Distinct Mechanisms Regulate TIMP-1 Expression at Different Stages of Phorbol Ester-Mediated Differentiation of U937 Cells†

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ABSTRACT: Upon exposure to 12-*O*-tetradecanoylphorbol 13-acetate (PMA), promonocyte-like U937 cells differentiate into macrophage-like cells and begin to express certain metalloproteinases and TIMP-1. We report here that distinct mechanisms regulate TIMP-1 production in PMA-treated U937 cells. TIMP-1 protein and steady-state mRNA levels increased about 10-fold in PMA-differentiated cells compared to undifferentiated cells. TIMP-1 transcription increased about 2.5-fold, but this stimulation was not detected until at least 48 h post-PMA. In contrast, the half-life for TIMP-1 mRNA increased about 3-fold and was detected at 8 h post-PMA. Using *in vitro* translation assays, we found that TIMP-1 mRNA from PMA-differentiated cells translated about 5-fold less efficiently than that from basal cells, suggesting structural differences in TIMP-1 mRNA in basal and differentiated U937 cells. Although primer extension and RNase protection analyses showed 5′ heterogeneity of TIMP-1 transcripts, all forms were equally stimulated in response to PMA-mediated differentiation. The poly(A) tail length of TIMP-1 mRNA, however, was longer in PMA-treated cells. Our findings suggested that up-regulation of TIMP-1 expression in PMA-treated U937 cells is mediated early by enhanced TIMP-1 mRNA stability, possibly related to increased poly(A) tail length, and later by an increase in transcription rate.

The tissue inhibitors of metalloproteinases (TIMPs)[1] are a family of proteins that can inhibit the catalytic activity of matrix metalloproteinases (MMPs). Thus far, three TIMPs have been isolated and cloned. TIMP-1, a 28 kDa glycoprotein, has activities apparently beyond that of an MMP inhibitor. For example, TIMP-1 was characterized as erythroid potentiating activity (Docherty et al., 1985) and can promote erythroid burst and colony formation (Avalos et al., 1988; Niskanen et al., 1988) and stimulate cell growth (Hayakawa et al., 1992). TIMP-2 is often coexpressed with 72 kDa gelatinase and is involved in gelatinase activation and inhibition (Howard et al., 1991a,b). TIMP-3 also inhibits many MMPs (Leco et al., 1994). TIMP-1 has been implicated in tissue remodeling associated with morphogenesis (Waterhouse et al., 1993), wound healing (Stricklin et al., 1993), and angiogenesis (Denhardt et al., 1993). TIMP-1 may act to neutralize excess activated MMPs in the extracellular space, thereby preventing unwanted or excessive matrix degradation (Banda, 1995). In contrast, underexpression of TIMP-1 is associated with destructive conditions and events, such as chronic ulcers (Bullen et al., 1995) and metastasis (Khokha & Denhardt, 1989; Waterhouse et al., 1993). Thus, the production of TIMP-1 needs to be carefully regulated to achieve a correct stoichiometric balance between enzyme and inhibitor.

Although many agents coordinately regulate TIMP-1 and MMPs, disparate expression of these proteins occurs in response to certain mediators and remodeling events. For example, in fibroblasts and alveolar macrophages, dexamethasone suppresses collagenase-1 expression without affecting TIMP-1 expression (Clark et al., 1987; Shapiro et al., 1991). Similarly, interferon-γ enhances MMP expression in keratinocytes without altering TIMP-1 production (Tamai et al., 1995). Retinoic acid inversely modulates TIMP-1 and collagenase-1 expression in fibroblasts, increasing TIMP-1 and decreasing collagenase-1 levels (Clark et al., 1987). *In vivo*, TIMP-1 and collagenase-1 are simultaneously expressed in poorly healing ulcers but in different locations and by different cell types (Saarialho-Kere et al., 1992). The net proteolytic activity within a tissue microenvironment depends on the spatial and temporal balance of TIMPs and MMPs. Thus, understanding the regulation of inhibitor expression is needed to appreciate the mechanisms controlling tissue remodeling.

Activated macrophages produce large quantities of MMPs and TIMPs; these cells are important in clearing debris and remodeling tissue at sites of inflammation. The U937 monocytic cell line differentiates into a population of macrophage-like cells in response to 12-*O*-tetradecanoylphorbol 13-acetate (PMA) (Minta & Pambrun, 1985). As we and our co-workers have shown, induction of MMP expression, particularly of collagenase-1 and 92 kDa gelatinase, and marked stimulation of TIMP-1 production are charac-

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[1] Abbreviations: TIMP, tissue inhibitors of metalloproteinase; MMP, matrix metalloproteinase; PMA, 12-*O*-tetradecanoylphorbol 13-acetate; CAT, chloramphenicol acetyltransferase.

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teristic features of differentiated U937 cells and human blood monocytes (Campbell et al., 1991b; Saarialho-Kere et al., 1993; Shapiro et al., 1993; Welgus et al., 1992). Here, we demonstrate that transcriptional and posttranscriptional mechanisms regulate TIMP-1 expression in PMA-differentiated U937 cells and that these processes operate at different stages during cellular differentiation. Our findings indicate that structural changes in TIMP-1 mRNA are associated with its enhanced production, and we suggest that these alterations may be related to increased TIMP-1 mRNA stability in differentiated U937 cells.

EXPERIMENTAL PROCEDURES

Cell Culture. U937 cells (Sundstrom & Nilsson, 1976) were obtained from the American Type Culture Collection (CRL 1593) and maintained in RPMI 1640 medium (GIBCO-BRL, Gaithersburg, MD) supplemented with 10% FCS (low endotoxin; <0.02 ng/mL; GIBCO-BRL), nonessential amino acids, L-glutamine, sodium pyruvate, 100 units/mL penicillin, and 100 μg/mL streptomycin. For induction of cell differentiation, U937 cells were plated at 5×10^5 cells/mL. PMA (Sigma Chemicals, St. Louis, MO) was dissolved in DMSO added to a final concentration of 8.0×10^{-8} M. Actinomycin D (Sigma) was dissolved in 100% ethanol and added to cultures to a final concentration of 5 μg/mL. Cycloheximide (Sigma) was dissolved in water and added to a final concentration of 10 μg/mL. Dexamethasone (Sigma) was added to a final concentration of 10^{-6} M.

Immunoassay. Conditioned medium was collected at various times after stimulation, and indirect enzyme-linked immunosorbant assay for TIMP-1 or collagenase-1 was used to quantify the levels of secreted TIMP-1 and collagenase-1 protein (Shapiro et al., 1993). These assays have nanogram sensitivity, are specific for TIMP-1 and collagenase-1, and recognize both free and complexed proteins. Due to minor cross-reactivity between human and bovine TIMP-1, conditioned medium samples containing 10% FCS were corrected for their small contribution of bovine TIMP-1 ($\leq$0.01 μg/mL immunoreactive material). This represented <2% of TIMP-1 produced by differentiated U937 cells and resulted in a negligible background. Standard curves for ELISA were generated with recombinant human TIMP-1 or purified human collagenase-1.

