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Induction of c-*sis* gene expression and synthesis of platelet-derived growth factor in human myeloid leukemia cells during monocytic differentiation

(HL-60 cells/U-937 cells/macrophage differentiation/c-*sis* induction)

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ABSTRACT **Phorbol esters induce the differentiation of human myeloid leukemia cells HL-60 and U-937 along the monocytic–macrophage lineage. This process has been associated with the induction of several cellular protooncogenes, including the c-*fos* and c-*fms* genes. We now report that phorbol ester-induced differentiation of the HL-60 and U-937 cells results in the induction of the expression of the c-*sis* platelet-derived growth factor 2 (PDGF-2) protooncogene. sis mRNA transcripts were not detectable in the uninduced cells but were detectable within 12 hr of phorbol ester induction. Concomitantly, the induced cells were shown to synthesize and secrete biologically active PDGF-like proteins, identified in the conditioned medium of the phorbol ester-treated cells by direct immunoprecipitation with PDGF antiserum. Addition of cycloheximide to phorbol ester-treated HL-60 cells superinduced sis mRNA transcripts. c-sis gene transcripts were also detected in freshly isolated human monocytes but not in human granulocytes or in HL-60 cells induced to differentiate along the granulocytic lineage. Activation of the c-*sis*/PDGF-2 gene in human hematopoietic cells during monocytic differentiation may serve in the mediation of physiologic functions of the differentiated cells by means of the secretion of potent PDGF-like mitogen.**

The transforming gene (v-*sis*) of the simian sarcoma virus (SSV), an acute transforming retrovirus of primate origin, encodes for the platelet-derived growth factor 2 (PDGF-2) chain of human PDGF (1–3). In SSV-transformed cells the v-*sis*/PDGF-2 gene product is processed into a disulfide-linked PDGF-2 homodimer (4) that is structurally, immunologically, and functionally similar to biologically active PDGF (5). These findings suggested that the transforming ability of SSV is mediated by the production of PDGF-like mitogen.

The cellular c-*sis* has also been shown to encode for the PDGF-2 chain (6, 7). c-sis/PDGF-2 transcripts have been demonstrated in human malignant cells of mesenchymal origin (8–10) whose normal counterparts are target cells to PDGF action (11). *sis* activation in these human malignant cells was accompanied by the synthesis and secretion of biologically active PDGF-like mitogen (9, 10, 12). These findings suggested the possibility that inappropriate expression of PDGF-related genes plays an important role in the processes leading certain cells of mesenchymal origin to malignant transformation.

Recent studies have shown that *sis* activation and synthesis and secretion of PDGF-like mitogen are not the prerogative of transformed cells that are target cells to PDGF action. For example, cultured vascular endothelial cells (13), activated human monocytes (14), and macrophages (15) have been shown to exhibit c-sis transcripts and to synthesize and secrete biologically active PDGF-like mitogen. Production of PDGF-like mitogen by these cells may be involved in important physiological processes, such as wound healing, by means of the paracrine stimulation of connective tissue cell migration (16–18), proliferation (11), and collagen synthesis (19) as well as in the development of the atherosclerotic plaque (20).

The human myeloid leukemia cell lines HL-60 (21) and U-937 (22) have been shown to terminally differentiate into monocytes–macrophages in the presence of phorbol 12-myristate 13-acetate (PMA) (23, 24). The induction of differentiation of these two cell lines has been well characterized (see ref. 25 for review). We and others have shown that untreated HL-60 cells do not exhibit detectable amounts of mRNA related to the c-*sis*/PDGF-2 sequences (26, 27) or produce PDGF-like mitogen (27).

We now report the induction of c-*sis* gene expression during the PMA-induced differentiation of the HL-60 and U-937 cells toward monocytes–macrophages. Concomitantly, the differentiating cells synthesize and secrete biologically active PDGF-like mitogen.

