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Binding of factor VIIa to tissue factor induces alterations in gene expression in human fibroblast cells: Up-regulation of poly(A) polymerase

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ABSTRACT **Tissue factor (TF) is the cellular receptor for an activated form of clotting factor VII (VIIa) and the binding of factor VII(a) to TF initiates the coagulation cascade. Sequence and structural patterns extracted from a global alignment of TF confers homology with interferon receptors of the cytokine receptor super family. Several recent studies suggested that TF could function as a genuine signal transducing receptor. However, it is unknown which biological function(s) of cells are altered upon the ligand, VIIa, binding to TF. In the present study, we examined the effect of VIIa binding to cell surface TF on cellular gene expression in fibroblasts. Differential mRNA display PCR technique was used to identify transcriptional changes in fibroblasts upon VIIa binding to TF. The display showed that VIIa binding to TF either up or down-regulated several mRNA species. The differential expression of one such transcript, VIIa-induced up-regulation, was confirmed by Northern blot analysis. Isolation of a full-length cDNA corresponding to the differentially expressed transcript revealed that VIIa-up-regulated gene was poly(A) polymerase. Northern blot analysis of various carcinomas and normal human tissues revealed an over expression of PAP in cancer tissues. Enhanced expression of PAP upon VIIa binding to tumor cell TF may potentially play an important role in tumor metastasis.**

Tissue factor (TF) is the cellular receptor for an activated form of clotting factor VII(a) and factor VIIa/TF complex is the physiological initiator of the coagulation cascade (1). Tissue factor is constitutively expressed in many cells including fibroblasts and pericytes in and surrounding blood vessel walls (2, 3). However, TF is not normally expressed in cells within vasculature, such as monocytes and endothelial cells. Regulation of TF gene by a large and diverse group of molecules (4) raises the possibility that TF may participate in biological processes other than hemostasis, particularly in angiogenesis and tumor metastasis. Recently, several studies (5–7) provided *in vivo* experimental evidences of a role for TF in tumor metastatic process. It has been suggested that the metastatic effect of TF could involve transduction of an angiogenic signal for vascularization of developing tumors (6).

Several recent findings suggest that TF could function as a true receptor. Specific serine residues in the cytoplasmic tail of TF have been shown to be phosphorylated in cells following stimulation with protein kinase C activator (8). Rottingen *et al.* (9) showed that the binding of VIIa to TF induced cytosolic Ca^{2+} signals in J82 cells and in other cell types. More recently, Masuda *et al.* (10) showed that several polypeptides were transiently phosphorylated on tyrosine residues when cultured human monocytes were incubated with VIIa. However, so far no reports are available on whether VIIa binding to TF alters the rates of synthesis and/or the steady state levels of any cellular mRNAs and of the corresponding proteins.

The present study is carried out to test the hypothesis that the occupancy of TF receptor by its ligand induces an intracellular signal(s) that will trigger a selective expression or suppression of cellular gene expression. We have used differential display PCR methodology to identify alterations in gene expression in fibroblasts upon VIIa binding to cell surface TF. The results indicate that the expression of several transcripts are altered by VIIa binding to cell surface TF and one such alteration is the induction of poly(A) polymerase (PAP) gene that plays an important role in processing mRNA. Our data also show that PAP gene expression is up-regulated in many human cancer tissues, particularly in colon carcinoma.

MATERIALS AND METHODS

Reagents. RNAmap kit for mRNA differential display PCR was purchased from GenHunter corporation (Brookline, MA). AmpliTaq polymerase was from Perkin–Elmer, [α-^{35}S]dATP and [α-^{32}P]dCTP (3,000 Ci/mmol; 1 Ci = 37 GBq) were from DuPont/NEN, nick translation kit was from Amersham, TRI reagent was from the Molecular Research Center (Cincinnati); T4 DNA ligase, DNase I, and restriction enzymes were from New England Biolabs or Promega. Human monocyte (phorbol 12-myristate 13-acetate-treated) cDNA library was obtained from CLONTECH.

