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Expression of estrogen receptor β exon-deleted variant mRNAs in ovary and uterine endometrium☆

Jiro Fujimoto*, Wen-Shu Sun, Ryou Misao, Hideki Sakaguchi, Ikumi Aoki, Hiroshi Toyoki, Teruhiko Tamaya

Department of Obstetrics and Gynecology, Gifu University School of Medicine, 40 Tsukasa-machi, Gifu City 500-8705, Japan

Abstract

Various estrogen receptor β exon-deleted variant (ER-β EDV) mRNAs were expressed in human ovary and uterine endometrium. Estrogen receptor β (ER-β) completely or partially deleted exon n is expressed as ER-β EnDV or En′DV, respectively. The mRNAs for ER-β single exon-deleted variant (EDV), ER-β E2DV, E4DV, E5DV and E6DV; for ER-β double exon-deleted variants, ER-β E1′ + 2DV, E4 + 5DV and E5 + 6DV; and for ER-β triple exon-deleted variants, ER-β E2′ + 3 + 4DV and E4 + 5 + 6DV were detected. In ER-β E2DV, E4 + 5DV, E5DV and E6DV mRNAs, the new stop codon is made in the exon following the deleted exon(s), and the new proteins may lack the corresponding domains. In ER-β E1′ + 2DV, E2′ + 3 + 4DV, E4DV, E4 + 5 + 6DV and E5 + 6DV mRNAs, the original stop codon is still present, and the new proteins may conserve the new short amino acid sequences surrounding the deleted exons. ER-β E1′ + 2DV, E2DV, E2′ + 3 + 4DV, E4DV, E4 + 5DV and E4 + 5 + 6 are unlikely to work as a transcription factors. On the other hand, ER-β E5DV, E6DV and E5 + 6DV lack only the ligand-binding domain, and might work as dominant positive or negative factors. Therefore, ER-β E5DV, E6DV and E5 + 6DV, constitutively expressed in human ovary and uterine endometrium might, in part regulate estrogen responsiveness.

Keywords: ER-β; Exon-deleted variants; Ovary; Uterine endometrium

1. Introduction

Growth and function of female reproductive organs, especially uterus and ovary, are partly regulated by estrogen [1]. Therefore, estrogen might reflect the expression and function of estrogen receptor (ER) in the target cells. Although de novo mutation of the ER-α gene is a rare event even in estrogen target tissues [2,3], ER-α single exon-deleted variants (EDVs), such as ER-α E2DV [4,5], E3DV [4,6], E4DV [5,7,8], E5DV [5,9,10], E6DV [6] and E7DV [4–7,11], as well as ER-α double EDV E4 + 7DV, were cloned from tumor cells. In addition, E4DV [8,12] and E5DV mRNAs [9] are expressed in normal tissues from human uterus and rat uterus and brain. ER-α EDVs are a common event in normal tissue.

Although multiple forms had been identified for other members of the nuclear receptor superfamily, the existence of only one ER was believed for about 10 years following the cloning of ER-α cDNA [13,14]. Then novel rat ER-β (rER-β) [15] and human ER-β (hER-β) [16,17] were identified in cDNA libraries from rat prostate and human testis, respectively. The hER-β consists of 530 amino acids [17]. The rER-β consists of 485 amino acids and distinctly expresses in epithelial cells of prostate, granulosa cells of ovary [18], and osteoblasts of bone [19]. Furthermore, ER-β has high affinity for estradiol-17β [18,20] and characteristics similar to ER-α in specific binding to various estrogenic substances and antagonists [18]. Although the phosphorylation site for mitogen-activated protein kinase is conserved in the activation function (AF)-1 region of ER-β as in ER-α [21], both AF-l and AF-2 regions of ER-β are shorter than those of ER-α. Furthermore, the transcription at an AP1 element was inhibited by estradiol and activated by antiestrogens via ER-β cascades [22]. This indicates some difference in the transcriptional efficiency and regulatory potential of the target genes. Human ER-β specifically expresses in testis, ovary, thymus, spleen [16], osteoblasts [23] and fetus [24].

Although dysfunctions of the genital tract were likely to occur only in female ER-α knockout mice, disruption of reproductive functional behavior associated with sterility and reduction of bone density in both males and females was observed [25,26]. It was later demonstrated that ER-α defects

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* Corresponding author. Tel.: +81-58-267-2631; fax: +81-58-265-9006.
E-mail address: jf@cc.gifu-u.ac.jp (J. Fujimoto).

