Thrombin participates in several regulatory events following injury as a result of its effects on blood coagulation and cell migration, proliferation, and differentiation. Protease nexin-1 (PN-1) is a potent thrombin inhibitor in the extracellular environment. Since injury-related factors are known to regulate the synthesis and secretion of PN-1, the inhibitor may serve to modulate the actions of thrombin during injury. Here we report the molecular mechanisms that underlie this regulation. In normal human fibroblasts, interleukin-1 (IL-1) β stimulated the synthesis and secretion of PN-1. The stimulation correlated with an increase in steady-state levels of PN-1 mRNA. Treatment of cells with both cycloheximide and IL-1 reduced the levels of PN-1 mRNA. Nuclear run-on assays indicated that IL-1 modestly increased the rate of PN-1 transcription. However, experiments with actinomycin D demonstrated that IL-1 significantly increased the half-life of the PN-1 mRNA. In contrast, dexamethasone (DXM) repressed the synthesis and secretion of PN-1 from fibroblasts. This effect correlated with a decrease in PN-1 mRNA. A sustained decrease in PN-1 mRNA was also seen when cells were treated with cycloheximide and DXM. In nuclear run-on assays, DXM functioned as a transcriptional repressor of PN-1 synthesis. Treatment of cells with actinomycin D showed that DXM did not affect mRNA stability. Thus, our experiments demonstrate that IL-1 and DXM, which function biologically in different fashions, regulate the synthesis of PN-1 by separate molecular mechanisms. While DXM directly regulates PN-1 at the level of transcription, IL-1 in the presence of ongoing protein synthesis regulates PN-1 production predominantly in a post-transcriptional fashion by increasing the half-life of the PN-1 mRNA.

Protease Nexin-1 (PN-1) is a 43-kDa serine protease inhibitor which is expressed and secreted from fibroblasts, astrocytes, and certain other extravascular cells (1-3). PN-1 in solution is a potent inhibitor of thrombin, urokinase, and plasmin (4). The protein acts as a pseudosubstrate for each protease, which results in an SDS stable complex between PN-1 and its target protease. Once formed, the complex binds back to the cell surface where it is rapidly internalized and degraded (5). In culture, extracellular PN-1 can differentially regulate serine proteases based on its localization in the microenvironment of the cell. PN-1 binds tightly to the cell surface and the extracellular matrix (ECM) (6), and this interaction accelerates its inhibition of thrombin and blocks its inhibition of urokinase or plasmin (7). Since the majority of PN-1 is likely to be present on the cell surface and the ECM in vivo, it is believed that PN-1 primarily functions as a thrombin inhibitor.

Inhibition of thrombin by PN-1 participates in the regulation of several activities of cultured cells. Thrombin is mitogenic for fibroblasts (8), smooth muscle cells (9), and astrocytes (10). Thrombin is also chemotactic for monocytes (11). More recently, thrombin was shown to retract neurites and stellate processes on neurons and astroglial cells, respectively (12-15). PN-1 can modulate or in some cases reverse this control by inhibiting thrombin. For example, addition of PN-1 to neuronal and astroglial cells treated with thrombin reverses the retraction process, and subsequently returns cells to their differentiated state (12, 14). Since thrombin is produced at sites of tissue injury, its effects on cells may facilitate components of the repair process. However, it is undoubtedly important to regulate thrombin in the extracellular environment of its target cells since uncontrolled actions of thrombin could likely compromise the complex events in tissue repair.

Several studies provide evidence that PN-1 (as well as the identical glia-derived nexin (16, 17)) may play important roles after injury. Following lesion of the sciatic nerve in rats, PN-1/glia-derived nexin is increased in regions of the nerve distal to the site of the lesion (18). Moreover, increased PN-1/glia-derived nexin occurs for a prolonged period in the hippocampus of gerbils following transient forebrain ischemia which produces delayed neuronal death (19). Recent studies showed that several injury-related cytokines and growth factors increased the expression of PN-1 from a neuroblastoma cell line (20). These findings provided evidence that the regulation of PN-1 may be linked to the process of injury and wound repair.

