Transcriptional Control of Estrogen Receptor in Estrogen Receptor-negative Breast Carcinoma

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Abstract
The mechanisms which control estrogen receptor (ER) expression in breast carcinoma have not been elucidated. The MCF-7 (ER-positive) and MDA-MB-231 (ER-negative) breast carcinoma cell lines have been used to examine ER gene expression. Northern blot analysis of mRNA isolated from these two cell lines demonstrates that MCF-7 cells express an expected 6.5-kilobase ER mRNA whereas MDA-MB-231 do not make detectable ER message. Reverse polymerase chain reaction, which offers greater sensitivity, confirms these Northern blot results. The nuclear run-on assay was used to examine directly transcription in these two cell lines. MCF-7 cells actively transcribe the ER gene whereas no ER transcription is detected in MDA-MB-231. Southern analysis of genomic DNA confirms that the ER gene is present in MDA-MB-231. We conclude that the ER gene is controlled transcriptionally in this ER-negative breast carcinoma line.

Introduction
ER expression is intimately associated with the biology of breast carcinoma. However, little is known about mechanisms which control ER expression. The cDNA for ER was originally cloned from the MCF-7 breast carcinoma cell line (1, 2). The receptor is an inducible trans-activator which becomes functional when bound to estradiol (3). The gene for ER has been localized to chromosome 6q24-27 (4). The ER gene has not been defined. The central focus of these experiments is to determine the mechanism for the lack of expression of ER in certain breast cancers. Two breast carcinoma cell lines were used in these experiments both of which were derived from malignant effusions. The MCF-7 cell line was used as a breast cancer line which has retained ER expression (8). MDA-MB-231 cells were used as a breast cancer line which lacks receptor expression.

Materials and Methods
Cell Lines. All cell lines were obtained from American Type Culture Collection, Rockville, MD. Cells were maintained in minimal essential medium (Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal bovine sera (Hyclone, Logan, UT), 25 mm 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 26 mm sodium bicarbonate, 5000 units/ml penicillin G (Gibco BRL), 5000 μg/ml streptomycin (Gibco BRL), and 6 μg/ml bovine insulin (Sigma Chemical Company, St. Louis, MO). Cells were incubated at 37°C in 5% CO₂.

DNA Isolation and Analysis. The HEO plasmid containing the estrogen receptor cDNA was obtained as a gift from Professor Pierre Chambon, Strasbourg, France. Plasmid DNA was isolated by the alkaline lysis method and purified by precipitation with polyethylene glycol as described previously (6). Genomic DNA was analyzed by Southern blotting as described previously (6).

RNA Isolation and Analysis. Polyadenylated RNA was isolated using the Fast-Track RNA isolation kit (Invitrogen, San Diego, CA) according to the recommendations of the supplier. Total cell RNA was isolated from cell lines which were washed once with phosphate-buffered saline and then lysed in RNA lysis buffer (200 μl NaCl-100 μl Tris-HCl pH 7.5-50 ml EDTA-1% sodium dodecyl sulfate -200 μg/ml proteinase K) and incubated at 37°C for 45 min. The lysates were extracted with an equal volume of phenol:chloroform (1:1) and the nucleic acid was precipitated with 2.5 volumes of ethanol. DNA was digested in a DNase buffer containing 100 units/ml DNase I (Boehringer-Mannheim, Indianapolis, IN), 200 units/ml RNase inhibitor, and 5 mg MgCl₂ at 37°C for 60 min. The RNAs were extracted with phenol:chloroform, and RNA was ethanol precipitated, resuspended in water, and stored at -20°C.

Reverse PCR was accomplished using the RNA amplification kit (Perkin-Elmer, Norwalk, CT). Primers used for amplification across the first splice site of the ER gene are TACTGCATCAGATCCAGGG and ATCAAATGTG- CACTGGTTGG. Primers for amplification across the last splice site are GCACCTGAAATCTCGAGA and TGGCTAAAGTGGTGCATGAT. The primer pair used to amplify actin mRNA is GCAATGGAAGAAGAGATCGC and ACATGCGCCCGGTTGAGAG.

Nuclear run-on assays were performed as described previously (7). Nuclei were labeled at 30°C for 15 min using [α³²P]-UTP. Hybridizations were performed at 42°C in 50% formamide. Filters were stringently washed in 0.1× 150 mM NaCl, 15 mM Na Citrate standard saline-citrate 0.5% sodium dodecyl sulfate at 65°C and were treated with RNase A to further increase specificity.

Results
The central focus of these studies is to determine the mechanism for the lack of expression of ER in certain breast cancers. Two breast carcinoma cell lines were used in these experiments, both of which were derived from malignant effusions. The MCF-7 cell line was used as a representative line which has retained ER expression (8). MDA-MB-231 cells were used as a breast cancer line which lacks receptor expression (9). Cytoplasmic RNA was isolated from these two cell lines to determine if ER mRNA is synthesized. Fig. 1 shows that MCF-7 cells make an expected 6.5-kilobase mRNA which hybridizes to an ER cDNA probe whereas this mRNA was not detected in MDA-MB-231 cells. An identical blot probed with actin confirms the presence of intact mRNA in both samples.