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lead to abnormalities in spermatogenesis [27] and sexual behavior [28] in males and abnormalities in sexual behavior in females [29]. The hER-α defect disease has been discovered in only one man. His symptoms were unfused epiphyses in the knees, demineralized bone, low sperm viability, hyperestrogenemia, slightly abnormal glucose tolerance, acanthosis nigricans, etc. [30]. As ER-β does not compensate for the defects that are observed in the ER-α knockout mouse or in man, ER-β might not conserve the same physiological functions as does ER-α, and may play a role of co-function with ER-α [31]. Therefore, ER-β EDV expression needs to be analyzed in target tissues.

Recently, several ER-β EDVs have been reported as follows: ER-β E2DV is observed in the majority of all ER-β-positive pituitary tumors [32]. ER-β E5DV lacks the ligand-binding domain, and is expressed in estrogen-independent breast cancers [33]. ER-β E8DVs (hERβ2 and hERβ3) and E1+2+3+4+5+6DVs (hERβ4 and hERβ5) were detected in breast cancers [34,35] and in colon cancers [36]. The significance of ER-β EDV expressions has been considered in breast, colon and pituitary tumors. However, the expression of ER-β EDVs in normal tissues needs to be confirmed, and then ER-β EDVs must be considered for their overall biophysiologic significance, as the ER-αEDVs have been. Therefore, we investigated the existence and significance of ER-β EDVs in normal ovary and uterine endometrium.

2. Materials and methods

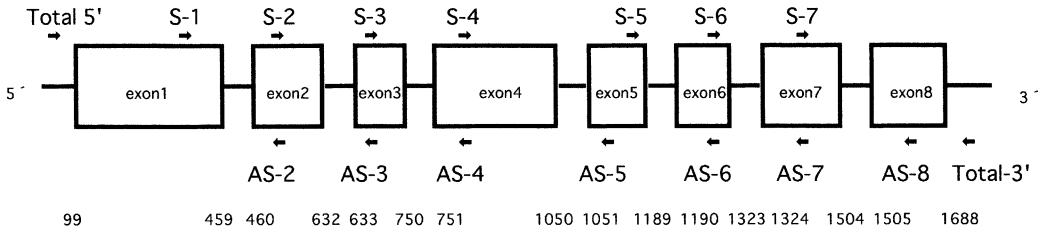
2.1. Patients

Consent for the following studies was obtained from all patients and the Research Committee for Human Subjects, Gifu University School of Medicine. Thirty patients ranging from 33 to 47 years of age underwent operation for uterine leiomyoma (30 cases) at the Department of Obstetrics and Gynecology, Gifu University School of Medicine, between January 1998 and 1999. None of the patients had received any preoperative therapy. A part of the tissues of normal ovary and uterine endometrium was snap-frozen in liquid nitrogen for the studies to determine ER-β EDV mRNA expressions, and a neighboring part of the tissues was submitted for histopathological study.

2.2. Reverse transcription and polymerase chain reaction (RT-PCR)

Total RNA was isolated from the tissues by the acid guanidium thiocyanate–phenol–chloroform extraction method [37]. Total RNA (3 μg) was reverse transcribed with Moloney murine leukemia virus reverse transcriptase (MMLV-RTase, 200 units, Gibco BRL, Gaithersburg, MD, USA) in 20 mM Tris–HCl, pH 8.4, 50 mM KCl, 2.5 mM $MgCl_2$, 0.1 mg/ml bovine serum albumin, 10 mM dithiothreitol, and 0.5 mM deoxynucleotides to generate cDNAs using random hexamer (50 ng, Gibco BRL) at 37 °C for 60 min. The RT reaction mixture was heated at 94 °C for 5 min to inactivate MMLV-RTase.

The specific primers for PCR were synthesized according to the published information on ER-β gene [17] as shown in Fig. 1. PCR for reverse transcribed cDNA, consisting of 1 min at 94 °C for denaturation, 1 min at 55 °C for annealing, and 1 min at 72 °C for extension, was carried out with templates and 0.1 μM specific primers using the Iwaki thermal sequencer TSR-300 (Iwaki Glass, Tokyo, Japan), with Vent DNA polymerase (New England Biolabs, Beverly, MA, USA) in 10 mM KCl, 20 mM Tris–HCl, pH 8.8, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100 and 0.15 mM deoxynucleotide phosphates for 30 cycles.