Northern Hybridization. Total cellular RNA was isolated by the guanidinium/phenol extraction method or by CsCl gradient centrifugation (Parks et al., 1988). Poly(A)$^+$ mRNA was isolated using the PolyATract mRNA Isolation System III (Promega, Madison, WI). Conditions for Northern hybridization and washes were as described (Parks et al., 1988). Briefly, unless otherwise noted, 10 μg of total RNA was denatured, fractionated through a 1.0% agarose−formaldehyde gel, transferred to a solid support, and hybridized overnight with either a 2.2 kb human collagenase-1 (Goldberg et al., 1986), 0.8 kb human TIMP-1 (Carmichael et al., 1986), or a 1.3 kb rat GAPDH (Fort et al., 1985) random-primed, ^{32}P-labeled cDNA probe. Filters were washed and visualized by autoradiography, and band intensity was quantified by densitometry.

Nuclear Runoff. The transcription rate of specific mRNAs was determined in isolated nuclei as described (Pierce et al., 1992). Briefly, 2.5×10^7 intact nuclei from untreated and PMA-treated U937 cells were incubated in transcription buffer containing 200 μCi of [α-^{32}P]GTP at 30 °C for 30 min About $(5-15) \times 10^6$ cpm of labeled RNA was routinely obtained with this procedure. Nascent RNA transcripts were extracted by the guanidinium thiocyanate/phenol technique, and equivalent counts of purified ^{32}P-labeled RNA were heat-denatured and hybridized to specific gel-purified, denatured cDNA inserts slotted on nitrocellulose. In addition to TIMP-1 cDNA, filters were slotted with a 1.9 kb β-actin cDNA, used as a control for unmodulated transcription, and a 2.5 kb human Alu-repeat fragment derived from the ϵ-globin gene (Wu et al., 1990), used as an indicator of total transcription. Following hybridization, filters were washed and exposed to film.

Plasmid Constructs. pBLCAT2 contains the −105/+51 region of the herpes simplex virus thymidine kinase promoter fused to a CAT reporter gene (Luckow & Schutz, 1987). pAPCAT2a is derived from pBLCAT2 and contains a tandem triplet of the collagenase-1 AP-1 site subcloned 5′ of the thymidine kinase promoter. The pG3/10H4.3 construct (Gewert et al., 1987) contains the murine TIMP-1 gene and was provided by Dr. Daniel Skup (McMasters University, Montreal, Canada). p-223/+190CAT contains a *Hin*dIII/*Bam*HI TIMP-1 gene fragment which includes 223 base pairs of the promoter region, exon 1, and 116 base pairs of intron 1 subcloned into pBLCAT2 cut with *Hin*dIII/*Bgl*2 which removed the thymidine kinase promoter. The *Hin*dIII/*Bal*I fragment of p-223/+729CAT was subcloned into *Hin*dIII/*Sma*I cut pBluescript II KS (Stratagene, La Jolla, CA) to create pBS-TIMPINT. The *Hin*dIII/*Bam*HI gene fragment (−223/+190) was subcloned into *Hin*dII/*Bam*HI cut pBLCAT2 to create p-223/+190TKCAT. p-223/+729CAT contains the TIMP-1 promoter, exon 1, and intron 1 and was made by subcloning the *Bam*HI fragment from pBS-TIMPINT into *Bam*HI/*Bgl*2 cut p-223/+190TKCAT.

A 118 base pair fragment of the human TIMP-1 gene containing the 5′ flanking region and all of exon 1 were amplified from genomic DNA using PCR. PCR was done using the following TIMP-1 specific primers: 5′-GGAG-GCCTGTGGTTTCCG-3′ and 5′-CTCTGGTGTCTCTCT-GGG-3′. The 118 base pair product was subcloned into *Sma*I cut pBluescript II KS or pSPT18 (Boehringer Mannheim, Indianapolis, IN) to generate pBS-TIMP118 or pSPT-TIMP118, respectively. Deep Vent DNA Polymerase (New England Biolabs, Beverly, MA) was used during the PCR step to minimize unwanted mutations, and constructs were sequenced with the Sequenase kit (United States Biochemical, Cleveland, OH) to verify the lack of mutations.

Transient Transfection of U937 Cells. U937 cells were transfected by a modification of the DEAE/dextran method as described (Grosschedl & Baltimore, 1985; Pierce et al., 1996). After transfection, cells were allowed to recover for 24 h before treatment with PMA. After recovery, cultures were divided equally, and cells were plated in medium with or without PMA. After 24 h, cells were harvested and washed twice with PBS. Transfected cells were lysed in 250 mM Tris, pH 7.8, by freeze-thawing. Lysates were incubated at 65 °C for 5 min and cleared of debris by centrifugation. Equivalent amounts of cleared lysate, normalized to total protein (Bradford protein assay; Bio-Rad Laboratories, Palo Alto, CA), were assayed for chloramphenicol acetyltransferase (CAT) activity using acetyl-CoA (Sigma) and [^{14}C]chloramphenicol essentially as described (Gorman et al., 1982). Reaction products were separated

by thin-layer chromatography and visualized by autoradiography. Percent acetylation was determined by counting the radioactivity in the appropriate spots on the chromatography plate.

In Vitro Translation. Equivalent amounts of total (CsCl-purified) or poly(A)$^+$ selected RNA were translated in a reticulocyte lysate or wheat germ extract (Promega) *in vitro* system in the presence of 0.6 mCi/mL L-[^{35}S]methionine or 0.3 mCi/mL L-[3,4,5-^{3}H]leucine. Translation reactions were performed as outlined by the manufacturer. Total incorporated counts were determined by TCA-precipitation. Radiolabeled, translated TIMP-1 protein was precipitated using a specific antibody (Shapiro et al., 1993). Equivalent volumes of each translation reaction were combined with immunoprecipitation buffer (1× PBS with 0.8% Triton X-100, 20 mM EDTA, pH 7.5, 1 mM PMSF, and 1 mg/mL BSA) and antibody. Samples were incubated overnight at 4 °C with gentle rocking. Antibody−antigen complexes were precipitated with protein A−Sepharose, washed twice with immunoprecipitation buffer (without BSA), and fractionated through a 6% denaturing SDS−polyacrylamide gel. Gels with ^{3}H-labeled products were impregnated with En^{3}Hance (Dupont-NEN, Boston, MA), and radiolabeled proteins were visualized by fluorography. For experiments with [^{35}S]methionine, gels were immediately dried, and products were visualized by autoradiography. The intensity of TIMP-1-specific bands was quantified by densitometry. The relative concentration of TIMP-1 mRNA in samples used for *in vitro* translation reactions was determined by Northern analysis. Translatability was defined as follows: (density of immunoprecipitated TIMP-1 band/total incorporated cpm)/(relative TIMP-1 mRNA levels).

