Rapid Transcriptional Down-Regulation of *c-myc* Expression during Cyclic Adenosine Monophosphate-promoted Differentiation of Leukemic Cells

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Abstract

Pharmacologic elevation of cyclic AMP (cAMP) promotes growth arrest and differentiation in a variety of transformed mammalian cells, including the HL-60 human promyelocytic leukemia cell line. However, mechanisms underlying this phenomenon are poorly understood. Because cellular oncogenes play a pivotal role in regulating proliferation and differentiation, we examined whether cAMP-promoted differentiation of HL-60 was preceded by a decrease in the expression of *c-myc*, a cellular oncogene both amplified and constitutively expressed in HL-60. We find that cyclic AMP elevation in HL-60 caused by three different pharmacologic regimens is followed by an abrupt, > 90% decrease in steady state *c-myc* mRNA levels within 3 h, well before detectable changes in proliferation and differentiation. This decrease, which occurs despite protein synthetic blockade, is attributable to transcriptional down-regulation of *c-myc* and is accompanied by changes in chromatin structure near *c-myc* promoter sites. Our findings establish that cAMP, a ubiquitous intracellular regulatory messenger previously known only to enhance gene transcriptional activity in higher eukaryotic cells, can also suppress transcription of a cellular oncogene, thereby suggesting a potential mechanism for cAMP-promoted differentiation.

Introduction

Intracellular elevation of the nucleotide cyclic adenosine monophosphate (cAMP), in addition to regulating specific metabolic pathways, elicits such complex changes as growth arrest and differentiation in some transformed mammalian cells—an often-described (1–6) but poorly understood phenomenon (7). Among the cell types which undergo cAMP-promoted differentiation is HL-60 (8, 9), a human promyelocytic leukemia cell line (10) that also differentiates along either monocytic or neutrophilic lines when exposed to such chemically diverse agents as dimethylsulfoxide (DMSO) (11), retinoic acid (12), or vitamin D₃ (13). Consequently, HL-60 is extensively used as a model for studying normal and leukemic hematopoietic differentiation.

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Of particular interest is the attenuated expression of *c-myc*, a cellular oncogene amplified 10- to 20-fold in HL-60 (14), that accompanies terminal differentiation caused by these three agents (15, 16), which suggests a potential role for continuous expression of *c-myc* in maintaining a transformed phenotype and preventing maturation. We therefore used HL-60 as a well-characterized model in which to examine the possible involvement of altered cellular oncogene expression in cAMP-promoted differentiation. We find that a surprisingly rapid and abrupt decrease in steady state *c-myc* messenger RNA (mRNA) levels, caused by transcriptional down-regulation and accompanied by *c-myc* chromatin structural changes, precedes cAMP-promoted growth arrest and differentiation in HL-60.

Methods

Materials, cell lines, and probes. Dibutyryl cAMP (dibcAMP)¹, cholera toxin (CT), isobutylmethylxanthine (IBMX), and actinomycin D were obtained from Sigma Chemical Co. (St. Louis, MO). The source of *Bordetella* adenylate cyclase toxin (BACT) was a dialyzed 4-M urea extract of intact phase I *Bordetella pertussis* organisms prepared as previously described (17). HL-60 was obtained from the American Tissue Culture Collection (Rockville, MD) and adapted to grow in Iscove's tissue culture medium that contained 100 U/ml penicillin and 100 µg/ml streptomycin with 15% heat-inactivated fetal calf serum. The human *c-myc* probe was a 1.6-kb Sst I restriction fragment encompassing the entire first coding exon (see Fig. 4); the β -actin probe was a 0.7-kb Bam HI–Eco RI fragment containing a 3' untranslated portion of β -actin (18); and the human thymidine kinase probe was pTK11 (19).

