Oligonucleotide Squelching Reveals the Mechanism of Estrogen Receptor Autologous Down-Regulation

S. Santagati, E. Gianazza, P. Agrati, E. Vegeto, C. Patrone, G. Pollio, and A. Maggi

Center Milan Molecular Pharmacology Laboratory and Laboratory for the Study of Arteriosclerosis (E.G.)
Institute of Pharmacological Sciences
University of Milan
I-20133 Milan, Italy

Antisense oligos complementary to the 5′-end, but not to the 3′-end, of the estrogen receptor (ER) messenger RNA caused a paradox accumulation of ER protein in MCF-7 cells. The same effect was observed after treatment of the cells with the corresponding sense oligos. The oligos interfering with ER down-regulation were demonstrated to specifically bind the ER with affinities in the nanomolar range. It is, therefore, proposed that the ER up-regulation induced by the oligos might be due to squelching of the ER (or ER-inducible proteins) from their binding site located in the 5′-end of the ER gene. We also report that transcriptionally inactive ER mutants can undergo down-regulation, and that in denaturing gels, the migration profile of ER-oligo and ER-estrogen-responsive element complexes are dissimilar. We, therefore, propose that ER can interact with DNA in different ways and at different binding sites. These observations might have important pharmacological consequences, since specific drugs could be devised to induce the ER conformation necessary to perform only selected tasks of the ER transcriptional repertoire. (Molecular Endocrinology 11: 938–949, 1997)

INTRODUCTION

17β-Estradiol (E_2) exerts its physiological effects on target tissues by interacting with its cognate estrogen receptor (ER), which is a member of a superfamily of ligand-regulated transcription factors (1–3). Several lines of evidence prove that after steroid binding, the hormone-receptor complex interacts with specific DNA target sequences, named estrogen-responsive elements (ERE), which are located in the promoter of estrogen-responsive genes (4–6). The activity of the hormone-receptor complex can then be further modulated by a series of proteins acting as coactivators or as repressors (7–10).

The concentration of ER is an important determinant of cellular responsiveness to steroids, as suggested by the observation that in mammals, ER expression fluctuates in a spatial-temporal fashion in response to a variety of endocrine stimuli. Further support for this view is provided by studies at the cellular level, demonstrating that receptor expression above a specific concentration threshold is required for ER-mediated events to occur (11, 12).

Due to the involvement of estrogens in an increasing number of pathologies (13, 14), the study of receptor regulation may have relevant pharmacological and therapeutical consequences. To date, a limited number of studies have addressed the mechanisms of ER regulation, which remain elusive. It is known that ER intracellular content may be regulated by selected hormonal signals (*e.g.* the heterologous down-regulation induced by progesterone) (15). In the majority of animal cells and tissues studied, the ER concentration is under a control primarily exerted by estrogens through a process termed autologous down-regulation or autologous up-regulation depending on the tissue being considered (16–18). The experimental evidence obtained to date seems to indicate that in cells of cancer origin, the activated ER is an integral part of this regulatory process. Saceda *et al.* (19) showed that at least part of the autologous down-regulation is associated with a decrease in the cell content of ER messenger RNA (mRNA); this, in turn, results from a decreased ER gene transcription rate in a process that is cycloheximide resistant and hormone concentration dependent. The model devised to explain these findings invokes an interaction of the ER with its own gene, similar to the mechanism by which this same transcription factor regulates any other target gene (20, 21). The activated ER, therefore, would bind selected regions in the pro-

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moter of its own gene, preventing its further transcription. The promoter of the ER gene, however, lacks the canonical ERE; this suggests that a novel mechanism of ER-DNA interaction may be involved.

To study the effect of estrogen on ER turnover, we used antisense oligonucleotides (aODN) (22, 23) as specific blockers of ER mRNA translation (24–26). It is well known that many variables are involved in the mechanism of action of aODN, and systematic studies on these variables in eukaryotic systems are still lacking; it is, then, difficult to predict the best targets for aODN. To select the most active oligos, a series of molecules was synthesized and tested in a cell line expressing ER (MCF-7 cells). Contrary to our expectations, none of the antisense oligonucleotides used was able to block ER synthesis, whereas two of them caused a significant increase in the ER protein content. In addition, ER up-regulation was observed with oligos complementary (sense) to the aODNs tested.

These results indicated that the physiological machinery responsible for maintaining the intracellular ER levels could be offset by the presence of selected DNA sequences. This prompted us to further investigate the mechanisms underlying ER up-regulation mediated by aODNs.

RESULTS

Selected Sense and Antisense Oligonucleotides Increase ER Protein Content in MCF-7 Cells

Four antisense molecules corresponding to translated and untranslated 5′ regions of the ER mRNA (Fig. 1A) and their complementary counterparts (sense) were tested in MCF-7 cells. The sequences selected for the initial studies were as follows: oligo1 (nucleotides −117/−99), GAC ATG CGC TGC GTC GCC; oligo2 (nucleotides 495/512), GCC AGT ACC AAT GAC AAG; oligo3 (nucleotides 1140/1157), TGT GCC TGG CTA GAG ATC; and oligo4 (nucleotides 4134/4151), GGG TCA GTG GGT TCT TTT. Care was taken to avoid homologies between the chosen sequences and other mRNAs deposited in EMBL and GenBank databases (with special attention to the mRNAs of the receptor superfamily members).

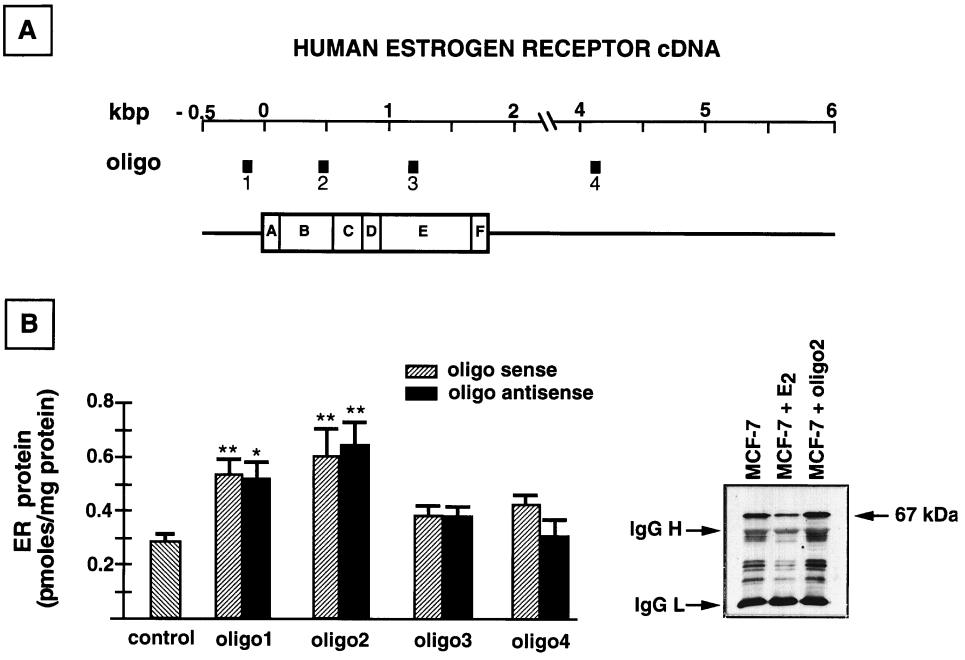

Fig. 1. Effects of Antisense and Sense Oligonucleotides on ER Protein Content in MCF-7 Cells

