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## INTRODUCTION

The proposed studies in this research grant focused on a Estrogen Receptor (ER) upregulated gene, Prothymosin  $\alpha$  (PT $\alpha$ ). PT $\alpha$  is a small highly acidic protein found in the nuclei of virtually all mammalian tissues. Its high conservation in mammals and wide tissue distribution suggest an essential biological role. While the exact mechanism of action of PT $\alpha$  remains elusive, the one constant has been its relationship with the proliferative state of the cell and its requirement for cellular growth and survival. Recently PT $\alpha$  was found to promote transcriptional activity by sequestering the anticoactivator, REA from the Estrogen Receptor (ER) complex. We observed that Estradiol (E<sub>2</sub>) upregulates PT $\alpha$  mRNA and protein expression. Further studies indicate that ER $\alpha$  regulates PT $\alpha$  gene transcriptional activity. The specific aims proposed in this grant addressed the functional relevance of PT $\alpha$  in breast cancer gene expression and proliferation and the mechanism of regulation of PT $\alpha$  by the ER.

## BODY

We have completed Task 1 and about 70% of Task 2 of the original proposal. These studies also resulted in the attached publication in the journal Oncogene. Some of our findings are described in the enclosed manuscript published in the journal Oncogene (Bianco and Montano, 2002). Major accomplishments described in this publication include (1) mutational analyses to further delimit the fragment required for Estrogen Receptor (ER) binding to and transcriptional activation of the PT $\alpha$  gene promoter (2) further verification of the role of PT $\alpha$  in estrogen-induced breast epithelial cell proliferation using quantitative double immunocytochemistry.

The role of PT $\alpha$  as a transcriptional regulator and other cellular function can be accomplished by further characterizing the PT $\alpha$ -interacting clones we have identified in the yeast two hybrid screenings. Sequence of one of the putative PT $\alpha$ -interacting clones indicates that its identity is Elongation Factor 1  $\beta$  (EF1 $\beta$ ). This factor is involved in the proposed link between translational control and cell growth regulatory pathways. We verified the interaction of EF1 $\beta$  with PT $\alpha$  using *in vitro* glutathione-S-transferase (GST)-pull down assays). The affinity column for these assays consisted of PT $\alpha$  expressed as a fusion protein with GST bound to a Glutathione-Sepharose beads. *In vitro* translated and radiolabeled EF1 $\beta$  was retained in the column indicating a direct interaction between EF1 $\beta$  and PT $\alpha$ . EF1 $\beta$  was not retained in a column consisting only of GST.

On the last year of funding we have redone the yeast two hybrid screenings and have identified another putative PT $\alpha$ -interacting clone. The interaction was verified *in vitro* using GST-pull down assays. The clone encodes a protein, CGI-48, of unknown function. Further studies are necessary to determine the functional implications of the interaction of PT $\alpha$  with clones isolated from yeast two hybrid screenings

As an alternative and also to verify findings from yeast two hybrid screenings we proposed to use mass spectrometry technology. A proteomics core facility in Cleveland Clinic is available to further characterize the multiprotein complex wherein PT $\alpha$  exist. It is likely that associated proteins are an integral part of the function of PT $\alpha$  and will be a key to understanding their biological function. We proposed to use conventional chromatography to reduce non-specific proteins by first enriching target proteins prior to

immunoaffinity purification. We will identify associated proteins using antibody-affinity chromatography. We have an antibody for PT $\alpha$  for use in affinity chromatography. After purification of protein complexes, the identity of associated proteins will be determined by mass spectrometry technology. A limiting factor in this experiment has been the quality of our PT $\alpha$  antibody. On the last year of funding we found that it may not be of sufficient quality for affinity chromatography as we were not able to immunoprecipitate. Thus further optimization of our immunoprecipitation procedure is required or another antibody may have to be generated.

Please note that we did not feel the need to examine the effectiveness of PT $\alpha$  antibody injections in inhibiting PT $\alpha$  activity and compare to antisense methodology as we have been able to inhibit PT $\alpha$  expression using antisense retroviruses. Antibody injections, which are technically very challenging, were proposed as an alternative approach should antisense technology not work. We also did not feel it was necessary to determine whether suppression of PT $\alpha$  expression results in loss of myc-dependent growth of MCF7 cells as our studies indicate that ER regulation of PT $\alpha$  is direct and does not require c-myc.

## **KEY RESEARCH ACCOMPLISHMENTS**

**A. The following were accomplished and reported in the Oncogene manuscript.**

Task 1. Determined the mechanism of regulation of Prothymosin  $\alpha$  gene transcriptional activity by the estrogen receptor (ER)

- constructed reporter constructs containing deletion mutants of the regulatory region of the PT $\alpha$  gene
- examined the activities of deletion mutant reporter constructs in MCF breast cancer cells in transfection assays
- biochemical analyses of the interactions of MCF7 breast cancer cell factors with the PT $\alpha$  gene regulatory regions

Task 2. Determined the functional importance of estrogen-mediated increase in Prothymosin  $\alpha$  expression on the mitogenic effects of estrogens in breast cancer cells

- determined time course for maximal induction of PT $\alpha$  expression by estrogens
- demonstrated that antisense retroviruses can inhibition of PT $\alpha$  expression
- determined whether suppressing PT $\alpha$  expression results in partial or complete loss of estrogen-dependent growth of MCF7 cells

**B. The following were accomplished but the data were not published:**

- identified PT $\alpha$  interacting clones using the yeast two hybrid system
- verified interactions using *in vitro* protein-protein interaction assays

**C. The following were not completed:**

- examine the effect of inhibition of PT $\alpha$  expression on the induction of reporter activity in MCF7 cells that also contain either serum responsive element (SRE)-, TPA-responsive element (TRE), or cAMP responsive element (CRE)-LacZ indicator plasmids

- further characterize clones isolated from yeast genetic screenings

### REPORTABLE OUTCOMES

Bianco NR and **Montano MM.** (2002) Transcriptional regulation of Prothymosin alpha gene by the Estrogen Receptor: Molecular mechanisms and functional implications. Oncogene, 21:5233-5244

### CONCLUSIONS

We have shown estrogen-stimulated gene transcription from PT $\alpha$  gene promoter-containing reporter constructs. We have delimited the region required for estrogen-mediated induction to a 43-bp fragment, and the mechanism for ER $\alpha$ -mediated activation most likely involves a complex interplay between ER $\alpha$  and other protein factors bound to this region. The transcriptional activation by estrogens appears to involve ER $\alpha$  binding to the PT $\alpha$  gene promoter. Estrogen treatment also resulted in increased PT $\alpha$  nuclear localization which in turn is correlated with increased cell proliferation. Our studies also indicate that PT $\alpha$  plays a role in E<sub>2</sub>-induced proliferation of breast cancer cells.

While the exact details of the mechanism of action of PT $\alpha$  remain elusive, it is clearly involved in the regulation of cell transformation and proliferation. Future studies on the PT $\alpha$  interacting proteins that we have identified should prove useful in further defining the biological role of PT $\alpha$ . The estrogen receptor (ER) is a ligand activated transcription factor and the identification of "primary" ER target genes is imperative for

understanding the basis for the proliferative action of ER in breast cancer cells. There are only a few candidate genes that appear to be under the direct regulation of the ER; much less genes that are associated with cell proliferative activity. PT $\alpha$  is a particularly strong candidate because its expression and intracellular localization appears to be regulated by estrogen, and down-regulation of PT $\alpha$  expression inhibits E<sub>2</sub>-induced breast cancer cell proliferation.

#### **PERSONNEL SUPPORTED BY THE GRANT**

Principal Investigator: Monica Montano, Ph.D.

