Differentiating agents regulate cathepsin B gene expression in HL-60 cells

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Abstract: We utilized HL-60 cells as a model system to examine the regulation of *ctsb* gene expression by differentiating agents. Inducers of monocytic differentiation [phorbol ester (PMA), calcitriol (D_3), and sodium butyrate (NaB)] and inducers of granulocytic differentiation [all-*trans* retinoic acid (RA) and 9-*cis* retinoic acid (9-*cis* RA)] increase *ctsb* mRNA levels in a dose-dependent manner as determined by Northern blot hybridization. D_3 and retinoids exert additive effects, suggesting that these agents act in part through distinct pathways. Actinomycin D decay experiments indicate that D_3, NaB, RA, and 9-*cis* RA do not alter mRNA stability. In contrast, PMA markedly increases the half-life of *ctsb* mRNA. In transient transfection assays, PMA and NaB both stimulate transcription of the luciferase reporter gene placed under the control of *ctsb* promoter fragments. Thus, inducers of HL-60 cell differentiation can regulate the expression of the *ctsb* gene at both transcriptional and posttranscriptional levels. *J. Leukoc. Biol.* 66: 609–616; 1999.

Key Words: *mRNA · transcriptional regulation · posttranscriptional regulation · monocyte · granulocyte*

INTRODUCTION

Cathepsin B (ctsb) is a lysosomal cysteine protease with a broad substrate specificity [1]. Ctsb is expressed in most tissues, but its levels vary widely among different tissues or cell types [2–6]. Macrophages and osteoclasts produce high levels of ctsb [7–12], as do malignant tumor cells of various origins [for a review, see ref. 13]. Several groups have reported increases in *ctsb* transcripts or ctsb activity in association with differentiation of specific cell types *in vivo* or *in vitro*. For instance, both ctsb activity and *ctsb* mRNA levels increase during rodent and calf myoblast-myotube differentiation [14–16]. *Ctsb* expression is also enhanced during the early stages of osteogenic differentiation in mouse mandibular condyles *in vitro* [17]. Maturation of human monocytes into macrophages *in vitro* is accompanied by an increase in ctsb activity [7]. Differentiation of the human U937 promonocytic cell line, induced by phorbol myristate acetate (PMA) or granulocyte-monocyte colony-stimulating factor, triggers an increase in *ctsb* mRNA and protein [18]. PMA increases *ctsb* mRNA levels and stimulates secretion of procathepsin B from colon carcinoma cells [19, and D. Keppler, personal communication]. Induction of monocytic differentiation of HL-60 cells by PMA increases ctsb protein and activity [20, 21]. Because HL-60 cells can be committed toward granulocytic or monocytic pathways with a variety of agents [22], we utilized these cells as a model system to investigate whether differentiating agents directly regulate the expression of the *ctsb* gene. We investigated the effects of PMA, calcitriol (D_3), sodium butyrate (NaB), all-*trans* retinoic acid (RA), and 9-*cis* retinoic acid (9-*cis* RA) on *ctsb* mRNA levels and transcriptional activity of the *ctsb* promoter in HL-60 cells.

MATERIALS AND METHODS

Reagents

The PMA and all-*trans* RA (Sigma Chemical Co., St. Louis, MO) were dissolved in dimethyl sulfoxide. The sodium butyrate, lithium chloride, actinomycin D, and cycloheximide (Sigma) were dissolved in water. The 9-*cis* RA (Sigma) and calcitriol (a kind gift of Dr. Milan R. Uskokovic, Hoffman La-Roche, Nutley, NJ) were dissolved in ethanol. The nitroblue tetrazolium (Sigma) was dissolved in phosphate-buffered saline containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM glucose.

Cells

The HL-60 cells (American Type Culture Collection, Rockville, MD) were maintained in RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum, 5 mM L-glutamine, 50 units/mL penicillin, and 50 μg/mL streptomycin at 37°C and 5% CO_2. The medium was replaced every 2–3 days.

Northern blots

Cells were seeded at 2 to 8 × 10^5/mL in 100-mm plates and treated as indicated in Results. Total cellular RNA was isolated using a modified guanidinium thiocyanate procedure [23]. Total RNA (10–20 μg) was denatured, fractionated on a 1% formaldehyde-agarose gel, and transferred onto a NYTRAN$^+$ membrane (Schleicher & Schuell). The blots were then hybridized overnight at 42°C with radiolabeled probes generated from randomly primed

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cDNAs for human *ctsb* (0.8 kb EcoRI fragment of pLC343 [24]) or chicken *gapdh* (1.2 kb *Pst*I fragment of pGAD-28 [25]). Northern blots were washed for 10 min at 45°C in 2× SSPE buffer [26]/0.2% sodium dodecyl sulfate, for 1 h at 60°C in 0.2× SSPE buffer/2% sodium dodecyl sulfate. The blots were initially exposed to Kodak X-OMAT film with intensifying screens at −70°C to produce autoradiographs. The blots were subsequently exposed to a phosphor screen and bands were quantitated with a PhosphoImager (Molecular Dynamics, Sunnyvale, CA). Results of *ctsb* expression were normalized based on *gapdh* expression. All treatment conditions were examined a minimum of three times.