Sense primers:		Antisense primers:	
Total 5' (1-20)	GTTGACAGCCATTATACTTGC	AS-2 (506-525)	TTGAACCTGGACCAGTAACA
S-1 (417-436)	TGGTGTGAAGCAAGATCGCT	AS-3 (652-671)	ACACTGATTTGTAGCTGGAC
S-2 (506-525)	TGTTACTGGTCCAGGTTCAA	AS-4 (779-798)	TTCTCTGTCTCCGCACAAGG
S-3 (652-671)	GTCCAGCTACAAATCAGTGT	AS-5 (1060-1079)	TTGGTCGAACAGGCTGAGCT
S-4 (779-798)	CCTTGTGCGGAGACAGAGAA	AS-6 (1288-1307)	CATGGCCTTGACACAGAGAT
S-5 (1167-1186)	GCTCCAGATCTTGTTCTGGA	AS-7(1410-1429)	CTCTTGGCAATCACCCAAAC
S-6 (1288-1307)	ATCTCTGTGTCAAGGCCATG	AS-8 (1601-1620)	TGATGGAGGACTTGCACCCG
S-7 (1410-1429)	GTTTGGGTGATTGCCAAGAG	Total-3'(1721-1740)	ACGCTTCAGCTTGTGACCTC

Fig. 1. Schematic of estrogen receptor-β gene and primers for polymerase chain reaction.

The ratios of ER-β exon-deleted variants mRNAs to ER-β wild type mRNA levels of were determined from three parts taken from each tissue, and the assay for each sample was carried out in triplicate.

2.3. DNA sequence analysis

PCR products for the ER-β DNA fragment were separated by 12% polyacrylamide gel electrophoresis, eluted in an elution buffer (0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, pH 8.0, and 0.1% sodium dodecyl sulfate) from the gel stained with ethidium bromide, and purified with a Quick Spin G-50 Sephadex Column (Boehringer Mannheim, Indianapolis, IN, USA). The single-strand DNA fragment was generated from purified PCR products with an AutoLoad Solid Sequencing Kit (Pharmacia Biotech, Uppsala, Sweden). The DNA extension with Cy5-labeled primer and the single-strand DNA fragment as a template was carried out with a Cy5 AutoRead Sequencing Kit (Pharmacia Biotech). The PCR products from ER-β exon-deleted variant mRNAs were analyzed with an ALFexpress DNA sequencer (Pharmacia Biotech).

3. Results

The mRNAs for ER-β single exon-deleted variants, ER-β E2DV, E4DV, E5DV and E6DV; for ER-β double exon-deleted variants, ER-β E1′ + 2DV, E4 + 5DV and E5 + 6DV; and for ER-β triple exon-deleted variants, ER-β E2′ + 3 + 4DV and E4 + 5 + 6DV were detected in human ovary and uterine endometrium as shown Fig. 2 for a representative case. The ratios of ER-β exon-deleted variants mRNAs to ER-β wild type mRNA in ovary and uterine endometrium are variable (Table 1) as good as those in ovarian cancers ($n = 12$) and uterine endometrial cancers ($n = 12$) (data not shown).

In ER-β E2DV, E4 + 5DV, E5DV and E6DV mRNAs, the new stop codon is formed in the exon following the deleted exon(s), and the new proteins may lack the corresponding domains, as shown in Fig. 3. Therefore, it can be assumed that ER-β E2DV conserves only exon 1, ER-β E4 + 5DV lacks the hinge and ligand-binding domains, and ER-β E5DV and E6DV lack the ligand-binding domain.

In ER-β E1′ + 2DV, E2′ + 3 + 4DV, E4DV, E4 + 5 + 6DV and E5 + 6DV mRNAs, the original stop codon is still present, and the new proteins may conserve the new short amino acid sequences surrounding the deleted exons (Fig. 3). Therefore, it can be assumed that ER-β E1′ + 2DV lacks the transactivation domain, E2′ + 3 + 4DV the DNA binding + hinge domains, E4DV the hinge domain, E4 + 5 + 6DV the hinge + ligand-binding domains, and ER-β E5 + 6DV the ligand-binding domain.