Proteins. Recombinant human VIIa, a gift from Novo-Nordisk (Copenhagen), was reconstituted in sterile water at a concentration of 1 mg/ml. Factor Xa was prepared by incubating purified factor X with Russell's viper venom followed by benzamidine Sepharose chromatography (11). Monospecific, polyclonal anti-human TF antiserum was raised in a rabbit (3) and the IgG fraction was separated by precipitation at 40% ammonium sulfate saturation, followed by DEAE/Affi-Gel-Blue chromatography.

Cell Culture. A fibroblast cell line, WI-38, was grown in T-75 flasks as described earlier (12). When cells reached 80–90% confluency, the monolayers were washed once with buffer A (10 mM Hepes, pH 7.5, containing 150 mM NaCl/4 mM KCl/11 mM glucose) containing 5 mM EDTA and then twice with the buffer not containing EDTA before the monolayers were subjected to specific treatments.

Tissue Specimens. A variety of normal and cancer tissues were obtained from Nissi Varki (Cancer Center Research

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This paper was submitted directly (Track II) to the *Proceedings* office.
Abbreviations: TF, tissue factor; VIIa, activated form of clotting factor VII; PAP, poly(A) polymerase.
Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF002990).
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Tissue Core, University of California, San Diego). These tissues, obtained from surgical specimens and in some cases from autopsies, were collected within an hour of removal. The tissue samples were flash frozen and stored at ultra low temperature (below −135°C). The existence of malignant cells in carcinoma tissues was confirmed by hematoxylin/eosin staining.

RNA Isolation and DNase I Treatment. Total RNA was isolated using TRI reagent according to the guidelines provided by the manufacturer. Total RNA, 25 μg in 100 μl of 10 mM Tris·HCl, 50 mM KCl, 1.5 mM $MgCl_2$, and 10 units of RNase inhibitor, was treated with 10 units of DNase I for 20 min at 37°C. Then, the RNA was extracted once with phenol/chloroform (1:1, vol/vol), precipitated with ethyl alcohol/0.3 M sodium acetate, and the pellet was resuspended in 20 μl of diethyl pyrocarbonate-treated water.

mRNA Differential Display. Differential display of mRNA was performed as described (13, 14) with slight modifications. Briefly, DNA free total RNA (0.2 μg in 20 μl) was reverse transcribed with Moloney murine leukemia virus reverse transcriptase (100 units) for 1 h at 37°C in the presence of dNTPs (each at 20 μM) and one of the four T_{12}MN oligonucleotides (1 μM) (M = G, C, or A; N = G, A, T, or C) that prime Poly(A) region of mRNAs. The cDNAs generated were then amplified by PCR using 0.1 volume of the above reverse transcribed mixture in the presence of [α-^{35}S]dATP. The reaction mixture (20 μl) contained 2 μM of dNTPs, 1 μM of the same arbitrary T_{12}MN oligonucleotide that was used in the reverse transcription reaction, 0.2 μM of a specific arbitrary 10-mer and 10 units of AmpliTaq DNA polymerase. Light mineral oil was overlaid and PCR reactions were performed in a Perkin–Elmer thermal cycler. The reaction cycles were: 94°C for 30 sec, 40°C for 2 min, 72°C for 30 sec for 40 cycles; 72°C for 5 min and soak at 4°C. One μl of loading dye was added to 5 μl of PCR samples and heated to 80°C for 2 min prior to loading on 6% polyacrylamide sequencing gel. The gels were run at 90 watts with constant current for 2 h. The gels were dried without fixation and exposed directly to the Reflection NEF 495 autoradiography film overnight at room temperature.

Band Recovery and Reamplification. After orienting the dried gel with the autoradiograph, the differentially expressed bands were excised from the dried gel. The gel slice was soaked in sterile water for 10 min and then boiled for 15 min. After centrifugation, the supernatant was transferred to a new tube and the DNA was precipitated in the presence of glycogen and sodium acetate. The pellet was rinsed with 85% ice cold ethyl alcohol and resuspended in 10 μl of water. Reamplification of these DNAs was performed using one-third of the recovered sample by PCR with the appropriate 5′ primers and 3′ primers. The conditions for the PCR were the same as the above except that dNTPs concentrations were increased to 25 μM and the radioisotope tracer was omitted from the reaction mixture. The PCR products were run on a 1.8% agarose gel to determine the size of each fragment. The DNA bands were excised from the gel and the DNAs were eluted from the gel by a standard freeze-squeeze method.