MATERIALS AND METHODS

Materials. Complete RPMI 1640 medium, fetal calf serum, and antibiotics were from M. A. Bioproducts (Walkersville, MD). RPMI 1640 medium free of cystine was prepared with "media kits" (GIBCO). PMA and cycloheximide (Chx) were from Sigma, and protein A bound to Sepharose CL-4B beads was from Pharmacia P-L Biochemicals. [^{35}S]Cysteine (specific activity >1000 Ci/mmol; 1 Ci = 37 GBq) and Amplify solution were from Amersham. Molecular mass protein markers were from Pharmacia and Bio-Rad.

Cells and Cell Treatments. Human monocytes were freshly isolated from whole blood of normal donors as described (28). HL-60 and U-937 cells were maintained in RPMI 1640 medium containing 10% fetal calf serum supplemented with penicillin (50 units/ml) and streptomycin (50 μg/ml). The cultures were incubated in a humidified 5% CO_2 in air atmosphere at 37°C. The protocols applied to metabolically label proteins secreted by the cultured cells varied according to the experiment. Exponentially grown HL-60 and U-937 cells in suspension culture were harvested, and the cell pellet was resuspended in serum-free/cystine-free RPMI 1640 medium supplemented with L-glutamine and penicillin/strepto-

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Abbreviations: PDGF, platelet-derived growth factor; PMA, phorbol 12-myristate 13-acetate; Chx, cycloheximide; SSV, simian sarcoma virus.

mycin. Equal suspension volumes containing 1.5×10^6 cells per ml were removed. PMA in phosphate-buffered saline (PBS) was added to a final concentration of 33 nM, whereas control cell suspension(s) received [^{35}S]cysteine and were incubated at 37°C for the periods of time indicated in the legends of Figs. 4 and 5. To inhibit protein synthesis, Chx was added to the cell culture to a final concentration of 10 μg/ml. Differentiation of the cells exposed to 33 nM PMA has been assessed (29).

Immunoprecipitation and Analysis of Proteins. Following the desired incubation period, the medium conditioned by the metabolically labeled cells was collected and prepared for and subjected to immunoprecipitation with PDGF antiserum as described (10). Wherever indicated, samples of equal volume were treated with normal preimmune rabbit serum, antiserum to PDGF, and PDGF antiserum in the presence of an excess of purified PDGF (500 ng) (competition experiment) as described (10). Immunoprecipitates were analyzed under nonreducing and/or reducing conditions on NaDodSO$_4$/16% acrylamide slab gels, followed by fluorography (30). Radioactivity incorporated into the protein bands of interest on the gels was quantitated as described (30).

Preparation of RNA and Hybridization. Total cellular RNA was isolated by the guanidine thiocyanate technique (31) and selected for poly(A)$^+$-containing RNA by one cycle of affinity chromatography on oligodeoxythymidilic acid cellulose as described (32). Total cellular RNA (20 μg) or poly(A)$^+$ RNA (8 μg) was subjected to electrophoresis on a formaldehyde-containing gel, transferred to nitrocellulose paper, and hybridized to ^{32}P-labeled 1.2-kilobase (kb) *Pst* I fragment of the v-*sis* gene purified from the pv-sis plasmid (33). In some experiments the blot was stripped of the v-sis probe by boiling for 5 min in water and then was hybridized to the 1.0-kb *Pst* I fragment of the v-*fms* gene purified from the pSM3 plasmid.

Assay for Mitogenic Activity of Cell-Conditioned Medium. Exponentially grown HL-60 and U-937 cells were harvested and washed twice in serum-free medium, and the cell pellets were resuspended in serum-free medium at 1×10^5 cells per ml and incubated for 5 hr at 37°C to allow for complete depletion of residual PDGF activity from the serum. The cells were then harvested and resuspended in fresh serum-free medium at 1×10^6 per ml. Twenty-milliliter aliquots of suspension cultures were incubated at 37°C for 12 or 24 hr. Incubation was in the absence or presence of 33 nM PMA. The conditioned medium of each suspension culture was clarified by centrifugation, freeze-dried, dissolved in 2 ml of 150 mM NaCl, and dialyzed for 24 hr at 4°C against 1 liter of 150 mM NaCl with two changes of the dialyzing solution. Protein content of the conditioned medium concentrates was estimated by the method of Lowry *et al.* (34) using human serum albumin as a standard. Aliquots of conditioned medium were heated at 100°C for 10 min and assayed for their ability to stimulate incorporation of [^{3}H]thymidine into the DNA of quiescent BALB/c-3T3 cells as described (35).

