Transcriptional regulation of plasminogen activator inhibitor type-1 mRNA in Hep G2 cells by epidermal growth factor

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ABSTRACT

Secretion of plasminogen activator inhibitor type-1 (PAI-1) by cultured cells is increased after exposure to specific cytokines and growth factors. We have shown previously that incubation of Hep G2 cells with epidermal growth factor (EGF) results in a marked increase in steady state levels of PAI-1 mRNA (Lucore, C.L., et al. (1988) J. Biol. Chem. 263, 15845 – 15848). The present study was undertaken to determine whether the regulation of expression of PAI-1 mRNA by EGF is mediated at the level of transcription and/or by post-transcriptional mechanisms. The rate of transcription of the PAI-1 gene measured by nuclear run-on assays was found to be increased within 2 h after stimulation of the cells with EGF (5 ng/ml) (3.2 fold increase relative to control, n = 2, range 3.0 – 3.4). It reached a maximum in 3 h, (9.2 fold increase relative to control, n = 2, range 8.8 – 9.6) and returned to baseline in 5 h. Exposure of the cells to EGF did not increase the rate of transcription of the glyceraldehyde-3-phosphate dehydrogenase gene. The half life of PAI-1 mRNA in Hep G2 cells was 120 min as determined by RNA blot analysis after exposure of the cells to actinomycin D to inhibit transcription. Stimulation of the cells with EGF did not result in significant change in the half life of PAI-1 mRNA. The results demonstrate that exposure of Hep G2 cells to EGF increases PAI-1 gene transcription.

INTRODUCTION

Plasminogen activator inhibitor type-1 (PAI-1), a glycoprotein of approximately 52 kDa, is the major physiologic inhibitor of tissue-type (t-PA) and urokinase-type plasminogen activators (1). It is present in numerous cell types including platelets, endothelial cells, hepatocytes, and fibroblasts (2- 6). PAI-1 activity in plasma attenuates the activity of t-PA after pharmacologic infusions of the activator for treatment of coronary thrombosis (7). PAI-1 activity in the plasma is elevated in association with deep venous thrombosis (8) and responds as an acute phase reactant with increases after surgery, trauma, or severe infection (8–11). PAI-1 elevations revert more rapidly to normal than those of other acute phase reactants, with plasma levels returning to baseline

as early as one day after surgery (9). PAI-1 is associated with extracellular matrix and is thought to regulate matrix turnover (12).

The activity of PAI-1 in conditioned media of diverse human cell types in culture increases after exposure of the cells to several identified factors. Increased activity appears generally to be preceded by an increase in the steady state level of PAI-1 mRNA. Steady state levels of PAI-1 mRNA are increased in endothelial cells exposed to transforming growth factor beta (TGF- β), lipopolysaccharide (LPS), interleukin-1, tumor necrosis factor (TNF) (13,14), or to thrombin (15); in fibroblasts exposed to TGF- β (16); and in rhabdomyosarcoma cells exposed to phorbol esters (17). The stimulatory effect of TGF- β , LPS, TNF and phorbol esters has been shown to be mediated, at least in part, by increased PAI-1 gene transcription (14,17,18). We have shown previously that epidermal growth factor (EGF) modifies steady state levels of PAI-1 mRNA in Hep G2 cells (19). To determine whether the effect of EGF on PAI-1 mRNA expression in Hep G2 cells is mediated by an increase in transcription, an increase in mRNA stability, or both, we performed nuclear run-on assays and mRNA half life studies.

EXPERIMENTAL PROCEDURES

Materials

Well differentiated human hepatoma cells (Hep G2) were obtained from the American Type Culture Collection (ATCC no. HB 8065) (20). The PAI-1 cDNA probe was provided by K. Kretzmer of the Monsanto Corp. (St. Louis, MO). Glyceraldehyde-3-phosphate dehydrogenase cDNA was obtained from the American Type Culture Collection (ATCC No. 57091). Serum-free media (Dulbecco's modified Eagles medium/Ham's nutrient F-12 with 25 mM HEPES) was obtained from the Washington University Tissue Culture Support Center. Minimum essential medium with Earle's salts and L-glutamine, penicillin (10,000 units/ml), and streptomycin (10,000 μ g/ml) were purchased from Gibco (Grand Island, NY); NuSerum and EGF from Collaborative Research (Bedford, MA); [35 S]-methionine, [α - 32 P]dCTP, and [α - 32 P]UTP from Amersham Corporation (Arlington Heights, IL); ultrapure ATP, CTP, and GTP from Pharmacia (Piscataway, NJ); RNase A, actinomycin D, and ultrapure phenylmethylsulfonyl fluoride (PMSF) from Boehringer

