# Changes in the Expression of Oncogenes Encoding Nuclear Phosphoproteins but Not c-Ha-Ras Have a Relationship to Monocytic Differentiation of HL 60 Cells

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*Abstract.* HL 60 cells were subcloned into variants with different sensitivities to the differentiation-inducing effect of 1,25 dihydroxyvitamin $D_3$ ($1,25(OH)_2D_3$), as shown by increased oxidative and phagocytic activity and the appearance of cytoplasmic $\alpha$-naphthyl butyrate esterase. Brief treatment (4 h) with $1,25(OH)_2D_3$ or 5-azacytidine (5-Aza CR) of sensitive sublines induced monocytic differentiation in a fraction of treated cells. The phenotypic differentiation induced by $1,25(OH)_2D_3$ was preceded by altered expression of oncogenes c-myc and c-fos, but not of c-Ha-ras or of the constitutively expressed p72 gene. Treatment with 5-Aza CR had similar effects but in addition increased the rate of transcription of the c-Ha-ras oncogene. In partially resistant sublines exposure to $1,25(OH)_2D_3$ or 5-Aza CR resulted in changes in the levels of c-myc and c-fos mRNA, but the expression of c-Ha-ras gene did not correlate with the phenotypic differentiation. When induction of differentiation in responsive cells was delayed by cycloheximide, a temporal correlation could be made between changes in the expression of the c-myc gene and differentiation. These results suggest that a reduction of the elevated levels of c-myc gene transcription in HL 60 cells is required for the initiation of monocytic differentiation, that the induction of c-fos gene expression is an early step in this process, and that the transient increase in the expression of c-Ha-ras gene by 5-Aza CR is not related to differentiation.

THE oncogene c-myc has been implicated in the control of cell replication (Kelly et al., 1983; Campisi et al., 1984; Kaczmarek et al., 1985), and its abnormal expression may account for the aberrant growth and poor differentiation of some tumor cells (Dalla Favera et al., 1982*a*; Taub et al., 1982; Alitalo et al., 1983; Klein, 1983; Little et al., 1983). However, the abnormal expression of other genes may also be necessary to permit the progression of the transformed cells to the expression of the fully malignant phenotype (Diamond et al., 1983; Land et al., 1983; Ruley, 1983).

The reversal of the neoplastic phenotype through differentiation is sometimes possible and occurs spontaneously in some human tumors, most strikingly in teratocarcinomas (Pierce and Wallace, 1971) and neuroblastomas (e.g., Knudson and Meadows, 1980). Human leukemic cells in culture have also provided interesting models of cell differentiation. For instance, HL 60 cells, derived from a patient with promyelocytic leukemia (Collins et al., 1977), can be induced to differentiate towards granulocytes (Collins et al., 1978) or monocytes (Rovera et al., 1979) by a variety of agents, though these chemically induced mature forms do not completely resemble the normal end cells of those pathways of maturation (Newburger et al., 1979; Pessano et al., 1983; Koeffler et al., 1984). HL 60 cells have a markedly amplified c-myc oncogene (Collins and Groudine, 1982; Dalla Favera et al., 1982*b*), which is overexpressed as compared with the oncogene in normal myeloid cells, but its expression is reduced by agents that induce granulocytic or monocytic differentiation of these cells (Westin et al., 1982; Reitsma et al., 1983; Grosso and Pitot, 1984, 1985). It would appear that high or inappropriate expression of the c-myc gene prevents the cell's entry into a differentiation program, but it has been argued that the declining expression of c-myc is a consequence, rather than a cause, of differentiation. Gonda and Metcalf (1984) observed that transcription of c-myc RNA decreases only at late stages of differentiation of murine myeloid leukemic cells, and Filmus and Buick (1985) concluded that changes in c-myc expression in HL 60 cells induced with dimethylsulfoxide can be attributed primarily to cell differentiation.

There are conflicting reports on the kinetics of induction of c-fos gene expression in leukemic cells. An early increase in the expression of c-fos mRNA was noted in phorbol-12-myristate-13-acetate (TPA)[1]-induced (Mitchell et al., 1985),

1. *Abbreviations used in this paper:* 5-Aza CR, 5-azacytidine; dThd, thymidine; NSE, nonspecific esterase; poly $A^+$, polyadenylated; TPA, phorbol-12-myristate-13-acetate; $1,25(OH)_2D_3$, $1\alpha,25$ dihydroxyvitamin $D_3$.

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but not in 1,25 dihydroxyvitamin $D_3$ ($1,25(OH)_2D_3$)-induced monocytic differentiation (Muller et al., 1985). On the other hand, experiments with the granulocyte-colony stimulating factor in the presence of actinomycin D (Gonda and Metcalf, 1984), or with TPA (Muller et al., 1984), led to the suggestion that the expression of the c-fos gene in mononuclear phagocytes is restricted to late stages of differentiation. These reports, therefore, raise questions regarding both the rapidity and the significance of the induction of c-fos gene to monocytic differentiation.

We have recently described a procedure that permits a closer analysis of the events associated with the initiation of the monocytic differentiation pathway (Studzinski et al., 1985*a*). A short exposure to the inducer in the absence of a serum supplement in the nutrient medium transiently decreases the steady state level of c-myc mRNA and is followed by phenotypically recognizable differentiation of a significant proportion of treated cells, at a time c-myc mRNA has returned to the pretreatment levels (Studzinski et al., 1985*a*). We now report a more detailed comparison of the kinetics of changes in the transcription rates of oncogenes c-myc and c-fos produced by treatment with two inducers of monocytic differentiation of HL 60 cells; a comparison of these changes with the expression of c-Ha-ras gene, since the ras family of genes is known to be related to cell proliferation rather than differentiation (e.g., Campisi et al., 1984); and the relationship of these changes to the subsequent appearance of the phenotypically more mature cells.

## Materials and Methods

### Cell Culture

The original stock cultures were HL 60 cells obtained from Dr. G. Rovera. These cells were subcloned in semisolid agar essentially as described by Boyd and Metcalf (1984), and variants with differing responses to induction of differentiation by $1,25(OH)_2D_3$ were selected and propagated in suspension as described (Studzinski et al., 1985*a*). As before, untreated cultures had viability of >90% (trypan blue exclusion) and a doubling time of 24–36 h.

### Assessment of the Differentiation State

The determination of markers of monocytic differentiation has been described in detail (Studzinski et al., 1985*a*). The time course of changes in the phenotype indicative of differentiation was routinely followed by Giemsa staining and by cytochemical determination of $\alpha$-naphthyl butyrate esterase (nonspecific esterase, NSE) activity (Yam et al., 1971). Two observers enumerated the proportion of NSE-positive cells on coded slides. These indices of differentiation correlated well with other markers for monocytes, such as reduction of nitro blue tetrazolium, phagocytosis, and adherence to substratum (Studzinski et al., 1985*a*).

### Determination of DNA Synthesis

The overall rate of DNA synthesis was determined by exposing aliquots of control and drug-treated cultures for 1 h to 5 $\mu$Ci/ml of [$^3$H]thymidine (dThd) (methyl-$^3$H, specific activity 62 Ci/mmol, ICN Biomedicals Inc., Irvine, CA). The samples were centrifuged and washed four times with phosphate-buffered saline, then three times with 1 ml ice-cold 5% trichloracetic acid. The acid-insoluble precipitate was washed three times with 1 ml 95% ethanol and dissolved in 200 $\mu$l 0.1 N NaOH. A 50-$\mu$l aliquot of this solution was added to 5 ml Biofluor (New England Nuclear, Boston, MA), and the radioactivity was determined in a Beckman LS-300 liquid scintillation counter (Beckman Instruments Inc., Palo Alto, CA).

