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Oestrogen regulates cathepsin D mRNA levels in oestrogen responsive human breast cancer cells

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ABSTRACT

A cDNA library was constructed from the mRNA of the ZR-75 oestrogen responsive human breast cancer cell line and screened for oestrogen regulated mRNA sequences. Of the recombinants isolated, 30 contained cDNA that corresponded to a single mRNA species of 2.1 kb that was induced between 10 and 15 fold by oestradiol treatment. The sequence of the entire open reading frame and 3'non-coding region of the mRNA was determined and shown to encode the aspartyl protease cathepsin D. The induction of cathepsin D mRNA is specific for oestrogen and is maximal at 3×10^{-10} M. Cathepsin D mRNA levels were increased by oestrogen in 3 oestrogen responsive breast cancer cell lines. Cathepsin D mRNA was expressed but not regulated in an oestrogen receptor negative breast tumour cell line, BT 20, and in 2 other malignant cell lines, Hela and A431.

INTRODUCTION

The growth of human breast cancer cells is often oestrogen responsive; both in vivo and in vitro. Approximately one third of breast cancer patients show objective clinical remission following oestrogen ablation or antioestrogen therapy (1). A number of human breast cancer cell lines have been established that contain oestrogen receptor and respond to oestrogens. These cell lines provide useful model systems for studying the mechanism of action of oestrogens and antioestrogens. They may also be used to understand the mechanisms involved in the stimulation of cell proliferation by steroid hormones and to identify reliable clinical markers of oestrogen responsiveness. Since the original demonstration that the growth of the MCF_7 cell line is stimulated by oestrogens (2), several different types of responses have been characterised including the regulation of certain mRNAs (3;4), specific proteins (5;6;7) and growth factor activities (8).

To define more precisely the responses of breast cancer cells to oestrogens, we have constructed cDNA libraries from 2 oestrogen responsive breast cancer cell lines (MCF_7 and ZR-75). These cDNA libraries have been screened for oestrogen regulated mRNA sequences by differential hybridisation.

In contrast to others (3) who identified only one relatively abundant regulated mRNA from the MCF_7 cell line using this technique, we have identified several mRNAs whose levels are regulated between 2 and 15 fold by oestrogens.

In this report, we show that one of the mRNAs regulated by oestrogens in the ZR-75 cell line codes for the lysosomal aspartyl protease Cathepsin D.

METHODS

Cell Culture

The MCF_7 (9), ZR-75 (10), T47D (11) and BT 20 (12) cell lines were maintained as described previously (5). The Hela (13) and A431 (14) cell lines were maintained in Dulbecco's modified Eagle's medium containing 5% foetal calf serum.

Cells were withdrawn from steroids and then stimulated as described previously (4) except that medium lacking phenol red was used and charcoal treated newborn calf serum was used instead of charcoal treated foetal calf serum.

RNA Preparation

Total cellular RNA, total cytoplasmic RNA and cytoplasmic poly(A)+ RNA was prepared as described previously (4).

cDNA Cloning

The ZR-75 cDNA library was constructed from 5ug of poly(A)+ RNA isolated from cells grown continuously in 10% foetal calf serum as described elsewhere (4). Briefly, cDNA was synthesised using reverse transcriptase and E. coli DNA polymerase. Following treatment of the cDNA with S1 nuclease, it was tailed with dG residues and annealed with dC tailed Kpn 1 digested pUC19. E. coli strain JM105 were transformed with the annealed DNA and colonies containing recombinant plasmids identified by plating on agar containing IPTG and X-gal. The cDNA library was screened as described previously (4).

Electrophoresis and Transfer of RNA to Nitrocellulose

RNA samples were denatured using formaldehyde (15) and then electrophoresed through a 1.2% agarose gel containing 2.2 M formaldehyde in 10 mM sodium phosphate (pH 7) for 400 volt hours. The gel was stained with ethidium bromide for 30 minutes and visualised on a transilluminator to ensure the integrity and equal loading of the RNA. Following equilibration for 2 x 30 minutes each in 20 x SSC, the RNA was transferred to nitrocellulose (16). Filters were baked at 80°C.