Cellular differentiation analysis

Ten thousand cells were plated into 2 mL of media in 24-well plates and treated with RA (10^{-7} M), D_3 (10^{-7} M), 9-*cis* RA (10^{-7} M), PMA (10^{-8} M), or NaB (5×10^{-4} M) for 24 h. The cells were then harvested and incubated in 0.2% nitroblue tetrazolium/0.15 μM PMA for 20 min at 37°C. Aliquots were counted for total number of cells and those containing formazan granules. All experiments were performed a minimum of four times.

Genomic *ctsb* clones and sequencing of the promoter region

pCBG1 genomic cosmid clone was a gift from Dr. Dunne Fong [27]. This clone contains the *ctsb* gene with approximately 6 kb of 5′ flanking sequences upstream of exon 1 [13]. A 2.2-kb SacI fragment from pCBG1, including 1.8 kb of 5′-flanking region, exon 1 (135 bp), and 0.3 kb of intron 1, was subcloned into pBluescript KS(+) (Stratagene) to generate pICB8. Two extensive, overlapping sets of nested deletion variants of this plasmid were constructed from either end of the insert in order to sequence both strands of the *ctsb* promoter region. The sequence has been submitted to Genbank (accession no. AF086639). pSCB1 was constructed by subcloning the 2.2-kb *ctsb* insert into promoterless pGL3-Basic vector (Promega) upstream of the beetle luciferase reporter gene (see Fig. 6). pSCB2 is similar to pSCB1, but does not contain the last 79 bp of exon 1 and intron 1 sequences (it was generated by removing *ctsb* sequences downstream of the first PstI site in exon 1). pSCB2 contains 1774 bp of 5′-flanking region and the first 56 bp of exon 1. pSCB8 is a nested deletion variant containing the last 105 bp of the insert in pSCB2, which includes 49 bp of 5′-flanking region and 56 bp of exon 1.

Transfections

After optimizing conditions to achieve pulse times of 34–38 ms (260–270 volts, 960 μF), we transiently transfected plasmids into HL-60 cells (6×10^6 cells in 300 μL) via electroporation [28] using a Gene Pulser (Bio-Rad). Cells from a single transfection were equally divided into two dishes, and agents known to stimulate *ctsb* mRNA levels were added to one of the dishes. All cells were harvested after 12–16 h, and equal amounts of protein from each sample were used to measure luciferase activity (with a kit from Promega). Results were normalized by comparing only paired dishes (treated versus untreated) from a single transfection [29–32]. NaB treatment can enhance transfection efficiency in fibroblasts [33, 34]. To rule out potential confounding effects in the HL-60 cells, we co-transfected a plasmid containing the SV40 promoter upstream of the renilla luciferase gene with the pSCB1, pSCB2, and pSCB8 plasmids before NaB treatment. For these transfections, both the beetle and renilla luciferase activities were assessed via a single tube dual luciferase assay (kit from Promega). Relative stimulation of expression of the CTSB promoter-beetle luciferase by NaB was normalized to the renilla activity.

RESULTS

We examined steady-state levels of *ctsb* mRNA in HL-60 cells upon treatment with various differentiating agents. The monocytic inducers PMA, D_3, and NaB and the granulocytic inducers RA and 9-*cis* RA increased *ctsb* mRNA levels in HL-60 cells (**Fig. 1**, **Table 1**). PMA exerted the most pronounced effect, increasing *ctsb* mRNA levels more than 20-fold relative to *gapdh* after 24 h. However, PMA also reduced *gapdh* mRNA levels, so that the absolute increase in *ctsb* mRNA was 7.5-fold. Prolonged treatment with PMA increased the *ctsb* levels further, while having no additional effect on *gapdh* mRNA levels. The other agents increased *ctsb* mRNA levels approximately two- to fourfold from 24 to 72 h, with no significant effect on *gapdh* mRNA levels. HL-60 cells expressed both the 2.2- and 4.0-kb forms of *ctsb* mRNA. Throughout the experiments, treatments did not significantly alter the ratio of the 2.2- and 4.0-kb mRNA forms.

