et al. [20] is termed 'C'. Letters D and E were skipped due to the existence of such upstream exons in human and/or chicken ERα gene and which are not homologous to any described mouse ERα exon. Coding exons are simply numbered from exon 1 (containing the translation initiation ATG) to exon 8.

All position numbers are according to the sequence of cDNA cloned by White et al. [20].

2.2. RACE

A modified RACE technique was used to amplify the 5′ ends of mERα mRNA variants. In essence, a long, highly labelled primer, complementary to the 252 nt region downstream of the initiating AUG from position +191 to +443 of mERα mRNA was prepared using a biotinylated template as described [29]. Total RNA (35 μg) from uterus and from liver were co-precipitated with the primer, resuspended in hybridisation buffer and hybridised overnight at 55°C. The primed RNA was precipitated and reverse-transcribed at 42°C using 50 U of Expand™ reverse transcriptase (Roche Diagnostics, Mannheim, Germany) following the instructions of the manufacturer. RNA in RNA/DNA hybrids was then degraded using DNase-free pancreatic RNAse. One half was then loaded on a denaturing polyacrylamide gel to check the primer extension pattern. The second half was tailed with dCTP using terminal deoxy transferase (Gibco-BRL, Eggenstein, Germany) as described by the manufacturer. Tailed cDNA was then amplified by PCR using upstream anchor primer (5′-GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3′) and downstream primers P1 (5′-GGCAGCCGGCGAGTTACAGA-3′) and P2 (5′-GCTCCCCGCGCACGCAGAGG-3′) located in exon 1. PCR products were subcloned in the TA cloning vector pCR™2.1 (Invitrogen, San Diego, CA, USA) and colonies were screened with oligonucleotides C1 (5′-ATCACACACCGCGCCACTCGATCAT-3′), specific for the C mRNA variant [20] and P3 (5′-CTCAGCA-GCGCGTGGGCCAC-3′) which is nested to primer P2. Clones that hybridised with the P3 probe but not with the C1 probe were sequenced.

2.3. RNA isolation

Adult female or male mice were killed by cervical dislocation. The uterus, brain, lung, kidney, femoral muscle, aorta, liver and testis were immediately removed and frozen in liquid nitrogen. Tissues were stored at −80°C before use. Total RNA was extracted from tissues of several mice using TRIzol reagent (Gibco-BRL, Eggenstein, Germany) as described by the manufacturer. RNA was dissolved in water, aliquoted and stored in −80°C. A quality of the RNA was checked by agarose gel electrophoresis. The ratio of absorbance at 260 and 280 nm was in the range between 1.8 and 1.9. The complete coding region of the estrogen receptor α cDNA (2010 bp) was consistently amplified from the isolated RNA, which was used for all experiments.

2.4. RT-PCR

Total RNA (2 μg) was reverse transcribed using Expand™ reverse transcriptase (Roche diagnostics) as described by the manufacturer with a primer 5′-ATGAACCAGCTCCCTATCTG-3′, which is complementary to exon 4 of the mERα gene. 10% of the resulting cDNA was used as a template in two rounds of PCR using nested upstream primers specific for different 5′ends of mRNA variants and downstream primers specific for exon 4 (5′-TTCAACATTCTCCCT-CCTCGGCGGTCTTTC-3′ and exon 3 (5′-TTCATCATGCCCACT-TCGTAACACT-3′). Sequences of nested PCR primers specific for various 5′UTRs were as follows: Exon A: 5′-AACTATGCGTG-CGCCTTCTCTAAT-3′, 5′-CTTCTCTAATCGCAGGCTCTACTC-T-3′. Exon B: 5′-TCCACCCCTGCAGCGAATCC-3′, 5-CAGCGA-ATCCAGCAGCGAAGACC-3′. Exon C: 5′-ATCACACACCGC-GCCACTCGATCAT-3′, 5-ATTCCTTCCTTCCGTCTTACTGTCT-CA-3′. Exon F1: 5′-TGCCCCCTCCTCTGCCATTGTCT-3′, 5′-TCTCTGGGCGACATTCTTCTCAA-3′. The primer complementary to the rat 0S mRNA variant [26] used for isolating and cloning of the mouse F mRNA variant was 5′-GAAAACACAAGGCTCCATGCT-3′. Amplified products were separated by agarose gel electrophoresis and transferred to Hybond N+ membrane (Amersham) with a Semi-Dry Electrotransfer unit (Bio-Rad). Membranes were hybridised under stringent conditions with ^{32}P end-labelled oligonucleotide probes specific for exons 1, 2 or 3 of the mERα gene.