Labelling of DNA and Hybridisation

Radiolabelled probes were synthesised either by nick-translation (17) or

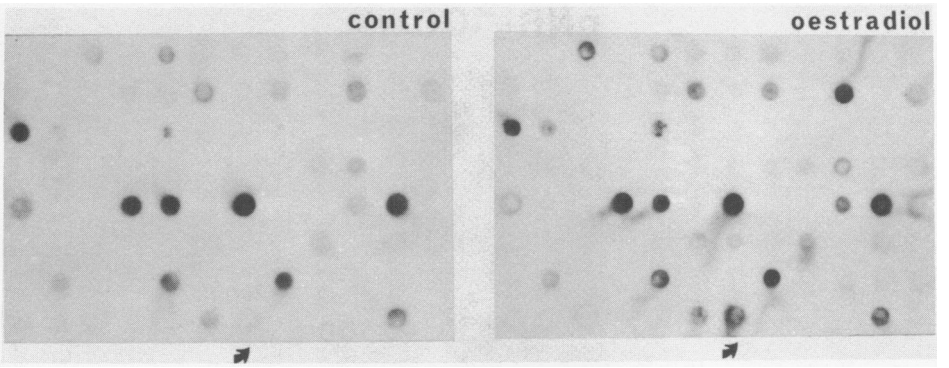

Figure 1. Detection of recombinants containing oestrogen regulated sequences. Bacterial colonies were grown in two identical arrays on nitrocellulose, lysed and immobilised. The duplicate filters were then hybridised either with ^{32}P-cDNA prepared from poly(A)+ RNA of withdrawn (A) or oestrogen treated (B) cells. Hybridised cDNA was detected by autoradiography. Arrows show a recombinant that hybridises more strongly to cDNA prepared from poly(A)+ RNA from oestrogen treated cells.

by transcription of cDNA fragments from pNR-100 clone 6 that had been inserted between promoters for SP6 and T7 polymerase in the plasmid pGEM 1 (18). Specific activities of at least 5 x 10^8 cpm per microgram for the DNA probes and 10^9 cpm per microgram for the RNA probes were obtained. Hybridisation was as described previously (19) for the nick-translated probes in prehybridisation and hybridisation solutions containing 50% formamide. In the case of RNA probes, the prehybridisations and hybridisations were at 65°C in solutions containing 50% formamide. The filters were washed at 65°C in 2 x SSC if hybridisation was with the nick-translated probe and at 75°C in 0.1 x SSC if hybridsation was with the RNA probe. The amount of radiolabelled probe hybridised was quantified by densitometric scanning of the autoradiographs.

RESULTS

Isolation of Oestrogen Regulated mRNAs from ZR-75 Cells

A cDNA library of 15,000 independant recombinants was constructed from mRNA prepared from oestrogen stimulated ZR-75 cells. The library was screened for oestrogen regulated mRNA sequences by differential screening using cDNA synthesised from mRNA that had been prepared from withdrawn and oestrogen treated cells. An example of the differential hybridisation obtained is shown in figure 1. Recombinants that hybridised differently to the 2 probes were

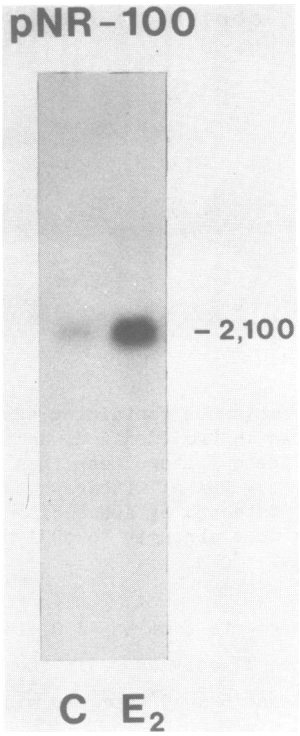

Figure 2. Oestrogen regulates a 2,100 nucelotide mRNA in ZR-75 cells. 10ug of RNA prepared from withdrawn and oestrogen treated ZR-75 cells were separated by electrophoresis and transferred to nitrocellulose. pNR-100 clone 2 (see Fig. 3) was labelled with ^{32}P by nick-translation and hybridised to the immobilised RNA. The size of the mRNA is shown on the right in nucleotides.

rescreened and then plasmid DNA was prepared, labelled by nick-translation and hybridised to Northern transfers of total RNA prepared from withdrawn and oestrogen treated cells.

