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Breast Cancer

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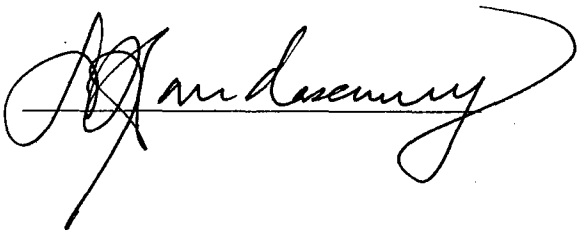
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13. ABSTRACT (Maximum 200 Words) Estrogen-dependent growth and metastasis of breast cancers is mediated through the estrogen receptor (ER). The objective of these studies is to produce, identify and demonstrate the utility of single chain antibodies (scFv) that interfere with ER action. These scFv are derived from the DNA binding domain (DBD) of the ER such that binding of a scFv to the ER DBD would prevent the interaction of the ER with estrogen response elements in the promoter of estrogen responsive genes. The estrogen dependent transcription of such genes results in the proliferation of breast cancer cells. To date several low affinity scFv have been developed. However difficulties in obtaining bacterial expression and correct folding of the scFv have been encountered. Optimizing the expression of scFv is now primarily a bacteriological problem as opposed to providing direct training in breast cancer research. I have therefore also begun to study the promoter of PI-9: an estrogen inducible inhibitor of granzyme B mediated apoptosis. Studying this gene will provide further insight into how breast cancer cells evade immune attack. I have found that the promoter of this gene contains a unique estrogen responsive unit that is capable of binding ER in both gel shift assays and DNase I footprinting.				
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FOREWORD

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Sacha Krieg
PI - Signature

Date

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Introduction

Approximately 40% of breast cancers are dependent upon the presence of estradiol-estrogen receptor complex for proliferation and metastasis. Preventing interaction of the estrogen receptor with its DNA response element would prevent estrogen dependent transcription of genes that are responsible for the proliferation of breast cancer cells. Over the past years I have been using phage antibody display technology to develop single chain antibodies (scFv) against the estrogen receptor DBD to prevent interaction of the estrogen receptor (ER). This scFv-ER interaction would therefore prevent the transcription of estrogen responsive genes. A problem we have encountered is the prevalence of amber mutations that reduce the yield of full length scFv, making appropriate biochemical studies of the scFv difficult. While the ultimate scFv will have application in inactivation of the estrogen receptor in breast cancer cells, optimizing scFv expression and folding have become problems in microbial genetics and protein chemistry, which do not provide much immediate exposure to breast cancer cells and to techniques directly applicable to breast cancer research. Although development of such a scFv is a scientifically interesting question, which we are still pursuing, with the agreement of my mentor Professor Shapiro, I have also begun to study an estrogen inducible inhibitor of Granzyme B mediated apoptosis, proteinase inhibitor 9 (PI-9). Since space in this annual report is limited, I will focus largely upon the PI-9 system. Studying the mechanism by which estrogen inhibits the ability of cells of the immune system to carry out immune surveillance and kill tumor cells is both highly relevant to breast cancer research and provides a more direct exposure to breast cancer cells and to techniques used in breast cancer research.

PI-9 is a serine protease inhibitor that irreversibly inhibits Granzyme B and was recently shown by Bird and coworkers to inhibit Granzyme B mediated apoptosis in MCF-7 breast cancer cells stably transfected with the PI-9 gene. The cell-mediated immune response plays a crucial role in immune surveillance resulting in the eradication of neoplastic cells and those infected with intracellular pathogens. Cytolytic lymphocytes kill neoplastic cells through a Granzyme B dependent pathway in which uptake of granzyme B by cancerous cells results in activation of a caspase cascade resulting in apoptosis of the target cell. PI-9 could provide a mechanism by which breast cancer cells evade immune attack. Recently, through the use of differential display technology, Dr. Hiroshi Kanamori a former Post Doc in Dr. Shapiro's lab, showed that the expression of PI 9 is upregulated in the presence of estrogen. This rapid induction was later shown to be a rapid and direct transcriptional effect of estrogen. For this reason I have been studying the promoter of this gene to ascertain how estrogen activates PI-9 transcription. The ability of estrogen to activate the transcription of PI-9 could provide an explanation for part of the ability of estrogen receptor positive breast cancer cells to evade immune surveillance. This project will supplement my training in breast cancer research with training in molecular biology techniques used in breast cancer research.

Body

Over the past two years I have been developing single chain antibodies (scFv) against the estrogen receptor DNA binding domain (ER DBD) to prevent interaction of the ER with its palindromic estrogen response element (ERE). This scFv-ER interaction would therefore prevent the transcription of estrogen responsive genes. The development of such an scFv has potential as a new approach for interfering with the activity of the ER in breast cancer cells. Using this methodology several potential scFv have been identified which bind with a modest affinity to the ER DBD. As indicated in the introduction, problems with amber mutations and expression levels as well as the production of scFv as insoluble inclusion bodies have significantly slowed characterization and application of the antibodies. While I understand that problems and difficulties are a universal part of thesis research, I became concerned that overcoming these problems was causing my training to largely focus on problems that involve microbiology and protein chemistry, and that this training, although useful, was not immediately relevant to breast cancer research. Although development of such a scFv is a scientifically interesting question, which we are still pursuing, with the agreement of my mentor Professor Shapiro, I have also begun to study an estrogen inducible inhibitor of Granzyme B mediated apoptosis, proteinase inhibitor 9 (PI-9). I will restrict this annual report to discussion of our PI-9 findings. Studying the mechanism by which estrogen inhibits the ability of cells of the immune system to carry out immune surveillance and kill tumor cells is both highly relevant to breast cancer research and provides a more direct exposure to technique used in breast cancer research.

PI-9 is an irreversible inhibitor of granzyme B and has been shown recently by Bird and coworkers (1) to inhibit granzyme B mediated apoptosis in MCF-7 breast cancer cells stably transfected with the PI-9 gene. Granzyme B plays a crucial role in the cell mediated immune response, a form of immunity responsible for immune surveillance of neoplastic cells. When a cytolytic cell recognizes a neoplastic cell Granzyme B is released, resulting in activation of a proteolytic caspase cascade and apoptosis of the neoplastic cell. By inhibiting Granzyme B, PI-9 prevents the cell mediated immune response from eradicating neoplastic cells. PI-9 was identified as an estrogen inducible gene product by Dr. Kanamori a former post-doc in the lab, further studies using both transcriptional and translational inhibitors suggested that upregulation of PI-9 message is a transcriptional effect of the estrogen receptor. At the cellular level the PI-9 gene was upregulated approximately 30-fold in the presence of estrogen, this level of estrogen induction is unusually large for human genes. Due to the extent of induction and data showing no mRNA stabilization effect we felt that the PI-9 promoter probably contained a consensus or multiple ERE's that closely resemble the consensus ERE in sequence. To determine the mechanism of PI-9 upregulation by estrogen I screened a human genomic lamda library to find the promoter for PI-9.

Promoter isolation and subcloning. Using a cDNA fragment of PI-9 containing the most 5' mRNA sequence known, 8 lamda clones were plaque purified from 500,000 phage. DNA from these 8 lambda clones was purified to obtain the promoter for PI-9. Using primers corresponding to either the T7 or SP6 primer sequences in the lamda arms and a 3' primer that would anneal to the most 5' known PI-9 sequence a 1.8 kB promoter

fragment was isolated and sequenced.

Identification of the minimal estrogen responsive unit (ERU) for PI-9. After sequencing the promoter a transcription factor database search was used to locate potential promoter elements responsible for estrogen inducibility of this gene. To assess estrogen inducibility of this promoter the full length promoter was cloned upstream of a luciferase reporter gene and transfected into several cancer cell lines, including MCF -7 breast cancer cells resulting in a 12 fold induction of the promoter (fig 1). Because of the unusually large estrogen induction we anticipated that the promoter of this gene would contain an ERE. Although no consensus ERE's were seen several imperfect ERE's were found upstream of the transcriptional start site. However mutational and deletional analysis of the promoter showed that these elements were not responsible for estrogen inducibility of the PI-9 gene (fig. 2). Based on these truncations it became apparent the the minimal promoter for PI-9 was found downstream of the transcriptional start site, therefore we began to focus on an unusual element found 200 base pairs downstream of the transcriptional start site (table 1). This element contains an everted half site, an imperfect ERE and two consensus ERE half sites spaced 13 base pairs apart (DR13).

Table 1.

