

Specific Inhibition of Estrogen Receptor Alpha Function by Antisense Oligodeoxyribonucleotides

ANTHONY H. TAYLOR,¹ J. HOWARD PRINGLE,² STEPHEN C. BELL,¹ and FAROOK AL-AZZAWI¹

ABSTRACT

We have tested the effect of a range of antisense oligodeoxyribonucleotides (ODN) directed against the human estrogen receptor alpha (ER α) on ER α protein expression and function. Antisense ER α ODN transfected into the ER α -positive human breast carcinoma cell line MCF7-K2 showed variable responses dependent on the oligo used. The most active antisense ODN (oligo 7) decreased the levels of ER α protein by 61% as measured by Western blot analysis. Exogenous 17 β -estradiol (17 β -E₂), but not 17 α -E₂, augmented this effect, with a threshold effect at 10⁻⁸ M 17 β -E₂. The inhibitory effect of antisense ER α oligo 7 was confirmed by measurement of functional ER α protein. ³H-17 β -E₂ binding to MCF7 cell extracts was inhibited to approximately 40% of control values in the presence of oligo 7. Antisense-transfected MCF7-K2 cell cultures produced a further 30% binding reduction in the presence of exogenous 17 β -E₂. An inhibitory effect on 17 β -E₂-dependent cell function was confirmed by the demonstration that ER α oligo 7-transfected MCF7-K2 cells failed to exhibit 17 β -E₂-stimulated cell proliferation. Exogenous 17 β -E₂ enhanced the inhibitory effect of the antisense ODN by increasing ODN transfection efficiency but without ER α catabolism via the proteosomal pathway, suggesting an effect of 17 β -E₂ on the plasma membrane and the existence of different ER α degradation pathways in the MCF7-K2 cell subclone. As 17 β -E₂ had no effect on ER α protein degradation, we conclude that the observed reduction of ER α protein levels is due solely to the presence of the antisense ER α ODN. Antisense ER α ODN molecules, therefore, may form the basis of effective therapies against ER α -dependent malignancies.

INTRODUCTION

NATURAL AND SYNTHETIC ESTROGENS exert their physiologic and pharmacologic effects on target tissues by interacting with the cognate estrogen receptor (ER α), a member of the steroid/thyroid hormone superfamily of nuclear transcription factors (Evans, 1988; O'Malley, 1990; Ribeiro et al., 1995). ER α antagonists that inhibit estrogen-dependent breast and endometrial pathologies while also providing ER α agonist activity on the skeleton and cardiovascular system (SERM) have been developed for the treatment of estrogen-deficient states in women (Mijatovic et al., 1999; Mitlak and Cohen, 1999; Poletti, 1999). Unfortunately, the reinitiation of uterine bleeding and increased breast tenderness are two of many symptoms of SERM therapy in women that cause discontinuation of long-term treatment (Schiff et al., 1998).

To inhibit the dysfunctional uterine bleeding and, to a lesser extent, breast tenderness, a progestin is often added to the hor-

mone replacement therapy (HRT). However, progestins often cause premenstrual-type symptoms that also cause patients to discontinue treatment (Al-Azzawi and Habiba, 1994). If the adverse effects on the endometrium of unopposed estrogens could be blocked, the addition of a progestin to a patient's HRT would not be needed, and problems associated with progestin use would be avoided. Simultaneously, the systemic effects of the estrogen would be maintained without the reinitiation of uterine bleeding. These data suggest that an alternative treatment method for estrogen-sensitive tissues might be useful.

As the expression and intracellular concentration of ER α are central to the progression of estrogen-dependent malignancies and there is clear evidence that ER α levels must achieve a critical level for ER α -mediated events to occur (Vanderbilt et al., 1987; Weeb et al., 1992), a reduction in ER α protein levels in a target tissue, such as the breast or uterus, to below a critical level might prevent the activation of ER α -dependent transcription pathways (Curtis and Korach, 1990).

¹Gynaecology Research Group, Department of Obstetrics & Gynaecology, and ²Department of Pathology, Faculty of Medicine and Biological Sciences, University of Leicester, Leicester, Leicestershire, LE2 7LX, England.

Antisense oligodeoxyribonucleotide (ODN) sequences used in gene therapy that show tissue specificity have been used to ameliorate the symptoms of some clinical conditions and to significantly improve quality of life (Yacyshyn et al., 1998; Sereni et al., 1999; Warzocha, 1999). Additionally, data suggest that many ER α -dependent tissues are amenable to transfection, with some studies showing specific inhibition of gene activity in laboratory animals (L'Horset et al., 1994; Arslan et al., 1995; Moreau et al., 1995; Charnock-Jones et al., 1997; Horn et al., 1998). Therefore, an attractive route to ameliorate the noncontinuation issue for SERM treatment regimens would be the use of topical antisense ODN gene therapy against the human ER α in the human uterus.

This study was designed to test the hypothesis that antisense ODN directed against the human ER α might reduce cellular ER α concentrations in a human cell line. To study the action of the antisense ODN, we have used a subclone of the well-characterized human breast cancer cell line MCF7. The data presented show that three antisense ER α ODN sequences inhibit ER α protein concentrations. The antisense effect is enhanced by exogenous 17 β -estradiol (17 β -E₂) through a mechanism that increases ODN uptake, which augments ER α clearance, but not through the normal MCF7 cell proteasome-dependent pathway (Alarid et al., 1999; Nawaz et al., 1999). One of the antisense ODN specifically inhibits 17 β -E₂-dependent MCF7 cell proliferation.

MATERIALS AND METHODS

ODN design and synthesis

Antisense ODN were designed to encompass the translation start site (-3 to 15 bp) and both coding and noncoding regions of the human ER α gene (Greene et al., 1986; Santagati et al., 1997). Control sequences were the respective sense strands, and for oligo 7, an additional control was the nucleotides contained in the antisense sequence arranged at random to generate a scrambled sequence. The antisense ODN, numbered oligo 1 to oligo 6, (Fig. 1) have been described elsewhere (oligo 1; GACATGCGCTGCGTCGCC; oligo 2, GCCAGTACCAATGACAAG; oligo 3, TGTGCCTGGCTAGAGATC; oligo 4, GGGTCAGTGGGTTCTTTT; oligo 5 GTGTCTCCGAGCCCGCTG; oligo 6 TGGACAGTAGCGAGTCAG [Santagati et

al., 1997]). To ensure the uniqueness of the sequences, they were aligned against all known human sequences on the sequence databases. All sequences were unique to human ER α except the antisense sequence designed to encompass the translation start site (oligo 7, GACCATGACCATGACCCT) that aligned with the rat and mouse ER α and the human, mouse, and rat estrogen receptor-related alpha1 (ERR-1 α) genes alone (White et al., 1987; Giguere et al., 1988; Shigeta et al., 1997). The ODN and RT-PCR primer sequences (Table 1) were commercially synthesized (Bioline Ltd., London, U.K.) and purified by both PAGE electrophoresis and HPLC prior to use.

Cell culture, transfection, and protein extraction

MCF7-K2 cells (Katzenellenbogen et al., 1987) obtained from the Breast Cancer Unit, Glenfield Hospital (Leicester, U.K.) were maintained in phenol red-free Dulbecco's modified Eagle's medium (DMEM) (GIBCO-BRL, Paisley, Scotland) containing 10% fetal bovine serum (FBS), 50 U/ml penicillin, 50 μ g/ml streptomycin, and 2 mM L-glutamine (GIBCO-BRL) in a humidified 5% CO₂ in air atmosphere. For transfection, MCF7-K2 cells were subcultured to 10-cm diameter plastic Petri dishes with the aid of trypsin-EDTA at a density of 4 \times 10⁶ cells/cm² in the above medium and allowed to adhere for 24 hours. Cells were then washed twice with sterile phosphate-buffered saline (PBS), covered with serum-free medium, and transfected with DNA-lipofectamine complexes at a 1:8 ratio according to the manufacturer's instructions (GIBCO-BRL).

