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Phorbol Ester Induces c-*sis* Gene Transcription in Stem Cell Line K-562

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The phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) induced megakaryoblastic differentiation and c-*sis* expression in the human hematopoietic stem cell line K-562. This induction occurred at the transcriptional level, as determined by a nuclear runoff transcriptional assay, and was not a generalized effect of TPA, since the treatment of other hematopoietic cell lines and normal peripheral blood lymphocytes with TPA did not result in the appearance of c-*sis* mRNA.

The study of cellular oncogenes (c-*onc*) has become an important tool in exploring the mechanisms of neoplasia (2). Many proto-oncogenes are also expressed in normal cells during growth and differentiation (7, 13, 21). Simian sarcoma virus bears in its structure the viral oncogene *sis* (v-*sis*) which has its homologous cellular counterpart, c-*sis* (7). Expression of this oncogene has been reported in glioblastoma and sarcoma cell lines (10, 22, 28), but it is also expressed in normal endothelial and placental cells (1, 13, 15). c-*sis* codes for the B chain of platelet-derived growth factor (4, 9, 16, 23, 27), a potent mitogen for connective tissue cells which is released from platelet alpha granules during clot formation (17). Recently, the sequence of a cDNA clone (from normal endothelial cells) coding for c-*sis* was reported (6). However, the expression of this oncogene has not been studied in megakaryocytes, the main source of platelet-derived growth factor in the organism, because it is extremely difficult to obtain enough megakaryocytes from normal bone marrow. We report here the expression of c-*sis* in the stem cell line K-562 during phorbol ester (12-*O*-tetradecanoylphorbol-13-acetate [TPA])-induced megakaryocytic differentiation of these cells (12, 24, 25). The c-*sis* transcript can be detected as early as 4 h after adding TPA to cultures, even though megakaryocytic differentiation could only be detected after day 2 of treatment. We demonstrate that c-*sis* expression may occur concomitantly with the megakaryocytic differentiation of K-562 cells rather than as result of nonspecific effects of TPA, since other cell lines and normal peripheral blood lymphocytes treated with TPA do not show c-*sis*-related transcripts. c-*sis* induction occurs at the transcriptional level as determined by a nuclear runoff assay.

The K-562 cell line was grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and penicillin-streptomycin. Log-phase K-562 cells were treated with TPA (Sigma Chemical Co., St. Louis, Mo.) (final concentration, 10^{-9} M) and kept in culture for variable lengths of time ranging from 1 h to 6 days. Megakaryoblastic differentiation was assessed by flow cytometric analysis with the monoclonal antibody 10E5 (5; kindly provided by B. Coller, State University of New York at Stony Brook) which recognizes the GpIIb-IIIa complex, a specific megakaryocytic marker expressed early in the differentiation of this lineage (19, 26). Cells positive for the GpIIb-IIIa complex were detected after 2 days of TPA treatment, and by 4 days, the majority of the cells expressed this antigen. Untreated cells were 10E5 negative (Fig. 1). This agrees with previous reports (12, 24, 25) in which megakaryoblastic differentiation of the K-562 cell line was achieved after 2 to 4 days of TPA treatment. By morphologic criteria megakaryoblastic differentiation could be observed within 3 days of TPA treatment.

Total cellular RNA was isolated by guanidine isothiocyanate extraction and cesium chloride centrifugation by the method of Chirgwin et al. (3). Briefly, 10^8 cells were washed twice in phosphate-buffered saline, lysed in 4 M guanidine isothiocyanate, layered on 5.7 M cesium chloride, and spun at 25,000 rpm in a Beckman SW 27 rotor at 20°C for 24 h. Pellets were suspended in 10 mM Tris hydrochloride (pH 7.6)–1 mM EDTA and ethanol precipitated. The final RNA precipitate was dissolved in water, and total cellular poly(A)$^+$ RNA was selected by affinity chromatography over oligo(dT)-cellulose columns. Poly(A)$^+$ RNA (4 μg) was size fractionated on a 1% agarose-formaldehyde gel and transferred to Gene-Screen Plus nylon paper (New England Nuclear Corp., Boston, Mass.), and the blot was prehybridized in 50% formamide–1% sodium dodecyl sulfate–1 M NaCl–10% dextran sulfate–50 mM Tris-hydrochloride (pH 7.5) at 43°C for 20 h. Hybridization was initiated by adding ^{32}P-labeled probes (v-*sis*, c-*myc*, or beta2-microglobulin labeled by the random priming technique [11]; specific activities, 10^9 cpm/μg) and sheared salmon sperm DNA (100 μg/ml) with incubation at 43°C for 24 h. Blots were washed three times in 0.3 M NaCl–0.03 M sodium citrate–0.17% sodium dodecyl sulfate at room temperature for 5 min and three times for 15 min each in 0.015 M NaCl–0.0015 M sodium citrate–0.1% sodium dodecyl sulfate at 50°C. The filters were exposed to Kodak XAR-5 film for variable periods in the presence of an intensifying screen. The v-*sis* probe is an *Sst*I-*Xba*I fragment representing the complete v-*sis* transforming gene of simian sarcoma virus (Oncor); the c-*myc* probe is a *Cla*I third exon cDNA clone; and beta2-microglobulin is a full-length cDNA clone (both probes kindly provided by K. Kelly, National Institutes of Health). mRNA hybridizing with the v-*sis* probe could be detected as early as 4 h after TPA treatment (Fig. 2, lane b). The highest level of v-*sis*-hybridizing transcripts was reached after 4 days, concomitant with the greatest number of differentiated cells, according to flow cytometric analysis. It was not possible to demonstrate c-*sis* transcripts in the poly(A)$^+$ mRNA isolated from untreated K-562 cells. The results are