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Mannheim (Indianapolis, IN); restriction endonucleases and plasmids from New England Biolabs (Beverly, MA); cycloheximide, α -amanitin, S-adenosyl methionine, and ultrapure RNase-free sucrose from Sigma Chemical Co. (St. Louis, MO); RNAzol B from Cinna/Biotech Lab. (Friendswood, TX) and 2-[tris(hydroxymethyl)methylamino]-1-ethanesulfonic acid (TES) from Aldrich Chemical Co., (Milwaukee, WI). Unless otherwise specified, all chemicals were of reagent grade and were purchased from Sigma.

Cell Culture

Hep G2 cells were grown to confluence in 100 mm or 150 mm cell culture dishes with minimum essential medium supplemented with 10% NuSerum, Earle's salts, L-glutamine (2 mM), penicillin (50 units/ml) and streptomycin (50 μ g/ml). Preconfluent cell monolayers were fed 24 hr prior to experiments with complete media. On the following day confluent monolayers were washed three times with phosphate-buffered saline and incubated for 4 h in serum-free media. EGF (5 ng/ml) was then added to the media of half the cell plates. The other half were maintained in serum-free media.

Preparation of cDNA Probes

A 0.9 kb PAI-1 cDNA probe was generated by digestion of PAI-1 cDNA with Eco RI and Sal I and isolated by batch affinity absorption with sodium iodide glass beads (Geneclean, Bio 101, Inc.). The glyceraldehyde-3-phosphate dehydrogenase probe (0.6 kb) was obtained by Eco RI/Pst I digestion. The cDNA probes were radiolabeled with [α - 32 P]dCTP by the random primer technique (Boehringer Mannheim) to a specific activity of 1.8×10^9 dpm/ μ g (21).

Preparation of RNA and Northern Blot Analysis

Hep G2 cells were harvested with a cell scraper, centrifuged at $500 \times g$ for 5 min at 4°C, drained, and stored at -70°C in Corning polypropylene centrifuge tubes. Total cellular RNA was extracted with the proteinase K technique (22) or with a single step acid guanidinium thiocyanate-phenol-chloroform extraction with RNAzol B (23). RNA was quantified by absorbance at 260 nm and assessed for structural integrity by electrophoresis in 1.2% agarose gels after denaturation with methylmercuric hydroxide. Northern blots were prepared as described previously (19) with 5 μ g of RNA and Genescreen membranes (DuPont-New England Nuclear). Hybridization was performed simultaneously with PAI-1 (320,000 dpm/ml) and glyceraldehyde-3-phosphate dehydrogenase (160,000 dpm/ml) cDNA probes for 18–24 h at 42°C with continuous agitation. Membranes were washed three times in a solution of $2 \times$ SSC (0.3M NaCl, 0.03M sodium citrate) with 1% SDS for 5 min at room temperature, once in a solution of $2 \times$ SSC with 1% SDS for 30 min at 65°C and twice in a solution of $0.2 \times$ SSC with 0.1% SDS for 30 min at 65°C. Individual bands were quantified by radioisotopic scanning (Ambis scanner, Automated Microbiology Systems, Inc.) with normalization of the PAI-1 signal to the glyceraldehyde-3-phosphate dehydrogenase signal to correct for unequal loading or transfer of RNA. Autoradiograms were prepared with Kodak XAR-5 film and Cronex intensifying screens at -70°C.