In the current study, we explored the mechanisms by which injury-related factors regulate the synthesis of PN-1 since this has not yet been examined. The low expression of PN-1 mRNA in neuroblastoma cells precluded their use in RNA analysis. We therefore conducted our studies on normal human neonatal foreskin fibroblasts (HF cells), since they strongly express the protease inhibitor at both the protein and mRNA level. We focused our attention on two factors.
that have a pronounced effect on the secretion of PN-1 from HF cells, and are known to affect injury-related processes. The first is interleukin-1 (IL-1), a polypeptide released by activated macrophages which plays a central role in promoting host responses to infection and inflammation (21). IL-1 stimulates the release of prostaglandin E2 in the development of fever (22) and induces the expression of collagenase and stromelysin, leading to the degradation of the ECM (23, 24). The second is nonsteroidal anti-inflammatory drug (NSAID). In contrast to IL-1, DXM is an anti-inflammatory agent, which has a therapeutic application as an immunosuppressive (25). Biochemically, it acts as an antagonist of IL-1 (26) and negatively regulates the genes for stromelysin and collagenase (24, 27).

Our results demonstrate that IL-1 and DXM have opposite effects on the expression of PN-1 from HF cells. While IL-1 stimulated the secretion of the protein, DXM strongly repressed it. These effects were related to changes in the mRNA for PN-1. Studies on the molecular mechanisms of regulation by these factors indicated that IL-1 acted mainly by increasing the half-life of the PN-1 mRNA, while DXM inhibited the rate of PN-1 transcription.

**EXPERIMENTAL PROCEDURES**

**Materials**—HF cells were purchased from the National Institute of Aging Cell Repository, number GM 08860, and cultured as described (28). DXM was purchased from Sigma, and IL-1β from Boehringer Mannheim. PN-1 was purified from serum-free medium conditioned by HF cells using an immobilized anti-PN-1 monoclonal antibody as described (29). Human α-thrombin was obtained from Calbiochem and was iodinated with Na<sup>125</sup>I using the chloroglycoul method as previously described (30). Dulbecco’s modified Eagle’s medium (DME), antibiotics, and fetal bovine serum were purchased from Life Technology, Inc.

**Measurements of Activity of Secreted and Cell-bound PN-1**—HF cells were seeded in a 3.8-cm<sup>2</sup> dish (Corning) in DMEM containing 10% fetal bovine serum and antibiotics and grown to confluence. Cells were incubated overnight in serum-free defined medium conditioned by HF cells using an immobilized anti-PN-1 monoclonal antibody as described (29). Human α-thrombin was obtained from Calbiochem and was iodinated with Na<sup>125</sup>I using the chloroglycoul method as previously described (30). Dulbecco’s modified Eagle’s medium (DME), antibiotics, and fetal bovine serum were purchased from Life Technology, Inc. Measurements of Activity of Secreted and Cell-bound PN-1

**Northern Blot Analysis**—Total RNA was isolated from cultured HF cells using the guanidinium thiocyanate procedure (32). RNA was electrophoresed in a 1.4% agarose gel containing 2.2 M formaldehyde, 20 mM HEPES, pH 7.8, and 1 mM EDTA and transferred overnight onto a nylon filter (Du Pont-New England Nuclear). PN-1 and human β-actin mRNA were analyzed using two complementary riboprobes. For PN-1, a 603-base pair cDNA was subcloned into the HinClI site of pBS (Stratagene), and a run-off transcript was synthesized using T<sub>4</sub> RNA polymerase (Pharmacia LKB Biotechnology Inc.) in the presence of 100 μCi of [α-<sup>32</sup>P]UTP (3000 Ci/mm) (NEN). For β-actin, a 1.1-kilobase cDNA fragment was subcloned into pBS, and a riboprobe was generated using T<sub>7</sub> RNA polymerase (Pharmacia). Both clones were a generous gift from Dr. Paul Ilsackson. Prehybridization and hybridization were carried out in 5 X SSC, 50% (v/v) formamide, 500 μg/ml denatured calf thymus DNA, 0.1% SDS, and 50% dextran sulfate. Hybridizations were performed at 60 and 65 °C for PN-1 and β-actin, respectively. Analysis of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was performed using a 1.2-kilobase fragment that was random primer labeled using Klenow (Boehringer Mannhein) and [α-<sup>32</sup>P]GTP (NEN). Hybridized filters were washed twice in 2 X SSC, 0.1% SDS for 10 min at room temperature followed by two washes each in 0.1 X SSC, 0.1% SDS for 20 min at 60 °C. Autoradiography was performed at −70 °C with an intensifying screen.