### Hybridization Probes

pMyc 7.4, a gift from Dr. G. Rovera, is a 1.2-kb cDNA fragment of the second and third exons of the c-myc gene inserted at the Pst I site of pBR322 (Watt et al., 1983). Plasmid pc-fos (human) containing an 8.8-kb DNA fragment inserted into the EcoR I site of pBR322 (Van Straaten et al., 1983) was cleaved to obtain the 2.7-kb Xho I-Nco I restriction fragment for use as a probe (Mitchell et al., 1985). Plasmid pc-ras contains a Bam HI 6.2-kb fragment of T24 DNA, a gift from Dr. M. Wigler (Taparowsky et al., 1982). Probe p72 is a cloned DNA fragment from the human tumor cell line A-549 ligated to the pML-TK-Bgl II plasmid by Dr. A. Pater. It contains an expressed portion of human tumor cell genome (Pater and Pater, 1984). The plasmids were grown and purified, and the probes were excised with the appropriate restriction enzymes, electroeluted from preparative 1% agarose gels using a unidirectional electroeluter (I.B.I. Inc., New Haven, CT), and labeled in vitro by nick translation. These procedures are described by Maniatis et al. (1982). Typically, $10^7$–$10^8$ cpm/$\mu$g DNA was prepared for use as a hybridization probe.

### Measurement of Cellular mRNA Content

For determination of RNA transcript levels by dot-blot hybridization total cellular RNA was extracted using the guanidinium isothiocyanate/hot phenol method, its integrity was checked by electrophoresis in a 1% agarose minigel, and polyadenylated (poly A$^+$) RNA was obtained using batch absorption to oligo(dT) cellulose (Maniatis et al., 1982), quantitated by hybridization to [$^3$H]-poly U (Bantle et al., 1976), and blotted onto Biotrans nylon membrane (ICN Biomedicals Inc.) at several dilutions according to the protocol supplied by the manufacturer. In preliminary experiments the amounts of RNA were determined that fall on the linear portion of the dose-density curve, after a standard autoradiographic exposure, and quantitation of blot intensity using a scanning densitometer, on two separate autoradiograms of each transfer.

Northern transfer of poly A$^+$ RNA onto the nylon membrane was performed after the RNA was denatured by being heated at 65°C for 5 min in 10 mM sodium phosphate (pH 6.5), 50% formamide, 2.2 M formaldehyde, 0.5 mM EDTA, and fractionated by electrophoresis in 1.0% agarose gel containing 1.1 M formaldehyde, 10 mM sodium phosphate at pH 6.5 (Meinkoth and Wahl, 1984). The blots were air dried, baked for 3–4 h at 80°C, and hybridized for ~20 h at 45°C to $1 \times 10^6$ cpm of nick-translated probes (specific activity $10^7$–$10^8$ cpm/$\mu$g DNA) per 1 ml of the hybridization buffer (0.05 ml/cm$^2$) and thoroughly washed as described previously (Studzinski et al., 1985*a*). Routinely, c-Ha-ras was hybridized along with either c-myc or c-fos mRNA for a direct comparison of cellular mRNA levels, which obviated possible RNA transfer problems.

Dot-blots performed on total cellular RNA gave results similar to those obtained with poly A$^+$ RNA, but these were not used in the experiments reported here. There was in general good agreement between results obtained with dot-blots and Northern transfers, and although some variation on individual blots was occasionally seen, the trends shown in the summaries of the data were evident in each experiment.

### Nuclear Transcription Assay

RNA chain elongation in isolated nuclei (McKnight and Palmiter, 1979) was performed by the method described by Sheffery et al. (1984) with the following modifications. Nuclei from $5 \times 10^7$ cells were isolated (Hallick and Namba, 1974) and counted in a hemocytometer. $2 \times 10^7$ nuclei were incubated for 20 min at 37°C in a reaction mixture containing 1 mM ATP, 0.25 mM GTP, 0.25 mM CTP, and 100 $\mu$Ci (33 nM) [$\alpha$-$^{32}$P]-UTP (3,000 Ci/mmol sp act). The labeled RNA was extracted, and equal amounts of acid-precipitable RNA radioactivity ($4 \times 10^6$ cpm) were used to hydridize 10 $\mu$g DNA from each probe, excised from plasmids, and blotted on Biotrans nylon membrane. The filters were thoroughly washed as described and exposed to X-AR2 x-ray films (Eastman Kodak Co., Rochester, NY) with an intensifying screen for ~72 h. Comparisons of blot intensity could be made only within each experiment, since the duration of exposure varied for the films shown in Figs. 5, 9, and 10. Background controls were performed by hybridization of the nuclear run off to filters containing immobilized pBR322 DNA and showed no significant hybridization, and $\alpha$-amanitin at 2 $\mu$g/ml added to the transcription reaction abolished the incorporation of the precursors into all mRNAs studied.

### Materials

$1,25(OH)_2D_3$ was a generous gift from Dr. Milan Uskokovic, Hoffmann-LaRoche, Nutley, NJ. 5-Azacytidine (5-Aza CR) and other biochemicals were obtained from Sigma Chemical Co., St. Louis, MO.

$1,25(OH)_2D_3$ and 5-Aza CR were used at concentrations that in preliminary experiments were shown to inhibit the DNA synthesis of HL 60 cells to ~20% of the control level, after a 4-h exposure of the cells to the compound.

## Results

### *Sequential Changes in Oncogene Expression in Subclones of HL 60 Cells Responsive and Resistant to the Induction of Monocytic Differentiation*

We chose $1,25(OH)_2D_3$ as the primary tool for examining the relationship between changes in the expression of c-myc and c-fos genes and monocytic differentiation of HL 60 cells because of its low cytotoxicity and its effectiveness as an inducer of transient phenotypic changes after brief contact with these cells (Studzinski et al., 1985*a*). Our previous studies have also shown that HL 60 cells obtained from different sources respond to the inducers of differentiation in a variable manner. We have therefore subcloned cultures of HL 60 cells in soft agar and selected a number of clones with a range of susceptibility to the differentiation-inducing activity of $1,25(OH)_2D_3$, including several sublines that do not show significant evidence of differentiation within 72 h after a 4-h pulse with $1,25(OH)_2D_3$ (e.g., clones HL 60-R3 and HL 60-R4), and some with intermediate sensitivity to $1,25(OH)_2D_3$ (e.g., clones HL 60-R3Z1 and HL 60-AB3), as judged by NSE activity, nitro blue tetrazolium reduction, phagocytosis, and the adherence to the substratum. Sublines that differentiated in response to this induction regimen (e.g., clones HL 60-R2AB2 and HL 60-R5AB2) showed an almost immediate decrease in c-myc RNA concentration (Fig. 1), followed rapidly by an activation of the expression of c-fos gene (Fig. 2). There were no detectable changes in the levels of c-Ha-ras mRNA (Fig. 3), or gene p72 mRNA and the overall rate of RNA synthesis (data not shown). In contrast, in sublines of HL 60 cells (e.g., clone HL 60-R4) that failed to differentiate in response to $1,25(OH)_2D_3$, the level of the c-myc mRNA remained essentially unchanged, and c-fos mRNA increased less than in cells that differentiated (Fig. 4 and Table I). More detailed results for three other sublines tested are summarized in Table II. It may be noted that DNA synthesis was inhibited in resistant sublines exposed to $1,25(OH)_2D_3$, which, together with transcription of c-fos gene, indicated that a failure of the compound to enter the cells was not the reason for the reduced ability of these cells to differentiate.