Isolation of Nuclei and Performance of Transcription Run-On Experiments

Nuclei from EGF stimulated and control cells were isolated simultaneously as described by Marzluff and Huang (24) with

a 2.1 M sucrose solution used for the centrifugation process. Transcription run-ons and isolation of nascent RNA transcripts were performed as described by Buonanno (25). Equal numbers of nuclei ($5-20 \times 10^6$) from stimulated and control cells were assayed in parallel in a 200 μ l reaction mixture containing 12.5% glycerol, 5.0 mM magnesium acetate, 120 mM KCl, 0.1 mM EDTA, 25 mM Tris-Cl (pH 7.5), 2.5 mM dithiothreitol, 0.05 mM PMSF, 0.025 mM S-adenosyl methionine, 0.5 mM each in ATP, CTP, GTP, and 500 μ Ci [α - 32 P]UTP (0.2 mM) for 45 min at 25°C. To compare the overall rate of transcription in nuclei from EGF stimulated and control cells the incorporation of [α - 32 P]UTP into trichloroacetic acid-precipitable material was monitored at 0, 15, 30, and 45 min as described by Marzluff and Huang (24). Reactions were terminated by the addition of 2.2 ml of a solution of 7 M urea, 2% sarkosyl, 0.35 M NaCl, and 10 mM Tris-Cl (pH 7.5). The DNA was then sheared by passage through 21 and 25 gauge syringe needles, and the RNA was isolated on a gradient of 5.7 M CsCl by ultracentrifugation at $100,000 \times g$ for 12–16 h at 20°C. To verify RNA polymerase II dependence, nuclei from Hep G2 cells stimulated with EGF for 3 h were preincubated with α -amanitin (2 μ g/ml) for 15 min prior to initiation of transcription run-on assays.

Dot blots of linearized, heat denatured plasmid DNA containing the cDNAs were prepared with a minifold apparatus (Schleicher and Schuell, Inc.) and nitrocellulose paper (Schleicher and Schuell, Inc.). To ensure conditions of DNA excess $5-20 \mu$ g of plasmid DNA were spotted onto the membranes. The membranes were prehybridized in $2 \times$ TESS buffer (0.01 M TES pH 7.4, 0.3 M NaCl, 10 mM EDTA, and 0.2% SDS) containing 200 μ g/ml of yeast tRNA for 12–18 hours at 65°C. Hybridization was performed for 48–72 h at 65°C in $2-5$ ml of $2 \times$ TESS buffer containing equal quantities of labeled RNA transcripts ($2-8 \times 10^6$ dpm/ml) derived from the stimulated or control nuclei. Membranes were washed three times in $2 \times$ SSC with 1% SDS for 5 min at room temperature, twice in $2 \times$ SSC for 30 min at 65°C, twice in $2 \times$ SSC with 40 μ g/ml RNase A for 2 h at 37°C, once in $2 \times$ SSC with 200 μ g/ml proteinase K for 30 min at 37°C, and once in $2 \times$ SSC with 1% SDS for 30 min at 65°C. Autoradiograms were prepared as described above. Signal intensity was quantified by scanning laser densitometry (Ultrosan XL, LKB) with subtraction of background hybridization to linearized pUC-18 DNA.

Determination of PAI-1 mRNA Half-life

Confluent monolayers of Hep G2 cells were washed and serum starved as described above. Cells were then incubated with serum-free media containing EGF (5 ng/ml) or serum-free media alone (control) for 3 h. Actinomycin D (10 μ g/ml final concentration) was then added (0 time) and the cells were harvested at specified times thereafter. Total cellular RNA was extracted with RNAzol B, quantified, and assessed for structural integrity as described above. Slot blots of alkali denatured (10 mM NaOH, 1 mM EDTA) total cellular RNA (5 μ g from cells exposed to EGF and 15 μ g from control cells) were prepared with a slot-blot apparatus (BioRad) and Zetaprobe membranes (BioRad). The membranes were prehybridized in a solution of 1 mM EDTA, 7% SDS, 0.5 M Na₂HPO₄, pH 7.2 for 5–15 min at 65°C. Hybridization was performed with a PAI-1 cDNA probe (320,000 dpm/ml) in fresh 1 mM EDTA, 7% SDS, 0.5 M Na₂HPO₄, pH 7.2 for 18–20 h at 65°C. Membranes were washed twice in a solution of 1 mM EDTA, 5% SDS, 40 mM Na₂HPO₄, pH 7.2 for 30 min at 65°C and twice in a solution of 1 mM EDTA, 1% SDS, 40 mM Na₂HPO₄, pH 7.2 for 30 min at 65°C. The slot blots were

quantified by radioisotopic scanning as described above. PAI-1 mRNA half-life was also measured by Northern blot analysis as described above to allow determination of the half-life of both the 3.2 kb and 2.2 kb PAI-1 mRNA transcripts individually.

