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Research Article

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1,25-Dihydroxyvitamin D_3 Modulates the Expression of a Lymphokine (Granulocyte-Macrophage Colony-stimulating Factor) Posttranscriptionally

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Abstract

We recently showed that $1,25(OH)_2D_3$ sensitively inhibited the expression of granulocyte-macrophage colony-stimulating factor (GM-CSF) in normal human mitogen-activated peripheral blood lymphocytes and in the human T lymphotropic virus I immortalized T cell line known as S-LB1 at the levels of both mRNA and protein. Using S-LB1 cells as a model system the present paper identifies at least in part the mechanisms by which $1,25(OH)_2D_3$ regulates the expression of GM-CSF. Time-course studies demonstrated that by 6 and 48 h of exposure of S-LB1 cells to $1,25(OH)_2D_3$ (10^{-8} M) the GM-CSF mRNA levels were reduced by 50 and 90%, respectively. Studies using cycloheximide as a protein synthesis inhibitor showed that the inhibitory action of $1,25(OH)_2D_3$ on GM-CSF expression was dependent on new protein synthesis. In vitro nuclear run-on assays demonstrated that $1,25(OH)_2D_3$ (10^{-8} M) did not change the rate of transcription of the GM-CSF gene. The $t_{1/2}$ of GM-CSF mRNA, however, was profoundly reduced by $1,25(OH)_2D_3$ when transcription was blocked by actinomycin D compared with the half-life of GM-CSF in the presence of actinomycin D alone ($t_{1/2}$, < 0.5 and 4 h, respectively). Taken together, these results demonstrate that $1,25(OH)_2D_3$ regulates expression of the lymphokine GM-CSF posttranscriptionally by influencing the stability of GM-CSF mRNA.

Introduction

Survival, proliferation, and differentiation of hematopoietic cells are dependent on colony-stimulating factors (CSF).[1] The granulocyte-macrophage (GM) CSF is produced by activated T lymphocytes and by mesenchymal cells stimulated by macrophage-derived tumor necrosis factor alpha and IL-1 (1–4). The natural and recombinant GM-CSF possess multilineage CSA, and also enhance mature cell function of neutrophils, macrophages, and eosinophils (5–8).

The hormonally active metabolite of vitamin D_3, $1,25(OH)_2D_3$, may play an important role as an immunohematopoietic regulatory hormone (9, 10). We and others have shown that activated macrophages synthesized hormonally active $1,25(OH)_2D_3$ (11, 12). Further, $1,25(OH)_2D_3$ sensitively and specifically inhibited GM-CSF mRNA in normal mitogen-activated T lymphocytes and in a human T cell lymphotropic virus 1 (HTLV-1) immortalized T lymphocyte line derived from a normal individual (S-LB1) (13).

The present study addresses the mechanism by which $1,25(OH)_2D_3$ controls expression of GM-CSF in human lymphocytes. Both transcriptional and posttranscriptional mechanisms control the regulation of gene expression in eukaryotic cells (14). For instance, studies showed that gamma-IFN regulated the expression of *c-myc* in the human lymphoblastic Daudi cells at the posttranscriptional level (15, 16). In contrast, a block to elongation of transcription was found to be responsible for the decreased expression of *c-myc* in the HL-60 promyelocytic leukemic cells when induced to differentiate by all-*trans* retinoic acid (17). We and others recently found that recombinant human tumor necrosis factor alpha regulated the expression of *c-myc* in the promyelocytic leukemia HL-60 cells and in Hela cells at the level of transcription (18–20).

Using S-LB1 cells as a model system we show in this study that the reduced expression of GM-CSF, which is mediated by $1,25(OH)_2D_3$ in T lymphocytes, is due to decreased stability of GM-CSF mRNA (posttranscriptional regulation).

Methods

Cell cultures and chemicals. The HTLV-1 immortalized T lymphocyte line S-LB1 (21) was maintained in suspension culture T-flask (Miles Laboratories, Inc., Naperville, IL) containing alpha medium (Flow Laboratories, Inc., Rockville, MD) and 10% fetal bovine serum (Irvine Scientific, Santa Ana, CA) in a humidified atmosphere of 7% CO_2. The $1,25(OH)_2D_3$ was dissolved in 100% ethanol to a stock concentration of 1×10^{-3} M and stored at −20°C. The final concentration of 10^{-8} M was obtained by diluting the stock solution in PBS. Actinomycin D (Boehringer Mannheim Diagnostics, Inc., Indianapolis, IN) was dissolved in 100% ethanol to a stock concentration of 1 mg/ml. Cycloheximide was purchased from Sigma Chemical Co. (St. Louis, MO). Cell viability was not affected in the various experimental protocols, as determined by trypan blue exclusion.