Of the 15,000 recombinants screened, 46 hybridised more strongly to RNA prepared from oestrogen treated cells. Thirty recombinant plasmids hybridised to a mRNA of approximately 2,100 nucleotides that was induced 10-12 fold by oestrogen. The hybridisation of one such recombinant is shown in figure 2. The 2,100 nucleotide RNA was designated pNR-100.

Other recombinant plasmids were identified that hybridised to oestrogen regulated mRNAs of different sizes. For example, 10 recombinants hybridised to a mRNA of 600 nucleotides and 4 recombinants hybridised to a mRNA of 1,800 nucleotides. These mRNAs will be described in more detail elsewhere.

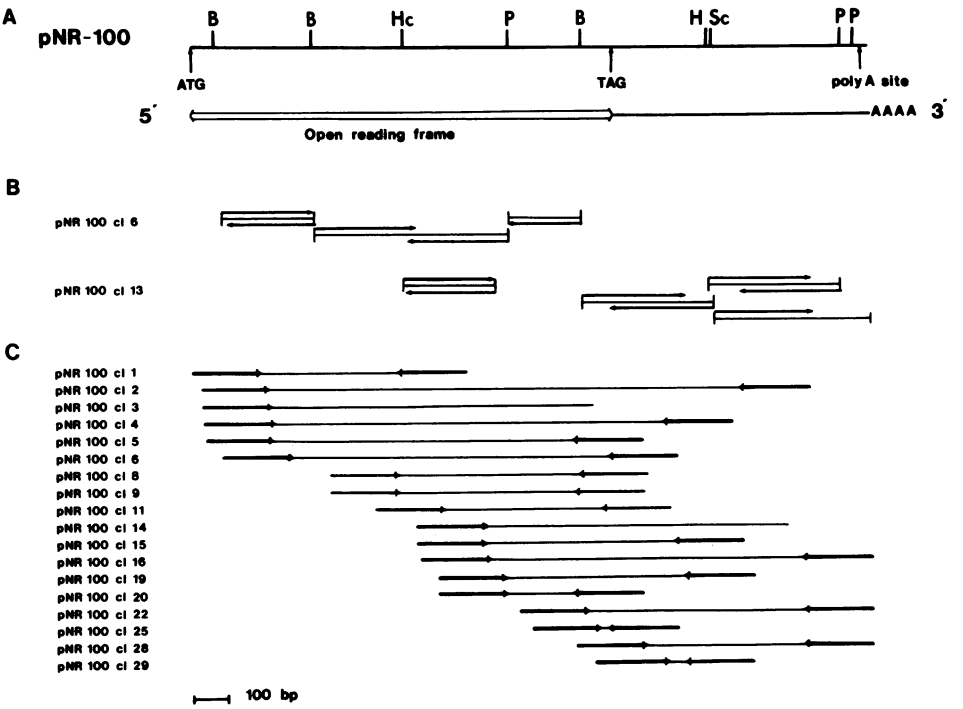

Figure 3. Sequencing strategy for pNR-100. A. Restriction map of pNR-100 mRNA. B, Bam HI; Hc, Hinc II; P, Pst I; H, Hind III; Sc, Sac I. The positions of the initiation (ATG), termination (TAG) and polyadenylation (poly A) sites are marked. The extent of the open reading frame is indicated below the restriction map. B, M13 recombinants used to generate single stranded templates for sequencing by chain termination: the bars represent the fragments of pNR-100 clone 6 or clone 13 subcloned into M13 mp 18 or mp 19 and the arrows above and below indicate the length and direction of nucleotide sequence obtained. C. Nucleotide sequence obtained by plasmid sequencing of pNR-100 recombinants: the lines show the part of the pNR-100 sequence contained within each recombinant. The arrows show the length of the nucleotide sequence obtained.