Inhibition of New Protein Synthesis by Cycloheximide

To determine whether de novo protein synthesis was required for the induction of PAI-1 mRNA by EGF, confluent monolayers of Hep G2 cells were preincubated with cycloheximide (25 μ g/ml) for 30 min prior to the addition of EGF (5 ng/ml). The cells were harvested after 3 h and RNA was extracted from the cells as described above. To determine whether cycloheximide inhibited new protein synthesis, Hep G2 cells were incubated in serum-free media or serum-free media containing cycloheximide (25 μ g/ml) for 30 min. Cells were washed three times with methionine-free media and incubated for 1 h in methionine-free media containing [35 S]-methionine (16.5–165 μ Ci/ml) with or without cycloheximide. The cells were washed three times with phosphate-buffered saline and cell lysates were prepared with a solution of 0.01 M Tris-Cl, pH 8.0, 0.02 M NaCl, 1 mM EDTA, 0.5% Triton X-100. [35 S] activity present in trichloroacetic acid (TCA) precipitates was quantified by scintillation counting (26). To rule out cycloheximide induced cell death, cells exposed to cycloheximide (25 μ g/ml) for 3 h were washed three times in methionine-free media, incubated for 1 h in methionine-free media containing [35 S]-methionine (21.5 μ Ci/ml) and then processed as described above.

RESULTS

Incubation of confluent monolayers of Hep G2 cells with EGF (5 ng/ml) for 3 h resulted in a 9.2 fold increase in transcription of the PAI-1 gene relative to transcription in control cells not exposed to EGF ($n = 2$, range 8.8–9.6) (Figure 1). Although stimulation with EGF for 3 h resulted in a modest increase in overall transcription (2.0 fold relative to control at 45 minutes, $n = 4$, $p = 0.08$ [paired t-test]) determined from the rate of incorporation of [32 P]UTP into trichloroacetic acid-precipitable material, transcription of the glyceraldehyde-3-phosphate dehydrogenase gene used as a control did not increase. Incubation of nuclei with α -amanitin (2 μ g/ml) inhibited the incorporation of [32 P]UTP into trichloroacetic acid-precipitable material by approximately 40%. After incubation with α -amanitin, transcripts encoding PAI-1 and glyceraldehyde-3-phosphate dehydrogenase were not seen, confirming the RNA polymerase II dependence of the in vitro transcription reactions (Figure 1).

To define the time course of PAI-1 gene transcription in Hep G2 cells, we performed nuclear run-on assays after 1, 2, 3, and 5 h of stimulation of the cells with EGF. Transcription of the PAI-1 gene increased after 2 h of stimulation with EGF (3.2 fold increase, $n = 2$, range 3.0–3.4), peaked in 3 h (9.2 fold increase, $n = 2$, range 8.8–9.6), and returned to baseline after 5 h (Figure 2). EGF did not increase the rate of transcription of the glyceraldehyde-3-phosphate dehydrogenase gene at any time point (data not shown). In every experiment, the plasmid DNA bound to the membrane was confirmed to be in excess of [32 P]-labeled nascent RNA transcripts.

To define the processing time of the PAI-1 primary transcript product (hnRNA), we compared the transcription time course in response to EGF to changes in the concomitantly measured steady state levels of PAI-1 mRNA. Northern blot analyses showed that steady state levels of both forms of mature PAI-1 mRNA (2.2 kb and 3.2 kb) increased after 2 h of stimulation

Figure 1: Regulation of PAI-1 gene transcription by EGF. Confluent Hep G2 cells were serum starved for 4 h. Cells were then incubated with EGF (5 ng/ml) for 3 h or maintained in serum free media. Transcription run-on experiments with isolated nuclei were then performed. Labeled nascent transcripts were hybridized to nitrocellulose membranes containing blotted PAI-1 and glyceraldehyde-3-phosphate dehydrogenase (GAP) cDNAs. The resultant autoradiograms were analyzed by densitometry with subtraction of background hybridization to linearized pUC-18 DNA. To confirm the RNA polymerase II dependence of results, nuclei from Hep G2 cells stimulated with EGF (5 ng/ml) were preincubated with α -amanitin (2 μ g/ml) for 15 min prior to assay of transcription in run-on experiments.