PDGF and PDGF Antiserum. Purified PDGF was prepared at our laboratories from clinically outdated human platelets as described (1, 35). Antiserum to purified PDGF was raised in rabbits as described (10).

RESULTS

PMA Induces c-*sis* Gene Expression in HL-60 and U-937 Cells. Uninduced HL-60 cells do not contain detectable amounts of sis mRNA. However, HL-60 cells exposed to PMA for 12 hr contain a 25S sis mRNA transcript (Fig. 1*A*). At the same time, a 28S fms mRNA transcript could be detected (Fig. 1*A*). The fms transcript has been specifically related to monocytic differentiation (29). Both transcripts increased progressively during a 24-hr PMA treatment (Fig. 1*A*). c-sis mRNA was also detected in PMA-treated U-937 cells (Fig. 1*B*, lane c) as well as in freshly isolated human monocytes and 14-day cultured monocytes (macrophages) (Fig. 1*B*, lanes a and b) but not in dimethyl sulfoxide-induced granulocytic HL-60 cells (Fig. 1*B*, lanes d and e). Similarly, no sis transcripts were detected in freshly prepared human granulocytes (data not shown). sis transcripts have also been reported by others in activated human monocytes and macrophages (14, 15).

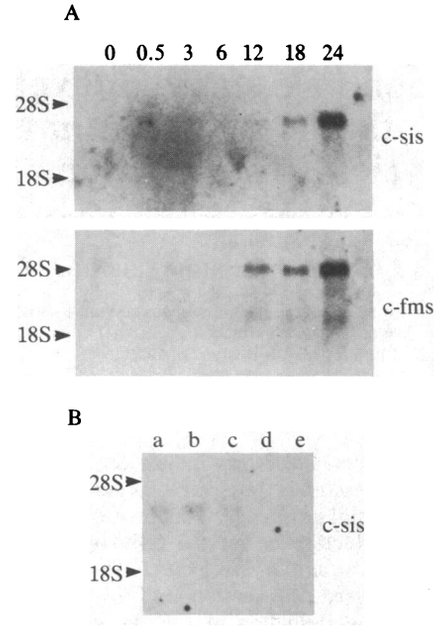

FIG. 1. (*A*) Time course of c-sis and c-fms mRNA induction in PMA-treated HL-60 cells. RNA was prepared from HL-60 cells treated with PMA for the periods of time indicated in hours. Poly(A)$^+$ RNA was first hybridized to a v-sis probe and then hybridized to a v-fms probe. (*B*) c-sis transcripts in hematopoietic cells. Total cellular RNA was hybridized to a v-sis probe. Lanes: a, freshly isolated human monocytes; b, 14-day cultured human monocytes (macrophages); c, U-937 cells treated with PMA for 24 hr; d and e, HL-60 cells exposed to 1.25% dimethyl sulfoxide for 24 hr and 120 hr, respectively. On the left, the 18S and 28S ribosomal RNA markers are indicated.

Chx Superinduces c-sis Transcripts. To examine the possible role of protein synthesis in the PMA-induced expression of the c-*sis* protooncogene, we monitored the appearance of sis mRNA in the presence of Chx. Fig. 2 shows that when Chx was added in cells already differentiated by a 24-hr exposure to PMA, sis mRNA increased by 4 hr after Chx treatment, peaked by at least 10-fold at 8 hr, and declined thereafter. In contrast, c-fms mRNA did not increase and by 24 hr of Chx treatment c-fms transcripts were undetectable (Fig. 2). No sis transcripts were detected in cells exposed to Chx alone (Fig. 2). Taken together these results suggest that the c-*sis* gene is a superinducible gene that is a primary target for PMA-regulated transcription.