Restriction maps of the cDNA inserts of the 30 recombinant plasmids hybridising to the 2,100 nucleotide mRNA were then obtained. The maps showed that the recombinants were derived from a single mRNA species and that they together covered approximately 2,000 nucleotides. The restriction map is shown in figure 3A.

Sequencing the pNR-100 mRNA

The nucleotide sequence of the mRNA was then obtained by sequencing the cDNA inserts. Figure 3 shows the sequencing strategy. Seven restriction

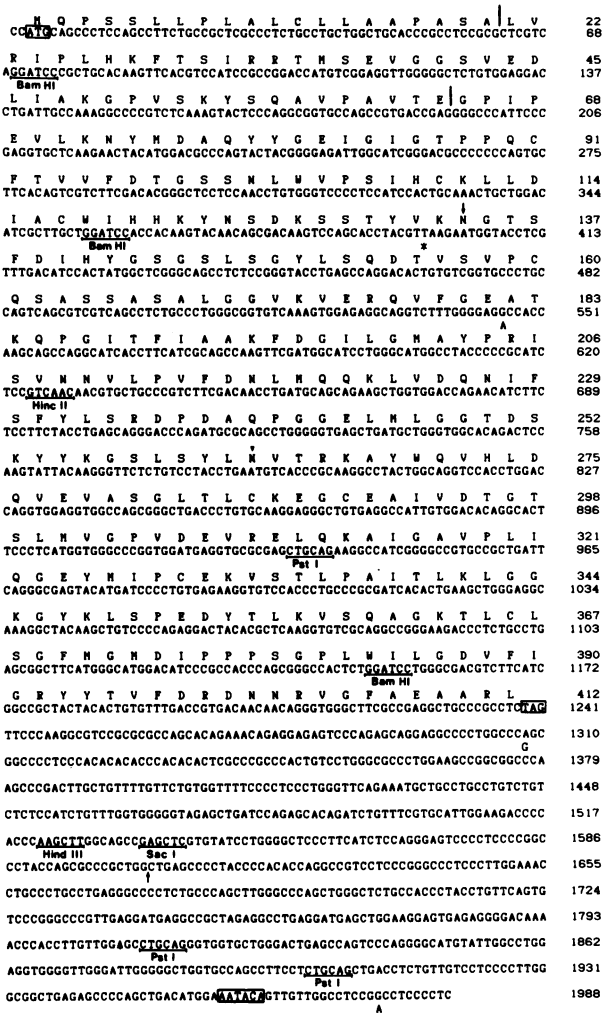

Figure 4. Nucleotide and deduced amino acid sequence of the pNR-100 RNA. The nucleotide sequence is shown on the bottom line and the deduced amino acid sequence (single letter code) of the major open reading frame is shown above. The initiation (ATG) and termination (TAG) codons of the open reading frame are boxed as is the putative polyadenylation signal, approximately 30 nucleotides from the 3' end of the mRNA. Possible polymorphisms identified during the sequencing of different recombinants are shown below the nucleotide sequence. The asterisk (position 398) and upward pointing arrow (position 1,604) mark the two differences between the pNR-100 sequence and human cathepsin D sequence reported by Faust et al (22). Bars mark the putative junctions between the end of the signal sequence and the beginning of the pro segment, and the end of the pro segment and the start of the mature protein. Downward pointing arrows mark the two potential glycosylation sites. The positions of the restriction sites shown in Fig. 3 are indicated.

fragments from two recombinants (pNR-100 clone 6 and pNR-100 clone 13) were subloned into M13 vectors and sequenced. This provided the sequence of the majority of the mRNA. Eighteen recombinants, derived from different regions of the mRNA sequence were used for plasmid sequencing using the 17bp and reverse sequence M13 primers. This provided a series of overlapping sequences from the ends of the cDNA inserts. The combination of the two techniques allowed almost the entire mRNA to be sequenced in both directions.