Figure 2: Time course of PAI-1 gene transcription. Confluent Hep G2 cells were serum starved for 4 h. Cells were then incubated with EGF (5 ng/ml) for 1–5 h or maintained in serum free media. Transcription run-ons, hybridization of nascent transcripts and quantitation of autoradiograms were then performed. Results at all intervals are averages of duplicate experiments with the exception of the value at 5 h. Representative autoradiograms at each interval are shown in the top portion of the figure.

with EGF and peaked at 3 h (Figure 3). Steady state levels of glyceraldehyde-3-phosphate dehydrogenase mRNA did not change. The increase in steady state levels of PAI-1 mRNA correlated temporally with the increase in PAI-1 gene transcription indicating rapid processing of PAI-1 hnRNA.

Figure 3: Increase of PAI-1 steady state mRNA after exposure of Hep G2 cells to EGF. Confluent Hep G2 cells were serum starved for 4 h and then incubated with EGF (5 ng/ml) for selected intervals. Northern blots were prepared with 5 μ g of total RNA and probed with a PAI-1 cDNA probe and a glyceraldehyde-3-phosphate dehydrogenase (GAP) cDNA probe. Molecular weight markers in kilobases are shown to the left.

To determine whether new protein synthesis is a necessary condition for the increased expression of PAI-1 mRNA in Hep G2 cells exposed to EGF, cycloheximide was used to inhibit translation. Pretreatment with cycloheximide inhibited [35 S]-methionine incorporation into protein by 93% and inhibited the increased expression of PAI-1 mRNA in response to EGF ($n = 4$) (Figure 4).¹ After treatment with cycloheximide for 3 h, Hep G2 cells were viable and capable of responding to stimuli recovering 90% of their protein synthetic capacity 1 h after cycloheximide washout. Exposure of cells to cycloheximide increased PAI-1 mRNA levels modestly, 1.3-fold above control cells ($n = 4$, range 1.2–1.4) (Figure 4).

To determine whether EGF stabilizes PAI-1 mRNA in Hep G2 cells actinomycin D was used as a transcriptional inhibitor. The half life of PAI-1 mRNA in Hep G2 cells under control conditions and after treatment with EGF was determined by slot blot analysis and by Northern blot analysis of total PAI-1 mRNA. The results are expressed as the percent of PAI-1 mRNA at time = 0 remaining after 1 and 2 hours of exposure of Hep G2 cells to actinomycin D to facilitate pooling of data from multiple experiments. The results shown in Figure 5A indicate that the half life of total PAI-1 mRNA under control conditions was 120 minutes ($r = 0.99$, $n = 6$), and 140 minutes after stimulation with EGF ($r = 0.99$, $n = 5$). Therefore, EGF does not stabilize PAI-1 mRNA transcripts.

The half life of the 3.2 kb and 2.2 kb PAI-1 mRNA transcripts was determined by Northern blot analysis. Treatment with EGF did not result in stabilization of either the 2.2 kb (Figure 5B) or 3.2 kb (Figure 5C) PAI-1 mRNA transcript. Under control

Figure 4: Inhibition of new protein synthesis blocks the effect of EGF on PAI-1 mRNA expression. Hep G2 cells were serum starved for 4 h and then incubated for 3 h with serum-free media (control), serum-free media with cycloheximide (25 μ g/ml), serum-free media with EGF (5 ng/ml), or preincubated with cycloheximide (25 μ g/ml) for 30 min prior to the addition of EGF (5 ng/ml) for 3 h. Northern blots were prepared with 5 μ g of total RNA and probed with a PAI-1 cDNA probe and a GAP cDNA probe. Molecular weight markers in kilobases are shown to the left. A representative blot from a total of four separate experiments is shown.

conditions the half life of the 3.2 kb PAI-1 mRNA transcript was 73 minutes ($r = 0.99$, $n = 4$) and the half life of the 2.2 kb transcript was 215 minutes ($r = 0.99$, $n = 4$).