A, ER cDNA target sequences: oligo1 (nucleotides −117/−99), GAC ATG CGC TGC GTC GCC; oligo2 (nucleotides 495/512), GCC AGT ACC AAT GAC AAG; oligo3 (nucleotides 1140/1157), TGT GCC TGG CTA GAG ATC; and oligo4 (nucleotides 4134/4151), GGG TCA GTG GGT TCT TTT. B, *Left*, ER content was measured by EIA in extracts of MCF-7 cells treated for 48 h with 1 nM E_2 in the absence or presence of each of the oligos listed in A (14 μM, final concentration). The *bars* represent the mean ± SEM of a minimum of eight independent experiments performed in duplicate. *, $P < 0.05$; **, $P < 0.01$ (*vs.* controls, by ANOVA followed by Fisher's test). B, *Right*, Oligo2-induced ER up-regulation as assessed by Western blotting. MCF-7 cells were treated for 48 h with either 10 nM E_2 or 14 μM ss_{oligo2}. Three hundred micrograms of whole cell extracts were immunoprecipitated and then run on SDS-PAGE (10% T) and analyzed by Western blotting using the anti-human ER antibody H_{222}. *Arrows* indicate the position of the 67-kDa ER polypeptide as well as that of the heavy (H) and light (L) chains of IgG from the immunoprecipitation step. The autoradiograph shows results representative of three separate experiments.

The activity of the oligos on ER synthesis was determined by enzyme immunoassay (EIA) quantitation of ER protein content in cell lysates after 48-h treatment with 14 μM oligonucleotides. Figure 1B, *left*, shows that in MCF-7 treated with oligo1 and oligo2 (both sense and antisense), the ER content was 73–107% higher than that in untreated control cells. Oligo3 and oligo4 did not modify the ER concentration with respect to that in controls.

Western blot analysis using the H_{222} antibody as a probe (Fig. 1B, *right*) confirmed the increase in ER protein in MCF-7. Forty-eight hours after oligo2 treatment (*right lane*), the intensity of the specific 67-kDa ER band (*arrow*) was 50–70% higher than that in controls (*left lane*). After exposure to E_2 (*middle lane*), its intensity was 30–40% lower than that in controls. As the same amount of protein was subjected to immunoprecipitation, the strongly reactive IgG bands (*arrows*) may be regarded as loading and blotting controls. A number of bands, of lower M_r, were detected in control and treated cell lysates. These may correspond to either cross-reacting components or intermediates in endocellular ER catabolism. The slight discrepancy in the extent of up-regulation, as quantitated by EIA or by densitometric scanning of Western blots, was attributed to both the different sensitivities of the two techniques and to the fact that the densitometric analysis had been restricted to the 67-kDa band.

To explain the ER intracellular accumulation caused by oligo1 and 2, we ruled out the hypothesis that this could occur via an interaction of the active oligos with the ER mRNA, reasoning that both sense and antisense had the same effect; therefore, a direct pairing with the mRNA sequence could not have occurred. An alternative explanation was provided by recent reports which postulated that intracellular receptors can control the transcriptional activity of their own genes by interacting with negative hormone-responsive elements located in the promoter or within the gene-coding sequence (20). If this were the case, the paradox effect observed with the oligonucleotides could have been due to the squelching of ER from the promoter of its own gene.

The observation that oligos promoting ER accumulation blocked E_2-induced down-regulation, whereas the inactive oligos did not have any influence on the autologous down-regulation event (as shown in Fig. 2), further indicated that the oligos hindered the physiological mechanisms of ER autologous down-regulation.

Regardless of the represented strand (sense or antisense), only oligos corresponding to the 5′-region of the ER complementary DNA (cDNA) were found to up-regulate ER. Thus, it was conceivable that this region was primarily involved in ER autologous down-regulation. To test this possibility, we studied E_2-induced down-regulation in cells transiently transfected with mutants of the mouse ER cDNA. COS-1 cells were, therefore, transfected with the full-length mouse cDNA (MOR 1–599), an in-frame deletion mutant of the mouse ER cDNA lacking the activation domain AF-1

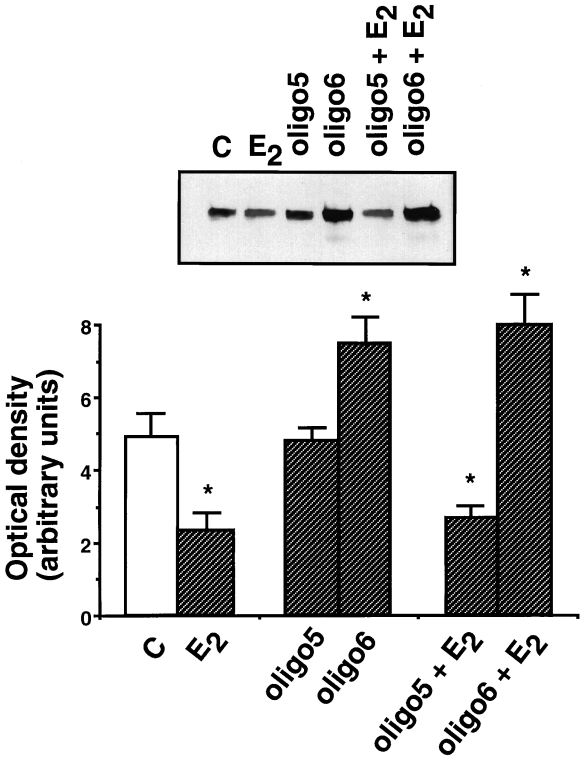

Fig. 2. Blockage of Estrogen-Induced Down-Regulation of ER by Oligos

MCF-7 cells were treated for 48 h with 10 nM E_2, 14 μM oligo, or both hormone and oligo. The sequences of the oligos were homologous to different regions of the 5′-end of the ER cDNA as follows: oligo5 (nucleotides 302/320), GTG TCT CCG AGC CCG CTG; and oligo6 (nucleotides 539/557), TGG ACA GTA GCG AGT CAG. ER protein levels were determined by autoradiographic scanning of films of four separate Western blots performed as described in Fig. 1. The autoradiograph shows a representative experiment. The *bars* represent the mean ± SEM.

(MOR 182–599) and the DNA-binding domain mutant C241A/C244A, which is transcriptionally inactive (27) but, in our hands, binds DNA. As expected, E_2 induced decreases in ER mRNA (−56%; Fig. 3B) and protein (−42%; Fig. 3A) when the full-length ER cDNA was transfected. However, with the ER mutant lacking the NH_2-terminus region (MOR 182–599), E_2 did not affect ER synthesis. This observation further underscored the hypothesis of a role in ER down-regulation of the cDNA sequences encoding residues 1–182 of the receptor.