Brief exposure of several of the HL 60 sublines to $5 \times 10^{-6}$ M 5-Aza CR, a concentration that produced a transient inhibition of DNA synthesis, showed that this nucleoside analogue also induces monocytic differentiation. Sublines of HL 60 cells partially or fully resistant to $1,25(OH)_2D_3$ were also partially resistant to the induction of differentiation by 5-Aza CR (Fig. 4 and Table I). Again, there was a good correlation between the degree of inhibition of c-myc expression by 5-Aza CR and the extent of subsequent differentiation, but such a relationship between the increased transcription of the c-Ha-ras gene and phenotypic differentiation was not evident. Expression of c-fos gene was increased less by 5-Aza CR in sublines with reduced proportion of differentiated cells, but the correlation with the expression of phenotypic markers of differentiation was less clear than for c-myc, suggesting that

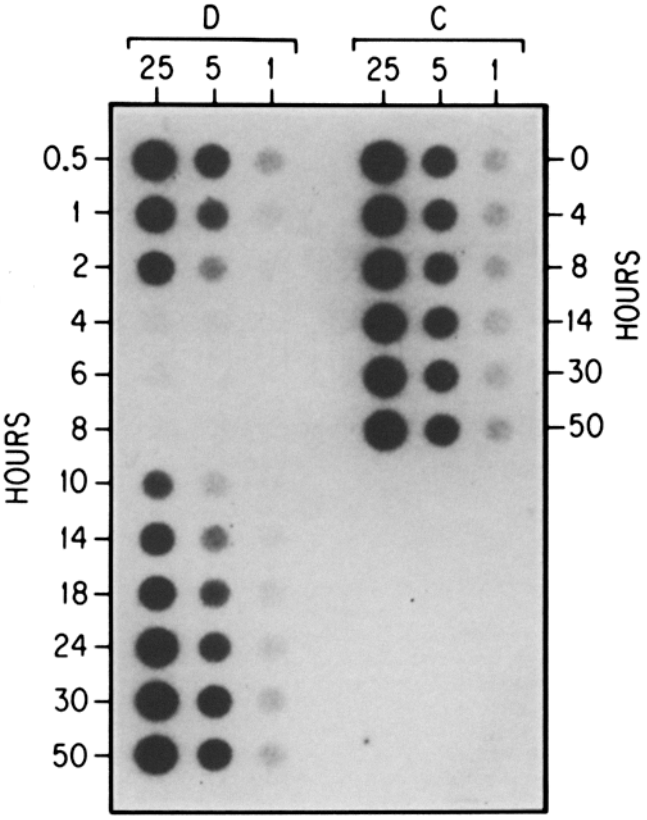


*Figure 1.* Dot-blot analysis of the levels of c-myc mRNA during and after a 4-h exposure of HL 60-R2AB2 cells to $4 \times 10^{-7}$ M $1,25(OH)_2D_3$ (rows marked *D*) and in untreated cultures (*C*). Three concentrations of poly $A^+$ RNA (25, 5, and 1 μg per well) were dot-blotted in rows marked by the duration (in hours) of the experiment from the initiation of the exposure to $1,25(OH)_2D_3$. $T_0$, time of addition of $1,25(OH)_2D_3$.

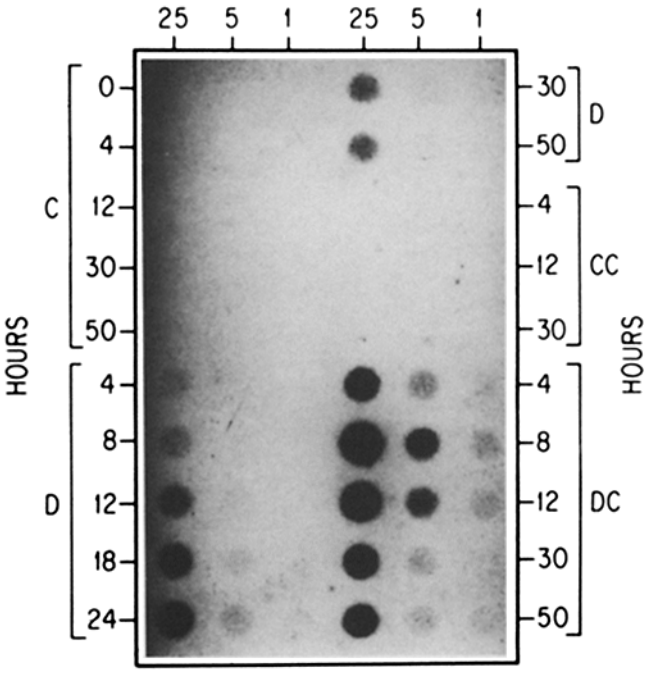


*Figure 2.* A dot-blot analysis of c-fos mRNA annotated as Fig. 1. In addition to untreated (*C*) and $4 \times 10^{-7}$ M $1,25(OH)_2D_3$-treated (*D*) groups, poly $A^+$ RNA was extracted from HL 60-R2AB2 cultures treated for 4 h with $7.1 \times 10^{-5}$ M (20 μg/ml) cycloheximide (*CC*) or $4 \times 10^{-7}$ M $1,25(OH)_2D_3$ together with $7.1 \times 10^{-5}$ M cycloheximide (*DC*). The blots D-4 h and D-8 h were more evident on the original autoradiogram.

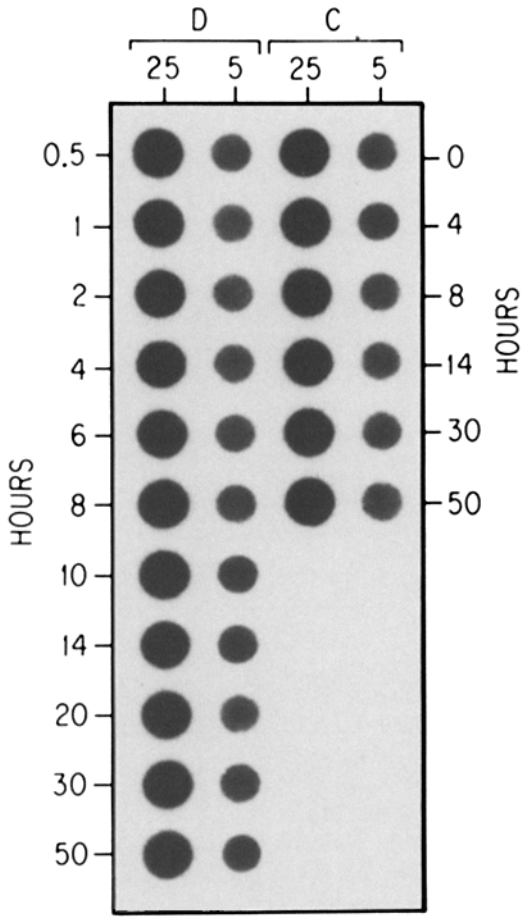


*Figure 3.* A similar dot-blot analysis of c-Ha-ras mRNA extracted from the $1,25(OH)_2D_3$-resistant subline HL 60-R3. The RNA blotted exactly as in the experiment shown in Fig. 1.

the activation of c-fos gene is a necessary but an insufficient step for differentiation (Fig. 4).