Northern Blot Analysis, Cloning, and Sequencing. Reamplified cDNA probes were labeled with [α-^{32}P]dCTP by nick-translation. Northern blot analysis was carried out using standard procedures. The differentially expressed transcripts were subcloned into Bluescript plasmid at *Eco*RV site after the addition of T overhangs (15). Subcloned inserts were isolated, confirmed for differential hybridization on Northern blots, and then sequenced using Sequenase Rapid Well DNA sequencing kit (United States Biochemical).

Construction of Human Colon Carcinoma cDNA Library and Isolation of a Differentially Expressed cDNA. Total RNA was extracted from human colon carcinoma tissue using TRI reagent. Poly(A)-rich RNA was isolated from the total RNA using an oligo(dT) cellulose column. Size selected (0.8 to >4.0 kb) double-stranded DNA was cloned into Uni-ZAP XR vector (Stratagene). Clones (2×10^6) of the unamplified library were screened with a radiolabeled band 11 cDNA insert (see *Results* for details) employing standard procedures. Twelve positive clones were obtained in the initial screening. In further screenings, the positive clones were plaque purified. Bluescript phagemid DNA was recovered from the Uni-ZAP with ExAssist helper phage (Stratagene) and the inserts were released by *Eco*RI and *Xho*I digestion. The cDNA inserts of several positive clones were partially sequenced using T3/T7 primers to obtain 5′ and 3′ terminal nucleotide sequences.

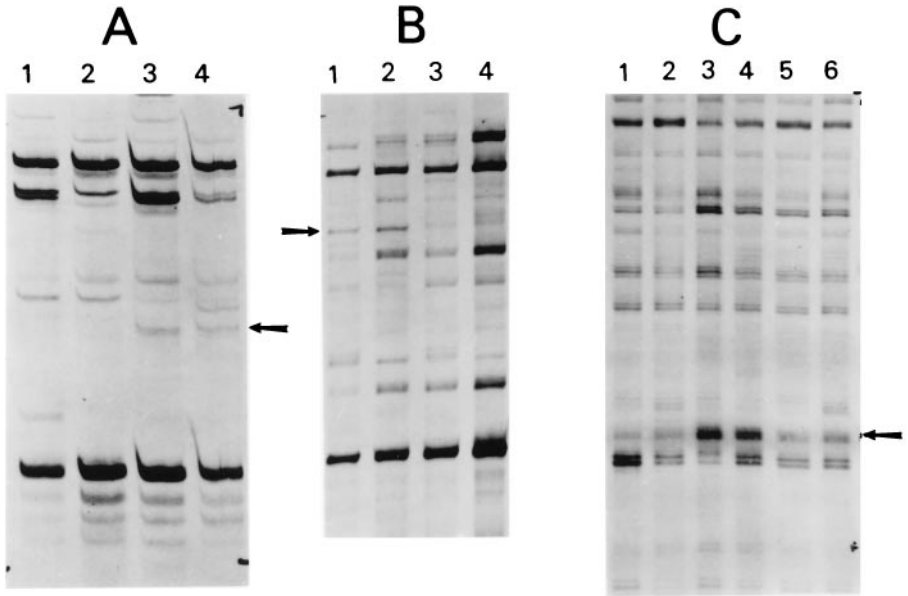

FIG. 1. Representative differential mRNA display PCR. Lanes 1 and 2, RNA from control cells; lanes 3 and 4, RNA from VIIa-treated cells; lane 5, RNA from Xa-treated cells; lane 6, RNA from anti-TF IgG-treated (200 μg/ml) cells prior to the addition of VIIa. The monolayers were treated for 90 min with a control buffer or the buffer containing VIIa or Xa, 10 nM. RNA samples were reverse transcribed and amplified with (*A*) T_{12}MC and 5′-AGCTGACCGT-3′; (*B*) T_{12}MA and 5′-GTGATCGGAC-3′; and (*C*) T_{12}MT and 5′-AGCCAGCGAA-3′ primers. The differentially displayed bands are identified by arrows.