The pNR-100 sequence is shown in figure 4. The orientation of the mRNA was established by the presence of poly(A) tracts in some of the recombinants. An AUG codon was present at the extreme 5' end of the cloned mRNA sequence and was in frame with a long open reading frame of 412 amino acids. The methionine is presumed (see below) to be the initiation codon. The pNR-100 mRNA has a long 3' noncoding region of 748 nucleotides. Three possible polymorphisms were identified within the mRNA sequence. At position 547 an A was found instead of a G in one of the 4 recombinants sequenced. A G was found instead of an A at position 1,308 in 1 out of 7 recombinants and an A was found instead of a G at residue 1,979 in 1 out of 3 recombinants. Only the first is found within the open reading frame and would result in a threonine being substituted for an alanine. A second, shorter open reading frame of 330 nucleotides was also identified. It extends from an AUG at position 1,430 to a UGA termination codon at position 1,762.

The nucleotide and deduced amino acid sequences were then used to search for identical or related sequences in a commercially available database (20). The deduced amino acid sequence showed significant homology to a number of aspartyl proteases including renin, chymosin and pepsin. The homology varied from 40 to 50%. The highest degree of homology, however, was to porcine cathepsin D (21). This is an intralysosomal aspartyl protease, whereas renin, chymosin and pepsin are secreted proteases. The homology with porcine Cathepsin D was 86% between amino acids 65 and 410 (equivalent to positions 1 and 339 in the porcine sequence). Subsequent comparison of the pNR-100 sequence with the human cathepsin D sequence (22) confirmed that the pNR-100 mRNA sequence encodes human cathepsin D.

The 5' end of the cloned sequence starts with an AUG codon which is the initiation codon in the cathepsin D mRNA sequence reported by Faust et al (22). Apart from the possible polymorphisms listed above, there were two differences from the sequence of Faust et al (22). T was replaced by G at position 398 but this does not result in a change to the protein sequence. In the 3' noncoding

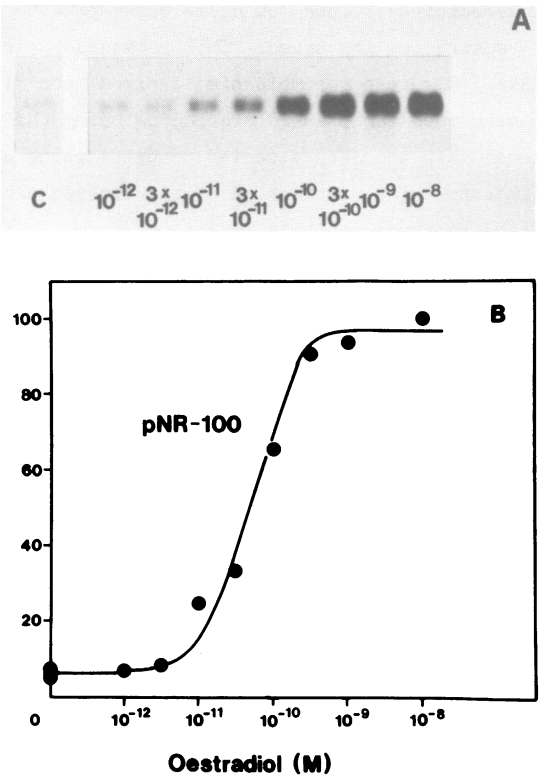

Figure 5. Effects of different concentrations of oestradiol on cathepsin D mRNA levels. ZR-75 cells were withdrawn for 5 days and then stimulated for 2 days with a range of oestradiol concentrations between 10^{-12} and 10^{-8} M. Total RNA was prepared and 2ug was separated by gel electrophoresis and then ransferred to nitrocellulose. The filter was hybridised with ^{32}P-labelled probe synthesised as described in the methods from a fragment of pNR-100 clone 6 inserted into the pGEM 1 vector. A. shows an autoradiograph of the filter and B. the amount of radiolabelled probe hybridised, as quantified by densitometry.

region, there are two G's at position 1,603-1,604 whereas there were 3 reported by Faust et al (22).

