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Alteration of the Expression of Human Estrogen Receptor Gene by Distamycin

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The effects of distamycin on the expression of the estrogen receptor gene were determined in the MCF7 human breast cancer cell line. Estrogen receptor (ER) RNA transcripts were analyzed by Northern blotting and RT-PCR using specific oligonucleotides for the 5′ upstream region and for ER cDNA. After *ex vivo* distamycin treatment of the cells the expression of the canonical ER mRNA isoform of 6.3 kb is strongly inhibited, without appreciable alteration of the accumulation of 5′ upstream ER mRNA isoforms. These results suggest that distamycin alters the transcriptional activity of the ER gene causing a change in the ratio between the canonical transcript and other isoforms containing 5′ upstream regions.

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INTRODUCTION

The estrogen receptor (ER) is a member of the steroid and thyroid hormone receptor superfamily, that elicits its biological functions as a ligand-dependent transcription factor [1]. Many cell biology and molecular biology investigations show that the expression of the ER gene is an excellent marker for both breast and endometrial differentiation states. With respect to ER expression, it is to be pointed out that recently published reports have described gene variants leading to different mRNA molecular species [2–4]. Alternative splicing of ER mRNA, particularly in breast cancer cell lines and tumors was also reported [2, 3, 5].

In light of (a) the demonstration that many breast and endometrial cancers are dependent on estrogen for growth and progression [6] and (b) the central role that ER plays in these neoplasias [7], the possibility of modulating the expression of the ER gene is of great interest.

In this respect, a number of investigations have proposed DNA-binding compounds exhibiting a certain degree of sequence-selectivity, as pharmacologically active agents able to alter the expression of selected genes [8, 9]. For instance, distamycin, a DNA minor groove-binding ligand, is able to induce conformational changes in DNA structure and to interfere with DNA–protein interactions (transcription initiation) [10–12] as well as with the activity of RNA polymerase II (transcriptional elongation) [13].

In the present study we analyzed the effects of distamycin on ER gene expression in the MCF7 human breast cancer cell line. ER gene expression was evaluated by both Northern blotting and RT-PCR. The MCF7 cell line was used since it expresses the ER protein at high level and it was previously tested for distamycin binding to the 5′ upstream region of ER gene [14]. The 5′ region of the human ER gene, containing the exon 1′ (coding for the untranslated 5′ region of the proposed ER mRNA isoforms 2 and 3 [15]), indeed contains an intron of 1.9 kb including a $(TA)_{26}$ stretch. This dinucleotide repeat is a typical DNA sequence which is recognized by distamycin [16–18]. This system appears therefore useful for determining the effects of DNA-binding of drugs recognizing AT-rich sequences on transcription.

MATERIALS AND METHODS

Drug

Distamycin was obtained from Sigma; stock solution of the drug (9.6 mM) was stored at −20°C in the dark and diluted immediately before use.

Cell culture conditions

The MCF7 human breast cancer cell line was cultured in MEM α-medium supplemented with 10%

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foetal bovine serum (FBS, Flow Laboratories), in a 5% CO_2 humidified atmosphere, as previously described [19].

Northern blotting

Total RNA was phenol–chloroform extracted from the cytoplasm of MCF7 cells. 10 μg of total RNA was loaded onto 1% agarose–formaldehyde gel, transferred to nylon membranes (Hybond-N) and hybridized with ^{32}P-labelled specific ER probes as previously described [15].

Oligonucleotide primers and RT-PCR amplification

The sequences and the relative positions of the primers used in the reverse transcriptase and in the polymerase chain reaction are reported in Table 1 and in Fig. 1; the experimental conditions were as previously described [15]. Reverse transcriptase (cDNA cycle kit, Invitrogen), 10 U, was used to synthesize (1 h, 42°C, 2×) a single stranded cDNA from 1 μg of total cytoplasmic RNA or 0.2 μg of poly(A)$^+$ RNA.

Table 1.