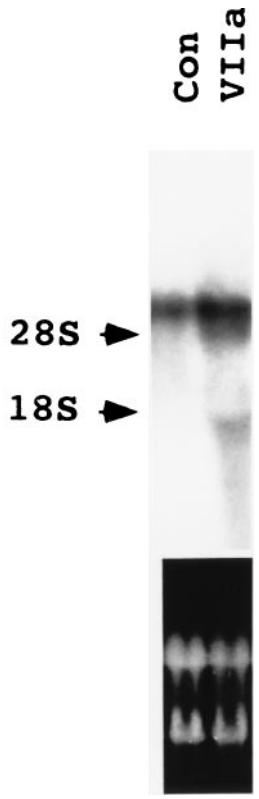

FIG. 2. Northern blot analysis confirming differential expression of a specific mRNA in VIIa-treated cells. WI-38 monolayers were treated with the control buffer or the buffer containing VIIa, 10 nM, for 90 min at 37°C. Total RNA was extracted from the cells and 15 μg of each RNA sample was used for Northern blot analysis. The blot was hybridized with a cDNA fragment of band 11 isolated from the display gel of Fig. 1*C*. (*Upper*) Northern blot analysis with band 11 cDNA. (*Lower*) Ethidium bromide staining of total RNA samples as controls for equal loading.

RESULTS

Differential mRNA Display. Fibroblast monolayers that constitutively express cell surface TF were cultured to 80–90% confluency in 75 mm^2 flasks and the monolayers were treated at 37°C with a control calcium containing buffer or the buffer containing VIIa, 10 nM. As additional controls, the monolayers were first incubated with anti-TF IgG, 200 μg/ml, for 20 min prior to the addition of VIIa or factor Xa was substituted for VIIa in the treatment. After a 90-min treatment period, total RNAs were isolated from the treated monolayers and the expression of mRNA was analyzed using combinations of 20 arbitrary 10-mers and four T_{12}MN primers. To minimize errors in the procedure and to avoid selection of artifact bands as differentially expressed transcripts, RNA samples prepared from two separate experiments were used as duplicates for each display. Each differential display lane yielded 35–60 discrete bands allowing evaluation of 2,800 to 4,800 mRNA species in total. Patterns of amplified cDNA species between the control and VIIa-treated samples were mostly identical, providing a reproducible uniform background over which specific differences could be observed (Fig. 1). In initial display, we identified 12 differentially expressed transcripts. To confirm the reproducibility of the differential expression, we repeated the display including reverse transcription/PCR amplification reactions using a new set of RNAs isolated from control and VIIa-treated cells on two different days. When the reactions were repeated for eight of the 12 selected bands, six of the eight bands showed reproducible differential expression. Two transcripts, designated as bands 11 and 16, were up-regulated, whereas four transcripts, designated as bands 12, 20, 27, and 28, were down-regulated upon VIIa binding to TF.

Northern Blot Analysis with Differentially Expressed Transcripts and Cloning of the Reamplified cDNA Fragments. The bands that showed VIIa-specific differential expression were excised from the gel, reamplified, labeled with [α-^{32}P]dATP, and used as probes in Northern blot analysis. The blots contained RNA isolated from the control and VIIa-treated WI-38 cells. Out of six probes tested, differential hybridization pattern was clearly evident with only one probe (band 11). With others, it was difficult to draw firm conclusions on their differential expression since the hybridization signals were either weak or hybridized to multiple mRNA species in both the control and VIIa-treated samples.

