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The regulation of uterine tissue factor by estrogen

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Tissue factor (TF) is a transmembrane protein that initiates coagulation and indirectly catalyzes the conversion of prothrombin to thrombin. We previously showed that treatment of immature rats with estradiol (E_2) stimulated a rapid increase in TF mRNA and protein in the uterus. Our current experiments using *in situ* hybridization show that the increase in TF mRNA occurred primarily in the stromal cell layer. The effect of E_2 to increase TF mRNA occurred in uterine organ cultures but not in separated epithelial and stromal cells *in vitro*. Thrombin and the phorbol ester, TPA, compounds which regulate TF expression in other cell types by activation of protein kinase C (PKC), increased TF mRNA in both uterine organ cultures and in separated uterine cells. The 5′ regulatory region of the TF gene was examined for the presence of an estrogen response element (ERE) using a plasmid, pTFCAT, containing −740 to +15 bp of the mouse TF promoter upstream of the chloramphenicol acetyltransferase (CAT) reporter gene. There was no response to E_2 in HeLa cells co-transfected with pTFCAT and a human ER construct, pHEO. In contrast, E_2 increased CAT activity in cells co-transfected with a positive-control plasmid, containing the consensus ERE cloned upstream of the thymidine kinase promoter-driven CAT gene, and pHEO. CAT activity was also increased by TPA in cells transfected with pTFCAT. In summary, E_2 induces TF mRNA in uterine organ culture indicating that systemic factors are not absolutely required for the effect. However, E_2 injection induces transudation of plasma prothrombin into the uterus where it may be converted to thrombin. Thus thrombin may contribute to E_2-induction of TF mRNA *in vivo*. An ERE was not identified in the 750 bp immediately 5′ to the transcription start site of the TF gene although a TPA-responsive element was present. It is postulated that E_2 may induce TF mRNA by multiple indirect pathways including stimulation of PKC and Jun and Fos transcription factors, and by generation of thrombin in the uterus.

Keywords: tissue factor; estrogen regulation; gene expression; rat uterus; estrogen response element; *in situ* hybridization

Introduction

Our earlier studies of TF and prothrombin as E_2-responsive uterine proteins showed that both are increased three- to fourfold within 3 h of administration of E_2 to immature rats (Henrikson *et al.*, 1990; Jazin *et al.*, 1990). We hypothesized that the increase of the two could result in an increased level of thrombin in uterine cells, where it could act as an E_2-induced growth factor (Henrikson, 1992). For this reason we have characterized the induction of TF in the uterus.

The primary physiological role of TF is in the initiation of blood coagulation. TF is a 263-residue integral membrane protein that serves as the receptor for Factor VII, the precursor of activated coagulation Factor VIIa. After activation of Factor VII to VIIa, the TF/Factor VIIa complex initiates the coagulation cascade (Nemerson, 1988). TF is expressed variably on the surface of cells, generally at epithelial cell surfaces, consistent with a role in hemostasis (Drake *et al.*, 1989). It is normally not expressed by intravascular cells, although it can be induced in endothelial cells and monocytes by a variety of inflammatory agents (Edgington *et al.*, 1991). TF is induced very rapidly by serum and growth factors in certain cell types in culture and is classified as an early response gene (Bloem *et al.*, 1989; Hartzell *et al.*, 1989; Herschman, 1991; Lau and Nathans, 1991). Factors that have been reported to increase TF rapidly in monocytes and endothelial cells include phorbol esters, thrombin, lipopolysaccharides, serum, TGF-β, and the cytokines interleukin-1 and tumor necrosis factor (Gregory *et al.*, 1989; Crossman *et al.*, 1990; Archipoff *et al.*, 1991; Edgington *et al.*, 1991). The role of the E_2-induced increase in uterine TF is unknown.

Our laboratory showed that induction of uterine TF mRNA by treatment with E_2 *in vivo* was inhibited by actinomycin but not cycloheximide, indicating a requirement for *de novo* RNA synthesis but not protein synthesis (Henrikson *et al.*, 1992). These results suggested that E_2 might stimulate transcription of the TF gene directly by interaction with promoter regulatory elements. An alternative possibility was that E_2 induced TF mRNA by activating protein kinase C (PKC) and stimulating increases in Jun and Fos transcription factors. Estrogen induces rapid expression of c-*jun* and c-*fos* proto-oncogene families in the rat uterus (Weisz and Bresciani, 1988; Loose-Mitchell *et al.*, 1988; Weisz *et al.*, 1990; Chiapetta *et al.*, 1992; Nephew *et al.*, 1993; Webb *et al.*, 1993). The Jun proteins form homodimers or heterodimers with Fos proteins and bind to AP-1 recognition sites on DNA (Angel and Karin, 1991), two of which are involved in transcriptional regulation of the human TF gene (Mackman *et al.*, 1990; 1991). Estrogen also stimulates phospholipase C-mediated generation of inositol phosphates and diacylglycerol (DAG) in the uterus (Ignar-Trowbridge *et al.*, 1991). DAG stimulates PKC, which phosphorylates substrates

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involved in cellular signaling (Angel and Karin, 1991).