Results shown in Table II also indicate that in responsive cells the inhibition of c-myc gene expression by $1,25(OH)_2D_3$ can be detected before the evidence of activation of the c-fos gene transcription, and, more strikingly, that both the inhibition of the c-myc gene and the NSE index of differentiation are transient, whereas the expression of the c-fos gene continues long after the inducer is removed. The increased levels of c-fos mRNA precede the appearance of NSE activity and peak a little earlier but persist longer. In one responsive subline (HL 60-R3Z1 cells, Table II) tested at 90 h, when no morphological or cytochemical evidence of differentiation remained after a 4-h exposure to $1,25(OH)_2D_3$, the level of c-fos mRNA was still greatly elevated as compared with the level in uninduced HL 60 cells.

Since cellular levels of mRNA are subject to changes due to altered degradative as well as transcriptional activity (Dani et al., 1984, 1985), we have confirmed that changes in oncogene mRNA levels are due to altered rates of transcription, using nuclei isolated from HL 60-R2AB2 cells treated with $1,25(OH)_2D_3$ (Fig. 5). The data show an early inhibition of transcription of the c-myc gene (~ 1 h) that is reversed shortly after the inducer is removed, an activation of the c-fos gene (at 2 h) that persists for the duration of this experiment, but no appreciable change in the transcriptional rates of genes c-Ha-ras and p72, a gene constitutively expressed in HL 60 cells (Pater and Pater, 1984).

### *Monocytic Differentiation Induced by $1,25(OH)_2D_3$ Is Delayed by Cycloheximide in Parallel with Its Effects on c-myc mRNA Levels*

The link between changes in oncogene expression and phenotypic changes in HL 60 cells was further examined in

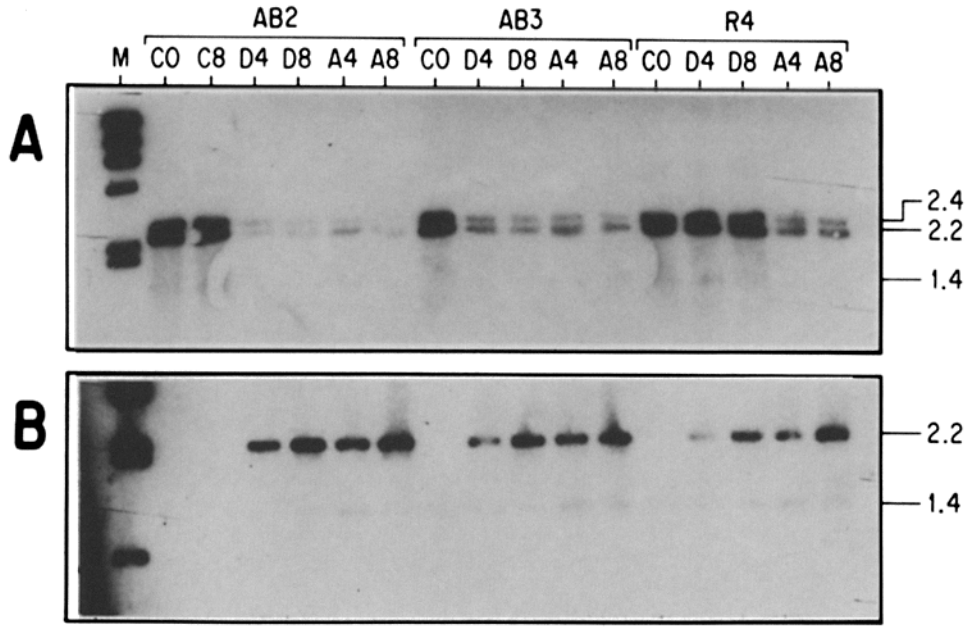


*Figure 4.* Northern blot analysis of oncogene mRNA levels after a 4-h exposure to either $4 \times 10^{-7}$ M $1,25(OH)_2D_3$ or $5 \times 10^{-6}$ M 5-Aza CR in three sublines of HL 60 cells with different sensitivities to the differentiation-inducing effects of $1,25(OH)_2D_3$. (*A*) 10 μg RNA was applied to each lane. The Northern blot was hybridized simultaneously to c-myc and c-Ha-ras probes. Note separation of the two c-myc transcripts (2.4 and 2.2 kb). The c-Ha-ras probe had low [$^{32}$P]-specific activity in this hybridization, but the c-Ha-ras transcript (1.4 kb) was more clearly visualized after a longer autoradiographic exposure (not shown) and served as an internal control for gel loading and the RNA transfer. (*B*) Northern blot hybridized to c-fos (2.2 kb) and c-Ha-ras (1.4 kb) probes. The lanes for both *A* and *B* are indicated as follows: *M*, size markers (Hind III digest of lambda phage); *C 0*, poly A$^+$ RNA from untreated cells; *D*, poly A$^+$ RNA from $1,25(OH)_2D_3$-treated cells; *A*, poly A$^+$ RNA from 5-Aza CR-treated cells. Designations 4 and 8 refer to the number of hours from the initiation of the experiment.

*Table I. Relationships between c-myc and c-fos mRNA Levels, as Determined by Dot Blots in Parallel with the Northern Gel Shown in Fig. 4, and the Subsequent Appearance of NSE Activity*

| Inducer | Time | HL 60-R2 AB2 cells | | | HL 60-R2 AB3 cells | | | HL 60-R4 cells | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | c-myc* | c-fos‡ | NSE§ | c-myc | c-fos | NSE | c-myc | c-fos | NSE |
| | *h* | | | | | | | | | |
| $1,25(OH)_2D_3$ ($1 \times 10^{-7}$ M) | 4 | 6.3 | 9.4 | | 22 | 3.7 | | 89 | 2.9 | |
| | 8 | 6.1 | 17.8 | | 13.1 | 10.8 | | 88 | 6.5 | |
| | 30 | | | 39 | | | 10 | | | 1 |
| 5-Aza CR ($5 \times 10^{-6}$ M) | 4 | 5.0 | 4.7 | | 23 | 4.3 | | 7.6 | 3.2 | |
| | 8 | 2.4 | 9.7 | | 5.1 | 5.6 | | 6.8 | 5.3 | |
| | 30 | | | 45 | | | 12 | | | 10 |

* Percent of control at 0 h. The mean values of two experiments are presented. $T_0$, addition of $1,25(OH)_2D_3$ or 5-Aza CR.

‡ Densitometer readings in arbitrary units.

§ Percentage of cells positive for NSE activity.