The amino acid sequence deduced from the short open reading frame between nucleotides 1,430 and 1,762 is not related to any protein sequence in the data base.

Characteristics of the Induction of Cathepsin D mRNA by Oestradiol

The concentration of oestradiol required to induce cathepsin D mRNA was determined. ZR-75 cells were withdrawn from oestradiol and them stimulated using a range of concentrations of oestradiol from 10^{-12} M to 10^{-8} M. Total

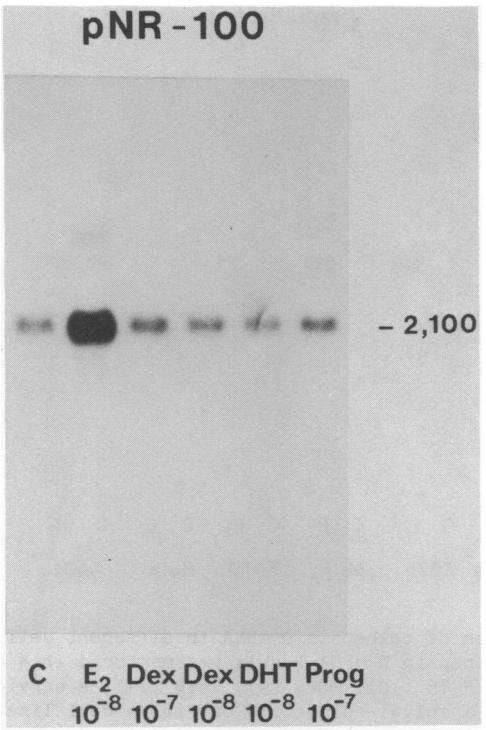

Figure 6. Effects of different steroids on the induction of cathepsin D mRNA. ZR-75 cells were withdrawn for 5 days and then stimulated for 2 days with oestradiol, 10^{-8} M, (E_2); dexamethasone, 10^{-7} and 10^{-8} M, (Dex); 5α-dihydrotestosterone, 10^{-8} M, (DHT); progesterone, 10^{-7} M, (Prog). Total RNA was prepared, and 2ug was separated by gel electrophoresis and then transferred to nitrocellulose. The filter was hybridised with a ^{32}P-labelled probe synthesised as described in the methods from a fragment of pNR-100 clone 6 inserted into the pGEM 1 vector. The size of the mRNA hybridising is shown on the right in nucleotides.

RNA was prepared, separated by electrophoresis and transferred to nitrocellulose. A ^{32}P-labelled RNA probe, synthesised from a fragment of pNR-100 clone 6 inserted into the pGEM 1 vector, was hybridised to the filter. The autoradiograph is shown in figure 5 together with a graph showing the quantitation of these results by scanning densitometry. Oestradiol increased the levels of cathepsin D mRNA at concentrations above 10^{-11} M. The increase is maximal at a concentration of 3×10^{-10} M. At this concentration the mRNA is increased 15 fold over the level in withdrawn cells.

The effects of different classes of steroid on the induction of cathepsin D mRNA were then determined (Fig. 6). ZR-75 cells were withdrawn from