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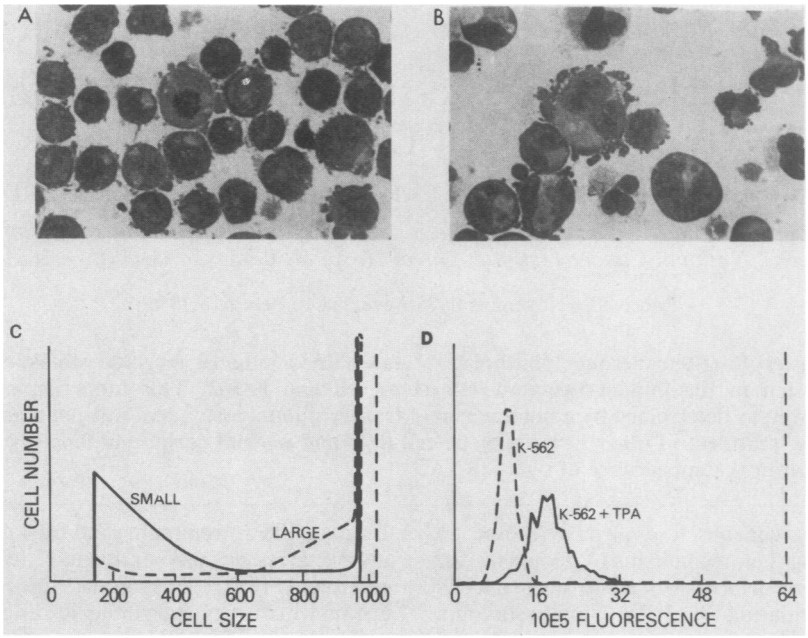

FIG. 1. K-562 differentiation. (A) Control K-562 cells (Wright stain); (B) K-562 cells treated with TPA for 4 days (same staining and magnification as in panel A); (C) light scatter histograms generated by flow cytometric analysis after fractionating TPA-treated K-562 cells by size; (D) control K-562 cells (solid line) and K-562 cells treated with TPA for 4 days (dashed line) were reacted with the monoclonal antibody 10E5 (specific for the GpIIb-IIIa complex of megakaryocytes).

not likely to be due to different amounts of RNA loaded since both untreated and TPA-treated cells possessed relatively equal amounts of beta2-microglobulin mRNA (Fig. 2). In our hands, the size of the v-*sis*-hybridizing mRNA was 3.3 kilobases. An mRNA of similar size was observed in HUT-102 cells and in two glioblastoma cell lines (data not shown).

After TPA treatment, two populations of K-562 cells could be seen: one comprised large cells with a polylobated nucleus and vacuoles similar to those found in megakaryoblasts; the other consisted of small cells difficult to distinguish morphologically from untreated K-562 cells (Fig. 1A and B). To look for differences in the amount of c-*sis* mRNA expressed in these populations, TPA-treated K-562 cells were size fractionated on discontinuous Percoll density gradients (Fig. 1C), and the poly(A)$^+$ mRNA was prepared for Northern blot analysis. The largest cells (morphologically similar to megakaryoblasts) contained approximately the same amount of c-*sis* mRNA as the small-cell population (morphologically undifferentiated) (Fig. 3). In addition to sharing c-*sis* expression, both the small-cell and the large-cell populations expressed equivalent levels of the 10E5 megakaryocytic marker, and both size populations were cell cycle synchronized and growth inhibited (data not shown).

To study whether expression of c-*sis* after TPA treatment was due to stabilization of low-abundance mRNA or to induction of c-*sis* transcription, we performed an in vitro nuclear runoff transcription assay by the method of Greenberg and Ziff (14). Nuclei were isolated from K-562 cells either untreated or treated for 4 days with 10^{-9} M TPA (10^8 cells per condition). DNA probes (500 ng per slot) were blotted onto nitrocellulose with a Schleicher & Schuell Slot Blotter. After overnight prehybridization, the slots were hybridized for 3 days at 40°C with ^{32}P-labeled runoff transcripts generated by in vitro incubation of 20×10^6 isolated nuclei in the presence of [^{32}P]UTP (New England Nuclear Corp.). The filters were then washed, air dried, and exposed to X-ray film as described above. Figure 4 demonstrates the absence of newly synthesized c-*sis* transcripts in untreated K-562 nuclei (lane a) and the synthesis of such transcripts by nuclei isolated from K-562 cells after 4 days of TPA treatment (lane b). Conversely, c-*myc* transcription was not altered by TPA treatment (Fig. 4, lanes a and b), but