*Table II. Levels of c-myc, c-fos, and [$^3$H]-dThd Incorporation in Sublines of HL 60 Cells Exposed to $4 \times 10^{-7}$ M $1,25(OH)_2D_3$ for 4 h and Allowed to Differentiate in Normal Medium*

| | Time (h) | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 4 | 6 | 8 | 10 | 12 | 14 | 18 | 24 | 30 | 50 | 90 |
| HL 60-R2AB2 | | | | | | | | | | | | | | |
| c-myc* | 100 | 66 | 44 | 2.5 | 2.0 | 1.2 | 23 | 49 | | 75 | 116 | 114 | 106 | |
| c-fos‡ | ND | | ND | | 20 | | 44 | | 46 | 45 | 44 | 35 | 34 | |
| dThd incorporation* | 100 | | 34 | | | | | | | | 80 | | | |
| NSE§ | 5 | | | 5 | | 6 | 21 | 24 | 25 | 28 | 38 | 51 | 26 | |
| HL 60-R3Z1 | | | | | | | | | | | | | | |
| c-myc | 100 | | | 3.1 | | 3.1 | | 9 | | 31 | | 65 | 87 | 106 |
| c-fos | ND | | ND | 10 | | 16 | | 25 | | 38 | 41 | 40 | 28 | 20 |
| dThd incorporation | 100 | | | 12 | | | | | | | 81 | | 93 | |
| NSE | 1 | | | 0 | | 1 | | 10 | | 15 | 21 | 29 | 19 | 5 |
| HL 60-R3 | | | | | | | | | | | | | | |
| c-myc | 100 | | | 109 | | 92 | | | | 103 | | | | 98 |
| c-fos | 3 | | | 2.5 | | 6 | | | | 2 | | | | 2 |
| dThd incorporation | 100 | | 62 | 62 | 60 | 75 | | | | 78 | 77 | | 93 | 96 |
| NSE | 0 | | | 0 | | | 0 | | | 0 | 1 | 0 | 1 | 0 |

Mean values of two separate experiments are shown. Oncogene mRNA levels were determined by serial dot-blots. $T_0$, addition of $1,25(OH)_2D_3$ or 5-Aza CR. ND, not detected.

* Percent of control at 0 time (addition of $1,25(OH)_2D_3$).

‡ Densitometer deflection in arbitrary units. The background control (pBR322) had no detectable absorption.

§ Percentage of the cells positive for NSE activity.

experiments in which the effects of the inducer were modulated by a simultaneous addition of cycloheximide at a concentration that inhibits protein synthesis to ~5% of the control rate after 4 h of exposure (Fig. 6). Comparison of control samples in Fig. 6, lanes *B–D* with samples from cycloheximide-treated cells in lanes *H–L*, and samples in lanes *E–G* ($1,25(OH)_2D_3$) with lanes *M–Q* ($1,25(OH)_2D_3$ and cycloheximide), shows that the addition of cycloheximide to the cultures increased the levels of both c-myc and c-Ha-ras mRNA. The increases in these mRNAs were most marked after 4 h of exposure to cycloheximide, when the drug was washed away, and control values were reached at ~12–18 h, when the rate of protein synthesis was ~60% of the control. Inhibition of protein synthesis is known to stabilize c-myc mRNA (Kelly et al., 1983; Dani et al., 1984). A rather similar increase in c-fos mRNA is illustrated in Figs. 2 and 7. In Fig. 7, the levels of c-fos mRNA that were not detectable in untreated cells (lanes *B–D*) became recognizable after 4 h of cycloheximide treatment (lane *H*), and the blots were more intense after the addition of cycloheximide in both Figs. 2 and 7.

In addition to such stabilization of oncogene mRNA by cycloheximide, these experiments demonstrate that cycloheximide delays the recovery from the $1,25(OH)_2D_3$-induced decrease in c-myc mRNA levels (Fig. 6, lanes *M–Q*), as previously shown for TPA-plus-cycloheximide-treated HL 60 cells (Mitchell et al., 1985). Additional experiments were carried out in which oncogene mRNA was quantitated by other Northern and multiple dot-blots, and the phenotypic evidence of differentiation was monitored in parallel. These data are summarized in Fig. 8, which shows the temporal relationship between the reduced cellular levels of the c-myc transcripts and the subsequent phenotypic differentiation, and the delay in both parameters due to the cycloheximide treatment.

The cycloheximide-induced delay in the inhibition of c-myc gene transcription was confirmed by the determination of nuclear transcription rates on oncogene mRNA in HL 60-R2AB2 cells treated with cycloheximide alone or with cyclo-

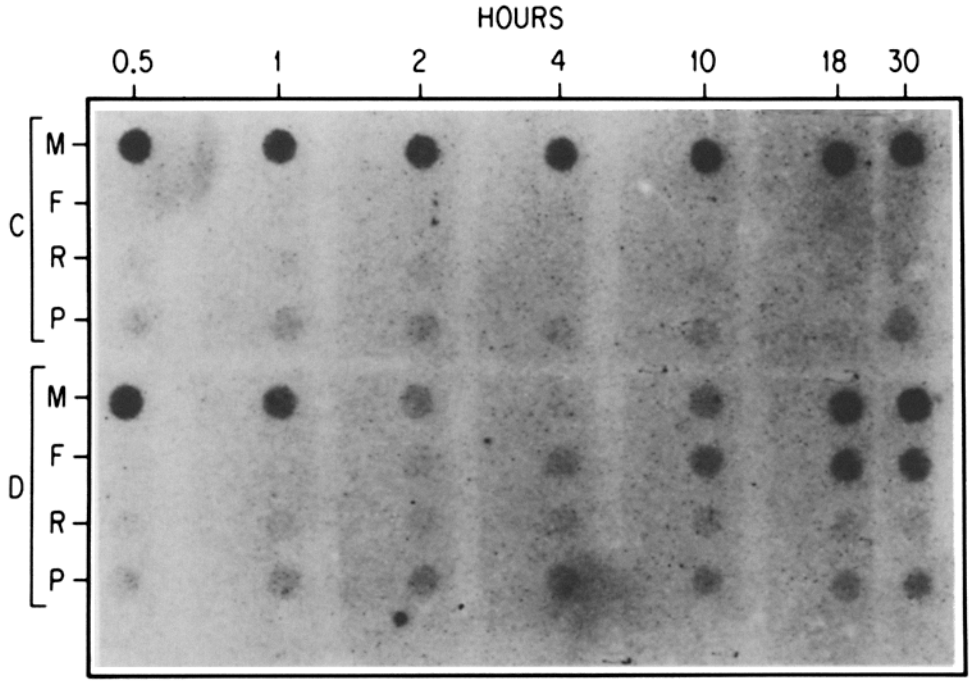


*Figure 5.* Dot-blots of RNA extracted from an isolated nuclei transcription system (HL 60 R2AB2 cells) and hybridized to probes immobilized on Biotrans nylon filters for c-myc (*M*), c-fos (*F*), c-Ha-ras (*R*), and p72 (*P*). The time at which nuclei were isolated after the addition of the inducer ($4 \times 10^{-7}$ M $1,25(OH)_2D_3$) is indicated in hours for untreated (*C*) and induced (*D*) cultures. The transcription of c-Ha-ras and p72 genes is essentially unchanged, and these blots provide controls for the upper panels.