## Regulation of prothymosin $\alpha$ by estrogen receptor $\alpha$ : molecular mechanisms and relevance in estrogen-mediated breast cell growth

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**Prothymosin  $\alpha$  (PT $\alpha$ ) is a small highly acidic protein found in the nuclei of virtually all mammalian tissues. Its high conservation in mammals and wide tissue distribution suggest an essential biological role. While the exact mechanism of action of PT $\alpha$  remains elusive, the one constant has been its relationship with the proliferative state of the cell and its requirement for cellular growth and survival. Recently PT $\alpha$  was found to promote transcriptional activity by sequestering the anticoactivator, REA from the Estrogen Receptor (ER) complex. We now report that Estradiol (E<sub>2</sub>) upregulates PT $\alpha$  mRNA and protein expression. Further studies indicate that ER $\alpha$  regulates PT $\alpha$  gene transcriptional activity. We have also delimited the region of PT $\alpha$  gene promoter involved in ER $\alpha$ -mediated transcriptional regulation and identified a novel ER $\alpha$ -binding element. Increased intracellular PT $\alpha$  expression in the presence of estrogens is accompanied by increased nuclear/decreased cytoplasmic localization. Increased nuclear expression of PT $\alpha$  is correlated with increased proliferation as measured by expression of Ki67 nuclear antigen. Conversely, inhibition of nuclear PT $\alpha$  expression in breast cancer cells using antisense methodology resulted in the inhibition of E<sub>2</sub>-induced breast cancer cell proliferation. Overall these studies underscore the importance of PT $\alpha$  in estrogen-induced breast cell proliferation. *Oncogene* (2002) 21, 5233–5244. doi:10.1038/sj.onc.1206545**

**Keywords:** estrogen; estrogen receptor  $\alpha$ ; prothymosin  $\alpha$

### Introduction

The estrogen receptor (ER) protein is essential for mediating the actions of estrogen in target tissues. The binding of estrogen initiates a process of receptor activation that includes the high affinity binding of ER to specific DNA sequences, termed estrogen response elements (EREs). The interaction of ER with EREs results in the modulation of specific gene expression, through which the physiological actions of estrogens

are manifested (reviewed in Aranda and Pascual, 2001). Estrogens acting via the ER dramatically escalate proliferative and metastatic activity in breast tumor cells, in part via the induction of growth factors, proteases, and basement membrane receptors (reviewed in Russo and Russo, 1998). However, the relative role of the induction of these genes on the proliferative effects of estrogens in breast cancer cells is not well-defined.

Prothymosin  $\alpha$  (PT $\alpha$ ) is a small highly acidic protein found in the nuclei of virtually all mammalian tissues (Clinton *et al.*, 1991; Gomez-Marquez and Segade, 1988; Goodall *et al.*, 1986; Manrow *et al.*, 1991; Palvimo and Linnala-Kannkunen, 1990; Watts *et al.*, 1989). Its high conservation in mammals and wide tissue distribution suggest an essential biological role. PT $\alpha$  expression correlates well with the proliferative activity of tissues (Eschenfeldt and Berger, 1986; Gomez-Marquez *et al.*, 1989; Rodriguez *et al.*, 1998; Sburlati *et al.*, 1991; Wu *et al.*, 1997). Recently, PT $\alpha$  was shown to be capable of transforming rodent fibroblast cells in a manner similar to Ras, suggesting that PT $\alpha$  may be an important downstream target for inducers of cellular transformation (Orre *et al.*, 2001). In breast cancer PT $\alpha$  appears to have some prognostic value. PT $\alpha$  expression is higher in tumor samples than in normal breast tissue (Tsitsilonis *et al.*, 1998), and the expression levels of PT $\alpha$  can be correlated with the proliferation status and metastatic potential of tumors (Magdalena *et al.*, 2000; Tsitsilonis *et al.*, 1998).

While PT $\alpha$  appears to play a role in cell proliferation, PT $\alpha$  also has an emerging role in the regulation of transcription. Recently Martini *et al.* (2000) reported that PT $\alpha$  is involved in the transcriptional repression by the anticoactivator factor, Repressor of Estrogen Receptor Activity (REA). PT $\alpha$  is able to promote ER transcriptional activity by sequestering REA from the ER complex. In addition the PT $\alpha$  protein has been localized in the nucleus (Clinton *et al.*, 1991; Manrow *et al.*, 1991; Palvimo and Linnala-Kannkunen, 1990; Wu *et al.*, 1997) and studies suggest that PT $\alpha$  binds specifically to linker histone H1 and cooperates in nucleosome assembly (Diaz-Jullien *et al.*, 1996; Gomez-Marquez and Rodriguez, 1998; Karetsov *et al.*, 1998), implicating a putative nuclear function related to chromatin remodeling. A role for PT $\alpha$  in the transcriptional activation process is supported by studies wherein cells

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overexpressing PT $\alpha$  exhibit more active chromatin and higher rates of transcriptional activity than control cells (Karetsov *et al.*, 1998).

The intracellular signaling pathways governing PT $\alpha$  expression are not well-defined. PT $\alpha$  mRNA levels are increased after serum restitution or after stimulation with various mitogens (Pineiro *et al.*, 2000; Zalvide *et al.*, 1992). Thus far, only two transcription factors have been proposed to positively regulate the PT $\alpha$  gene promoter, E2F (Eilers *et al.*, 1991; Szabo *et al.*, 1993) and *c-myc* (Desbarats *et al.*, 1996; Gaubatz *et al.*, 1994). PT $\alpha$  expression has been proposed to be under the direct control of *c-myc* through a DNA element known as the E-box, however other findings do not confirm this observation (Mol *et al.*, 1995). Studies indicate that *c-myc* expression is not necessary for PT $\alpha$  expression (Loidi *et al.*, 1999). It has been reported that the transcription factor elongation factor (E2F) which is involved in the regulation of genes important in DNA replication and cell cycle regulation also activates PT $\alpha$  gene transcriptional activity (Eilers *et al.*, 1991; Szabo *et al.*, 1993). PT $\alpha$  has also been reported to be negatively regulated by p53, further supporting its potential role in proliferation of the cell (Zhao *et al.*, 2000). The PT $\alpha$  protein is phosphorylated although the physiological relevance of this finding is not known (Perez-Estevez *et al.*, 1997; Sburlati *et al.*, 1993).

We report that estrogens upregulate PT $\alpha$  mRNA and protein levels in breast cancer cells. Estradiol upregulates PT $\alpha$  gene transcription and we have delimited a novel region involved in this transcriptional activation. Our results indicate that ER $\alpha$  is part of the transcriptional complex that binds to PT $\alpha$  gene promoter and suggest direct transcriptional regulation of this gene by ER $\alpha$ . PT $\alpha$  induction by estrogens appears to be involved in estrogen induction of breast cancer cell proliferation.

## Results

### Identification of PT $\alpha$ as an estrogen-regulated gene

Shown in Figure 1a (left panel) is a Northern blot using the human PT $\alpha$  cDNA as a probe. We found that PT $\alpha$  1.4 kb mRNA is present at 2–3-fold higher levels in estradiol (E<sub>2</sub>)-treated cells when compared to control cells 24 h after treatment. No increase in PT $\alpha$  mRNA was observed in the presence of the antiestrogen ICI182,780 (ICI). Upregulation of PT $\alpha$  by E<sub>2</sub> was evident even with cycloheximide pretreatment, suggesting that intervening protein synthesis is not required. The increase in PT $\alpha$  mRNA in response to estrogens is reflected at the protein level wherein we also observe a 2–3-fold increase (Figure 1a, right panel).

### Induction of Prothymosin $\alpha$ gene transcriptional activity by estrogens

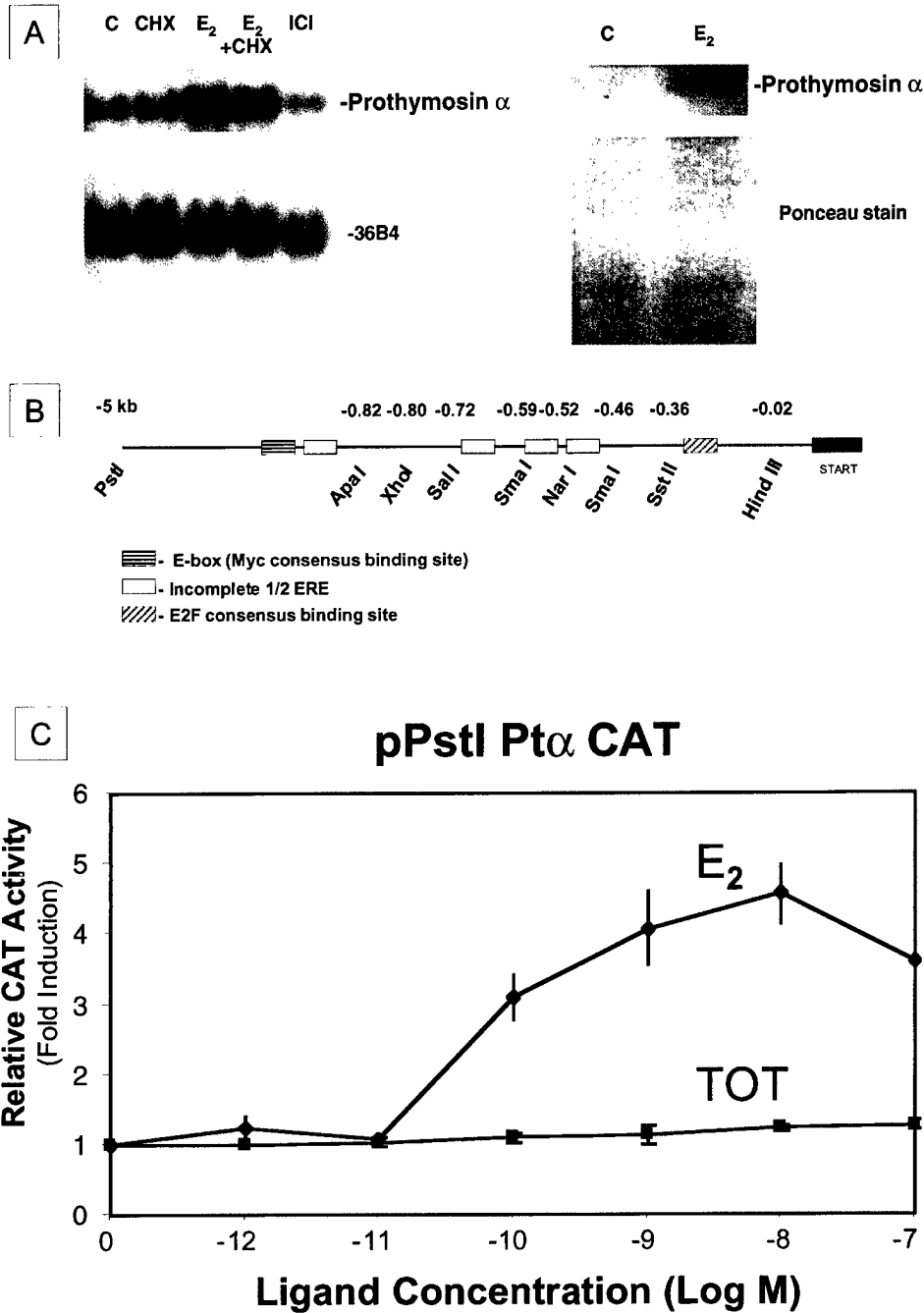
To determine if estrogen regulation of PT $\alpha$  expression occurs at the transcriptional level a reporter construct containing the 5' regulatory region of the PT $\alpha$  gene,