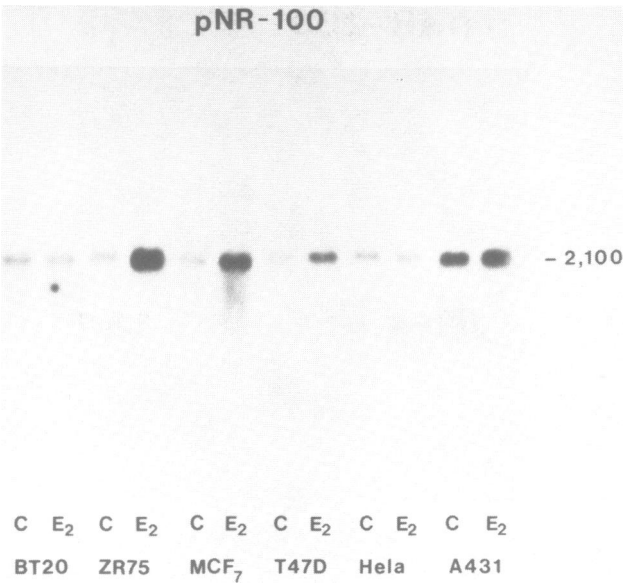

Figure 7. Regulation of cathepsin D mRNA in different cell lines. The effect of oestradiol on cathepsin D mRNA levels was measured in 4 breast cancer cell lines, BT 20 (12), ZR-75 (10), MCF_7 (9), T47D (11), a cervical carcinoma cell line, Hela (13) and a vulval epidermoid carcinoma cell line, A431 (14). In all cases, cells were withdrawn from oestradiol for 5 days and stimulated with 10^{-8} M oestradiol for 2 days. Total RNA was prepared and 5ug was separated by gel electrophoresis and then transferred to nitrocellulose. The filter was hybridised with ^{32}P-labelled probe synthesised as described in the methods from a fragment of pNR-100 clone 6 inserted into the vector pGEM 1.

oestrogen and then treated with oestradiol (10^{-8} M), the synthetic glucocorticoid dexamethasone (10^{-7} and 10^{-8} M), the androgen 5α-dihydrotestosterone (10^{-8} M) and progesterone (10^{-7} M). In this experiment the medium containing progesterone was changed twice rather than once daily as it has been reported that progesterone is metabolised by breast cancer cells (23). Total RNA was prepared from the cells and the cathepsin D mRNA levels determined as for the previous experiment. Oestradiol was the only steroid to increase cathepsin D mRNA levels. In this experiment, oestradiol increased cathepsin D mRNA levels eight fold whereas none of the other steroids increased the cathepsin D mRNA by more than 10%.

Expression and Regulation of Cathepsin D mRNA in other Cancer Cell Lines

To establish whether the regulation of cathepsin D mRNA levels by ostrogen is restricted to the ZR-75 cell line, mRNA levels were measured in two other oestrogen responsive cell lines ((MCF_7 and T47D), one oestrogen receptor

negative cell line (BT 20), and two cell lines established from other human malignancies (Hela and A431). The results of these experiments are shown in figure 7. Oestradiol increased cathepsin D mRNA levels in all three oestrogen receptor positive cell lines (9 fold in the ZR-75 cell line, 7 fold in the MCF_7 cell line and 3 fold in the T47D cell line). The cathepsin D mRNA levels in the BT 20 cell line are similar to those found in the withdrawn oestrogen responsive cell lines, but oestradiol had no effect on the levels. Similar results have also been obtained with the oestrogen receptor negative MDA-MB 231 breast cancer cell line (unpublished data).

Oestradiol did not affect the levels of cathepsin D mRNA in two cell lines derived from malignant cervical and vulval epithelial cells. In Hela cells, the mRNA level was similar to the levels found in the withdrawn breast cancer cell lines whereas in the A431 cell line, the level was about half that of oestrogen stimulated ZR-75 cells.

DISCUSSION

In this report, we have shown that cathepsin D mRNA levels are regulated by oestradiol in oestrogen responsive breast cancer cell lines. This regulation appears to be a specific oestradiol mediated response, as other classes of steroids do not affect cathepsin D mRNA levels. Moreover, the induction is probably mediated by the oestrogen receptor as concentrations of 10^{-10} - 10^{-9}M maximally stimulate cathepsin D mRNA levels. These concentrations are similar to those required to fully occupy the oestrogen receptor of ZR-75 and MCF_7 cells (10).