DISCUSSION

Type 1 plasminogen activator inhibitor (PAI-1) is a physiologically important component of the fibrinolytic system. Its biosynthesis is highly regulated in cultured cells. For example, in cultured rat ovarian granulosa cells PAI-1 production is suppressed by both follicle-stimulating hormone and leutinizing hormone (27). Glucocorticoids increase PAI-1 production in human fibroblasts (28), fibrosarcoma cells (29), mammary carcinoma cells (30), and rat HTC cells (31,32). A steroid responsive enhancer element in the 5'-flanking DNA of the PAI-1 gene regulates its expression in rat FT02B hepatoma cells (33). Recently, we observed that epidermal growth factor (EGF) increases steady-state levels of PAI-1 mRNA and secretion of PAI-1 protein by Hep G2 cells in a dose- and time-dependent manner (19). The increase in steady-state levels of PAI-1 mRNA observed could have been attributable to increased transcription, increased stability of mRNA, or both. In bovine aortic endothelial (BAE) cells TGF- β , TNF- α , and LPS increase PAI-1 gene transcription but do not stabilize PAI-1 mRNA (14). In the present study, regulation of expression of the PAI-1 gene by EGF was characterized in vitro with nuclear run-on reactions. The results demonstrate that exposure of Hep G2 cells to EGF increases PAI-1 gene transcription in a time dependent fashion. The close temporal correlation of the increase of PAI-1 gene transcription and the increase of steady state mRNA levels indicates that nascent transcripts are processed rapidly.

¹ Initial results with cycloheximide failed to reflect inhibition of the effect of EGF on steady state levels of PAI-1 mRNA in Hep G2 cells (19). However, as noted in the present report, pre-incubation with cycloheximide was shown to be inhibitory. Subsequent repeat studies demonstrated inhibition even without pre-incubation ($n = 4$).

Figure 5: Effect of EGF on the half life of PAI-1 mRNA. Confluent, serum starved Hep G2 cells were incubated with EGF (5 ng/ml) or maintained in serum free media (controls) for 3 h. Actinomycin D (10 μ g/ml) was then added (0 time) and the cells were harvested at the specified times. Slot blots (5 μ g) and Northern blots (10–15 μ g) of total cellular RNA were probed with a PAI-1 cDNA probe and then analyzed by radioisotopic scanning. Panel A represents the time course of decay of total PAI-1 mRNA. Each data point represents the mean one standard deviation of six control experiments or 5 experiments performed on cells treated with EGF. Panels B and C show the time course of decay of the 2.2 kb PAI-1 mRNA transcript and 3.2 kb PAI-1 mRNA transcript, respectively. Each point represents data (mean $\pm$ one standard deviation) from 4 Northern blot experiments with control cells and 3 Northern blot experiments with EGF-treated Hep G2 cells.

EGF has previously been shown to stabilize mRNA of β -actin, β -tubulin, and the EGF receptor in human KB cells (34). Treatment of Hep G2 cells with EGF did not result in a significant change in PAI-1 mRNA half-life. The results indicate that EGF regulates PAI-1 mRNA at the level of transcription.

Transiently expressed genes, such as those encoding various lymphokines, proto-oncogenes, and cytokines, contain AU-rich sequences in the 3' untranslated regions of their mRNA (35,36). These AU-rich sequences impart instability to the mRNA molecule presumably mediated by ribonucleases. Modification of the β -globin mRNA to contain a portion of the GM-CSF AU-rich 3' untranslated region shortens its half-life from hours to less than 30 min (35). The two PAI-1 mRNA transcripts are the result of alternative cleavage-polyadenylation of the 3' untranslated region (37). Interestingly, the 3.2 kb transcript contains AU-rich sequences within the 3' untranslated region which may render the transcript unstable relative to the 2.2 kb mRNA which does not contain these sequences.

Epidermal growth factor and transforming growth factor alpha (TGF- α) are small polypeptides that share approximately 30% structural homology (38). Epidermal growth factor has been identified in platelets (39). TGF- α has been identified in several cell types including monocyte/macrophages and liver cells (38,40). In liver, TGF- α may function as an inducer of hepatocyte regeneration through an autocrine mechanism (40). Both EGF

and TGF- α bind to the EGF receptor and stimulate the receptor's tyrosine kinase activity (38). Because the EGF receptor is localized to the plasma membrane, regulation of PAI-1 gene transcription requires transduction of a signal to the nucleus. The mechanisms by which this occurs are not yet clearly understood. Attenuation of the EGF-induced increase in PAI-1 mRNA in Hep G2 cells by pretreatment of the cells with cycloheximide in the present study suggests that this effect may be mediated by the protein products of other genes, possibly immediate response genes. EGF has been shown to induce transcription of the c-jun, c-fos, and c-myc genes in cultured mouse and rat fibroblasts (41,42). In contrast, cycloheximide does not inhibit the increase of steady state levels of PAI-1 mRNA in Hep G2 cells induced by TGF- β (43). Accordingly, the stimulatory effects of TGF- β appear to be independent of new protein synthesis and to be mediated by mechanism(s) different than those involved with EGF.