Next, the reamplified cDNA fragment of band 11 was cloned into the pBluescript SK+/− phagemid at *Eco*RV site after adding T overhang at 3′ end. Inserted cDNAs were isolated, radiolabeled, and used as a probe in Northern blot analysis to confirm the VIIa-specific up-regulation of the transcript. Several individual cDNA clones generated from band 11 showed an enhanced hybridization signal to a specific mRNA transcript of ≈4.5 kb in VIIa-treated cells (Fig. 2). In several experiments, we also observed a faint but specific hybridization to a mRNA transcript corresponding to 2.3 kb, in addition to the 4.5 kb, in VIIa-treated cells. Cloned cDNA fragments of band 11 that showed specific differential hybridization patterns on the Northern blots were sequenced. The nucleotide sequence showed that the band 11 transcript was flanked by mRNA mapping 10-mer primer sequences at the 5′ end and putative polyadenylation signals (T_{12}MN) at 3′ end (Fig. 3). Searching the GenBank and EMBL DNA databases using BLAST program revealed no homology sequences in the data bank.

Isolation of Full-Length cDNA for Band 11. A human monocyte, phorbol 13-myristate 12-acetate perturbed, λ gt11 cDNA library was screened with a cDNA probe generated from band 11. In an initial screening, one positive cDNA clone (≈0.7 kb) was obtained. The insert was isolated, radiolabeled, and used as a probe to confirm the differential expression in Northern blot analysis. The hybridization pattern of this probe was similar to that of hybridization pattern observed with the cDNA probe directly generated from the PCR product of band 11 (see Fig. 2). The 3′ sequence of this clone was identical to the sequence of band 11 cDNA fragment. However, the 0.7-kb cDNA insert also had no ORF. Rescreening of the monocyte library with the 0.7-kb insert did not yield any additional positive clones.

Next, we screened human colon carcinoma cDNA library to isolate a full-length cDNA corresponding to band 11 since a high level expression of this gene was found in human colon carcinoma tissue (see below). By screening 2×10^6 clones with

AGCCAGCGAA GAAAAAAGAT GGTCATACTA ACAGGTGAAA TGTACAAGGT GTCTGTGTGT
TTTGTGTAGC TTCAGAGTTA GATTGAAATT ACCAGGCACA GATTTAGTCT TGTCATTTTG
TTTACACATT GGGGAAAACA ATTCAGTTTA TTAAACGTTT CATGTAACTG CACCCAAGTT
TTGCCAAGCT GGAAACTTGG ACCTTTTCTG TGTAGTGACT TTTTAATTAT AGTTTTCATA
ACCTGGAGAT CAGACTGTTG CTTTCGCATG ATGTATGTAG TGTCTCATGA CTGGAGTTTG
CTTTGTTTTA TAGTATCTGT ACTCCTTGTA TTTTTCAAGA GCTATTTTGT AAACAGATGA
TGTATTTCTC CATTGAAAAC ACAATAAAAA AAAACAGCAC AAAAAAAAAA AA

FIG. 3. Nucleotide sequence of band 11 transcript. Flanking primer sequences are underlined.