Cathepsin D is a lysosomal aspartyl endopeptidase (24) that has been identified in and purified from a wide variety of tissues. While its normal biological function is thought to be the catabolism of cellular proteins, it may also play a role in tissue remodelling processes such as metamorphosis in amphibia (25).

The biological significance of the regulation of cathepsin D by oestradiol is unclear. Oestrogen may regulate cathepsin D levels in normal mammary epithelial cells and this regulation could be required during proliferation or involution of mammary epithelial cells during lactation. Alternatively, cathepsin D levels may not be significantly regulated in normal mammary epithelial cells but only become regulated in malignant epithelial cells perhaps as a result of the increase in the oestrogen receptor levels that is postulated to occur during preneoplasia.

The activity and biosynthesis of cathepsin D is regulated by steroids in

the rat uterus (26) and it has been postulated that this regulation is involved in processes such as blastocyst implantation and post partum involution of the uterus. Although both progestins and oestrogens increase cathepsin D in the rat uterus, the effect of progesterone is relatively specific while the increase following oestrogen treatment parallels the general increase in protein synthesis (26). The regulation of cathepsin D in the rat uterus therefore differs from its regulation in human breast cancer cell lines.

Work by ourselves (5) and others (3;6;7;8; 27) has previously attempted to identify oestrogen regulated proteins in breast cancer cells. Such proteins might provide useful markers of oestrogen responsiveness and may be useful for studying the mechanism of action of oestrogens and antioestrogens. Some of the regulated proteins correspond to known transforming growth factors (8), proteins related to known growth factors (28), proteases (27, 29) or steroid receptors (7), whereas the identity of others such as the 24K (6) and the 160K (30) proteins is unknown. The 46K oestrogen regulated secreted protein(referred to also as 50K and 52K) may be cathepsin D. Cathepsin D can be secreted (31) and it has been reported that the 46K protein has protease activity with an acidic pH optimum (29). The molecular weight of the pro-cathepsin D protein, deduced from its sequence, is 42,608. This would probably be increased to approximately 46,000 by the addition of two N-linked oligosaccharide chains. However, the amino acid composition of the 46K protein has recently been reported (32) and is significantly different from the amino acid composition deduced from the cathepsin D sequence and the measured amino acid composition of human cathepsin D prepared from liver (33). Notably the 46K protein has fewer glycine and more valine, leucine and isoleucine residues than human cathepsin D. Furthermore, cathepsin D is found in a wide variety of cell types, whereas the expression of 46K protein is much more limited (34). Thus, the relationship between the two proteins remains to be established.

A further reason for identifying oestrogen regulated mRNAs in oestrogen responsive breast cancer is that such RNAs may encode proteins that are involved in mediating the effects of oestrogens on malignant breast epithelial cells. The synthesis and secretion of proteases such as plasminogen activator (27), capthepsin B (35) and MEP (major excreted protein) (36) are elevated in transformed cells. The finding that cathepsin D is regulated by oestrogens and the observation that cathepsin D can be secreted by breast cancer cells (31) raises the possibility that the increased cathepsin D levels in the presence of oestrogen contribute to an increased growth or dissemination of tumour cells by acting on the extra-cellular matrix (37).

It has also been reported that cathepsin D stimulates DNA synthesis and mitosis in the rat liver (38). It is therefore possible, that cathepsin D could directly alter the proliferation of tumour cells. Such a hypothesis may now be tested using transfection techniques to modify cathepsin D expression in breast cancer cells.

The regulation of cathepsin D in human breast cancer cells may provide a versatile experimental system for studying the mechanism of oestrogen action. Cloned genes may be manipulated and reintroduced into responsive cells in culture, and cathepsin D is one of the few oestrogen responsive genes that has been cloned from these cells. Cathepsin D is also expressed but not regulated both in a number of oestrogen receptor negative breast cancer cell lines and in cell lines derived from other types of tumour. The factors required to confer oestrogen responsiveness may therefore be identified by introducing putative regulatory molecules such as the oestrogen receptor, into cultured cells expressing cathepsin D.

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