FM	5′-GCATGATGGCATCATTAACAT-3′
RE	5′-AGTTGCACTGACCGTGATTGGG-3′
F2	5′-CCGTAAGACTATGATTCACAG-3′
R4	5′-GCTGGATCAAGAACGTCT-3′
F1	5′-GACGCATGATATACTTCACC-3′
R1	5′-GCAGAATCAAATATCCAGATG-3′
R2	5′-TAGGGCCATCCCAGATGCTTTGGT-3′
E4	5′-GCTTCGATGATGGGCTTACTGACCAACCTGGCA-3′
E8	5′-CAGCAGGTCATAGAGGGGCACCACGTTCTTGCA-3′

RNA was pretreated at 37°C for 10′, with 2 U or 0.5 U RQ1 DNase, respectively, which was subsequently denatured by incubation at 70°C for 10′. A fifth of each cDNA product was used to amplify fragments of ER cDNA in the presence of 2.5 U Taq polymerase (Cetus, Perkin Elmer). PCRs were performed with the same amount of cDNA, by performing 30 cycles on a Violet Thermal Cycler. The cycles were 30″ at 94°C (denaturation), 60″ at 55°C (annealing) and 60″ at 72°C (extension). A tenth of each PCR product was electrophoresed in 1% agarose gel, blotted onto nylon

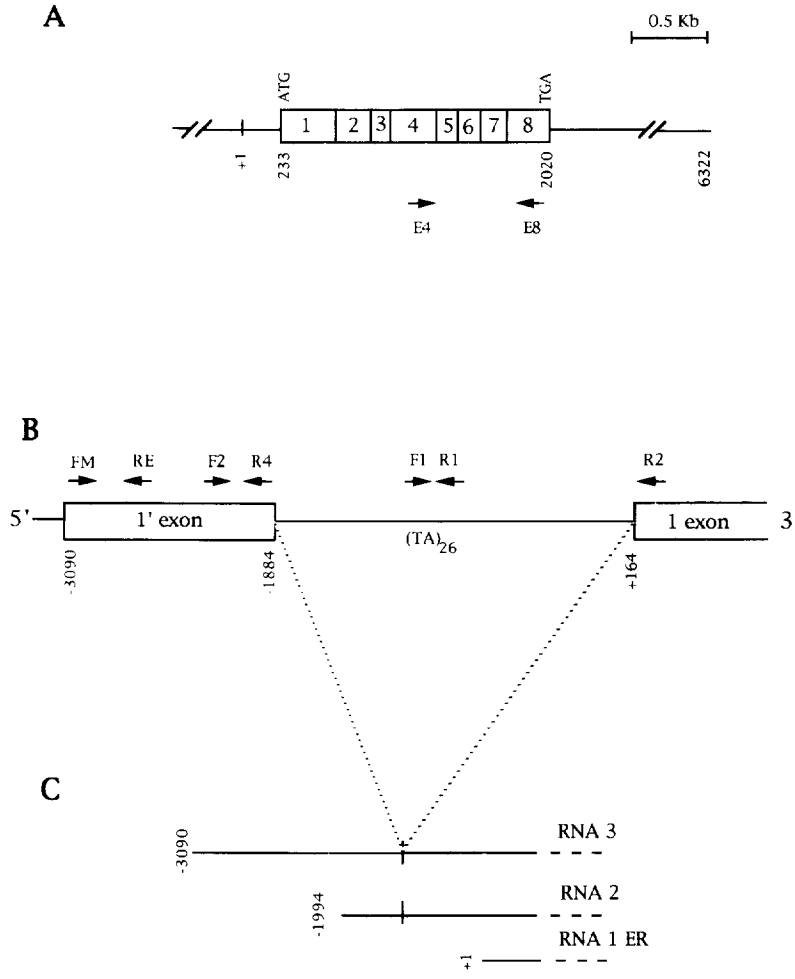

Fig. 1. (A) Location of E4 and E8 primers within the cDNA sequence corresponding to the human ER mRNA. The canonical ER mRNA [22] is comprised between nucleotide +1 to +6322. (B) Scheme of the 5′ upstream region of ER gene with the location of the oligonucleotides used as RT-PCR primers. (C) Molecular events proposed to be responsible for the generation of three ER RNA isoforms.

membrane using standard procedures [20] and hybridized with 7×10^6 cpm of ^{32}P-labelled specific ER probes.