pPstI PT $\alpha$  CAT (Figure 1b) (Szabo *et al.*, 1993) (Mol *et al.*, 1995), was transfected into an ER positive breast cancer cell line MCF7 (Figure 1c). After introduction of pPstI PT $\alpha$  CAT, cells were treated with increasing concentrations of E<sub>2</sub> and the antiestrogen trans-hydroxytamoxifen (TOT). Analyses of reporter activity reveal a dose dependent increase in PT $\alpha$  gene transcriptional activity in response to E<sub>2</sub> but not TOT (Figure 1c).

The role of ER $\alpha$  in the transcriptional regulation of the PT $\alpha$  gene was examined in two ER $\alpha$  negative cell lines transfected with an expression vector for wild type ER $\alpha$ . A significant increase in transcriptional activity of the pPstI PT $\alpha$  CAT reporter construct in the presence of estradiol (E<sub>2</sub>) was observed in human breast cancer MDA-MB-231 cells when cells were cotransfected with an expression vector for ER $\alpha$  (Figure 2a). A slightly higher activation of PT $\alpha$  gene transcriptional activity by estrogens was evident in Hec-1B human endometrial cancer cells when compared to MDA-MB-231 cells. TOT did not activate transcription from the PT $\alpha$  gene reporter construct in any of these cell lines. No increase in the activity of the control pCAT3 promoter vector was observed with E<sub>2</sub> or TOT (Figure 2a).

### Identification of PT $\alpha$ gene promoter regions required for activation by estrogens

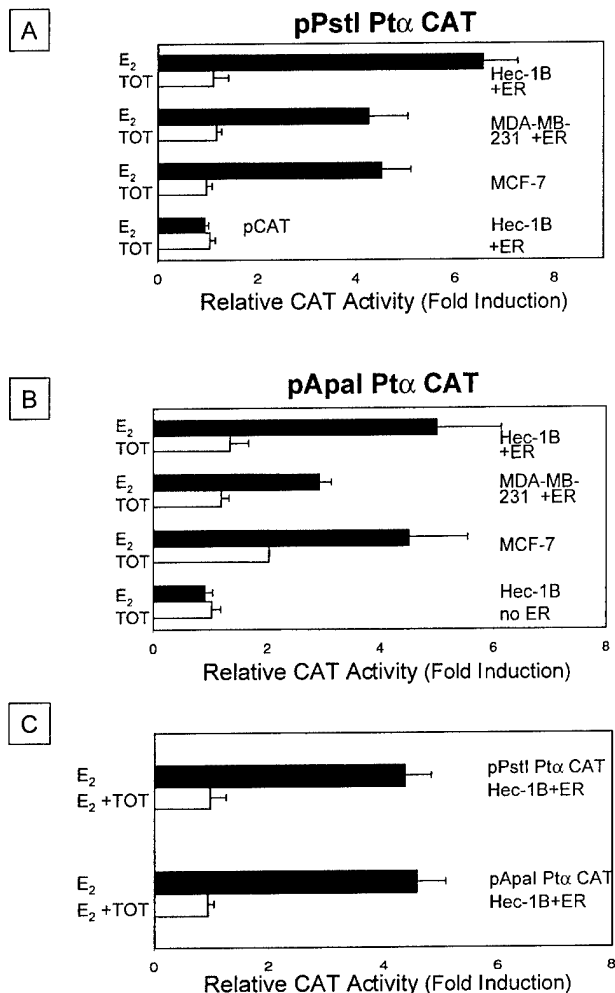
To determine the promoter region(s) involved in estrogen activation of PT $\alpha$  gene transcriptional activation, a reporter construct containing the 5' regulatory region of the PT $\alpha$  gene from the *ApaI* site located –0.819 kb from the transcriptional start site, pApaI PT $\alpha$  CAT, was introduced into MCF7, MDA-MB-231, and Hec-1B cells. Specifically we wanted to determine if the E-box located at –1.173 kb of the PT $\alpha$  gene promoter was required for E<sub>2</sub>-induced increase in PT $\alpha$  gene transcriptional activity (Figure 1b). Our results indicate that E<sub>2</sub> induced an increase in pApaI PT $\alpha$  CAT activity and the region between –5 and –0.819 kb of the human PT $\alpha$  gene is not required for transcriptional induction by E<sub>2</sub> (Figure 2b). Note that there were no differences in the basal activities of the pPstI PT $\alpha$  CAT and pApaI PT $\alpha$  CAT constructs (data not shown). Similar observations were made in the three cell lines examined. These results suggest that the induction of PT $\alpha$  gene transcriptional activity by E<sub>2</sub> is not mediated through the E-box in the –5 kb promoter region of the PT $\alpha$  gene. Thus it is unlikely that estrogen-stimulated increase in PT $\alpha$  expression is exerted through increased expression of *c-myc* in response to estrogens (Dubik and Shiu, 1988) and binding of *c-myc* to the E-box in this region. Cells transfected with the empty control pCMV vector (lacking the ER $\alpha$  cDNA) did not show an E<sub>2</sub>-mediated increase in pApaI PT $\alpha$  CAT activity (Figure 2b). The antiestrogen TOT blocked E<sub>2</sub>-mediated induction of both reporters (Figure 2c). These findings suggest a requirement for the ER $\alpha$  in E<sub>2</sub>-mediated transcriptional activation of PT $\alpha$  gene activity.



**Figure 1** Regulation of PT $\alpha$  gene expression by estrogens at the transcriptional level. (a) Left panel; Total RNA was collected from MCF7 parental cells 24 h after treatment with control ethanol vehicle (C), 4 h pretreatment with cycloheximide, (CHX,  $10^{-5}$  M), estradiol (E<sub>2</sub>,  $10^{-8}$  M), E<sub>2</sub> ( $10^{-8}$  M)+CHX pretreatment, or ICI 182,780 (ICI,  $10^{-7}$  M) as indicated. Equal amounts (20  $\mu$ g) of total RNA were separated by electrophoresis. The blot was probed with the random primer labeled PT $\alpha$  cDNA. As an RNA loading control, the same blot was reprobed with 36B4 cDNA. Right panel: Western blot analyses of PT $\alpha$  protein levels in MCF7 cells in the absence (C) or presence of E<sub>2</sub>. Whole cell lysates were collected 24 h after treatment, electrophoresed on SDS-PAGE gels, transferred to nitrocellulose filters, probed with PT $\alpha$  polyclonal antibody, and visualized using horseradish peroxidase-conjugated secondary antibody. The lower figure shows the Ponceau S-stained blot to show equal loading. The autoradiographs shown in (a) are representative of three separate experiments. (b) The PT $\alpha$  gene promoter region (c) MCF7 cells were transfected with the PT $\alpha$  gene reporter construct, PstI PT $\alpha$  CAT, along with a  $\beta$ -galactosidase internal control reporter to correct for transfection efficiency. Cells were then treated for 24 h with control ethanol vehicle (C) or varying concentrations of E<sub>2</sub> and TOT as indicated. Cell extracts were prepared and analysed for CAT activity and  $\beta$ -galactosidase activity. Values are the means  $\pm$  s.e.