Synthesis of c-sis/PDGF-2 Proteins by PMA-Treated HL-60 Cells. Induction of the expression of the *sis*/PDGF-2 gene in PMA-treated HL-60 cells was accompanied by the synthesis and secretion of biologically active PDGF-like proteins. Their identification was accomplished by immunoprecipitation with PDGF antiserum from the conditioned medium of metabolically labeled HL-60 cells. In our initial study, the HL-60 cells were labeled in serum-free medium for 18 hr in the presence of 33 nM PMA. The conditioned medium of the labeled cells was subjected to immunoprecipitation with specific PDGF antiserum, and the precipitate was analyzed by NaDodSO$_4$ gel electrophoresis under nonreducing and

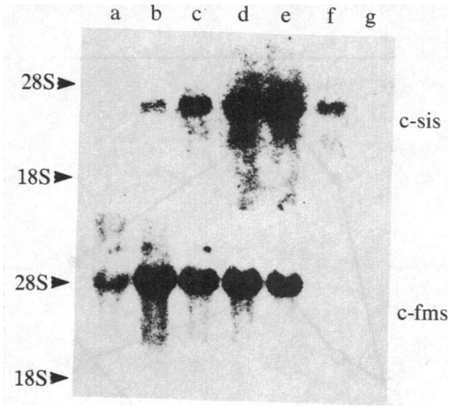

FIG. 2. Effect of Chx on c-sis and c-fms mRNA expression in PMA-treated HL-60 cells. Cultures of HL-60 cells, treated with PMA for 24 hr, received Chx and incubation was continued at 37°C. Cells were harvested after 4 hr, 6 hr, 8 hr, and 24 hr of Chx treatment. Control cells were treated for 24 hr with either PMA or Chx alone. Total cellular RNA was hybridized to a v-sis or v-fms probe. Lanes: a, untreated cells; b, 24 hr of PMA; c, 28 hr of PMA, 4 hr of Chx; d, 30 hr of PMA, 6 hr of Chx; e, 32 hr of PMA, 8 hr of Chx; f, 48 hr of PMA, 24 hr of Chx; g, 24 hr of Chx. The 28S band on lane a represents nonspecific hybridization of the v-*fms* probe to rRNA.

reducing conditions (Fig. 3). Under nonreducing conditions the immunoprecipitate contained proteins of 32 kDa and 30 kDa (Fig. 3, lane b). These proteins are within the molecular range of biologically active, unreduced PDGF (1). They did not precipitate with nonimmune serum (lane a), and their precipitation by the antiserum was prevented by the addition of excess PDGF (lane c). Upon reduction, the 32-kDa and 30-kDa proteins were converted to their monomeric 14-kDa

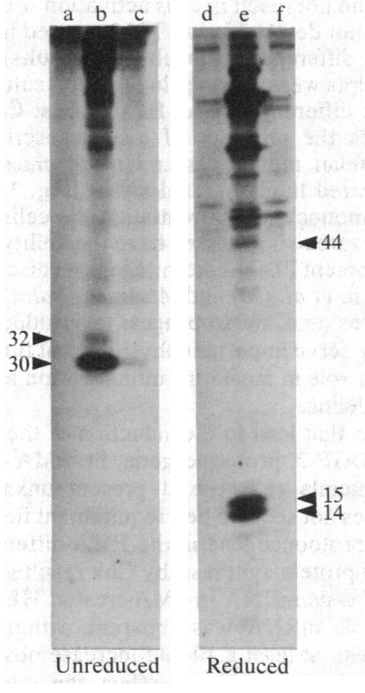

FIG. 3. Immunoprecipitation of sis/PDGF-2 proteins using specific PDGF antiserum. Control (untreated) and PMA-treated HL-60 cells were labeled with 250 μCi of radioactive cysteine per ml for 18 hr, and the immunoprecipitates from the conditioned medium were analyzed for PDGF-like proteins. Lanes: a and d, immunoprecipitation with normal serum; b and e, immunoprecipitation with PDGF antiserum; c and f, immunoprecipitation with PDGF antiserum in the presence of excess purified PDGF (500 ng).