Interestingly, E_2 induced down-regulation of the transcriptionally inactive ER mutant C241A/C244A of the DNA-binding domain, and this effect was of the same magnitude as that seen with the wild type receptor.

To further investigate the mechanism of aODN-dependent up-regulation, a series of experiments was undertaken with the most active of the oligos tested, oligo2.

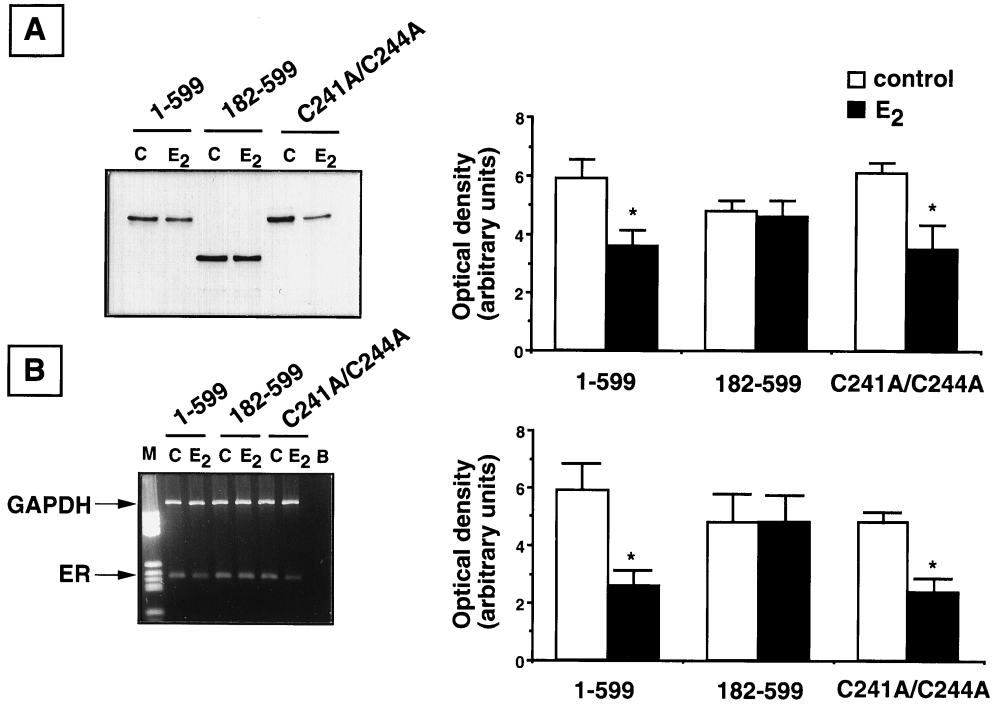

Fig. 3. Effects of ER Deletions on the Autologous Down-Regulation of ER mRNA and ER Protein

COS cells were transfected with 0.5 μg/ml of plasmids containing either the wild type ER cDNA (MOR 1–599) or two mutated forms, MOR 182–599 and MOR C241A/C244A. After treatment of the cells with 1 nM E_2 for 48 h, the ER protein and ER mRNA levels were measured as described in *Materials and Methods*. A, *Left*, Representative immunoblot developed with the anti-ER H_{222} antibody. The M_r of the stained bands are 67 kDa for 1–599 and C241A/C244A, and 47 kDa for 182–599. A, *Right*, Densitometric analysis of ER protein levels after normalizing the data for βgal activity. B, *Left*, Agarose gel of the ER-RT PCR amplified DNA stained with ethidium bromide. The M_r marker is *Hae*III-cut pBR322 DNA. The size of the amplification products is 600 bp for GAPDH and 218 bp for ER. B, *Right*, Densitometric analysis of the amplified product levels after normalization with the GAPDH, which was used as an internal control. Data are expressed as the mean ± SEM of three separate experiments. *, $P < 0.01$ *vs.* controls, by ANOVA followed by Fisher's test.

Gel Mobility Assay Argues for a Direct Interaction between ER and Oligo2

We first assessed by electrophoretic gel shift assay (EMSA) whether a direct interaction between oligo2 and ER protein could occur. The oligo2 retardation on gel, induced by ER, was compared with that of the canonical target for ER, the ERE. All of the studies were performed using MCF-7 nuclear extracts as the source of ER.

With oligo2 (Fig. 4; A, antisense; B, sense; C, double strand), we observed several retarded bands; only one of these was competed off by cold oligo2 (*arrow*). When other oligos, such as those previously shown not to interfere with ER accumulation (*e.g.* oligo4) or scrambled oligos, were used, none of the retarded bands could be competed off (data not shown). We, therefore, concluded that the binding between ER and oligo2 (sense and antisense) was specific.

The binding affinities of ER for single (ss) and double (ds) stranded oligo2 were evaluated by saturation analysis, as described in *Materials and Methods*. The data in Fig. 4 were obtained by plotting the integrated areas from the densitometric scanning of the relevant bands according to Ligand (28). The ds oligo2 bound ER with higher affinity than the ss oligo2 (ds_{oligo2}: K_d = 42 nM; $sense_{oligo2}$: K_d = 139 nM; $antisense_{oligo2}$: K_d = 423 nM). In all cases, however, the K_d values for oligo2-ER interaction were significantly lower than those for ds_{ERE}-ER (1.6 nM; Fig. 4D).

To prove that the shifts of ^{32}P-labeled oligo observed were indeed due to the binding to ER, we checked whether the band corresponding to the oligo-receptor complex was supershifted in the presence of the specific anti-ER H_{222} antibody. As shown in Fig. 5, with both ds_{oligo2} and $sense_{oligo2}$ the antibody caused a supershift of the complex comparable to that observed with ER-ERE. With $antisense_{oligo2}$ we observed a smear of the retarded band, probably due to low affinity within the ER-$antisense_{oligo2}$ complex. The inactive oligo, oligo5, was not shifted in the presence of ER. To further prove that ER was responsible for the supershift reported above, we also tested nuclear extract from COS-1 cells, transfected or not with ER cDNA. No retarded band could be observed with extracts of COS-1 cells not expressing ER (see Fig. 8).