Crystalline 17 β -E₂ and 17 α -E₂ were purchased from Sigma-Aldrich (Poole, Dorset, U.K.), and the proteasome inhibitor MG-132 was from CN Biosciences (Nottingham, U.K.). E₂ and MG-132 solutions in ethanol and dimethylsulfoxide (DMSO), respectively, were diluted in serum-free medium and added to cultures at the indicated concentrations. To control for possible solvent effects, control cultures contained 0.1% ethanol or 0.2% DMSO in serum-free medium. After a further 24-hour incubation, cells were washed with sterile PBS and scraped from the plastic substratum in GETD buffer (10% glycerol, 1.5 mM EDTA, 10 mM Tris-HCl, pH 7.6, 10 mM dithiothreitol [DTT]) in the absence of protease inhibitors and subjected to five rounds of freeze-fracture. Protein concentrations were measured using the Bradford microassay (Bradford, 1976) (Bio-Rad Laboratories, Hemel Hempstead, Hertfordshire, U.K.), with bovine serum albumin (BSA) as standard and samples

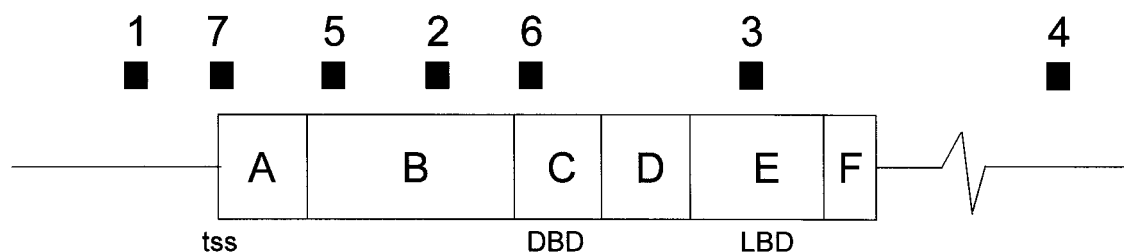


FIG. 1. Schematic representation of antisense ODN designed against human ER α . ER α target sequences oligo 1-oligo 6 are depicted by the numbered boxes corresponding to translated and nontranslated regions of the human ER α mRNA sequence. The sequences of oligo 1-oligo 7 correspond to nucleotides -117--99, 495/512, 1140-1157, 4134-4151, 302-320, 539-557, and -3-15 of the human ER α cDNA, respectively (Santagati et al., 1997). The various regions of the ER α gene are labeled A, B, C, D, E, F, following established nomenclature. DBD, DNA-binding domain; LBD, ligand-binding domain.

TABLE 1. PCR PRIMER SEQUENCES USED IN RT-PCR ANALYSIS

Primer	Sequence ^a								Expected size
ER left	GAG	ACA	TGA	GAG	CTG	CCA	AC		380 bp
ER right	CCA	AGA	GCA	AGT	TAG	GAG	CA		
β -actin left	GTT	GCT	ATC	CAG	GCT	GTG	CT		284 bp
β -actin right	GGC	CAT	CTC	TTG	CTC	GAA	GT		

^aSequences are designated 5' to 3' (Heikinheimo et al., 1995).

stored at -20°C for radioligand binding and Western blot analysis.

Western blot analysis

Total cellular protein (12 μg) was separated on 12% precast denaturing SDS-PAGE gels (Novex, San Diego, CA) at 125 V constant voltage for 1.5 hours. After transfer to nitrocellulose membranes (Hybond-C) (Amersham PLC, Amersham, Buckinghamshire, U.K.) in transfer buffer (12 mM Tris, 96 mM glycine, 20% methanol), membranes were blocked with 3% nonfat milk powder in PBS for 20 minutes. Membranes were then incubated at 4°C for 18 hours with monoclonal primary ER α antibody (clone 05-394, Upstate Biotechnology, Lake Placid, NY) diluted to 2 $\mu\text{g}/\text{ml}$ in the blocking solution. After five washes with deionized water, membranes were incubated with secondary antimouse horseradish peroxidase (HRP)-linked antibody (1:3000) (Amersham) in 3% blocking solution at ambient temperature for 1.5 hours. After a further five washes with water, a 5-minute wash with 0.05% Tween-PBS, and a further five washes with water, the protein-antibody complexes were visualized with enhanced chemiluminescence (ECL) detection, according to the manufacturer's protocol (Amersham), and exposure to x-ray film (Kodak Biomax ML) (Integra Biosciences Ltd, Letchworth, Hertfordshire, U.K.) for up to 5 minutes. In some experiments, human recombinant ER α (Affinity Bioreagents Inc., Golden, CO) was used to create a curve of standards within the assay. The densities of the visualized bands were obtained by video-assisted densitometry (NIH Image program Version Beta 3b, 1998, modified for Windows 95/98 by Scion Corporation; <http://www.scioncorp.com>) and corrected for by the amount of β -actin (1:5000, clone AC-74, Sigma) from replica blots.

Radioligand binding

$^3\text{H-E}_2$ binding to MCF7-K2 extracts was performed essentially as described (Campisi et al., 1993). Briefly, initial studies to find maximum binding capacity used 200–300- μg aliquots of MCF7-K2 extract in triplicate adjusted to give a volume of 250 μl in GETD buffer and incubated with a range of $^3\text{H-E}_2$ (79.5 Ci/mmol) concentrations (Amersham) in GETD buffer at 4°C for 18 hours. Nonspecific binding was determined in the presence of 100-fold excess of unlabeled $17\beta\text{-E}_2$ in duplicate. Bound radioactivity was separated from free radioactivity by incubation with 500 μl dextran-coated charcoal in PBS for 20 minutes at 4°C , followed by centrifugation at 1800g for 20 minutes. An aliquot (500 μl) of the supernatant was counted by β -scintillation counting for 5 minutes. Maximum binding was

found to be 6×10^4 cpm of $^3\text{H-E}_2$, and this amount of $^3\text{H-E}_2$, 200 μg cell extract, and a 100-fold excess of unlabeled $17\beta\text{-E}_2$ were used in subsequent binding assays, with separation and counting as described.

Fluorescence photomicroscopy

Cells were plated at a density of 5×10^4 to polytetrafluoroethane-coated multispot slides (Hendley, Loughton, Essex, U.K.) contained in a sterile 10-cm Petri dish. After 24-hours attachment, the cells were transfected with fluorescein isothiocyanate (FITC)-labeled antisense oligo 7 in lipofectamine, as described, and immediately treated with up to 10^{-6} M $17\beta\text{-E}_2$ in serum-free medium. After 24 hours, cells were washed twice with PBS and then fixed with 4% paraformaldehyde in serum-free medium for 5 minutes. After two further washes with PBS, the slides were mounted in aquamount (BDH, Poole, Dorset, U.K.) and stored at 4°C in the dark until photographed on a Zeiss Axioplan II microscope fitted with a fluorescent attachment at 530–585-nm excitation onto Fujichrome ASA800 film.

RNA preparation and RT-PCR analysis

MCF7-K2 cells were transfected or nontransfected (controls) and treated with $17\beta\text{-E}_2$ as described. After 24 hours, cells were washed twice with PBS and lysed in TRIZOL reagent (GIBCO-BRL). Total RNA was prepared according to the manufacturer's instructions and stored at -80°C . Total RNA (1 μg) was electrophoresed on 1.2% agarose gels to ensure that the RNA was intact.

The primer sequences for ER α and β -actin and assay conditions showing amplification within the exponential part of the amplification have been described elsewhere (Heikinheimo et al., 1995) (Table 1). For RT-PCR analysis, 2 μl total cellular RNA was reverse transcribed and subjected to PCR using ExpeRT 100 PCR kits (ThermoHybaid, Teddington, Middlesex, U.K.) according to the manufacturer's one-tube protocol. The level of amplicon was corrected by that produced with specific β -actin primers. The RT conditions were 48°C for 30 minutes, 95°C for 2 minutes. PCR was performed with 10 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 68°C for 1 minute, followed by 25 further cycles with the exception that primer extension at 68°C for 1 minute was extended for 5 seconds on each further cycle. Finally, the reaction was incubated at 68°C for 10 minutes to fully extend all amplified products. Amplification of the housekeeping gene β -actin was similar, except the annealing temperature was 54°C . Thirty or forty percent of the reaction mixture was separated on 1.8% agarose gels impreg-

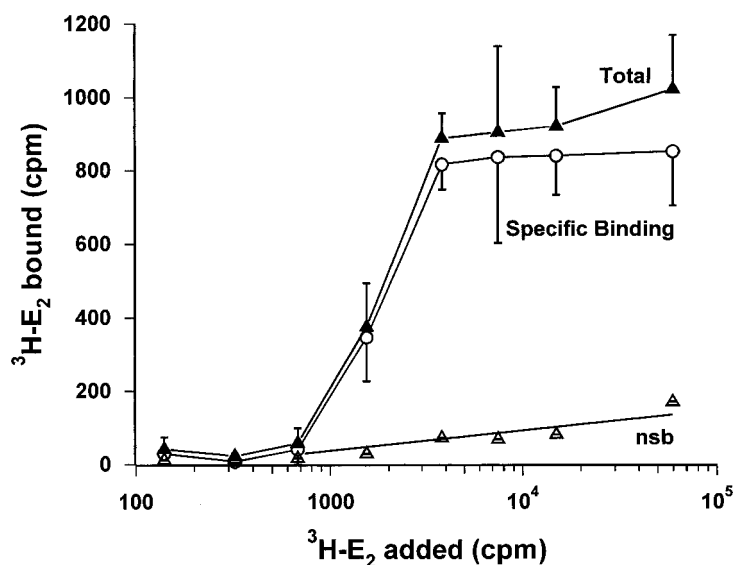


FIG. 2. Characterization of $^3\text{H-E}_2$ binding to MCF7-K2 cell extracts. Binding of $^3\text{H-E}_2$ to MCF7-K2 extracts, showing total (solid triangles), specific (open circles), and nonspecific binding (open triangles). Data are presented as mean \pm SD from four experiments performed in triplicate. Error bars are not shown when encompassed by the symbol.

nated with 0.5 $\mu\text{g/ml}$ ethidium bromide, and the amount of signal was measured by video-assisted densitometry.