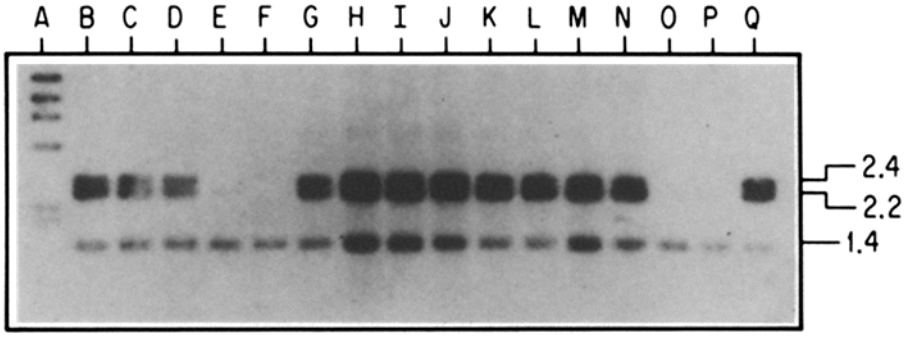


*Figure 6.* Northern blot analysis of the time sequence of the effects of a 4-h exposure of HL 60-R5AB2 cells to $4 \times 10^{-7}$ M $1,25(OH)_2D_3$ and/or $7.1 \times 10^{-5}$ M cycloheximide on cellular poly $A^+$ RNA transcripts that hybridize to the c-myc probe (transcript size 2.4 and 2.2 kb) and to a c-Ha-ras probe (1.4 kb transcript). 10 μg poly $A^+$ RNA was applied to each lane. Lane *A*, Hind III digest of $^{32}P$-labeled lambda phage, size markers. Lanes *B–D*, RNA from untreated cultures, extracted at 4 h (lane *B*), 8 h (lane *C*), and 12 h (lane *D*) from the initiation of the experiment. Lanes *E–G*, cultures treated with $1,25(OH)_2D_3$ for 4 h, mRNA extracted at 4 h (lane *E*), 8 h (lane *F*), and 12 h (lane *G*). Lanes *H–L*, cultures exposed to cycloheximide for 4 h, mRNA extracted at 4 h (lane *H*), 8 h (lane *I*), 12 h (lane *J*), 18 h (lane *K*), and 24 h (lane *L*). Lanes *M–Q*, cultures treated with $1,25(OH)_2D_3$ in the presence of cycloheximide for 4 h. RNA extracted at 4 h (lane *M*), 8 h (lane *N*), 12 h (lane *O*), 18 h (lane *P*), and 24 h (lane *Q*).

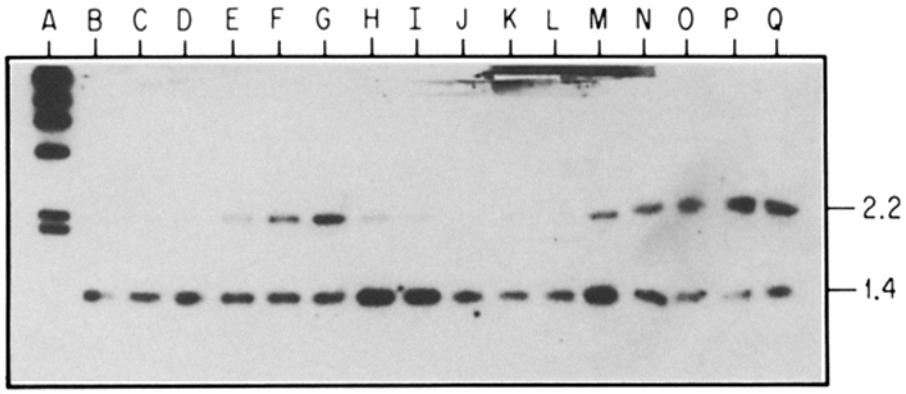


*Figure 7.* Northern blot analysis of mRNA transcripts from c-fos (2.2 kb) and c-Ha-ras (1.4 kb) genes, performed in parallel with the experiment shown in Fig. 6. The RNA samples were electrophoresed and annotated on the autoradiogram exactly as shown for Fig. 6.

heximide plus $1,25(OH)_2D_3$ (Fig. 9). Cycloheximide alone had no detectable effect on the transcriptional rates, whereas the transient inhibition of transcription of c-myc gene by $1,25(OH)_2D_3$ shown in Fig. 5 (2–10 h) was delayed when cycloheximide was added to $1,25(OH)_2D_3$ during the 4-h induction period. In contrast, nuclear transcription of c-fos gene was not delayed by the exposure to cycloheximide and $1,25(OH)_2D_3$ (compare Figs. 5 and 9). These data, taken together, are consistent with the hypothesis that the inhibition of the transcription of c-myc gene is the signal that initiates a

pathway that leads to the phenotypic differentiation of HL 60 cells, but not with the view that increased transcription of c-fos plays such a role.

### *Cycloheximide Does Not Modulate the Induction of Monocytic Differentiation or the Inhibition of c-myc Gene Transcription by 5-Aza CR*

It is possible to argue that the delay in phenotypic differentiation produced by the addition of cycloheximide to the induction system was due to a slower progression of the cells along the differentiation pathway whereas protein synthesis was impaired by the residual effects of cycloheximide exposure. That this was not the case was shown by experiments carried out as described in the preceding section but in which 5-Aza CR was substituted for $1,25(OH)_2D_3$ (Figs. 10–12). Nuclear transcription assays indicated that changes in the transcription rates of oncogenes c-myc, c-fos, and c-Ha-ras produced by 5-Aza CR were not altered by a simultaneous exposure of differentiation-sensitive cells to cycloheximide (Fig. 10). Although cycloheximide increased mRNA stability, as shown on the Northern blot (Fig. 11) and summarized in Fig. 12, when cellular protein synthesis resumed (at ~12 h) the levels of c-myc and c-fos mRNA in cycloheximide-exposed cultures became identical to the levels found in cells induced with 5-Aza CR alone. Significantly, 5-Aza CR-induced phenotypic differentiation was not delayed by the addition of cycloheximide, suggesting that a fixed interval of time separates inhibition of c-myc gene transcription and phenotypic differentiation.

An increase in the expression of c-Ha-ras gene was also noted after exposure to 5-Aza CR (Fig. 10, lanes *4* and *8*, and Fig. 11, lanes *F–H*). Since this was not observed when HL 60 cells were induced to differentiate with $1,25(OH)_2D_3$ or several other inducers of HL 60 cell differentiation (data in preparation), it does not appear to be a differentiation-related event.