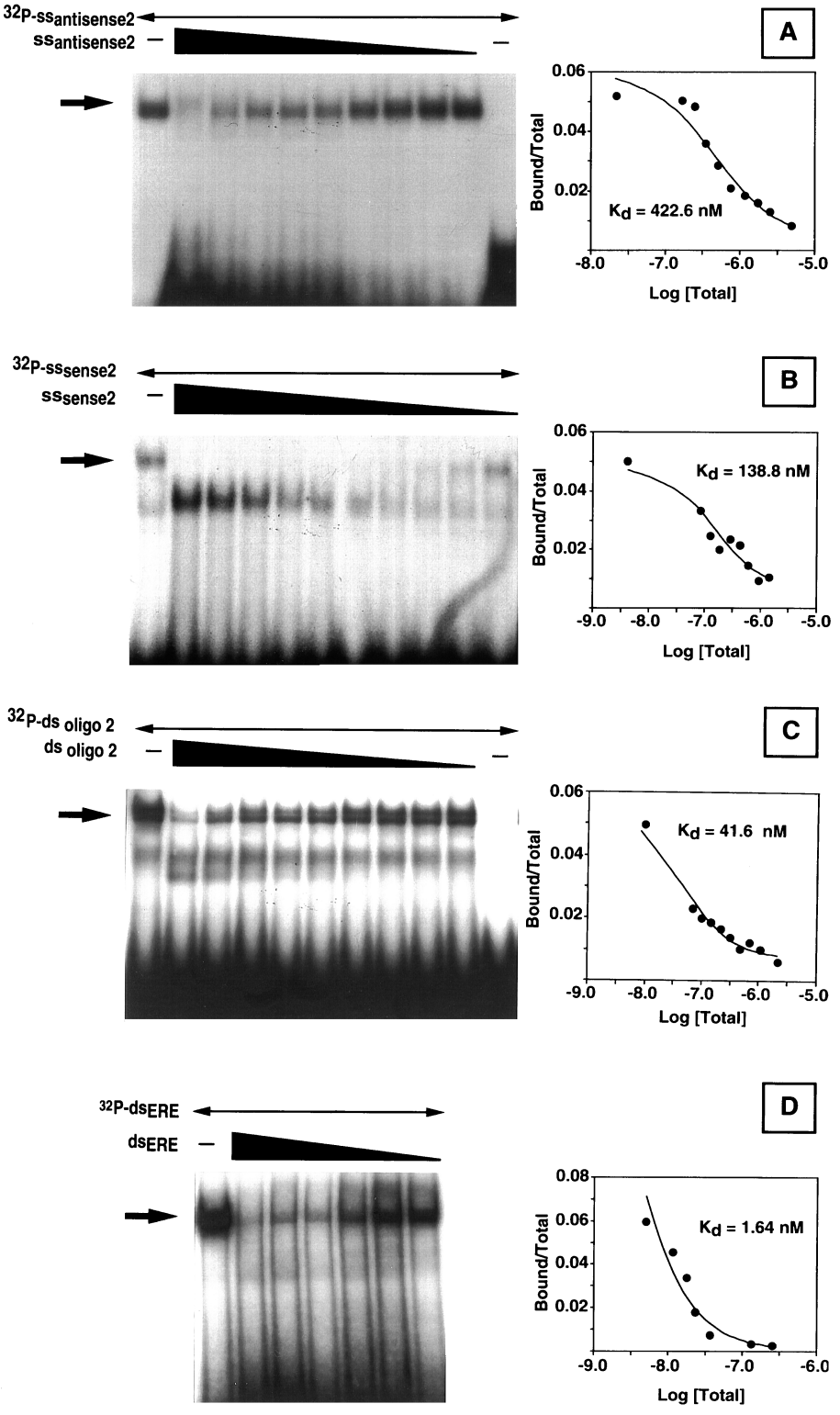

Fig. 4. Affinities of Oligo2 and ERE for ER as Assessed by EMSA

Nuclear extracts were incubated with ^{32}P-labeled $ss_{antisense2}$ (A), ss_{sense2} (B), ds_{oligo2} (C), and ds_{ERE} (D) in the absence (−) or presence of decreasing concentrations of the corresponding unlabeled oligo (from 1 μM to 4 nM). *Arrows* indicate the bands competed off by the unlabeled oligo. The time of exposure to the autoradiography films varied between 8 h (ER-ERE) and 3 days (ER-oligos). The exposure time was selected to ensure that the signals were within the range of linearity for the densitometric scanning. The binding parameters were calculated with the program Ligand (28). The [Total] concentration represents the total amount of labeled and unlabeled oligo in the incubation mixture. The homologous competition curves were plotted according to the Ligand program. This experiment was repeated three times with different receptor preparations, with superimposable results.

Estrogens Do not Repress Transcription of the p-oligo2-GL2B-Luciferase Reporter Gene

To assess whether the interaction between ER and oligo2 resulted in transcriptional modulation, the oligo2 sequence was cloned in the promoterless luciferase vector pGL2B, which was analyzed in the ER-negative HeLa cells by cotransactivation assay with or without cotransfected ER. Figure 6 shows that with the empty pGL2B promoter, in the presence of E_2-activated ER, the luciferase activity (LUC) was very low and not significantly higher than that in cells not expressing ER. The promoter containing oligo2 had over 2-fold higher basal transcription activity, and the presence of the unliganded receptor doubled LUC. The increased LUC due to ER presence, however, could not be modulated by E_2 (at high concentration, 100 nM). Similar results were obtained with transfection of empty or oligo2-containing pGL2B in MCF-7 cells (data not shown). In these ER-expressing cells the accumulation of LUC was almost 5-fold higher in oligo2-pGL2B- than in pGL2B-transfected cells. Also in this case, the presence of 100 nM E_2 did not cause any further accumulation of the reporter protein.

These results suggest that ER may bind oligo2, causing increased transcription of its downstream genes; this interaction, however, differs from the ER-ERE interaction, which is characteristically sensitive to the presence of the hormone (Fig. 6, *right panel*). It is worth stressing that these findings do not comply with the view of oligo2 representing a negative response element.

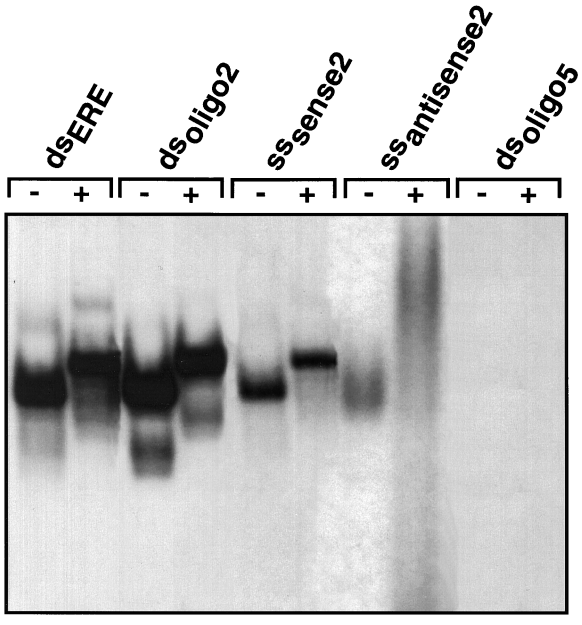

Fig. 5. Supershift of the ER-Oligonucleotide Complexes

Equal amounts of MCF-7 nuclear extracts were tested in a supershift assay using DNA probes consisting of ^{32}P-labeled ds_{ERE}, ds_{oligo2}, ss_{sense2}, $ss_{antisense2}$, and ds_{oligo5} in the absence (−) or presence (+) of monoclonal antibody H_{222}.

Oligo2-ER Complex Migration across a Transverse Urea Gradient

The experiments described above proved the specific interaction of both ss- and ds_{oligo2} with ER. We further compared the characteristics of oligo2-ER and ERE-ER interaction by investigating the electrophoretic behaviors of these complexes under denaturing conditions.