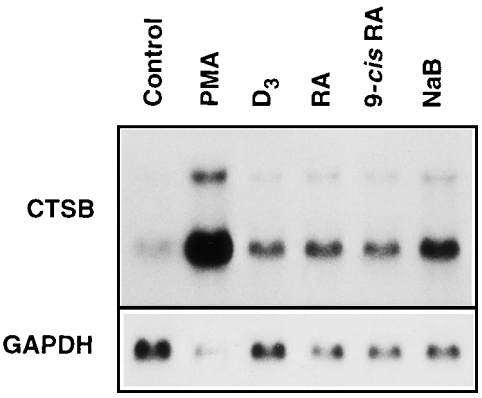

Fig. 1. Cathepsin B expression in HL-60 cells. Cells were treated for 24 h with 10^{-8} M PMA, 10^{-7} M D_3, 10^{-7} M RA, 10^{-7} M 9-*cis* RA, or 5×10^{-4} M NaB. Total cellular RNA (10 μg/lane) was hybridized with a *ctsb* cDNA probe as described in Materials and Methods. Blots were stripped and rehybridized with a *gapdh* cDNA probe as a loading control. The relative stimulation of *ctsb* mRNA levels was quantified for each agent (see Table 1).

Because all of the agents exerted an effect by 24 h of treatment, we performed a shorter-term kinetic assay. *Ctsb* mRNA levels increased as early as 3 h after PMA, RA, 9-*cis* RA, or NaB treatment and rose progressively throughout the duration of the assay (**Fig. 2**). D_3 had a more delayed action, with *ctsb* mRNA increasing by 12 h of treatment. The lack of a response within 1 h of treatment suggested that the agents tested may affect *ctsb* gene expression indirectly. Indeed, protein synthesis is required for the full extent of stimulation of *ctsb* expression by PMA. Co-treatment with PMA and the protein synthesis inhibitor cycloheximide (10 μg/mL) reduced the level of *ctsb* mRNA at 24 h as compared to PMA treatment alone (data not shown). However, cycloheximide by itself increased the level of *ctsb* mRNA approximately fourfold (data not shown; see Discussion). The other inducing agents, when used in combination with cycloheximide, neither increased nor reduced *ctsb* mRNA levels compared with cycloheximide alone.

TABLE 1. Relative Stimulation of *ctsb* mRNA Levels at 24, 48, and 72 h[a]

Treatment	24 h	48 h	72 h
PMA	21.6 ± 10.1 (7)	17.8 ± 2.3 (3)	39.9 ± 4.6 (3)
D_3	1.6 ± 0.4 (12)	1.8 ± 0.3 (8)	3.4 ± 0.3 (4)
RA	2.5 ± 0.9 (12)	1.7 ± 0.3 (8)	3.2 ± 1.1 (4)
9-*cis* RA	2.8 ± 0.4 (13)	2.1 ± 0.3 (8)	2.5 ± 0.5 (4)
NaB	4.1 ± 1.3 (7)	1.4 ± 0.0 (3)	n.d.[b]

[a] Mean increase ± standard deviation. Number of repeats is indicated in parentheses. Levels of *ctsb* mRNA in each experiment were calculated relative to control (vehicle-treated) sample in each blot. Fold stimulation is normalized for *gapdh* levels.—[b]Not determined.

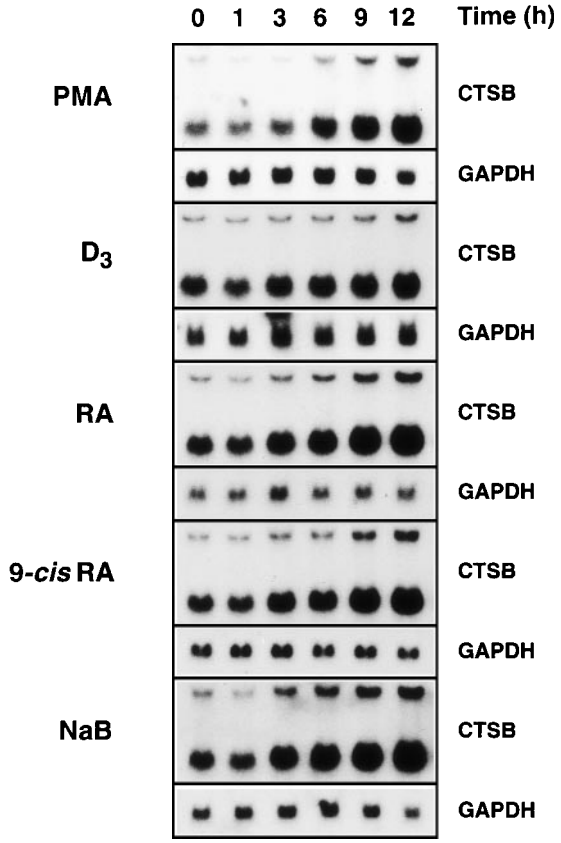

Fig. 2. Kinetics of *ctsb* mRNA expression in response to PMA, D_3, RA, 9-*cis* RA, or NaB. HL-60 cells were treated with 10^{-8} M PMA, 10^{-7} M D_3, 10^{-7} M RA, 10^{-7} M 9-*cis* RA, or 5×10^{-4} M NaB, for 1–12 h as indicated. Northern blotting was performed as described in Figure 1.