Identification of the minimal estrogen responsive unit for PI-9

<u>Construct</u>	<u>Fold Estrogen Induction</u>
ACTGGGGGGACCCTGACCTGACC(N)₁₃TGACC	4
GGGGACCCTGACCTGACC(N)₁₃TGACC	6
TGACCTGACC(N)₁₃TGACC	3
TGACC(N)₁₃TGACC	2
Consensus ERE sequence: GGTCAANNNTGACC	
Those nucleotides located in the imperfect ERE are underlined, nucleotides involved in everted or direct repeats are in bold.	

Further mutational analysis of the two internal half sites either separately or in combination showed no estrogen inducibility. Based on these findings the minimal ERU for PI-9 was determined to be an imperfect ERE directly adjacent to two consensus direct repeats spaced by 13 nt.

Assessment of the ability of the estrogen receptor to interact with the PI-9 ERU. The minimal ERU for PI-9 is an interesting element in that it is not a consensus ERE or a multimerization of imperfect ERE's which was anticipated because of the fold induction by estrogen. In most promoters an induction of this size typically occurs when multiple ER complexes are interacting with a series of imperfect ERE's. The ER has been shown by Chambon and coworkers to be capable of interacting with direct repeats although with a lower affinity than with the palindromic consensus ERE. Because of there are no nucleotides between the imperfect ERE and the DR13 it seems as though it would be difficult for multiple ER complexes to bind this sequence. To determine how the estrogen receptor is interacting with this element I performed electrophoretic mobility shift assays (EMSA) using flag epitope tagged estrogen receptor (fhER) purified from CHO-S cells transiently transfected with fhER using lipofectamine 2000. A 60 base pair

end-labeled probe containing the minimal ERU was used for the shifts, for comparison a 60 base pair consensus ERE probe was also constructed. In comparison to the consensus ERE the PI-9 ERU binds the estrogen receptor about 3 times less well. However there were two shifted estrogen receptor complexes, both of which are supershifted with the anti-flag M2 antibody suggesting that two ER dimers are capable of binding this element (fig. 3). To ascertain which portions of this minimal estrogen responsive element are physically interacting with the ER the same ERU was placed into pGEM and using restriction digest a 140 base pair probe for footprinting was created. This probe was then used for DNase I digests. Footprinting experiments show two protected regions, one encompassing the imperfect ERE and the direct repeat directly 3' to the ERE and the other the most distal direct repeat (fig. 4). These data indicate that both the imperfect ERE and the direct repeat ERE half sites are occupied by the estrogen receptor.

Conclusions. PI-9 expression is upregulated by estrogen at the level of transcription through a novel ERU located downstream of the transcriptional start site. This ERU consists of an imperfect ERE directly adjacent to two consensus direct repeats spaced by 13 basepairs. EMSA analysis shows that there are two ER complexes interacting with this region. Additionally, footprinting studies showed that both the imperfect ERE and DR13 are protected from DNaseI digest by estrogen receptor.

The ability of estrogen to rapidly upregulate expression of a protein which can prevent the cell mediated immune system from recognizing and eliminating neoplastic cells could provide a mechanism by which breast cancer cells can evade the immune response in an estrogen dependent manner.

References

1. Bird, C., Sutton, V., Sun, J., Hirst, C., Novak, A., Kumar, S., Trapani, J. and P. Bird. 1998. Selective regulation of apoptosis; the cytotoxic lymphocyte serpin proteinase inhibitor 9 protects against granzyme B-mediated apoptosis without perturbing the Fas cell death pathway. *Molecular and Cellular Biology*. 18(11): 6387-6398.
2. Kanamori, H., Krieg, S., Mao, C., Di Pippo, V, Wang, S., Zajchowski, D., and D.J. Shapiro. 2000. Proteinase Inhibitor 9, an Inhibitor of Granzyme B-mediated Apoptosis, Is a Primary Estrogen-inducible Gene in Human Liver Cells. *The Journal of Biological Chemistry*. 275(8):5867-5873.

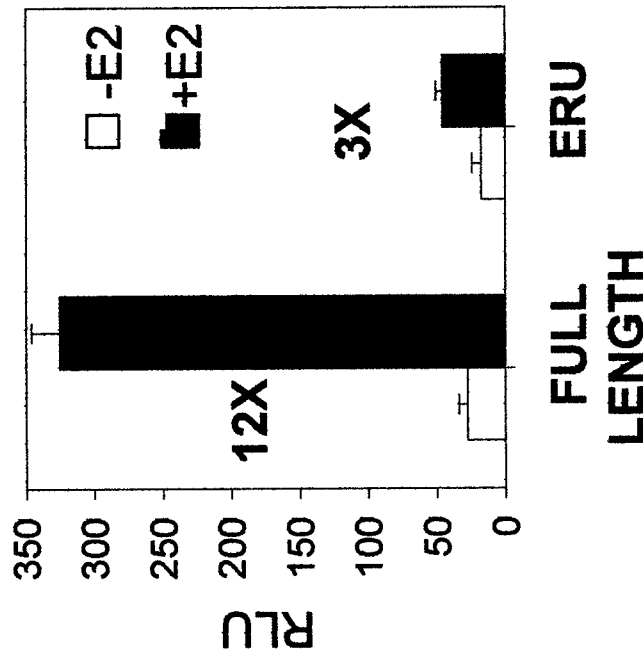


Figure 1. Transient transfection of PI-9 promoter constructs in MCF-7 human breast cancer cells. Either the full length PI-9 promoter or the minimal ERU was transfected and activity was assayed using the dual luciferase system.

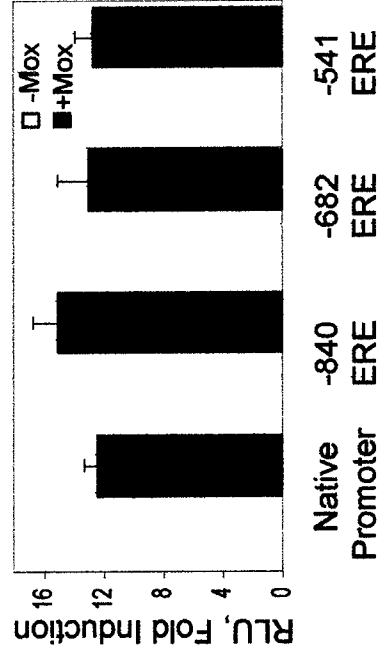


Figure 2. Mutational analysis of the upstream 5' imperfect ERE's. Mutation of the imperfect ERE's found at either -840, -682 or -541 had no influence on estrogen inducibility. Furthermore mutation of these elements in combination had no influence on estrogen inducibility.

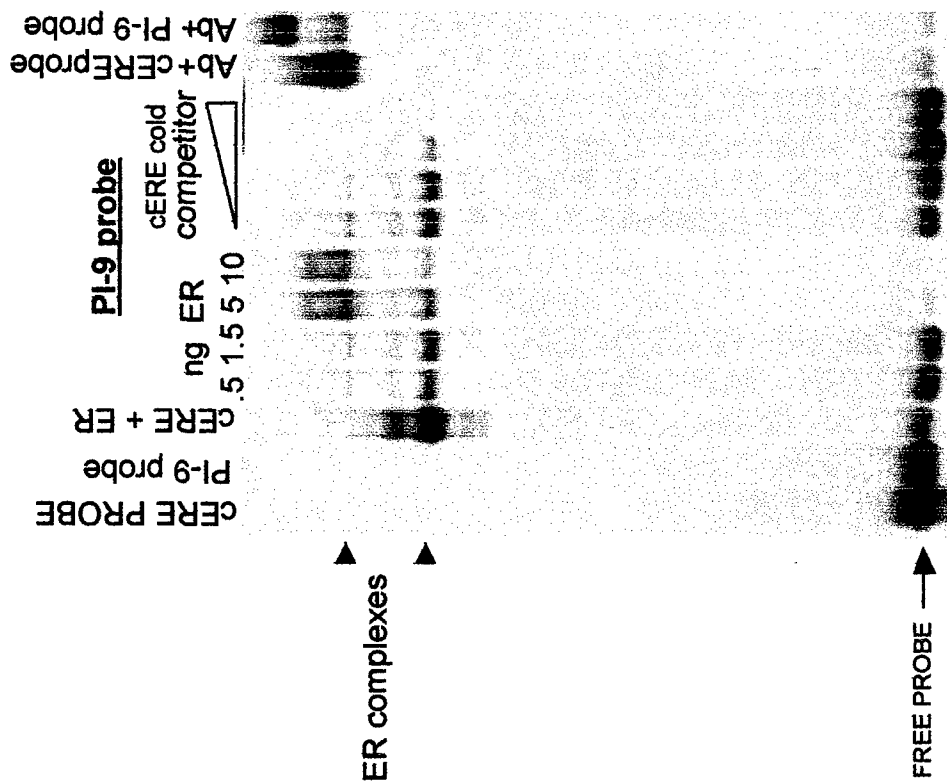


Fig. 3 EMSA of PI-9 ERU with purified estrogen receptor. Pure ER was incubated either with the PI-9 ERU or the consensus ERE (cERE) endlabelled probe and separated on a nondenaturing 6% gel.

