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Effects of 1-β-D-Arabinofuranosylcytosine on Proto-oncogene Expression in Human U-937 Cells

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SUMMARY

We have previously demonstrated that declines in c-myc expression precede the induction of c-fos and c-fms transcripts during monocytic differentiation of human leukemia (HL-60 and U-937) cell lines. The present study has monitored the effects of 1-β-D-arabinofuranosylcytosine (ara-C) on proto-oncogene expression in U-937 cells. The results demonstrate that ara-C inhibits both U-937 proliferation and c-myc expression in a concentration- and time-dependent manner. At non-toxic concentrations of ara-C, these decreases in c-myc RNA occur in the absence of changes in the level of actin transcripts. The results also demonstrate that ara-C increases c-fos but not c-fms expression. Similar findings have been obtained with retinoic acid. Furthermore, although both agents induce a more mature U-937 phenotype, ara-C is a relatively weak inducer of these cells. These findings would suggest that the changes in proto-oncogene expression induced by ara-C may be related to induction of differentiation or the inhibitory effects of this agent on proliferation.

ara-C is one of the most effective agents in the treatment of human acute myelogenous leukemia (1). This agent incorporates specifically into DNA and not RNA of murine and human leukemic cells (2, 3). The incorporated ara-C residue behaves as a relative chain terminator and the extent of ara-C incorporation into DNA correlates with inhibition of DNA synthesis (4, 5). This inhibition of replicative DNA synthesis by ara-C results in DNA fragmentation (6). The extent of (ara-C) DNA formation also bears a significant relationship with loss of leukemic cell clonogenic survival (2, 3, 7). These findings have suggested that incorporation of ara-C into leukemic cell DNA is responsible for inducing cytotoxicity. The effects of this agent at a molecular level, however, remain unclear.

The inhibition of DNA synthesis by sublethal doses of ara-C is associated with differentiation of human HL-60 promyelocytes to monocyte-like cells (8). ara-C also induces terminal differentiation of human K562 erythroleukemia cells (9). Treatment of K562 cells with ara-C results in increased heme synthesis, accumulation of α-, γ-, ε-, and ζ-globin RNA, and enhanced cell surface expression of glycophorin (10). Other studies have demonstrated that the human ML-1 myeloblastic leukemic cell line is induced by ara-C to differentiate along the monocytic lineage (11). Furthermore, ara-C induces differentiation of the U-937 monocyte-like cell line and enhances the differentiating effects of retinoic acid on these cells (12). Taken together, these findings suggest that ara-C is capable of inducing changes in gene expression associated with the appearance of a more differentiated leukemic cell phenotype.

The present work has monitored the effects of ara-C on proto-oncogene expression in U-937 cells. Previous studies using leukemic cells have demonstrated that induction of monocytic or myeloid differentiation is accompanied by decreases in c-myc transcripts (13–16). Furthermore, we have previously shown that differentiation along the monocytic lineage is associated with induction of the c-fos and c-fms proto-oncogenes (15). The present results demonstrate that increasing concentrations of ara-C progressively inhibit U-937 proliferation and c-myc expression. These effects of ara-C on c-myc RNA levels are also dependent on duration of drug exposure. The results also demonstrate that ara-C increases expression of the c-fos proto-oncogene. These findings are similar to those obtained with retinoic acid, an agent known to induce a more mature U-937 cell phenotype.