This study examines the mechanism for the E_2-induced increase in TF mRNA by: (1) determining whether effects of E_2 on TF mRNA observed *in vivo* can be duplicated *in vitro*, (2) identifying cell types in the uterus in which TF mRNA is increased by E_2, (3) determining the role of agonists that stimulate PKC, such as thrombin and the phorbol ester, TPA, on induction of TF mRNA in the uterus, (4) examining a potential direct effect of E_2 on transcriptional regulatory elements of the TF gene using transient transfection assays with a mouse TF promoter.

Results

Modulation of TF mRNA levels in uterine organ culture

Regulation of TF mRNA levels was examined in short-term uterine organ cultures. In addition to E_2, effects of thrombin and TPA were tested since they have been shown to induce TF mRNA in endothelial cells and monocytes (Archipoff *et al.*, 1991; Edgington *et al.*, 1991). An example of quantitation of TF mRNA using the RNase protection assay is shown in Figure 1, where addition of increasing amounts of total RNA to the assay resulted in a linear increase in signal. Treatment of uteri with E_2, thrombin, or TPA increased TF mRNA 1.6-, 1.8-, and 1.7-fold, respectively (Figure 2, $P<0.05$). A comparable 2.4-fold increase in TF mRNA was obtained in rats treated with E_2 *in vivo* (Figure 2, $P<0.05$). Thus, TF is responsive to E_2 *in vivo* and in organ culture. Thrombin and TPA, agents that stimulate PKC, also increase uterine TF mRNA.

Localization of TF mRNA by in situ *hybridization*

In situ hybridization experiments were performed to determine the distribution of TF mRNA in the control and E_2 treated uterus. A weak hybridization signal for TF mRNA was observed in the epithelial, stromal, and myometrial layers of the uterus in saline-treated rats (Figure 3A and C), although this signal was only slightly greater than the nonspecific signal obtained using a TF sense probe as control (Figure 3E). In contrast, an intense hybridization signal for TF mRNA was observed in the stromal layer of E_2-treated rats whereas the signal in the epithelial and myometrial layers was unchanged (Figure 3, B and D). These results identify the stromal layer as the major site of the E_2-induced increase in TF mRNA.

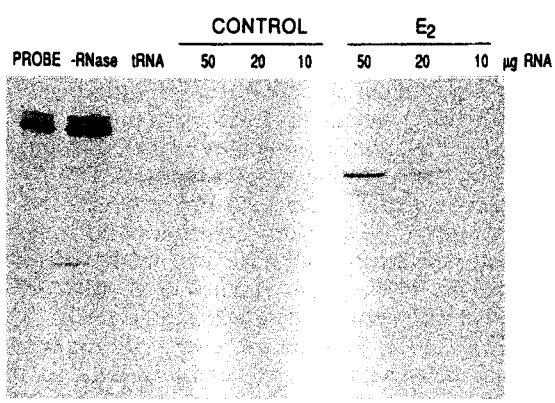

Figure 1 RNase protection assay to quantitate TF mRNA levels. Assay of 10, 20 and 50 µg total RNA from uteri of control rats or rats treated with E_2 (see Materials and methods) resulted in a linear increase in the protected fragment. TF riboprobe not subjected to incubations (probe), and riboprobe carried through the entire protocol but without incubation with RNase (−RNase) are shown as well as a tRNA control to detect nonspecific hybridization.

Modulation of TF mRNA in stromal and epithelial cell cultures

To further examine the regulation of uterine TF *in vitro*, effects of E_2, thrombin, and TPA were examined in primary cultures of stromal and epithelial cells from immature rats. In stromal cells thrombin and TPA induced 2.0- and 1.9-fold increases in TF mRNA, respectively (Figure 4). In epithelial cells thrombin and TPA induced 1.8- and 1.5-fold increases in TF mRNA, respectively (Figure 5). E_2 had no significant effect on TF mRNA in stromal cells or epithelial cells (Figures 4 and 5). The failure of E_2 to induce TF mRNA in cell cultures contrasts with the positive effect observed in organ cultures (Figure 2) and the increase in TF mRNA detected in the stroma by *in situ* hybridization after E_2 injection *in vivo* (Figure 3). The ER level measured in one of the stromal cell cultures was 20.5 fmol/mg protein. This level was higher than that simultaneously assayed in E_2-responsive MCF7 breast cancer cells (15 fmol/mg protein). These results indicate that culture of separated uterine cells does not maintain E_2-responsiveness of TF expression despite the presence of ER.