We verified that the differentiating agents that increase *ctsb* mRNA levels act in a dose-dependent manner at the 24-h time point (**Fig. 3**). The optimal dose of PMA for induction of *ctsb* mRNA was 10 nM. At this concentration, more than 90% of cells were adherent, a characteristic of HL-60 cells undergoing differentiation toward the macrophage phenotype [22]. The

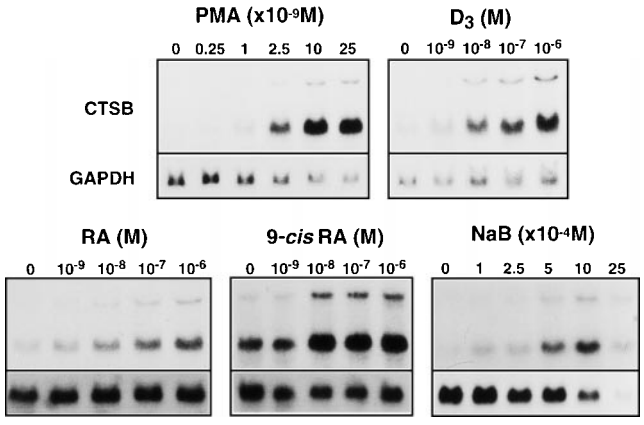

Fig. 3. PMA, D_3, RA, 9-*cis* RA, and NaB elicit a dose-dependent increase in *ctsb* mRNA levels. HL-60 cells were treated for 24 h with PMA, 9-*cis* RA, or NaB and for 48 h with D_3 or RA at the concentrations indicated. Northern blotting was performed as described in Figure 1. For all treatments, the upper box contains the *ctsb* mRNA bands, and the lower box contains the *gapdh* mRNA bands.

monocyte inducers D_3 and NaB and the granulocytic inducers RA and 9-*cis* RA increased the cytoplasm/nucleus ratio of HL-60 cells but did not induce an adherent phenotype at the times and concentrations tested, as reported previously [22]. D_3 and 9-*cis* RA stimulated *gapdh*-normalized *ctsb* mRNA levels maximally at 10^{-7} M, whereas RA increased *ctsb* mRNA levels further at 10^{-6} M (Fig. 3). Maximal stimulation of *ctsb* expression by NaB was seen with a 10^{-3} M concentration. We observed morphological alterations suggestive of apoptosis (vacuolization and cell fragmentation) after 24-h treatment with 10^{-6} M D_3, RA, and 9-*cis* RA, and with NaB concentrations above 5×10^{-4} M.

To assess the extent of cell differentiation upon treatment with the above agents, we performed nitroblue tetrazolium assays. The ability to reduce nitroblue tetrazolium is a biochemical marker of maturation of HL-60 cells. Treatment of these cells with the monocytic inducers D_3 and NaB and the granulocytic inducers RA and 9-*cis* RA for 24 h resulted in differentiation of fewer than 1% of cells (**Table 2**) as assessed by this assay. This suggests that these agents do not induce full differentiation of HL-60 cells within 24 h at the concentrations tested. However, prolonged treatment for 5 days resulted in differentiation of 63–83% of cells. Thus, the regulation of *ctsb* gene expression by these factors is not secondary to terminal differentiation, but likely occurs before the activation of signals necessary for differentiation. Despite the lack of formazan granule staining of the PMA-treated cells (Table 2), both the 24- and 120-h treatments resulted in morphological changes, notably complete adherence and spreading of the cells. Therefore, PMA already induces some degree of differentiation of HL-60 cells toward the monocyte/macrophage pathway as early as 24 h.

We performed actinomycin D decay assays (**Fig. 4A**) to determine whether differentiation-inducing agents act by altering *ctsb* mRNA stability as opposed to increasing the level of transcription from the gene. In the absence of prior treatment, *ctsb* mRNA levels rapidly decreased upon addition of 5 μg/mL actinomycin D (with a half-life of ~3 h). D_3, RA, 9-*cis* RA, and NaB exerted no significant effect on the decay of *ctsb* mRNA. In contrast, PMA increased *ctsb* mRNA stability under these conditions, with approximately 80% of transcripts remaining after 8 h (Fig. 4B), resulting in an approximate half-life of 24 h. Because actinomycin D can induce apoptosis in HL-60 cells [35], we repeated the assay with lower doses of this agent. We

TABLE 2. Differentiation of HL-60 Cells[a]

Treatment	24 h	120 h
Control	<1	<1
PMA	<1[b]	2 ± 1[b]
D_3	<1	82 ± 2
RA	<1	63 ± 2
9-*cis* RA	<1	80 ± 9
NaB	<1	83 ± 3

[a]Cells were treated for either 24 or 120 h as described in Materials and Methods. The degree of differentiation is expressed as the percentage of cells with formazan precipitates after staining with nitroblue tetrazolium. All treatment conditions were examined four times, and the mean ± standard error is displayed.—[b]After PMA treatment, most cells were morphologically differentiated (i.e., adherent and spread), although few formazan precipitates were seen.