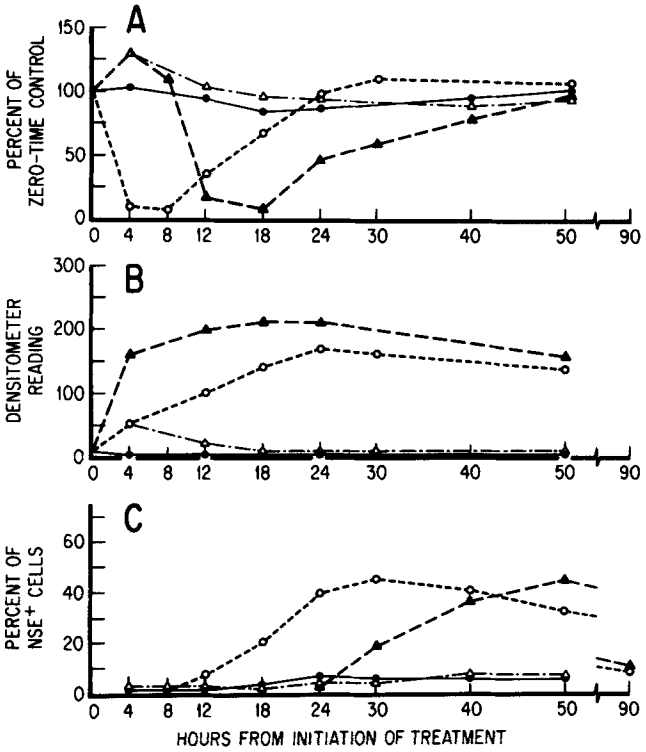


*Figure 8.* Modification by cycloheximide (CHX) of the time relationships between the $1,25(OH)_2D_3$-induced events: (*A*) the reduction in cellular c-myc mRNA levels; (*B*) the increased levels of c-fos mRNA; (*C*) the phenotypic differentiation assessed by NSE activity. The data were obtained in three separate experiments, analyzed by dot-blots at each time point (mean values were used to construct the graphs), and confirmed at selected time points by Northern blot transfers, examples of which are shown in Figs. 6 and 7. ●, untreated cells. ○, $1,25(OH)_2D_3$, 0.4 μm. △, CHX, 20 μg/ml. ▲, $1,25(OH)_2D_3$ + CHX.

## *Discussion*

The experiments were designed to determine to what extent changes in c-myc and c-fos gene expression fit the role of the signal that initiates monocytic differentiation of HL 60 cells. Our experimental system permits examination of cellular changes in the absence of the chemical inducer. Brief exposure of sensitive cells to the inducing agent inhibits c-myc transcription within 1 h (Fig. 1), followed shortly by induction of c-fos expression and a delayed appearance of markers of phenotypic differentiation (Figs. 8 and 12). The recovery of a high rate of c-myc transcription after removal of $1,25(OH)_2D_3$ has been noted (Reitsma et al., 1983; Studzinski et al., 1985*a*), but we show here more precise kinetics of this recovery, and a constant relationship between these events (the inhibition and the recovery of transcription of the c-myc gene) and the expression and disappearance of the phenotypic differentiation markers. The appearance of maturation characteristics

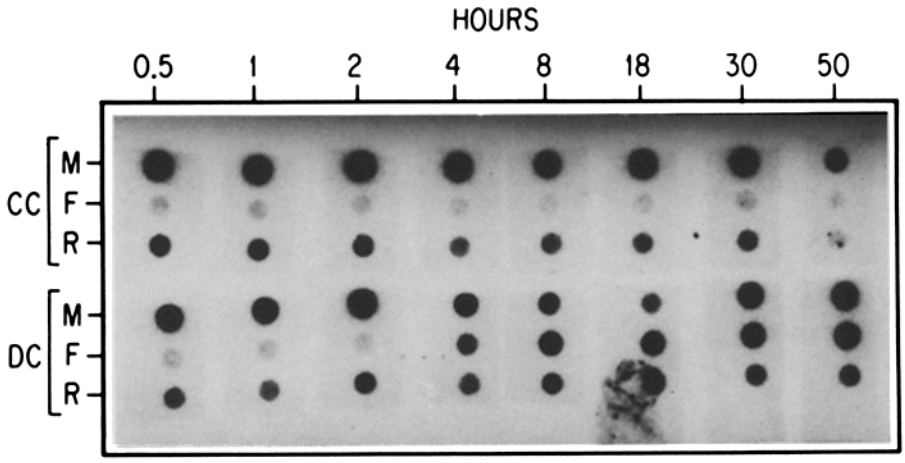


*Figure 9.* Dot-blot analysis of $^{32}P$-labeled RNA transcribed in nuclei isolated from HL60-R2AB2 cells exposed to $7.1 \times 10^{-4}$ M cycloheximide (*CC*) or $4 \times 10^{-7}$ M $1,25(OH)_2D_3$ (*DC*) for 4 h. 10 μg myc (*M*), fos (*F*) and c-Ha-ras (*R*) DNA was blotted on the membrane and hybridized to $4 \times 10^6$ cpm of acid-precipitable $^{32}P$-labeled RNA as described in Materials and Methods.

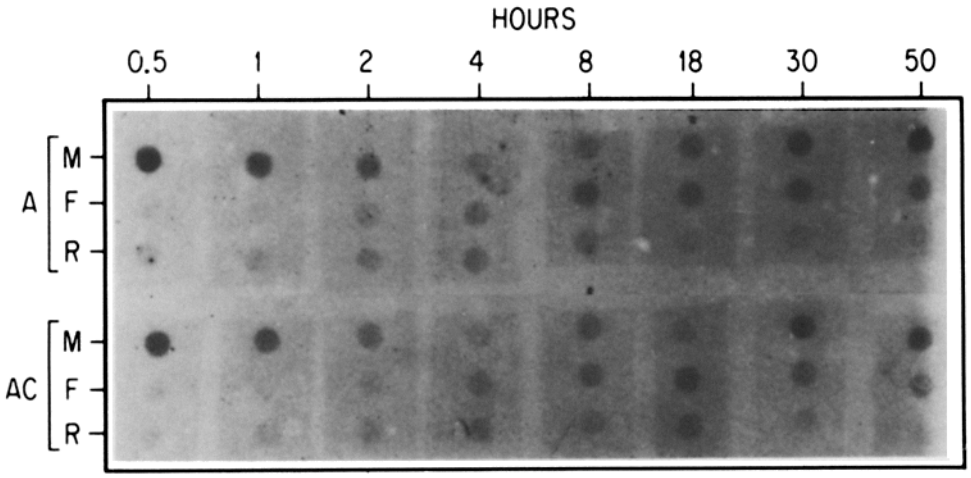


*Figure 10.* Dot-blot analysis of transcripts produced by nuclei isolated from HL 60-R2AB2 cells treated for 4 h with $5 \times 10^{-6}$ M 5-Aza CR in the absence (*A*), or presence (*AC*), of $7.1 \times 10^{-5}$ M cycloheximide, performed as described in the legend to Fig. 9.

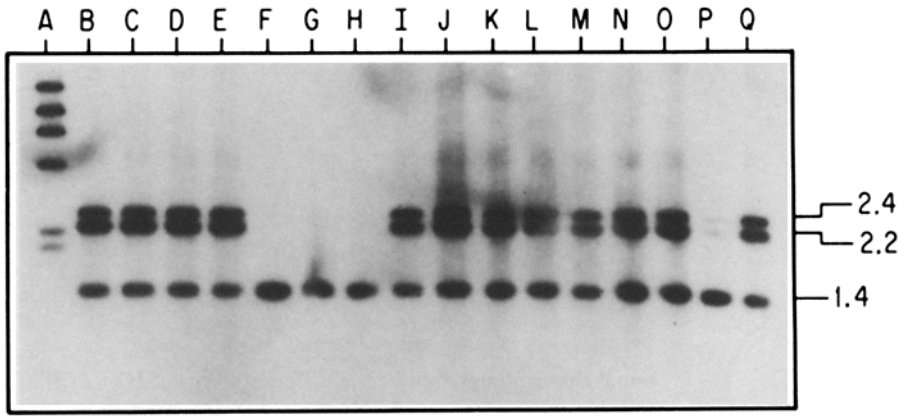


*Figure 11.* Northern blot analysis of the time sequence of changes in the levels of c-myc (2.4 and 2.2 kb) and c-Ha-ras (1.4 kb) transcripts in HL 60-R5AB2 cells treated for 4 h with $5 \times 10^{-6}$ M 5-Aza CR and/or $7.1 \times 10^{-5}$ cycloheximide (CHX). 10 $\mu$g poly $A^+$ RNA was applied to each lane. Lane *A*, Hind III digest of $^{32}$P-labeled lambda phage. Lanes *B–E*, mRNA extracted from untreated cells at 4 h (lane *B*), 8 h (lane *C*), 12 h (lane *D*), and 24 h (lane *E*). Lanes *F–I*, mRNA extracted from 5-Aza CR-treated cultures. Lanes *J–M*, cultures exposed to CHX only. Lanes *N–Q*, cultures treated with 5-Aza CR in the presence of CHX. In each series the time points were 4, 8, 12, and 24 h.