RESULTS

We have previously demonstrated that distamycin differentially affects the PCR amplification of selected ER genomic sequences [14]. In particular, distamycin inhibits the generation of a PCR product of an ER region containing the $(TA)_{26}$ stretch located in the upstream intron [21], whereas being unable to prevent PCR-amplification of ER gene regions lacking TA-rich sequences.

The effects of distamycin on the expression of the ER gene was first studied by Northern blotting. MCF7 cells were cultured for 1 day in the absence or in the presence of 400 and 600 μM distamycin; after this *ex vivo* treatment total cytoplasmic RNA was isolated, Northern blotted and hybridized to the specific cDNA probe pOR15 [15]. As shown in Fig. 2, the hybridization signal, corresponding to the 6.3 kb canonical ER mRNA, is (a) strongly reduced when RNA is isolated from MCF7 cells treated with 400 μM distamycin and (b) completely abolished as a result of treatment with 600 μM distamycin. This result indicates that distamycin causes a strong inhibition of the accumulation of ER mRNA in MCF7 cells.

A second question we wanted to address is related to the selectivity of distamycin treatment for different ER gene regions. With respect to this point, we have previously demonstrated that at least three different ER-specific transcript RNAs are generated in MCF7 cells [15]: ER mRNA 1, proposed as the canonical ER mRNA [22]; and ER RNA 2 and 3, two other isoforms different from the canonical one in their 5′ region, including an upstream exonic portion [15]. With respect to this last feature of the transcription pattern of the human ER gene, two major transcription starting sites were found, at -1994 and at -3090 respectively [15]. The $(TA)_{26}$ stretch is located between the -1994 and the canonical $(+1)$ transcription site.

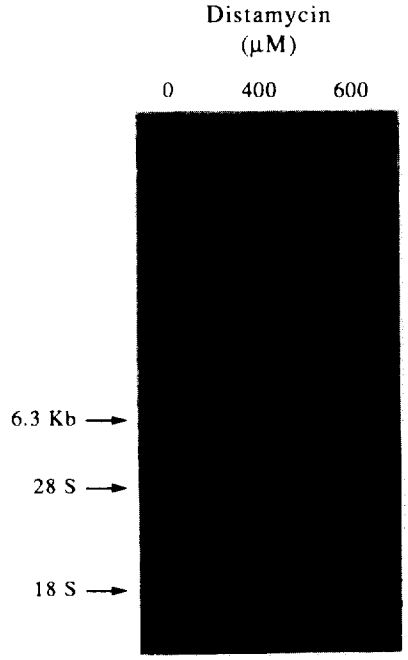

Fig. 2. Northern blotting analysis of human ER mRNA. Total RNA from MCF7 cells cultured in presence (400, 600 μM) or in absence (0 μM) of distamycin was electrophoresed on a 1% agarose gel, transferred to a nylon membrane and hybridized with ER cDNA ^{32}P-probe. The size (kb) of the autoradiographic signals and position of 28S and 18S rRNA are indicated.

In order to investigate the effects of distamycin on the accumulation of these less abundant upstream transcripts [15], we have performed RT-PCR analysis, as this technique is far more sensitive than Northern blotting. RNA was (a) isolated from MCF7 cells cultured for 1 day without distamycin or in the presence of 400 and 600 μM distamycin and (b) reverse transcribed with either the E8 ER primer, located within the exon VIII, or the R2, R4, RE primers, specific for the 5′ upstream region as indicated in Fig. 1. PCR on the cDNA obtained by the reverse transcription was performed using the E4, F2 and FM PCR primers.