Primer Extension. Primer extension analysis was done by a modification of the method of Boorstein and Craig (1989). Briefly, 100 μg of total RNA was hybridized overnight at 55 °C to the ^{32}P-labeled oligonucleotide primer PET-1 (5′-CTCTGGTGTCTCTCTGGG-3′), specific to exon 1 of TIMP-1, in 20 μL of hybridization buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, pH 8.0, and 0.3 M NaCl). Hybrids were ethanol-precipitated and dissolved in reverse transcription buffer [1 mM each dATP, dCTP, dGTP, and dTTP, 50 μg/mL actinomycin D, 1 mM DTT; 0.8 unit/μL RNasin, 1X transcription buffer (supplied by GIBCO-BRL)]. Primers were extended at 42 °C for 1 h with 200 units of Superscript reverse transcriptase (GIBCO-BRL). Extension reactions were ethanol-precipitated, washed with 70% ethanol, dried, suspended in 10 μL of 0.1 M NaOH, and heated 5 min at 65 °C. Sample volumes were adjusted to 20 μL with water, ethanol-precipitated, washed twice with 70% ethanol, and suspended in 10 μL of denaturing gel loading buffer (98% formamide, 10 mM EDTA, and 0.025% each xylene cyanol FF and bromophenol blue). Samples were separated through a 5% polyacrylamide, 7 M urea sequencing gel. The gel was dried and autoradiographed at −70 °C with an intensifying screen. Extension products were sized against Klenow-filled, ^{32}P-labeled *Msp*I fragments of pGEM-4Z (Promega).

RNase Protection Assay. Probes for RNA protection assays were *in vitro* transcribed using the MAXIscript kit (Ambion, Austin, TX). All transcription reactions were done according to the manufacturer's instructions using [α-^{32}P]-UTP. The pTRI-GAPDH template (Ambion) was transcribed with T7 RNA polymerase to generate a 403 nucleotide antisense murine GAPDH cRNA control probe. pBS-TIMP118 or pSPT-TIMP118 was linearized with *Eco*RI prior to *in vitro* transcription with either T7 or SP6 RNA polymerase, respectively, generating either a 190 or a 180 nucleotide antisense human TIMP-1 cRNA probe. RNase protection was done using the Direct Protect kit (Ambion) as described by the manufacturer. Briefly, 10^7 untreated or PMA-treated U937 cells were lysed in equivalent volumes of lysis solution. Total RNA was purified from equal amounts of lysate, and RNA was resuspended in lysis solution. A total of 10^5 cpm of ^{32}P-labeled, *in vitro* transcribed antisense TIMP-1 or GAPDH cRNA was added to each tube, and samples were incubated overnight at 37 °C to allow hybridization. Unhybridized RNA was digested by diluting the samples with RNase buffer, adding a cocktail of RNase T1 and RNase A, and incubating the reaction for 1 h at 30 °C. To stop the digestion, sarcosyl and proteinase K were added to each sample, and reactions were incubated for 30 min at 37 °C. Protected RNA fragments were precipitated by addition of an equal volume of isopropyl alcohol. The RNA pellet was suspended in gel loading buffer, heat-denatured, and separated on a 5% polyacrylamide/8 M urea sequencing gel. Protected products were sized against Klenow-filled, ^{32}P-labeled *Msp*I fragments of pGEM-4Z (Promega).

Polyadenylation Assay. Ten micrograms of total RNA was extracted once with phenol/chloroform/isoamyl alcohol (25:24:1), and the aqueous phase was ethanol-precipitated. The dried pellet was resuspended in 10 μL of DEPC-treated water and either oligomer $(dT)_{15}$ or oligomer RH-1 (5′-GGCTATCTGGGACCGCAGGGACTGCCA-3′), specific for TIMP-1 mRNA just 5′ of the translation stop codon, or both were added to the sample. Samples were heated to 85 °C for 5 min and slow-cooled to 25 °C to allow hybridization of the oligomers. An equal volume of 2× RNase H buffer (40 mM Hepes, pH 8.0, 100 mM KCl, 20 mM $MgCl_2$, and 1 mM DTT) containing 40 units of RNasin (Promega) was added to the each tube. Samples were incubated for an additional 30 min at 30 °C prior to the addition of 2 units of RNase H. RNase H digestion was done at 30 °C for 1 h, followed by 15 min at 37 °C. Reactions were stopped by extraction with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1), and the aqueous phase was ethanol-precipitated. RNA pellets were washed twice with 70% ethanol and dried under vacuum. Pellets were suspended in 10 μL of RNA gel loading buffer (5% glycerol, 0.1 mM EDTA, and 0.04% each bromophenol blue and xylene cyanol FF) and processed for Northern analysis.

RESULTS

Delayed Activation of TIMP-1 Transcription in Response to PMA-Mediated Differentiation. In an earlier study, we reported that TIMP-1 protein and mRNA levels increase progressively from low basal levels beginning about 8 h after initiation of PMA-mediated differentiation of U937 cells (Shapiro et al., 1993). As demonstrated in experiments completed for the studies reported here, secreted TIMP-1 protein and steady-state mRNA levels increased to about 9-fold by 48 h after PMA treatment of U937 cells (Figure 1A). During this same period, as we reported (Shapiro et al., 1993), induction of collagenase-1 expression is detected at about 8 h post-PMA, increases progressively, and peaks by 48 h post-PMA. As we demonstrated by nuclear runoff