Materials and Methods

Cell culture. U-937 cells (obtained from Dr. Brice Weinberg, University of North Carolina) were maintained at a density of 2–3 × 10^5 cells/ml in RPMI 1640 medium (GIBCO Laboratories, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Hazelton Research Products, Denver, PA), 2 mM L-glutamine (Gibco) in a 5% CO_2 humidified atmosphere at 37°. The U-937 cells were grown

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ABBREVIATIONS: ara-C, 1-β-D-arabinofuranosylcytosine; NBT, nitroblue tetrazolium; SSC, 0.15 M sodium chloride, 0.015 M sodium citrate; SDS, sodium docecyl sulfate; kb, kilobase.

in suspension culture in the presence of ara-C, retinoic acid or both agents (Sigma Chemical Co., St. Louis, MO). Fresh media and drug were added at 0 hr and again at 24 hr. Retinoic acid was dissolved in 100% ethanol and diluted 1000-fold in medium to a final concentration of 10^{-6} M retinoic acid (0.1% EtOH). Maturation was assessed by NBT reduction as previously described (17). Viability was monitored by trypan blue exclusion.

Northern blot analysis. U-937 total cellular RNA was purified by the guanidine thiocyanate-cesium chloride method (18) and analyzed by electrophoresis of 20 μg of RNA through 1% agarose-formaldehyde gels followed by Northern blot transfer to nitrocellulose filters (19). The filters were prehybridized at 42° for 16–24 hr in buffer consisting of 50% formamide, 5× SSC, 0.1% SDS, 5× Denhardt's solution, and 200 μg/ml of salmon sperm DNA. The RNA blots were then hybridized for 16–24 hr at 42° with 2×10^6 cpm of ^{32}P-labeled probe/ml of hybridization buffer (same as the prehybridization buffer, except with 1x Denhardt's). After hybridization, the blots were washed twice in 2× SSC, 0.1% SDS at room temperature for 60 min and then washed twice in 0.1× SSC, 0.1% SDS at 55° for 30 min. The blots were then dried and exposed to X-ray film with an intensifying screen at −70°. DNA probes were purified by preparative gel electrophoresis and nick-translated to specific radioactivities of approximately 5×10^8 cpm/μg of DNA. The 1.6-kb ClaI/EcoRI fragment of the human c-myc 3′ exon was purified from the pMC41-3 RC plasmid (20). The 1.5-kb p52C-EcoRI fragment of the HL-60 N-ras gene was purified from a reconstructed pBR322 plasmid (21). The 2.0-kb PstI fragment of the chicken β-actin gene was purified from the pA1 plasmid (22). The 2.7-kb XhoI/NcoI fragment of the c-fos gene was obtained from the pc-fos (human)-1 plasmid (23). The 1.0-kb PstI fragment of the v-fms gene was purified from the pSM3 plasmid (24).

Results

U-937 cells were exposed for 24 hr to concentrations of ara-C ranging from 10^{-8} to 10^{-5} M. The effects of ara-C on c-myc and actin gene expression are illustrated in Fig. 1. The c-myc transcripts were detectable in untreated U-937 cells. Although 10^{-8} M ara-C had no detectable effect on the level of c-myc RNA, increasing concentrations of this agent progressively decreased c-myc expression (Fig. 1A). In contrast, ara-C had less of an effect on the level of actin transcripts (Fig. 1B). U-937 cell growth was partially slowed by 10^{-8} M ara-C, whereas 5×10^{-7} M ara-C was cytostatic without decreasing viability (see legend to Fig. 1). In contrast, 10^{-6} and 10^{-5} M ara-C resulted in cell lethality, and this effect may have contributed to declines in levels of both c-myc and actin RNA. Thus, in order to avoid the possible effects associated with loss of cell viability, subsequent experiments were performed with 5×10^{-7} M ara-C.

Fig. 2 illustrates the effects of 5×10^{-7} M ara-C on c-myc expression during 48 hr of drug exposure. In this experiment, there was no detectable effect following 6 hr of drug treatment, whereas a partial decrease in the level of c-myc transcripts was observed at 12 and 24 hr (Fig. 2A). These transcripts were nearly undetectable by 48 hr, whereas there was little effect on actin RNA levels (Fig. 2B). The effects of ara-C on c-myc expression were therefore dependent upon both drug concentration and duration of exposure. Furthermore, ara-C treatment decreased expression of the c-myb proto-oncogene in a manner similar to that observed for c-myc transcripts (data not shown).