S-LB1 cells were exposed to either actinomycin D (5 μg/ml), cycloheximide (20 μg/ml), or control alpha medium with 10% fetal bovine serum. The RNA synthesis was studied by labeling S-LB1 cells (1×10^6/ml) with 0.5 μCi [^{14}C] uridine for 1 h at 37°C (triplicate wells per point), washing the cells twice in PBS, precipitating the cells on ice with 5% TCA for 10 min, washing twice with 5% TCA, and heating for 60 min at 80°C. A 200-μl aliquot from each sample was mixed with Aquasol (New England Nuclear, Boston, MA) and counted in a scintillation photometer. Protein synthesis was determined by suspending the cells (75,000 cells/microtiter well) in methionine-free Earle's medium (Gibco, Grand Island, NY) with 5% FCS, and by exposing them

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1. *Abbreviations used in this paper:* GM-CSF, granulocyte-macrophage colony-stimulating factor; HTLV-1, human T cell lymphotropic virus-1; SSC, standard saline citrate; [α-^{32}P]UTP, [α-^{32}P]uridine-5'-triphosphate.

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to 1 μCi [^{35}S] methionine for 90 min. The cells were harvested with a MASH-harvester, and isotope uptake was determined with liquid scintillation photometry (S1800; Beckman Instruments, Inc., Fullerton, CA).

DNA. The GM-CSF cDNA probe (0.9 kb, Eco RI-Bam HI) was derived from plasmid pCSF-2 (reference 2, a generous gift of S. Clark, Genetics Institute, Boston), the p53 probe (Hind III-Eco RI, 1.76 kb) from plasmid pR4-2 (22), the β-actin DNA (Eco RI-Bam HI, 700 bp) from plasmid pHFβA-3' ut (23), and the β-globin DNA (Bam HI, 1.8 kb) from plasmid pβ4.4 (24). The IL-2 receptor cDNA was kindly provided by T. Nikaido (Aichi Cancer Center Research Institute, Nagoya, Japan). When used as probes, the DNA inserts were oligolabeled (random primed) as described (25).

RNA blot technique. For cytoplasmic RNA, freshly harvested cells were suspended in hypotonic buffer (10 mM Tris-HCl [pH 7.4], 1 mM KCl, and 3 mM $MgCl_2$), and were lysed with 0.3% NP-40. Nuclei were removed by centrifugation. Cytoplasmic RNA was extracted by the phenol-chloroform method as essentially described (26) and quantified by absorbance at 260 nM. RNA blotting was performed essentially as described (27). Samples were denatured at 65°C for 10 min, size-separated by an agarose formaldehyde gel (1% agarose [Bethesda Research Laboratories, Gaithersburg, MD], 50 mM Na acetate, 10 mM Na_2 EDTA, 200 mM 3-(4-morpholino) propane sulfonic acid, and 2.2 M formaldehyde), and transferred to nylon membrane filters (ICN Biomedicals Inc., Irvine, CA). Filters were dried, baked at 80°C in vacuo for 2 h, and then prehybridized for 16–24 h. Hybridizations with ^{32}P-labeled DNA (1×10^6 cpm/ml) were performed at 42°C for 16–24 h in a solution containing 50% (vol/vol) formamide, 2× standard saline citrate (SSC) (1× SSC is 150 mM NaCl and 15 mM sodium citrate), 5× Denhardts (1× Denhardts is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA), 0.1% SDS, 1 mM EDTA, 10% (vol/vol), dextran sulfate (Sigma Chemical Co.) (500,000 mol wt), and 100 μg/ml salmon sperm DNA (Sigma Chemical Co.). Filters were washed to a stringency of 0.1× SSC, 1% SDS at 65°C, and exposed for 24–48 h at −70°C on Kodak XAR-5 film. Intensity of bands of hybridization were determined on autoradiograms exposed for various durations using a laser densitometer.