**Nuclear Transcriptional Assay**—Nuclei were isolated by the method of McKnight (33), and transcription reactions were performed according to Linial (34), with the following modifications. Reaction mixtures contained fibroblast nuclear extract (18), 5 X Tris-HCl, pH 8.0, 12.5 mM MgCl<sub>2</sub>, 750 mM KCl, and 1.25 mM each of ribonucleoside triphosphates ATP, CTP, GTP, 250 μCi of [α-<sup>32</sup>P]UTP, and 40 units/μl RNase inhibitor (Promega) in a final volume of 250 μl. The reaction was carried out for 30 min at 30 °C. Following DNease I (Life Technology, Inc.) treatment, the reaction was adjusted to a final concentration of 0.1 M TEDTA, 1 mM Tris-HCl, pH 7.5, and 0.1 mg/ml proteinase K. The reaction was extracted with an equal volume of phenol/ chloroform, then back extracted with 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, pH 5.0, 0.1% SDS, and extracted a final time with phenol/ chloroform. The aqueous layer was removed and made 2 M with ammonium acetate. The RNA was precipitated with 2.5 volumes of ethanol, washed once with 75% ethanol, dried briefly, and resuspended in 100 μl of diethyl pyrocarbonate-treated water. Unincorporated radiolabeled nucleotides were removed by passing the suspension through a Sephadex G-50 RNAse-free spin column (Boehringer Mannheim). Samples were analyzed to ensure equal amounts of radioactivity. Samples were heated at 90 °C for 5 min, then directly added to the hybridization mix.

**Nonradioactive anti-sense PN-1 (603 bases) and β-actin RNA (1100 bases) were synthesized in an in vitro transcription reaction, but were not slot blotted on a nylon filter as routinely done in Northern blots.** Samples were analyzed to ensure equal amounts of radioactivity. Samples were heated at 90 °C for 5 min, then directly added to the hybridization mix.

**Message Stability Experiments**—HF cells were grown to confluence in a 60-mm culture dish, incubated in serum-free medium, and subsequently pretreated with either IL-1 for 90 min or DXM for 3 h. Following treatment, 10 μg/ml of actinomycin D (Sigma) was added directly to the cell culture medium. RNA was extracted at specific times and the mRNA for PN-1 and β-actin were analyzed by Northern hybridization analysis.

**RESULTS**

**Effects of IL-1 and DXM on Secreted and Cell-bound PN-1**—To examine effects of IL-1 and DXM on PN-1 expression, aliquots of medium from control or treated HF cells were incubated with [α-<sup>32</sup>P]labeled thrombin and analyzed for PN-1-[α-<sup>32</sup>P]thrombin complexes, as described under “Experimental Procedures.” Fig. 1A shows that PN-1-thrombin complexes appear as a doublet. The formation of both bands was blocked by a monoclonal antibody against PN-1, demonstrating that both bands contain PN-1 (data not presented). The faster migrating band likely represents a clipped form of the PN-1-thrombin complex, which probably occurs during the incubation process of the assay. In the presence of IL-1, PN-1 activity increased in the cell culture medium by 12 h, and this increase continued throughout the 48-h treatment (Fig. 1A). Densitometry measurements showed that IL-1 treatments increased PN-1 activity 9-fold at 24 h and approximately 20-fold at 48 h (Fig. 1B). This increase in activity correlated with an increase in PN-1 synthesis as shown by immunoprecipitation of PN-1 from culture medium of cells previously labeled with [35S]methionine (data not presented). Throughout these studies, the above activity assay for PN-1 was employed since it was more sensitive and linear over a greater PN-1 concentration range than available immunosassays. Since IL-1 is a known mitogen for fibroblasts (35), we questioned if the up-
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FIG. 1. Regulation of PN-1 secretion by IL-1 and DXM. Confluent HF cells were incubated in serum-free medium and treated with either IL-1 (5 ng/ml) or DXM (1 μM). Cells incubated in serum-free medium alone were used as a control. Culture medium was collected at selected time points and assayed for PN-1 activity as described under "Experimental Procedures." Panel A, [125I]-thrombin plus fibroblast purified PN-1 (lane 1); [125I]-thrombin alone (lane 11). Arrows indicate molecular weight markers. Panel B, PN-1 activity was quantitated by densitometric measurements of [125I]-thrombin-PN-1 complexes from the autoradiogram shown in panel A.