The RT-PCR analyses demonstrate that distamycin leads to sharp reduction (at 400 μM concentration) or lack (at 600 μM) of the canonical ER transcript [Fig. 3 (lanes e, h and m)], completely in agreement with the Northern blotting analysis shown in Fig. 2. By contrast distamycin does not block transcription directed by the 5′ upstream region (see Fig. 3). This was proven by the fact that RT-PCR amplification is obtained when FM/RE and F2/R4 primers are used. These results were reproducibly obtained in four independent RT-PCR amplifications.

In order to determine whether transcription is blocked in distamycin-treated cells at the level of the $(TA)_{26}$ stretch, the experiment shown in Fig. 4 was performed. The cDNA produced by reverse transcription of MCF7 RNA with the R2 primer was amplified with the F2/R2 primers. Since this protocol leads to the coamplification of non-specific sequences [23], we previously demonstrated that among the obtained PCR products only one, exhibiting the expected size, is able to hybridize to the human ER probe pBLCAT8ERCAT1 [Fig. 4(A)].

When F2/R2 RT-PCR was performed with samples from MCF7 cells or distamycin-treated MCF7 cells, it is evident that the ER specific PCR product is sharply reduced in samples from distamycin-treated cells, suggesting that the transcription directed by the 5′ upstream region of the human ER gene is blocked at the level of the $(TA)_{26}$ stretch by distamycin treatment.

These results strongly suggest that binding of distamycin to the TA rich region located within an intronic portion of the human ER gene induces alterations in gene transcription.

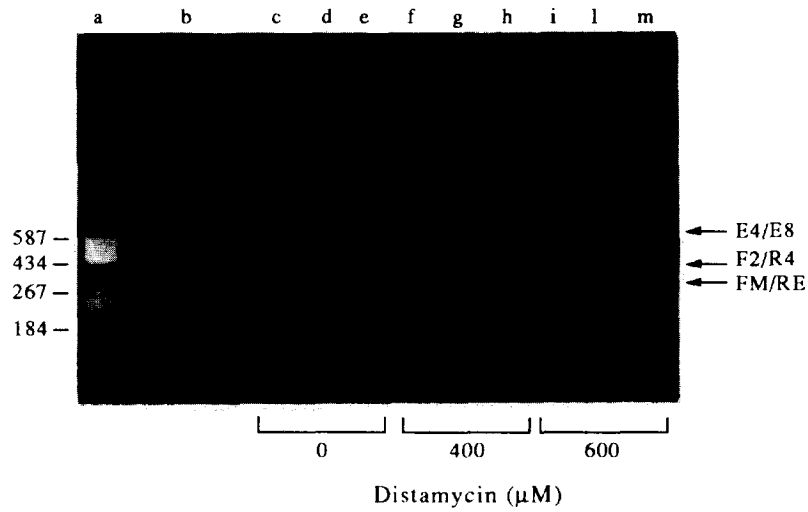

Fig. 3. Effects of distamycin on ER gene transcription studied by RT-PCR. MCF7 breast cancer cells were cultured for 24 h in the absence (0) or in the presence of the indicated concentrations (400, 600 μM) of distamycin. Cells were collected, lysed, total RNA was isolated and RT reactions were performed using the E8, R4, RE oligonucleotides as primers. cDNAs were amplified by PCR using the E4 and E8 (e, h, m), the F2 and R4 (d, g, l) and the FM and RE (c, f, i) primers. The ER-specific PCR products are arrowed. The specificity of the ER RT-PCR products was confirmed by hybridization to ER specific probes (data not shown). (a) Molecular weight marker (Hae III restricted pBR322); (b) negative control of PCR reactions.

DISCUSSION

The expression of the ER gene has been studied in several cancer cell lines [2] and tumors [3, 5]. However, few studies have been published on the effects of anticancer drugs on its expression, although ER plays a central role in growth and progression of human cancers.

In this paper we have examined the effects of distamycin on the expression of the human ER gene by analysing the accumulation of ER RNA transcripts, including canonical ER mRNA [22] and two other previously identified isoforms [15].