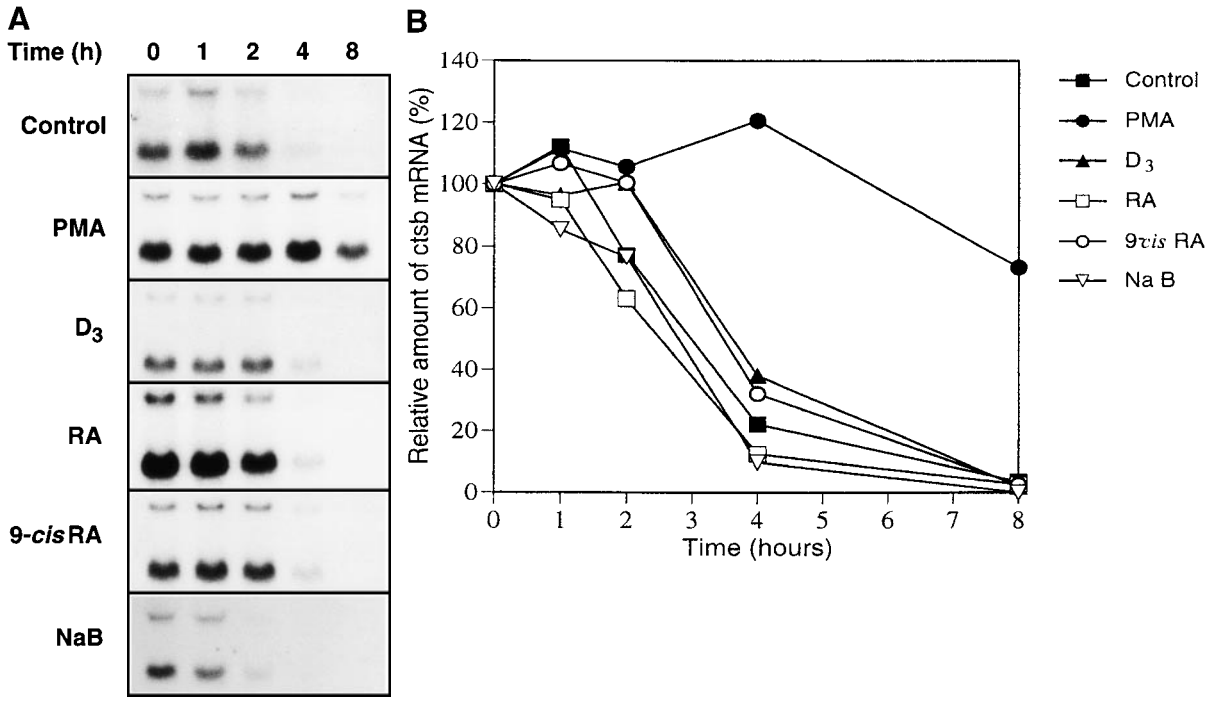

Fig. 4. PMA increases the stability of *ctsb* mRNA in HL-60 cells. HL-60 cells were pretreated for 24 h with PMA, 9-*cis* RA, or NaB and for 48 h with D_3 or RA at the concentrations indicated in Figure 1. Cells were then treated with 5 μg/mL actinomycin D for the times indicated. (A) Northern blotting was performed as described in Figure 1. (B) The relative amount of *ctsb* mRNA (% of levels at time zero) was plotted vs. time.

obtained similar results with actinomycin D concentrations as low as 0.1 μg/mL (data not shown).

D_3, RA, and 9-*cis* RA act by binding to VDR, RAR, and RXR (vitamin D, retinoic acid, and retinoid X receptor) members of the steroid/nuclear receptor family of transcription factors [36]. These factors have been shown to form homo- and heterodimers, with different binding affinities for various promoter elements [37, 38]. Therefore, we examined the combined effect of D_3 and RA or D_3 and 9-*cis* RA on steady-state *ctsb* mRNA levels 48 h post-treatment. The effect of D_3 and retinoids was more pronounced than either agent alone, and was additive (threefold stimulation with D_3 and RA or D_3 and 9-*cis* RA vs. 1.3- to 2-fold stimulation for each agent alone; **Fig. 5**). Therefore, the two classes of agents are likely to act, in part, through different pathways.

With the exception of PMA, all agents tested here did not alter *ctsb* RNA stability in actinomycin D assays. Therefore, our next experiments tested the hypothesis that these agents increase transcription of the *ctsb* gene. For these studies, we sequenced a 1.8-kb fragment of genomic DNA containing the promoter region of the human cathepsin B gene. As reported previously, the promoter region contains no canonical TATA or CAAT box immediately upstream of the transcription initiation site [39] but contains several Sp1 sites. A search for consensus VDR and RAR responsive elements revealed no matches. There are numerous potential AP1 and AP2 sites and three potential NF-κB sites.