when levels of c-myc RNA are increasing to pretreatment levels indicates that high levels of transcription of the c-myc gene do not interfere with the concurrent phenotypic differentiation of HL 60 cells. However, the transient nature of such differentiation shows that the downregulation of c-myc transcription must be maintained for the progression of the process. The presence of a constant interval of ~24 h between the decrease in c-myc mRNA levels and the appearance of differentiation markers suggests that other genes are switched on and link the original signal to the recognizable phenotypic changes.

There are no previous reports of induction of c-fos gene by $1,25(OH)_2D_3$ or 5-Aza CR. Several groups studied its induction in HL 60 cells by TPA. Muller et al. (1984) reported that c-fos expression is restricted to the late stages of differentiation, and Mitchell et al. (1985) observed a rapid (20–30 min) increased in c-fos mRNA in both U-937 and HL 60 cells exposed to TPA. This was later confirmed by Muller et al. (1985), but this group did not observe a rapid induction of c-fos RNA by $1,25(OH)_2D_3$ and concluded that this phenomenon is specific for treatment with TPA. In their system, reduced expression of c-myc RNA was observed later than the rapid changes in c-fos RNA, but changes in message stability have not been excluded in their experiments. In time course studies with $1,25(OH)_2D_3$ and 5-Aza CR presented here, both cellular levels and transcriptional rates of c-fos mRNA increased 2–4 h after the inducer was added and remained elevated for at least 90 h after the inducer was withdrawn (Table II, Figs. 2, 5, 8–10, and 12). Thus, in our system, the induction of the expression of c-fos gene by $1,25(OH)_2D_3$ or 5-Aza CR has an intermediate position in the sequence of differentiation-related events, and the rapid increase in c-fos mRNA observed by others may be a peculiarity of TPA.

The persistence of the increased rates of transcription of c-fos gene, long after the inducer has been removed and the cultures show decreasing proportions of differentiated cells, has some interesting implications. First, it shows that the activity of this gene need not be followed by phenotypic differentiation. Second, the presence of c-fos mRNA in cultures that reverted to the undifferentiated phenotype demonstrates that the differentiated cells were not simply lost from the culture or totally outgrown by the cells that failed to differentiate, but rather remained in a phenotypically undifferentiated form.

The transient "superinduction" of c-Ha-ras gene by 5-Aza CR does not appear to be related to differentiation, since it is not observed after the induction of differentiation by other compounds. This finding is interesting, however, because activation of the related c-Ki-ras gene has been linked to oncogenic transformation (Taparowsky et al., 1982) and to cell proliferation (e.g., Campisi et al., 1984), but not to the inhibition of DNA synthesis by a chemotherapeutic drug.

Only a few of the events associated with the initiation of differentiation and the selection of a lineage pathway in HL 60 cells exposed to antimetabolites are known at this time,

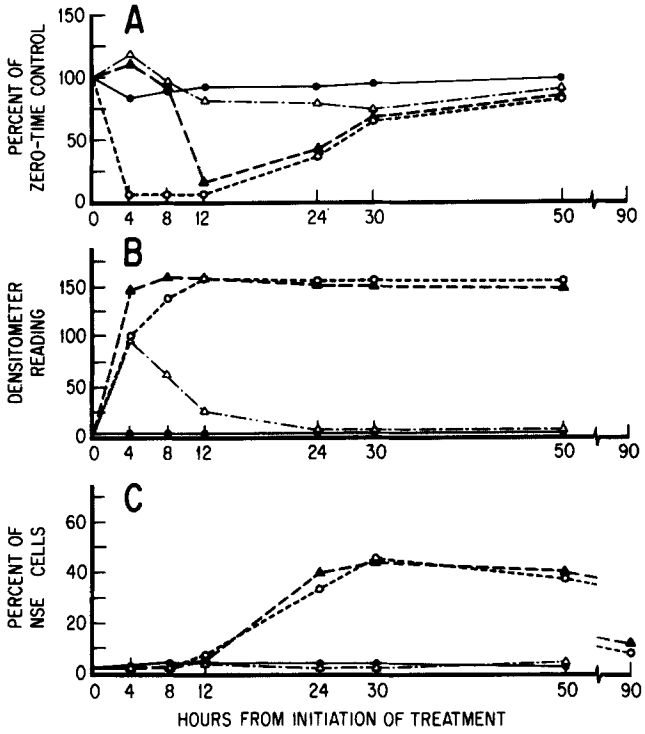


*Figure 12.* Summary of data obtained in three dot-blot experiments and confirmed at selected time points by Northern transfer determinations of mRNA levels as described in the legend to Fig. 8, but after treatment of HL 60 cells with $5 \times 10^{-6}$ M 5-Aza CR in place of $1,25(OH)_2D_3$. ●, untreated cells. ○, Aza CR, 5 μM. △, CHX, 20 μg/ml. ▲, Aza CR + CHX. CHX, cycloheximide.

but the following scenario could serve as a framework for further experiments. Uninduced HL 60 cells have a highly amplified c-myc gene with a correspondingly high expression (Westin et al., 1982). If the excess of the c-myc protein product present in HL 60 cells exerts a strong pressure on these cells to traverse rapidly the proliferative cycle (Kaczmarek et al., 1985), the failure to pause at various restriction points (Pardee, 1974) may make these cells unresponsive to the signals that require a more prolonged residence in a portion of the cell cycle sensitive to the natural inducers of differentiation. Such phase-specific enhancement of sensitivity to induction occurs in different portions of the HL 60 cell cycle with different inducers (Yen and Albright, 1984; Boyd and Metcalf, 1984; Studzinski et al., 1985*b*). Temporary normalization of the elevated levels of the c-myc product in the cell by an antimetabolite or a hormone would then permit the activation of differentiation-related events, including the transcription of c-fos and esterase genes, which result in the appearance of the early features of monocytic differentiation.

Our results suggest that inhibition of the transcription of c-myc gene serves as a signal for the initiation of differentiation of HL 60 cells and that inhibition of c-myc gene expression must be maintained to ensure a commitment to differentiation. Increased expression of the c-fos gene is one of the early steps that lead to monocytic differentiation and it provides the earliest available evidence of that process but does not fit the role of a signal. Involvement of other genes in differentiation of leukemic cells, and the mechanisms of lineage selection and progress along the chosen pathway, remain to be elucidated.

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