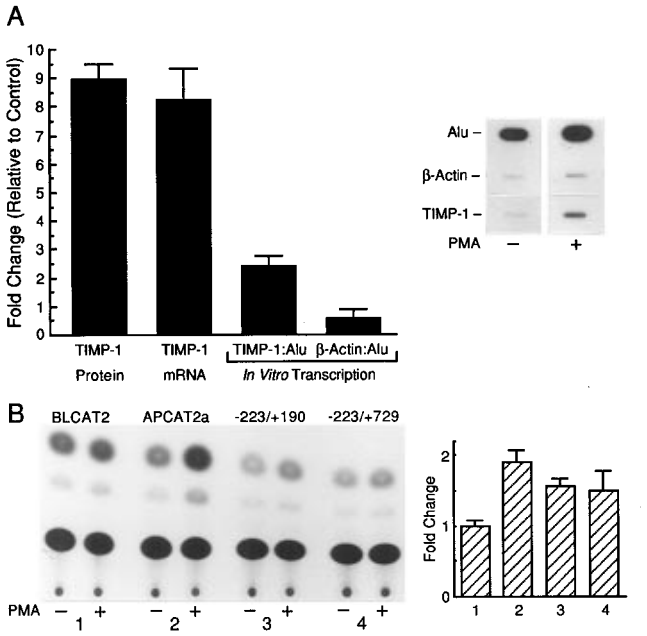

FIGURE 1: TIMP-1 transcription. Transcription of the TIMP-1 gene was assessed by nuclear runoff, and TIMP-1 promoter activity was in transiently transfected cells. (A) U937 cells were treated with PMA for 48 h. Medium was collected for immunoassay (TIMP-1 Protein), and the cell layer was harvested for isolation of total cytoplasmic RNA for Northern hybridization (TIMP-1 mRNA) and nuclei for runoff assay (*In Vitro* Transcription). Assays were done as described under Experimental Procedures. For the nuclear runoff assay, filters were slotted a 2.5 kb genomic fragment of the ϵ-globin gene containing two Alu repetitive elements as an indicator of overall transcription and with cDNAs for β-actin and TIMP-1. A representative autoradiogram is shown. The histogram shows the mean $\pm$ standard deviation of data for each parameter from six experiments expressed relative to time-matched control values. The signal for TIMP-1 transcription was normalized to that for Alu, TIMP-1 mRNA levels were normalized to GAPDH mRNA, and protein levels were normalized to total protein. (B) U937 cells were transfected with pBLCAT2, pAPCAT2a, p-223/+190CAT, or p-223/+790CAT and were treated with PMA 24 h later. Cells were harvested and lysed at 48 h after the start of PMA exposure, and CAT activity was assessed using 50 μg of cell lysates. pBLCAT2 served as a control for unmodulated activity, and pAPCAT2a served as a positive control for PMA induction. The histogram shows the means $\pm$ SEM of four determinations for each construct. A fold change equal to 1.0 indicates no stimulation by PMA treatment.

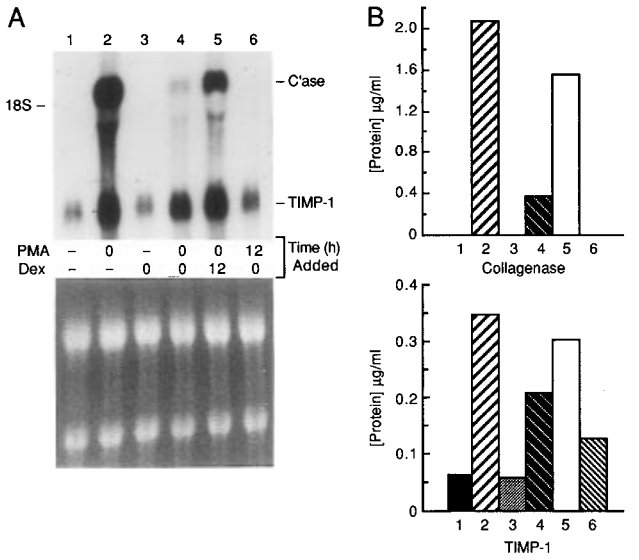

FIGURE 2: Regulation of TIMP-1 and collagenase-1 regulated by dexamethasone. (A) U937 cells were treated with PMA and/or dexamethasone for 24 h, and total RNA was analyzed by Northern hybridization. The time at which PMA or dexamethasone were added is indicated. (B) Secreted levels of TIMP-1 (bottom graph) and collagenase-1 (top graph) were determined by immunoassay of conditioned medium from the same U937 cells. The lane numbers at the top of the autoradiogram correspond to the numbers underneath the bars in the histogram.

assay, the rate of collagenase-1 transcription at 8, 12, and 24 h post-PMA paralleled the increases in steady-state mRNA levels and the concentration of secreted enzyme, but at these same times, we detected no change in TIMP-1 transcription even though its mRNA and secreted protein levels had increased significantly by 24 h post-PMA (Shapiro et al., 1993). The disparity in these earlier findings lead to more carefully examination of the mechanisms regulating TIMP-1 expression during monocytic differentiation. By nuclear runoff assay, low levels of TIMP-1 transcription were detected in untreated U937 cells, and these increased about 2.5-fold at 48 h of PMA-mediated differentiation (Figure 1A). Consistent with our previous findings (Shapiro et al., 1993), we did not detect an increase in TIMP-1 transcription by nuclear runoff earlier than 48 h post-PMA (data not shown).

We used TIMP-1 promoter constructs in transient transfection assays to further assess transcriptional activation in response to PMA-mediated differentiation. The human and murine TIMP-1 genes are quite homologous between positions -223 and $+190$, and this region responds to stimulators of TIMP-1 in other cell types (Campbell et al., 1991a; Edwards et al., 1992). Transcription from p-223/+190CAT and p-223/+729CAT increased about 1.5-fold at 24 h post-PMA (Figure 1B). Consistent results were seen for each construct in six separate experiments. The small difference between TIMP-1 stimulation detected by nuclear runoff and CAT activity assays may be due to the sensitivities of the methods or to influences from other regions of the endogenous gene. Regardless, both assays indicated that TIMP-1 transcription increased after PMA-mediated differentiation of U937 cells. Notably, whereas steady-state mRNA levels increased about 9-fold by 48 h post-PMA, TIMP-1 transcription, as determined by nuclear runoff or CAT assay, increased only about 2-fold (Figure 1A). Thus, increased TIMP-1 expression upon U937 differentiation is not fully accounted by an increase in gene transcription.

Dexamethasone Has Distinct Effects on TIMP-1 and Collagenase-1 Expression. Glucocorticoids differentially affect TIMP-1 and collagenase-1 expression in both fibroblasts (Clark et al., 1987) and alveolar macrophages (Shapiro et al., 1991). To determine if U937 cells behave similarly, various combinations of dexamethasone and PMA were used to stimulate U937 cells for 24 h (Figure 2, compare lanes 1 and 3). Dexamethasone did not modulate basal levels of TIMP-1 protein or mRNA (Figure 2, lane 3). However, when added together with PMA and assessed 24 h later (lane 4), dexamethasone markedly inhibited collagenase-1 expression but only slightly reduced TIMP-1 production. When dexamethasone was added 12 h after PMA (lane 5), TIMP-1 mRNA levels were not affected, but collagenase-1 mRNA dropped by about 50% relative to that seen with PMA alone (lane 2). In contrast, a 12 h pretreatment with dexamethasone before addition of PMA completely blocked PMA-mediated induction of collagenase-1 and abrogated much of the