2.5. Sequence analysis and alignment

All sequence alignments were computer-assisted using the Gap pairwise comparison procedure in Wisconsin Package Version 10.0, Genetics Computer Group (GCG), Madison, WI, USA.

2.6. S1 nuclease assay

A modified S1 nuclease protection assay was performed as described [30]. As probe templates fragments obtained in RACE or RT-PCR experiments cloned into the TA cloning vector pCRTM2.1 (Invitrogen, San Diego, CA, USA) were used. The probe templates were amplified from the vector using either biotinylated M13 or M13 reverse primers. Biotinylated PCR products were bound to streptavidin-coated magnetic beads (Dynal, Great Neck, NY, USA) as recommended by the manufacturer and the non-biotinylated DNA strands were eluted with 0.2 M NaOH. A primer complementary to the 3′ end of the exon one (5′-GTAGAAGGCGGGAGGGCCG-GTGTCGCGCA-3′) was then annealed to the single-stranded DNA and extended using T7 DNA polymerase in presence of [α^{32}-P]dCTP (3000 Ci/mmol). Thus the resulting probes were complementary to the whole exon 1 and the complete isolated 5′UTR specific sequences. Extended products were denatured with 0.2 N NaOH and purified on an 8% denaturing polyacrylamide–urea gel. This probe (10^4 cpm) was co-precipitated with 30 μg of total RNA and then dissolved in 20 μl hybridisation buffer. Samples were denatured at 70°C for 10 min and hybridised overnight at 55°C. S1 digestions were then carried out in the presence of 300 units of S1 nuclease for 1 h at 30°C and after precipitation, the samples were separated on 4% polyacrylamide–urea gels and exposed to film.

3. Results

3.1. Multiple mRNA variants of the mERα

Sequence alignment of mouse ERα mRNA [20] with various human and chicken ERα mRNA variants predicted that a conserved common acceptor splice site exists at position +119 in exon 1 of the mouse ERα gene. In order to investigate if more than one mERα mRNA variant occurs in mouse by alternative splicing of upstream exons to the splice acceptor site at +119, S1 nuclease protection assay was performed using a probe spanning the region +1 to +652 of the original mERα mRNA described by White et al. [20] (renamed as variant C in this study), with total mRNA from several mouse tissues. Two protected fragments were observed in all tissues; one corresponding to mERα mRNA variant C (totally protected probe) and another shorter fragment corresponding in size to the cDNA from position +119 to +652 (Fig. 1). The presence of a strong signal for the shorter protected fragment in all tissues tested indicated an existence of unknown mERα mRNA variant(s) in addition to the mERα C mRNA variant. A similar experiment with equivalent results was performed with a longer S1 probe spanning the region +1 to +1270 (data not shown).

In order to isolate further 5′ end(s) of unknown mERα mRNA variant(s) a modified technique of RACE was performed. A long, highly radioactive labelled primer spanning the region of 252 nucleotides downstream of the initiator ATG in the first exon of the mouse ERα gene was used. In addition to the previously described variant C, the 5′ends of three different mERα mRNA variants (A, F, H) were cloned from uterus and liver total RNA isolated from adult female mouse. Variant A corresponds to the genomic sequence that is directly upstream of the first coding exon of mERα gene. A