Growth curves

Stock cultures of MCF7-K2 cells were subcultured for three passages and maintained in E_2 -deficient medium. This medium consists of phenol red-free DMEM containing 5% charcoal-stripped FBS (batch AHM9371, Hyclone, Logan, UT), 50 U/ml penicillin, 50 $\mu\text{g/ml}$ streptomycin, and 2 mM L-glutamine. For the actual growth experiments, cells were plated on 96-well plates at a seeding density of 5000 cells per well. After 24 hours, the cells were transfected in the absence of serum (as described) for 24 hours. The medium was then exchanged for phenol red-free DMEM medium containing 1% charcoal-stripped FBS, 50 U/ml penicillin, 50 $\mu\text{g/ml}$ streptomycin, 2 mM L-glutamine, and 0.12 U/ml bovine insulin supplemented with up to 10^{-6} M of either $17\alpha\text{-E}_2$ or $17\beta\text{-E}_2$ (Malet et al., 1988). The

medium was changed daily for up to 7 days. At the end of the culture period, cell density was assessed with the XTT formazan dye production assay (Roche Diagnostics, Ltd., Lewes, East Sussex, U.K.) for 2 hours, according to the manufacturer's instructions. XTT absorption was measured at 490 nm with a Bio-Rad model 450 ELISA plate reader with a reference absorbance set at 595 nm.

RESULTS

Characterization of MCF7-K2 cells

To ensure that the phenotype of the MCF7-K2 cells used in this study corresponded with that detailed in the literature, $^3\text{H-E}_2$ binding, proteosomal degradation, and the growth characteristics of these cells were assessed. Binding of $^3\text{H-E}_2$ to MCF7-K2 cell extracts showed the presence of a functional $\text{ER}\alpha$ (Fig.

TABLE 2. $^3\text{H-E}_2$ BINDING TO MCF7 CELL EXTRACTS OF NONTRANSFECTED AND TRANSFECTED CULTURES TREATED WITH EXOGENOUS ESTRADIOL

E_2 dose (M)	Control		Scrambled		Antisense	
	fmol/mg	%	fmol/mg	%	fmol/mg	%
0	16.9 \pm 2.3 ^a	100	14.1 \pm 3.5	100	7.0 \pm 2.5**	100
10^{-9}	18.3 \pm 2.3	108	9.9 \pm 2.5*	70	5.6 \pm 1.5**	80
10^{-8}	17.3 \pm 2.6	102	9.0 \pm 2.5*	64	1.0 \pm 0.6**	13****
10^{-7}	12.1 \pm 2.4	71	10.0 \pm 2.6	71	1.0 \pm 0.6**	14****
10^{-6}	12.5 \pm 1.6	74	8.7 \pm 2.6	62	1.2 \pm 1.0**	18***

^aData are presented as the amount of $^3\text{H-E}_2$ binding in fmol/mg of cellular protein and are the mean \pm SEM of three independent experiments performed in triplicate.

* $p < 0.05$, ** $p < 0.01$, transfected compared with nontransfected cultures, ANOVA with Tukey's significant difference test. To indicate the proportional effect of added E_2 , data are also normalized to untreated cultures and presented as % of untreated control.

*** $p < 0.01$, **** $p < 0.001$, E_2 -treated compared with untreated, two-way ANOVA with Tukey's significant difference test.

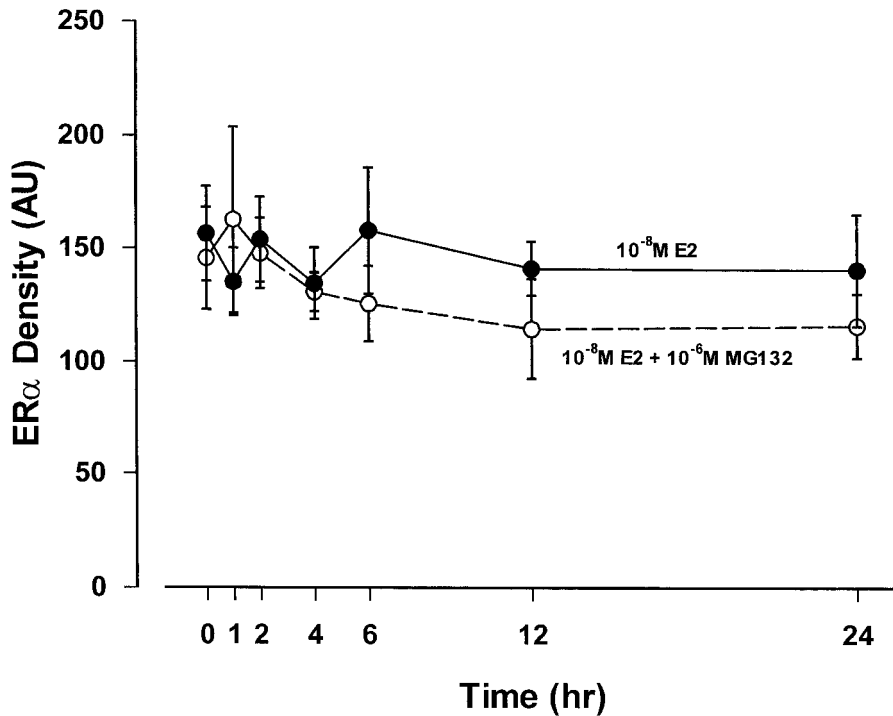


FIG. 3. MCF7-K2 ER α protein is not degraded via the proteasome. Protein extracts (12 μ g) from MCF7 cells treated with 17 β -E $_2$ (10^{-8} M) in the absence (solid circles) or presence of MG-132 (open circles) for the times indicated were subjected to Western blot analysis as described in Materials and Methods. Data are the mean \pm SD of six experiments performed in triplicate ($n = 6$) (data were not significantly different, ANOVA with Tukey's significant differences test).

2). Specific $^3\text{H-E}_2$ binding increased with dose and reached a plateau at $\sim 10^4$ cpm, with no appreciable increase in nonspecific binding up to 6×10^4 cpm (Fig. 2). The calculated K_d and B_{max} were 3.9 ± 1.5 nM and 32 ± 3.4 fmol/mg protein ($n = 6$), respectively. As $^3\text{H-E}_2$ at 6×10^4 cpm did not show appreciable increased nonspecific binding, the amount of total $^3\text{H-E}_2$ used in subsequent binding assays to show that the antisense ER α ODN might also affect functional ER α protein (Table 2) was set to 6×10^4 cpm.

Because exogenous 17 β -E $_2$ enhances ER α protein degradation in MCF7-K1 but not in MCF7-K2 cells through the proteasome (Aronica and Katzenellenbogen, 1993; El Khissi and Leclercq, 1999; Ing and Ott, 1999), we postulated that 17 β -E $_2$ -induced ER α protein degradation in MCF7-K2 cells would not occur. To test this possibility, MCF7-K2 cells were treated with 10^{-8} M 17 β -E $_2$ (the dose that significantly enhances antisense ER α oligo 7 action and ER α degradation through the proteasome in MCF7-K1 cells) for up to 24 hours in the presence and absence of the proteasome inhibitor MG-132 (Fig. 3). 17 β -E $_2$ had no effect on the degradation of ER α over the duration of the experiment (Fig. 3). The addition of the proteasome inhibitor MG-132 to 17 β -E $_2$ -treated cultures had no effect on ER α protein levels (Fig. 3). This model suggests that any reduction in ER α protein levels observed would, therefore, be due to a direct effect of antisense ODN action and not 17 β -E $_2$ -induced proteasomal degradation of receptor.