Since retinoic acid has previously been shown to induce differentiation of U-937 cells (12), we compared the effects of that agent and ara-C on proliferation and c-myc expression. Retinoic acid (10^{-6} M) partially inhibited U937 cell growth, whereas ara-C (5×10^{-7} M) completely blocked proliferation (Fig. 3). The combination of these agents yielded results similar

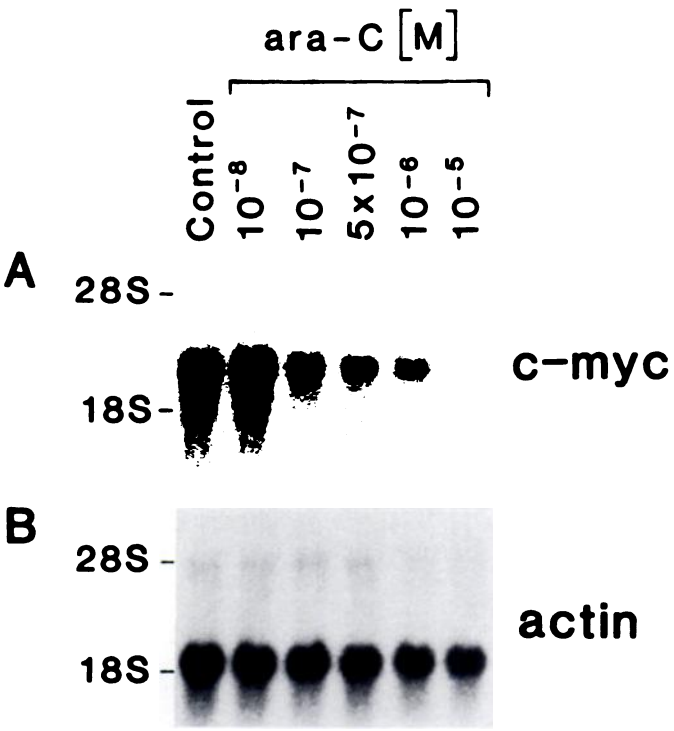

Fig. 1. Effects of ara-C at varying concentrations on c-myc and actin expression in U-937 cells. U-937 cells were grown in logarithmic phase at 5×10^5/ml. ara-C was added at the indicated concentrations for 24 hr. The cells were then harvested for purification of total cellular RNA and analysis by Northern blots using the following ^{32}P-labeled probes: A, the 1.6-kb ClaI/EcoRI fragments of the human c-myc 3′ exon; and B, the 2.0-kb PstI fragment of the chicken β-actin gene. The mean cell counts × 10^5 (percentage viability) at 24 hr were: control, 13.0 (100); 10^{-8} M ara-C, 10.8 (100); 10^{-7} M ara-C, 5.0 (100); 5×10^{-7} M ara-C, 5.0 (100); 10^{-6} M ara-C, 5.1 (93); and 10^{-5} M ara-C, 4.3 (80). The intensity of the hybrids was determined by scanning densitometry. The peaks were weighed in duplicate and the results are expressed as percentage of control for c-myc (actin): 10^{-8} M ara-C, 95.8 (86.0); 10^{-7} M ara-C, 37.3 (81.6); 5×10^{-7} M ara-C, 30.8 (80.0); 10^{-6} M ara-C, 20.6 (65.4); and 10^{-5} M ara-C, 2.5 (57.5).

to that obtained with ara-C alone. When monitoring for effects on c-myc expression, retinoic acid induced a more rapid down-regulation of these transcripts than did ara-C (Fig. 4). c-myc RNA levels decreased by 3 hr of retinoic acid treatment, whereas 12–24 hr of ara-C exposure were required for comparable effects. Furthermore, the more rapid declines in c-myc expression induced by retinoic acid were slowed by combining these agents (Fig. 4). Finally, neither ara-C nor retinoic acid decreased expression of the N-ras gene (data not shown), thus suggesting that these agents selectively inhibit expression of the c-myc gene.