Transcriptional activity of the TF promoter

Examination of the mouse TF promoter sequence for transcriptional regulatory elements (Figure 6A)

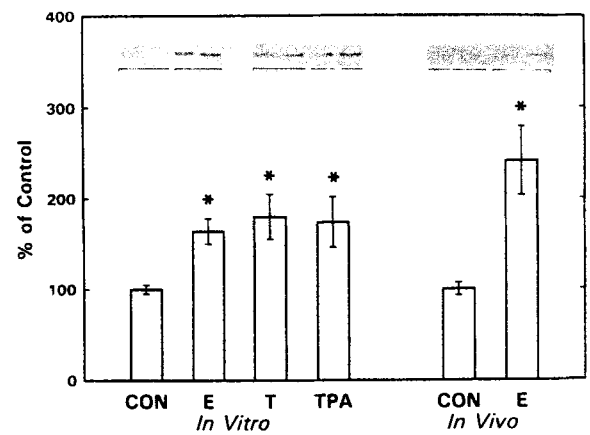

Figure 2 Modulation of TF mRNA levels in uterine organ culture. TF mRNA levels were measured by RNase protection assay in uteri cultured for 2 h with E_2 (10 nM), thrombin (T; 100 nM), TPA (100 nM), or an equivalent amount of ethanol as control (CON). Results are expressed as a percentage of TF mRNA levels in control cultures. The specific TF signal obtained by RNase protection in two representative cultures for each treatment is shown at the top of the figure. Twenty or 30 µg of total RNA was assayed in each experiment. The graph shows the means ± s.e.m. of four cultures per treatment (* = $P<0.05$).

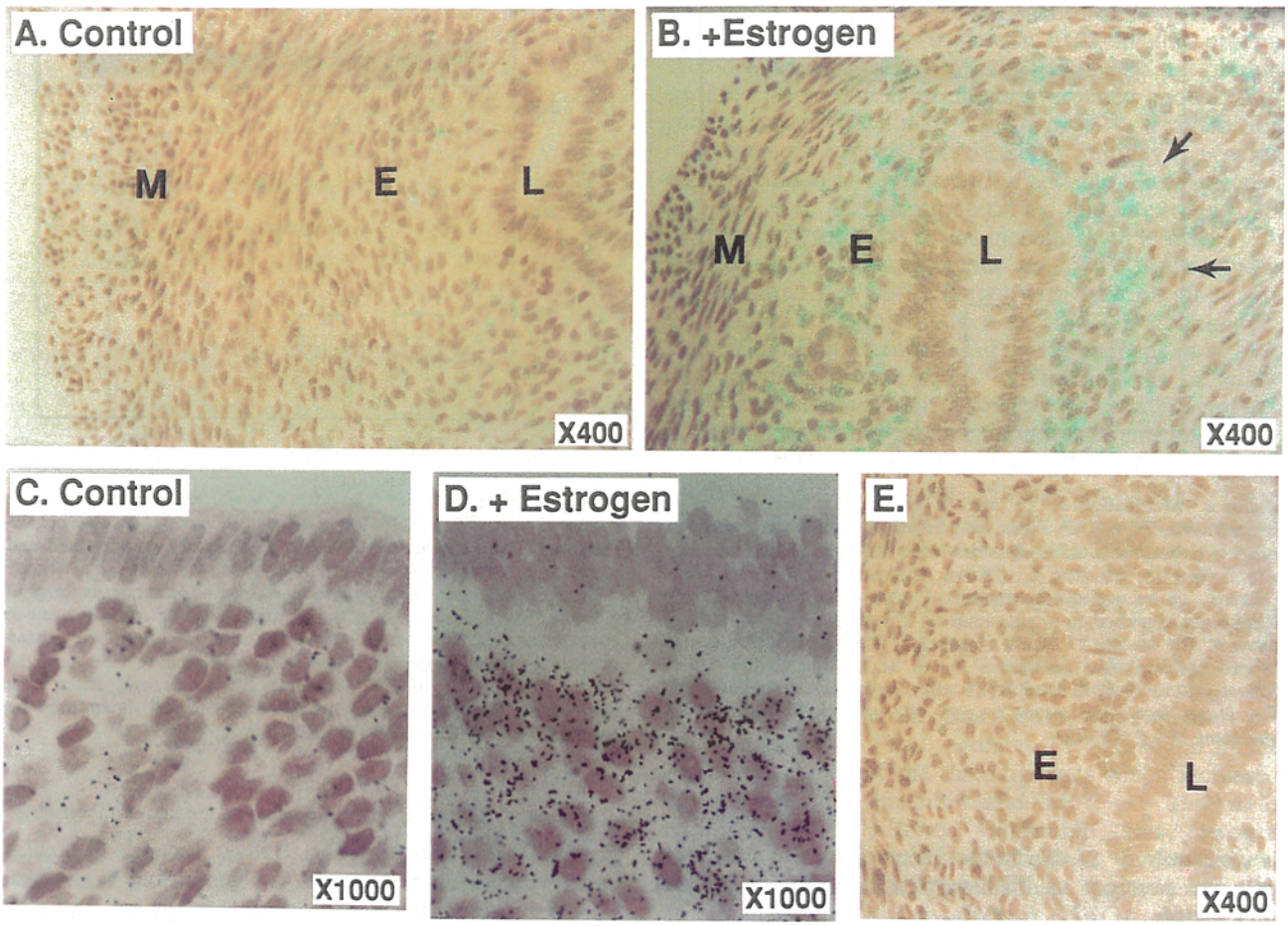

Figure 3 *In situ* hybridization of uterine TF mRNA. Uteri from E_2-treated or control rats were processed for *in situ* hybridization as described in Materials and methods. (**A-D**) show hybridization with an anti-sense TF riboprobe while (**E**) shows a low level of non-specific background signal with the sense TF riboprobe. The signal in control uteri (**A** and **C**) is not significantly different from background (**E**). In E_2-treated uteri there is a significant increase in signal above background in the stromal cells of the endometrium (E) (arrows in **B**) but not in the epithelial cell layer (immediately adjacent to the uterine lumen, L) or myometrial cell layer (M). Sections were photographed under dark field using polarized light epiluminescence (**A**, **B** and **E**) in which positive hybridization appears as bluish grains or under bright field (**C** and **D**) in which positive hybridization appears as black grains. Original magnification is shown in each panel.