In vitro nuclear run-on assay. For run-on experiments, nuclei were isolated by resuspending the cells in hypotonic buffer (10 mM Tris-HCl [pH 7.4], 1 mM KCl, 3 mM $MgCl_2$) and lysing the cells with 0.3% NP-40. The nuclei were washed twice in hypotonic buffer, resuspended in nuclear storage buffer (40% glycerol, 50 mM Tris-HCl [pH 8.3], 5 mM $MgCl_2$, and 0.1 mM EDTA), and stored at −70°C. Transcription of nuclei and RNA isolation were performed as described (28). Briefly, the nuclei (1×10^8) were thawed and incubated for 10 min at 26°C in reaction buffer containing 200 μCi [α-^{32}P]uridine-5'-triphosphate ([α^{-32}]UTP, 3,000 Ci/mmol) (ICN Biomedicals Inc.), and the labeled RNA was hybridized for 3 d to Southern blots of restriction enzyme digests of the various clones. The blots were washed to a final stringency of 0.1× SSC, 0.1% SDS, and washed again at room temperature for 30 min in RNase (10 μg/ml, in 2× SSC). The filters were washed again at room temperature for 15 min in 2× SSC.

Results

1,25(OH)$_2$D$_3$ regulates mRNA levels of GM-CSF in S-LB1 cells (Fig. 1). Exposure of S-LB1 cells for various lengths of time (0.5–48 h) to 1,25(OH)$_2$D$_3$ (10^{-8} M) showed a time-dependent decrease in the levels of GM-CSF mRNA (Fig. 1, *top*). A 48-h exposure resulted in an ~ 90% reduction of GM-CSF mRNA levels (lane *6*) compared with the untreated control sample (lane *1*); and a 50% reduction was observed after a 6-h exposure (lane *4*), as determined by densitometry readings. Ethidium bromide staining of the formaldehyde agarose gel was used to assure that comparable amounts of RNA were used for each experimental point (Fig. 1, *middle*). Also, the 1.4-kb IL-2 receptor mRNA showed little change in S-LB1 cells cultured with 1,25(OH)$_2$D$_3$ (Fig. 1, *bottom*). We also determined whether 1,25(OH)$_2$D$_3$ might regulate the expression of the β-actin and p53 genes. The protooncogene p53 is often present in actively proliferating cells, but is undetectable or expressed at low levels in resting cells (29). The S-LB1 cells constitutively express the p53 gene. The 1,25(OH)$_2$D$_3$ (10^{-8} M for 48 h) reduced mRNA levels of β-actin and p53 by approximately twofold compared with untreated control samples (data not shown). These results suggest that 1,25(OH)$_2$D$_3$ regulates mRNA levels of different genes. The viability of S-LB1 cells was not affected at any of the experimental points, as determined by trypan blue exclusion.