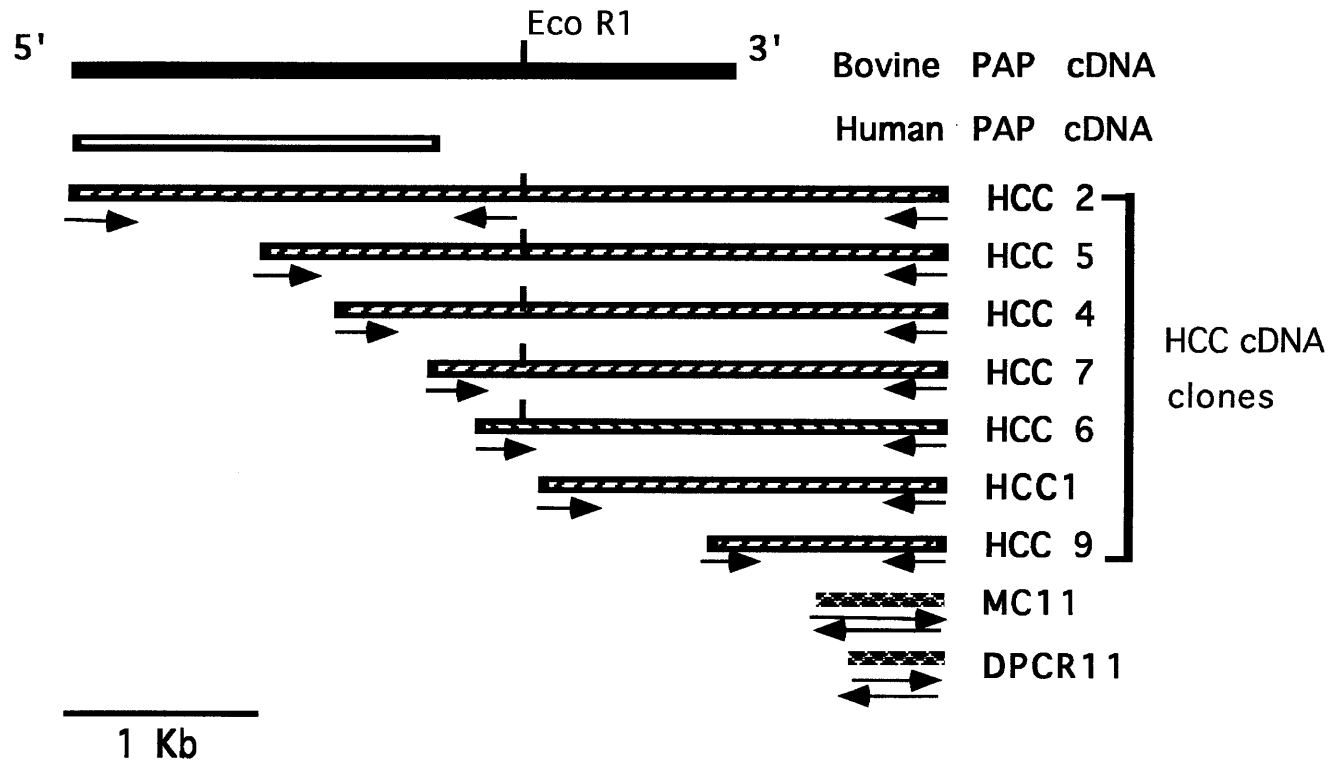

FIG. 4. The schematic representation of cDNA clones that were isolated from human colon carcinoma (HCC) cDNA library by using MC11 cDNA probe. The direction of arrows and the length of arrows under each clone represent the direction of the sequence and the length of the sequence obtained. Top bars represent the length of known cDNA sequences for the bovine PAP (solid) and the human PAP (open). DPCR 11 denotes a band 11 cDNA and MC11 denotes a cDNA clone corresponding to band 11 isolated from phorbol 13-myristate 12-acetate-treated human monocyte cDNA library.

the 700-bp insert isolated from phorbol 13-myristate 12-acetate-perturbed monocytic cDNA library yielded 12 positive clones. The estimated inserts size varied from 1.1–4.5 kb. Several of these inserts were sequenced partially to obtain 5′ and 3′ terminal nucleotide sequences. The limited sequences obtained showed a near 100% homology with human PAP (16) and ≈97% homology with bovine PAP (17). The longest PAP cDNA isolated was ≈4,500 bp, and was likely to be a full-length since it started with a 5′ untranslated region and ended with a Poly(A) tail at 3′. This cDNA clone was ≈2.5 kb and ≈1.1 kb longer than the previously isolated PAP cDNAs from human (16) and bovine (17) sources, respectively (Fig. 4).

To further strengthen our observations and to show that PAP was indeed differentially expressed in VIIa-treated cells, the full-length PAP cDNA insert was isolated, radiolabeled, and used to probe the RNA isolated from the control and VIIa-treated cells by Northern blot analysis. The data showed that PAP message was up-regulated in VIIa-treated cells (Fig. 5).

Increased Expression of Band 11 Transcript (PAP) in Cancer. RNA was isolated from a variety of normal and cancerous tissues and analyzed by Northern blot. The blot was probed with the 400-bp band 11 cDNA fragment that was originally isolated from the display. As seen in Fig. 6, the specific hybridization signal for a 4.5-kb mRNA transcript was much stronger for RNA isolated from cancer tissues than RNA isolated from their benign counterparts. The data also showed a faint hybridization of band 11 transcript to a 2.3-kb mRNA transcript in cancer tissues but not in normal tissues. The expression of band 11 transcript was particularly striking in colon carcinoma compared with a low level expression found in benign colon tissue. The increased expression of the band 11 transcript was also evident in many other malignant tissues, such as breast, ovary, and pancreas.