indicated no consensus ERE in the 755 nt clone used in this study or in 1100 nt of a mouse TF promoter clone reported previously (Mackman *et al.*, 1992). However, sequences corresponding to 'half arms' of the palindromic ERE are present at positions −110 (TGACC), −307 and −626 (GGTCA) (Figure 6A). ERE half sites separated by considerable distances have been shown to confer E_2-responsiveness in other systems (Kato *et al.*, 1992) and were, therefore, tested in the TF promoter. In addition, a sequence located at positions +16 to +28, which contains four mismatches relative to the consensus ERE (TF(ERE)/TKCAT; Figure 6A), was tested since non-consensus sequences have been shown to confer E_2-responsiveness to other genes (Berry *et al.*, 1989; Wahli *et al.*, 1989; WuPeng *et al.*, 1992). Two AP-1 sites and an NF-κB-like site, located between positions −218 and −176, are 100% conserved in the mouse and human promoters (Mackman *et al.*, 1989, 1992) and are necessary for transcriptional activation in response to bacterial endotoxin (Mackman *et al.*, 1991) (Figure 6A). Sp-1 sites are present, two of which occur at comparable sites in the human TF promoter (Mackman *et al.*, 1989) in a region required for basal transcriptional activity (Mackman *et al.*, 1990).

The transcriptional activity of the mouse TF promoter was quantified in HeLa cells co-transfected with TF-CAT constructs and a human ER-expression plasmid, pHEO. E_2 had no effect on CAT activity in cells co-transfected with pHEO and pTFCAT containing positions −740 to +15 of the promoter. However, treatment of cells with TPA induced a 3.8-fold increase in CAT activity (Figure 6B). E_2 increased CAT activity 4.5-fold in cells co-transfected with pHEO and the consensus ERE construct but did not stimulate CAT activity in cells transfected with pTF(ERE)/TKCAT containing the non-consensus potential TF ERE (Figure 6C). In pTFCAT E_2 caused a 50% decrease in CAT activity. Although the mechanism for this reduction by E_2 is not understood, previous studies have shown that E_2 in the presence of pHEO tends to reduce basal activity from pBLCAT2 constructs (Wu-Peng *et al.*, 1992). The results indicate that E_2 does not interact directly with regulatory elements within the 750 bp

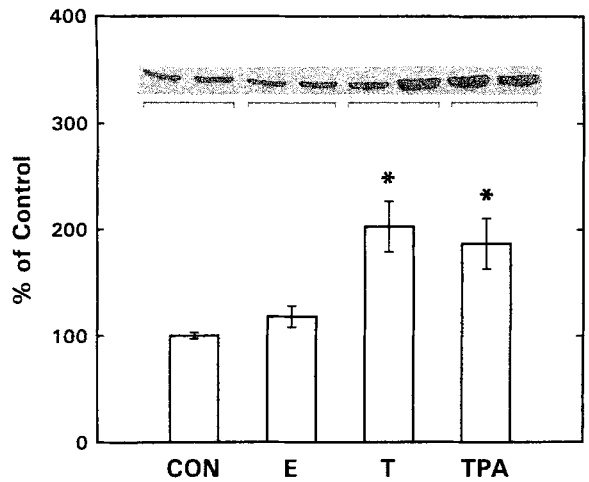

Figure 4 Modulation of TF mRNA levels in cultures of uterine stromal cells. TF mRNA levels were measured by RNase protection assay in stromal cells cultured as described in Materials and methods and treated for 2 h with E_2 (10 nM), thrombin (T; 100 nM), TPA (100 nM), or an equivalent amount of ethanol as control (CON). Results are expressed as a percentage of TF mRNA levels in control cultures. The specific TF signal obtained by RNase protection assays in two representative cultures is shown at the top of the figure. Between 10 to 20 μg of total RNA was assayed in each experiment. The graph shows the means ± s.e.m. of seven to 10 cultures per treatment (* = $P < 0.01$).