Although cycloheximide alone did not appreciably increase PAI-1 mRNA levels in Hep G2 cells after 3 h (1.3 fold increase, Figure 4) or 6 h of incubation (43), it markedly increases steady state levels of PAI-1 mRNA in endothelial cells, fibroblasts, and rhabdomyosarcoma cells (13,14,16,17). In endothelial cells the effect has been shown to be mediated primarily by an increase in PAI-1 gene transcription (14). Thus, regulation of PAI-1 mRNA in Hep G2 cells observed in the present study differs from

that of other cell types consistent with the possibility that short half-life negative regulatory factor(s) are important in these other cell types. The modest increase in PAI-1 mRNA levels elicited by cycloheximide alone in Hep G2 cells is likely to reflect inhibition of ribonuclease production with subsequent mild prolongation of the PAI-1 mRNA half-life (44). Our results indicate that EGF induces PAI-1 mRNA levels in Hep G2 cells by transcriptional mechanisms and that this induction is dependent upon new protein synthesis.

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REFERENCES

- Loskutoff, D. J., Sawdey, M., and Mimuro, J. (1988) in *Progress in Thrombosis and Hemostasis* (Coller, B. ed) Vol. 9, pp. 87–115, W.B. Saunders, Philadelphia.
- Erickson, L. A., Ginsberg, M. H. J., and Loskutoff, D. J. (1984) *J. Clin. Invest.* 74, 1465–1472.
- van Mourik, J. A., Lawrence, D. A., and Loskutoff, D. J. (1984) *J. Biol. Chem.* 259, 14914–14921.
- Laiho, M., Saksela, O., Andreasen, P. A., and Keski-Oja, J. (1986) *J. Cell Biol.* 103, 2403–2419.
- Sprengers, E. D., Princen, H. M. G., Kooistra, T., and Van Hinsbergh, V. W. M. (1985) *J. Lab. Clin. Med.* 105, 751–758.
- Knudsen, B. S., Harpel, P. C., and Nachman, R. L. (1987) *J. Clin. Invest.* 80, 1082–1089.
- Lucore, C. L., and Sobel, B. E. (1988) *Circulation* 77, 660–669.
- Kruihof, E. K. O., Gudinchet, A., Bachmann, F. (1988) *Thromb. Haemost.* 59(1), 7–12.
- Kluft, C., Verheijen, J. H., Jie, A. F. H., Rijken, D. C., Preston, F. E., Sue-Ling, H. M., Jespersen, J., and Aasen, A. O. (1985) *Scand. J. Clin. Lab. Invest.* 45, 605–610.
- Colucci, M., Paramo, J. A., and Collen, D. (1985) *J. Clin. Invest.* 75, 818–824.
- Engelbrechtsen, L. F., Kierulf, P., and Brandtzaeg, P. (1986) *Thromb. Res.* 42, 713–716.
- Mimuro, J. and Loskutoff, D. J. (1989) *J. Biol. Chem.* 264, 5058–5063.
- Schleef, R. R., Bevilacqua, M. P., Sawdey, M., Gimbrone, M. A., and Loskutoff, D. J. (1988) *J. Biol. Chem.* 263, 5797–5803.
- Sawdey, M., Podor, T. J., and Loskutoff, D. J. (1989) *J. Biol. Chem.* 264, 10396–10401.
- Dichek, D. and Quertermous T. (1989) *Blood* 74, 222–228.
- Lund, L. R., Riccio, A., Andreasen, P. A., Nielsen, L. S., Kristensen, P., Laiho, M., Saksela, O., Blasi, F., and Dano, K. (1987) *Embo. J.* 6, 1281–1286.
- Mayer, M., Lund, L. R., Riccio, A., Skou, J., Nielsen, L. S., Stacey, S. N., Dano, K., and Andreasen, P. A. (1988) *J. Biol. Chem.* 263, 15688–15693.