The migration across a transverse urea gradient, as introduced by Creighton (29) correlates the unfolding of a protein molecule to a reduction in its electrophoretic mobility. The typical transition from the high to the low mobility form occurs over a defined interval of the chaotrope concentration, and the overall curve displays a sigmoidal shape. This technique may be applied to the study of interacting systems; different

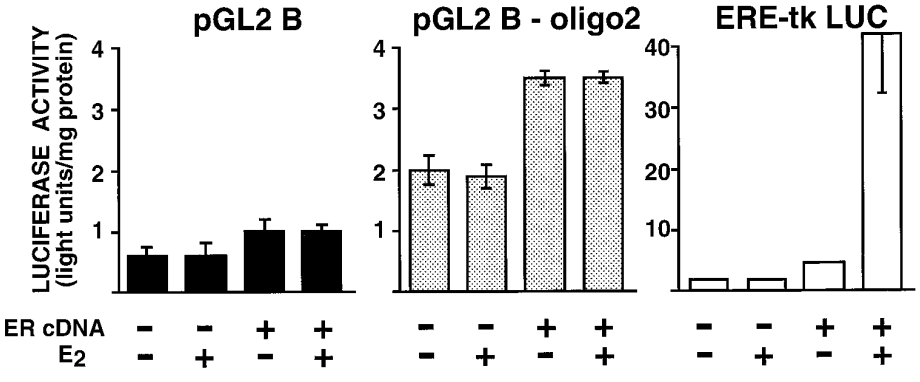

Fig. 6. Effect of Transient Transfection of ER on the Transcription of a Promoter Driven by Oligo2 or ERE

HeLa cells were transfected with the ER expression vector (1 μg) or mock plasmid together with either pGL2B plasmid or the same plasmid containing the oligo2 sequence (1.5 μg). As a control for transcription efficiency, all samples were cotransfected with 0.5 μg pCMV-βgal. Assays of luciferase and βgal were performed as described in *Materials and Methods* 48 h after addition of the hormone (100 nM E_2). All transfections were performed in triplicate. *Bars* represent the mean ± SEM of two separate experiments.

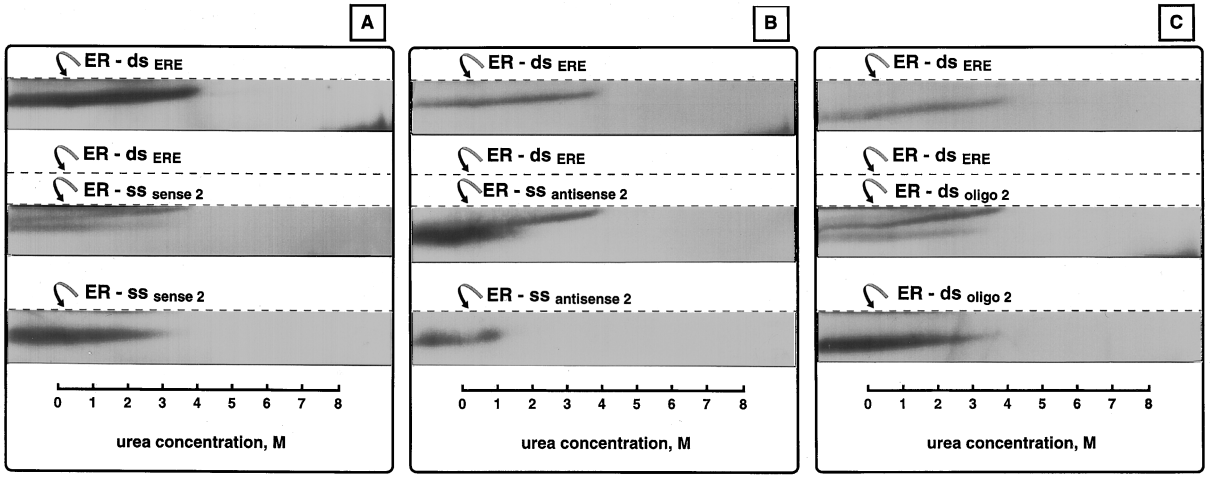

Fig. 7. Electrophoresis across a Transverse Urea Gradient (0–8 M) of [^{32}P]Oligo2 and [^{32}P]ERE Complexes with ER

In each panel the behavior of a given oligonucleotide (ss_{sense2} in A; $ss_{antisense2}$ in B; ds_{oligo2} in C) is compared with that of ds_{ERE}. The *top sections* show the dissociation/denaturation curve of ds_{ERE}-ER. The *bottom sections* show the curves for the various oligo2 forms complexed with ER. The *middle sections* correspond to gels in which ds_{ERE}-ER and each specific oligo2-ER complex were loaded in parallel troughs. The positions of application trenches are indicated by the *dashed lines*.

profiles of migration are observed depending upon the homo- or heteromultimeric nature of the complex (30). We, therefore, ran a series of experiments aimed at studying the behavior under denaturing conditions of the ss- and ds_{oligo2} as well as ds_{ERE} once complexed with ER.

Panels A–C in Fig. 7 compare the profiles of migration across increasing urea concentrations of ER-ds_{ERE} and the complexes between ss- and ds_{oligo2} with ER. The end of the radioactive tracing corresponds to the urea concentration at which ^{32}P-labeled oligo and protein dissociate. This occurs at urea concentrations of about 4 M for [^{32}P]ds_{ERE}-ER, 3 M for both [^{32}P]ss_{sense2} and [^{32}P]ds_{oligo2}-ER, and around 1 M for [^{32}P]$ss_{antisense2}$-ER. These figures prove the interaction between ds_{ERE} and ER to be much stronger than that between oligo2 and ER, in agreement with the K_d values calculated by EMSA.

Further examination of the dissociation/denaturation curves of the three forms of ER-oligo2 complex shows a migration profile parallel to the application trench (Fig. 7, A–C, *bottom*). This implies that receptor-DNA dissociation occurs before any appreciable unfolding of the molecule. On the contrary, the migration of the ER-[^{32}P]ds_{ERE} complex slows down around 0.5 M urea (A-C, *top*), indicating that some unfolding occurs (presumably outside the receptor domain binding [^{32}P]ds_{ERE}) before the complex dissociates. The unfolding of the ER molecule is thus likely to occur stepwise. Under the selected experimental conditions, however, we cannot actually monitor the behavior of ER, but can do so for the DNA-ER complex, although only up to the point of its dissociation.

In the unfolding assay the various domains within the ER molecule appear to behave independently. These data, therefore, support the view that [^{32}P]ds_{ERE} and oligo2 interact differently with the ER molecule. Moreover, the various domains appear to be differentially stabilized by the binding of their ligands. Examples of both features (ligand stabilization and stepwise unfolding) have been previously described for model systems (30–34).

Both the effective urea concentrations and the peculiar shapes of the dissociation/denaturation curves were evaluated in independent replicas of each run, and the behavior of each form of oligo2 was compared with that of ER-ds_{ERE} in experiments (*middle section* of A–C) in which the reference and the test complexes were migrated in the same gel from two parallel trenches.