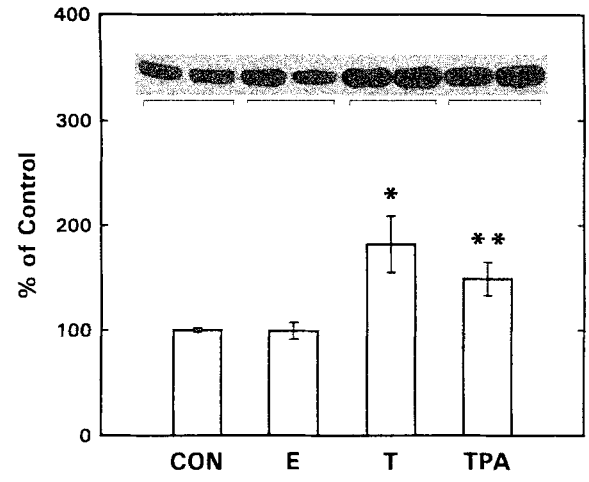

Figure 5 Modulation of TF mRNA levels in cultures of uterine epithelial cells. TF mRNA levels were measured by RNase protection assay in epithelial cells cultured as described in Materials and methods and treated for 2 h with E_2 (10 nM), thrombin (T; 100 nM), TPA (100 nM), or an equivalent amount of ethanol as control (CON). Results are expressed as a percentage of TF mRNA levels in control cultures. The specific TF signal obtained by RNase protection assays in two representative cultures is shown at the top of the figure. Between 12 to 15 μg of total RNA was assayed in each experiment. The graph shows the means ± s.e.m. of six cultures per treatment (* = $P < 0.05$, ** = $P < 0.01$).

immediately 5′ to the transcription start site but suggest the presence of TPA-responsive elements within this region.

Discussion

We have previously shown that increased TF is an early response to E_2 administration in the immature rat uterus. The purpose of the work presented here was to better characterize the cellular and molecular aspects of TF production in the uterus. Studies *in vivo* confirmed our previous finding that treatment with E_2 resulted in increased TF mRNA in the uterus. Using a sensitive RNase protection assay for TF mRNA, we extended that observation to uterine organ culture, where treatment with E_2, thrombin, or TPA resulted in increased TF mRNA. *In situ* hybridization studies indicated that a significant increase in TF mRNA occurred in the stroma following E_2 treatment. In contrast to *in vivo* studies and uterine organ culture studies, E_2 did not increase TF mRNA in isolated stromal and epithelial cells. However, both thrombin and TPA increased TF mRNA in each cell type. A 750 nt fragment of the mouse TF promotor driving a CAT reporter gene was not responsive to E_2 but was responsive to TPA, suggesting that E_2 does not act directly on regulatory sequences of the TF promoter to increase transcription.

There are a number of potential roles for the E_2-induced increase in TF in the uterus. First, increases in TF may be a mechanism to insure hemostasis during increased blood flow to the uterus in response to estrogen. In addition, because TF is induced rapidly in response to serum and growth factors in a number of cells, it has been postulated to play some role in cell growth (Lau and Nathans, 1991). Finally, TF may contribute to uterine growth by generating thrombin. E_2 injection results in an influx of plasma proteins including prothrombin into the uterus (Henrikson *et al.*, 1990) due to an E_2-induced increase in vascular permeability. Thrombin could be generated from this source either by the increased TF or autocatalytically. Thrombin, which is a potent mitogen in fibroblasts, endothelial cells, and other cell types, may then act as a paracrine factor to stimulate uterine growth (Henrikson, 1992).

In situ hybridization experiments demonstrate that the stroma is the major site of the E_2-induced increase in TF mRNA. Localization studies of TF protein and activity in the uterus using immunocytochemistry and clotting assays showed that E_2 increased TF in both the epithelium and stroma (Henrikson *et al.*, 1994). These contrasting results may be explained by differences in sensitivities of the assays. In epithelial cells a small increase in TF mRNA which is not large enough to be detected by *in situ* hybridization could result in the synthesis of an amount of TF protein that can be readily detected by the more sensitive clotting assay or by immunocytochemistry. Nevertheless, the results of *in situ* hybridization demonstrate that the stroma is the major site of the E_2-induced increase in TF mRNA.

E_2 did not increase TF mRNA levels in cultured stromal or epithelial cells. In contrast, both TPA and thrombin were effective, suggesting that culture conditions maintained responsiveness of the TF gene, but may not have been adequate to maintain responsiveness to E_2. In support of this possibility, there have been numerous reports of failure to reproduce the mitogenic effects of E_2 observed *in vivo* in cultured uterine cells (Murphy, 1991). Interactions between