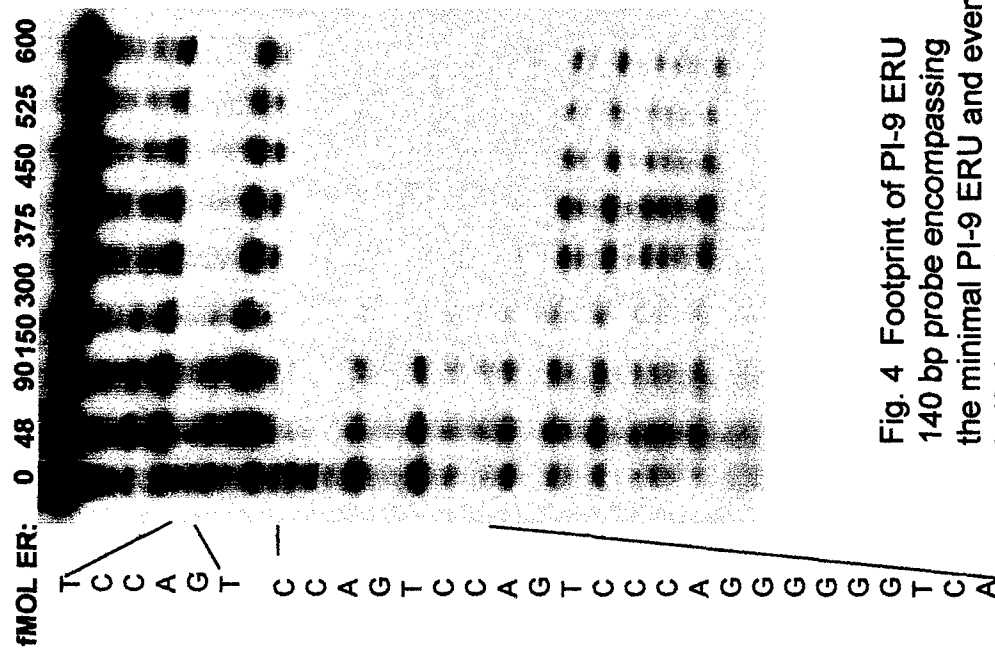


Fig. 4 Footprint of PI-9 ERU 140 bp probe encompassing the minimal PI-9 ERU and everted half site was incubated with purified ER and digested with DNase I.

Appendix

Key research accomplishments in the past year

- Identification of the PI-9 minimal estrogen response unit
- EMSA of the PI-9 ERU
- Footprinting the PI-9 ERU

Reportable outcomes

Kanamori, H. *, Krieg, S. *, Mao, C., Di Pippo, V, Wang, S., Zajchowski, D., and D.J. Shapiro. 2000. Proteinase Inhibitor 9, an Inhibitor of Granzyme B-mediated Apoptosis, Is a Primary Estrogen-inducible Gene in Human Liver Cells. *The Journal of Biological Chemistry*. 275(8):5867-5873.

*Coequal first authors

Abstract presentation at DOD Breast Cancer Research Program Meeting (June, 2000)

Invited speaker, platform presentation, DOD Breast Cancer Research Program

Abstract presentation, (June, 2000) Endocrine Society meeting

ESTROGEN INDUCIBLE GENE EXPRESSION IN CANCER CELLS

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Estrogen-dependent growth and metastasis of breast cancers is mediated through the estrogen receptor (ER). The aim of these studies is to produce, identify and demonstrate the utility of single chain antibodies (scFv) that interfere with ER action. These scFv are derived against the DNA binding domain (DBD) of the ER such that binding of a scFv to the ER DBD would prevent the interaction of the ER with estrogen response elements in the promoter of estrogen responsive genes. The estrogen dependent transcription of such genes results in the proliferation of breast cancer cells. To date low affinity scFv have been developed, however difficulties in bacterial expression and correct folding have been encountered. Optimizing the expression of scFv has become a bacteriological problem as opposed to providing training in breast cancer research. For this reason we have begun to study genes which are regulated by estrogen as possible targets for potential repressors. One such gene product, PI-9, was recently found to be an estrogen inducible inhibitor of granzyme B mediated apoptosis. Since expression of PI-9 in MCF-7 breast cancer cells prevents apoptosis caused by cytolytic lymphocytes, overexpression of PI-9 could provide a mechanism by which neoplastic cells evade immune surveillance. This gene product shows a 30-fold estrogen induction in HepG2 cells stably transfected with estrogen receptor. This estrogen induction of PI-9 expression was confirmed to be a transcriptional effect of estrogen. To ascertain how the estrogen receptor is upregulating PI-9 expression at the transcriptional level, a lambda phage library was screened and clones containing the promoter region were isolated and characterized. When this promoter region was placed upstream of a luciferase reporter a 15-fold increase in luciferase activity was seen in the presence of the estrogen, moxestrol. Several potential estrogen responsive elements in the promoter were analyzed to determine what region confers estrogen inducibility. Analysis of the promoter led to the identification of an unusual estrogen responsive unit responsible for estrogen induction. Electrophoretic mobility shift assays show that the estrogen receptor is capable of binding this element, although with a lower affinity than it exhibits for the consensus ERE. An upstream AP-1 site also appears to contribute to the overall activity of the gene but not to estrogen inducibility. Identification of PI-9 as an estrogen dependent gene product in cancer cells could provide one mechanism by which cancer cells evade elimination by the immune system.

ANALYSIS OF THE PROTEINASE INHIBITOR-9 PROMOTER, AN ESTROGEN INDUCIBLE INHIBITOR OF GRANZYME B-MEDIATED APOPTOSIS

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Although estrogens and other hormones have important effect on immune system functions, the molecular basis of many of these effects is not well understood. Using differential display we identified proteinase inhibitor-9 as an mRNA upregulated by estrogen in a human hepatoblastoma cell line (HepG2) stably transfected with the estrogen receptor (ER). PI-9 is an irreversible inhibitor of the protease granzyme B. PI-9 has been shown by Bird and coworkers to inhibit granzyme B mediated apoptosis. Granzyme B is found in granules that are produced by cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells. CTLs and NK cells use perforin and granzyme B-containing granules, in addition to the Fas mediated pathway, to initiate apoptosis of target cells that are neoplastic or infected with intracellular pathogens. Studies using cycloheximide and actinomycin D suggested that this estrogen effect on PI-9 gene expression was transcriptional. To more directly identify the mechanism of PI-9 mRNA estrogen induction, we isolated a human genomic clone containing the PI-9 promoter region, identified a putative transcriptional start site, and carried out transient transfections using PI-9 promoter-luciferase reporter gene constructs in HepG2 cells. When this promoter region was placed upstream of a luciferase reporter a 15-fold increase in luciferase activity was seen in the presence of the estrogen, moxestrol. Several potential estrogen responsive elements in the promoter were analyzed to determine what region confers estrogen inducibility. Mutational analysis of potential upstream, imperfect, estrogen response elements (EREs) suggested that an upstream imperfect ERE was not responsible for this estrogen induction. Deletion analysis of the promoter led to the identification of an unusual downstream estrogen responsive unit responsible for estrogen induction consisting of an imperfect ERE and two direct repeats. An upstream AP-1 site also appears to contribute to the overall activity of the gene, but not to estrogen inducibility. Electrophoretic mobility shift assays show that the estrogen receptor is capable of binding this element, although with a lower affinity than it exhibits for the consensus ERE. Raloxifene and ICI 182,780 both acted as antagonists for reporter gene expression. 4-hydroxytamoxifen (OHT), the active metabolite of the widely used selective estrogen receptor modulator, tamoxifen, was a potent inducer when the PI-9 promoter region was introduced into the cells by transient transfection. In contrast, OHT was a weak inducer of expression from the chromosomal PI-9 gene. The estrogen regulation of PI-9 gene expression could provide one mechanism by which estrogen influences the function of the cell mediated immune system.