For analysis of promoter activity, various fragments from the *ctsb* promoter region were inserted into pGL3-Basic vector (Promega) upstream of the luciferase reporter gene (**Fig. 6A**). The first of the resulting plasmids, pSCB1, contains a 2.2-kb *ctsb* fragment, including 1.8 kb of 5′-flanking region, exon 1 (135 bp), and 339 bp of intron 1. Because we thought that the splice donor site at the end of exon 1 might interfere with expression of luciferase, pSCB2 was generated, where the last 79 bp of exon 1 and intron 1 were removed, leaving 1774 bp of 5′-flanking region and 56 bp of exon 1. We also constructed pSCB8, a deletion variant containing 49 bp of 5′-flanking region and 56 bp of exon 1. We transfected each of the above plasmids into HL-60 cells via electroporation and measured luciferase activity in protein extracts from cells treated for 12–16 h with PMA, D_3, 9-*cis* RA, and NaB and paired untreated cells from the same transfection. We observed a consistent approximately sevenfold induction of luciferase expression by

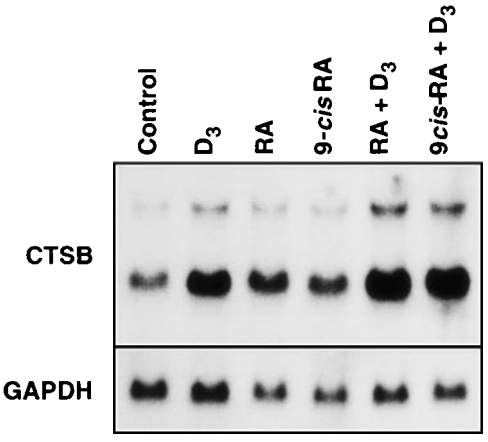

Fig. 5. D_3 and retinoids exert additive effects on *ctsb* mRNA expression. HL-60 cells were treated for 48 h with D_3, RA, and 9-*cis* RA (each at 10^{-7} M) singly or in combinations as indicated. Northern blotting was performed as described in Figure 1.