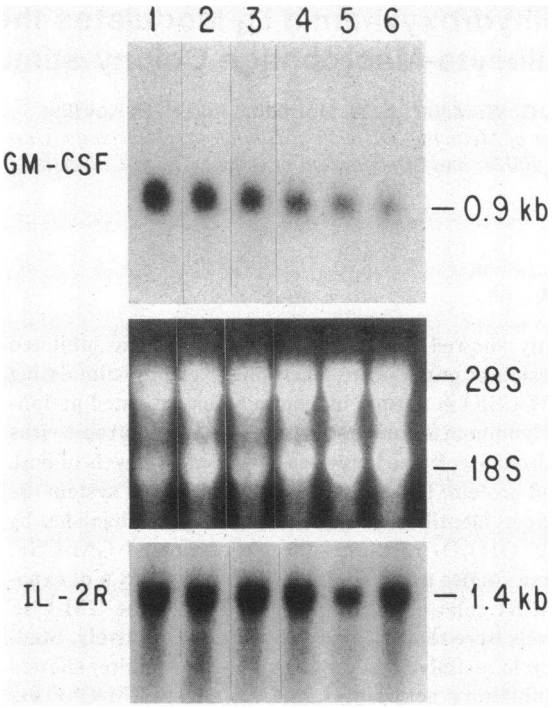

Figure 1. Regulation of GM-CSF mRNA levels in S-LB1 cells by 10^{-8} M 1,25(OH)$_2$D$_3$ (time course): lane *1*, control (no 1,25[OH]$_2$D$_3$); lane *2*, 0.5-h exposure; lane *3*, 2-h exposure; lane *4*, 6-h exposure; lane *5*, 24-h exposure; lane *6*, 48-h exposure. (*Top*) RNA blot of hybridization with a cDNA probe for GM-CSF. A single band could be detected at 0.9 kb which is consistent with GM-CSF mRNA. (*Middle*) Ethidium bromide staining of the 28S and 18S RNA of the formaldehyde agarose gel of the RNA blot shown in the upper panels. (*Bottom*) The Northern blot was rehybridized with a cDNA probe for IL-2 receptor and the 1.4-kb IL-2 receptor (IL-2R) band is shown. Analyses were performed as described in Methods. Each lane contains 20 μg cytoplasmic RNA.

Influence of protein synthesis on the regulation of GM-CSF by 1,25(OH)$_2$D$_3$ in S-LB1 cells (Fig. 2). To determine the role of new protein synthesis in the regulation of GM-CSF mRNA levels in S-LB1 cells with exposure to 1,25(OH)$_2$D$_3$, the cells were pretreated for 45 min with 20 μg/ml cycloheximide (a protein synthesis inhibitor) and then exposed to 10^{-8} M 1,25(OH)$_2$D$_3$ for 6 h. As a control, S-LB1 cells were cultured with cycloheximide alone (20 μg/ml for 6.5 h). Cycloheximide blocked > 85% of protein synthesis in S-LB1 cells as measured by [^{35}S]methionine incorporation. As shown in Fig. 2, inhibition of new protein synthesis abolished the inhibitory effect of 1,25(OH)$_2$D$_3$ on GM-CSF mRNA accumulation (lane *4*). The

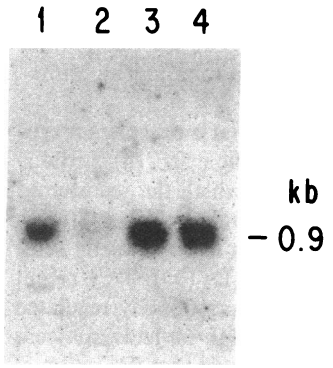

Figure 2. Influence of the inhibition of new protein synthesis on the action of 10^{-8} M $1,25(OH)_2D_3$ on GM-CSF mRNA levels in S-LB1 cells. New protein synthesis was blocked by 20 μg/ml cycloheximide. Cytoplasmic RNA (20 μg/lane) was extracted and analyzed by RNA blot technique using a cDNA probe for GM-CSF. Lane *1*, control S-LB1 cells (no cycloheximide, no $1,25[OH]_2D_3$); lane *2*, S-LB1 cells exposed for 6 h to $1,25(OH)_2D_3$ alone; lane *3*, S-LB1 cells treated for 6.5 h with cycloheximide alone; lane *4*, RNA from S-LB1 cells after treatment with cycloheximide for an initial 30 min and exposure to $1,25(OH)_2D_3$ for an additional 6 h. Analyses were performed as described in Methods.

cycloheximide alone increased GM-CSF mRNA levels by approximately twofold (lane *3*) compared with the untreated control sample (lane *1*).

Level of regulation of GM-CSF by $1,25(OH)_2D_3$ in S-LB1 cells (Figs. 3–5). The $1,25(OH)_2D_3$ might regulate expression of GM-CSF at either the transcriptional or posttranscriptional level. In vitro transcriptional run-on assays were performed to determine if the regulation was at the level of transcription (Fig. 3). In this assay in vitro transcripts are generated by elongation of previously initiated RNA chains in the presence of [α^{32}P]UTP. As demonstrated in Fig. 3, levels of GM-CSF transcripts did not change in S-LB1 cells that were treated with 10^{-8} M $1,25(OH)_2D_3$ for either 6 or 48 h compared with the untreated control sample (lane *1* in each panel of Fig. 3). Also, almost no change in the rate of β-actin transcripts was observed (lane *2* of each panel). Furthermore, nonspecific hybridization to β-globin DNA did not occur (lane *3*). No nonspecific hybridization to plasmid DNA was seen (Southern blot contained both the eukaryotic inserts [GM-CSF, β-actin, and β-globin] and the restriction-digested, linearized plasmids). Similar results were observed in another run-on transcriptional assay (data not shown).