regulation in PN-1 activity might be due to an increase in cell number. However, no differences in cell number between control and IL-1-treated cultures were observed (data not presented).

In contrast, HF cells treated with DXM secreted less PN-1 activity into the medium (Fig. 1A). This effect was apparent by 12 h, when levels of PN-1 activity were half that of untreated cells (Fig. 1B). By 48 h, PN-1 activity in the medium was reduced approximately 7-fold. DXM did not have a repressive effect on overall protein synthesis, since the activity of plasminogen activator inhibitor type-1 in the medium increased after DXM treatment, as previously reported (36) (data not presented).

Earlier studies showed that PN-1 binds to the cell surface and ECM of fibroblasts (6). This interaction accelerates its ability to inactivate thrombin, but blocks its ability to inhibit urokinase and plasmin (7). Because PN-1 binds very tightly to the cell surface and ECM, it is believed that the majority of PN-1 is localized there in vivo. Therefore, it was important to examine the effects of IL-1 and DXM on levels of cellular and ECM-bound PN-1. As observed above for PN-1 in the medium, cellular and ECM-bound PN-1 was increased when cells were exposed to IL-1, and decreased when treated with DXM (Fig. 2A). Densitometry measurements showed that the increase in PN-1 by IL-1 and decrease by DXM paralleled the changes in PN-1 in the medium (Fig. 2B).

We also examined the effects on PN-1 secretion of other growth factors and cytokines known to be influential during injury response processes. As Fig. 3 shows, only tumor necrosis factor-α (TNFα) seemed to have a significant effect on the secretion of PN-1 from HF cells. IL-1 is known to stimulate the secretion of IL-6 from fibroblasts (37). Since IL-6 produced only a slight increase in PN-1 secretion, it did not mediate the effect of IL-1. Recent findings showed that transforming growth factor-β (TGFβ) and platelet-derived growth factor stimulate the secretion of PN-1 in cultured brain cells (20). Interestingly, in HF cells neither factor produced a significant effect. However, it is important to note that the growth factors and cytokines used in this study were added to HF cells at only one concentration. Therefore, we cannot rule out the possibility that these factors may affect PN-1 synthesis at different dosages.

Effects of IL-1 and DXM on Steady-state Levels of PN-1 mRNA—To determine if IL-1 or DXM affected PN-1 mRNA levels, we performed Northern blots on RNA from HF cells. PN-1 mRNA was identified with a [3P]-labeled PN-1 riboprobe. PN-1 mRNA was quantitated by densitometry and standardized to the mRNA of β-actin. Cells treated with IL-
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FIG. 2. Analysis of PN-1 activity bound to the cell surface and the ECM. HF cultures were treated with either IL-1 or DXM for 24 and 48 h. Following treatment, cells were rinsed and incubated with [125I]-thrombin (300 ng/ml) as described under “Experimental Procedures.” Panel A, autoradiogram of [125I]-thrombin-PN-1 complexes representing the amount of active PN-1 bound to the cell surface and ECM of HF cells. Panel B, cell and ECM-bound PN-1 were quantitated by densitometric measurements of [125I]-thrombin-PN-1 complexes at 24 and 48 h.