MCF7-K2 cells treated with vehicle (0.1% ethanol) in 1% charcoal-stripped serum proliferated weakly compared with untreated cultures (Fig. 4) but with a population doubling time of 7–8 days. The observed doubling time is similar to that found in the literature (Bocuzzi et al., 1994). Similarly, cells treated

with 10^{-8} M 17 β -E $_2$ did not proliferate above that of untreated cell cultures or vehicle-treated cultures, whereas MCF7-K2 cells showed an increased proliferation rate (~ 2.5 –3 days) in response to exogenous 17 β -E $_2$ (Fig. 4).

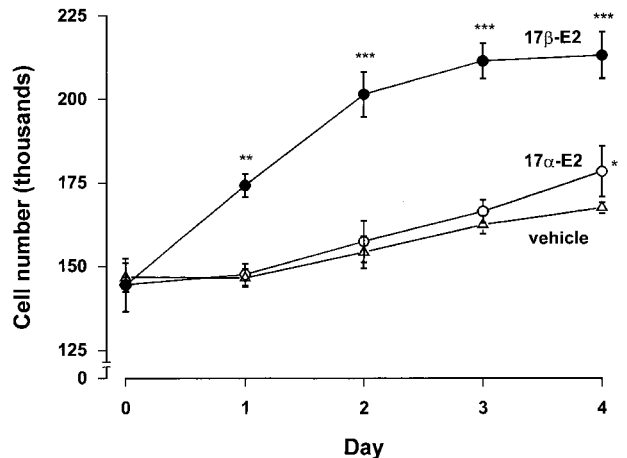


FIG. 4. Characterization of 17 β -E $_2$ -dependent MCF7-K2 cell proliferation. MCF7-K2 cells (5000) plated in estrogen-deficient medium (10% charcoal-stripped serum) for 3 days were treated with vehicle (0.1% ethanol) (open triangles), 10^{-8} M 17 α -E $_2$ (open circles), or 10^{-8} M 17 β -E $_2$ (solid circles) for a further 4 days. Medium was changed on days 1 and 3. Growth was assessed using XTT formazan dye conversion at 490 nm. Data are the mean \pm SD of three experiments performed in quadruplicate ($n = 3$) (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ANOVA with Tukey's significant differences test compared to day 0).

These data suggest that MCF7-K2 cells are an appropriate model to study the effect of antisense ODN on the levels of ER α mRNA, protein, $^3\text{H-E}_2$ binding, and proliferation in response to $17\beta\text{-E}_2$ (Katzenellenbogen et al., 1987; Read et al., 1989).

Antisense ER α ODN decrease ER α protein expression

To test the hypothesis that antisense ER α ODN inhibits ER α protein synthesis, the levels of ER α protein in MCF7-K2 cell extracts transfected with antisense ER α ODN were analyzed by Western blot analysis. MCF7-K2 cells produce a single immunoreactive signal with a relative molecular mass of $\sim 66\text{--}67$ kDa (Fig. 5A). After densitometric analysis and normalization against untreated controls (Fig. 5B), the data showed that lipofectamine-treated cells and cells transfected with either sense or scrambled ER α ODN sequences had no significant effect on the expression of ER α in MCF7-K2 cells. Oligos 1–6 had no effect on the levels of ER α protein (Fig. 5B), but oligo 7 reduced the

levels of ER α in MCF7-K2 cells by $61\% \pm 14\%$ ($n = 6$) (ANOVA).

To ensure that the decrease in cytoplasmic ER α levels was not an artifact caused by aberrant $17\beta\text{-E}_2$ in the culture system altering ER α degradation, transfected MCF7-K2 cells were treated with 10^{-8} M $17\beta\text{-E}_2$ in an attempt to induce similar reductions in ER α concentrations regardless of the oligo transfected. The four sense and antisense oligonucleotides (oligos 2, 3, 5, and 6, Fig. 1) previously used to demonstrate autologous downregulation of ER α on ER α expression in MCF7 cells (Santagati et al., 1997) showed no effect on ER α protein levels in the presence of $17\beta\text{-E}_2$ (Fig. 5C). However, cells transfected with oligo 1, oligo 4, or oligo 7 subsequently treated with exogenous $17\beta\text{-E}_2$ (Fig. 5C) showed significant ER α protein level reductions of $36\% \pm 7\%$ for oligo 1 ($n = 6$) (Student's *t*-test) and $62\% \pm 10\%$ ($n = 6$) (Student's *t*-test) for oligo 4 and $78\% \pm 4\%$ for oligo 7, respectively.

Because the antisense ODN directed toward the translational start site of human ER α (oligo 7) showed an inhibitory action

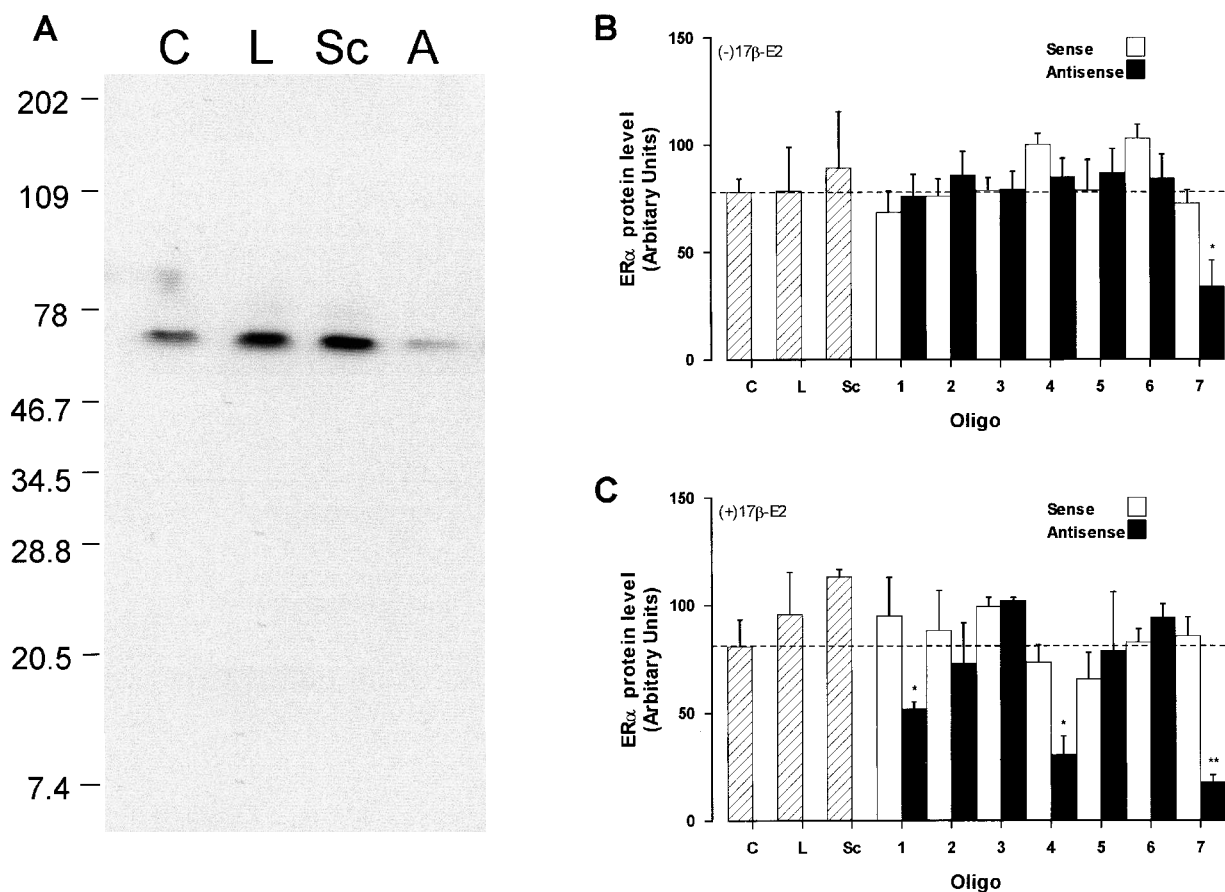


FIG. 5. Antisense oligos 1, 4, and 7 decrease ER α protein levels. (A) A representative ER α protein Western blot of protein extracts from untreated MCF7-K2 cells (C), cells treated with lipofectamine (L), cells transfected with scrambled oligo 7 (Sc), or cells transfected with antisense oligo 7 (A). Migration markers for the indicated molecular mass markers (in kDa) are shown at left. The blot is representative of six separate experiments. (B) Untreated MCF7-K2 cells (C), cells treated with lipofectamine alone (L), or cells transfected with sense (open bars) or antisense oligos (solid bars) or scrambled oligo 7 (Sc) and treated with vehicle (0.1% ethanol) for 24 hours were subjected to Western blot analysis as described in Materials and Methods, and the amount of ER α protein obtained was normalized against signals obtained from related β -actin Western blots. (C) Parallel cultures transfected and treated in a similar manner, except all cultures were treated with 10^{-8} M $17\beta\text{-E}_2$. Data are the mean \pm SD ($n = 6$) (* $p < 0.05$, ** $p < 0.01$, Student's unpaired *t*-test).

on ER α expression both in the absence and the presence of 17 β -E $_2$, only this antisense molecule was tested further.