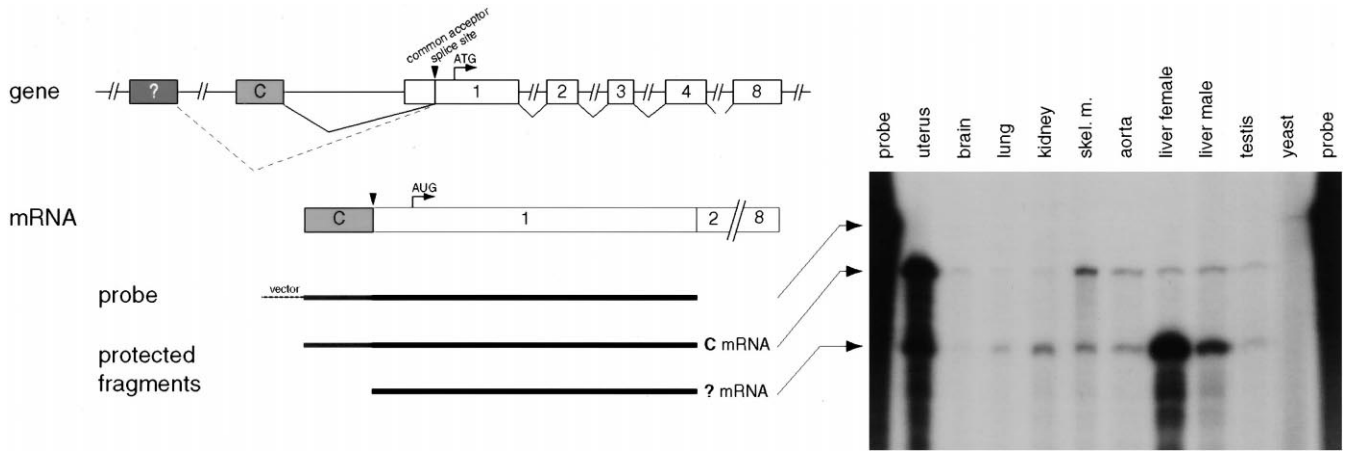

Fig. 1. Scheme of mERα gene and mRNA as described by White et al. [20] with the potential upstream untranslated exon represented by a dark grey rectangle with '?' and the dashed line representing splicing to a common acceptor splice site located in the first translated exon. The translation initiation AUG is depicted. S1 nuclease assay probe spanning the region of mERα mRNA from the transcription start site up to the end of exon 1 and including non-homologous vector sequence at its 3′ end and presumed protected fragments are outlined below. On the right the autoradiogram of the experiment is shown.

homologous human ERα mRNA was the first ERα mRNA cloned [6] (Fig. 2). Part of the sequence of the mERα H mRNA variant was almost identical to the rat ERα mRNA variant isolated from liver [31] but extended further in 5′ direction (Fig. 2). Although only six nucleotides of mRNA variant F were isolated, it was present in 20% of clones generated by RACE.

The murine mRNA variant A isolated in RACE experiments showed homology to the human ERα A mRNA variant, which is transcribed from a promoter located upstream of the first translated exon of human ERα gene. As another human mRNA variant (B) is transcribed from the promoter located upstream of promoter A [28], we were interested if an analogous promoter exists in mouse. The intronic region, spanning approximately 2 kbp between the originally described first exon and the first translated exon of the mouse ERα gene, was cloned and sequenced. Alignment with the corresponding promoter region of the human ERα gene revealed homology to the A and B promoters and associated exons of the hERα gene [28]. This suggested that promoter B might also be functional in mouse. RT-PCR with mRNA from uterus and liver using primers complementary to this B homologous region and downstream primers located in the translated exon 2 confirmed the expression of this mERα B mRNA variant in these two tissues (data not shown). Additionally, a PCR product longer than expected was amplified with primers specific for exon B. Sequencing analysis revealed an mRNA variant generated by utilisation of another donor splice site located 100 bp downstream of the donor splice site in exon B that is conserved between human and mouse. This longer variant was called B2.

As two mouse ERα mRNA variants (C and H) showed high homology to rat sequences (Fig. 2) we decided to investigate if the other two known rat ERα mRNA variants 0N and 0S [25,26] have counterparts in mouse. RT-PCR analysis of total RNA from mouse uterus and liver using upstream primers complementary to the sequences of rat 0N and 0S ERα mRNA variants [25,26] confirmed the presence of a corresponding ERα 0S mRNA variant [26] in mouse. However, the rat ERα 0N mRNA variant [25] was not detected in these tissues, which might be due to the low homology of the used rat 0N primers to the mouse sequences. Unexpectedly, two