RNA extraction, dot-blot and northern hybridization. For dot-blot hybridization, total cytoplasmic RNA was extracted (20) from 10⁶ cell aliquots of HL-60, transferred onto nitrocellulose (20), and hybridized with nick-translated probes (1–2 × 10⁸ cpm/µg) (20, 21). Hybridization conditions were 42°C in 5 × standard saline citrate (SSC) and 50% formamide for 18 h; washing conditions were 65°C in 0.1% sodium dodecyl sulfate (SDS), 0.1 × SSC for 1 h. Autoradiograms were prepared using Kodak XAR film and Kodak Lightening-Plus screens at –70°C for various times. For northern blot hybridization studies, RNA was extracted from 1 × 10⁷ cells using the guanidinium isothiocyanate technique (22), polyadenylated RNA selected on oligothymidylic acid–cellulose affinity columns (23) and 5 µg electrophoresed in a 1% agarose–formaldehyde gel, transferred to nitrocellulose (24), and probed as described above.

In vitro nuclear run-off transcription assay. Nuclei from 5 × 10⁷ HL-60 cells were incubated in the presence of [³²P]UTP (25), labeled RNA isolated, and 2 × 10⁶ cpm hybridized to filters (25) to which 5 µg of the indicated plasmid DNA had previously been bound (26). Hybridization

1. Abbreviations used in this paper: BACT, *Bordetella* adenylate cyclase toxin; CFU-GM, colony-forming units of granulocyte–macrophage precursors; CT, cholera toxin; dibcAMP, dibutyryl cAMP; IBMX, isobutylmethylxanthine.

was carried out at 65°C for 36 h in 10 mM *N*-Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid, pH 7.4, 0.2% SDS, 10 mM EDTA, 0.3 M NaCl, and 200 µg/ml *Escherichia coli* RNA. Filters were washed in 2 × SSC at 65°C for 1 h and autoradiograms made.

Actinomycin chase study of *c-myc* stability. Aliquots containing 5 × 10⁶ cells were removed 10 and 40 min after addition of 10 µg/ml actinomycin D. The aliquots were rapidly chilled, pelleted, and washed once in 20 ml ice-cold phosphate-buffered saline. RNA was then immediately extracted by the guanidinium isothiocyanate technique (22), and northern blots were prepared and probed for *c-myc* as described above. Hybridization signals were quantitated from autoradiograms by scanning video densitometry.

DNase I hypersensitive site analysis. Nuclei from 1 × 10⁸ cells were washed, resuspended in digestion buffer (60 mM KCl, 15 mM NaCl, 15 mM Tris, pH 7.4, 0.5 mM DTT, 0.25 M sucrose, 3 mM MgCl₂, and 0.05 mM CaCl₂) and a range of DNase I (Worthington Biochemical Corp., Freehold, NJ) concentrations from 0 to 10 U/ml, and incubated 10 min at 37°C (27). Digestion was stopped by the addition of 1% SDS and 20 mM EDTA. Each sample was then digested with 1 mg/ml proteinase K at 37°C overnight. DNA was isolated by repeated organic extractions and ethanol precipitation. Samples were then digested to completion with Bgl II, and 10 µg was electrophoresed on a 0.8% agarose gel (27). A graded series of four digestions from each treatment group, chosen by ethidium bromide staining to reflect comparable extents of overall DNA degradation among the three groups, was re-electrophoresed on 0.8% agarose, hybridized (27) with the 1.6-kb *c-myc* probe previously described, and autoradiograms made.

Results

In preparatory experiments (not shown) we first defined the time course of the cAMP effect upon HL-60 proliferation. Because pharmacologic agents that elevate cAMP may have unrelated,

nonspecific effects, in our studies we used three cAMP induction regimens with mutually exclusive mechanisms of action: (a) 500 µM dibcAMP, a lipophilic analogue of cAMP that crosses cell membranes; (b) 25 ng/ml CT, a stimulant of membrane-bound adenylate cyclase with 500 µM IBMX, which is a cAMP phosphodiesterase inhibitor; and (c) 150 µg protein/ml of a crude bacterial extract containing BACT. This last agent, produced by *B. pertussis*, is a membrane-invasive adenylate cyclase (17) that penetrates a variety of mammalian cells (28), including HL-60 (9), and generates large intracellular accumulations of authentic cAMP from intracellular ATP stores. The three regimens affected HL-60 proliferation identically: pulse [³H]thymidine incorporation into TCA-precipitable material was minimally affected through 6 h, but declined to 50% that of untreated cells by 12 h, 20% by 24 h, and < 3% from days 2 through 5. The similar effects of three cAMP induction regimens demonstrate a cAMP-specific effect upon HL-60 proliferation.