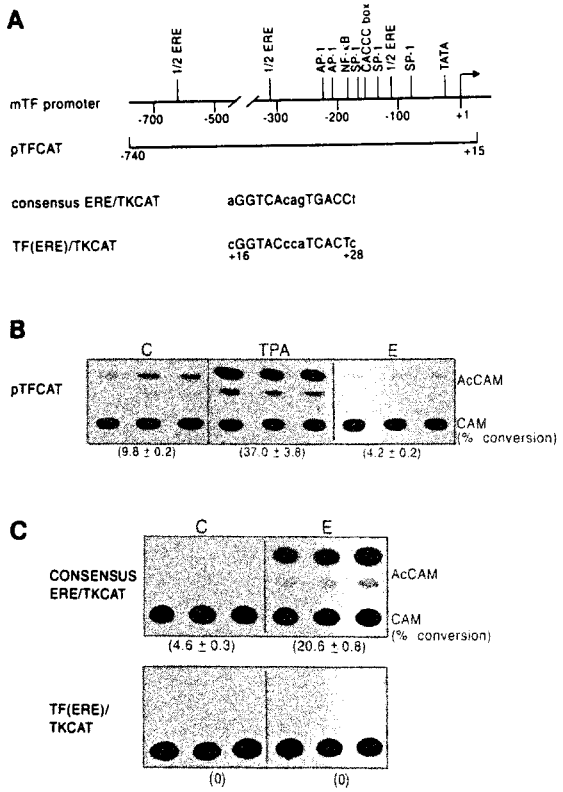

Figure 6 Transcriptional regulation of the mouse TF promoter. A: Representation of the 756 bp mTF clone and CAT constructs used in transfection experiments. Positions of transcriptional regulatory elements are shown. Numbering is relative to the transcription start site at +1 and corresponds to the previously reported mouse TF promoter sequence (Mackman *et al.*, 1992). B and C: Effect of TPA (200 nM) and E_2 (10 nM) or ethanol as control (C) on HeLa cells co-transfected with 2 μg CAT constructs, 800 ng E expression plasmid pHEO and sufficient pBSK- DNA to adjust DNA to 25 μg. CAT assays were performed on cell extracts using 20 μg protein in experiments shown in panel B and 200 ng protein in experiments shown in C. In cells transfected with TF(ERE)/TKCAT a similar lack of E_2-responsiveness was observed when higher amounts of cell extract (14 μg) was assayed (not shown).

uterine cells may be necessary for increases in TF mRNA in response to E_2.

It is intriguing that steroids regulate TF expression in both the rat and the human uterus. Progestins induced a dramatic increase in TF in decidualized stromal tissue of the human uterus. While E_2 synergistically augmented progestin induced increases in TF mRNA and protein, it was ineffective alone (Lockwood *et al.*, 1993a,b). The increase in TF in the immature rat uterus is estrogen-specific since it does not occur in response to progesterone or other steroids (Henrikson *et al.*, 1992). Differences in hormone specificity may represent species differences or differences in differentiation between the immature rat uterus and decidualized human stroma. Immunohistochemistry detected substantial TF in human stromal cells, particularly in decidualized endometrium, but no staining in epithelium (Lockwood *et al.*, 1993a). These results are consistent with the relatively lower levels of TF mRNA detected in the epithelium of the rat uterus by *in situ* hybridization. It is possible that steroids regulate hemostasis by modulating TF activity in both rodents and humans.

A functional ERE was not identified in the 750 bp mouse TF promoter construct used in the present study. A consensus ERE sequence was also not present in 1100 bp of an independently isolated mouse promoter clone (Mackman *et al.*, 1992). Transfection experiments were undertaken initially because experiments *in vivo* suggested a possible direct effect of E_2 on the TF promoter by two criteria: (1) Actinomycin prevented the increase in TF mRNA in response to E_2, indicating that *de novo* RNA synthesis was required. (2) Co-treatment with cycloheximide and E_2 resulted in a superinduction of TF mRNA levels, suggesting that synthesis of new proteins was not necessary (Henrikson *et al.*, 1992). However, a recent study demonstrated that a number of protein synthesis inhibitors including cycloheximide have effects on cells independent of their activity as protein synthesis inhibitors (Edwards and Mahadevan, 1992). These effects included induction of c-*fos* and c-*jun*, which in our experiments (Henrikson *et al.*, 1992) may have stimulated transcription of the TF gene (see below). Therefore, protein synthesis-dependent pathways may be involved. Alternatively, an unidentified ERE may be present in a region of the TF gene not tested or require uterine-specific transcription factors not present in the HeLa cells used in the transfection experiments.

It is likely that PKC regulates TF expression in the uterus since each of the three agonists which increased TF mRNA, E_2, thrombin and TPA, are known to modulate PKC activity. Thrombin and estrogen stimulate phospholipase C-mediated hydrolysis of phophatidylinositol-4,5-bisphosphate to generate the second messengers diacylglycerol (DAG) and inositol triphosphate (Ignar-Trowbridge *et al.*, 1991; Van Obberghen-Schilling and Pouysségur, 1993). Inositol triphosphate mobilizes Ca^{2+} from intracellular stores while DAG activates PKC. TPA mimics the actions of DAG to stimulate PKC (Angel and Karin, 1991). PKC mediates some of its effects on gene transcription by increasing expression of c-*jun* and c-*fos* transcription factors, which bind to AP-1 promoter elements (Angel and Karin, 1991). Estrogen also directly increases expression of c-*jun* and c-*fos* (Loose-Mitchell *et al.*, 1988; Weisz and Bresciani, 1988; Weisz *et al.*, 1990; Chiapetta *et al.*, 1992; Nephew *et al.*, 1993; Webb *et al.*, 1993), and this may represent another mechanism whereby uterine TF levels are increased. PKC also acts through NF-κB promoter elements (Lenardo and Baltimore, 1989), and studies of the human TF promoter showed that AP-1 sites and an NF-κB-like site that are 100% conserved in the human and mouse TF promoters are involved in transcriptional regulation in response to LPS in monocytes (Mackman *et al.*, 1991). Overall, studies on transcriptional regulation of the TF promoter suggest a complex mechanism of control that depends on both the agonist and cell type examined (Mackman *et al.*, 1990, 1991; Cui *et al.*, 1994). We postulate that E_2 may elicit at least some of its effects on uterine TF mRNA by activation of PKC and induction of Jun and Fos transcription factors.