Previous work has demonstrated that both ara-C and retinoic acid induce U-937 cells to express a more mature monocytic phenotype (12). In the present study, approximately 30% of the ara-C-treated cells reduced NBT, whereas retinoic acid induced this cytochemical marker of differentiation in nearly 90% of the cells (Table 1). The combination of these agents was similar to that obtained with retinoic acid alone (Table 1). Although the ability to reduce NBT is indicative of a more differentiated phenotype, increases in c-fos and c-fms expression have also been associated with induction along the monocytic lineage (15). In this regard, treatment of U-937 cells with ara-C and/or retinoic acid increased levels of the 2.2- and 3.5-kb c-fos

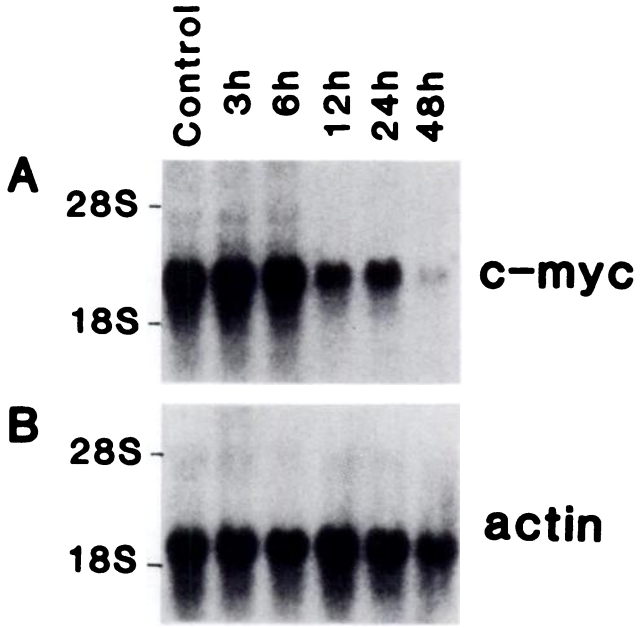

Fig. 2. Effects of duration of ara-C exposure on c-myc and actin expression. U-937 cells in logarithmic phase were exposed to 5×10^{-7} M ara-C for the indicated times. Total cellular RNA was then prepared for Northern blot analysis following hybridization with the c-myc (A) and actin (B) probes. The intensities of the c-myc (actin) hybrids as determined by scanning densitometry and expressed as percentage of control were: *3h*, 111.0 (89.2); *6h*, 105.7 (83.6); *12h*, 45.3 (107.6); *24h*, 32.3 (100.7); *48h*, 8.1 (79.8).

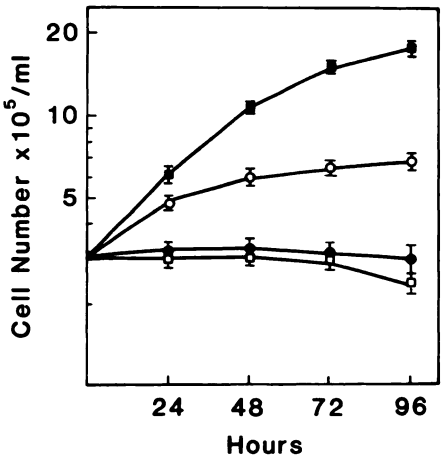

Fig. 3. Effects of 5×10^{-7} M ara-C and 10^{-6} M retinoic acid on U-937 cell proliferation. U937 cells in logarithmic phase at 3×10^5/ml were grown in the absence (■) and presence of 5×10^{-7} M ara-C (●), 10^{-6} M retinoic acid (○), or 5×10^{-7} M ara-C/10^{-6} M retinoic acid (□). Cell number was determined at the indicated times. Results are expressed as the mean ± standard deviation for three determinations.

transcripts. A representative experiment is shown in Fig. 5. In contrast, there was no detectable induction of c-fms RNA following treatment of these cells with either agent alone or in combination (data not shown).