The lack of detectable ER mRNA in MDA-MB-231 cells could be due to a lack of transcription, abnormal processing of primary transcript, degradation of message, or an inability to transport message to the cytoplasm. To determine if there were low levels of ER mRNA or possibly partially processed nuclear RNA, a reverse PCR technique was used to identify ER mRNA. Primers were constructed across the first and last introns of the ER mRNA. As a control, primers across the first splice site of γ-actin were also synthesized. These primers were used to amplify cDNA synthesized using random primers and reverse transcriptase from total cell RNA from MCF-7 and MDA-MB-231 cells. Fig. 2 shows the results of this experiment. When using RNA from MCF-7 cells, an expected 650-base pair fragment using the primer pair across the first intron is amplified. No band is detected when RNA from MDA-MB-231 cells is used. Similarly, a 469-base
TRANSCRIPTION OF ER IN BREAST CARCINOMA

Fig. 1. Northern blots of ER mRNA. Polyadenylated cytoplasmic RNA was isolated from MCF-7 and MDA-MB-231 cells. RNA was resolved on 1% agarose-formaldehyde gels and blotted to Nytran membrane. Identical blots were probed with ER cDNA (left) or actin cDNA (right).

Fig. 2. Evaluation of ER RNA with PCR. Primers were constructed across the first (ERSPL1) and last (ERSPL7) splice sites of the ER gene. Additionally, a set of primers across the first splice site of γ-actin (ActinSPL1) were used as a control. cDNA was synthesized from total cell RNA using random primers and reverse transcriptase. The cDNA was then amplified using PCR. The sizes of amplified regions from cDNA corresponding to the first splice site of ER, seventh splice site of ER, and actin splice site are 650, 469 and 400 base pairs, respectively. Markers are φX174 HindIII digests.

Fig. 3. Nuclear run-on. Nuclei were isolated from MCF-7 or MDA-MB-231 and were labeled with [α-32P]UTP for 15 min at 30°C. RNA was extracted and used as a probe on slot blots in which 5 µg of actin DNA, λ DNA, ER cDNA, DNA upstream of the ER transcriptional start site, and a CAT plasmid were each immobilized on Nytran membrane. After hybridization, filters were stringently washed and treated with RNase A to increase specificity.

Fig. 4. Southern blots of genomic DNA. Genomic DNA from MCF-7 or MDA-MB-231 cells was digested with EcoRI or HindIII and probed with the ER cDNA. Identical restriction patterns are obtained in both cell lines. These results are also identical to those of previous reports. These data, however, do not exclude the possibility of subtle muta-

positive control. λDNA, a 900-base pair fragment of the ER 5'-flanking region cloned in a CAT plasmid, and a CAT expression plasmid served as negative controls. Fig. 3 shows the results of the nuclear run-on assay. Whereas actin is actively transcribed to the same extent in both cell lines, ER gene appears to be transcribed only in MCF-7 cells. λ, a region upstream of the ER-transcribed region and a CAT-derived plasmid are all negative as expected. These results confirm the lack of expression of ER in MDA-MB-231 cells is due to a lack of transcription of the gene.

One simple explanation of these results would be that both alleles of the ER gene are deleted in MDA-MB-231. Previous studies have not detected a deletion of the ER gene by restriction analysis (10). Fig. 4 shows the results of a Southern blot of genomic DNA from MCF-7 or MDA-MB-231 cells digested with EcoRI or HindIII and probed with the ER cDNA. Identical restriction patterns are obtained in both cell lines. These results are also identical to those of previous reports. These data, however, do not exclude the possibility of subtle muta-

pair DNA fragment is expected using primers across the seventh splice site of the ER mRNA. This band is seen only with RNA from MCF-7. A 400-base pair band is expected when using primers across the γ-actin splice site. RNA from both MCF-7 and MDA-MB-231 gave the expected PCR product. Although not proof, these results nevertheless suggest that the lack of ER mRNA in MDA-MB-231 was not due to degradation or abnormal nuclear transport. Abnormal splicing could still be a possibility but is unlikely since identical results are obtained for two different ER splice sites.

To further substantiate these results, nuclear run-on assays were performed to examine directly the transcription of the ER gene. Nuclei were isolated from MCF-7 and MDA-MB-231 cells and in vitro RNA synthesis using [α-32P]UTP was carried out for 15 min at 30°C. RNA was then isolated from these nuclei and used as a probe on slot blots in which ER cDNA was immobilized. Actin cDNA was used as a
tions within the gene or even sizable deletions which might occur within introns which may affect transcription.

Discussion

The expression of estrogen receptor plays a central role in the biology of breast cancer. ER-positive tumors tend to have a less aggressive phenotype than cancers which lack receptor expression (11). Little is known about the regulation of ER expression in breast carcinoma. These studies were undertaken to clarify the mechanism whereby ER-negative cancers fail to express receptor. By utilizing a breast cancer cell line model, we have shown that the estrogen receptor gene is controlled transcriptionally in the ER-negative breast cancer line MDA-MB-231. Although it is possible that other mechanisms may exist to control ER expression, these data clearly demonstrate that transcriptional control is one mechanism which is responsible for the ER-negative phenotype.

Two possibilities exist for the transcriptional control of ER. ER-positive and ER-negative cells may have intrinsic differences in transcriptional machinery defined by a difference in trans-acting transcription factors. Alternatively, there may be cis alterations of the ER promoter in ER-negative cells which result in a transcriptionally inactive promoter. No such alterations have yet been defined at the level of Southern blot analysis. However, there may be subtle mutations not identified by Southern analysis or possibly deletions of important control regions which lie outside regions examined. For example, there could be deletions of important control regions within the ER introns or far upstream of the transcriptional start site which would not be identified in the Southern analysis performed here.

These data allow the focus of subsequent work to center on the ER promoter. Further work is presently under way to functionally map the ER promoter. A more detailed description of the mechanism which silences ER expression in certain breast carcinomas. These studies will also need to be expanded to include fresh cancer isolates to show that this breast cancer cell line model is representative of in situ carcinomas. Understanding ER promoter control in breast cancer will help to clarify the relationship between ER expression and the biology of breast carcinoma.

References

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