(Supported by NIH grant HD-16720 and USAMRMC Breast Cancer Research Program Predoctoral Fellowship DAMD 17-98-1-8197 to S.K.)

Proteinase Inhibitor 9, an Inhibitor of Granzyme B-mediated Apoptosis, Is a Primary Estrogen-inducible Gene in Human Liver Cells*

(Received for publication, July 26, 1999, and in revised form, November 18, 1999)

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Although liver is an estrogen target tissue, the number of hepatic genes known to be directly induced by estrogen is very small. We identified proteinase inhibitor 9, or PI-9, as being rapidly and strongly induced by estrogen in an estrogen receptor-positive human liver cell line (HepG2-ER7). Since PI-9 mRNA was also induced by estrogen in a human liver biopsy sample, PI-9 is a genuine estrogen-regulated human gene. PI-9 is a potent inhibitor of granzyme B and of granzyme B-mediated apoptosis. Estrogens induced PI-9 mRNA within 2 h, PI-9 mRNA levels reached a plateau of 30–40-fold induction in 4 h, and induction was not blocked by cycloheximide, indicating that induction of PI-9 mRNA is a primary response. The antiestrogen *trans*-hydroxytamoxifen was a partial agonist for PI-9 mRNA induction, whereas the antiestrogen ICI 162,780 was a pure antagonist. Western blot analysis showed that estrogen strongly increases PI-9 protein levels. Inhibition of transcription with actinomycin D resulted in identical rates of PI-9 mRNA decay in the presence and absence of estrogen. We isolated genomic clones containing the PI-9 promoter region, identified a putative transcription start site, and carried out transient transfections of PI-9-luciferase reporter gene constructs. The estrogen, moxestrol, elicited a robust induction from the PI-9-luciferase reporter. Mutational inactivation of three potential imperfect estrogen response elements in the PI-9 5'-flanking region had no effect on moxestrol estrogen receptor induction.

The intracellular actions of estrogen are mediated by estrogen receptors (ERs).¹ Although the effects of estrogens in ovary, uterus, breast cancer cells, and osteoblasts have been the sub-

ject of intensive study, much less is known of estrogen actions in the human liver, which is an estrogen target tissue. The levels of a few liver proteins including some coagulation factors, sex hormone binding globulin, angiotensinogen, somatomedin C, and apolipoprotein A are altered after oral administration of estrogen to women (1), but in most cases the responses are relatively small, and it is not clear whether these are primary or secondary responses to estrogen. Since these few genes are unlikely to account for the physiologic effects of estrogen in liver, we used a differential display approach (2) to identify RNAs that were induced by estrogen. Differential display is distinguished from other methods in that it does not involve a preconceived idea of the genes or pathways likely to be regulated.

Although early passages of the widely used HepG2 cells, a human hepatoblastoma cell line (3), contained ER (4), this cell line subsequently lost receptor and became ER-negative. To restore ER expression, HepG2 cells were stably transfected to express ER α , which is the ER subtype normally found in liver (5). Using these cells (termed HepG2-ER7), we identified proteinase inhibitor 9 (PI-9) as an estrogen-inducible gene.

PI-9 is a potent inhibitor of the protease, granzyme B (6, 7). Granzyme B is found in granules that are produced by cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells. CTLs and NK cells use perforin and granzyme B-containing granules (8, 9) as well as the Fas-mediated pathway (10) to destroy target cells that are neoplastic or infected with intracellular pathogens (11, 12). After entry into target cells, granzyme B cleaves the precursor forms of several caspases and induces apoptosis (13–15). PI-9 inhibits apoptosis mediated by either purified granzyme B and perforin or by CTLs (7). Although PI-9 does not inhibit most caspases, it does inhibit caspase 4 (7), a member of the caspase subfamily involved in the maturation of interleukins.

Although the ability of PI-9 to modulate apoptosis mediated by CTLs and by NK cells make this an important system for study, this work represents the first study of the regulation of PI-9 gene expression. We demonstrate that PI-9 mRNA is rapidly and strongly induced by estrogen and that the induction of PI-9 transcription is a direct, primary, effect of estrogen. Since PI-9 mRNA was also induced by estrogen in several independently isolated ER positive lines of HepG2 cells and was induced by estrogen in a human liver biopsy specimen, PI-9 mRNA is one of a handful of human mRNAs shown to exhibit large, order of magnitude, increases as a primary response to estrogen. Although estrogen strongly induces transcription of a construct containing the PI-9 promoter region linked to a luciferase reporter gene, the estrogen induction of PI-9 transcription is not mediated by estrogen response elements in the PI-9 5'-flanking region.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) AF200209.

¶ These researchers should be considered co-equal first authors.

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¹ The abbreviations used are: ER, estrogen receptor; CTL, cytotoxic T lymphocyte; NK, natural killer; RT, reverse transcriptase; PCR, polymerase chain reaction; E₂, 17 β -estradiol; PI-9, proteinase inhibitor 9; CMV, cytomegalovirus; DPE, downstream promoter element; ERE, estrogen response element; MOX, moxestrol.

EXPERIMENTAL PROCEDURES

Differential Display—Differential display was modified and performed as we recently described (2). Briefly, total cell RNA was prepared using guanidine thiocyanate extraction and centrifugation through cesium chloride (16). Before reverse transcription, the RNAs were DNase-treated. Primer I (5'-TTGTTGTTAACTTGTGTTA-3') was used for reverse transcription, and the cDNAs were fractionated on a Bio-Rad Prep A purification column to remove the primer used for reverse transcription. Primer Q (5'-CAGCGGAGACAGAGGAAG-3') was 5'-end-labeled using T4 kinase. Arbitrary primer AP02 (5'-GG-GAAGCTTGCTAAGACTAGC-3') was added to the reaction, and PCR was performed in a 25- μ l reaction.

Cell Culture—The HepG2, human hepatoma cell line (Ref. 3; The Wistar Institute, ATCC number HB8065) was transfected with pSV2neo/CMV-ER expression vector as a calcium phosphate-DNA coprecipitate essentially as described (17), except that the ER cDNA was from the HEGO plasmid (18). Cells were cultured in Eagle's minimal essential medium (Life Technologies, Inc.) supplemented with 1 mM HEPES, 2 mM glutamine, 0.1 mM Eagle's nonessential amino acids, 1.0 mM sodium pyruvate, 50 μ g/ml gentamicin, 10% fetal bovine serum, and 10 nM ICI 164,384 during selection in 1000 μ g/ml G418 (Life Technologies). Stable ER-expressing clones were identified by immunocytochemical and Western blot analyses (19) using anti-ER antibodies kindly provided by G. Greene (University of Chicago). Using a whole cell binding assay for tritiated 17 β -estradiol, we determined that the HepG2-ER7 clonal isolate contains approximately 30,000 ER sites/cell. HepG2-ER7 cells were routinely cultured in Dulbecco's modified Eagle's medium, 10% dextran-coated charcoal-treated fetal bovine serum. Three independent clones of HepG2 cells expressing FLAG epitope-tagged hER α were isolated using the bicistronic system essentially as we recently described for ER-positive HeLa cell lines (20). The HepG2ER clones were selected in Dulbecco's modified Eagle's medium, 10% dextran-coated charcoal-treated fetal bovine serum containing 800 μ g/ml G418 and 50% HepG2-conditioned medium.

Northern Blotting—cDNA coding for PI-9 was cloned by RT-PCR using the Takara RNA LA PCR kit (PanVera, Madison, WI) according to the manufacturer's instructions. Using total RNA from cells treated with 17 β -estradiol, RT-PCR was carried out using PI-9 5' (5'-GTG-GCAGGCCCTGCATCA-3') and 3' (5'-CACCCCTTTATGGCGATGA-3') primers. The amplified PCR product (nucleotides 87–1240 of PI-9 cDNA, GenBank[®] accession Number L40378) was subcloned into the pGEM-T vector (Promega, Madison, WI), and its identity was confirmed by sequencing. The plasmid was then digested with *ScaI* and used as a template for *in vitro* transcription using SP6 RNA polymerase to generate an RNA probe containing 421 nucleotides of the PI-9 coding sequence (Fig. 1B). Total cell RNA was prepared using Trizol reagent (Life Technologies) in combination with DNase treatment. 10 μ g of total RNA was glyoxal-treated, run on a 1% agarose gel, transferred to a BIODYNE PLUS membrane (Pall, BioSupport Division, Port Washington, NY), and hybridized to either the RNA probe described above or to an actin probe used as an internal standard. Quantitation of PI-9 mRNA levels was either by PhosphorImager analysis or by densitometry of bands on x-ray film.