Evaluation of the Size of the ER [^{32}P]ds_{oligo2} Complex

The data accumulated to date show that ds_{oligo2} and ERE interact with ER differently. To determine whether accessory proteins were participating in the establishment of the oligo2-ER complex, we determined the size of the ER-oligo2 complex according to the method of Ferguson (35). Samples were run in gels of different polyacrylamide concentrations (T%) along with M_r markers, as described in *Materials and Methods*. The larger the protein size, the more pronounced was the decrease in protein mobility due to the increasing sieving of the polyacrylamide matrix. The $\log_{10}$ of protein mobility was plotted against the corresponding gel concentrations, and the slope of the resulting straight line, defined as K_R, was computed. The molecular size of the test sample, inversely proportional to K_R, may be estimated by comparing its mobility to that of a series of standards (Fig. 8).

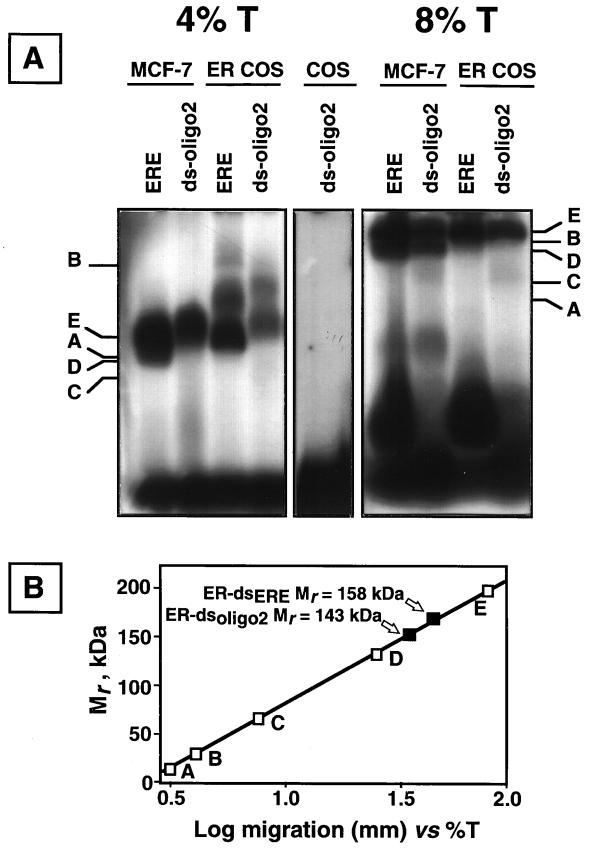

Fig. 8. Evaluation of the Molecular Sizes of the Complexes between ER and ds_{oligo2} by Analysis of the Electrophoretic Migration Data according to the Method of Ferguson

Nuclear extracts from MCF-7 and ER-COS cells were run after incubation with either [^{32}P]ds_{ERE} or [^{32}P]ds_{oligo2}. A, Comparison of their migration in 4% T (left) and 8% T (right) polyacrylamide gels. In the *middle*, a COS-1 nuclear extract is shown as a negative control. The *horizontal ticks* mark the positions of the molecular size markers: A, α-lactoglobulin; B, carbonic anhydrase; C, BSA monomer; D, BSA dimer; and E, BSA trimer. In B, the molecular sizes of the complexes are assessed by plotting, according to the method of Ferguson, M_r *vs.* retardation coefficients (slope of the curve correlating $\log_{10}$ migration with the percentage of T in the polyacrylamide matrix).

Extracts of COS-1 transfected with ER-cDNA (ER-COS) or MCF-7 used as a source of ER were incubated with either [^{32}P]ds_{ERE} or [^{32}P]ds_{oligo2}. The analysis of both cell lines allowed cross-checking of the molecular size of the ER complexed with each oligo in cells constitutively expressing the hormone receptor (MCF-7 cells) or overexpressing its exogenous cDNA (COS-1 cells).

In untransfected COS-1 cells no retarded band could be observed (Fig. 8, *middle section*). With extracts from ER-COS and MCF-7, the size of the ER-ds_{ERE} complex was evaluated at 158 kDa, whereas that of the ER-ds_{oligo2} complex corresponded to 143 kDa (Fig. 8B). The difference in size between the two complexes is accounted for by the different lengths of the two test oligonucleotides (33 nucleotides for ds_{ERE} *vs.* 18 nucleotides for ds_{oligo2}).

These figures, therefore, indicate that both oligonucleotides bound either the dimeric form of the activated ER or its monomeric form plus an accessory protein with a M_r around 67 kDa; no sign of different migrations of the complexes from the two cell lines could be detected.

DISCUSSION

The present study stems from the observation of a paradoxical increase in ER content in MCF-7 cells treated with oligonucleotides aimed at blocking ER mRNA translation. Our results are in agreement with those of a previous study by Williard *et al.* (36), in which ER activity was demonstrated to be significantly increased in cells expressing antisense ER RNA.

It could be hypothesized that the effects observed in the presence of oligonucleotides are linked to the mechanism of ER autologous down-regulation, which, according to Kaneko *et al.* (20) and Burnstein *et al.* (21), occurs via a transcriptional repression exerted by estrogen (or glucocorticoid) receptors on the ER (or glucocorticoid receptor) genes. This effect would be mediated by specific DNA sequences, acting similarly to the negative response elements well characterized for glucocorticoid and thyroid hormone receptors (37). In this perspective, the oligos we describe would compete with the target gene for ER, thereby decreasing the efficacy of ER repression. In agreement with this hypothesis is the finding that ER specifically binds oligos 1 and 6 (data not shown) and oligo2, which are active in determining the receptor up-regulation, and it does not interact with the inactive oligos (oligos 3, 4, and 5). However, a number of other observations argue against the hypothesis of ER acting as a repressor: 1) the oligos determining ER up-regulation have a sequence quite dissimilar from one another and certainly very different from any ERE described to date; 2) autologous down-regulation of the ER was observed with the ER mutant C241A/C244A, which is transcriptionally inactive; 3) the activity of a reporter gene controlled by a promoter containing oligo2 failed to show any negative transcriptional activity of ER. Indeed, the transcription from this promoter was slightly increased in the presence of ER and was hormone independent. ER, therefore, seems to modulate the transcription of its own gene via an alternative mechanism.

The gel shift analysis carried out under denaturing and nondenaturing conditions suggests that ER interacts differently with oligo2 and ERE. The migration across the urea gradient clearly shows that the binding to ds_{oligo2} prevents the change in ER conformation observed at about 0.5 M urea with the

ER-ERE complex. In addition, ERE competes for binding to the receptor with all forms of oligo2, while the opposite does not apply to oligo2, even for very high concentration ratios (up to a 10,000-fold molar excess; data not shown). It is, therefore, conceivable that oligo2 and ERE do not bind the same ER domain; however, the binding to ERE hinders the oligo2-binding site.

On the basis of what we described above, it could be proposed that autologous down-regulation of ER occurs via a novel mechanism of receptor-DNA interaction that is unrelated to the mechanism of hormone-dependent transcriptional repression. This is further supported by the fact that full antagonists of the ER receptor, such as ICI 182,780, which allows the formation of transcriptionally unproductive ER-ERE complexes, are extremely efficient in causing ER down-regulation (38, 39).