In summary, the fact that E_2 induces TF mRNA in uterine organ culture indicates that systemic factors are not absolutely required for the effect of E_2 observed *in vivo*. However, thrombin stimulates TF mRNA *in vitro* and because thrombin increases in the uterus after injection of E_2, it may also contribute to increased

levels of TF mRNA *in vivo*. The effect of E_2 in the uterus is selective in that the major increase in TF mRNA occurs in the stroma. An ERE was not identified in 750 bp immediately 5′ to the transcription start site of the TF gene. However, a TPA-responsive element was present within this region. Although direct interaction of E_2 with regulatory elements in other regions of the TF gene has not been excluded, it is likely that at least some of E_2 effects on TF are mediated indirectly by stimulation of PKC and Jun and Fos transcription factors.

Materials and methods

Animals

Female Wistar rats at 18 to 20 days of age were obtained from the Griffin Laboratory of the Wadsworth Center for Laboratories and Research. They were maintained according to NIH guidelines and euthanized by cervical dislocation. Procedures were approved by the Institutional Animal Welfare Committee. In some experiments rats were injected i.p. with 1 μg 17β-estradiol (E_2) in a 1% ethanol-0.14 mM NaCl solution or with vehicle in control animals.

Uterine cultures

For organ culture, uteri from four untreated rats were cut into four pieces and placed in 10 ml of a basal medium (BM), which consisted of a 1:1 (vol/vol) mixture of Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (Ham) (Life Technologies, Grand Island, NY) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL fungizone, 1 mM pyruvate, and 2 mM glutamine. Cultures were gassed with 95% O_2-5% CO_2 in 20 ml glass scintillation vials, capped tightly, and incubated for 2 h at 37°C with gentle rotation on a platform shaker. Pieces of uteri were snap frozen on dry ice and stored at −80°C until processed for RNA analysis.

Epithelial and stromal cells were isolated from the uteri of 30 immature rats using the method of McCormack and Glasser (1980) and grown in BM containing 10% fetal bovine serum. Stromal cells were distributed into five T25 flasks and grown for 1 week until confluent. Cells were then removed by trypsinization and replated in eight T75 flasks. Cells were grown for 1 week to confluence before use in experiments. Epithelial cells were distributed into eight T25 flasks that had been coated with 1% gelatin to enhance cell attachment and grown for 1 week to confluence before use in experiments. The extent of contamination of epithelial cultures by stromal cells was assessed by visual inspection 3 to 5 days after the cultures were initiated. Contamination was not more than 5%. Our observations agree with the original validation of the uterine cell separation method (McCormack and Glasser, 1980), indicating insignificant contamination of the epithelial cell fraction with stromal cells and stromal cell fractions containing approximately 10% epithelial cells. The medium was replaced with serum-free BM for 1 h at the start of experiments and then replaced with serum-free BM containing 10 nM E, 100 nM thrombin, or 100 nM TPA. Ethanol was added to control and thrombin-treated cultures (0.2%) to control for addition of E_2 and TPA in ethanol. After 2 h the medium was removed, the flasks rinsed with PBS, and cells processed for RNA analyses (see below). In one experiment levels of ER were measured in stromal cell cultures by an ER EIA assay (Abbott, Chicago, IL), using a rat anti-ER antibody as a solid phase capture antibody and a rat anti-ER monoclonal antibody conjugated to horseradish peroxidase as a detection antibody.

RNA analysis

Total RNA was prepared from uterine tissue by the guanidinium thiocyanate method followed by centrifugation through a cushion of CsCl (Chirgwin *et al.*, 1979; Henrikson *et al.*, 1992). RNA was isolated from cultures of epithelial and stromal cells by RNAzol B (Biotecx Laboratories, Houston, TX), which uses a single-step acid guanidinium thiocyanate-phenol-chloroform extraction procedure described by Chomczynski and Sacchi (1987).

The RNase protection assay used to quantitate levels of specific mRNAs and preparation of ^{32}P-labeled riboprobes was done exactly as described (Gilman, 1989). The TF riboprobe was prepared by T7 polymerase transcription using as template a 149 bp fragment of rat TF cDNA (Edgington *et al.*, 1991; +236 to +384 relative to the transcription start site in the mouse TF promoter Hartzell *et al.*, 1989) cloned into the BamHI/PstI sites of pGEM4Z (Promega, Madison, WI) and linearized with BamHI in the multiple cloning site. In all experiments the specific signals measured in RNase protection assays were quantitated by direct counting on a beta-detector (Betagen Intelligenetics, Mountainview, CA).

Levels of the mRNA for glyceraldehyde-3-phosphate dehydrogenase (GA3PD), were measured by RNase protection assay of RNA from uterine organ cultures and from stromal cells to test for uniformity in amount of total RNA added to the TF RNase protection assays (determined by optical density). Normalization of TF mRNA levels to GA3PD mRNA levels did not alter relative changes in TF mRNA in response to various treatments and, therefore, non-normalized data are presented. GA3PD mRNA levels were not determined in RNA samples from epithelial cell cultures because of limiting numbers of cells and RNA obtained for analysis. The template for preparation of the GA3PD riboprobe was made by cloning a 100 bp fragment of the rat GA3PD cDNA (positions +211 to +310, Tso *et al.*, 1985) into the pCR vector (InVitrogen, San Diego, CA). The fragment was generated by reverse transcriptase-polymerase chain reaction from liver RNA. The GA3PD antisense riboprobe was generated by transcription with T7 polymerase as described (Gilman, 1989) using BamHI-linearized template.