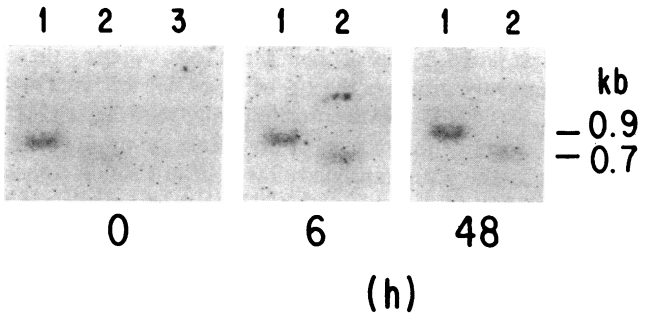

Figure 3. Transcriptional run-on analysis of GM-CSF in isolated nuclei of S-LB1 cells untreated or treated with (10^{-8} M) $1,25(OH)_2D_3$ for 0, 6, and 48 h. Analysis was performed as described in Methods. Autoradiograms show hybridization of ^{32}P-labeled transcripts to GM-CSF DNA (lanes *1*, 0.9-kb bands); β-actin DNA, positive control (lanes *2*, 0.7-kb bands), and β-globin DNA, negative control (lane *3*). The DNA inserts (10 μg) were size-separated from their plasmid vectors by electrophoresis on an agarose gel and transferred to a nylon membrane filter. Autoradiogram was exposed to film for 3.5 d.

Posttranscriptional regulation was examined by determining changes in GM-CSF mRNA levels induced by 10^{-8} M $1,25(OH)_2D_3$ when transcription was blocked by 5 μg/ml actinomycin D. Actinomycin D blocked > 95% of transcription in S-LB1 cells as measured by [^{14}C]uridine incorporation. Initially, the $t_{1/2}$ of GM-CSF mRNA was determined in the presence of actinomycin D alone (Fig. 4 *A, top*). The GM-CSF mRNA had a $t_{1/2}$ of 4 h. The $t_{1/2}$ of GM-CSF mRNA was reexamined when transcription was blocked by actinomycin D and the cells were exposed simultaneously to $1,25(OH)_2D_3$ (Fig. 4 *B, top*). Addition of $1,25(OH)_2D_3$ to these cells resulted in a rapid and profound decrease of levels of GM-CSF mRNA; the $t_{1/2}$ of GM-CSF mRNA under these conditions was < 30 min. Ethidium bromide staining of the formaldehyde agarose gel was used to assure that similar amounts of RNA were used (Fig. 4 *B, bottom*). These results suggest that $1,25(OH)_2D_3$ regulates the expression of GM-CSF at the posttranscriptional level. Fig. 5 summarizes the densitometry readings of the autoradiograms shown in Figs. 1 and 4, *A* and *B*.

Discussion

The secosteroid $1,25(OH)_2D_3$, which is the most active metabolite of vitamin D_3, effectively reduces the expression of the