1 contained a higher level of PN-1 mRNA (Fig. 4A). This increase was observable as early as 3 h, and continued throughout the 24 h, when IL-1-stimulated cells contained four times the amount of PN-1 mRNA as control cells as determined by densitometry scanning of the data in Fig. 4A. In contrast, cells exposed to DXM contained lower amounts of PN-1 mRNA compared to control cells (Fig. 4B). The decrease in mRNA was first observed at 6 h and was sustainable throughout the 48-h treatment, when DXM-treated cells contained approximately seven times less PN-1 mRNA than control cells. For both IL-1 and DXM, these results correlated with the effects observed on PN-1 activity, although quantitative differences in protein and mRNA levels were not comparable in IL-1 samples.

Next, we examined the mechanism by which IL-1 and DXM regulated PN-1 mRNA levels. First, we determined if IL-1 and DXM were direct regulators of PN-1 transcription, or if they functioned through the induction of a second regulatory factor. To do this, HF cells were pretreated with the protein synthesis inhibitor, cycloheximide. At a concentration of 2 µg/ml, cycloheximide inhibited the incorporation of [35S]methionine into HF cell proteins by 85% (data not presented). In the presence of cycloheximide, cells contained a greater amount of PN-1 mRNA (Fig. 5A and B). When DXM was added subsequently to cycloheximide-treated cells, the message for PN-1 was reduced at 48 h, although not to the same extent as when cells were treated alone with DXM (compare lanes 2 and 4 in Fig. 5A). This implies that in the absence of new protein synthesis, DXM directly regulates the transcription of PN-1. The results obtained in IL-1 and cycloheximide-treated cells were more complex. At 24 h, cells treated with both factors contained less PN-1 mRNA than when treated with either IL-1 or cycloheximide alone (compare lane 4 to lanes 2 and 3 in Fig. 5B). This result suggests that IL-1 does not directly regulate PN-1 transcription, but most likely requires the de novo synthesis of another protein to implement its effects.

DXM and IL-1 Transcriptionally Regulate the Synthesis of PN-1—To further analyze the effects of IL-1 and DXM on PN-1 mRNA, we performed nuclear run-on transcriptional assays on HF nuclei. The conventional approach in these assays utilizes a slot-blotting apparatus to immobilize either
linearized complementary DNA or in vitro synthesized anti-sense RNA onto a filter. In our hands, the slot-blotting technique gave variable results. We therefore employed an alternative method in which in vitro synthesized anti-sense RNA corresponding to either PN-1 or β-actin was first run on a mini-agarose gel and then transferred by capillary action onto a nylon filter (refer to “Experimental Procedures”). Strips of nylon containing both transcripts were then probed with radiolabeled nuclear RNA extracted from either treated or untreated cells. Fig. 6A shows the results of our transcriptional assays at 3, 6, and 12 h when cells were exposed to either IL-1 or DXM. In the presence of DXM, the rate of PN-1 transcription was decreased 2–3 fold at 6 h, but returned to basal levels by 12 h. This result correlated well with the time at which we first saw a decrease in the steady-state levels of PN-1 message (Fig. 4B). We are unable to explain, however, how such a transient decrease in transcription, which peaked at 6 h, could account for the continuing decrease in PN-1 mRNA observed in the Northern blots. We performed additional transcriptional assays with cells that were treated with DXM for 24 h, and again saw no difference in the rate of PN-1 transcription compared to control cells (data not presented). When HF cells were treated with IL-1 the rate of PN-1 transcription was increased about 50% at 3 h (Fig. 6A). This correlated with the time at which PN-1 mRNA increased in the Northern blots. At 6 h there also appeared to be a slight increase in PN-1 transcription, but when PN-1 signals were standardized to β-actin, rates of PN-1 transcription were identical in IL-1-treated and control cells. In addition, there was no differences in the transcriptional rate of the PN-1 gene at 12 h between IL-1- and control treated cells. When these experiments were repeated, similar results were observed with both DXM and IL-1.

**FIG. 3. Regulation of PN-1 activity by injury-related factors.** Culture medium from HF cells was collected 24 h after the addition of the indicated factors and assayed for PN-1 activity as described under “Experimental Procedures.” The factors IL-1, TNFa, IL-3, IL-6, epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF A/B), and TGFβ type 1 were used at concentrations of 5 ng/ml, except for DXM which was used at a concentration of 1 μM. Note that IL-1, and to a lesser degree, TNFa were the most potent inducers of PN-1 activity. In addition, no other factors decreased PN-1 activity to the same extent as DXM.