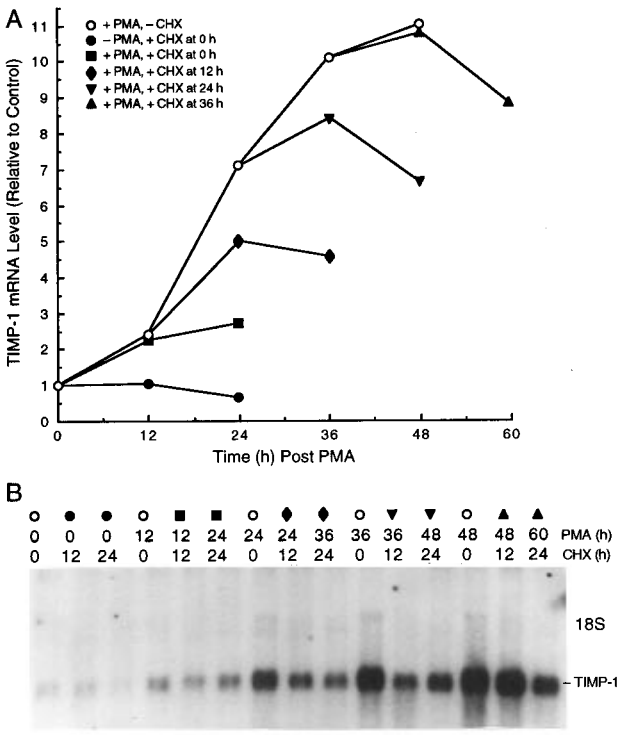

FIGURE 3: *De novo* protein synthesis is required for maximal TIMP-1 mRNA induction. U937 cells were treated with PMA for various times prior to the addition of 10 μg/mL cycloheximide (CHX). Cells were harvested 12 or 24 h after the addition of cycloheximide, and mRNA levels were assessed by Northern hybridization. (A) Graph showing the relative level of TIMP-1 mRNA after PMA treatment alone (open circles) and after treatment with cycloheximide (filled symbols). (B) Autoradiogram of Northern blot showing TIMP-1 mRNA levels after the various treatments. The lengths of time that PMA and cycloheximide were in the culture are indicated. For example, '0, 0' means the culture received no PMA or cycloheximide, and '36, 12' means cycloheximide was added at 36 h post-PMA and RNA was isolated 12 later.

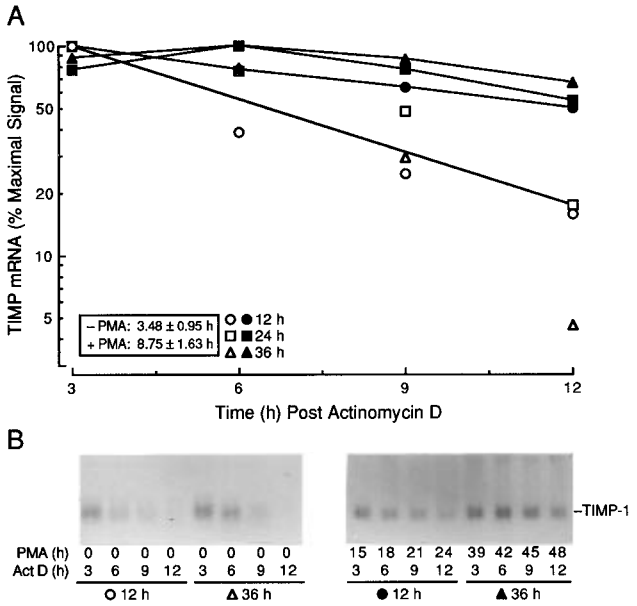

FIGURE 4: TIMP-1 mRNA is stabilized in PMA-differentiated U937 cells. Control U937 cells (open symbols) or cells treated with PMA for 12, 24, or 36 h (filled symbols) were exposed to actinomycin D for 12 h. Cells were harvested at 0−12 h after the addition of actinomycin D, and TIMP-1 and GAPDH mRNA levels were assessed by Northern hybridization. (A) The autoradiographic signal for TIMP-1 mRNA was normalized to that for GAPDH mRNA and expressed relative to the signal for TIMP-1 mRNA in the 36 h post-PMA, no actinomycin D sample. (B) Shown is the autoradiogram of the 12 and 36 h samples.

stimulation of TIMP-1 mRNA and protein levels. These findings indicate that collagenase-1 and TIMP-1 are regulated by distinct mechanisms during PMA-mediated differentiation of U937 cells.

Protein Synthesis Is Required for Maximal TIMP-1 Induction. Although we detected increased TIMP-1 mRNA levels at 12−16 h post-PMA (Shapiro et al., 1993), we saw no increase in TIMP-1 transcription until 48 h post-PMA (Figure 1). These observations suggest that TIMP-1 expression in differentiated U937 cells may be controlled by time-dependent mechanisms. To begin to characterize the temporal regulation of TIMP-1 in differentiated monocytes, we treated U937 cells with cycloheximide at different times after addition of PMA. Cycloheximide added at any time after the onset of PMA differentiation only partially diminished the increase of TIMP-1 mRNA (Figure 3). The degree to which cycloheximide blocked stimulation of TIMP-1 mRNA was time-dependent, being progressively less effective when added later after the start of PMA treatment (Figure 3). These findings suggest that *de novo* protein synthesis is required for maximal increases of TIMP-1 mRNA in PMA-mediated U937 differentiation but is not needed for early (0−12 h) stimulation.

TIMP-1 mRNA Is Stabilized Early in PMA-Differentiated U937 Cells. The discrepancy between steady-state mRNA levels and transcription rate at 48 h post-PMA suggests that a posttranscriptional mechanism is involved in stimulating TIMP-1 expression in U937 cells. To determine if the half-life of TIMP-1 mRNA changes with time after PMA addition, U937 cells were treated with actinomycin D at different times after the onset of differentiation, and TIMP-1 mRNA decay was observed over the next 12 h. At all times examined, the TIMP-1 mRNA half-life was the same in PMA-treated cells (Figure 4A), and the mRNA was about 3 times more stable in PMA-treated cells than in untreated cells. The earliest time we observed TIMP-1 mRNA stabilization was 8 h post-PMA (data not shown). The half-life of TIMP-1 mRNA was about 3.5 h in untreated cells and about 9 h in PMA-treated U937 cells, similar to that in cells at 48 h post-PMA (Shapiro et al., 1993). These findings suggest that the early PMA-mediated increase in TIMP-1 mRNA expression occurs by a posttranscriptional mechanism.

Translational Efficiency of TIMP-1 mRNA. Because specific sequences and secondary structures that influence mRNA stability can also affect translation (see Discussion), we assessed the translatability of TIMP-1 mRNA isolated from untreated and PMA-differentiated cells. TIMP-1 mRNA from untreated U937 cells translated about 3−5-fold more efficiently than that isolated from PMA-treated cells (Figure 5). Essentially identical findings were obtained with total RNA or purified poly(A)$^+$ mRNA translated in rabbit reticulocyte lysate or wheat germ extract (Figure 5). Collagenase-1 mRNA from PMA-treated U937 cells translated with equal efficiency at all times examined (data not shown). Although the finding that TIMP-1 mRNA from basal cells translated more efficiently than that from PMA-stimulated cells seems paradoxical, we interpret these data to indicate that intrinsic changes in the structure of TIMP-1 mRNA are introduced in response to PMA-mediated differentiation of

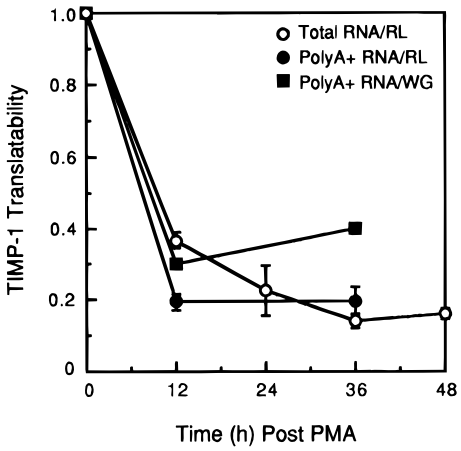

FIGURE 5: In vitro translation of TIMP-1 mRNA. Total RNA or poly(A)$^+$ mRNA was isolated from untreated (0 h) or PMA-treated (12, 24, 36, 48 h) U937 cells and was translated in reticulocyte lysate (RL) or wheat germ extract (WG). Translatability was defined as stated under Experimental Procedures. The data shown are the mean ± standard deviation of three determinations for each experiment.