Quantitation of PI-9 mRNA Levels in a Human Liver Biopsy Specimen—This experiment was carried out with informed consent from the patient and approval by the Committee on Human Ethics of the University of Tokyo. The biopsy sample was washed in medium (Dulbecco's modified Eagle's medium plus 10% CD-fetal bovine serum) then incubated for 5.5 h in medium either containing 1 μ M moxestrol or lacking moxestrol. RNA was extracted, and 1 μ g of RNA was analyzed by RT-PCR using the PI-9-specific primers described above in the section on Northern blotting and a β -actin primer pair (CLONTECH, Palo Alto, CA). The reverse transcription reaction was carried out at 42 °C for 50 min followed by 99 °C for 5 min and 5 °C for 5 min. The first PCR cycle was performed at 94 °C for 2 min followed by 28 PCR cycles at 94 °C for 30 s, 48 °C for 30 s, and 72 °C for 90 s. An aliquot (5%) of the RT-PCR product was run on a 0.8% agarose gel and photographed after staining with ethidium bromide.

Western Blot Analysis—To prepare a polyclonal antibody to PI-9, an *NcoI/ApaI* fragment encoding amino acids 41–315 was made blunt-ended, cloned into PET-21b (Novagen, Milwaukee, WI) that had been digested with *SalI* and *NotI*, and made blunt-ended. The fusion protein contained a T7-Tag at its N terminus and a His tag at its C terminus. After transformation, the protein was expressed in *E. coli* BL21DE3, and the protein was purified from inclusion bodies under denaturing conditions in one step using nickel nitrilotriacetic acid-agarose (Qiagen, Santa Clarita, CA). Crude serum from a rabbit immunized with the

purified PI-9 fragment was fractionated by precipitation with 50% ammonium sulfate followed by 40% ammonium sulfate (19).

HepG2 cells were broken by three freeze-thaw cycles followed by centrifugation at 45,000 revolutions/min for 20 min at 4 °C to remove cell debris. SDS-polyacrylamide gel electrophoresis and Western blotting were carried out as described (19), and antibodies were detected using ECL reagent (Amersham Pharmacia Biotech) with the manufacturer's protocol and following modifications. The membrane was blocked with 3% nonfat milk. Incubations with antibodies were for 1 h with a 30-min wash between incubations followed by a final wash for 1 h.

Isolation of Genomic PI-9 Clones—500,000 phage from a human λ EMBL3 SP6/T7 genomic library derived from human peripheral blood leukocytes (CLONTECH, Palo Alto, CA) were grown, transferred to nylon filters (Micon Separations, Westboro, MA), and hybridized at 65 °C for 16 h in 10% dextran sulfate, .05 M Tris-HCl (pH 7.6), 1% sodium dodecyl sulfate, 1 M NaCl, and 100 μ g/ml denatured salmon sperm DNA. The filters were washed at 65 °C in 1 $\times$ SSC (0.15 M NaCl and 0.015 M sodium citrate) and 0.2% SDS followed by a 0.1 $\times$ SSC and 0.1% SDS wash at 65 °C. The probe used for initial screening corresponded to nucleotides 53 to 1205 of the PI-9 cDNA, and the probe used for plaque purification of candidate-positive phage corresponded to nucleotides 53 to 297 (PI-9 cDNA sequence, GenBank[®] accession number L40378). The probes were labeled by random hexamer priming with [α -³²P]CTP (random-primed DNA-labeling kit, Roche Molecular Biochemicals). After plaque purification, the PI-9 promoter was isolated from the phage by PCR and cloned into the pGEM-T vector (Promega). Sequencing of the promoter was carried out using the BIG DYE terminator cycle sequencing kit (Perkin-Elmer). The promoter sequence was deposited in GenBank[®], (accession number AF200209).

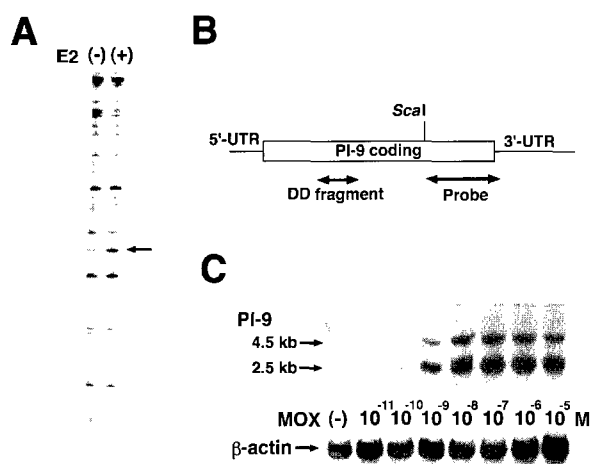
Identification of the Transcription Start Site of PI-9—To confirm the identity of the transcription initiation site, we carried out RT-PCR using the primers shown in Fig. 6. 1 μ g of total RNA from YT cells was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (Life Technologies) with a primer corresponding to nucleotides 2015–2028 in the promoter sequence (GenBank[®] accession number AF200209). PCR using one-quarter of the RT reaction as template was carried out using *Taq* polymerase (Life Technologies) with 3' primers (described in the legend to Fig. 6) and the same 5' primer used for the RT reaction, giving a ~470-base pair product. RT-PCR products were separated by electrophoresis on a 1% agarose gel and visualized by staining with ethidium bromide.

Generation of Estrogen Response Element (ERE) Mutations in the PI-9 Promoter—Mutations were generated using the Stratagene quick change kit. In each case the potential ERE sequence (see Fig. 7 legend) was mutated to a *HindIII* site in the context of the full-length promoter region in the pGL3 promoter plasmid. Because the promoter contains regions with high GC content, to increase the generation of full-length mutant DNAs, GC melt (CLONTECH, Palo Alto, CA) was added to 1 M. Thermocycling parameters were altered from the manufacturer's protocol using an annealing temperature of 48 °C and an extension time of 12.5 min. The presence of the desired mutation was confirmed by DNA sequencing.

Transfections—Transfections were performed using HepG2 cells maintained in Dulbecco's modified Eagle's medium (Life Technologies), 10% charcoal dextran-treated fetal bovine serum, and penicillin-streptomycin. Transfections were done in 6-well plates using calcium phosphate coprecipitation (21) with 3.8 μ g of PTZ18U as carrier DNA, 25 ng of pRLSV40 as an internal standard (Promega), 15 ng of CMVhER, and 100 ng of the indicated PI-9 promoter-luciferase reporter plasmid. The PI-9 promoter region was cloned into the *NheI* and *BglII* sites of the PGL3 promoter plasmid (Promega). The promoter fragment was generated by PCR using *Pfu* turbo (Stratagene, La Jolla, CA) and primers containing either *NheI* or *BglII* restriction sites. After shocking the HepG2 cells with 20% glycerol, moxestrol was added to 10⁻⁸ M. Cells were harvested 48 h after glycerol shock, and lysates were assayed using the dual luciferase assay kit according to the manufacturers protocol (Promega).

RESULTS

Identification of PI-9 as an Estrogen-inducible Gene In Human Liver Cells—HepG2-ER7 cells were maintained in 10⁻⁷ M 17 β -estradiol (E₂) or ethanol vehicle for 48 h, the cells were harvested, and the RNAs were analyzed by differential display. The intensity of one band was markedly increased in the sample from E₂-treated cells (Fig. 1A, arrow). The band was isolated from the gel, reamplified, subcloned into a plasmid vector,



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FIG. 1. Estrogen induces PI-9 mRNA. A, differential display of the RNA from HepG2-ER7 cells treated with (+ lane) or without (- lane) E_2 . Cells were treated with 10^{-7} M E_2 for 48 h and harvested, and total cell RNA was extracted and subjected to differential display as we recently described (2). To conserve space, the bottom section of the gel is not shown. The arrow indicates the induced band. B, schematic representation of the probe used for Northern blotting. A box denotes the coding sequence of PI-9 cDNA. Arrows under the sequence denote the fragment isolated by differential display (DD fragment, nucleotides 286–440) and the RNA fragment used as a probe in the Northern blots (Probe, nucleotides 820–1240). UTR, untranslated region. C, dose-response curve for moxestrol induction of PI-9 mRNAs. The HepG2-ER7 cells were maintained for 48 h in the indicated concentrations of medium containing either MOX or the ethanol vehicle (-), and RNAs were isolated and analyzed by Northern blotting as described in "Experimental Procedures." The 4.5- and 2.5-kilobase PI-9 transcripts are indicated by arrows (upper panel). β -actin was used as an internal standard.

and sequenced. Data base analysis revealed that the clone was identical to nucleotides 286–440 of PI-9 mRNA (Fig. 1B).