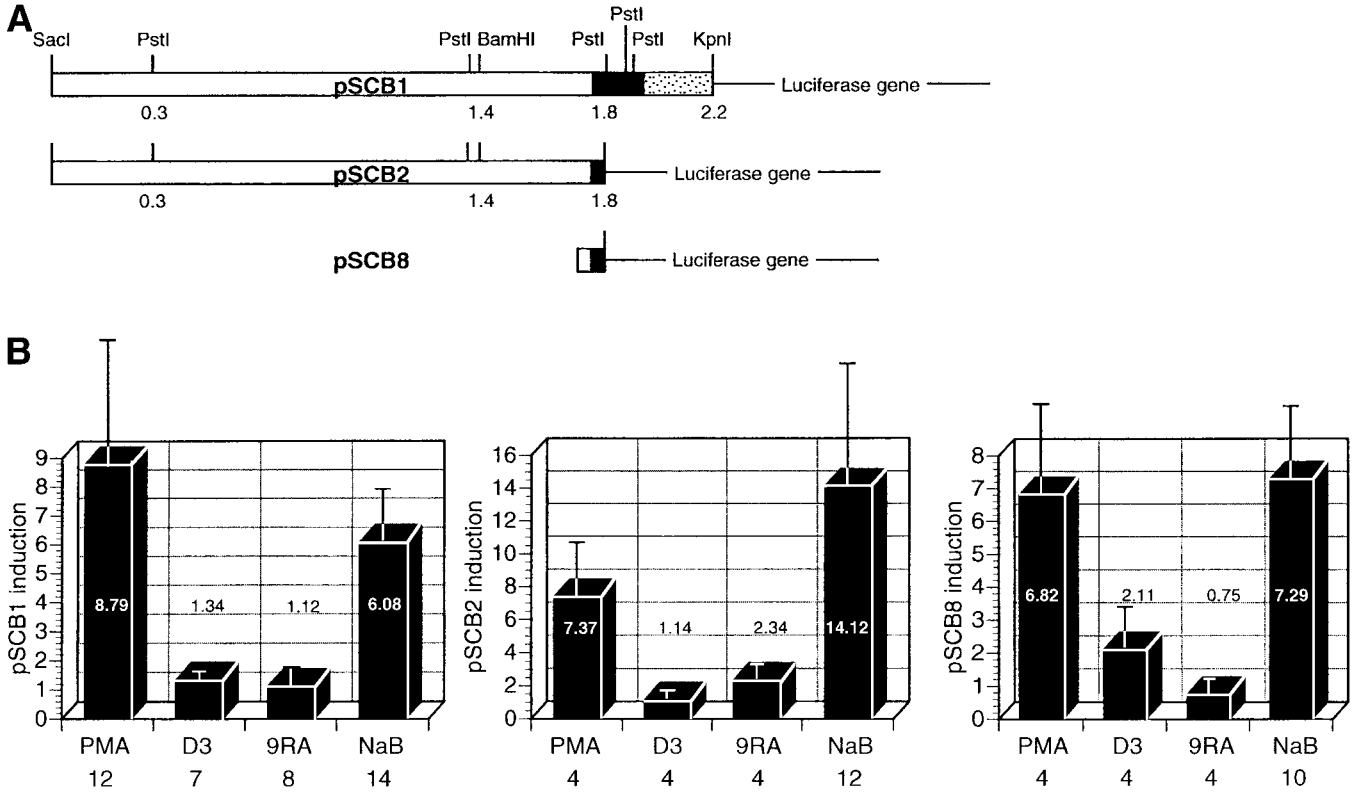

Fig. 6. Induction of luciferase activity after transient transfection of HL-60 cells with pSCB1, pSCB2, and pSCB8. (A) The pSCB1, pSCB2, and pSCB8 plasmids were generated as described in Materials and Methods. The regions upstream of exon 1 are depicted by the open rectangles; the black rectangles represent part or all of exon 1; and the stippled rectangle represents the first 339 bp of intron 1. (B) Plasmids were transfected into HL-60 cells via electroporation, and cells from a single transfection were equally divided into two dishes. One dish of each set was treated with 10^{-8} M PMA, 10^{-7} M D_3, 10^{-7} M 9-*cis* RA, or 5×10^{-4} M NaB, and the other dish was treated with vehicle only. All cells were harvested after 12–16 h. For the experiments involving PMA, D_3, and 9-*cis* RA treatments, equal amounts of protein from each sample were used to measure luciferase activity. For the experiments involving NaB treatment, dual luciferase assays were performed on all the samples, as described in Materials and Methods. Results are expressed as fold induction after treatment compared with the control dish from the same transfection, and shown as mean induction ± standard deviation. The number of transfections is shown beneath each column.

PMA for all three plasmids and a 6- to 14-fold induction during treatment with NaB (Fig. 6B). D_3 and 9-*cis* RA did not significantly increase luciferase expression in this assay.

DISCUSSION

We demonstrate that various agents that induce HL-60 cells to differentiate toward a monocytic or granulocytic phenotype increase steady-state levels of *ctsb* mRNA. Several groups have reported that this protease is expressed at elevated levels during differentiation of certain cell types, perhaps contributing to tissue remodeling that takes place at that time. In addition, ctsb levels remain high in a subset of differentiated cells that exhibit extensive lysosomal or secreted proteolytic activity. Some of the best-documented examples are in the monocyte cell lineage [7–12, 40]. Both PMA and 9-*cis* RA stimulate *ctsb* mRNA expression in the monocytic THP-1 cell line [Troen et al., unpublished data]. In the HL-60 model system, PMA-induced differentiation toward the monocyte/macrophage pathway is paralleled by an increase in ctsb protein and activity [20, 21]. Our study supports this earlier observation because PMA exerts a pronounced effect on *ctsb* mRNA levels. Reddy et al. [12] have shown that the cysteine proteases cathepsins B, L, and S are synthesized and secreted at high levels by monocyte-derived macrophages cultured on elastin-coated dishes and play an active role in the dissolution of this extracellular matrix protein. Cathepsin B is also expressed at high levels by macrophages associated with the invasive edge of human colon tumors [J. McKerrow, personal communication]. These observations are consistent with the fact that PMA, which induces an adherent macrophage-like phenotype in HL-60 cells, elicits a more pronounced increase in ctsb levels than do D_3 and NaB, which induce a nonadherent, monocyte-like phenotype [22].

The up-regulation of ctsb during differentiation of both monocytic and granulocytic cells may reflect a general up-regulation of lysosomal proteases to accommodate the increased turnover of proteins in these cells compared with their precursors. However, lithium chloride, which also can induce granulocytic differentiation of HL-60 cells, failed to increase *ctsb* mRNA levels (data not shown). Inducing agents promote various degrees of differentiation toward a mature cell lineage. Perhaps LiCl-treated HL-60 cells express a less differentiated phenotype than do HL-60 cells treated with RA or 9-*cis* RA. Like ctsb, the lysosomal aspartic protease cathepsin D is induced in HL-60 cells treated with inducers of both monocytic

and granulocytic differentiation [41]. In the latter case, the regulation of cathepsin D mRNA expression by D_3 and RA was found to be at least partially independent of differentiation. Because the effects of differentiating agents on *ctsb* transcript levels occur within 12–24 h, i.e., before the development of formazan granules in the nitroblue tetrazolium assay, these effects are not secondary to terminal differentiation of HL-60 cells. Therefore, the *ctsb* gene may be targeted by cellular factors that are activated or induced directly by the treatments and/or the early stages of differentiation.