4. Discussion

ER-α E2DV protein has no DNA binding since it is truncated before the two Zn fingers and has been found to have only mild dominant negative properties on ER-α wild type (ER-α WT) [4,5]. ER-α E3DV protein inhibits the transcriptional activity of ER-α WT as a dominant negative receptor [4,6]. ER-α E4DV protein has no in vitro DNA binding, hormone binding, or transactivating function, and thus exhibits no dominant negative activity [8]. Among ER-α EDVs, only ER-α E5DV protein is transcriptionally active without ligand-binding [9], mediated by AF-1 in the N-terminal domain, in transiently transfected cells [38] and yeast [39], being related to tamoxifen-resistant growth of breast cancer (MCF-7) cells [40]. Furthermore, relative overexpression of ER-α E5SV contributes to secondary spreading of gynecological cancers as a dominant positive property, regardless of estrogen dependency [41]. ER-α E6DV mRNA is expressed at a low level together with moderately expressed ER-α E3DV mRNA in MCF-7 breast cancer cells [6]. ER-α E7DV protein binds to DNA without transactivating function, exerting dominant negative actions in yeast but not HeLa cells [4,11]. Moreover, all the ER-α variants lacking one or more exons have been detected in the same RNA preparation not only from tumors and MCF-7 cells but also from the corresponding normal tissue, thus making the involvement of ER-α EDV in tamoxifen-resistant transformation rather unlikely [42,43]. The relative amount of splicing variant (ER-α E2DV, E3DV, E4DV, E5DV, E7DV and

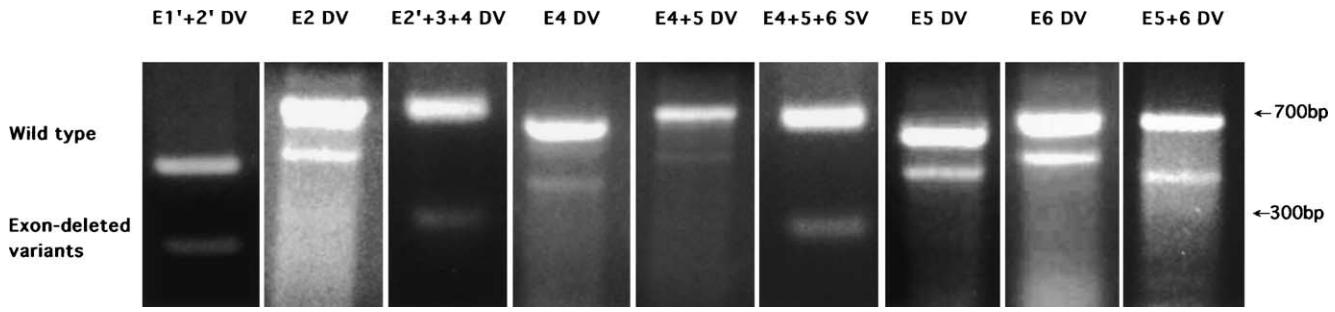


Fig. 2. Expression of estrogen receptor β exon-deleted variant mRNAs in uterine endometrium. Amplified PCR products from a representative case (case 4 as shown in Table 1) of uterine endometrium was electrophoresed with 1.2% agarose gel in a 100 V constant voltage field.

Table 1
The ratio of estrogen receptor β exon-deleted variant mRNAs to wild type mRNAs in ovary and uterine endometrium