Discussion

Previous studies with human leukemic cell lines (U-937, HL-60, ML-1) have demonstrated that induction of differentiation is associated with declines in c-myc expression (13–16). A sequential relationship has been observed between addition of

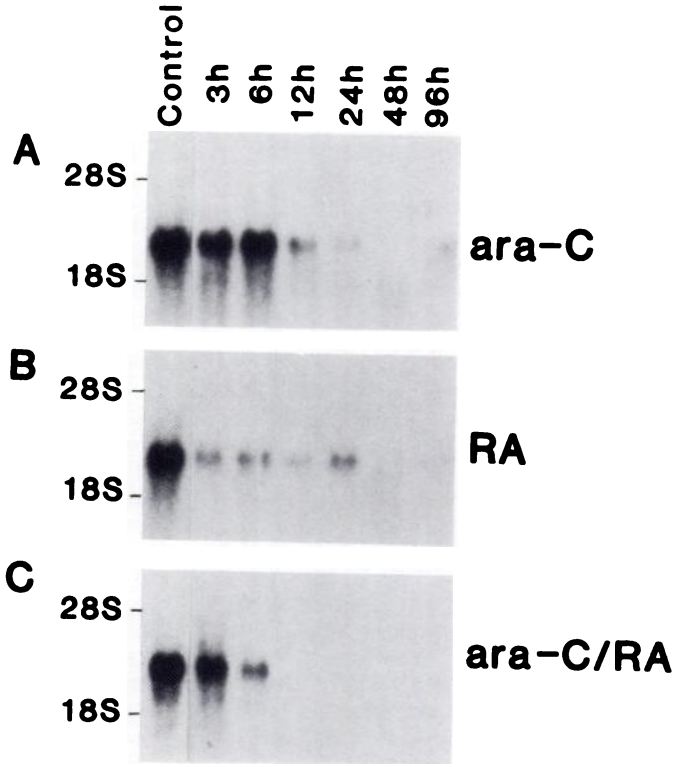

Fig. 4. Effects of ara-C and retinoic acid (*RA*) on c-myc expression. U-937 cells were exposed to 5×10^{-7} M ara-C, 10^{-6} M retinoic acid, or a combination of these agents. Total cellular RNA was harvested at the indicated times and monitored for c-myc expression by Northern blot analysis. The intensities (percentage of control) of the c-myc hybrids with RNA from ara-C, retinoic acid, and ara-C/RA-treated cells were: 92.9, 26.2, and 80.7 at *3h*; 101.2, 24.4, and 26.0 at *6h*; 21.7, 12.7, and 4.3 at *12h*; 8.7, 19.4, and 0 at *24h*; 0, 5.2, and 0 at *48h*; and 0, 4.3, and 0, respectively, at *96h*.

TABLE 1

Effects of ara-C and retinoic acid on percentage of NBT-positive U937 cells

Results are expressed as the mean ± standard error of two experiments (96 hr) each performed in quadruplicate.

	Per cent NBT positive
Control	4.9 ± 1.0
ara-C (5×10^{-7} M)	31.7 ± 6.4
Retinoic acid (10^{-6} M)	88.8 ± 1.2
ara-C/retinoic acid	92.6 ± 0.9

inducer, decreases in c-myc expression, slowing of proliferation, and appearance of the differentiated phenotype (14). We have previously shown, however, that the relationship between c-myc expression and cessation of proliferation varies when using different inducers of both myeloid and monocytic differentiation (16). These findings would suggest that, although c-myc expression decreases with induction of differentiation, this event is not necessarily related to the loss of proliferative capacity.