Specific 17 β -E $_2$ -induced inhibition of ER α protein levels

MCF7-K2 cells transfected with antisense ER α oligo 7 and treated with graded doses of 17 β -E $_2$ showed a marked reduction in ER α protein levels, with a threshold and maximal 36% \pm 9.3% ($n = 6$) inhibitory effect occurring at 10 $^{-8}$ M 17 β -E $_2$ (Fig. 6). In contrast, control MCF7-K2 cells (data not shown) and MCF7-K2 cells transfected with the control scrambled ER α ODN did not show significantly reduced protein concentrations when treated with 10 $^{-8}$ M 17 β -E $_2$ (4% \pm 11.0%) (Fig. 6).

Because both E $_2$ isomers can interact either in a nonspecific manner with the plasma membrane (Nemere and Farach-Carson, 1998) or specifically via a putative membrane ER (Razandi et al., 1999) of 17 β -E $_2$ -responsive cells, the enhancing effect of exogenous 17 β -E $_2$ on antisense oligo 7 reduction of ER α protein levels could be attributable to these phenomena. To test this hypothesis, MCF7-K2 cells transfected with a control scrambled (Fig. 7A) or antisense ER α oligo 7 sequence (Fig. 7B) were treated with 17 β -E $_2$ or its inactive isomer 17 α -E $_2$. As expected, 17 β -E $_2$ significantly enhanced the degradation of ER α by 30%–50% (compare Fig. 7B with Figure 6). Equivalent doses of 17 α -E $_2$ had no effect on the relative levels of ER α (Fig. 7B). Neither E $_2$ isoform affected the ER α protein concentrations of MCF7-K2 cells transfected with the scrambled ER α oligo 7.

These data suggest that 17 β -E $_2$ enhances the inhibitory effect of the antisense ER α oligo 7 on ER α levels in MCF7-K2 cells by a mechanism that assists in blocking the production of nascent protein rather than increased degradation of existing ER α protein.

Antisense ER α ODN inhibits 3 H-E $_2$ binding to MCF7 cytoplasmic extracts

MCF7-K2 cells transfected with the antisense ER α ODN showed a significant 58.2% decrease in 3 H-E $_2$ binding to cell extracts (Table 2). Cells transfected with the control scrambled ER α oligo 7 sequence and in the absence of exogenous 17 β -E $_2$ showed a nonsignificant 16.5% inhibition of 3 H-E $_2$ binding compared with control (Table 2). These data indicate that the antisense oligo 7 decreases either the ability of MCF7-K2 cell extracts to bind 3 H-E $_2$ or the levels of cytoplasmic ER protein.

In a parallel study, MCF7-K2 cells were transfected with scrambled or antisense ER α oligo 7 sequences and incubated with 10 $^{-9}$ –10 $^{-6}$ M 17 β -E $_2$ (Table 2). Binding to nontransfected control cell extracts was significantly inhibited by high doses of 17 β -E $_2$ (25.7% at 10 $^{-6}$ M), suggesting competition of unlabeled 17 β -E $_2$ with 3 H-E $_2$ for MCF7-K2 ER α (Table 2). 3 H-E $_2$ binding to extracts from cells transfected with the scrambled and antisense ER α oligo 7 was also reduced by 29.9% \pm 6.2% and 82.0% \pm 5.0% ($n = 9$) (ANOVA), respectively, in the presence of 10 $^{-6}$ M exogenous 17 β -E $_2$ compared with the respective controls. The amount of 3 H-E $_2$ bound to extracts from scrambled oligo 7-transfected cultures treated with 10 $^{-6}$ M 17 β -E $_2$ was not significantly different from the amount bound to similarly treated control cultures (9.9 \pm 2.6 compared with 12.5 \pm 1.6 fmol/mg protein) ($n = 9$) (ANOVA). However, the amount of 3 H-E $_2$ bound after 17 β -E $_2$ inhibition with 10 $^{-6}$ M 17 β -E $_2$ in an antisense ODN-transfected culture was significantly lower (1.2 \pm 1.0 vs. 9.9 \pm 2.6 or 12.5 \pm 1.6 fmol/mg protein) ($n = 9$) (ANOVA). These data suggest that 17 β -E $_2$ enhances the inhibitory effect of antisense ER α ODN on 3 H-E $_2$ binding to ER α protein.

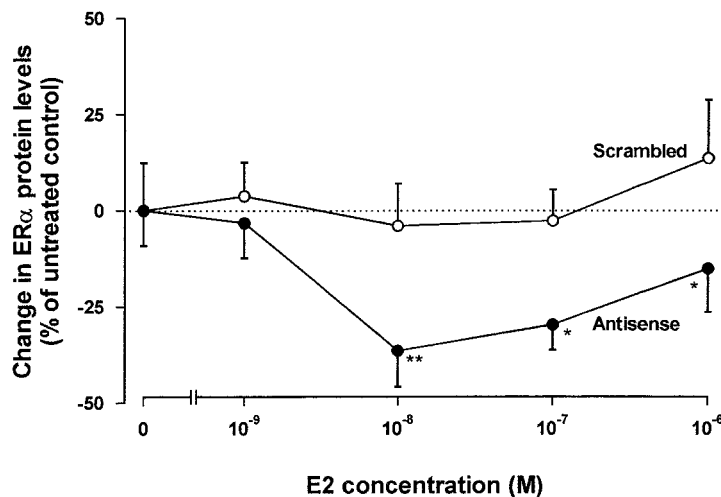
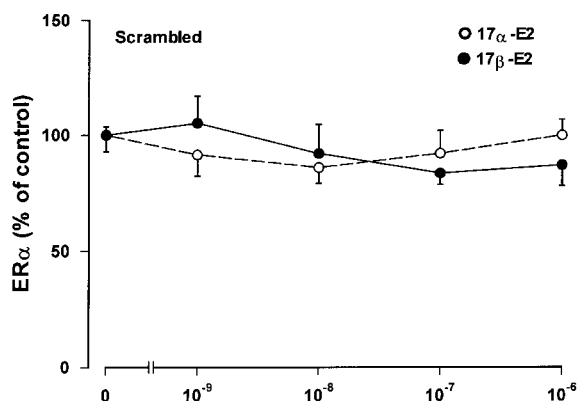


FIG. 6. 17 β -E $_2$ enhances the inhibitory effect of antisense ER α ODN on ER α protein levels. Control MCF7-K2 cells (dotted line) or cells transfected with scrambled (open circle) or antisense ER α oligo 7 (solid circles) were incubated with the indicated concentrations of exogenous 17 β -E $_2$ for 24 hours. Cell lysates (12 μ g) were subjected to Western blot analysis as described in Materials and Methods. The data were then corrected for the amount of β -actin protein and normalized to the relevant control. Data are the mean \pm SD of four experiments performed in triplicate ($n = 4$) (* $p < 0.05$, ** $p < 0.01$, two-way ANOVA with Tukey's significance difference test).

A



B

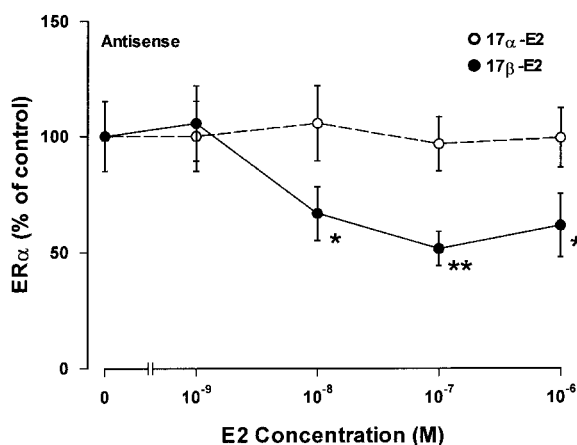


FIG. 7. 17 β -E₂ but not 17 α -E₂ enhances the inhibitory effect of antisense ER α ODN on ER α protein levels. Protein extracts (12 μ g) from MCF7 cells transfected with scrambled ODN (A) or antisense ER α ODN (B) and treated with the indicated doses of 17 β -E₂ (solid circles) or 17 α -E₂ (open circles) for 24 hours were subjected to Western blot analysis as described in Materials and Methods. Data are the mean \pm SD of two experiments performed in triplicate ($n = 2$) (* $p < 0.05$, ** $p < 0.01$, Student's paired t -test compared with untreated control).