U937 cells. These structural changes may mediate stabilization of TIMP-1 mRNA in U937 cells.

Analysis of the 5′ End of TIMP-1 mRNA. Heterogeneity occurs at the 5′ end of the murine TIMP-1 mRNA in fibroblasts (Campbell et al., 1991a; Coulombe et al., 1988; Ponton et al., 1992), and similar heterogeneity of TIMP-1 mRNA in U937 cells might influence its translatability *in vitro* and stability *in vivo*. To assess 5′ heterogeneity, we used primer extension analysis with RNA isolated from untreated and PMA-treated cells. A primer specific for sequences in exon 2 (PET-2: 5′-CAGAAGGCCGTCT-GTGGGTGGGGTGGGACACA-3′) resulted in numerous bands corresponding to premature termination of the extension reaction (data not shown). These findings suggested that a high degree of secondary structure exists in the region of exons 1 and 2 of the TIMP-1 mRNA. Therefore, we used another primer (PET-1) specific for sequences just 5′ of the exon 1/exon 2 splice junction. Presumably, this oligomer would hybridize to sequences upstream of the area of inferred secondary structure. (For presentation, the nucleotide in the human TIMP-1 5′ flanking region corresponding to the putative start site, which generated the 65 nucleotide product, has been designated +1.) Primer extension with PET-1 produced a faint triplet of extension products approximately 61−65 nucleotides long and a prominent triplet of about 34−37 nucleotides (Figure 6). No bands of these same sizes were seen in the "No RNA" and "tRNA" control reactions. Thus, these primer extension data indicate two putative transcription start site clusters located about 63 and 35 nucleotides upstream of the exon 1/exon 2 junction. Notably, the bands arising from the downstream start site cluster appeared to be induced in response to PMA treatment (Figure 6).

Analysis of the 5′ End of TIMP-1 mRNA. Because the 61−65 nucleotide primer extension products were faint (Figure 6) and because secondary structure was implied by the primer extension reactions with PET-2 (data not shown), we used RNase protection to better characterize the 5′ end of the TIMP-1 mRNA. Consistent with our primer extension results, RNase protection using two probes to the 5′ end of TIMP-1 mRNA (Figure 7, lanes 8−18) produced specific

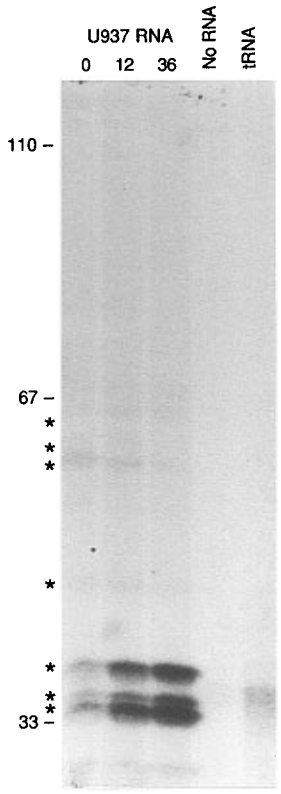

FIGURE 6: Analysis of the 5′ end of TIMP-1 mRNA by primer extension. Total RNA from untreated U937 cells (0) or cells treated with PMA for 12 or 36 h (12, 36) was hybridized to a radiolabeled oligomer (PET-1) specific to TIMP-1 mRNA, and the primer was extended with reverse transcriptase. The products were separated through a sequencing gel. An autoradiogram representative of three experiments is shown. TIMP-1-specific bands are marked by asterisks. The numbers at the left correspond to the position of the size markers. The smear in the tRNA control lane was not specific since it was not detected in other experiments and migrated differently from the small TIMP-1-specific bands.

protected products 61−65 nucleotides, 36−37 nucleotides, and 26 nucleotides long (marked by asterisks). However, unlike the primer extension results, the signal for all RNase protected products was increased in RNA samples from PMA-treated cells. RNase protection with the GAPDH probe verified that equal amounts of U937 RNA were used in each reaction (Figure 7, lanes 1−7). The shorter protection products in the GAPDH reactions were due to single nucleotide mismatches between the murine and human sequences. The nonspecific bands of different sizes detected in reactions without sample RNA (lanes 8 and 14), like the PET-2 primer extension (data not shown), imply that secondary structure forms at the 5′ end of the TIMP-1 mRNA. Thus, these findings, in agreement with the primer extension data, indicate heterogeneity in the 5′ end of TIMP-1 mRNA originating from the use of two putative transcription start site clusters. However, because the relative levels of all transcripts increased proportionately in PMA-treated cells, these data do not support the idea that increased stability *in vivo* or decreased *in vitro* translatability of TIMP-1 mRNA is mediated by structural changes at the 5′ end of the transcript.

TIMP-1 Polyadenylation. Because polyadenylation can affect the stability and translation of mRNA (Gorlach et al., 1994; Sachs, 1991), we used an RNase H assay to determine if TIMP-1 mRNA was differentially polyadenylated in untreated and PMA-treated U937 cells. Total RNA from