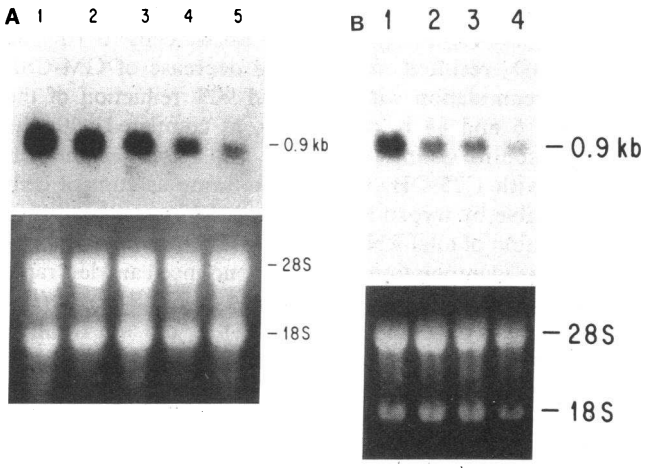

Figure 4. Posttranscriptional analysis of GM-CSF mRNA regulation by $1,25(OH)_2D_3$. (*A, top*) Determination of the $t_{1/2}$ of GM-CSF mRNA in S-LB1 cells by RNA blot technique. S-LB1 cells were exposed for various durations to 5 μg/ml actinomycin D and the cytoplasmic RNA (20 μg/lane) of these cells were hybridized with a GM-CSF cDNA probe. Lane *1*, control (no actinomycin D); lane *2*, 0.5-h exposure; lane *3*, 2-h exposure; lane *4*, 6-h exposure; and lane *5*, 10-h exposure. The $t_{1/2}$ of GM-CSF was determined by the decay of GM-CSF mRNA levels. (*Bottom*) Ethidium bromide stain of the 28S and 18S RNA on the formaldehyde agarose gel. (*B, top*) Determination of the $t_{1/2}$ of GM-CSF mRNA in the presence of $1,25(OH)_2D_3$ when transcription was blocked by actinomycin D. S-LB1 cells were exposed to 10^{-8} M $1,25(OH)_2D_3$ and 5 μg/ml actinomycin D. Cytoplasmic RNA (20 μg/lane) was extracted and analyzed by Northern blot technique as described in Methods. Lane *1*, control RNA of S-LB1 cells (no $1,25[OH]_2D_3$, no actinomycin D); lane *2*, 0.5-h exposure to both agents; lane *3*, 2-h exposure; and lane *4*, 6-h exposure to both agents, respectively. (*Bottom*) Ethidium bromide staining of 28S and 18S RNA on the formaldehyde agarose gel of the RNA blot shown in the top panel.

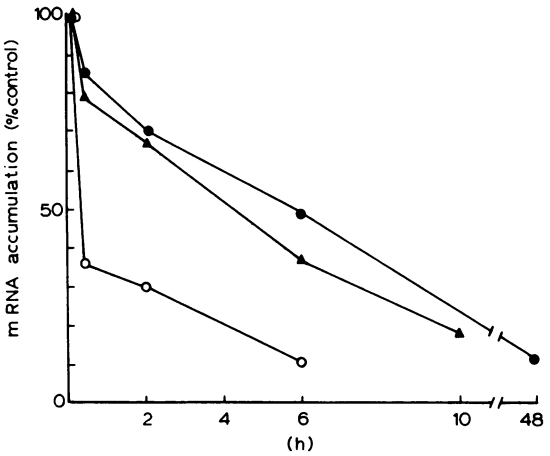

Figure 5. $t_{1/2}$ of GM-CSF mRNAs from S-LB1 cells treated with either $1,25(OH)_2D_3$ (●), actinomycin D alone (▲), or with both agents simultaneously (○). Autoradiograms shown in Figs. 1 and 4 *A* and *B* were quantitated by densitometry using the control samples (no $1,25[OH]_2D_3$, no actinomycin) as a reference for 100% GM-CSF mRNA accumulation.

hematopoietic growth factor GM-CSF in both normal human T lymphocytes and the HTLV-1 immortalized T cell line S-LB1 at both the levels of mRNA and protein (13). In the present paper we extended this observation by studying at least in part the mechanism of regulation of GM-CSF gene expression by $1,25(OH)_2D_3$. Exposure of S-LB1 cells to 10^{-8} M $1,25(OH)_2D_3$ resulted in a marked decrease of GM-CSF mRNA accumulation with a 50 and 90% reduction of the mRNA at 6 and 48 h, respectively, as compared with the untreated control sample (Fig. 1, *top*). Cell viability in the cells cultured with $1,25(OH)_2D_3$ were the same as control cells (> 95% viable by trypan blue staining). We recently showed that inhibition of total RNA by $1,25(OH)_2D_3$, as measured by [^{14}C]uridine incorporation, was less pronounced and less rapid than the decrease of GM-CSF mRNA in S-LB1 cells (13). Furthermore, we found that $1,25(OH)_2D_3$ did not decrease levels of the 1.4-kb band of the IL-2 receptor mRNA (13). In the present study, $1,25(OH)_2D_3$ (10^{-8} M for 48 h) reduced mRNA levels of β-actin and p53 by approximately twofold, which suggests that $1,25(OH)_2D_3$ can affect mRNA levels of several genes in S-LB1 cells.