**FIG. 4. Northern analyses of steady-state levels of PN-1 mRNA.** Confluent HF cells were incubated in serum-free medium and treated with either IL-1 for up to 24 h (panel A), or DXM for up to 48 h (panel B). Serum-free medium alone was used as a control. Total RNA was extracted from cells at various times, and 10-μg aliquots of RNA were analyzed by Northern hybridization. Steady-state levels of PN-1 mRNA were detected with a [α-32P]UTP anti-sense RNA probe specific to PN-1 sequences. The amount of RNA loaded in each well was adjusted to the signal of human β-actin mRNA.

The Stability of the PN-1 Message Is Enhanced by IL-1, But Not by DXM—Although the above experiments indicated that DXM and IL-1 were transcriptional regulators of the PN-1 gene, the effects were transient, and the effects with IL-1 were small. The effects in the transcriptional assays could not explain the greater differences observed in PN-1 mRNA or protein levels. Therefore, to address if DXM or IL-1 could also regulate PN-1 in a post-transcriptional fashion, we examined the stability of the PN-1 message in the presence of the transcriptional inhibitor, actinomycin D. Treatment of cells with DXM did not affect the stability of the PN-1 mRNA (Fig. 7A). In contrast, IL-1-treated cells maintained a greater amount of PN-1 mRNA in the absence of on-going transcription, as compared to control cells (Fig. 7B). This increase in mRNA was indicative of PN-1 message stability. Densitometric measurements were made to approximate the degree to which PN-1 mRNA was stabilized in the presence of IL-1.

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FIG. 5. Northern analyses of PN-1 mRNA: effect of cycloheximide. Confluent HF cells were incubated in serum-free medium and treated with 2 μg/ml of cycloheximide to test the effects of DXM or IL-1 in the absence of protein synthesis. Lanes 1, serum-free medium alone was used as a control; lanes 2, addition of either DXM (panel A) or IL-1 (panel B) alone; lanes 3, cycloheximide alone; lanes 4, cells were preincubated with cycloheximide for 2 h prior to addition of either DXM (panel A) or IL-1 (panel B). Cells were harvested at either 24 h (panel B) or 48 h (panel A) and RNA was prepared. Approximately 10 μg of total RNA was analyzed by Northern hybridization. Because steady-state levels of β-actin mRNA in HF cells increased in the presence of cycloheximide, the amount of RNA loaded in each well was standardized rather than to the mRNA of GAPDH. The bar graphs were derived from densitometric measurements of PN-1 and GAPDH ratios for each lane.

**DISCUSSION**

The repair process following injury is orchestrated by a series of events involving immune cell trafficking, ECM degradation/rebuilding, and cell migration and proliferation. Many of the cellular activities involved are regulated in part by the serine protease thrombin. Thrombin is mitogenic for fibroblasts and smooth muscle cells (8, 9). It is also chemotactic for monocytes (11), and it induces the secretion of endothelin from vascular endothelial cells (38). If left unregulated the actions of thrombin may lead to a pathological condition. For example, there is growing evidence that thrombin contributes to the inflammatory processes associated with the development of atherosclerosis (39). Likewise, thrombin has been localized to senile plaques in Alzheimer’s patients (40), which may contribute to the neurodegenerative state in that disease, since thrombin is known to retract processes on neurons and astrocytes (12-15). Thus, in normal repair processes following injury, the activity of thrombin must be maintained in a proper balance, in order for cells to return to homeostatic conditions. The regulation of thrombin’s activity is primarily mediated by both activators and inhibitors of the serine protease.

One molecule which functions as a potent inhibitor of thrombin, and which likely regulates its activities in vivo, is PN-1. Unlike the other thrombin inhibitors anti-thrombin III and heparin cofactor II which are localized in the circulatory system, PN-1 resides in the microenvironment of the cell rather than in plasma. In fact, earlier work in our laboratory showed that PN-1 is localized primarily to the ECM and the cell surface of fibroblasts (6). This result implied that PN-1 most likely functions physiologically to regulate thrombin at or near the surface of cells. Because thrombin is generated at sites of vascular injury, the presence of PN-1 may be necessary to control the actions of thrombin at the cellular level.