In situ *hybridization*

Uteri from E_2-treated or control rats were fixed in 4% paraformaldehyde-0.1 M phosphate buffer overnight at 4°C and then transferred to 70% ethanol until they were sectioned. Sections were processed for *in situ* hybridization exactly as described (Mackman *et al.*, 1993). To prepare riboprobes, a 700 bp EcoRI fragment spanning the 5′ portion of the rat TF cDNA (kindly provided by L. Feng) was cloned into pGEM9Z (Promega, Madison, WI) to create pRatTF2375. PvuII/SacI and PvuII/HindIII fragments from pRatTF2375 were isolated and used as templates for the generation of antisense and sense TF riboprobes in the presence of [^{35}S]UTP (>1200 Ci/mMol; Amersham, Arlington Heights, IL) using T7 and Sp6 polymerase, respectively. Consecutive sections were analysed using either an antisense TF riboprobe to identify mRNA, or a sense TF riboprobe to examine non-specific hybridization. All slides were exposed for 12 weeks at 4°C. Photomicrographs were taken either under bright field or under dark field using polarized light epiluminescence.

Transfection experiments

A fragment of the mouse TF promoter from positions −740 to +15 relative to the transcription start site was obtained by screening a genomic library prepared from liver of Balb/C mice cloned in the lambda EMBL-3 vector. The fragment was cloned into the EcoRI/KpnI sites of plasmid Bluescript (Stratagene) and designated 482Pro. The clone was

sequenced on both strands using Sequenase. There were differences in sequence at six positions from that of a mouse TF promoter fragment obtained from an NIH 3T3 genomic library (three gaps and four substitutions; Mackman *et al.*, 1992), none of which occurred in recognition sites for transcription factors described in Figure 6A. The promoter fragment was prepared for ligation into the chloramphenicol acetyl transferase (CAT) expression plasmid, pBLCAT3 (Luckow and Schütz, 1987), at the XbaI/XhoI sites of the polylinker by digesting 482Pro at the 3′ end with the KpnI neoschizomer, Asp718, blunt-ending with Klenow, and then digesting at an XbaI site 5′ to the fragment in the plasmid polylinker. The construct was designated pTFCAT. Using a mouse TF promoter to elucidate TF regulation by E_2 in the rat is justified because of the high degree of sequence homology of TF genes among various species and a similar effect of E_2 to increase TF *in vivo* in the immature mouse uterus (our unpublished results). The amino acid sequence of TF is highly conserved in rabbit, mouse and human (Andrews *et al.*, 1991) and partial sequencing of rat TF cDNA indicates a high degree of amino acid identity with mouse TF (94% and 89% identity in regions spanning amino acids 45 to 93; N. Mackman, unpublished and 241 to 294; Zioncheck *et al.*, 1992, respectively. The numbering of amino acids is from the methionine at position one of mouse TF according to Mackman *et al.*, 1992).

A consensus ERE and a potential TF ERE, shown in Figure 6A, were constructed by synthesizing complementary oligonucleotides containing HindIII sites at the 5′ ends, annealing and cloning into the HindIII site upstream of the thymidine kinase (TK) promoter in CAT expression plasmid pBLCAT2 (Luckow and Schütz, 1987).

Calcium phosphate transfection of DNA into HeLa cells was carried out as previously described (Pentecost *et al.*, 1990). Each 60 mm plate received 2 μg CAT construct together with 0.2 to 0.8 μg pHEO, a human ER expression clone (Druege *et al.*, 1986, previously described as pKCR2-ER in Green *et al.*, 1986). pHEO contains the complete ER open reading frame inserted in the eukaryotic expression vector pKCR2 downstream from the SV40 early promoter. Co-transfection with pHEO was necessary since HeLa cells lack functional ER. Sufficient Bluescript SK− was added to bring the total DNA to 25 μg. Cells were treated with E_2 (10 nM) or TPA (200 nM) in ethanol, or ethanol alone, with a final concentration of ethanol in cultures of 0.05%. Cells were harvested 22–24 h after treatment and processed for CAT assays as described (Pentecost *et al.*, 1990). All points in each figure were derived from plates transfected at the same time ($n = 3$ per treatment). The amount of cellular protein used for CAT assays was that expected to give 50% chloramphenicol conversion based on test assays of samples expected to contain the highest CAT activity. The conversion of chloramphenicol to acetylated forms was quantified by direct counting on a β-scope analyzer. The small degree of variation in CAT activity between replicate transfections (Figure 6B and 6C) indicated that transfection efficiency was relatively uniform.

Statistical analyses

Results from RNase protection assays were normalized to levels in control cultures (set to 100%) and then analysed by one-way analysis of variance followed by Duncan's multiple range test to detect differences between individual means.

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