Our study further showed that inhibition of new protein synthesis by cycloheximide abolished the effect of $1,25(OH)_2D_3$, which indicates that the action of $1,25(OH)_2D_3$ on GM-CSF expression is dependent on new protein synthesis. Cycloheximide alone increased GM-CSF mRNA levels twofold. This suggests the presence of a protein that destabilizes GM-CSF mRNA in S-LB1 cells, or suggests that degradation of mRNA requires its active translation (30). A similar superinduction by cycloheximide has been found in other transiently expressed genes such as *c-myc* (31–33).

The $t_{1/2}$ of GM-CSF mRNA was ~ 4 h, which demonstrates that GM-CSF is a moderately long-lived mRNA in S-LB1 cells. Other investigators recently showed that GM-CSF is a short-lived mRNA ($t_{1/2}$ ~ 30 min) in PHA-stimulated peripheral blood T lymphocytes, whereas in phorbol-diester–induced peripheral blood T lymphocytes the $t_{1/2}$ was > 2 h (34). One explanation for this discrepancy might be the different modes of stimulation of the T lymphocytes that produce GM-CSF. The S-LB1 cells are HTLV-1 immortalized T lymphocytes and constitutively express GM-CSF, whereas normal T lymphocytes produce GM-CSF only upon mitogenic stimulation.

Transcriptional and posttranscriptional mechanisms regulate the expression of genes in eukaryotic cells (14). Little is known about the exact mechanism by which the secosteroid $1,25(OH)_2D_3$ regulates expression of eukaryotic genes. Studies showed that a variety of cells, including hematopoietic cells, contain specific receptors for $1,25(OH)_2D_3$ (35). Very recent studies showed that $1,25(OH)_2D_3$ transcriptionally regulated *c-myc* expression in the promyelocytic HL-60 leukemia cells (36). Our recent study clearly demonstrated that the action of $1,25(OH)_2D_3$ on the expression of GM-CSF in human T lymphocytes was mediated by a specific receptor for this steroid (13). In the case of glucocorticoids, transcriptional activation of gene expression was found to be mediated by an interaction of hormone-receptor complexes with specific DNA sequences, and negative regulation might be likely to be affected by similar hormone-receptor complex–DNA interactions (for review, see reference 37). In a parallel fashion the $1,25(OH)_2D_3$ receptor complex might interact with specific sequences of the GM-CSF gene, and thus transcriptionally regulate the expression of GM-CSF. However, our in vitro nuclear run-on assay revealed that $1,25(OH)_2D_3$ did not alter the rate of GM-CSF transcription in S-LB1 cells (Fig. 3). By contrast, a sharp decrease of GM-CSF mRNA levels occurred in the S-LB1 cells exposed to $1,25(OH)_2D_3$ when transcription and therefore new mRNA production of these cells were blocked by actinomycin D (Figs. 4 *B* and 5). These results suggest that $1,25(OH)_2D_3$ posttranscriptionally regulates the expression of GM-CSF by influencing the stability of mRNA. Of note, but unexplained, is that GM-CSF mRNA concentrations decreased in S-LB1 in a biphasic manner after exposure of the cells to either actinomycin D, $1,25(OH)_2D_3$, or most prominently actinomycin D plus $1,25(OH)_2D_3$.

Several other studies have found that GM-CSF mRNA levels can be regulated posttranscriptionally by exposure of lymphocytes to 12-0-tetradecanoylphorbol 13-acetate (34) and exposure of macrophages to LPS and several other agents (38). Furthermore, GM-CSF mRNA as well as a variety of other lymphokines, including IL-2 and -3 and lymphocytotoxin, have conservation of adenosine-thymidine–rich sequences in the 3′ untranslated region of their genes (34). Altering these regions markedly prolongs the $t_{1/2}$ of GM-CSF mRNA (34). Perhaps $1,25(OH)_2D_3$ posttranscriptionally modulates GM-CSF by a direct or indirect effect on adenosine-uridine–rich sequences in the 3′ untranslated GM-CSF mRNA. Another possible mechanism by which $1,25(OH)_2D_3$ might regulate the gene expression of GM-CSF at the posttranscriptional level is by an accelerated degradation of GM-CSF mRNA such as by the (2′–5′) A synthetase/RNA L pathway (for review, see reference 39).

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