To determine levels of *c-myc* mRNA during cAMP-dependent HL-60 differentiation we performed quantitative dot-blot hybridization of total cytoplasmic RNA. In response to the three cAMP induction regimens described above, *c-myc* RNA levels decline abruptly by 3 h and remain < 10% that of control throughout the assay period of 3 d (Fig. 1 A). The cAMP effect on *c-myc* expression is not simply due to generalized RNA degradation because steady state levels of β-actin are unaffected (Fig. 1 B). The decrease in total cytoplasmic *c-myc* RNA detected in the dot-blot assay reflects polyadenylated RNA levels (Fig. 1 C). This cAMP-dependent decrement in *c-myc* RNA levels does not depend upon ongoing protein synthesis (and, by implication, altered translational expression of other genes) because it occurs

Figure 1. *c-myc* and β-actin mRNA in HL-60 after cAMP induction. (A and B) Cytoplasmic RNA was prepared from 10⁶ cell aliquots of HL-60 treated as indicated, serially diluted twofold, and transferred onto nitrocellulose. (A) Dot-blot hybridized with human *c-myc* probe (map in Fig. 4). Control: culture medium (Iscove's, with 15% fetal calf serum); dibcAMP: 500 µM dibcAMP; CT + IBMX: 25 ng/ml CT and 500 µM IBMX; BACT: 140 µg/ml protein of a dialyzed urea extract containing BACT; BACT + cycloheximide (CH): 140 µg/ml BACT with 2 µg/ml cycloheximide added 1 h previously. (B) Blots from A re-probed with a 3' untranslated portion of β-actin. Results were identical for regimens B and C (not shown). (C) Northern blot of polyadenylated RNA after cAMP induction. Oligo-dT-selected RNA was isolated from cells before treatment (a) and 4 h after addition of either 500 µM dibcAMP (b) or culture medium (c), and northern blots hybridized with the *c-myc* (A) or β-actin (B and C) probes described.

Figure 2. In vitro nuclear runoff transcription. Nuclei from 5×10^7 HL-60 cells before treatment and after 4 h in the presence or absence (control) of 500 μ M dibcAMP were incubated with [32 P]UTP, labeled RNA isolated, and 2×10^6 cpm hybridized to filters to which 5 μ g of the indicated plasmid DNA had previously been bound and autoradiograms made. Columns designated pSV2neo and pUC18 are vector controls containing no insert; *c-myc* and β -actin are these intact plasmids containing, respectively, the *c-myc* and β -actin clones described in Fig. 1. hu-TK is a plasmid containing a complementary DNA clone of human thymidine kinase, pTK11.

despite the presence of 2 μ g/ml cycloheximide, which blocks $> 90\%$ of [3 H]leucine incorporation into HL-60 cells (Fig. 1 A). Cells treated with BACT plus cycloheximide had decreased viability by day 1, so data beyond 5 h are not shown; cycloheximide alone induced *c-myc* levels.

c-myc RNA levels are regulated either at the level of transcription (29) or by modulation of *c-myc* RNA degradation (30), which is normally very rapid (half-life of 10 min) (31). To assess the former possibility, we used an in vitro nuclear runoff assay that detects the presence of active polymerases along a specific gene (25). After 4 h of exposure to dibcAMP, *c-myc* transcription is suppressed by $> 90\%$ as quantitated by densitometry, an extent commensurate with the decrease in steady state RNA levels (Fig. 2). In contrast, transcription of two other genes, β -actin and thymidine kinase, is relatively unaffected. To rule out the possibility that posttranscriptional alterations contribute to the decrease in steady state *c-myc* RNA, we also assayed the stability

of *c-myc* mRNA by northern blot hybridization of RNA isolated at intervals after the addition of 10 μ g/ml actinomycin D to block RNA synthesis. In cells incubated 4 h either with or without 500 μ M dibcAMP, *c-myc* mRNA half-life is the same: ~ 10 min (Fig. 3).