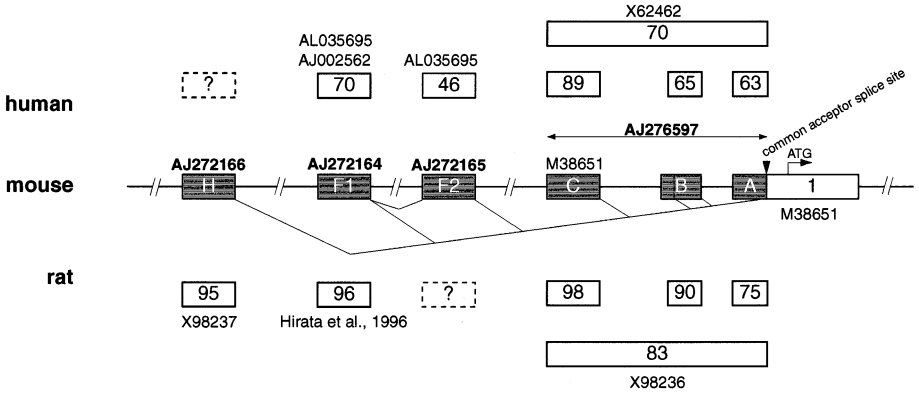

Fig. 2. Presentation of the alignment of the 5′ region of the human, mouse and rat estrogen receptor α genes. Various upstream untranslated exons (A–H) are depicted as boxes. EMBL nucleotide sequence database accession numbers or other sources of the sequences used are shown (new sequences in bold type). Numbers within the boxes represent the percentage of identity between mouse and human or mouse and rat sequences respectively. The question mark ('?') is used where no sequence data are available. Observed splicing of mouse ERα upstream exons is depicted as thin lines.

products were amplified with primers complementary to the rat 0S mRNA variant. Sequence analysis of both fragments revealed a sequence highly homologous to the rat ERα mRNA variant 0S [26] but with a sequence inserted between the common splice site and the region homologous to 0S in the longer fragment. This 109 bp sequence, as well as the corresponding part of the longer sequence, is highly homologous to the recently described hERα F mRNA variant [28] and therefore we termed these two new sequences F1 (shorter sequence) and F2 (109 bp inserted sequence)(Fig. 2). Alignment with a contig (EMBL: AL035695) from the human chromosome 6, where the human ERα gene is localised, revealed that regions homologous to F1 and F2 are located approximately 60 kbp apart in the human genome and therefore are two separate exons. In mouse, exon F1 is either spliced directly to the common acceptor splice site or to exon F2 and then to the common acceptor splice site +119 (Fig. 2). Furthermore, the six nucleotides isolated in the RACE experiment are derived from exon F2 and are present upstream of the donor splice site. It is not clear if this is an artefact of RACE, as RT-PCR analysis consistently amplified the entire F2 region. As the F2 exon is part of the F1F2 mRNA variant the F2 mRNA variant cannot be distinguished by PCR using an F2 specific primer. Thus experiments were performed using the F1 specific primers and S1 probes complementary to the F1F2 mRNA variant. For simplicity we refer to this longer variant in the following text as 'F'.

Alignment of various upstream exons of mouse ERα gene with their equivalents from human and rat and the proposed genomic organisation of the 5′ region is presented in Fig. 2.

3.2. Tissue-specific expression of mERα mRNA variants

RT-PCR analysis was used to determine if ER-α mRNA variants are differentially expressed in various mouse tissues. Variants A, C and F were detected in all tissues tested. However, mRNA variant B was not detected in lung and aorta and mRNA variant H was present only in liver and testis. The