Case number	E1′ + 2′DV	E2DV	E2′ + 3 + 4DV	E4DV	E4 + 5DV	E4 + 5 + 6DV	E5DV	E6DV	E5 + 6DV
Ovary									
1	0.28 ± 0.36	0.28 ± 0.27	0.24 ± 0.29	0.19 ± 0.25	0.24 ± 0.30	0.33 ± 0.24	0.33 ± 0.36	0.33 ± 0.36	0.27 ± 0.28
2	0.23 ± 0.33	0.31 ± 0.33	0.37 ± 0.40	0.25 ± 0.37	0.27 ± 0.24	0.50 ± 0.38	0.36 ± 0.30	0.47 ± 0.34	0.36 ± 0.33
3	0.18 ± 0.34	0.19 ± 0.24	0.31 ± 0.24	0.26 ± 0.25	0.43 ± 0.36	0.37 ± 0.25	0.32 ± 0.28	0.19 ± 0.30	0.44 ± 0.43
4	0.31 ± 0.24	0.39 ± 0.29	0.32 ± 0.33	0.31 ± 0.29	0.28 ± 0.26	0.32 ± 0.28	0.36 ± 0.33	0.32 ± 0.27	0.32 ± 0.27
5	0.46 ± 0.37	0.37 ± 0.34	0.25 ± 0.23	0.31 ± 0.26	0.25 ± 0.22	0.34 ± 0.26	0.34 ± 0.27	0.32 ± 0.26	0.29 ± 0.19
6	0.19 ± 0.35	0.33 ± 0.23	0.16 ± 0.22	0.19 ± 0.24	0.22 ± 0.28	0.39 ± 0.39	0.28 ± 0.23	0.18 ± 0.25	0.19 ± 0.26
Mean ± S.D.	0.27 ± 0.32	0.31 ± 0.27	0.28 ± 0.28	0.25 ± 0.26	0.28 ± 0.27	0.37 ± 0.29	0.33 ± 0.28	0.30 ± 0.30	0.31 ± 0.29
Uterine endometrium									
1	0.22 ± 0.15	0.45 ± 0.28	0.32 ± 0.18	0.38 ± 0.32	0.38 ± 0.20	0.49 ± 0.29	0.41 ± 0.25	0.25 ± 0.22	0.32 ± 0.21
2	0.41 ± 0.36	0.37 ± 0.33	0.29 ± 0.20	0.20 ± 0.19	0.28 ± 0.19	0.34 ± 0.24	0.39 ± 0.24	0.34 ± 0.18	0.28 ± 0.26
3	0.29 ± 0.35	0.33 ± 0.22	0.26 ± 0.19	0.19 ± 0.17	0.22 ± 0.28	0.27 ± 0.22	0.24 ± 0.20	0.38 ± 0.33	0.26 ± 0.17
4	0.13 ± 0.21	0.35 ± 0.26	0.19 ± 0.30	0.19 ± 0.26	0.19 ± 0.26	0.36 ± 0.21	0.35 ± 0.23	0.40 ± 0.35	0.41 ± 0.28
5	0.24 ± 0.22	0.26 ± 0.24	0.35 ± 0.31	0.28 ± 0.20	0.29 ± 0.26	0.24 ± 0.27	0.50 ± 0.33	0.23 ± 0.23	0.33 ± 0.25
6	0.41 ± 0.37	0.31 ± 0.36	0.26 ±0.29	0.36 ± 0.34	0.25 ± 0.22	0.36 ± 0.30	0.35 ± 0.25	0.43 ± 0.35	0.42 ± 0.35
Mean ± S.D.	0.28 ± 0.28	0.34 ± 0.27	0.28 ± 0.24	0.27 ± 0.25	0.27 ± 0.23	0.34 ± 0.25	0.37 ± 0.25	0.34 ± 0.27	0.33 ± 0.24

E3 + 4DV) mRNAs in breast carcinomas was recently observed to be comparable to that measured in healthy mammary gland tissue, which leads to the concept that the regulation of ER-α transcripts by alternative splicing may be involved in ER-α gene expression control independently from cellular transformation [42].

ER-β E5DV might work as a dominant positive variant [33]. ER-β E8DVs might form a heterodimer with ER-α [34], leading to an altered estrogen response [36]. Furthermore, several different point mutations and 21 bp deletions detected in ER-β were not associated with anorexia and bulimia nervosa [44]. Additionally, in rat, ER-β in-frame 54 bp insertion was detected in the ligand-binding domain, and works as a dominant negative variant [45–47].

In the present study, the mRNAs for ER-β single exon-deleted variants, ER-β E2DV, E4DV, E5DV and E6DV; for ER-β double exon-deleted variants, ER-β E1′ + 2DV, E4 + 5DV and E5 + 6DV; and for ER-β triple exon-deleted variants, ER-β E2′ + 3 + 4DV and E4 + 5 + 6DV were detected in human ovary and uterine endometrium (Fig. 2).