17 β -E₂ enhances antisense ER α ODN effect by increasing ODN uptake

To better understand how 17 β -E₂ increases the efficacy of antisense ER oligo 7, MCF7-K2 cells were transfected with FITC-labeled antisense ER α oligo 7 and treated with graded doses of 17 β -E₂ (Fig. 8). The amount of fluorescence is low in untreated cells (Fig. 8A) but increases with 17 β -E₂ treatment and reaches a maximum at about 10⁻⁸ M 17 β -E₂ (Fig. 8C). We also noted that at low doses of 17 β -E₂ and shorter times, fluorescence is confined to the plasma membrane and cytoplasm (data not shown). At higher doses and longer times, fluorescence signal accumulates in the nucleus. These data indicate that 17 β -E₂ enhances antisense ER α oligo 7 transfection efficiency to MCF7-K2 cells.

Antisense ER α ODN has no effect on ER α transcription

To discount the hypothesis that 17 β -E₂ affects ER α transcription in antisense ER α oligo-transfected MCF7-K2 cells, we measured the levels of ER α mRNA using RT-PCR (Fig. 9). The RT-PCR method, including the number of cycles, was validated to ensure the number of cycles was in the linear range for both ER α and β -actin (data not shown) (Heikinheimo et al., 1995). Antisense ER α oligo 7 had no effect on ER α mRNA levels when compared with ER α mRNA levels in scrambled oligo 7 and control cultures (Fig. 9A). Additionally, 17 β -E₂ had no significant effect on the levels of ER α in any of the control or treated MCF7-K2 cultures (Fig. 8A). Densitometry measurements of amplicon levels showed an apparent decrease in ER α mRNA levels when compared with control and scrambled ODN-transfected cultures, but the decrease was not found to differ statistically (Fig. 9B).

MCF7-K2 cell proliferation is enhanced by 17 β -E₂ and inhibited by antisense ER α ODN

Initial studies of MCF7-K2 cell proliferation treated with 17 β -E₂ at any serum concentration, cell density, and duration of culture showed these cells to be unresponsive to 17 β -E₂ (data not shown). However, after three passages of stock cultures in low-E₂-containing growth medium, MCF7-K2 cells showed both time-dependent (Fig. 4) and dose-dependent responses to 17 β -E₂ but not to 17 α -E₂ (data not shown). Parallel cultures transfected with scrambled oligo 7 or antisense ER α oligo 7 showed no alteration in the basic nonproliferation pattern when treated with 17 α -E₂ (Fig. 10A). In contrast, MCF7-K2 cells transfected with scrambled oligo 7 and treated with graded doses of 17 β -E₂ continued to proliferate (Fig. 10B). MCF7-K2 cells transfected with antisense oligo 7 showed a departure from the normal 17 β -E₂ growth patterns, with a dose-dependent loss of cells from the culture (Fig. 10B). The effect of antisense oligo 7 action on MCF7-K2 cell proliferation was maintained for at least 4 days in culture. Cells transfected with either scrambled or antisense oligo 7 ODN initially showed growth-inhibitory effects within the first 24 hours (Fig. 10C). When medium containing 10⁻⁸ M 17 β -E₂ was reintroduced to the cultures on day 2, MCF7-K2 cells transfected with scrambled oligo 7 or sense oligo 7 (data not shown) reinitiated cell proliferation and continued to proliferate at the same rate as non-transfected control cultures (Fig. 10C). However, MCF7-K2 cells transfected with antisense oligo 7 no longer had the capacity to proliferate in response to 10⁻⁸ M 17 β -E₂ (Fig. 10C). These data suggest that antisense oligo 7 inhibits the production of ER α in MCF7-K2 cells, which in turn inhibits 17 β -E₂-dependent MCF7-K2 cell proliferation.

DISCUSSION

Characterization of MCF7-K2 cells

There are several reports on the characteristics of MCF7 cells that show altered phenotypes in numerous laboratories. The main types of MCF7 cell are characterized by the expression of a functional ER α , but cellular response to exogenous estrogen (usually

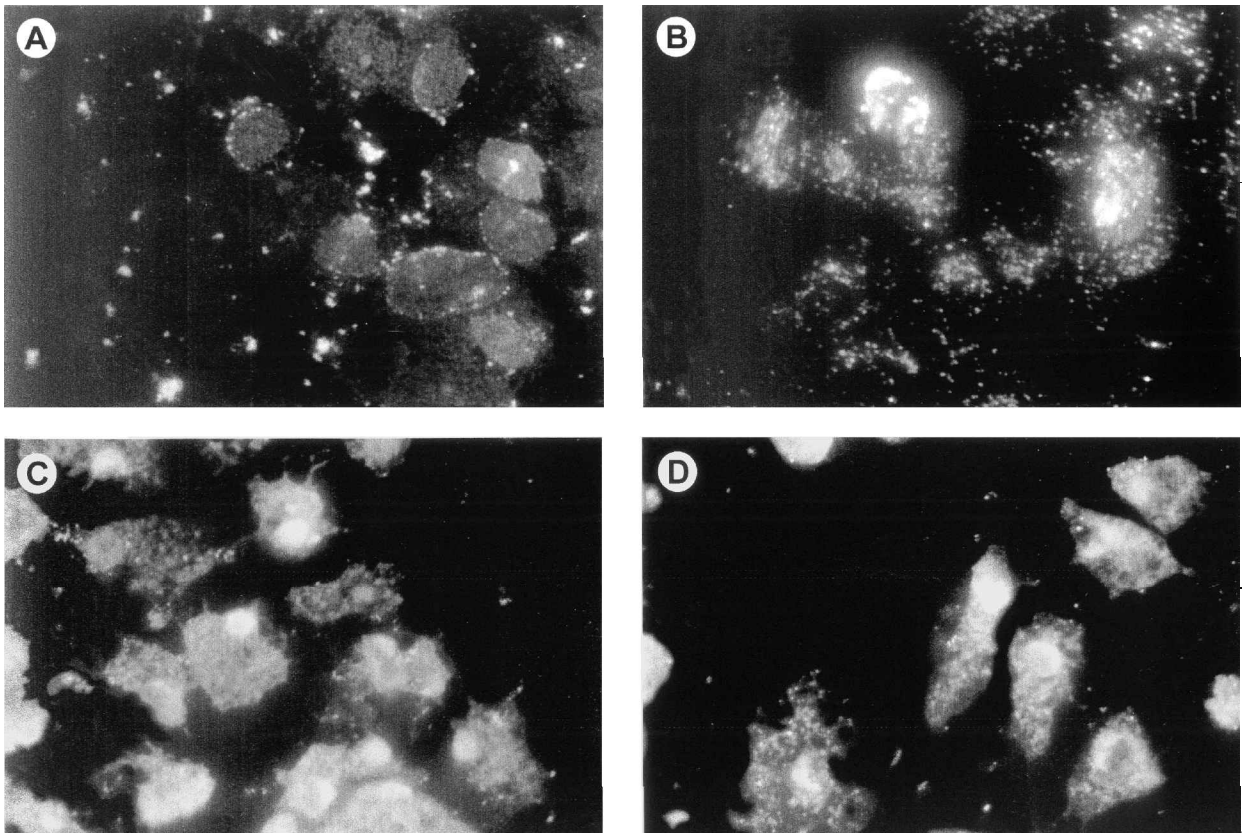


FIG. 8. 17β -E₂ enhances the cellular uptake of antisense ER α oligo 7. MCF7 cells grown on glass slides were transfected with FITC-labeled antisense ER α oligo 7 alone (A) or treated with 10^{-10} M (B), 10^{-8} M (C), or 10^{-6} M 17β -E₂ (D) for 24 hours. Cells fixed with paraformaldehyde were washed in PBS and photographed at 400 \times original magnification to ASA800 film. Photomicrographs are representative of two separate experiments.