Recently, we showed that the synthesis of PN-1 from a neuroblastoma cell line is regulated by several injury-related factors (20). This result provided evidence that PN-1 plays a role in processes related to injury/inflammation. In this report we investigated the molecular mechanisms that underlie this regulation. Our studies were performed on HF cells for two reasons. First, these fibroblasts are early passage cells which more closely resemble the characteristics of cells in vivo, compared to immortalized cell lines. Second, unlike the neuroblastoma cell line, HF cells express sufficiently high constitutive levels of PN-1 mRNA to permit studies on its regulation. To investigate the molecular mechanisms underlying PN-1 regulation, we specifically focused on two factors, which in HF cells regulated the synthesis of PN-1 in opposite fashions. At concentrations of 5 ng/ml, IL-1 up-regulated the secretion of PN-1 from HF cells, while 1 μM concentrations of DXM, inhibited the secretion of PN-1. Levels of PN-1 secretion after IL-1 treatment were approximately 20-fold higher than in untreated cells over a 48-h treatment. IL-1 also increased the levels of PN-1 bound to the cell surface and ECM. These increases were related to changes in the steady-state levels of mRNA for PN-1. Results employing the
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FIG. 6. Nuclear run-on transcriptional assay. Nuclear run-on assays in HF nuclei were performed as described under “Experimental Procedures.” The incorporation of [α-32P]UTP into RNA for each sample was similar. The autoradiograms in panel A demonstrate the transcriptional rate of the PN-1 gene at 3, 6, and 12 h following addition of either IL-1 or DXM. Panel B, nuclear run-on assay where labeled RNA was hybridized to anti-sense RNA corresponding to PN-1 sequences (lane 1), sense PN-1 RNA (lane 2), and RNA corresponding to 900 bases of the 3‘ end of the rhinovirus 14 genome (lane 3). The plasmid containing 3′ sequences was a generous gift of Stephen Todd. Panel C, anti-sense RNA corresponding to β-actin sequences was hybridized to labeled RNA extracted from cells incubated for 5 h in either serum-free medium (lane 1), or in serum-free medium containing 10 μg/ml actinomycin D (lane 2).

protein synthesis inhibitor, cycloheximide, indicated that IL-1 requires the de novo synthesis of a secondary factor to mediate its effects. Nuclear run-on assays performed after 3 h of treatment demonstrated that IL-1 slightly up-regulated the transcription of the PN-1 gene. But at 6 h after treatment the transcriptional rate returned to basal levels. This early increase in transcriptional rate appeared insufficient to explain the gradual increases we observed in steady-state levels of PN-1 mRNA, or the large amount of protein being secreted from HF cells over time. We therefore rationalized that IL-1 could also be functioning in a post-transcriptional fashion. We utilized the transcriptional inhibitor, actinomycin D, to determine whether PN-1 mRNA could be stabilized in the presence of IL-1. Our results indicated that IL-1 caused an increase in the half-life of the PN-1 mRNA. From these results we can conclude that IL-1 functions mainly to stabilize PN-1 mRNA, which in turn results in a greater amount of PN-1 secretion from HF cells.

The mechanisms regulating mRNA stability are not well understood. There is growing evidence, however, that regulatory elements residing in the 3′-untranslated region contribute to the instability of an mRNA, since short-lived lymphokine and proto-oncogene mRNAs have in common a conserved region of repeating AUUUA sequences in that region (41). These sequences are thought to direct rapid degradation of the mRNA, either by acting as a target site for an endonuclease, or by serving as a signal to direct the mRNA to a degradation complex present in the cytoplasm. Although, the PN-1 mRNA is not short lived, it nevertheless contains two AUUUA sequence elements in its 3′-untranslated region (42). Recently, a factor has been identified, which when induced by tumor necrosis factor, binds to a single AUUUA sequence in the glucose transporter mRNA, and thereby increases the half-life of that mRNA (43). We speculate that the mRNA of PN-1 might also be stabilized by a similar protein when cells are treated with IL-1.