Estrogen Induces PI-9 mRNA—To determine whether PI-9 mRNA was truly estrogen-inducible, we treated the HepG2-ER7 cells with increasing concentrations of the estrogen, moxestrol, which liver cells metabolize more slowly than 17β -estradiol (22). RNA was isolated and analyzed by Northern blotting using an RNA probe corresponding to the 3'-end of the PI-9 protein-coding region (Fig. 1B). In agreement with earlier reports (6, 23), two PI-9 mRNAs approximately 2.5 and 4.5 kilobases in length were detected (Fig. 1C). PI-9 mRNA levels were quite low in the absence of moxestrol. Induction of both PI-9 mRNAs was readily detected at 1 nM moxestrol, and induction was maximal at 10 nM moxestrol. Although the low basal level of PI-9 mRNA makes precise quantitation difficult, quantitation of the RNA bands indicates that moxestrol induces PI-9 mRNA 30–40-fold.

To determine if the moxestrol induction of PI-9 mRNA was a general property of HepG2 human hepatoma cells, we examined the ability of three additional lines of ER-positive HepG2 cells produced using a different protocol based on the production of a bicistronic ER mRNA (20) and prepared in a different laboratory than the one that isolated HepG2-ER7 cells. Moxestrol strongly induced PI-9 mRNA in all three ER positive HepG2 cell lines (data not shown).

PI-9 Is an Estrogen-inducible Gene in Human Liver—These studies employed a transformed, established human liver cell line. Although HepG2 cells are a widely used model for human liver, we wished to more directly examine the ability of moxestrol to induce PI-9 in human liver. A portion of a biopsy sample was obtained from a 61-year old female patient with a diagno-

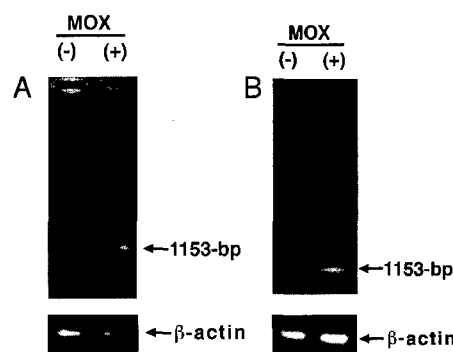


FIG. 2. Moxestrol induces PI-9 mRNA in human liver. HepG2-ER7 cells (panel A) or the human liver biopsy sample (panel B) were incubated for 5.5 h in medium either containing 1 μ M MOX or lacking MOX. RNA was extracted, and 1 μ g of RNA was analyzed by RT-PCR as described under "Experimental Procedures" using a PI-9-specific or a β -actin-specific primer pair as described under "Experimental Procedures." The products were analyzed by electrophoresis, and the samples were run on separate gels.

sis of autoimmune hepatitis. Tissue samples were incubated with and without moxestrol, and RNA was isolated and analyzed by RT-PCR (Fig. 2B). For comparison, RT-PCR was also carried out using the same protocol on an RNA sample from control and moxestrol-treated HepG2 ER-7 cells (Fig. 2A). Densitometric quantitation of the band from the actin internal standard indicated that the actin mRNA level was 1.4-fold higher in the moxestrol-treated biopsy sample than in the untreated control biopsy sample. PI-9 mRNA was virtually undetectable in the control minus moxestrol sample, and precise quantitation of its level by densitometry was therefore difficult. Moxestrol treatment increased the level of PI-9 mRNA by >5-fold. After correcting for the slight increase in the level of expression of the actin control, the fold induction of PI-9 by moxestrol in the human biopsy sample is at least 4-fold and may actually be much higher. These data demonstrate that estrogen induces PI-9 gene expression in human liver.

Antiestrogens Interfere with Moxestrol Induction of PI-9 mRNA—To investigate the effects of antiestrogens on PI-9 gene expression, *trans*-hydroxytamoxifen or ICI 182,780 was added to the culture medium with and without moxestrol. *Trans*-hydroxytamoxifen acted as a partial agonist, inducing low levels of PI-9 mRNA and only partially blocking moxestrol-mediated induction of PI-9 mRNAs (Fig. 3A). These data are consistent with several reports that tamoxifen can be a partial agonist in human liver (22, 24, 25). In contrast, a 100-fold excess of the pure antiestrogen ICI 182,780 completely blocked induction of PI-9 mRNAs by moxestrol (Fig. 3B). These data demonstrate that an estrogen-ER complex is required for induction of PI-9 mRNAs. Dexamethasone did not induce PI-9 mRNA and had little or no effect on moxestrol induction of PI-9 RNA (Fig. 3C).

Induction of PI-9 mRNA Is a Direct or Primary Effect of Estrogen—Steroid hormone-regulated genes are often classified by whether their expression is directly controlled by the hormone-receptor complex or whether their regulation is an indirect or secondary effect requiring prior expression of other hormone-regulated genes (reviewed in Ref. 26). To analyze the nature of the estrogen induction of PI-9 mRNA, we determined the time course of induction and the effect of blocking protein synthesis. The induction of PI-9 mRNA in HepG2-ER7 cells was detected as early as 2 h after the addition of moxestrol to the medium, and PI-9 mRNA levels reached a plateau within 4 h (Fig. 4A). When moxestrol was present, the fully induced level of PI-9 mRNA was maintained for at least 48 h (Fig. 4A). The rapid metabolism of E_2 by liver cells enabled us to examine

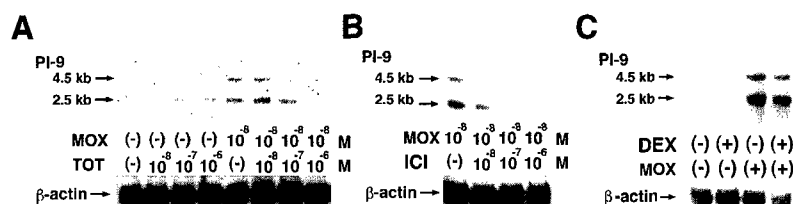


FIG. 3. **Antiestrogens interfere with the induction of PI-9 mRNA.** The indicated concentrations of *trans*-hydroxytamoxifen (TOT) (panel A) or ICI 182,780 (panel B) or 10^{-7} M dexamethasone (DEX, panel C) with or without 10^{-8} M MOX were added to the culture medium, and the cells were maintained for 24 h. RNA was extracted, and the samples were analyzed by Northern blot hybridization as described under "Experimental Procedures".

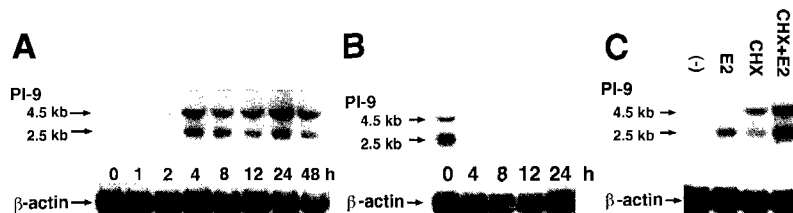


FIG. 4. **Induction of PI-9 mRNA is a direct effect of estrogen.** A, time course of moxestrol-mediated induction of PI-9 mRNA. The HepG2-ER7 cells were maintained in 10^{-7} M moxestrol for the indicated times (0, 1, 2, 4, 8, 12, 24, and 48 h), and RNA was analyzed by Northern blotting. B, decay profile of PI-9 mRNA. PI-9 mRNA was induced by treating the cells with 10^{-7} M E_2 for 24 h. Cells were then washed three times with phosphate-buffered saline to remove E_2 and maintained in estrogen-free medium for the indicated times (0, 4, 8, 12, and 24 h), and RNA was extracted and analyzed by Northern blotting. C, cycloheximide does not block moxestrol induction of PI-9 mRNA. The cells were maintained in medium either containing or lacking 50 μ g/ml cycloheximide (CHX) for 30 min, then 10^{-6} M E_2 (CHX + E_2) or ethanol vehicle (CHX) was added, and the cells were maintained for 6 h. RNAs were extracted and analyzed by Northern blot analysis. (—), ethanol vehicle only.

the decline in PI-9 mRNA on removal of E_2 from the culture medium. After removal of E_2 from the culture medium, PI-9 mRNA levels declined rapidly, returning to near basal levels in 24 h (Fig. 4B), indicating that continuous exposure to estrogen is required for maintaining the induced level of PI-9 mRNAs. Inhibition of protein synthesis with cycloheximide resulted in superinduction of PI-9 mRNA, a phenomenon observed with numerous mRNAs (27, 28). Cycloheximide did not block the estrogen-mediated induction of PI-9 mRNA. When E_2 and cycloheximide were both present, PI-9 mRNA levels were high and were approximately the levels expected if E_2 and cycloheximide exhibited additive effects (Fig. 4C). The unusually rapid induction of PI-9 mRNA and the inability of cycloheximide administration to block induction indicate that the induction of PI-9 mRNA is a direct or primary effect of estrogen.