In the present study, ara-C arrested U-937 cell growth and decreased levels of c-myc RNA. The effects of ara-C on c-myc expression were both concentration and time dependent. The decreases in steady state c-myc RNA levels induced by a nonlethal concentration of ara-C, however, appear to be selective since this agent had little effect on actin gene expression. Previous studies have similarly demonstrated that treatment

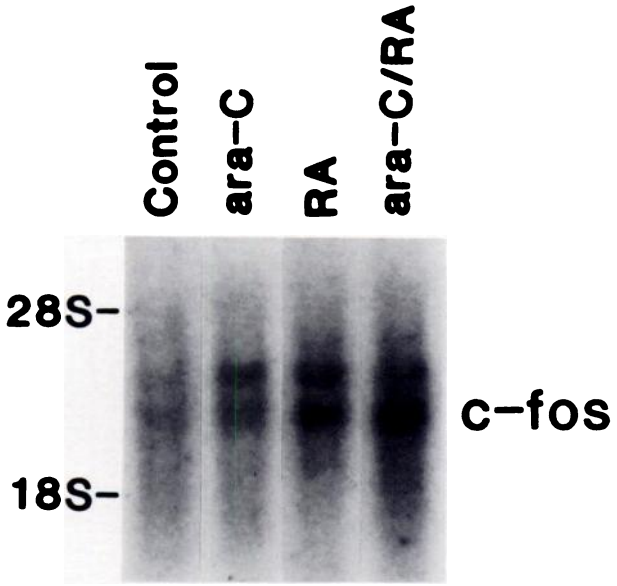

Fig. 5. Effects of ara-C and retinoic acid (*RA*) on c-fos expression. U-937 cells were treated with 5×10^{-7} M ara-C, 10^{-6} M retinoic acid, or a combination of these agents for 96 hr. Total cellular RNA was harvested and hybridized to the ^{32}P-labeled c-fos probe. The relative intensities of the c-fos hybrids were compared to control using densitometric tracings. ara-C, retinoic acid and ara-C/retinoic acid increased the level of c-fos RNA by 1.4-, 1.6- and 2.3-fold, respectively. Similar results were obtained in three separate experiments.

of HL-60 cells with multiple inducers of myeloid or monocytic differentiation has little effect on levels of actin and N-ras RNA (15, 16). Finally, c-myc expression is regulated at a transcriptional level during HL-60 differentiation (25), whereas post-transcriptional mechanisms control c-myc RNA levels during induction of the F9 embryonal carcinoma cell lines (26, 27). Studies monitoring mechanisms of c-myc regulation, however, have not as yet been performed with ara-C or other inhibitors of DNA synthesis.

Other reports have indicated that ara-C induces a more differentiated U-937 phenotype and potentiates the effects of retinoic acid on this cell line (12). Our findings have similarly demonstrated that ara-C increases the percentage of NBT-positive U-937 cells, although this effect was not as pronounced as that achieved with retinoic acid. Since NBT reduction is a cytochemical marker of differentiation, we also studied the expression of other proto-oncogenes which are known to be induced during the differentiation. In this respect, we have previously demonstrated that monocytic differentiation of U-937 or HL-60 cells induced by 12-*O*-tetradecanoylphorbol-13-acetate is associated with decreases in c-myc expression and induction of the c-fos and c-fms proto-oncogenes (15). In the present study, ara-C decreased c-myc expression and increased the level of c-fos transcripts in U-937 cells. Similar findings were obtained with retinoic acid. However, neither agent induced c-fms expression. Thus, ara-C, like retinoic acid, is capable of inducing only certain changes in proto-oncogene expression which are associated with monocytic differentiation.

