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A SINGLE NUCLEOTIDE SUBSTITUTION IN THE D DOMAIN OF ESTROGEN RECEPTOR cDNA CAUSES AMINO ACID ALTERATION FROM Glu-279 TO Lys-279 IN A MURINE TRANSFORMED LEYDIG CELL LINE (B-1 F)

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Summary—A polymerase chain reaction method was carried out to address the possible presence of estrogen receptor (ER) mutations in murine transformed cell lines. The segment of ER cDNA coding the C and D domains was amplified and cloned into pUC 19. Mammary carcinoma cell line (SC-3) did not show any mutation in this segment. However, the sequence analysis of ER in the Leydig cell line (B-1 F) revealed a single base change at acidic Glu-279 (GAA) to basic Lys-279 (AAA) compared with murine uterus ER cDNA. The biological significance of this mutation is discussed.

INTRODUCTION

Since the steroid receptors were cloned [1], various techniques have been applied to identify the mutation of receptor molecules. Some mutations have been successfully identified in androgen receptor [2, 3]. However, the possibility has not been fully addressed as to whether or not there is a variant estrogen receptor (ER) in nontransformed or transformed target cells. Since a polymerase chain reaction (PCR) technique has been proved to be very useful in the detection of mutations [4], we utilized this method to analyze ER molecules in murine transformed cell lines. In our previous study, one of the murine transformed Leydig cell lines, termed as B-1 cells, was found to contain the so-called 5S form of ER even in the absence of estrogen [5]. This unoccupied 5S ER has the ability to be tightly bound to the nuclei, although the full biological ability for ER to express the gene activation can be elicited by complexing with estrogen [5]. In addition, one of the mouse mammary carcinoma (Shionogi Carcinoma 115) cell lines, termed as SC-3 cells, was observed to have ER, as evidenced by biochemical techniques, but to fail to be growth-stimulated by estrogen [6]. Therefore, it seems to be interesting to obtain more information about the ER from these two cell lines at the molecular level. We report here a single base mutation in ER mRNA extracted from a Leydig cell line (B-1 F subcloned from B-1 cells).

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EXPERIMENTAL

Cell culture and RNA extraction

The procedures for cloning and subsequent culturing of murine transformed Leydig cells (B-1 F) [7] and mammary carcinoma cells (SC-3) [6] have been described previously. Total cellular RNA was isolated by extraction with guanidium chloride and ultracentrifugation through 5.7 M cesium chloride, as reported previously [7]. The poly(A)-rich fraction was obtained by passing through an oligo-dT cellulose column (Pharmacia, Uppsala, Sweden).

cDNA preparation and PCR

Poly(A)-rich RNA was reverse-transcribed into cDNA by avian myeloblastosis virus reverse transcriptase according to the manufacturer's instructions (Promega, Madison, WI, U.S.A.). PCR was carried out with cDNA product equivalent to 0.1 μg mRNA in 25 μl of 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 15 mM $MgCl_2$, 0.01% gelatin containing 5 pmol each of the ER primers Nos 1 and 2, 0.2 mM each deoxyribonucleotide and 0.625 U of Taq DNA polymerase (Perkin–Elmer/Cetus, Norwalk, CT, U.S.A.) [4]. The primers were synthesized by DNA synthesizer. The sense ER primer No. 1 (5′-ATGGAGTCTGCCAAGGAGACT-3′)

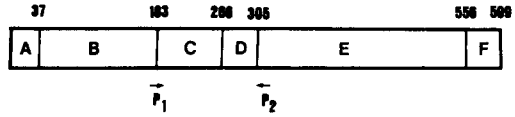

Fig. 1. Domain structure of murine ER. The 6 domains (A–F) are depicted according to the result reported by White *et al.* [7]. The numbers above the diagram indicate the number of amino acids from the N-terminal. The positions of the primers (P_1 and P_2) are also indicated below the diagram by the arrows.

corresponds to murine ER cDNA 729–749 [8]. ER primer No. 2 (5′-CAAGGCAGGGCTATTCTTCTT-3′) represents the anti-sense strand of murine ER cDNA sequence 1107–1127 (Fig. 1). Each cycle of amplification consisted of a 1 min denaturation at 94°C, followed by a 1 min annealing at 55°C and a 1 min polymerization at 72°C. After 30 cycles, the amplified segments (399 bp) were electrophoresed and subcloned into the Hinc II site of the pUC 19 cloning vector by the standard method. Then, cloned plasmids (6-8 clones for each sample) were sequenced by the dideoxy-mediated chain-termination method [9].

RESULTS

When the PCR products were electrophoresed on 1.2% low-temperature melting agarose gel, the amplified segment with the expected size of 399 bp was always visualized by staining the gel with ethidium bromide (data not shown); consistent with the previous results that both SC-3 and B-1 F cells contain ER [5-7]. Therefore, the nucleotide sequence was analyzed as described under Experimental. Comparison of ER cDNA sequences coding the C and D domains of SC-3 cells and murine uterus [8] showed a perfect sequence identity. Analysis of 6 independent clones gave the same results. On the contrary, a single base change was observed in B-1 F ER at nucleotide 1025, approximately the mid portion of the D domain (Fig. 2). The G (wild type) to A (B-1 F) mutation resulted in a conversion of Glu-279 (GAA) to Lys-279 (AAA) (Table 1). Except for this mutation, the nucleotide sequence of the C and D domains of ER mRNA from B-1 F cells was exactly the same as that in murine uterine ER mRNA. These results were confirmed by analyses of 8 independent clones.