Estrogen Does Not Stabilize PI-9 mRNA—In addition to its widely studied ability to increase rates of gene transcription, estrogen can act posttranscriptionally to alter mRNA stability (29). To examine the decay of PI-9 mRNA in the presence and absence of estrogen, we employed the most widely used method for analyzing mRNA degradation, inhibition of transcription with actinomycin D. After actinomycin D treatment, the rate of PI-9 mRNA decay was the same when the cells were maintained in the presence and absence of estrogen (Fig. 5). Since actinomycin D can sometimes induce artifacts in the measurement of mRNA decay rates (30, 31), it was important to confirm these data using another inhibitor of RNA synthesis. Estrogen also failed to alter the half-life of PI-9 mRNA when 100 μ M 5,6-dichlorobenzimidazole riboside was used to inhibit RNA synthesis (data not shown). We therefore conclude that estrogen does not stabilize PI-9 mRNA.

Estrogen Induces Transcription of PI-9—Although cellular PI-9 mRNA is strongly estrogen-inducible and the data of Fig. 5 indicates that estrogen does not increase the stability of PI-9 mRNA, it was important to directly test the idea that estrogen induces PI-9 gene transcription. We therefore screened a human phage library, isolated genomic PI-9 clones, and sequenced the promoter region. To determine the transcription initiation site, we carried out an initial primer extension analysis, which suggested that PI-9 mRNA contained a long ~480-

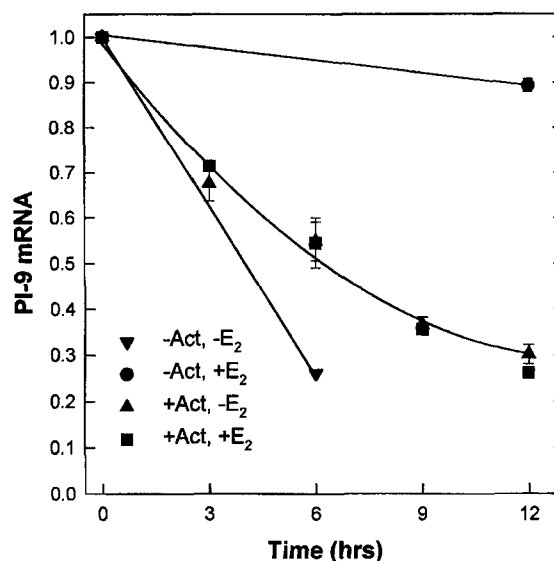


FIG. 5. **Estrogen does not alter the degradation rate of PI-9 mRNA.** PI-9 mRNA was preinduced by treating the HepG2-ER7 cells with 20 μ M E_2 for 72 h, the cells were rinsed three times and maintained in 20 μ M E_2 , 20 μ M E_2 plus 4 μ M actinomycin D, or 4 μ M actinomycin D alone. RNA was isolated at the indicated times, analyzed by Northern blotting using an actin internal standard, and quantitated by PhosphorImager analysis. A single line is drawn through the + E_2 + actinomycin D (squares, + E_2 , +Act) and - E_2 + actinomycin D (triangles, E_2 , +Act) points as the data points overlap. The data represent the average of three independent experiments plus or minus the S.E., except for the minus estrogen point (- E_2 , -Act), which is the average of two experiments.

nucleotide 5'-untranslated region. The unusually high GC content of the proposed 5'-untranslated region made it impossible to carry out primer extension using primers closer to the putative start site (data not shown). We therefore identified a candidate transcription start site by comparison to consensus transcription initiation elements and confirmed its identity by RT-PCR using 5' primers just upstream and just downstream of the putative start site. The putative transcription initiation

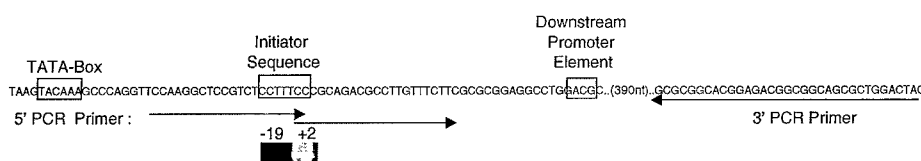


FIG. 6. **Localization of the PI-9 transcription start site.** Reverse transcription was carried out as described under "Experimental Procedures." 25% of the reverse transcriptase reaction was used for PCR using either primer -19 (upstream of the putative transcriptional start site) or primer +2 (downstream of the start site). The visualized band is correctly sized at ~470 base pairs. The three sequence elements characteristic of a transcriptional start site are boxed. The consensus sequence for the elements specifying a start site are: TATA, TATAAA; initiator, PyPy AN AT PyPy, where Py is pyrimidine; DPE, GACG.

region contained all three elements known to specify a eukaryotic transcription start site, a TATA box, an initiator element, and a downstream promoter element (DPE) (boxed sequences, Fig. 6). The TATA box and the initiator sequence differ from their respective consensus sequences by one nucleotide, and the DPE contains the consensus GACG sequence. In addition, the spacing of these three elements strongly suggests that they are functional. The TATA box and initiator are 27 nucleotides apart, and the DPE is 32 nucleotides downstream of the initiator. Since functional vertebrate initiator elements are usually 25–30 nucleotides downstream of the TATA box (32) and functional DPEs are 28–34 nucleotides downstream of the initiator (32), the TATA box, initiator, and DPE are correctly spaced to form a functional PI-9 initiation site.

To confirm the location of the initiation site, we used a method we described previously (33) in which PCR is carried out with primers just upstream and just downstream of the candidate site. If the transcription start site has been correctly identified, the sequence corresponding to the upstream primer will not be reverse-transcribed into cDNA and will not yield a PCR product. PCR using the downstream primer, which should hybridize to a transcribed sequence, should result in a PCR product. As predicted from the sequence data, a strong band of the correct size PCR product was obtained with a primer corresponding to nucleotides +2 to +22 (the numbering is derived from the position of the initiator element), and there was no detectable PCR product using a 5' primer corresponding to nucleotides -19 to +2 (Fig. 6). Taken together, the sequence and spacing of the initiation site elements and the PCR data localize the transcription start site to this region.

To determine whether moxestrol-liganded ER induced transcription from the PI-9 promoter, we prepared a construct in which a 1.8-kilobase DNA containing the PI-9 promoter region was linked to a luciferase reporter gene and carried out transfections in HepG2 cells. Expression of the reporter gene driven by the PI-9 promoter region was induced 12-fold by moxestrol-ER (Fig. 7, *Native Promoter*), clearly demonstrating that transcription of the PI-9 gene is estrogen-regulated in HepG2 cells.

Mutational Inactivation of Imperfect EREs in the 5'-Flanking Region Does Not Block Moxestrol Induction of PI-9—The PI-9 5'-flanking region lacks consensus EREs. We identified 3 imperfect EREs in the PI-9 5'-flanking region as candidate sequences that might be responsible for moxestrol-ER induction of PI-9 transcription. These imperfect EREs differed from the consensus ERE by 2 or 3 nucleotides (Fig. 7 legend). Each of the candidate EREs was mutated to a *HindIII* site. The resulting 6 or 7 mismatches out of the 10 nucleotides in the half-sites of the potential EREs would certainly destroy interaction of any of the mutated sequences with the estrogen receptor. In transient transfections, mutations of each of the EREs did not decrease overall promoter activity and had no effect on moxestrol-ER induction of the promoter (Fig. 7). Although the 3 candidate EREs are 150–200 nucleotides apart, making it unlikely that they would exhibit synergistic interac-

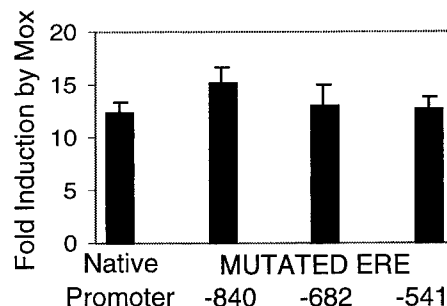


FIG. 7. **Moxestrol induces PI-9 transcription.** HepG2 cells were transiently transfected with the native PI-9 promoter-luciferase construct or with constructs in which one of the three candidate EREs was altered by mutation. Transfections contained 15 ng of CMV-hER, 25 ng of pRLSV40 internal standard, and pTZ18U carrier to 4 μ g of total DNA. Nucleotides in the candidate EREs and in the mutated EREs that differ from the consensus ERE palindrome are underlined below. The ERE sequences and the mutated EREs were: -840 ERE, GGTGAAtgaAAACC to AAGCTtgAAAAC; -682 ERE, TGTCaAatTGAAG to AAGCTatTGAAG; -541 ERE, GGTCAattTGAAA to AAGCTttTGA-AA. Fold induction represents the increase in luciferase activity for the wild-type promoter and for each mutant in the presence of 10 nM moxestrol, with the -moxestrol value set equal to 1. In the presence of moxestrol, total luciferase activity of each of the mutants was similar to the activity of the wild-type promoter. The data represent the mean $\pm$ S.E. for four separate transfections.

tions, we also mutated these EREs two at a time, in all possible combinations. The resulting double mutants also did not reduce moxestrol induction of the reporter gene (data not shown).