The present study showed that DXM caused a decrease in the amount of active PN-1 secreted and bound to HF cells and the ECM. The decrease in activity correlated well with reduced steady-state levels of PN-1 mRNA over the 48-h treatment. Cycloheximide experiments suggested that DXM did not require the synthesis of a secondary factor to mediate its repressive effects. Nuclear run-on assays demonstrated that DXM acted at the transcriptional level to repress the synthesis of PN-1. The rate of PN-1 transcription was repressed 2–3-fold in the presence of the glucocorticoid for 6 h. Interestingly, our nuclear run-on assays performed at 12 and 24 h showed no difference in the transcriptional rates of PN-1 synthesis between untreated and DXM-treated cells. Since Northern analysis indicated that the steady-state levels of PN-1 mRNA continually decreased over a 48-h period, it seems probable that an additional mechanism is involved to regulate PN-1 synthesis. However, experiments with actinomycin D showed no evidence that DXM functions to regulate the stability of the PN-1 mRNA. Thus, our results indicate that DXM acts primarily as a transcriptional repressor of the PN-1 gene, but may also function by a second, yet unidentified, regulatory mechanism.

The manner in which IL-1 and DXM regulate PN-1 synthesis is significant in light of how these factors are known to
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Fig. 7. PN-1 messenger RNA stabilization analyses. Confluent HF cells were incubated in serum-free medium and treated with either DXM for 3 h (panel A) or IL-1 for 90 min (panel B) prior to the addition of actinomycin D (10 μg/ml). RNA was prepared at selected time points and 5-μg aliquots of total RNA were analyzed by Northern hybridization. The graph represents the effect of IL-1 on PN-1 mRNA stabilization and was derived from PN-1 densitometric measurements for each time point indicated in panel B. Quantitative measurements of mRNA stability could not be derived from densitometric ratios of PN-1 to β-actin since the decay rate of β-actin mRNA appears to be faster than that of PN-1.

function biologically. IL-1 is synthesized and released from activated macrophages, and its activities are associated with inflammation. These include the stimulation of: (a) fibroblast proliferation (35); (b) specific hepatic acute-phase proteins (44); (c) B-cell growth (45); (d) collagenase and prostaglandin E2 production by fibroblasts (22, 23); and (e) procoagulant activity in endothelial cells (46). In contrast, DXM, which mimics the actions of glucocorticoids released from the adrenal gland, produces anti-inflammatory effects. It blocks prostaglandin synthesis (47), modulates the fibrinolytic system by activating the synthesis of plasminogen activator inhibitor type 1 (36), and inhibits the expression of metalloproteinases, stromelysin, interstitial collagense, and 92-kDa type IV collagenase (24, 48). DXM is also a direct negative regulator of IL-1 synthesis in U-937 cells (49).

In view of how these factors function, and the results we obtained, two final points can be made. First, the up-regulation of PN-1 appears to be associated with inflammatory processes directed by cytokines like IL-1. Perhaps then, the increase in PN-1 is required during injury in order to modulate the cellular activities of thrombin. In addition, anti-inflammatory processes directed by glucocorticoids function to down-regulate the synthesis of PN-1. This type of regulation may be necessary when cells specifically require the activities of proteases in processes related to wound repair. Second, the differential expression of PN-1 by IL-1 and DXM is controlled by two separate regulatory mechanisms. Up-regulation of PN-1 by IL-1 is mediated in a post-transcriptional fashion by increasing the half-life of the PN-1 mRNA. This type of regulation provides HF cells a means of quickly synthesizing and secreting PN-1 during an inflammatory response. In contrast, repression of PN-1 synthesis by DXM occurs at the transcriptional level. In this fashion, glucocorticoids can modulate the activity of PN-1 during anti-inflammatory processes. This mode of modulation may be temporally regulated, when glucocorticoids make their way to the injury site, or may work in concert with cytokines to help maintain the needed balance between proteases and protease inhibitors.

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