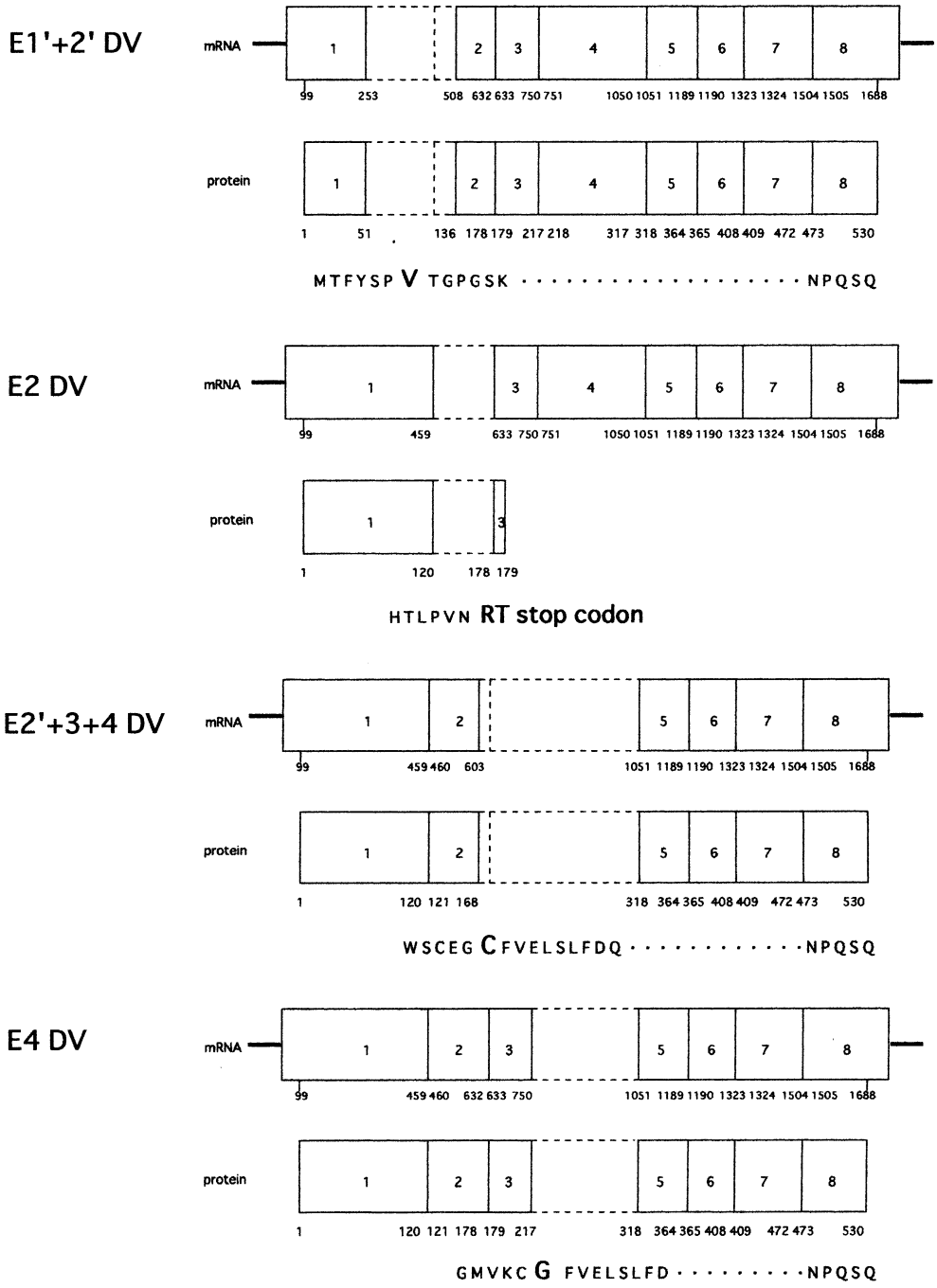


Fig. 3. Schematic of estrogen receptor-β exon-deleted variant mRNAs and proteins.