Moxestrol Induction of PI-9 Protein—To determine whether moxestrol induces PI-9 protein, HepG2-ER7 cells were maintained in medium containing or lacking moxestrol, total protein was isolated, and PI-9 protein levels were determined by Western blot analysis. As a positive control, we used extracts from YT cells. These NK cells contain substantial levels of PI-9 (7), which may protect them from the granzyme B they produce. Moxestrol strongly induced PI-9 protein in the HepG2-ER7 cells (Fig. 8, *HepG2 ER7*). After treatment with Moxestrol, PI-9 levels in HepG2-ER7 cells are significantly lower than levels in YT cells. The upper band in YT cells is probably the PI-9-granzyme B complex. This tight complex has been shown to persist after SDS gel electrophoresis (7).

DISCUSSION

Estrogen Induction of PI-9 Is a Rapid and Direct Effect—Because HepG2 cells rapidly metabolize 17β -estradiol (22), in most experiments we used the more slowly metabolized estrogen, moxestrol. 17β -Estradiol and moxestrol elicited similar inductions of PI-9 mRNA (compare 0 h in Fig. 4B with 10 nM MOX in Fig. 1C; and data not shown). Since moxestrol induced PI-9 mRNA in a human liver biopsy specimen and in three additional independently isolated lines of HepG2 cells stably transfected to express ER, PI-9 is an estrogen-inducible gene in human liver. The 30–40-fold estrogen induction of PI-9 mRNA in HepG2-ER7 human liver cells is unusually large. Although several estrogen-inducible human mRNAs have been identi-



FIG. 8. Moxestrol increases the level of PI-9 protein in HepG2-ER7 cells. The cells were maintained for 48 h in the absence ($-Mox$) or presence ($+Mox$) of 10 nM moxestrol, harvested, and lysed, and a whole cell extract was prepared. Protein from the HepG2-ER7 cell extract (50 μ g) or from the YT cell extract (20 μ g) were separated by SDS-PAGE and analyzed by Western blot as described under "Experimental Procedures." Comparison to molecular weight markers confirmed that PI-9 represented the main band seen in the YT cells. The lower band seen in YT cells may represent a PI-9 degradation product. GRB, granzyme B.

fied, there are only a handful of mRNAs with the constellation of properties exhibited by the PI-9 gene. The properties that make PI-9 a useful new model for regulation of gene expression by estrogen include the following. The ability of PI-9 to inhibit the apoptotic activator, granzyme B, gives PI-9 a known biological role; PI-9 is inducible in a human tissue, and the induction of PI-9 can be studied in cultured human cells; PI-9 is a primary or direct estrogen-regulated gene; induction of PI-9 mRNA is both rapid and rapidly reversible; PI-9 mRNA exhibits unusually large, order of magnitude increases in levels in response to estrogens. These properties of PI-9 gene expression combined with the ability of PI-9 protein to protect cells against granzyme B-mediated apoptosis (7) establish the PI-9 system as an unusually attractive human model for studies of gene regulation by estrogen.

Estrogen Induces PI-9 Transcription—The rapidity with which estrogen induces PI-9 mRNA and the failure of cycloheximide to block induction demonstrated that the induction of PI-9 mRNA is a direct or primary effect of estrogen. To identify the mechanism by which estrogens induce PI-9 mRNA, we carried out two types of studies. Studies using RNA synthesis inhibitors indicated that estrogens did not alter the half-life of PI-9 mRNA, suggesting that regulation of PI-9 gene expression was transcriptional. To study PI-9 gene expression more directly, we isolated genomic clones of PI-9, identified the region of transcription initiation, and used transient transfections to show that moxestrol strongly induced expression of a construct containing the PI-9 promoter regions linked to a luciferase reporter gene. Although the 5'-flanking region of the PI-9 gene contains 3 potential EREs, one of which differs from the consensus ERE by only 2 nucleotides, mutations that destroyed these candidate EREs had no effect on moxestrol induction. The robust estrogen induction of PI-9 gene transcription is therefore not mediated by upstream EREs, which represent the classical mechanism of ER action.

Potential Roles of PI-9—The high level of PI-9 in CTLs and the inhibition of granzyme B activity by PI-9 led to the recent proposal that one role of PI-9 is protection of CTLs and NK cells against apoptosis mediated by leakage of their own granzyme B from granules (7). Consistent with this possibility is the presence of high levels of PI-9 in YT cells and the presence of a putative PI-9-granzyme B complex in YT cells (Ref. 7; Fig. 5). Because the YT cells we employed did not activate a transfected ERE-containing reporter gene (data not shown) and were therefore ER negative, we were unable to evaluate the ability of estrogen to regulate PI-9 gene expression in YT cells. Analysis of commercially available RNA samples from tissues whose estrogen status is unknown showed that PI-9 mRNA is also present at high levels in lung and placenta and to a lesser extent in testis, spleen, thymus, and peripheral blood leukocytes (6, 23). One interesting question is whether the high

levels of PI-9 mRNA in human placenta (23) are due to induction of PI-9 gene expression by the high levels of estrogen to which the placenta is exposed.

CTLs and granzyme B are important mediators of graft *versus* host disease (34). However, there is as yet no information on the role of estrogen in liver transplant rejection. A more direct relationship between estrogen and apoptosis induced by CTLs exists for chronic aggressive hepatitis. Apoptosis, induced when CTLs recognize viral antigens displayed on the surface of infected cells, appears to be the major factor in the liver damage observed in chronic aggressive hepatitis (35). Iwamura (36) describes the importance of estrogen in protecting liver cells from immunologically mediated attack. A high level of serum estrogen was associated with a less severe level of hepatic injury caused by hepatitis. In a large clinical study, interferon therapy for hepatitis was far more effective in premenopausal women than in postmenopausal women (37). Higher estrogen levels were proposed as the major factor enhancing interferon therapy in premenopausal women (37). Estrogen depletion after menopause or bilateral ovariectomy tends to aggravate chronic hepatitis, even though the disease has been inactive (38). In addition, estrogen was used therapeutically to successfully treat women with chronic aggressive hepatitis, resulting in regression to the inactive state (38). Although estrogen metabolism by liver cells makes it difficult to determine the minimum concentration of estrogen in the cell culture medium that effectively induces hepatic PI-9 mRNA (Fig. 1), the levels of circulating estrogen in women during at least part of the menstrual cycle and pregnancy (39) are likely to be sufficient to induce substantial expression of hepatic PI-9.

Recently Bird and co-workers (6, 7) concluded that it was unlikely PI-9 evolved to protect against a direct hit by a CTL. Instead, they propose that PI-9 neutralizes lower levels of misdirected granzyme B that may inadvertently threaten a bystander cell or CTL. They conclude that it will be difficult to develop a test or assay for this type of indirect protection (7). If such an assay could be developed, future studies relating PI-9 regulation by estrogen to the sensitivity of liver cells to granzyme B-mediated apoptosis would shed light on the role and importance of ER in PI-9 activity and on the function of the immune system.

These studies extend the area of estrogen action to a new class of proteins important in immune system function and in apoptosis. Estrogen induction of PI-9 in human liver cells may have significant biological roles in modulating the extent of hepatic injury caused by hepatitis (viral and autoimmune) and by graft *versus* host reaction in liver transplants. Our work describes a potential point of intersection between the action of sex steroid hormones at the gene level and a key function of the immune system, induction of apoptosis.

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