The results reported here suggest that distamycin has a well defined activity on the ER genomic unit. Distamycin indeed suppresses the accumulation of ER

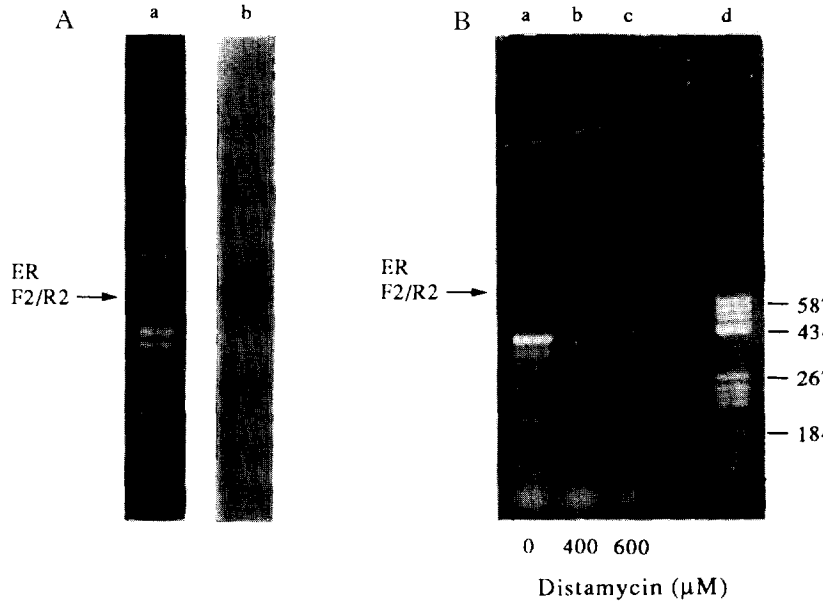

Fig. 4. Effects of distamycin on ER gene transcription studied by RT-PCR. (A) Southern blotting of F2/R2 RT-PCR product and hybridization to an ER-specific probe. In lane (a) agarose gel electrophoresis is shown; the ER-specific RT-PCR product (F2/R2) obtained from MCF7 cells RNA is arrowed; the hybridization between the Southern blotted F2/R2 PCR product, shown in lane (a), and the ER specific probe pBLCAT8ERCAT1 [15] is represented in lane (b). (B) RT-PCR performed with the F2 and R2 primers, using RNA of MCF7 cells treated for 1 day with 0 μM (a), 400 μM (b) and 600 μM (c) distamycin. The ER-specific PCR product is arrowed. Molecular weight marker (Hae III restricted pBR322) is shown in lane (d).

RNA isoform 1, being ineffective in inhibiting transcription of the ER isoforms 2 and 3. One possibility is that this drug forms a complex with a high degree of selectivity for the $(TA)_{26}$ DNA sequence present in the upstream region of the ER gene, leading to a decrease in ER canonical transcription. Not surprisingly, a small ligand, such as distamycin, could associate with the minor groove of DNA blocking RNA polymerase II mediated transcription [13]. The significant finding presented here is that distamycin treatment leads to a change in the ratio between ER transcripts.

In our opinion the data reported here are of interest from both theoretical and practical points of view. From the theoretical point of view, we would like to underline that, up to now, the question of the significance of multiple ER RNA transcripts in human tissues is an unresolved issue [15, 24]. Therefore, the experimental system described here could be of interest for determining the biological roles, if any, of the different ER RNA isoforms previously described [15]. Furthermore, our data encourage us to investigate (a) the presence of ER protein isoforms different from the canonical one or ER-related peptides with regulatory function(s) and (b) the effect of distamycin on the levels of ER protein and its biological activity.

From the practical point of view, it should be mentioned that inhibition of ER mRNA could have important phenotypic effects on breast tumor cells. For instance, the invasiveness of MCF7 cells through reconstituted basement membrane depends from estrogen receptor functions [25]. Accordingly, the modulation of ER gene expression by anticancer drugs such as distamycin could be of interest in order to induce ER down-regulation, possibly leading to a control of invasiveness of malignant breast cancer cells.

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