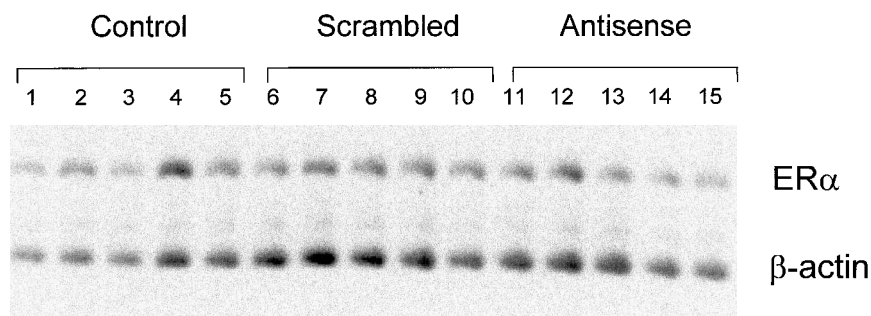
17β -E₂) differs markedly. For example, MCF7-K1 and MCF7-K2 cells both contain functional ER α that bind 17β -E₂. However, in MCF7-K1 cells, 17β -E₂ causes a number of effects, including ER α degradation through the proteasome pathway, activation of promoter ERE (estrogen responsive elements) by receptor transactivation, inhibition of ER α transcription and hence a reduction in the steady-state levels of ER α mRNA, and stimulation of 17β -E₂-dependent proliferation. In contrast, MCF7-K2 cells only show some of these properties (Read et al., 1989). To eliminate the confounding effects that 17β -E₂ administration might have on the potential mode of action of the antisense ODN used in this study, we used the MCF7-K2 cells. To ensure that the MCF7-K2 cells performed as described in the literature, we performed radioligand binding, proteosomal degradation, and growth analyses before subjecting the cells to transfection protocols. As predicted, the MCF7-K2 cells showed the expected features of this cell line's phenotype, including a proliferative response to 17β -E₂.

Using the MCF7-K2 cells, we have demonstrated that three specific antisense ER α ODN directed against the human ER α gene specifically and efficiently inhibit ER α protein synthesis. Two of the oligos, oligo 1 and oligo 4, appear to work only in the presence of exogenous 17β -E₂, whereas oligo 7 inhibited ER α protein levels in both the absence and presence of 17β -E₂. More detailed studies with antisense oligo 7 show that both ER α protein and ³H-E₂ binding were decreased by 50%–60%, suggesting that both methods measured the quantity of synthe-

sized ER α . The values for K_d and B_{max} for ³H-E₂ binding to MCF7-K2 cell extracts were similar to that reported by MacIndoe et al. (1982) but crucially different from those of others (Gyling and Leclercq, 1988; Otto, 1995). This discrepancy is probably due to the subclone of MCF7 cells used in the present studies. Additionally, the relative molecular mass of immunoreactive MCF7 ER α protein was identical to that reported previously, with no additional isoforms or detectable proteolytic products (El Khissin et al., 1997; Santagati et al., 1997).

The other antisense molecules directed against the human ER α (oligos 1 and 4) did not show an effect on ER α protein levels or affect the ability of the protein to bind radioligand (data not shown) in the absence of 17β -E₂ but showed significant reductions in ER α levels when exposed to 17β -E₂ (Fig. 5). These data do not correlate with the data obtained by others (Santagati et al., 1997). In the studies of Santagati et al. (1997) MCF7 ER α protein levels increased in response to antisense ODN transfection with oligos 1 and 2, which are directed against the 5' region of the human ER α cDNA. In the present study, ER α levels remained constant in four of six cases and showed inhibitory effects on ER α protein levels, but only in the presence of exogenous 17β -E₂. Oligos 1 and 4 showed no change in ER α levels in the absence of 17β -E₂, but significant reductions in ER α protein levels were observed in the presence of 10^{-8} M 17β -E₂ (Fig. 5). The reason for the discrepancy between these data is presumably due to the type of MCF7 cells

A



B

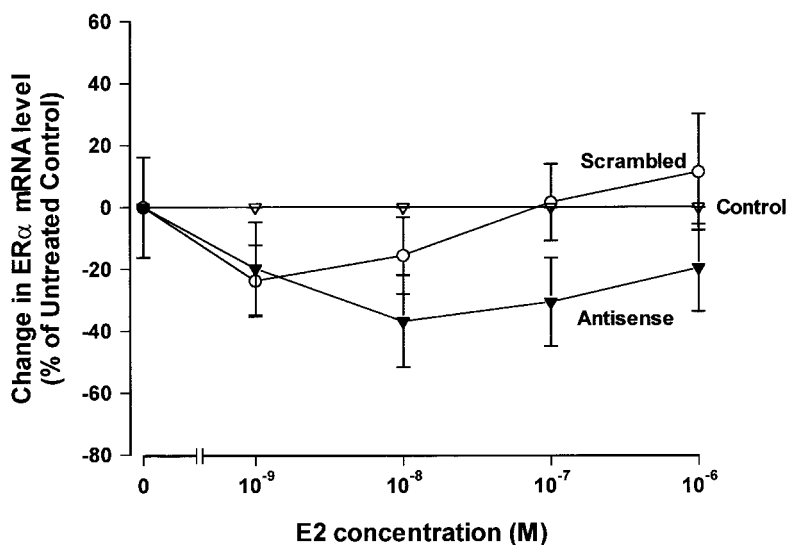


FIG. 9. Antisense ER α oligo 7 has no effect on levels of ER α mRNA. (A) A representative ethidium bromide-stained agarose gel of RT-PCR products for ER α and β -actin. MCF7 cells transfected with scrambled or antisense ER α ODN were untreated (lanes 1, 6, 11) or treated with 10^{-9} M (lanes 2, 7, 12), 10^{-8} M (lanes 3, 8, 13), 10^{-7} M (lanes 4, 9, 14), or 10^{-6} M 17β -E $_2$ (lanes 5, 10, 15) for 24 hours. Extracted total RNA (1 μ g) was subjected to RT-PCR with the primers shown in Table 1 and subjected to electrophoresis on 1.8% agarose gels impregnated with 0.5 μ g/ml ethidium bromide. The image is representative of three separate experiments. (B) The densities of the bands shown in A were quantitated by video-assisted densitometry and corrected for the levels of β -actin. Data were normalized to nontransfected control cultures (the zero line) and are the mean \pm SD for three experiments in triplicate ($n = 3$) (data were not significantly different, two-way ANOVA with Tukey's significance difference test).

used. The MCF7 cells used by Santagati et al. (1997) are probably different from the MCF7-K2 cells used in our studies, as several clones of the MCF7 cell line exist (Read et al., 1989). These data suggest that the use of antisense ODN directed against endometrial ER α would perhaps require individual testing on a patient biopsy before any clinical trial.

Both the MCF7-K1 and MCF7-K2 cell clones contain high levels of ER α , but only the MCF7-K1 cells exhibit autologous downregulation of the ER α in response to 17β -E $_2$ (Santagati et al., 1997). Although MCF7-K2 cells contain ligand-binding forms of the ER, 17β -E $_2$ does not affect ER α mRNA or protein levels (Read et al., 1989). We were unable to demonstrate a decrease in

ER α protein or mRNA levels in response to exogenous 17β -E $_2$ (Figs. 3 and 9 and Table 2), confirming that MCF7-K2 cells were used in the present study. However, using MCF7-K2 cells was important for this study because the autologous downregulating effect of exogenous 17β -E $_2$ on ER α expression that is seen in MCF7-K1 cells could have masked any modulation of ER α expression by the antisense ODN molecules. In other words, we can conclude that the inhibitory effect on the antisense ODN on ER α protein levels in the MCF7-K2 cells presented herein is due solely to the presence of the antisense ODN.