The decline in c-myc expression may also be related to the effects of ara-C on proliferation. Only 30% of the ara-C-treated U937 cells were NBT positive after 96 hr of exposure. In contrast, c-myc expression was significantly decreased by 12–24 hr of ara-C treatment. One explanation for these findings is that most of the cells were induced to differentiate, whereas only a subpopulation reduced NBT. Another explanation could be that, at nontoxic ara-C concentrations, the decrease in c-myc RNA is related to an arrest of growth and the associated cell cycle changes. Since this agent inhibits replicative DNA synthesis and transition from G_1 to S phase, the cell cycle effects of ara-C might explain the lag time observed before decreases in c-myc expression. In this regard, we have obtained similar findings with other inhibitors of S phase DNA synthesis, such as 5-fluorodeoxyuridine. Although previous studies have indicated that the level of c-myc RNA is constant throughout the cell cycle (28), perhaps agents which arrest cells in G_0/G_1 may thereby inhibit expression of this gene. Finally, certain cells arrested in G_1 phase may then have the capacity to undergo differentiation and express the mature phenotype.

References

1. Frei, E., J. Bickers, J. Hewlett, M. Lane, W. Leary, and R. Talley. Dose schedule and antitumor studies of arabinosylcytosine. *Cancer Res.* **29**:1325–1332 (1969).
2. Kufe, D., P. Major, E. M. Egan, and P. Beardsley. Correlation of cytotoxicity with incorporation of ara-C into DNA. *J. Biol. Chem.* **255**:8997–9000 (1980).
3. Major, P., E. M. Egan, G. Beardsley, M. Minden, and D. Kufe. Lethality of human myeloblasts correlates with the incorporation of ara-C into DNA. *Proc. Natl. Acad. Sci. USA* **78**:3225–3239 (1981).
4. Major, P., E. M. Egan, D. Herrick, and D. Kufe. The effect of ara-C incorporation on DNA synthesis in cells. *Biochem. Pharmacol.* **31**:2937–2940 (1982).
5. Kufe, D., D. Munroe, D. Herrick, E. Egan, and D. Spriggs. Effects of 1-β-D-arabinofuranosylcytosine incorporation on eukaryotic DNA template function. *Mol. Pharmacol.* **26**:128–134 (1984).
6. Fram, R., and D. Kufe. DNA strand breaks caused by inhibitors of DNA synthesis: 1-β-D-arabinofuranoxylcytosine and aphidicolin. *Cancer Res.* **42**:4050–4053 (1982).
7. Kufe, D., D. Spriggs, E. M. Egan, and D. Munroe. Relationships between ara-CTP pools, formation of (ara-C)DNA and cytotoxicity of human leukemic cells. *Blood* **64**:54–58 (1984).
8. Griffin, J., D. Munroe, P. Major, and D. Kufe. Induction of differentiation of human myeloid leukemic cells by inhibitors of DNA synthesis. *Exp. Hematol. (Copenh.)* **10**:744–781 (1982).
9. Luisi-DeLuca, C., T. Mitchell, D. Spriggs, and D. Kufe. Induction of terminal differentiation in human K562 erythroleukemia cells by arabinofuranosylcytosine. *J. Clin. Invest.* **74**:821–827 (1984).
10. Watanabe, T., T. Mitchell, E. Sariban, K. Sabbath, J. Griffin, and D. Kufe. Effects of 1-β-D-arabinofuranosylcytosine and phorbol ester on differentiation of human K562 erythroleukemia cells. *Mol. Pharmacol.* **27**:683–688 (1985).
11. Takeda, K., J. Minowada, and A. Bloch. Kinetics of appearance of differentiation-associated characteristics in ML-1, a line of human myeloblastic leukemia cells, after treatment with 12-*O*-tetradecanoylphorbol-13-acetate, dimethyl sulfoxide, or 1-beta-D-arabinofuranosylcytosine. *Cancer Res.* **42**:5152–5158 (1982).
12. Chomnienne, C., N. Balitrand, and J. Abita. Effect of 1-β-D-arabinofuranosylcytosine on differentiation of U-937 cells. ICTS Med. Sci. **11**:731–732 (1983).
13. Westin, E. H., F. Wong-Staal, E. P. Gelmann, R. Dalla Favera, T. S. Papas, J. A. Lautenberger, A. Eva, A. R. Premkum, E. Reddy, S. R. Tronick, S. A. Aaronson, and R. C. Gallo. Expression of cellular homologues of retroviral onc genes in human hematopoietic cells. *Proc. Natl. Acad. USA* **73**:2490–2494 (1982).
14. Reitsma, P., P. Rothberg, S. Astrin, J. Trial, Z. Bar-Sharit, A. Hall, S. Teitelbau, and A. Kahn. Regulation of myc gene expression in HL-60 leukemia cells by a vitamin D metabolite. *Nature (Lond.)* **306**:492–494 (1983).
15. Sariban, E., T. Mitchell, and D. Kufe. Expression of the c-fms pro-oncogene during human monocytic differentiation. *Nature (Lond.)* **316**:64–66 (1985).
16. Watanabe T., E. Sariban, T. Mitchell, and D. Kufe. Human c-myc and N-ras expression during induction of HL-60 cellular differentiation. *Biochem. Biophys. Res. Commun.* **126**:999–1005 (1985).
17. Yam L., C. Li, and W. Crosby. Cytochemical identification of monocytes and granulocytes. *Am. J. Clin. Pathol.* **55**:283–290 (1971).
18. Chirgwin, J., H. Przybla, R. MacDonald, and W. Rutter. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**:5294–5299 (1979).
19. Thomas, P. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc. Natl. Acad. Sci. USA* **77**:5201–5205 (1980).
20. R. Dalla Favera, E. P. Gelmann, S. Martinotti, G. Franchini, T. Papas, R. C. Gallo, and F. Wong-Staal. Cloning and characterization of different human sequences related to the onc gene (v-myc) of avian myelocytomatosis virus (NC29). *Proc. Natl. Acad. Sci. USA* **73**:6497–6501 (1982).
21. Murray, M., J. M. Cunningham, L. F. Parada, F. Dautry, P. Lebowitz, and