Several members of the steroid/nuclear receptor family of transcription factors mediate the action of D_3 and retinoids on gene transcription. HL-60 cells express the retinoid receptors RARα, RARβ, and RXRα [42, 43] and the vitamin D receptor [44, 45]. These receptors have been shown to form homo- and hetero-dimers, with different binding affinities for various promoter elements [37, 38]. RA binds primarily to RARα, whereas 9-*cis* RA can bind to RARα, RARβ, and RXRα [36]. In our study, the effects of D_3 and retinoids on *ctsb* gene expression are additive. Therefore, the two classes of agents may act in part via distinct pathways, which converge on the *ctsb* promoter. By sequence analysis, we have not detected consensus responsive elements for vitamin D or retinoic acid receptors in the promoter region of *ctsb* up to 1.8 kb upstream of the transcription start point. Moreover, in transient transfection assays D_3 and 9-*cis* RA failed, within 16 h, to induce luciferase expression under the control of the same promoter region. Taken together with their delayed effect on *ctsb* expression, this suggests that D_3, RA, and 9-*cis* RA may act indirectly by activating other factors that regulate *ctsb* gene transcription. These factors may bind further upstream in the *ctsb* gene or require a longer latency period than was allowed in transient transfection assays. For the latter case, Sp1 may be a candidate mediator of the effect of D_3 because D_3-induced differentiation of HL-60 cells markedly increases Sp1 binding to DNA, and a 96-h treatment of HL-60 cells leads to the increased production of the full-length Sp1 protein [46].

NaB may stimulate *ctsb* mRNA levels directly or indirectly because NaB can enhance transcription via discrete elements [47–49] or via histone hyperacetylation and opening of the chromatin structure [50–52]. The results of our transient transfection assays confirm that NaB stimulates transcription of the *ctsb* gene. However, whether discrete butyrate response elements are involved is still unknown because we were unable to detect significant similarities with promoter regions where such elements have been described. If NaB-responsive elements exist in the *ctsb* promoter, at least one of them must be located within the 49 bp upstream of exon 1 and the 56 bp of exon 1 present in pSCB8. Maiyar and Norman [53] have demonstrated that NaB increases VDR activity in primary chick kidney cells, which raises the possibility that D_3 and NaB can act in concert to regulate *ctsb* gene expression in HL-60 cells.

The mechanism of action of PMA is unique in that it appears to involve both stabilization of *ctsb* mRNA (as shown by actinomycin D decay assays) and transcriptional induction (as demonstrated by transient transfection assays). This dual effect may account for the marked increase in *ctsb* transcript levels found in PMA-treated HL-60 cells compared with cells treated with the other agents. Sequence analysis of the *ctsb* promoter reveals the presence of numerous potential responsive elements for PMA, including AP1, NF-κB, and Sp1 sites. It is interesting that the pSCB8 plasmid responds to PMA treatment even though no AP1 or NF-κB sites are present. Mutation of Sp1 sites in the CTSB promoter reveal that they play a significant role in the basal transcription of the gene [S. Yan, I. Berquin, B. Troen, and B. Sloane, unpublished results]. Because phorbol ester treatment of K562 cells increases Sp1 expression and DNA binding [54], we are currently assessing whether individual Sp1 binding sites are required for induction of transcription by PMA.

Actinomycin D has been reported to induce apoptosis at concentrations similar to those generally used for RNA synthesis inhibition [35]. Within 19 h of treatment with 0.1 μg/mL actinomycin D, 60% of HL-60 cells undergo apoptosis, and treatment with concentrations above 0.5 μg/mL induces 100% apoptosis [35]. We observed the stabilization of *ctsb* mRNA within 4 h of actinomycin D treatment at 0.1 μg/mL, i.e., at a time before overt apoptosis. Although differentiating HL-60 cells do undergo some degree of apoptosis [55], we observed stimulation of *ctsb* gene expression in response to PMA, D_3, RA, 9-*cis* RA, and NaB treatments at doses and exposure times where apoptosis was not apparent. Nonetheless, because ctsb is elevated in several physiological and pathological processes where apoptosis is involved, an intriguing possibility is that this protease is regulated by factors related to the apoptotic response.

In summary, inducers of monocytic and granulocytic differentiation increase the levels of *ctsb* transcripts in HL-60 cells before the achievement of a fully differentiated phenotype. This indicates that these differentiation inducers may regulate *ctsb* gene expression either directly or indirectly through a signal transduction cascade triggered by the treatment. D_3 and retinoids exert additive effects, suggesting that these agents stimulate *ctsb* gene expression through distinct pathways. The monocyte/macrophage inducer PMA is the most potent of the agents tested, and modulates *ctsb* expression in a dual fashion at both transcriptional and posttranscriptional levels. NaB also induces transcription of *ctsb* promoter fragments in transient transfection assays. Future efforts will be directed at determining whether the effect of NaB is mediated via discrete responsive elements. If such is the case, we will initiate a search for potentially new transcription binding factors acting on these elements.

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