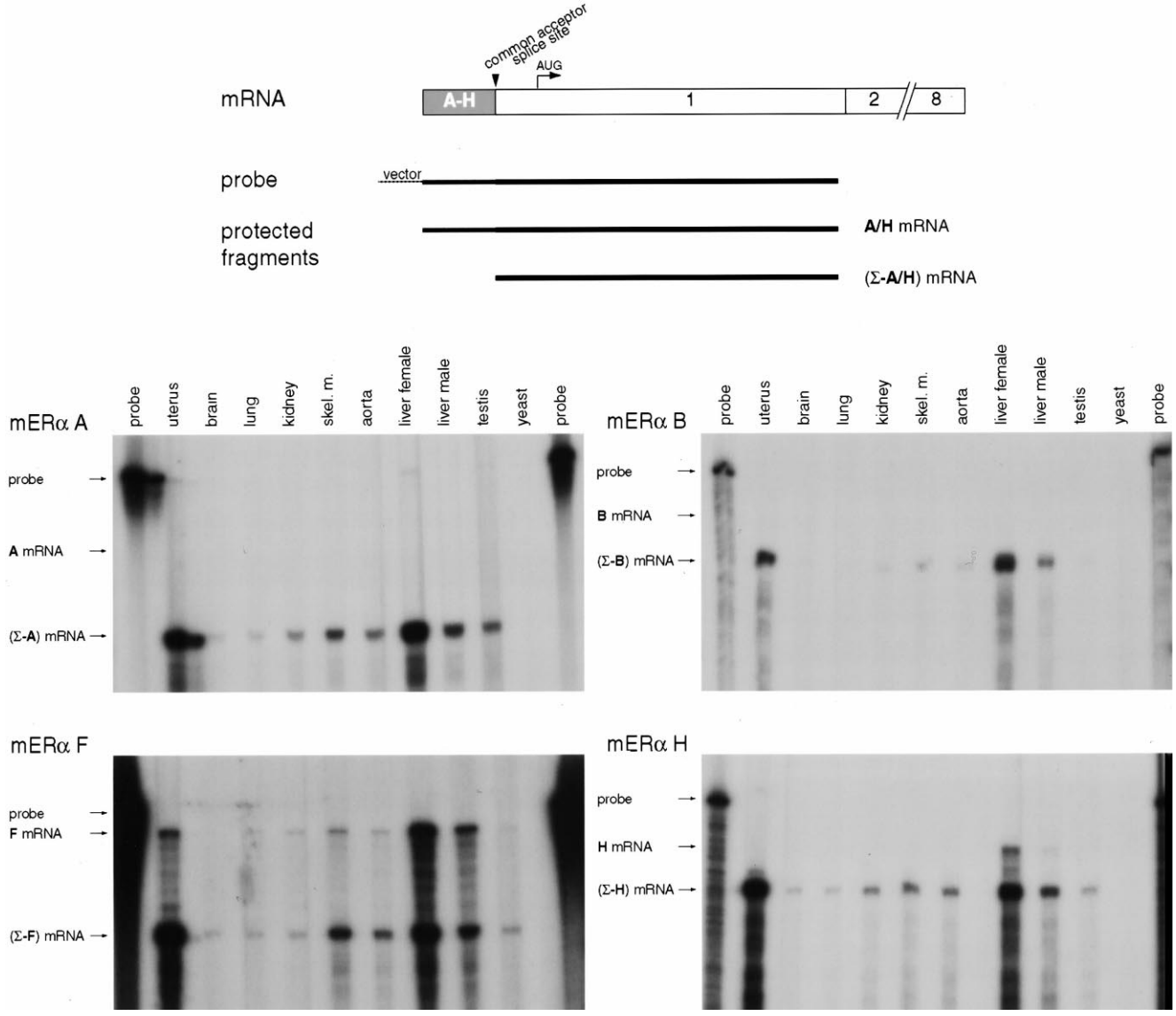

Fig. 3. S1 nuclease protection assay using probes complementary to the five new mERα mRNA variants. Top: a generalised diagram of an mRNA variant, the region covered by the probe and expected protected fragments is shown. Autoradiograms of S1 nuclease protection assay with probes complementary to mERα mRNA variants A, B, F and H are shown.

Table 1
RT-PCR and S1 analysis of the tissue distribution of mRNA variants

Tissue/variant	A	B	C	F	H	Total
Uterus	+	+	53	28	−	81
Brain	+	+	49	+	−	49
Lung	+	−	9	26	−	35
Kidney	+	+	6	27	−	33
Skeletal muscle	+	+	60	16	−	76
Aorta	+	−	40	12	−	52
Liver female	+	+	7	49	28	84
Liver male	+	+	13	41	6	60
Testis	+	+	32	16	+	48

Presence or absence of mERα mRNA variants in various tissues as observed in RT-PCR and S1 nuclease protection experiments: − mRNA variant observed neither in RT-PCR nor S1 analysis; + mRNA variant detected by RT-PCR only; Number stand for the relative abundance (in percent) of a particular mRNA variant in a tissue as estimated from S1 nuclease protection assay experiments (quantification: specific protected fragment+common protected fragment = 100%).
In the column 'total' the sum of the relative abundance of all mRNA variants in a tissue is calculated.

expression profiles of the various mRNA variants in particular tissues are summarised in Table 1.

As RT-PCR does not provide a quantitative determination of transcripts in cells, S1 nuclease protection assays were used to estimate the relative abundance of mERα mRNAs in a panel of mouse tissues (Fig. 3). The probes used contained the specific part of the particular mRNA variant followed by a sequence complementary to exon 1 and short vector sequence at the 5′ end to distinguish digested from undigested probe (see Fig. 3). Hence two protected fragments were anticipated, a fragment corresponding to fully protected probe, i.e. corresponding to the particular mERα mRNA variant, and a second shorter protected fragment corresponding to exon 1, which is common to all mRNA variants. This allows an estimation of the relative abundance of the particular mRNA variants in tissues by comparing the signal intensities for those two fragments. Variants A and B were not detectable by S1 nuclease protection assay indicating that their levels of expression in the tissues tested are very low. Variant C was expressed in all tissues examined and was the major mRNA variant present in uterus, brain, and skeletal muscle. Also, the mRNA variant F was detected in all tissues evaluated except brain and represents the predominant mRNA variant in liver. The H mERα mRNA variant was also detected by S1 nuclease protection assay only in male and female liver. Results of quantification using densitometric analysis together with RT-PCR analysis are summarised in Table 1.