- Riccio, A., Lund, L. R., Sartorio, R., Lania, A., Andreasen, P. A., Dano, K., and Blasi, F. (1988) *Nucleic Acids Res.* 16, 2805–2824.
- Lucore, C. L., Fujii, S., Wun, T., Sobel, B. E., and Billadello, J. J. (1988) *J. Biol. Chem.* 263, 15845–15848.
- Knowles, B. B., Hoew, C. C., and Aden, D. P. (1980) *Science* 209, 497–499.
- Feinberg, A. P. and Vogelstein (1984) *Annal. Biochem.* 137, 266.
- Dillmann, W. H., Barrieux, A., Neeley, W. E., and Conteras, P. (1983) *J. Biol. Chem.* 258, 7738–7745.
- Chomczynski, P. and Sacchi, N. (1987) *Anal. Biochem.* 162, 156–159.
- Marzluff, W. F. and Huang, R. C. (1984) in *Transcription and Translation: A Practical Approach* (Hames, D. B. and Higgins, S. F. eds.) pp. 89–129, IRL Press, Oxford.
- Buonanno, A., and Merlie, J. P. (1986) *J. Biol. Chem.* 261, 11452–11455.
- Bostrom, K., Boren, J., Wettsten, M., Sjoberg, A., Bondjers, G., Wiklund, O., Carlsson, P., and Olofsson, S. O. (1988) *J. Biol. Chem.* 263, 4434–4442.
- Ny, T., Bjersing, L., Hseuh, A. J. W. and Loskutoff, D. J. (1985) *Endocrinology* 116, 1666–1668.
- Crutchley, D. J., Conanan, L. B., and Maynard, J. R. (1981) *Ann. NY Acad. Sci.* 370, 609–616.
- Andreasen, P. A., Pyke, C., Riccio, A., Kristensen, P., Nielsen, L. S., Lund, L. R., Blasi, F. and Dano, K. (1987) *Mol. Cell Biol.* 7, 3021–3025.
- Busso, N., Berlin, D., Failly-Crepin, C. and Vassalli, J. D. (1987) *Cancer Res.* 47, 364–370.
- Cwikel, B. J., Barouski-Miller, P. A., Coleman, P. L., and Gelehrter, T. D. (1984) *J. Biol. Chem.* 259, 6847–6851.
- Loskutoff, D. J., Roegner, K., Erikson, L. A., Schleef, R. R., Huttenlocher, A., Coleman, P. L. and Gelehrter, T. D. (1986) *Thromb. Haemost.* 55, 8–11.
- Van Zonneveld, A.-J., Curriden, S. A., and Loskutoff, D. J. (1988) *Proc. Natl. Acad. Sci. USA* 85, 5525–5529.
- Jinno, Y., Merlino, G. T., and Pastan, I. (1988) *Nucleic Acids Res.* 16, 4957–4966.
- Shaw, G. and Kamen R. (1986) *Cell* 46, 659–667.
- Caput, D., Beutler, B., Hartog, K., Thayer, R., Brown-Shimer, S., and Cerami, A. (1986) *Proc. Natl. Acad. Sci. USA* 83, 1670–1674.
- Ny, T., Sawdey, M., Lawrence, D., Millan, J. L., and Loskutoff, D. J. (1986) *Proc. Natl. Acad. Sci. USA* 83, 6776–6780.
- Derynck, R. (1988) *Cell* 54, 593–595.
- Oka, Y. and Orth, D. N. (1983) *J. Clin. Invest.* 72, 249–259.
- Mead, J. E. and Fausto, N. (1989) *Proc. Natl. Acad. Sci. USA* 86, 1558–1562.
- Cutry, A. F., Kinneburgh, A. J., Krabak, M. J., Hui, S. W., and Wenner, C. E. (1989) *J. Biol. Chem.* 264, 19700–19705.
- Quantin, B. and Breathnach, R. (1988) *Nature* 334, 538–539.
- Fujii, S., Lucore, C. L., Hopkins, W. E., Billadello, J. J., Sobel, B. E. (1989) *Am. J. Cardiol.* 63, 1505–1511.
- Almendral, J. M., Sommer, D., MacDonald-Bravo, H., Burckhardt, J., Perera, J., and Bravo, R. (1988) *Mol. Cell. Biol.* 8, 2140–2148.