Developmental activation of gene transcription is accompanied by changes in chromatin structure. These are detectable both as an overall increase in DNase sensitivity of DNA in coding regions (32) and as localized DNase hypersensitive sites near the 5' end of the coding region and on regulatory sequences (33). To determine whether such chromatin structural changes also accompany cAMP-dependent transcriptional down-regulation of *c-myc*, we compared DNase I hypersensitive site patterns of *c-myc* at three stages during cAMP-promoted differentiation of HL-60. By 4 h, when *c-myc* transcription was first found to be attenuated, dibcAMP-treated cells demonstrated a relative resistance of *c-myc* DNA to DNase I digestion when compared with that of untreated cells (Fig. 4). By day 2, after growth arrest and differentiation had occurred, the intensities of hypersensitive sites II and III were markedly decreased relative to those of hypersensitive sites I and V (Fig. 4). Similar changes in *c-myc* hypersensitive site patterns were seen in cells treated with CT and IBMX (not shown).

Discussion

The *c-myc* gene encodes a nuclear phosphoprotein that may function as a cell cycle-specific "competence factor" permitting traversal of the G_1 phase of the cell cycle (34). *c-myc* is constitutively expressed in a variety of transformed cells, including HL-60 (14), and transiently expressed during normal hematopoietic differentiation of human granulocyte precursors (35). Moreover, *c-myc* expression is attenuated in HL-60 cells terminally differentiated with retinoic acid (15), DMSO (15), and vitamin D₃ (16), suggesting a potential role for continuous *c-myc* expression in maintaining a transformed phenotype. However, the growth inhibition, differentiation, and decline in *c-myc* expression that follows treatment with these agents all occur gradually over several days (29, 36), and therefore do not exclude the possibility that decreased *c-myc* expression is simply a consequence, not a cause, of growth arrest and differentiation. In contrast, cAMP causes growth arrest and differentiation of HL-60 within 24 h (8, 9). Our finding that a rapid transcriptional down-regulation of *c-myc* precedes these cAMP-specific effects supports a per-

Figure 3. (A) Actinomycin D chase study of *c-myc* RNA stability. RNA was isolated from cells incubated 4 h in the presence or absence (control) of 500 μ M dibcAMP at the indicated times after addition of 10 μ g/ml actinomycin D. Northern blots were hybridized with the *c-myc* probe depicted in Fig. 4. (B) Effect of dibcAMP on rate of decay of *c-myc* hybridization signal. Open boxes represent the intensity of the *c-myc* signal in control cells, and closed boxes that of dibcAMP-treated cells as quantitated from the autoradiograms shown in A. Error bars show the SE of triplicate densitometric determinations.

Figure 4. DNase I hypersensitive sites in HL-60 *c-myc* chromatin after cAMP induction. Nuclei were isolated from 2×10^7 HL-60 cells before, 4 h after, and 2 d after exposure to 500 μ M dibcAMP and digested with increasing concentrations of DNase I, which are designated by the direction of the arrow. DNA was isolated and exhaustively digested with Bgl II. A graded series of four comparable digestions from each treatment group was electrophoresed in 0.8% agarose, and probed with the P^{32} -labeled, nick-translated 1.6-kb *c-myc* probe indicated. The first lane on the left contains size markers. Bgl II generates a 6.0-kb band in DNA from untreated nuclei (see line drawing). With increasing DNase I digestion, specific subbands, representing hypersensitive sites and identified with roman numerals, are generated. Nomenclature for the subbands is that previously used by Siebenlist et al. (38). The minor sites III₁ and III₂ are not clearly resolved and are labeled III. In the line drawing, open bars indicate untranslated, and cross-hatched bars translated, *c-myc* exons.