Deletional analyses of the 819 bp region was performed to further delimit the region of the PT $\alpha$  promoter required for ER $\alpha$  transcriptional activation.

These experiments were conducted in Hec-1B endometrial carcinoma cells wherein we observe the highest activation from the pPstI PT $\alpha$  CAT reporter construct



**Figure 2** Activation of PT $\alpha$  CAT requires ER $\alpha$ . MDA-MB-231, MCF7 breast cancer cells and Hec-1B endometrial cancer cells were transfected with the PT $\alpha$  gene reporter construct, (a) PstI PT $\alpha$  CAT or (b) ApaI PT $\alpha$  CAT, along with an expression vector for the wild type human estrogen receptor. pCAT vector (lacking PT $\alpha$  promoter) and cmv5 vector (lacking ER cDNA) were used as controls. In (c) Hec-1B cells were transfected with ER $\alpha$  expression vector along with the PstI PT $\alpha$  CAT or ApaI PT $\alpha$  CAT reporter vectors. To normalize for transfection efficiency, cells were transfected with a  $\beta$ -galactosidase internal control reporter. Cells were then treated for 24 h with control ethanol vehicle (c), E<sub>2</sub> ( $10^{-8}$  M) and/or TOT ( $10^{-7}$  M) as indicated. Cell extracts were prepared and analysed for CAT activity and  $\beta$ -galactosidase activity. Values are the means  $\pm$  s.e. from three or more separate experiments

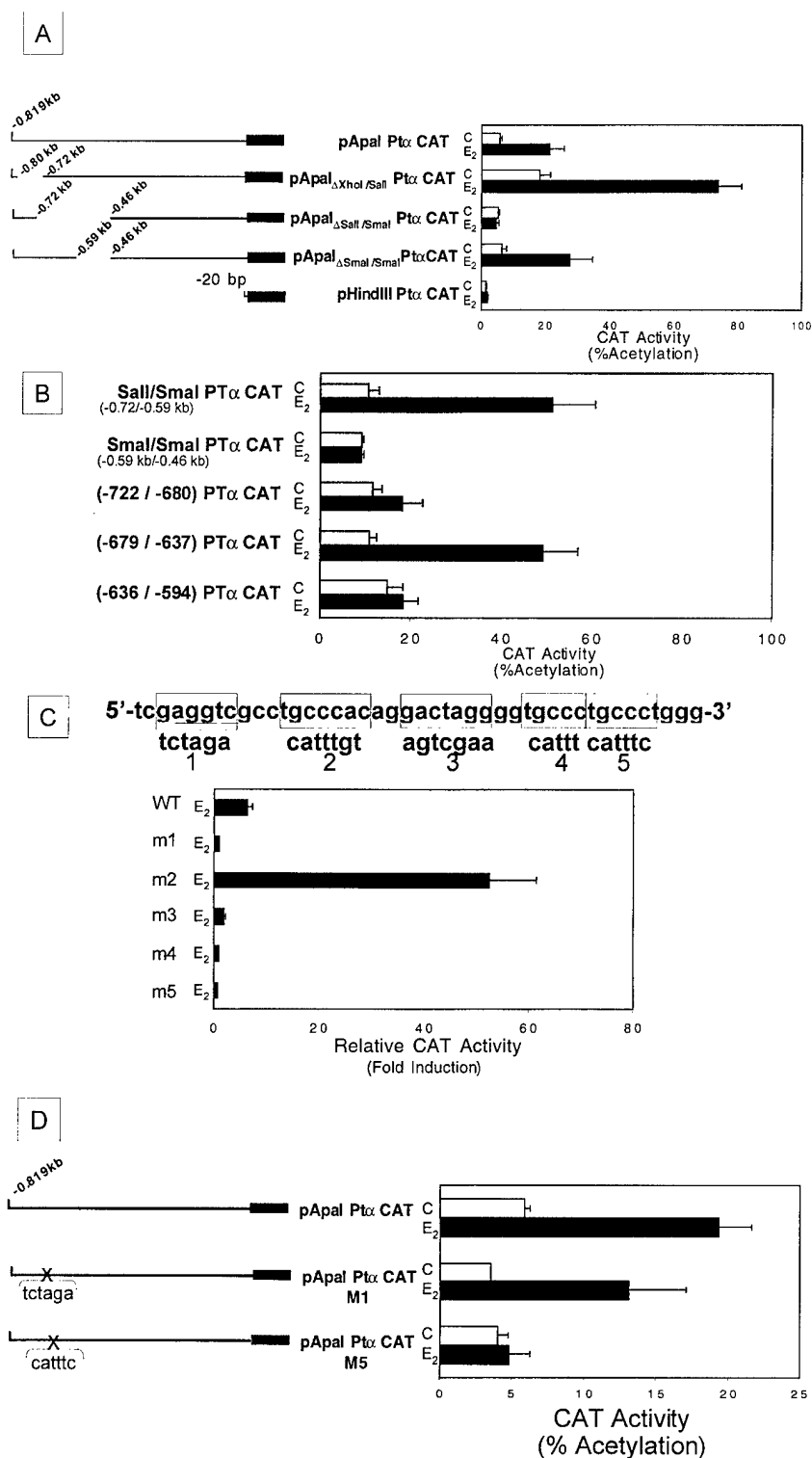
among the three cell lines examined. Using several pApaI PT $\alpha$  CAT deletion mutants we identified a 260 bp fragment flanked by the *SalI* ( $-722$  bp) and *SmaI* ( $-460$  bp) sites as important for estrogen induction of PT $\alpha$  promoter activity (Figure 3a). While deletion of the *XhoI/SalI* fragment significantly increased basal reporter activity, E<sub>2</sub> was still able to induce reporter activity about fourfold over basal activity. When the fragment encompassed by *SalI/SmaI* was broken into two parts, *SalI/SmaI* ( $-0.72/-0.59$  kb) *SmaI/SmaI* ( $-0.59/-0.46$  kb), and introduced into the pCAT3 promoter vector, E<sub>2</sub>-induced

CAT reporter activity only in the *SalI/SmaI* ( $-0.72/-0.59$  kb) fragment (Figure 3b). The fold-induction from the p*SalI/SmaI* PT $\alpha$  CAT cannot be attributed to change in basal activity as this parameter was not significantly different among the pPstI PT $\alpha$  CAT, pApaI PT $\alpha$  CAT, and p*SalI/SmaI* PT $\alpha$  CAT reporter constructs.

The *SalI/SmaI* ( $-0.72/-0.59$  kb) region of the PT $\alpha$  gene was divided into three 43 bp fragments and oligonucleotides were synthesized corresponding to these fragments. Oligonucleotides were cloned into the pCAT3 reporter vector upstream of the heterologous SV40 promoter. The reporter constructs were transfected into Hec-1B cells along with an expression vector for ER $\alpha$ . Similar fold-inductions with E<sub>2</sub> were observed with the ( $-679/-637$ )-PT $\alpha$  CAT reporter constructs as that observed with the pPstI PT $\alpha$  CAT reporter (Figure 3b). No significant induction over basal activity by E<sub>2</sub> was evident with reporter constructs containing the other two fragments,  $-722/-680$  and  $-636/-594$ . Of note, there were no differences in basal activity between the *SalI/SmaI* fragment and each of the 43 bp fragments it contains.

Sequence analysis of the  $-679/-637$  fragment indicates no consensus sequence for binding sites for any known transcription factors except for an incomplete half-ERE at  $-677$  to  $-672$  (Figure 3c). While there are reports of ER binding to half-EREs, there are also reports of binding of other transcription factors to the half-ERE that results in the regulation of ER-mediated gene transcription (Chen *et al.*, 1998; Garnier *et al.*, 1997; Klinge *et al.*, 1997). Also of interest are three repeats of a TGCCC element in this region, one next to the half-ERE and two sequentially ordered at the 3' end (Figure 3c). To analyse the role of the incomplete half-ERE, these six residues were mutated, introduced into the pCAT3 vector (mut 1) and transfected into Hec-1B cells along with an expression vector for ER $\alpha$ . In these experiments we observe no activation of reporter activity upon mutation of the half-ERE (Figure 3c). Mutations introduced  $-659$  to  $-639$  (mut 3, 4 and 5) also disrupted E<sub>2</sub> fold-induction. The magnitude of response to estrogens did not change with mut 2, however a significant decrease in basal activity was observed resulting in a higher fold-induction relative to wild type PT $\alpha$   $-679/-637$  fragment. Our mutational analyses also indicate that the two TGCCC elements at the 3' end are important for estrogen activation.