The fact that ER uses two different mechanisms of DNA interaction to induce the transcription of target genes and the repression of its own is not totally surprising. It is quite possible that, once activated by the cognate hormone, ER binds the responsive elements to modulate the transcription of the target genes, which are then inactivated by still undescribed modifications. In this transcriptionally inactive form, however, the receptor could acquire the capability to interact, directly or via specific adapter proteins, with its own gene to hinder its own transcription. Supporting this view is the observation that the phenomenon of down-regulation requires a longer time than transcription to start. Our study did not allow us to prove that accessory proteins take part in binding of the ER to oligo2; in the migration study performed according to the method of Ferguson (35), no distinguishable difference in size for the complexes ER-oligo2 and ER-ERE was observed regardless of the ER protein source (MCF-7 or COS-1 transfected with ER). We hypothesize, therefore, that a homodimer of the receptor is responsible for binding the ER gene; however, we cannot rule out the hypothesis that in the complex with oligo2, ER heterodimerizes with a protein with a M_r very close to 67 kDa. Other reports on steroid receptors (estrogen or progesterone) suggest that they can modulate transcriptional events in a conformation that does not require the presence of the DNA-binding domain (40, 41).

A better understanding of this phenomenon might have important pharmacological consequences, as certain drugs could be devised to induce the specific conformation aimed at carrying out a selected task.

The squelching assay with short oligonucleotide sequences homologous to the 5′-segment of the ER gene here described can be used to identify the targets of ER in autologous regulation and might be relevant for clarification of the mechanism of ER activity.

MATERIALS AND METHODS

Chemicals

Oligodeoxynucleotides were synthesized by Pharmacia Biotech (Cambridge, UK). E_2 was purchased from Sigma Chemical Co. (St. Louis, MO). Cell culture reagents were obtained from Flow Laboratories (Irvine, Scotland), Merck (Darmstadt, Germany), and Life Technologies (Madison, WI). All other reagents were molecular biology grade from Boehringer Mannheim (Mannheim, Germany) and Merck unless otherwise specified. Plasticware was obtained from Corning (Corning, NY). RPMI 1640 and DMEM were purchased from Sigma and Life Technologies (Paisely, UK), respectively. FBS was obtained from Imperial (Unipath, UK). The expression vector containing the human ER cDNA was provided by G. Greene (University of Chicago, Chicago, IL) (42). The pUHC-β-galactosidase (pUHC-βgal) was obtained from Promega. ER mutants (38) were a gift from M. Parker.

Cell Culture

MCF-7 and COS-1 cells were grown in RPMI 1640 without phenol red (Sigma, Milan, Italy) supplemented with 10% charcoal-stripped FBS (DCC-FBS) (43). The cells were grown in 100-mm petri dishes containing 10 ml culture medium. About 1×10^6 cells were seeded/dish. The medium was replaced every 4 days. Once a week, at semiconfluence, the cells were split by treatment with trypsin (0.5 g/liter trypsin and 0.2 g/liter EDTA) for 5 min at 37 C and resuspension in fresh medium at a 1:20 dilution.

To test aODN activity, the cells were plated in 2.5-cm wells (0.3×10^6 cell/well) and grown in phenol red-free medium supplemented with 10% DCC-FBS. To limit the degradation of the oligonucleotides by serum nucleases, the FBS was heat inactivated at 65 C for 45 min (44). As some degradation still occurred, as evaluated by PAGE, 14 μM nucleotides, dissolved in water, were added every 24 h. MCF-7 cells were treated in parallel with several oligonucleotides for 48 h. E_2 was diluted in phenol red-free RPMI at a final concentration of 10^{-8} M/0.01% ethanol; 0.01% ethanol was added to controls. ER quantitation was performed in duplicate on high salt buffer extracts as previously described (26). ER content was quantified immunoenzymatically (ER-EIA kit, Abbott Laboratory, North Chicago, IL) and normalized for the protein concentration, as evaluated by Bradford's method (45).

Transient Transfection Assay

E2-Induced Down-Regulation in COS Cells Transfected with ER Twenty-four hours before transfection, 2×10^5 cells were plated in 2.5-cm wells containing 3 ml phenol red-free RPMI 1640 medium supplemented with 10% DCC-FBS. Six hours before addition of the $CaPO_4$-DNA mixture, the medium was replaced with DMEM with 10% DCC-FBS containing 1 nM E_2 or its solvent. In a typical experiment, the cells were transfected using the following DNA concentrations: 200 μl of a suspension of 0.05 mg/ml ER cDNA (or its mutants), 0.05 mg/ml of the gene pUHC-βgal (as control for transfection efficiency), and 0.12 mg/ml carrier DNA (pGEM3z) in 1.8 ml medium. Sixteen hours after addition of the precipitate, the medium was discarded and, after a few washes with RPMI 1640, replaced with phenol red-free RPMI 1640 containing 1% DCC-FBS (with or without E_2). Forty-eight hours later, the medium was removed, the cells were washed several times with PBS, and cell extracts were prepared according to the method of Patrone *et al.* (46). βGal activity was measured as previously reported (47). The protein content was measured according to the method of Brad-

ford (45). Each experiment was repeated at least three times on duplicate samples.

Assessment of ER Transactivation Activity of oligo2-pGL2B For plasmid construction, the oligo2-pGL2B-luciferase reporter was generated by insertion of ds_{oligo2} into the *Sma*I site of the pGL2B plasmid (Promega).

Transfections Cells were plated at high density, as described above (HeLa cells were grown in Modified Eagle's Medium). Cells were transfected with 1.5 μg oligo2-pGL2B-luciferase (or pGL2B-luciferase), 0.5 μg pCMV-βgal, and 1 μg ER expression plasmid or mock plasmid. All transfections were performed in triplicate. After a 2-h transfection, the cells were washed, and fresh medium containing either 1 nM E_2 or its solvent was added. After 48-h incubation, luciferase and βgal were measured as previously described (48).

Gel Retardation Assay

The EMSA was performed as described by Bettini *et al.* (49). The oligonucleotides were labeled by incorporation of [γ-^{32}P]ATP (Amersham, Aylesbury, UK) with T_4 kinase (Promega) to a specific activity of about 10^8 dpm/μg DNA. The ^{32}P-labeled oligos ($\sim$10,000 cpm/lane) were incubated with the nuclear extract (5–8 μg protein) in the presence of 1 μg *Hae*III-cut pBR322 for about 15 min at 4 C before adding the competing cold nucleotides. The receptor concentration was chosen to ensure a ratio between the oligo bound and total oligo lower than 10% as required by Munson and Rodbard (28). The binding reaction was then carried to equilibrium with an incubation time of 20 min at room temperature. Addition of the DNA dye [50% (vol/vol) glycerol, 0.25% (wt/v) bromophenol blue, and 0.25% (wt/v) xylene cyanol] ended the reaction. The entire incubation mixture (10 μl) was loaded onto nondenaturing polyacrylamide gels (6% T and 2.5% C). The gels were run with TBE buffer (90 mM Tris-HCl, 90 mM boric acid, and 10 mM EDTA) for about 2 h. Gels were then dried with a gel dryer (Slab Dryer, Savant, Holbrook, NY) and exposed to x-ray film (Kodak RP, Eastman Kodak, Rochester, NY) for about 16 h. The gel retardation analysis was performed on several samples to which the same unlabeled competitor was added in various concentrations (4 nM to 1 μM) in the presence of a fixed concentration of [^{32}P]oligo. After densitometric scanning of the autoradiograph, the results were evaluated as homologous competition models by the program Ligand (to calculate the apparent affinity of ER for the DNA) (28).