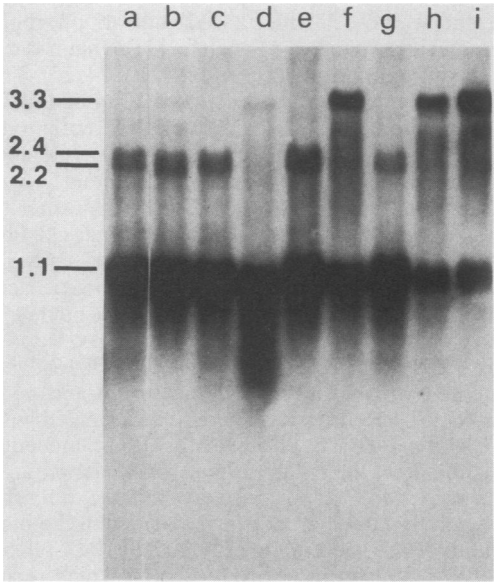

FIG. 2. Time course showing c-*sis* (3.3 kilobases), c-*myc* (2.2 and 2.4 kilobases), and beta2-microglobulin (1.1 kilobase) mRNA. Lanes: a and b, 1 and 4 h, after adding TPA, respectively; c, e, and g, control K-562 cells 1, 2, and 3 days after seeding, respectively; d, f, h, and i, K-562 cells 1, 2, 3, and 4 days after TPA treatment, respectively. Size in kilobases is on the left.

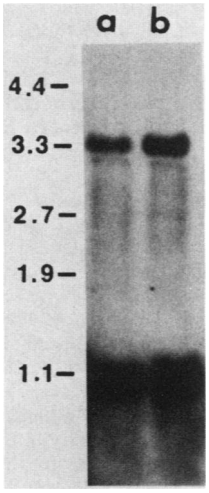

FIG. 3. TPA-treated K-562 cells were size selected on Percoll gradients, and mRNA was extracted and hybridized with the v-*sis* probe. Lanes: a, small cells; b, large cells. Numbers on left are in kilobases.

steady-state c-*myc* mRNA declined to undetectable levels after TPA treatment (Fig. 2, lane d). These data are concordant with the observed inhibition of cell growth (4a, 18) after TPA and the induction of differentiation. Such posttranscriptional regulation of the c-*myc* gene has been reported by others (8).

The final question we addressed was whether c-*sis* activation is specific to megakaryocytic differentiation of K-562 cells or is a nonspecific effect of TPA. To test this, we treated several cell lines (the T-acute lymphoblastic leukemia cell lines CEM, 8402, MOLT-4; the Burkitt's lymphoma cell line Raji; the promyelocytic cell line HL-60; and the myelomonocytic cell line U-937) and normal peripheral blood lymphocytes with TPA for 3 days. Of these, only U-937 cells expressed barely detectable c-*sis* mRNA after TPA treatment (less than 10% the amount seen in K-562 cells) (data not shown). In itself, this finding is not surprising since c-*sis* has been shown to be expressed in activated monocytes (20). These data confirm that the c-*sis* expression seen in TPA-treated K-562 cells is not due to a generalized, nonspecific effect of TPA, but may instead occur in conjunction with the TPA-driven megakaryoblastic differentiation of these cells.

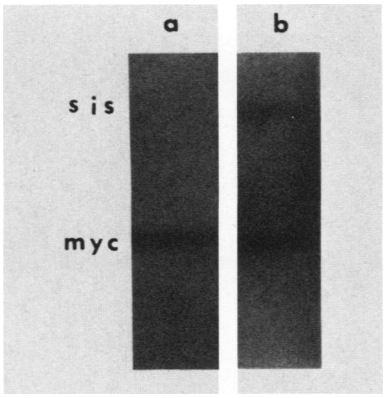

FIG. 4. Nuclear runoff transcription assay. Lanes: a, control K-562 cells; b, K-562 cells treated with TPA for 4 days.

Megakaryocytic differentiation of K-562 cells after TPA treatment has been reported (24). Since megakaryocytes are very difficult to obtain, we took advantage of the ability of TPA to induce megakaryocytic differentiation in K-562 cells to study expression of the c-*sis* gene. The induction of c-*sis* transcription is an early event in the differentiation of K-562 cells, even though the highest amount of c-*sis* mRNA is detectable 4 days after the addition of TPA. If TPA is mimicking endogenous or exogenous factors initiating normal bone marrow stem cell differentiation toward the megakaryocytic lineage (19), induction of c-*sis* transcription may be a very early event in this process. The data reported here are the first demonstrating the induction of c-*sis* in a previously transcriptionally silent cell and describe a suitable system for the study of mechanisms regulating expression of this oncogene.

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