To further verify the importance of this novel estrogen response element, we introduced mutations 1 and 5 into the context of the natural PT $\alpha$  ApaI promoter. This would allow us to better assess the physiological relevance of this element on the natural promoter. When pApaI PT $\alpha$  pCAT (mut 1) and pApaI PT $\alpha$  pCAT (mut 5) were transfected into Hec-1B cells, the E<sub>2</sub> responsiveness was completely blocked with mut 5 but not mut 1 (Figure 3d). Thus the 3' end of the PT $\alpha$   $-679/-637$  fragment is

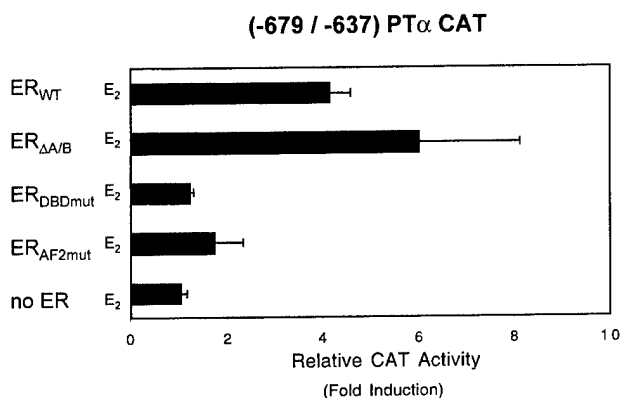


**Figure 3** Identification of PT $\alpha$  gene promoter regions involved in activation by estrogens. (a) The PT $\alpha$  gene reporter construct, pApal PT $\alpha$  CAT, or deletion mutants were transfected into Hec-1B cells along with an expression vector for the ER $\alpha$ . (b) PT $\alpha$  gene reporter constructs containing the region encompassed by *Sall/SmaI*, *SmaI/SmaI*, or fragments of the *Sall/SmaI* regions were transfected into Hec-1B cells along with an expression vector for the ER $\alpha$ . (c) Wild type and mutant (-679/-637)-PT $\alpha$  CAT reporter constructs were transfected into Hec-1B cells along with an expression vector for ER $\alpha$ . The half-ERE is underlined in the wild type sequence. The corresponding mutant is underneath the wild type sequence. (d) Wild type and mutant Apal PT $\alpha$  CAT reporter constructs were transfected into Hec-1B cells along with an expression vector for ER $\alpha$ . In (a), (b), (c), and (d) cells were treated for 24 h with control ethanol vehicle (C) or E $_2$  ( $10^{-8}$  M) as indicated. Cell extracts were prepared and analysed for CAT activity and  $\beta$ -galactosidase activity. Each value represents the mean of three or more separate determinations  $\pm$  s.e.m.

important for estrogen activation. While the incomplete half-ERE may be important for transcriptional activation in the context of a heterologous SV40 promoter, it does not appear to be necessary in the context of the natural promoter.

*Identification of ER $\alpha$  functional domains involved in activation of PT $\alpha$  gene transcription*

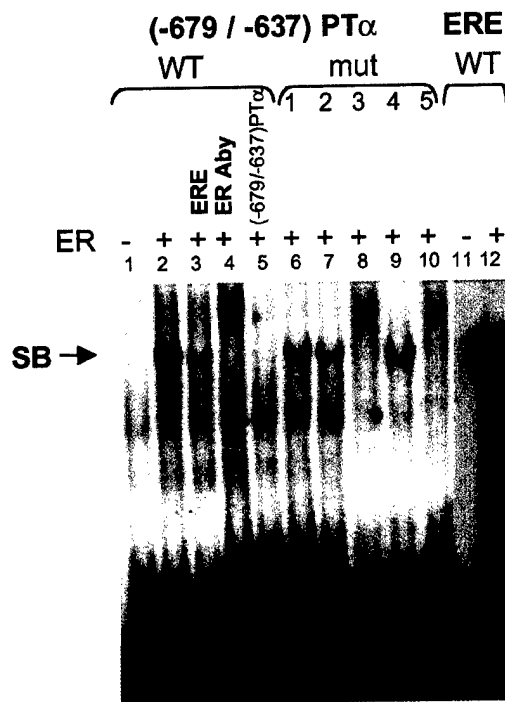
Further insight into the mechanism of ER $\alpha$  regulation of PT $\alpha$  gene transcriptional activation can be obtained from studies identifying the functional domains of the ER $\alpha$  required for PT $\alpha$  gene transcriptional activation. We examined the ability of ER $\alpha$  mutants with impaired activation function, hormone binding ability, or DNA binding ability to activate PT $\alpha$  gene reporter constructs. These experiments were conducted in Hec-1B cells where low levels of endogenous ER $\alpha$  allowed us to assess mutant ER $\alpha$  function. No activation of the (-679/-637)-PT $\alpha$  CAT reporter construct was observed with expression vectors for ER $\alpha$  with mutations in the DNA binding domain and AF2 region (Figure 4). ER $\alpha$  with three mutations in the DNA binding domain (HE82) that converts DNA binding specificity from an ERE to Glucocorticoid Receptor Response Element (GRE) (Mader *et al.*, 1989) did not induce enhancer activity. However deletion of the A/B domain, which has been shown to disrupt Activation Function 1 (AF-1) of the ER (Ali *et al.*, 1993) did not significantly affect the ability of ER $\alpha$  to mediate activation of the PT $\alpha$  gene promoter reporter construct. Our data suggest that the DNA binding and AF2 domains, but not the AF1 region, are required for transcriptional activation of PT $\alpha$  gene.



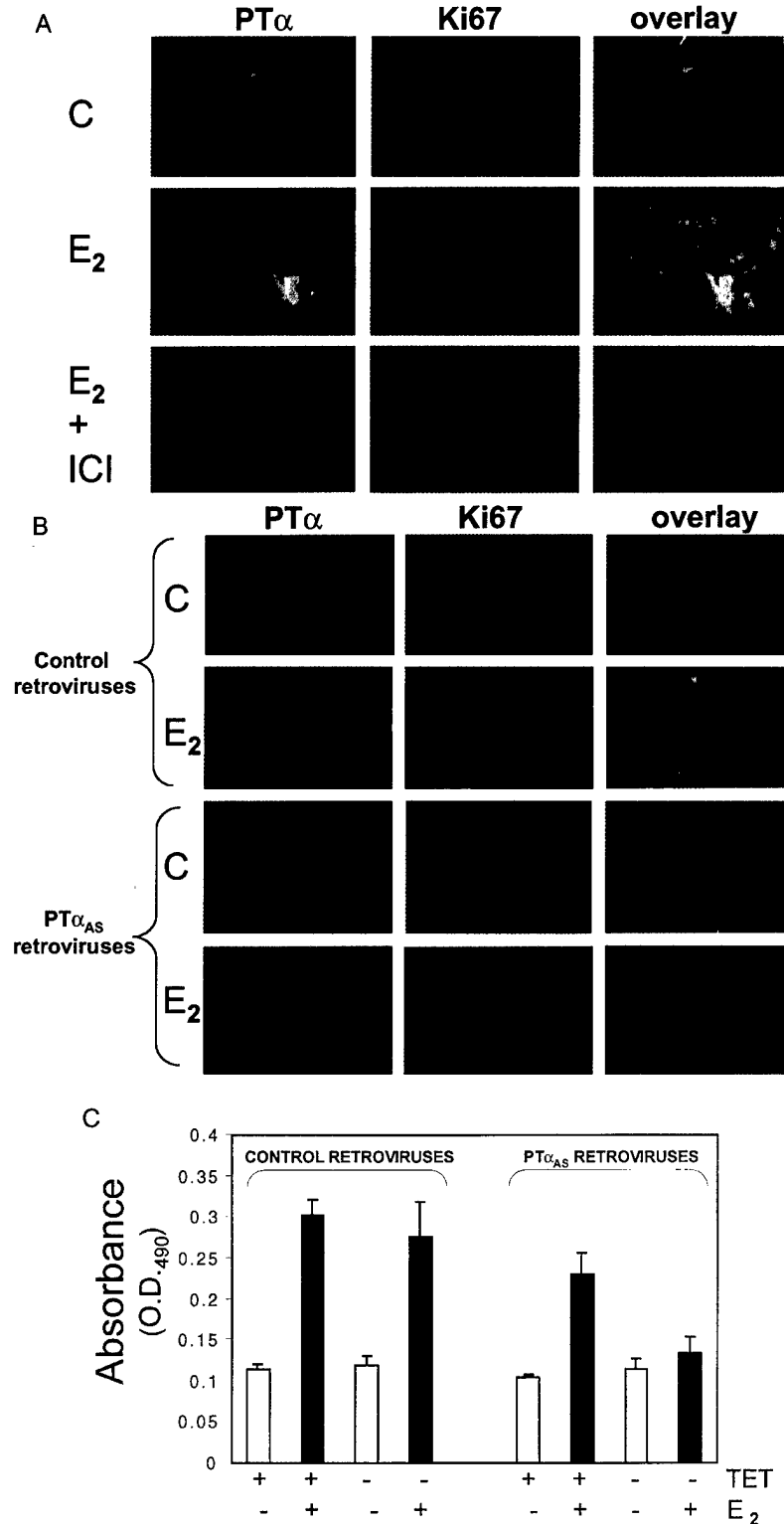
**Figure 4** Identification of functional domains of ER $\alpha$  involved in the activation of PT $\alpha$  gene transcriptional activity. The PT $\alpha$  gene reporter construct, (-679/-637)-PT $\alpha$  CAT, along with expression vectors for wild type or mutant ER $\alpha$  were transfected into Hec-1B cells. Cells were then treated for 24 h with control ethanol vehicle (c) or E<sub>2</sub> (10<sup>-8</sup> M) as indicated. Cell extracts were prepared and analysed for CAT activity and  $\beta$ -galactosidase activity. Each value represents the mean of three or more separate determinations  $\pm$  s.e.m.