Bandshift Assay

The labeled probes (10,000 cpm/lane) were incubated with 2 μg MCF-7 nuclear extract in a buffer consisting of 10 mM HEPES (pH 7.9), 100 mM KCl, 1 mM dithiothreitol, 0.5 mM EDTA, 2.5 mM $MgCl_2$, 6% glycerol, and 2% Ficoll. The reaction was allowed to proceed at room temperature for 10 min. The addition of the antibody (100 ng) was made before mixing with the labeled probe. The samples were subjected to electrophoresis (150 V at room temperature) in a nondenaturing 5% T polyacrylamide slabs in 0.5 $\times$ Tris-borate-EDTA for 4 h.

Electrophoresis across a Transverse Urea Gradient

The complexes between hormone-saturated ER and the four oligonucleotides were run across 0- to 8-M urea gradients according to the method of Creighton (29). The polyacrylamide matrix had a concentration of 6.5% T and 4% C in TBE buffer. The gels were cast against GelBond PAG foils (FMC, Rockville, MD) in a 0.5-mm thick cassette (18–1013-74, Pharmacia, Uppsala, Sweden); one or two parallel sample application trenches were shaped with 170 $\times$ 2.5-mm^2 strips of embossing tape glued to the glass plate. A 4-ml concentration plateau of the 8-M urea mix was poured first, followed by an 8-ml urea gradient delivered from a two-chamber mixer (18–1019-87, Pharmacia) and a further concentration plateau from the solution without urea. The gels were run in a horizontal electrophoresis chamber (Multiphor, LKB Pharmacia). To minimize radioactive contamination, according to the suggestion of Kleine *et al.* (50), 1% agarose strips in 4 $\times$ TBS (5 $\times$ 15 $\times$ 250 mm^3) were substituted for electrodic solutions and paper wicks. Under a constant voltage of 100 V/11 cm and at a temperature of 15 C, the run of a single sample lasted 80 min. When the behavior of a different oligonucleotide complex was compared with that of ERE, the latter was applied first in the more cathodal trench, whereas the second sample was loaded after a 30-min run, once the free oligonucleotide had migrated past the more anodal trench. The sample volume was 130 μl, containing 20 μg of nuclear proteins (in 20 μl), 75 μl EMSA buffer (48), 1 μg *Hae*III-cut pBR322 (in 1 μl), 2 μl [^{32}P]oligo to a specific activity of about 10^{-8} dpm/μg DNA, and 13 μl H_2O; the mixture was incubated for 30 min at room temperature, then added with 22 μl DNA dye [50% (vol/vol) glycerol, 0.25% (wt/vol) bromophenol blue, and 0.25% (wt/vol) xylene cyanol].

The Ferguson Plot

The molecular size of ER-oligonucleotide complexes was evaluated on polyacrylamide gels (4% and 8% T), cast in TBE buffer according to the method of Ferguson (34). The 250 $\times$ 125 $\times$ 0.5-mm^3 slabs, supported on GelBond PAG foils, were polymerized in halves at the two gel concentrations. Sample and marker proteins (α-lactalbumin, carbonic anhydrase, and BSA plus ovalbumin, which, however, exhibited an anomalous behavior in the presence of borate ions; all markers from Sigma Chemical Co., St. Louis, MO) were dissolved in high salt buffer; 7 μl were applied/lane, corresponding to 7 μg of the markers and 5–8 μg of the nuclear proteins. The gels were run at 15 C for 90 min in a Multiphor chamber (Pharmacia) at 100 V/11 cm. The marker proteins were stained with Coomassie blue R, whereas the ER-oligonucleotide complexes were detected by autoradiography.

Western Analysis of the ER Protein

Sample preparation was the same for MCF-7 and COS-1 cells, except that MCF-7 lysates were first subjected to an immunoprecipitation step, as previously reported (51). Thirty micrograms of proteins were immunoprecipitated, then denatured and loaded onto a 10% SDS-polyacrylamide slab gel. After electrophoretic migration, the proteins were transferred to Hybond-C Extra nitrocellulose (Amersham) in a Trans-Blot apparatus (Bio-Rad, Hercules, CA). Blots were stained with Red Ponceau (Sigma, St. Louis, MO) to assess the efficiency of protein transfer. The filters were saturated for 16 h with 5% milk proteins and 0.2% Tween-20 in TBS (50 mM Tris and 150 mM NaCl) before incubation with the anti-ER monoclonal antibody H_{222} (Abbott Laboratories, North Chicago, IL). Immunoreactive proteins were detected after incubation with a peroxidase-conjugated secondary antibody (rabbit anti rat-IgG antibody, Vector Laboratories, Burlingame, CA) through ECL reaction (Amersham) and exposure to Hyperfilm-MP (Amersham).

Semiquantitative Analysis of ER mRNA by reverse transcription-PCR

Total cell RNA was isolated with the Bio/RNA-X Cell kit (Bio/Gene, Kimbolton Cambs, UK) using 1 ml RNA-X reagent for 5–10 $\times$ 10^6 cells. One microgram of RNA was reverse transcribed using oligo(deoxythymidine)$_{12-18}$ and Moloney murine leukemia virus reverse transcriptase (HT Biotechnology, Cambridge, UK) as previously described (46). One tenth of the reaction of cDNA was amplified in a 100-μl mixture con-

taining 2.5 U DynaZyme-DNA polymerase (Finenzyme OY, Espo, Finland), the buffer provided by Finenzyme, 0.2 μM deoxy-NTP, and 100 pmol PCR primers, the 18-mers 5′-AGC GTG TCT CCG AGC CCG-3′ and 5′-TGC ACA GTA GCG AGT CTC-3′ for human ER. Fifty picomoles of a set of primers for the constitutively expressed enzyme GAPDH (the 24-mers 5′-CCA CCC ATG GCA AAT TCC ATG GCA-3′ and 5′-TCT AGA CGG CAG GTC AGG TCC ACC-3′) were used in each reaction as an internal control for the amount of mRNA transcribed and amplified. After denaturing at 94 C for 2 min, PCR amplification was performed for 32 cycles (94 C for 15 sec, 48 C for 20 sec, and 72 C for 30 sec), followed by a final extension step (72 C for 3 min). The duration and temperature of the PCR cycles were experimentally optimized to fall into the exponential phase of the amplification (52).

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Address requests for reprints to: Dr. A. Maggi, Center Milan Molecular Pharmacology Laboratory, Institute of Pharmacological Sciences, University of Milan, I-20133 Milan, Italy. E-mail: maggia@isfunix.farma.unimi.it.

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