R. A. Weinberg. The HL-60 transforming sequence: a ras oncogene coexisting with altered myc genes in hematopoietic tumors. *Cell* **33**:749–757 (1983).
22. Cleveland, D. W., M. Lopata, R. MacDonald, N. Cowan, W. Rutter, and M. Kirschner. Number and evolutionary conservation of alpha- and beta-tubulin and cytoplasmic beta- and gamma-actin genes using specific cloned cDNA probes. *Cell* **20**:95–105 (1980).
23. Curran, T., W. MacConnell, F. van Straaten, and I. Verma. Structure of the FBJ murine osteosarcoma virus genome: molecular cloning of its associated helper virus and the cellular homolog of the v-fos gene from mouse and human cells. *Mol. Cell. Biol.* **3**:914–921 (1983).
24. Roussel, M., C. Rettenmier, A. Look, and C. Sherr. Cell surface expression of v-fms coded glycoproteins is required for transformation. *Mol. Cell. Biol.* **4**:1999–2009 (1984).
25. Grosso, L., and H. Pitot. Transcriptional regulation of c-myc during chemically induced differentiation of HL-60 cultures. *Cancer Res.* **45**:847–850 (1985).
26. Dony, C., M. Kessel, and P. Gruss. Post-transcriptional control of myc and p53 expression during differentiation of the embryonal carcinoma cell line F9. *Nature* (*Lond.*) **317**:636–639 (1985).
27. Dean, M., R. Levine, and J. Campisi. c-myc regulation during retinoic acid induced differentiation of F9 cells is posttranscriptional and associated with growth arrest. *Mol. Cell. Biol.* **6**:518–524 (1986).
28. Thompson, C., P. Challoner, P. Neiman, and M. Groudine. Levels of c-myc oncogene mRNA are invariant throughout the cell cycle. *Nature* (*Lond.*) **314**:363–366 (1985).

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