DISCUSSION

In many receptor-mediated processes, occupancy of the receptor by its ligand alters the rate of synthesis and steady state levels of many cellular RNAs and the corresponding proteins. The present study is the first to provide evidence for the binding of VIIa to TF receptor leads to specific alterations in cellular gene expression in cultured fibroblasts. In this study, we employed differential mRNA display method to identify VIIa-induced specific gene alterations. To avoid isolating mRNAs whose differential expression might have been simply related to artifacts in the lengthy procedure rather than specific for VIIa treatment, we routinely performed differential display PCR using two different RNA samples obtained on two different days. Further, in several display runs, we also included RNA samples obtained from the monolayers treated with factor Xa and the monolayers treated with anti-TF IgG before the monolayers were exposed to factor VIIa as additional controls. More importantly, we repeated differential display analysis two or three times for each potential "positives" and only those that were reproduced consistently were considered as potential transcripts whose expression was altered by factor VIIa binding to TF. Even with such stringent conditions, only one of the six selected differentially expressed transcripts showed VIIa-specific differential expression on Northern blot analysis. However, this finding was not entirely unexpected. Differential display signals not confirmed by Northern blot analysis were relatively common due to undetectable signals (18). It is possible that a PCR-amplified product from a display band can contain a number of distinct multiple cDNA fragments, similar in size, but derived from different genes. The problem can be confounded further if the transcript that shows differential hybridization is present in low abundance.

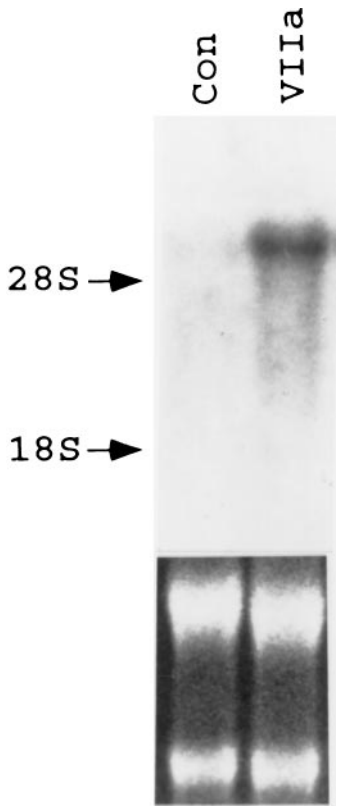

FIG. 5. Increased expression of PAP mRNA in fibroblasts treated with VIIa. WI-38 monolayers were treated with VIIa as described in Fig. 2. Total RNA was extracted from the cells and 15 μg of each RNA sample was used for Northern blot analysis. The blot was hybridized with a full-length PAP cDNA insert. Lane 1, RNA from control cells; lane 2, RNA from VIIa-treated cells. Bottom panel shows ethidium bromide staining of total RNA samples as controls for equal loading.

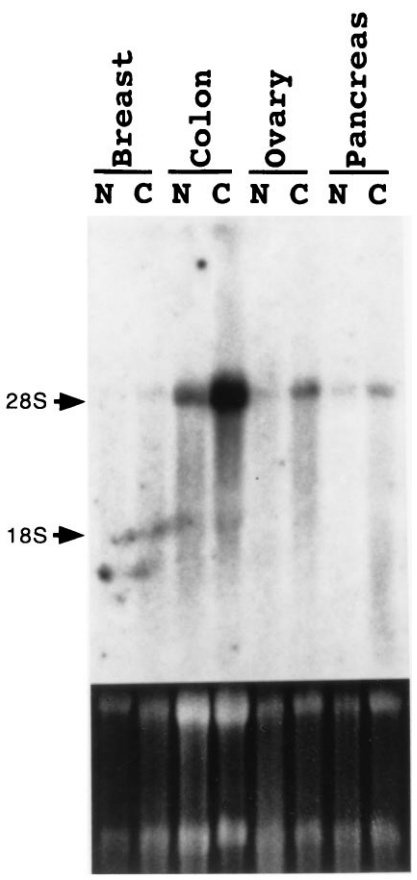

FIG. 6. Northern blot analysis of various cancer and benign tissues for the expression of band 11 transcript. Ten micrograms of total RNA from each tissue was loaded and the blot was hybridized with the 400-bp band 11 cDNA fragment. (*Bottom*) Ethidium bromide staining of total RNA samples as controls for equal loading. N, normal tissue; C, cancer tissue.