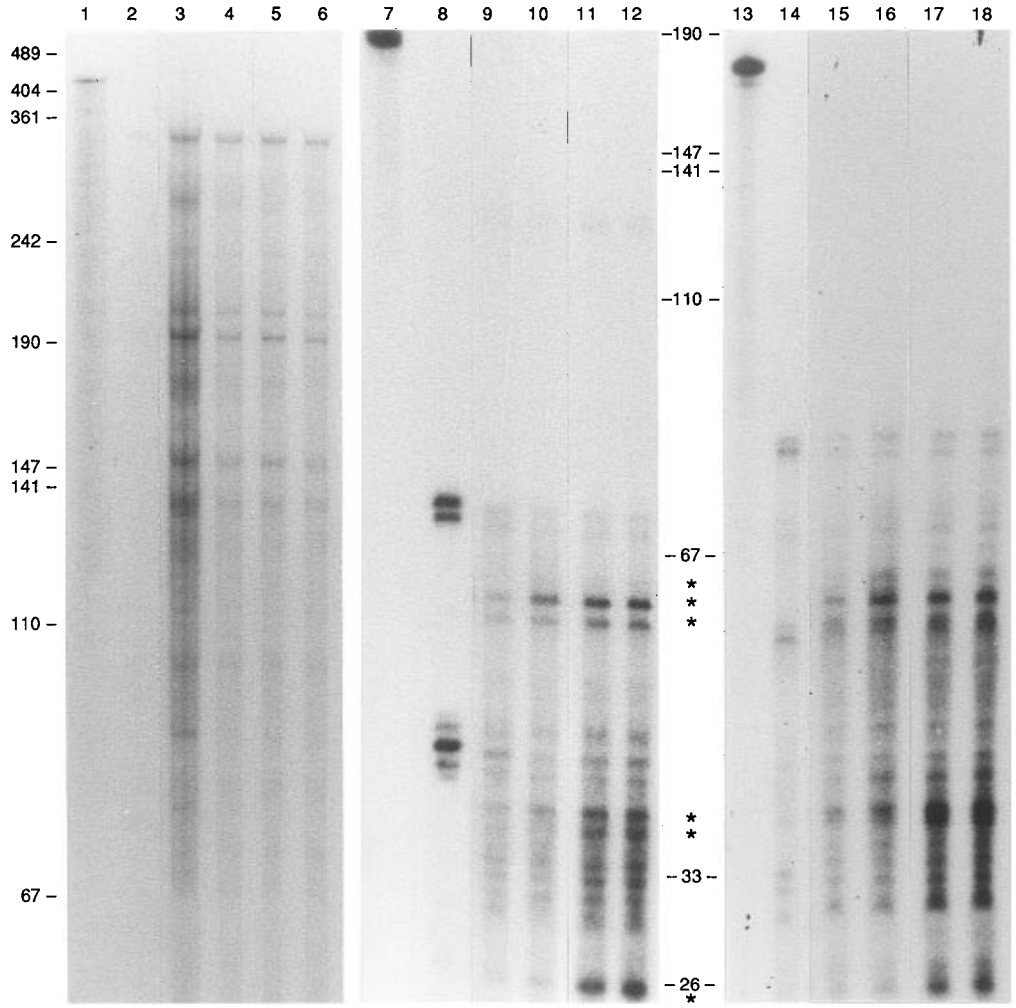

FIGURE 7: Analysis of the 5′ end of TIMP-1 mRNA by RNase protection assay. Total RNA from control U937 cells (lanes 3, 9, 15) or cells treated with PMA for 12 h (lanes 4, 10, 16), 36 h (lanes 5, 11, 17), or 48 h (lanes 6, 12, 18) was hybridized to a probe specific to GAPDH (lanes 1−6) or to probes transcribed from two different plasmids specific to TIMP-1 (lanes 7−12 and 13−18). Probes incubated with no RNase added indicate the size and quality of the undigested probes (lanes 1, 7, 13). Radiolabeled probes incubated without target RNA (lanes 2, 8, 14) show the size of nonspecific bands. The smaller products seen in the GAPDH assay are due to mismatches between the human and murine sequences. Specific TIMP-1 products are indicated by asterisks. Shown are autoradiographs representative of three experiments. The numbers adjacent to the gels correspond to the migration of size markers.

untreated and PMA-treated U937 cells was incubated with RNase H in the absence or presence of oligomer $(dT)_{15}$ or RH-1, and cleaved products were resolved on a 2.0% denaturing agarose gel and analyzed by Northern hybridization (Figure 8). TIMP-1 mRNA from 12 h and 36 h PMA-treated cells was larger than that from control U937 cells (upper band a in first set of lanes). This size difference was eliminated when RNA samples were treated with RNase H in the presence of either oligomer $(dT)_{15}$ alone (lower band a in second set of lanes), RH-1 alone (bands b and c in third set of lanes), or in the presence of both oligomers (bands a, b, and c in fourth set of lanes). Band c may be due to cleavage at an unintended oligomer RH-1 hybridization site. Presumably, the loss of the observed TIMP-1 mRNA size differences seen in the control reactions (with RNase H alone) is accounted for by the loss of the poly(A) tail in reactions containing RNase H in combination with oligomer $(dT)_{15}$ and RH-1. Therefore, these results indicate that the TIMP-1 mRNA poly(A) tail is longer in PMA-treated U937 cells than in untreated cells, and suggest that polyadenylation may be related to both decreased translatability *in vitro* and enhanced transcript stability in PMA-treated cells.

DISCUSSION

We report that TIMP-1 expression is increased during monocytic differentiation by both transcriptional and post-transcriptional mechanisms. In addition, our data demonstrate that these mechanisms occur during distinct phases of U937 differentiation. Whereas TIMP-1 mRNA is stabilized shortly after the onset of differentiation, increased endogenous TIMP-1 transcription is not detected until 48 h after exposure to PMA. We found that the poly(A) tail was longer in TIMP-1 transcripts from differentiated U937 cells than those from control cells. We suggest that the increase in TIMP-1 mRNA stability is related to increased polyadenylation and that these events are related to the process of differentiation.

Increased TIMP-1 transcription was not detected in a previous study (Shapiro et al., 1993), but because transcription was assessed before 48 h post-PMA, any stimulation was likely missed. The nuclear runoff and CAT assay data presented here demonstrate that TIMP-1 transcription is stimulated in U937 cells and by 48 h in response to PMA treatment (Figure 1). The increase in transcription observed (2.5-fold), however, may not fully account for the increase

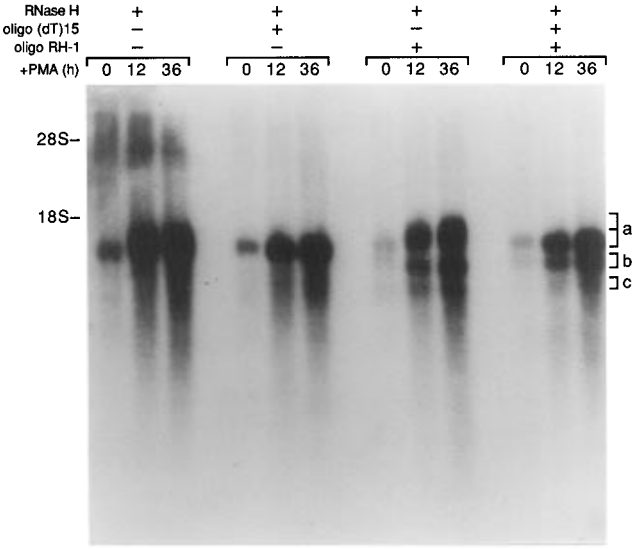

FIGURE 8: Polyadenylation of TIMP-1 mRNA. Total RNA from control U937 cells (0 h) or cells treated with PMA for 12 or 36 h was hybridized to a $(dT)_{15}$ oligomer and/or RH-1, a TIMP-1-specific oligomer. Hybrids were digested with RNase H, and reaction products were analyzed by Northern hybridization with a full-length TIMP-1 cDNA.

in TIMP-1 steady-state mRNA levels (~9-fold) (Figure 1). Because we also determined that TIMP-1 mRNA is stabilized in PMA-differentiated U937 cells (Figure 4), we conclude that both mechanisms lead to stimulation of inhibitor production. We cannot, however, determine accurately the relative contribution of transcriptional and RNA stabilization to increases in TIMP-1 steady-state mRNA levels.