*Biochemical analyses of interactions of MCF7 breast cancer cell factors with the Prothymosin  $\alpha$  gene promoter region*

Gel shift assays were then conducted to dissect the DNA-protein complex(es) that occur within the 43 bp region that mediate ER $\alpha$  regulation. <sup>32</sup>P end-labeled (-679/-637)-PT $\alpha$  oligonucleotides were incubated with purified recombinant ER $\alpha$ . A representative autoradiograph is shown in Figure 5 and indicates the presence of one major DNA-protein complex as a shifted band (SB). The specificity of the DNA-protein interactions was verified using competitive gel shift assays with unlabeled (-679/-637)-PT $\alpha$  oligonucleotide. The DNA-protein complex was supershifted by ER $\alpha$  antibody and could be competed off by unlabeled consensus ERE. Thus estrogen transcriptional activation appears to be mediated through ER $\alpha$  binding to PT $\alpha$  gene promoter elements. However, ER $\alpha$  binding to the PT $\alpha$  promoter element is much weaker than ER $\alpha$  binding to the ERE (Figure 5, lanes 11-12).



**Figure 5** Identification of elements involved in ER $\alpha$  binding to PT $\alpha$  gene fragment (-679/-637). Gel mobility shift assays were performed using a double stranded oligomer containing the wild type or mutant -679/-637 region of the human PT $\alpha$  gene along with purified ER $\alpha$  protein. Wild type and mutant <sup>32</sup>P(-679/-637)-PT $\alpha$  were incubated with purified recombinant ER $\alpha$  (lanes 2-10) in the absence or presence of 100-fold excess of unlabeled ERE, 100-fold excess of unlabeled -679/-637 fragment, or monoclonal ER $\alpha$  antibody. In lanes 11 and 12, <sup>32</sup>P-ERE was incubated in the absence or presence of recombinant ER $\alpha$ , respectively. Equal c.p.m. and ng amounts of <sup>32</sup>P(-679/-637)-PT $\alpha$ , <sup>32</sup>P(-679/-637)mut-PT $\alpha$ , and <sup>32</sup>P-ERE or equal amounts of recombinant ER $\alpha$  were used in the binding reactions. The autoradiographs are representative of three separate experiments



**Figure 6** Inhibition of PT $\alpha$  expression attenuates E<sub>2</sub>-induced breast cancer cell proliferation. (a) MCF7 cells were fixed for immunostaining 24 h after treatment with control ethanol vehicle (C), 10<sup>-8</sup> M E<sub>2</sub>, 10<sup>-8</sup> M E<sub>2</sub> + 10<sup>-7</sup> M ICI 182,780 as indicated. In (b) and (c) MCF7 cells were infected with control or antisense PT $\alpha$  retroviruses. Two days after infection cells were treated with vehicle or 10<sup>-8</sup> M E<sub>2</sub>. (b) Cells were fixed for immunostaining 24 h later or (c) cell number was determined 5 days later using the CellTiter 96 Aqueous One Solution Proliferation Assay. Cells were viewed under a fluorescent microscope at 200 $\times$  magnification. Background staining, as measured using control IgG, was subtracted from images. Values for cell number are expressed relative to the absorbance in control cells grown in the presence of tetracycline (which is set at 1). Values are the means  $\pm$  s.e. from two separate experiments with triplicate wells for each group

Further studies were conducted to determine the binding sites for ER $\alpha$ . Gel shift analyses indicate that ER $\alpha$  binding is disrupted upon mutations at 659/–653 (mut 3) and a TGCCC element at –645/–640 (mut 5) (Figure 5). These results are consistent with the involvement of these regions in ER $\alpha$ -mediated transcriptional activation. Mutation of the half-ERE at –677/672 did not disrupt ER $\alpha$  binding which is consistent with our observation that this element is not necessary for ER $\alpha$  activation in the context of the natural promoter. Mut 4, while not affecting ER $\alpha$  binding, is associated with decreased ER $\alpha$ -mediated activation. It is likely that this site may bind to accessory factors required for ER $\alpha$ -mediated activation.

#### *Determination of the relevance of ER $\alpha$ regulation of PT $\alpha$ transcriptional activity on breast cancer growth*

We then determined if estrogen-induced PT $\alpha$  expression can be correlated with increased proliferation. For these studies we used immunofluorescence staining to determine if the same cells that express PT $\alpha$  are also proliferating. PT $\alpha$  has been proposed to move between nuclear and cytoplasmic compartments (Enkemann *et al.*, 2000). However the relative importance of nuclear and cytoplasmic localization on its proliferative effects has not been examined. We used expression of Ki67 as a measure of proliferation status of the cells as well as a control for nuclear staining (Iatropoulos and Williams, 1996). Twenty-four hours after treatment, we see an increase in the number of cells that show primary nuclear (and decreased cytoplasmic) localization of PT $\alpha$  in the presence of estrogens, which was decreased when antiestrogen ICI182,780 was added (Figure 6a). Ki67 staining is highest in cells wherein PT $\alpha$  is primarily nuclear.

A self-contained tetracycline-regulated retroviral vector system (Paulus *et al.*, 1996) was used to express antisense PT $\alpha$  (PT $\alpha$ <sub>AS</sub>). In the presence of tetracycline, expression of PT $\alpha$ <sub>AS</sub> is inhibited. An ensuing 25 ± 7% and 48 ± 10% decrease in PT $\alpha$  expression in control and E<sub>2</sub> treated cells, respectively, was observed in MCF7 cells after infection with PT $\alpha$ <sub>AS</sub> retroviruses (Figure 6b). Infection with PT $\alpha$ <sub>AS</sub> retroviruses decreased PT $\alpha$  nuclear staining and Ki67 expression normally observed 24 h after treatment with E<sub>2</sub>. To confirm this, we also used a proliferation assay. While control cells show the expected increase in cell number after 6 days treatment with E<sub>2</sub>, cells infected with PT $\alpha$ <sub>AS</sub> retroviruses showed no increase in proliferation in the presence of E<sub>2</sub> (Figure 6c). These findings suggest that induction of PT $\alpha$  expression by E<sub>2</sub> plays a role in E<sub>2</sub>-mediated breast cancer growth induction.

#### **Discussion**

In summary, we have shown estrogen-stimulated gene transcription from PT $\alpha$  gene promoter-containing reporter constructs. We have delimited the region

required for estrogen-mediated induction to a 43 bp fragment, and the mechanism for ER $\alpha$ -mediated activation most likely involves a complex interplay between ER $\alpha$  and other protein factors bound to this region. The transcriptional activation by estrogens appears to involve ER $\alpha$  binding to the PT $\alpha$  gene promoter. Estrogen treatment also resulted in increased PT $\alpha$  nuclear localization which in turn is correlated with increased cell proliferation. Our studies also indicate that PT $\alpha$  plays a role in E<sub>2</sub>-induced proliferation of breast cancer cells.

We observed PT $\alpha$  to be a gene upregulated in E<sub>2</sub>-treated cells using Northern blot analyses. This increase was not observed with the antiestrogen TOT. Similarly Garnier *et al.*, using differential display report that PT $\alpha$  mRNA expression was also enhanced in neuroblastoma cells after estrogen treatment (Garnier *et al.*, 1997). The present studies now show that the increase in PT $\alpha$  mRNA in the presence of E<sub>2</sub> is also evident at the protein level. This protein has been of considerable interest to us due to its role in cellular proliferation. It is a highly acidic nuclear protein widely expressed in all cell types. While the exact mechanism of action of PT $\alpha$  remains elusive, the one constant has been its requirement for cellular growth and survival (Eschenfeldt and Berger, 1986; Gomez-Marquez *et al.*, 1989; Rodriguez *et al.*, 1998; Sburlati *et al.*, 1991; Wu *et al.*, 1997). Recently, PT $\alpha$  was shown to be capable of transforming rodent fibroblast cells in a manner similar to Ras, suggesting that PT $\alpha$  may be an important downstream target for inducers of cellular transformation (Orre *et al.*, 2001). PT $\alpha$  can serve as a marker for both breast cancer and hepatocarcinomas, and several malignant tissues have increased levels of PT $\alpha$  (Magdalena *et al.*, 2000; Pineiro *et al.*, 2000; Tsitsilonis *et al.*, 1998). Another source of interest for us is that PT $\alpha$  has also been shown to enhance ER transcriptional activity (Martini *et al.*, 2000), further validating its connection to estrogens.