DISCUSSION

The present results clearly demonstrate the existence of a variant ER. It is not completely conclusive whether this mutation is present in all ER transcripts derived from B-1 F cells. However, the observation that 8 independent clones have the same mutation would strongly suggest that a major part of ER transcripts in B-1 F cells have this mutation. Furthermore, it is quite unlikely that this mutation has arisen via an error in the PCR procedure, since the misjudgment of the nucleotide sequence in the PCR experiment is made maximally at the rate of 1/400 nucleotides [10].

The biological significance of the mutation identified in this study is currently unknown. Estrogen effects on cell proliferation [11] and

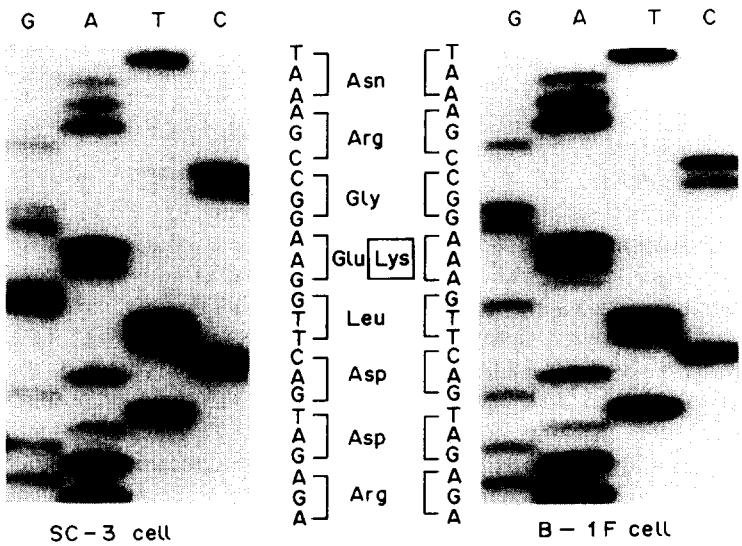

Fig. 2. Single base mutation in B-1 F ER cDNA. Autoradiograms of DNA sequencing gels from coding strands of SC-3 and B-1 F cells are shown.

Table 1. Partial amino acid sequence comparisons of ER D domains within the region of the single base mutation

ER	Amino acid alignment	Ref.
	[269] [279] [282]	
Murine uterus	Leu[a]-Lys-His-Lys-Arg-Gln-Arg-Asp-Asp-Leu-Glu-Gly-Arg-Asn	White *et al.* [8]
Murine B-1 F cells	*[b] - * - * - * - * - * - * - * - * - * - * - * - * - * -Lys- * - * - *	This study
Murine SC-3 cells	* - * - * - * - * - * - * - * - * - * - * - * - * - * - * - * - * - *	This study
Rat uterus	* - * - * - * - * - * - * - * - * - * - * - * - * - * - * - * - * - *	Koike *et al.* [19]
Human MCF-7	* - * - * - * - * - * - * - * - * - * - * - * -Gly- * - * - * -Gly	Green *et al.* [12]
Chick oviduct	Met - * -Gln- * - * - * - * - * -Glu-Glu-Glu-Asp-Ser - * - *	Krust *et al.* [20]

[a]Indicates the number from the N-terminal of murine ER.
[b]Indicates the conserved amino acid among various ERs.

gene expression [5] have been observed to be demonstrable in a murine transformed Leydig cell line. The size of ER mRNA has been also found to be similar to that of uterine ER mRNA [7]. However, biochemical analyses have revealed unusual but interesting characteristics of this ER, such as the tight association with nuclei as a 5S unoccupied form [5]. In this relation, it should be pointed out that amino acid alignment (Arg-Asp-Asp-Leu-Lys) induced by a single base mutation resembles the potential nuclear transfer signal (Arg-Pro-Gln-Leu-Lys) [12]. In addition, Glu-279 is well conserved among various ERs except for chick ER where Glu-279 is replaced by acidic Asp (Table 1). Moreover, the position of the mutation (Glu-279) is very closely localized to a sequence of amino acids bearing a similarity to a nuclear signal sequence present in the SV40 large T antigen [13]. These duplicated nuclear signal sequences in the D domain of this mutated ER might confer the ability to be tightly associated with nuclei even in the absence of estrogen. The formation of the unoccupied 5S ER might be related to this mutation. These possibilities are currently under investigation in our laboratory.

Some mutation of ER in SC-3 cells was anticipated, since we have been unable to demonstrate estrogen effects in terms of cell proliferation [6] as well as gene expression (data not shown). According to these biological and biochemical data on ER in SC-3 cells, we examined the C and D domains of ER molecules. However, the nucleotide sequence revealed the lack of any mutation. Thus, the lack of estrogen effects may be due to a mutation in a region not examined by the present PCR or to a post-receptor defect in SC-3 cells.

ER mutation has not been well characterized [14–16]. Recently, a single base difference at Val-400 (GTG) to Gly-400 (GGG) has been reported in human breast cancer specimens when compared with the nucleotide sequence of ER originally reported in MCF-7 cells [16]. However, this sequence has also been identified in ER isolated from a human leukocyte genomic library [17]. This discrepancy has been shown to have arisen by a cloning artifact. Tora *et al.* [18] reported that a wild type of ER contains Gly-400. Nonetheless, further studies are definitely required using many samples to discuss the biological significance of ER mutation.

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