Our data demonstrate that stabilization of TIMP-1 mRNA occurs relatively early after the onset of U937 differentiation, by 8 h post-PMA, and does not change thereafter. New protein synthesis may not be required for early events since TIMP-1 mRNA levels are increased to the same level at 12 h post-PMA regardless of the presence of cycloheximide (Figure 3). Because cycloheximide alone does not increase TIMP-1 mRNA levels relative to the untreated control, up-regulation requires a PMA-mediated mechanism (Figure 3). However, consistent with the delayed increase in TIMP-1 transcription, *de novo* protein synthesis, perhaps of a labile transcription factor, is required to achieve maximal levels of TIMP-1 mRNA at later times of PMA-mediated differentiation (Figure 3). Thus, the posttranscriptional and transcriptional mechanisms regulating TIMP-1 expression during U937 differentiation may be dissociated from each other in a time-dependent manner.

While we observed stabilization of TIMP-1 mRNA in differentiated U937 cells, no change in TIMP-1 mRNA stability has been reported in other cell types (Alitalo et al., 1990; Overall et al., 1991). In one study, no difference in TIMP-1 mRNA half-life was observed between control and TGF-β-treated fibroblasts (Overall et al., 1991). In the other study, which used PMA-differentiated K562 cells, TIMP-1 mRNA was induced 50-fold, whereas TIMP-1 transcription increased only 10-fold (Alitalo et al., 1990). This suggests that a posttranscriptional mechanism functions in PMA-treated K562 cells as well. Although these authors concluded that enhanced stability was not involved in increased TIMP-1 expression in K562 cells, the actinomycin D incubation time they used (2 h) was too brief to observe any decay of TIMP-1 mRNA (Alitalo et al., 1990). Thus, while cell-specific differences in regulation are likely, posttranscriptional regulation of TIMP-1 may be a common mechanism leading to increased production of inhibitor in hemopoietic cells.

We found that TIMP-1 mRNA from PMA-differentiated U937 cells translated 3−5 times less efficiently than that from undifferentiated cells in cell-free translation systems (Figure 5). Because these data seemed to conflict with the observed increase in TIMP-1 expression during PMA-mediated differentiation (Figure 1), we reasoned that the decrease in translatability *in vitro* reflected either changes in the structure of TIMP-1 mRNA between untreated and PMA-treated cells or peculiarities of the cell-free systems due to translation in a non-native environment. In contrast to what has been found in fibroblasts (Campbell et al., 1991a; Coulombe et al., 1988), our RNase protection assays demonstrated that all TIMP-1 transcripts are induced equally by PMA treatment (Figure 7). Thus, we conclude that the observed effects on *in vitro* translation and mRNA stability are not mediated by altered heterogeneity at the 5′ end of TIMP-1 mRNA, as suggested by others (Edwards et al., 1992; Waterhouse et al., 1990).

Poly(A) tail length can influence translational efficiency differently in an *in vivo* versus an *in vitro* setting (Drummond et al., 1985). Significantly, Drummond (1985) found that polyadenylation of mRNAs transcribed *in vitro* had distinct effects on the ability of the transcripts to be translated in *Xenopus* oocytes versus cell-free systems. Polyadenylation enhanced both the stability and translational efficiency of transcripts injected into oocytes but, similar to our finding, decreased their translatability in both wheat germ extracts and reticulocyte lysates. Notably, longer poly(A) tails caused a large decrease in translation efficiency *in vitro*, while having little effect on translational efficiency in oocytes. Our RNase H assay (Figure 8) indicated that the poly(A) tail length of TIMP-1 mRNA is longer in PMA-differentiated cells than in untreated cells. Therefore, we suggest that the decreased *in vitro* translatability of TIMP-1 mRNA from PMA-differentiated cells is due to its increased polyadenylation. In addition, increased polyadenylation may contribute to enhanced TIMP-1 mRNA stability in PMA-treated cells. Indeed, both increased mRNA stability and translational efficiency correlate with mRNA polyadenylation (Sachs, 1991).

Others have reported that posttranscriptional stabilization of mRNA is involved in the regulation of various genes during PMA-mediated U937 cell differentiation (Sherman et al., 1990; Wager & Assoian, 1990; Weber et al., 1989). The mechanism responsible for enhanced TIMP-1 mRNA polyadenylation and stability observed in PMA-treated cells is unclear, but because U937 cells become nonproliferative after PMA treatment, stimulated TIMP-1 expression may be related to the cessation of cell division. In an asynchronously dividing population of cells, some cells will be in S or M phase, during which transcription of many genes is disrupted or repressed (Brooks, 1995; Hartl et al., 1993; Martinez-Balbas et al., 1995), while the remainder will be in G1 or G2. Thus, in undifferentiated, proliferating U937 cells, the input of newly transcribed TIMP-1 mRNA may be disrupted during DNA replication and mitosis. When treated with PMA, U937 cells exit the cell cycle and enter G0, during which the population becomes synchronized, and TIMP-1 transcription would proceed uninterrupted. Hence, at any given time, independent of the transcription rate, the average

age of the TIMP-1 mRNA population in proliferating U937 cells will be older than that in nonproliferating, differentiated cells. Given that shortening of the poly(A) tail by a 3′ exonuclease is a first step in the decay of many mRNAs (Ross, 1995), a population of older mRNAs will have shorter poly(A) tails. In agreement with this prediction, the average poly(A) tail length of TIMP-1 mRNA in undifferentiated U937 cells was shorter than that in PMA-differentiated cells (Figure 8).

TIMP-1 mRNA stabilization in PMA-treated U937 cells might also be related to the use or regulation of distinct mRNA degradation pathways functioning in dividing versus nondividing, differentiated cells. Along these lines, c-*myc* mRNA is degraded by two distinct pathways in undifferentiated versus DMSO-differentiated HL-60 cells (Swartwout & Kinniburgh, 1989). In proliferating HL-60 cells, c-*myc* is predominantly degraded by a 3′ exonucleolytic pathway involving poly(A) shortening, while in differentiated cells, it is degraded more rapidly by a putative pathway involving endonucleolytic cleavage (Swartwout & Kinniburgh, 1989). In U937 cells, a ribonuclease activity exists that is down-regulated by PMA-mediated differentiation and is involved in TGF-β1 mRNA stabilization (Wager & Assoian, 1990). Thus, whereas the increased length of the poly(A) tail of TIMP-1 mRNA may be a consequence of exiting the cell cycle, the stability of the mRNA in differentiated cells may be prolonged by both increased poly(A) tail length and PMA-mediated modulation of nucleolytic pathways.

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