A specific transcript that was shown to be up-regulated in VIIa-treated cells (band 11) hybridized to two mRNA species of 4.5- and 2.3-kb on Northern blot analysis. Although the band 11 transcript hybridizes to the 4.5-kb transcript in RNA isolated from the control cells (not treated with VIIa), the intensity of the hybridization signal is lower in the controls compared with the VIIa-treated samples. The sequence of band 11 transcript did not match to any known sequences in the GenBank and EMBL databases. However, since the differential display method provides clones that contain only extreme 3′ regions of mRNA, the lack of homology with known sequences does not eliminate the possibility that the band 11 transcript may be homologous to known cDNAs whose 3′-untranslated region sequences were unavailable. Indeed, isolation a full-length cDNA clone corresponding to the band 11 transcript from human colon carcinoma cDNA library revealed that the VIIa-specific transcript we isolated from the differential display is the product of an already known gene, PAP.

PAP, both the mRNA and the protein, in mammalian cells exists in multiple forms (16). Northern blot analysis with RNA from HeLa cells and calf thymus showed three bands, 4.5, 2.4, and 1.3 kb (19). This could explain why our band 11 cDNA hybridized to two different mRNA species of 4.5 and 2.3 kb.

PAP plays a critical role in the synthesis of messenger RNA in eukaryotic cells. The main function of PAP is the addition of adenylate residues onto mRNA 3′ end following cleavage (20, 21). The length of polyadenylation is directly correlated to mRNA stability (22). Poly(A) tail shortening and its ultimate removal leads to mRNA degradation. In short lived mRNA, such as c-fos, poly(A) tail shortening is exceptionally rapid (23). Most of the oncogene and cytokines mRNA are also short lived. It is possible that increased expression of PAP could increase the polyadenylation of these mRNAs thereby increase their stability that ultimately leads to increased translation products. Such a mechanism would potentiate the action of many short-lived cytokines synthesized by tumor cells. However, further experiments are needed to substantiate that the observed increase in PAP in the cancer tissue is due to increased expression of PAP by the cancer cells and not the surrounding stromal cells or endothelial cells.

Recent studies showed that TF plays a role in angiogenesis and tumor metastasis (24). However, it is controversial whether TF coagulant function is required for tumor cell dissemination. Bromberg *et al.* (6) reported that a nonmetastatic human cell line became metastatic upon transfection with retrovirus carrying human TF cDNA to overexpress TF. Since metastases also occurred in mice injected with the melanoma cells expressing the extracellular TF mutant that was defective in its coagulant function, it had been suggested that the metastatic effect of TF in the severe combined immunodeficient mouse model did not involve products of the coagulation cascade (6). However, this assumption may not be valid since the extracellular TF mutant can bind to VIIa and the resultant TF/VIIa complexes were defective but not completely devoid of the coagulant function (25). Moreover, Fischer *et al.* (26) showed that the thrombin generation is necessary for the TF-dependent hematogenous metastasis (26). In a recent study, Ruf and Mueller (27) provided evidence that TF/VIIa has to be catalytically active for TF-dependent tumor cell metastasis.

In conclusion, our observation that the binding of VIIa to TF induces intracellular signaling was consistent with the earlier observations that VIIa binding is required for TF-mediated signal transduction events such as generation of cytosolic calcium transients (9) and transient tyrosine phosphorylation of several polypeptides (10). Up-regulation of PAP upon VIIa binding to TF suggests that the binding of VIIa to cell surface TF not only initiates the coagulation cascade but also transduces signal(s) that may play an important role in angiogenesis and tumor metastasis.

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