4. Discussion

This study indicates that the mERα gene is transcribed from at least five different promoters. Even though the existence of several promoters of human, chicken and rat ERα genes has already been demonstrated [22,24,27,28], the sequences of mouse upstream exons have remained unknown so far. Various mRNA splice variants within the coding region of mouse ERα were reported [32]. However, no information on the organisation of the 5′ region of the mERα gene has been available to date. Sequence alignment of newly described exons indicates a high degree of homology between human, rat and mouse 5′ UTRs. Various promoter utilisation and alternative splicing generate mRNA variants that differ in their 5′ untranslated regions. All these mRNAs can be translated into the same 66 kDa ERα protein. The presence of multiple promoters seems to be a general feature of steroid/thyroid hormone/retinoic acid receptors. Each cell type possesses a different composition of transcription factors, cofactors and other regulatory proteins that selectively direct utilisation of various promoters. Presumably multiple promoters are used to achieve various levels of expression of mERα protein needed for the particular cell function.

S1 analysis of several mouse tissues revealed a tissue specific expression for all mRNA variants. The abundance of mRNA variants is very different in all tissues tested. Surprisingly, although the mRNA A variant is highly expressed in several human tissues and in breast cancer cell lines [28], in mouse tissues it was detected only by RT-PCR and not by S1 nuclease protection assay. Though the sequence alignment of mouse and human promoter A region show high homology, recognition sequences required for utilisation of this promoter may have been lost during evolution of the mouse. Variant B is expressed in the tissues tested at levels not detectable by S1 nuclease protection assay and it is also not a major mRNA variant in human [28]. Murine variant H it is almost identical to the rat ERα mRNA variant isolated from liver by Freyschuss and Grandien [31]. As demonstrated in this study by S1 analysis, use of this promoter is restricted to liver in mouse. The expression profile of this variant in rat has not been reported. The level of expression of variant H is different between the two sexes as in female liver it forms almost one third of total mERα mRNA while in the male liver it is a minor mERα mRNA variant. Interestingly, variant H is not homologous to the human mRNA variant E [28], which was also detected exclusively in human liver by S1 nuclease protection assay. Presumably, the H promoter is responsive only to transcription factors that are expressed only in this tissue.

Variants C and F are expressed in all tissues tested (in both mouse and human) and are the major mRNA variants present in mouse. Variant C is highly expressed in uterus, testis, brain, skeletal muscle and aorta, where it is the major mERα mRNA variant. Unlike the mERα C, variant F is predominant in liver of both sexes. Interestingly, the ratio of variants C and F in uterus and testis are similar. Both are reproductive tissues and might possess a similar array of transcription factors and other cofactors that can activate this promoter. Variants C and F may generate a basal level of ERα protein that is required for the physiological functions of cells and a temporal requirement of higher ERα levels can be regulated by switching on other promoters in a particular tissue.

The estimation of the relative abundance of mRNA variants shows that the sum of the relative amounts of variants A, B, C, F and H does not account for the strong signal obtained for protected exon 1 fragment in S1 analysis in all tissues except uterus and female liver. It is therefore likely that other unknown upstream exons or other alternatively spliced mRNA variants exist in other tissues. The fact that they were not detected by RACE suggests that these putative mRNA variants are expressed at very low levels in uterus and in liver, which were used as a source of mRNA for RACE. The total levels of mERα expression differ between tissues. ERα is highly expressed in uterus and liver and to a lower extent in

skeletal muscle and aorta. However in other tissues mERα expression is hardly detectable by S1 nuclease protection assay.

In this study five new mERα mRNA variants and a likely genomic organisation of the 5′ region of the mERα gene are presented. Mice are often used as a model to study the role of estrogens in mammals. The sequences of the new upstream exons presented and their adjacent regions contribute to the understanding of the mechanisms of estrogen action in various physiological and pathological conditions.

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