and 15-kDa forms (Fig. 3, lane e), which is consistent with the disulfide-linked dimeric nature of PDGF (1). These polypeptides did not precipitate by nonimmune serum (Fig. 3, lane d) and underwent competition from the antiserum with excess PDGF (Fig. 3, lane f).

To investigate the relative period required for PMA to induce synthesis of the sis/PDGF-2 proteins in the HL-60 cells, we monitored the secretion of these proteins by immunoprecipitation with PDGF antiserum. The results are shown in Fig. 4*A*. The 30-kDa, 12-kDa, and 11.5-kDa polypeptides are observed in the medium of the treated cells 6 hr after addition of PMA to the cell culture (Fig. 4*A*, lane b). They were not present in the medium of cells treated with PMA for 4 hr, suggesting that the production of these PDGF-like proteins commenced 4–6 hr after addition of PMA to the cell cultures. These proteins were not present in control, uninduced HL-60 cells. Synthesis and secretion of these proteins continued for up to 24 hr of treatment, with a peak observed between 12 and 24 hr of treatment (Fig. 4*A*, lanes b). This is demonstrated in Fig. 4*B*, which shows the incorporation of radioactivity in the PDGF-like proteins. A polypeptide of 24 kDa was present in the immunoprecipitates derived from the medium of 24-hr PMA-treated HL-60 cells. This 24-kDa polypeptide may represent a further processed PDGF-like product, similar to the 24-kDa PDGF-like polypeptide identified in the lysates of SSV-transformed marmoset cells (4) and in human glioblastoma and fibrosarcoma cells (10).

Mitogenic Activity of PDGF-Like Proteins Secreted by PMA-Treated HL-60 and U-937 Cells. Conditioned medium derived from PMA-treated HL-60 and U-937 cells exhibited biological activity similar to that induced by PDGF. As shown in Fig. 5, these preparations were capable of stimulating the incorporation of [^{3}H]thymidine in cultured BALB/c-3T3 (clone A31) cells. Medium derived from cultures of untreated control HL-60 and U-937 cells did not exhibit significant PDGF-like activity (Fig. 5). The PDGF-like activity of the conditioned medium was stable to heating at 100°C for 10 min, a property similar to that described for human PDGF (35). The specificity of the PDGF-like activity in the conditioned medium of PMA-treated cells was further established by its inhibition with IgG (40 μg) derived from specific PDGF antiserum (Fig. 5).

PDGF-like activity could be detected as early as 6 hr after PMA treatment, and it increased progressively during a 24-hr treatment (Fig. 5). This is in agreement with the immunoprecipitation data (Fig. 3) that demonstrated the secretion of PDGF-like proteins by the PMA-induced HL-60 cells. The activity of the PMA-treated U-937 cells was lower than that secreted by the PMA-treated HL-60 cells (Fig. 5). These biologic data correlate with hybridization results that showed significantly lower amounts of sis mRNA in PMA-treated U-937 cells compared to that of PMA-treated HL-60 cells (results not shown).

Cellular extracts obtained from PMA-treated HL-60 and U-937 cells were subjected to heat treatment at 100°C for 10 min, and the clarified supernatants were assayed for PDGF-like activity. The activity of cellular extracts (data not shown) was significantly lower than that shown in the conditioned medium of these cells (Fig. 5), suggesting that the PDGF-like proteins synthesized by the PMA-treated cells are secreted rapidly.