Regarding the transcriptional regulation of PT $\alpha$ , there are still many unanswered questions. PT $\alpha$  mRNA levels are increased after serum restitution or after stimulation with various mitogens, further supporting its role in the cell cycle (Pineiro *et al.*, 2000; Zalvide *et al.*, 1992). However, levels of PT $\alpha$  do not vary significantly during the cell cycle making it hard to assign it to a particular cellular process (Pineiro *et al.*, 2000). While there is an E2F binding site between –323 and –316, the role of this site in E2F-induction of PT $\alpha$  gene transcriptional regulation has not been specifically tested (Eilers *et al.*, 1991; Szabo *et al.*, 1993). Our results suggest that this site is not necessary for ER $\alpha$  regulation. While the E-box at –1.173 kb of the PT $\alpha$  gene promoter has been proposed to mediate *c-myc* induction, *c-myc* activation of PT $\alpha$  expression has been put into doubt (Desbarats *et al.*, 1996; Gaubatz *et al.*, 1994; Mol *et al.*, 1995). Studies with antisense *c-myc* suggest that PT $\alpha$  expression is not completely dependent on *c-myc*. While *c-myc* is a well-known ER target gene (Dubik and Shiu, 1988) the present studies indicate that it is unlikely that E<sub>2</sub>

upregulation of PT $\alpha$  occurs indirectly through E<sub>2</sub>-mediated induction of *c-myc* expression. Our studies suggest that intervening protein synthesis is not required for the increase in PT $\alpha$  mRNA expression in the presence of E<sub>2</sub>. Moreover, deletion of the E-box did not affect E<sub>2</sub>-mediated upregulation of PT $\alpha$  gene transcriptional activity. Another group has shown that E<sub>2</sub> does not regulate the expression of *N-myc*, the neural counterpart of *c-myc* in neural cells, wherein E<sub>2</sub> has been demonstrated to upregulate PT $\alpha$  expression (Garnier *et al.*, 1997). Additional supporting evidence for direct transcriptional regulation of the PT $\alpha$  gene by the ER is that the increase in PT $\alpha$  expression was observed 1 h after estrogen treatment in neuroblastoma cells (Garnier *et al.*, 1997).

While there are no full EREs for binding of the ER $\alpha$  in the PT $\alpha$  promoter, there are several half-palindromic EREs that may be able to promote transcription (Chen *et al.*, 1998; Klinge *et al.*, 1997). However, the incomplete half-ERE (AGGTCG) in the 43 bp region does not appear to be essential for ER $\alpha$  regulation of PT $\alpha$  gene promoter or for ER $\alpha$  binding. The 3' end of the 43 bp region important for ER $\alpha$  transcriptional activation and binding does contain a direct TGCCC (or GCCCT) repeat. Since a GCCCT direct repeat has been shown to bind Sp1 (Dennig *et al.*, 1995) and Sp1 can interact with ER $\alpha$  to transactivate genes (Safe, 2001), we investigated the possibility that this element may recruit ER $\alpha$  through Sp1 interaction. However, gel shift analysis revealed no binding of Sp1 to this element or significant effects on ER $\alpha$  activation (data not shown).

During the preparation of this manuscript, there was a report from another group examining the regulation of PT $\alpha$  by estrogens (Martini and Katzenellenbogen, 2001). Their studies indicate the involvement of two half-EREs in the regulation by estrogens. These half-EREs, located at -886 to -861 and -588 to -560, were also proposed (but not examined) by Garnier *et al.* (1997) to be involved in estrogen regulation of PT $\alpha$  expression in neuroblastoma cells. While mutational analyses support the involvement of these two half-EREs (Martini and Katzenellenbogen, 2001), the half-EREs were not cloned upstream of a heterologous promoter to indicate enhancer activity. Our results indicate that when the *SmaI/SmaI* region (containing the half-EREs at -588 to -560) was cloned upstream of a heterologous promoter no E<sub>2</sub>-mediated activation was evident. In addition when the half-ERE at -886 to -861 was deleted (compare *ApaI* PT $\alpha$  CAT with *PstI* PT $\alpha$  CAT) we did not see a significant decrease in E<sub>2</sub>-mediated activation. Thus the decrease in promoter activity resulting from mutation of the two half-EREs may be attributed to disruption of intermolecular interactions in the promoter region, rather than promoter context-independent transcriptional regulation. The differences between these two reports may be also attributed to methodological differences.

Overall our findings reveal an interesting cross-talk between ER $\alpha$  and PT $\alpha$ . As mentioned above it has been shown that PT $\alpha$  can influence ER transcriptional

activity (Martini *et al.*, 2000). PT $\alpha$  does not directly interact with ER but appears to sequester the anti-coactivator factor REA from the ER transcriptional complex. ER is then able to interact with its coactivators. Certain aspects of PT $\alpha$  structure may be useful in understanding the transcriptional regulatory function of PT $\alpha$ . Because of the high acidity of the protein (Palvimo and Linnala-Kankkunen, 1990) it is unlikely to be a DNA binding protein. There are structural similarities between PT $\alpha$  and nuclear proteins known to be involved in chromatin activity, and it has been reported that PT $\alpha$  interacts specifically with histone H1 (Diaz-Jullien *et al.*, 1996; Gomez-Marquez and Rodriguez, 1998; Karetsoy *et al.*, 1998). PT $\alpha$  is proposed to play a role in nucleosome assembly (Diaz-Jullien *et al.*, 1996; Gomez-Marquez and Rodriguez, 1998; Karetsoy *et al.*, 1998), implicating a putative nuclear function related to chromatin remodeling. Transcriptional studies suggest a role for histone H1 in the high compaction of DNA and hence the general repression of transcription; other studies suggest H1 to be involved in the transcriptional repression of a selected group of genes (reviewed in Crane-Robinson, 1999; Wolfe *et al.*, 1997) PT $\alpha$  may play a role in transcriptional activation by associating with histone H1 and releasing histone H1 from chromatin. By functioning as a histone receptor (histone sink), PT $\alpha$  may allow access of the basal transcription factors to the DNA template. Regardless of the exact mechanism of PT $\alpha$  regulation of ER transcriptional activity, the regulation of PT $\alpha$  expression by E<sub>2</sub> adds another level of complexity and may represent a positive feedback loop for E<sub>2</sub>-ER $\alpha$  regulation of transcription.

Our studies suggest that estrogens may be involved in the regulation of PT $\alpha$  localization. While the localization of PT $\alpha$  have been initially reported to be primarily nuclear, more recent reports also indicate cytoplasmic localization. However, none of these studies have compared PT $\alpha$  localization in the absence and presence of mitogenic agents. We observe an increase in PT $\alpha$  nuclear localization in the presence of estrogens which translates to increased cell proliferation. Conversely, infection with PT $\alpha$ <sub>AS</sub> retroviruses results in decreased estrogen-induced nuclear PT $\alpha$  localization and decreased cell proliferation. These findings also suggest that the increase in PT $\alpha$  protein expression in the nucleus may be attributed to the transcriptional effects of estrogen on PT $\alpha$  gene transcription.

While the exact details of the mechanism of action of PT $\alpha$  remain elusive, it is clearly involved in the regulation of cell transformation and proliferation. The estrogen receptor (ER) is a ligand activated transcription factor and the identification of 'primary' ER target genes is imperative for understanding the basis for the proliferative action of ER in breast cancer cells. There are only a few candidate genes that appear to be under the direct regulation of the ER; much less genes that are associated with cell proliferative activity. PT $\alpha$  is a particularly strong candidate because its

expression and intracellular localization appears to be regulated by estrogen, and down-regulation of PT $\alpha$  expression inhibits E<sub>2</sub>-induced breast cancer cell proliferation.

## Materials and methods

### Chemicals and materials

Cell culture media was purchased from GIBCO (Grand Island, NY, USA). Calf serum was from Hyclone Laboratories (Logan, UT, USA) and fetal calf serum from Sigma Chemical Company (St. Louis, MO, USA). 17 $\beta$ -Estradiol (E<sub>2</sub>), trans-hydroxytamoxifen (TOT), and cycloheximide (CHX) was obtained from Sigma Chemical Company. ICI182,780 was obtained from Tocris (Ballwin, MO, USA). Custom oligonucleotides were purchased from Genosys (Grand Island, NY, USA).