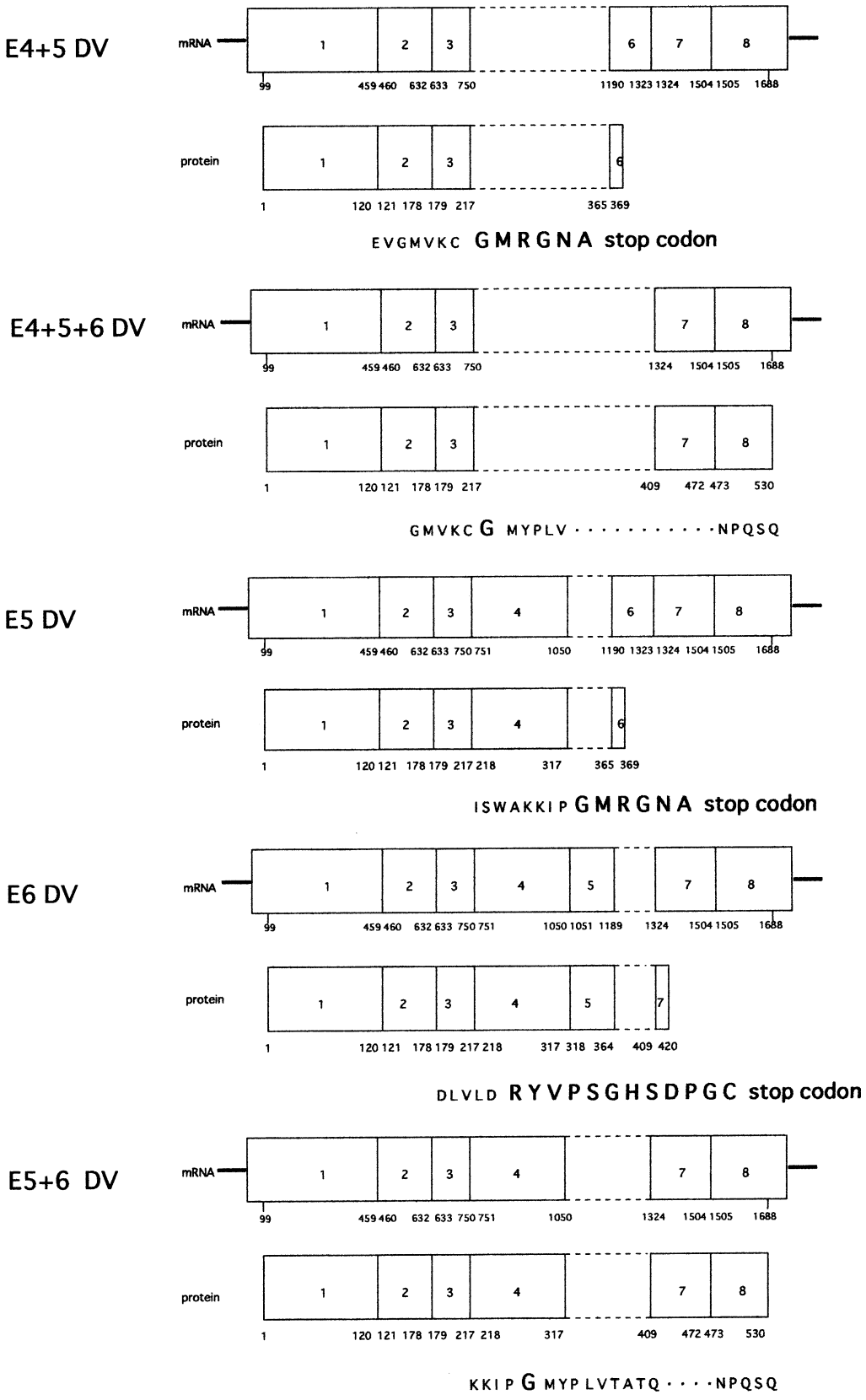


Fig. 3. (*Continued*).

ER-β E2DV might not be an essential protein. ER-β E1′ + 2DV lacks the transactivation domain, E2′ + 3 + 4DV the DNA binding + hinge domains, E4DV the hinge domain, and E4 + 5 + 6DV, the hinge + ligand-binding domains. Therefore, it is not likely that they work as transcription factors. On the other hand, ER-β E5DV, E6DV and E5 + 6DV lack the ligand-binding domain, and might work as dominant positive or negative factors. Therefore, ER-β E5DV, E6DV and E5 + 6DV constitutively expressed in human ovary and uterine endometrium might in part regulate estrogen responsiveness.

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