DISCUSSION

The studies described above have shown that PMA-induced differentiation of human myeloid leukemia cells along the monocytic–macrophage pathway is associated with the activation of the c-*sis*/PDGF-2 protooncogene and the ability of the differentiated cells to synthesize and secrete PDGF-like

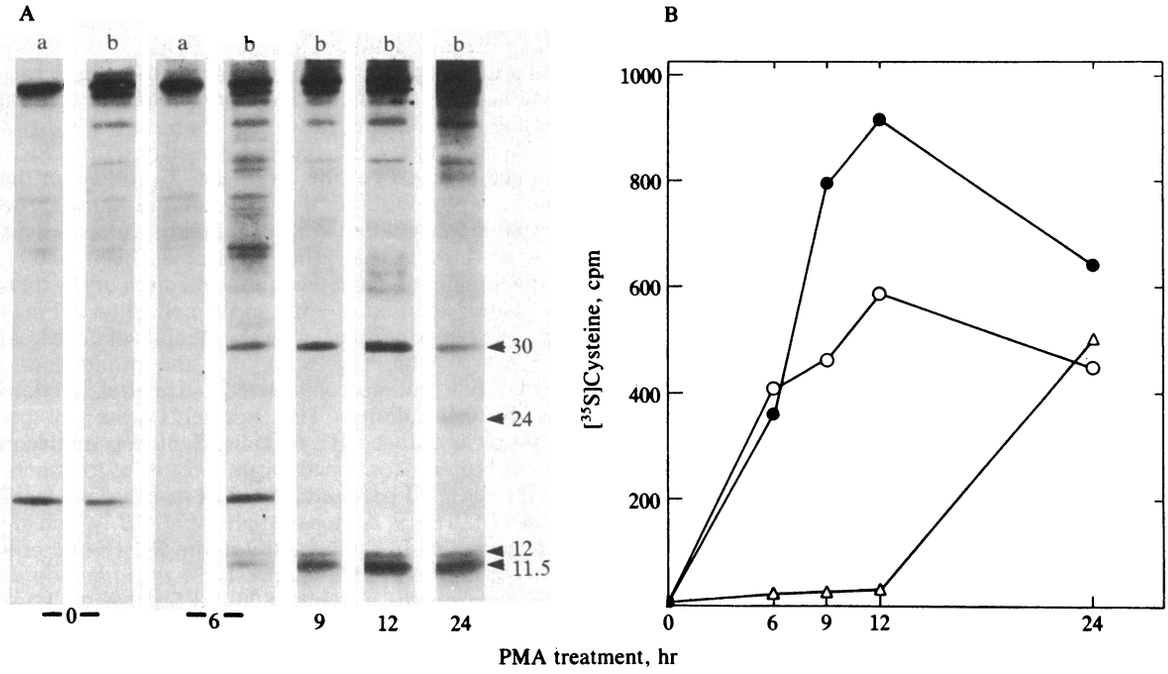

FIG. 4. Secretion of sis/PDGF-2 as a function of period of PMA treatment. Exponentially grown HL-60 cells were placed in serum-free/cystine-free medium (1.5×10^6 cells per ml) supplemented with L-glutamine and antibiotics. Aliquots of the cell suspension were placed in T25 plastic flasks. Two flasks received no treatment, whereas five flasks received PMA. All culture flasks were incubated at 37°C. Radioactive cysteine (300 μCi/ml) was added to each cell culture 3 hr prior to termination of incubation. (*A*) Radioimmunoprecipitation of secreted proteins. Metabolically labeled conditioned medium was collected, clarified, and immunoprecipitated with normal serum (lanes a) or PDGF antiserum (lanes b). Immunoprecipitates were analyzed by $NaDodSO_4$ gel electrophoresis and fluorography. (*B*) Incorporation of radioactivity by the secreted sis/PDGF-2 proteins. Following fluorography, incorporation of [^{35}S]cysteine into bands of interest on the dry gels was measured as described (30). ●, The 11.5- and 12-kDa polypeptide; △, 24-kDa polypeptide; ○, 30-kDa polypeptide.

mitogen. Activation of the *sis* gene in the myeloid leukemia cells apears to be part of the complex process associated with the PMA-induced monocytic differentiation.