### Northern blot analyses

Total RNA was isolated using Trizol (GIBCO-BRL, Rockville, MD, USA). Gel purified reamplified PT $\alpha$  cDNA was random primer labeled using the Ready-to-Go DNA labeling kit from Pharmacia (Piscataway, NJ, USA) for Northern analysis. Twenty  $\mu$ g of total RNA was separated by electrophoresis, transferred to nitrocellulose support and hybridized with random primer labeled cDNA (Cho *et al.*, 1991). Full-length cDNA for human PT $\alpha$  was obtained from ATCC (Manassas, VA, USA). Quantitative analysis was performed on a Macintosh computer using the public domain NIH Image program (developed at the US National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/nih-image/>).

### Western blot analyses

Whole cell extracts were prepared from breast epithelial MCF7 cells as previously described (Wrenn and Katzenellenbogen, 1993). Proteins were separated by electrophoresis on 15% SDS-polyacrylamide gels and transferred electrophoretically onto 0.2 micron nitrocellulose membranes. Blots were incubated with anti-PT $\alpha$  polyclonal antibody (1:2000 dilution, ImmunDiagnostik, Bensheim, Germany) and goat anti-rabbit IgG secondary antibody (1:30 000 dilution) for detection by chemiluminescence (Super Signal West Femto, Pierce, Rockford, IL, USA). Quantitative analysis was performed on a Macintosh computer using the public domain NIH Image program (developed at the US National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/nih-image/>).

### Plasmid construction and mutagenesis

All cloning was done using standard techniques (Ausubel *et al.*, 1992; Sambrook *et al.*, 1989). The reporter vectors pPstI PT $\alpha$  CAT (containing the 5' regulatory region of the PT $\alpha$  gene from the PstI site located -5 kb from the transcriptional start site) and pApaI PT $\alpha$  CAT (containing the 5' regulatory region of the PT $\alpha$  gene from the ApaI site located -0.819 kb from the transcriptional start site) were obtained from Dr Shelby Berger (National Cancer Institute, Bethesda, MD, USA). Deletions of the pApaI PT $\alpha$  CAT constructs were constructed using the available restriction sites.

Reporter constructs containing fragments of the SalI/SmaI region were constructed using the following oligonucleotides

with their complement: PT $\alpha$  (722-680): 5'-cgcgtcgactgactggccgacggacgacgacctgatgtgggcaaa-3' PT $\alpha$  (679-637): 5'-cgcgtcgaggtgcctgccacaggactaggggtgccctgccctggg-3' PT $\alpha$  (636-594): 5'-cgcgaggggagggatggcagagggcggggccag-gtcccc-3' Each oligonucleotide and their complement were annealed, gel purified and cloned into the MluI/XhoI-digested pCAT3 promoter vector (Promega, Madison, WI, USA). Oligonucleotides containing mutations: PT $\alpha$  (679-637)mut 1: 5'-cgcgtctctagagcctgccacaggactaggggtgccctgccctggg-3' PT $\alpha$  (679-637)mut 2: 5'-cgcgtcgaggtgcctgccattttaggactaggggtgccct-gcctggg-3' PT $\alpha$  (679-637)mut 3: 5'-cgcgtcgaggtgcctgccaca-gagtcgaaggtgccctgccctggg-3' PT $\alpha$  (679-637)mut 4: 5'-cgcgt-cgaggtgcctgccacaggactaggggatttgcctggg-3' PT $\alpha$  (679-637)mut 5: 5'-cgcgtcgaggtgcctgccacaggactaggggtccccatttca-gg-3' were annealed to their complement, gel purified, and cloned into MluI/XhoI-digested pCAT3 promoter vector. The first four nucleotides from the 5' end of the oligonucleotides were added for cloning into the pCAT3 vector.

The expression vectors for the wild type human ER $\alpha$  (pCMV5-ER), ER $\alpha$  Activation function mutant ER $\alpha_{\Delta A/B}$ , ER $\alpha$  DNA binding mutant ER HE82 (E203G/G204S/A207V) which changes DNA binding specificity to a GRE, and ER $\alpha$  Activation Function 2 mutant ER $\alpha_{AF2mut}$  (L540Q/E542A/D545A) have been described previously (LeGoff *et al.*, 1994; Montano and Katzenellenbogen, 1997; Montano *et al.*, 1996; Wrenn and Katzenellenbogen, 1993; Mader *et al.*, 1989). The plasmid pCMV $\beta$  (Clontech, Palo Alto, CA, USA) which encodes the  $\beta$ -galactosidase gene, was used as an internal control for transfection efficiency in all experiments.

### Cell culture and transfections

MCF7, MDA-MB-231 and Hec-1B cells were maintained and transfected as previously described (Montano and Katzenellenbogen, 1997). Cells were seeded for transfection in 100-mm dish in Improved Minimum Essential Media (IMEM) minus phenol red containing 5% CDGS. Cells were transfected as previously described (Montano *et al.*, 1996), and using 2  $\mu$ g of PT $\alpha$  gene promoter reporter constructs, 10 ng of ER $\alpha$  expression vector, and 0.2  $\mu$ g pCMV $\beta$ -galactosidase internal control plasmid.  $\beta$ -galactosidase activity, which was measured to normalize for transfection efficiency, and CAT activity were assayed as previously described (Montano *et al.*, 1996).

### Gel shift assays

Human recombinant ER $\alpha$  was obtained from PanVera Corp. (Madison, WI, USA). The single stranded oligomers, representing wild type and mutant PT $\alpha$  (679-637) were annealed to their complement oligonucleotides. Double stranded oligomers were gel purified on a nondenaturing 4.5% polyacrylamide gel run in 1 $\times$ TBE. The ability of purified protein to bind to the PT $\alpha$  gene promoter fragments was analysed using standard gel mobility shift assays (Montano *et al.*, 2000). Briefly 700 fmol of recombinant ER $\alpha$  was mixed with 1 ng of end-labeled PT $\alpha$  gene oligomer in the presence of 0.4  $\mu$ g/ $\mu$ l dIdC, 20 mM HEPES (pH 7.9), 200 mM KCl, 10 mM MgCl<sub>2</sub>, 2 mM DTT, 2 mM EDTA, 20% glycerol, 1  $\mu$ g/ $\mu$ l BSA and incubated at room temperature for 20 min. The specificity of binding was assessed by competition with excess unlabeled double stranded PT $\alpha$  gene fragment. The presence of ER $\alpha$  in DNA-protein complexes was verified using supershift assays with ER $\alpha$  antibody, H222 (Abbott Laboratories, Chicago, IL, USA). The non-denaturing gels used to analyse the protein-DNA complexes were run as described previously (Montano *et al.*, 2000).

#### Retroviral-mediated transfection and immunostaining

Retroviruses were made by transfecting PA317 cells using the CaPO<sub>4</sub> coprecipitation method with the pBPSTR1 plasmid alone (to make control retroviruses) or pBPSTR1 containing PT $\alpha$  cDNA in the antisense orientation (Paulus *et al.*, 1996). PA317 media containing retroviruses was collected 48 h later and passed through a 0.45 micron filter. Breast epithelial cell lines were infected with retrovirus-containing supernatants in the presence or absence of 3  $\mu$ g/ml tetracycline. When tetracycline was added, expression of the viral gene was inhibited.

PT $\alpha$  protein expression was examined by immunostaining using PT $\alpha$  antibody. Cells infected with control or antisense PT $\alpha$  retroviruses were grown on coverslips and subsequently fixed in 4% paraformaldehyde. After blocking with serum, samples were incubated with anti-PT $\alpha$  polyclonal antibody and goat, anti-rabbit IgG Alexa 488 fluorescence secondary antibody. As a negative control duplicate sections were immunostained with nonspecific rabbit IgG. Proliferating cells were identified by immunostaining using Ki67 IgG monoclonal antibody (Lab Vision) and goat, anti-mouse Alexa 594 secondary antibody (Molecular Probes). Semi-quantitative analysis was performed on a Macintosh computer using Adobe Photoshop 6.0 software. Mean luminosity of 20 cells from three separate experiments was measured and averaged with background subtracted out from each field.

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#### Proliferation assays

$1 \times 10^5$  cells were seeded in a 24 well plate. Two days after plating, fresh media was added containing hormones. Fresh media with hormones was added every two days. Cell number was determined five days after initial hormone treatment using the CellTiter 96 Aqueous One Solution Proliferation Assay (Promega, Madison, WI).

#### Abbreviations

PT $\alpha$ , prothymosin  $\alpha$ ; ER $\alpha$ , Estrogen Receptor  $\alpha$ ; E<sub>2</sub>, estradiol; TOT, trans-hydroxytamoxifen; ERE, estrogen response element

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