Although 17β -E $_2$ increases the degradation of ER α protein in MCF7-K1 cells in a dose-dependent and time-dependent manner

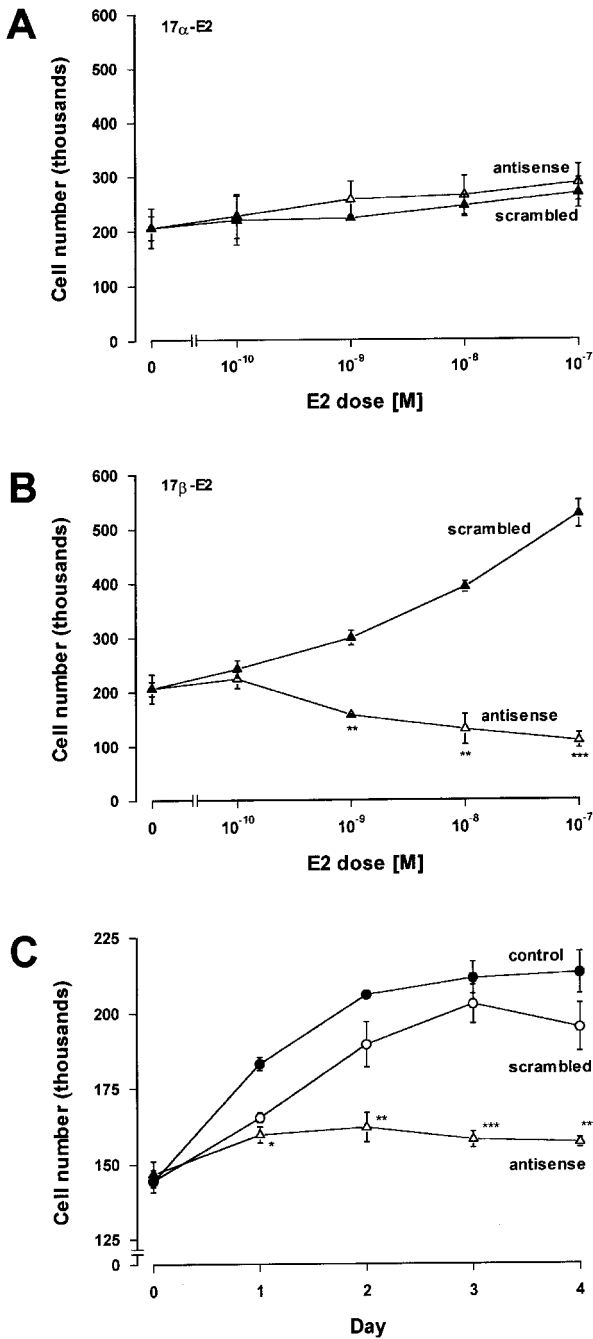


FIG. 10. Antisense ER α oligo 7 inhibits 17 β -E₂-dependent proliferation of MCF7-K2 cells. MCF7-K2 cells (5000) plated in estrogen-deficient medium (1% charcoal-stripped serum) for 3 days were transfected with either scrambled oligo 7 (solid triangles) or antisense oligo 7 (open triangles) and treated with the indicated doses of 17 α -E₂ (A) or 17 β -E₂ (B) for a further 4 days. Medium was changed on alternate days. (C) MCF7-K2 cells (5000) were allowed to attach and grow for 24 hours. On day 0, cells were either not transfected (solid circles), transfected with scrambled oligo 7 (open circles), or transfected with antisense oligo 7 (open triangles) for 24 hours. On day 1, the medium on all cultures was changed for one containing 1% charcoal-stripped serum supplemented with 10⁻⁸ M 17 β -E₂. This medium was left in contact with the cells for the duration of the experiment. Growth was assessed using XTT formazan dye conversion at 490 nm. Data are the mean \pm SD of three experiments performed in quadruplicate ($n = 3$) (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ANOVA with Tukey's significant differences test).

(Aronica and Katzenellenbogen, 1993; Ing and Ott, 1999) via the proteasome (Alarid et al., 1999; Nawaz et al., 1999), this hormone also increases plasmid DNA transfer to MCF7 cells (Jain and Gerwitz, 1998). This suggests that 17 β -E₂ may have an effect on the MCF7 cell via the plasma membrane. Although a specific membrane ER has been reported recently (Nemere and Farach-Carson, 1998), there is also evidence that 17 β -E₂ interacts with the plasma membrane in a nonspecific manner (Dufy et al., 1979) and that other steroids directly interact with the plasma membrane (Zinder and Dar, 1999). These data suggest that the addition of exogenous 17 β -E₂ could enhance the effect of some antisense molecules through two or more mechanisms. First, 17 β -E₂ could interact with a plasma membrane ER to activate the degradation of existing ER protein, or second, the hormone might interact with fundamental components of the plasma membrane to increase transfection efficiency or alter the physical characteristics of the lipofectamine-DNA complex to alter transfection efficiency. In our hands, 17 β -E₂ had no effect on endogenous ER α protein expression and clearly is unable to induce ER α turnover via the proteasome pathway. Because ER α mRNA levels are not affected but protein levels decrease in the presence of antisense ER α ODN, we can conclude that antisense ER α ODN efficiently binds to the ER α mRNA, translation of nascent ER α protein is blocked, and endogenous ER α is cleared by a nonproteosomal pathway in MCF7-K2 cells.

The FITC-labeled ODN uptake experiment (Fig. 8) showed that 17 β -E₂ enhanced ODN uptake and, therefore, transfection efficiency. Therefore, 17 β -E₂ must alter the physical properties of the transfection process by interfering with either the liposome-DNA complex or the MCF7 cell plasma membrane to increase passage of DNA into the cell.

17 β -E₂ enhanced FITC-labeled antisense ER α ODN (oligo 7) uptake and nuclear accumulation by MCF7-K2 cells. Theoretically, antisense ODN should be assimilated by cells through an episomal pathway and, therefore, should not appear in the nucleus (Cooper, 1996). However, coating of DNA molecules with cationic liposomes, such as lipofectamine, may protect naked DNA and retarget the DNA through an alternative pathway to the MCF7-K2 nucleus, as is seen in keratinocytes (White et al., 2000). This raises the possibility that first contact for the antisense ODN is heteronuclear RNA and not mature cytoplasmic RNA. This may explain why the levels of ER α mRNA showed an apparent, but not significant, decrease in the presence of antisense oligo 7 ER α ODN (Fig. 9B). However, as ER α mRNA concentrations were not significantly different, an RNase H-dependent RNA degradation mechanism (Schlingensiepen and Schlingensiepen, 1997) involving the destruction of ER α mRNA is not implicated. The observed decrease in ER α protein levels with antisense oligo 7 is, therefore, presumably due to a steric blockade at the ER α translational start site.

The addition of exogenous 17 β -E₂, but not 17 α -E₂, to cultures augments both decreased ER α protein concentration and ³H-E₂ binding to MCF7 extracts, suggesting that 17 β -E₂ increases the transfection efficiency of the antisense ER α ODN. Additionally, 17 β -E₂ does not override the antiproliferative effect of antisense oligo 7 but appears to enhance the antiproliferative effect. This is presumably due to increased transfection efficiency, leading to further reductions in ER α protein levels and, hence, ER α -dependent proliferation.

The ER α ODN described are not the first antisense molecule to be tested in our eventual goal, the uterine endometrium. An-

tisense ODN directed against Hox a10, Hox a11, calcitonin, and other target genes in laboratory animals have been described (Zhu et al., 1998; Bagot et al., 1999; Kunishige et al., 1999; Malayer et al., 1999). This is to our knowledge, however, the first report of the use of antisense ODN against a human steroid receptor nuclear transcription factor. This is an important step forward because steroid receptors are implicated in numerous pathologic conditions, and the data presented may help in the elucidation of new gene targets. However, the diversity of steroid receptor-mediated effects suggests that significant systemic toxicity, a major concern in gene therapy (Cotter, 1997), could occur with the use of antisense ER α oligo 7 in future human gene therapy clinical trials.

Although we envisage that antisense oligo 7 or similar molecules will be used solely as a topical application to the endometrial layer of the uterus and, thus, will have reduced toxic effects compared with a systemic application, there are two pieces of extra data that suggest any systemic toxic effect of a topically applied antisense ER α might be negligible. First, the application of β -galactosidase by transfection of the mouse uterus failed to elicit more than a few millimeters of penetration (Chamock-Jones et al., 1997), and second, the female ER α knockout mouse does not exhibit gross systemic anatomic anomalies (Couse and Korach, 1999). These data suggest that an antisense ODN would fail to penetrate further than a few millimetres into the human endometrium, and should it enter the vasculature, systemic organ damage would be limited. Additionally, unmodified oligonucleotides are rapidly destroyed in blood, suggesting that antisense ER α ODN, such as the ones described herein, would not cause significant toxicity (Agrawal et al., 1997).

Accordingly, this paper has important implications for the control of diseases that involve transcriptional regulators, such as the steroid receptors, and especially diseases that are enhanced by 17 β -E₂ through ER α . The data presented may lead to effective therapies for such diseases. The data presented also suggest that antisense ER α ODN have the potential to help unlock the mechanism of ER α synthesis and protein stability in target cells and help to dissect the relative roles of ER α and ER β in 17 β -E₂-dependent cells.

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Address reprint requests to:

Dr. Farook Al-Azzawi
Senior Lecturer, Consultant Gynaecologist
Gynaecology Research Group
Department of Obstetrics & Gynaecology
Faculty of Medicine and Biological Sciences
University of Leicester
Robert Kilpatrick Clinical Sciences Building
Leicester Royal Infirmary
Leicester, Leicestershire
LE2 7LX England

E-mail: fa2@le.ac.uk

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