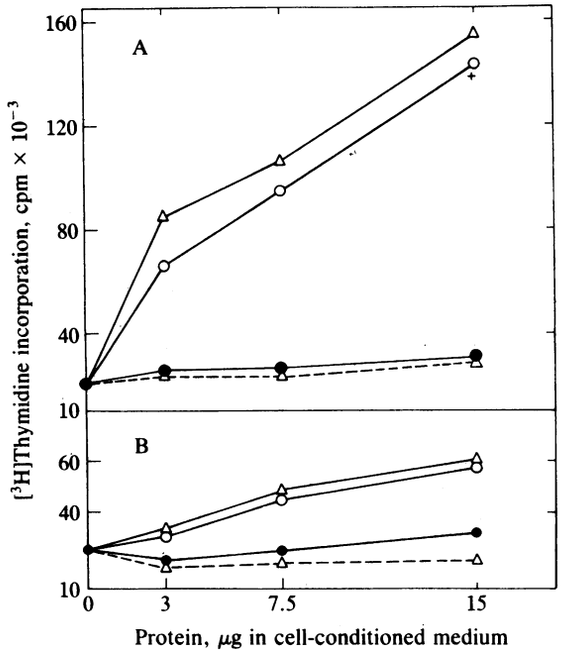

FIG. 5. Mitogenic activity of the sis/PDGF secreted proteins. (*A*) Medium conditioned by HL-60 and PMA-treated HL-60 cells. (*B*) Medium conditioned by U-937 and PMA-treated U-937 cells. △—△, PMA for 24 hr; ○—○, PMA for 12 hr; ●—●, without PMA (24 hr); △- - -△, PMA for 24 hr and anti-PDGF IgG.

In the absence of monocytic differentiation, PMA treatment alone did not result in c-*sis* activation. For example, sis mRNA was not detectable in a PMA-treated human cell line that does not differentiate (unpublished results). Similarly, no c-sis transcripts were detected in dimethyl sulfoxide-induced granulocytic differentiation of HL-60 cells. Consistent with these data is the presence of c-sis transcripts in freshly prepared human monocytes and macrophages but not in freshly prepared human granulocytes (Fig. 1*B*). It appears that during monocytic differentiation the cells are endowed with the activated *sis* gene and have the ability to synthesize and secrete potent PDGF-like mitogen. As discussed recently by Shimokado *et al.* (15) and Martinet *et al.* (14), the ability of monocytes and macrophages to produce PDGF-like mitogen may serve important physiological functions, which have a major role in mediating inflammation and connective tissue remodeling.

The events that lead to the induction of the expression of the c-*sis*/PDGF-2 protooncogene in PMA-differentiating myeloid leukemia cells are at present unknown. Protein synthesis does not seem to be a requirement for the induction of the c-*sis* protooncogene in the PMA-differentiated cells. Inhibition of protein synthesis by Chx resulted in the superinduction of c-sis mRNA in PMA-treated HL-60 cells. The increase in sis mRNA was apparent within 4 hr of Chx treatment, with at least a 10-fold increase observed at 8 hr. Also, Chx treatment did not affect the c-fms mRNA in PMA-treated HL-60 cells during the 8-hr treatment. Mitchell *et al.* (36) reported the superinduction of c-fos mRNA by Chx in PMA-treated U-937 cells. Superinduction of c-sis mRNA in HL-60 cells may reflect stabilization of sis transcripts, resulting from the inhibition of the synthesis of a labile ribonuclease by Chx, as suggested for the superinduction of c-fos mRNA (36). Another alternative is that Chx prevents

the synthesis of a labile repressor protein (37) allowing for the accumulation of high concentrations of sis transcripts.

The observations described here may provide a model for the investigation of the mechanism leading to *sis*/PDGF-2 protooncogene activation during PMA-induced monocytic differentiation of precursor myeloid leukemia cells.

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