missive or causal role for extinction of *c-myc* expression in promoting functional maturation of this leukemic cell line. Indeed, the recent demonstration that antibodies to the *c-myc* protein suppress DNA synthesis in HL-60 nuclei (37) implies that cAMP-dependent attenuation of *c-myc* expression can by itself directly cause growth arrest.

Investigating the mechanism behind rapid extinction of *c-myc* expression in HL-60 after cAMP elevation, we found a transcriptional down-regulation accompanied by diminished expression of DNase I hypersensitive II sites and III (29, 38). These sites are located just upstream of, respectively, promoter sequences previously designated as P₀ (29) or P₁ and P₂ (39). Moreover, preliminary experiments utilizing exon-specific probes indicate that down-regulation of *c-myc* is caused by a block to transcriptional elongation between exons I and II (unpublished observations). Strikingly similar changes accompany HL-60 differentiation caused by DMSO (38, 40) and retinoic acid (29), but occur much more slowly—over days rather than within 4 h. In view of these mechanistic similarities, the relative speed with which cAMP attenuates *c-myc* transcriptional activity suggests that DMSO and retinoic acid—structurally unrelated substances with no known role in the regulation of normal cellular functions—might ultimately act through a mechanism dependent upon cAMP, an endogenous intracellular signalling substance. The observation that retinoic acid, DMSO, and phorbol esters also increase nucleosol-associated cAMP-dependent protein kinase activity in HL-60 (41) supports this hypothesis.

The role of cAMP in regulating such cytosolic metabolic pathways as glycogenolysis is well characterized. In contrast, understanding of mechanisms underlying such complex phenom-

ena as cAMP-dependent growth arrest and differentiation, which likely involve changes in gene expression, remains rudimentary (7). Though cAMP alters gene expression in mammalian cells (42), previous investigations have only shown cAMP capable of stimulating transcriptional activity of differentiation-specific genes, such as prolactin and growth hormone in rat pituitary cells (43). We demonstrate a rapid, cAMP-specific decrement in HL-60 *c-myc* RNA levels mediated by transcriptional down-regulation—to our knowledge the first example of this phenomenon in higher eukaryotic cells. The detection of concomitant *c-myc* chromatin structural changes further emphasizes that cAMP attenuation of *c-myc* is effected at the genomic level. Thus, our studies extend previous correlations between exposure to cAMP analogues and decreases in steady state levels of oncogene protein (44) and RNA (45), and raise the possibility that transcriptional modulation of oncogene expression may, in part, mediate cAMP-dependent differentiation in other transformed cell types. One hypothetical mechanism envisions cAMP simultaneously promoting transcription of genes encoding proteins characteristic of terminal differentiation, while repressing transcription of genes, such as *c-myc*, which may permit continuous proliferation and block differentiation.

Our findings in HL-60 leukemic cells may also have relevance to normal hematopoiesis. Several agents that increase intracellular cAMP, including monocyte-derived prostaglandins of the E series (PGEs), inhibit proliferation of colony-forming units of granulocyte-macrophage precursors (CFU-GM) in a soft agar cloning assay (46, 47). Monocytes and macrophages have on this basis been implicated as participating in intrinsic negative feedback control of normal myelopoiesis (47). Human bone

marrow cells exposed to growth factors that promote CFU-GM growth transiently, but strongly express *c-myc* 24 h later, when cells morphologically identifiable as promyelocytes predominate (35). cAMP-promoted transcriptional down-regulation of *c-myc*, were it to occur in normal as well as HL-60 leukemic promyelocytes, could mediate suppression of CFU-GM growth in response to cAMP elevation. Thus, cAMP, which is generated by a receptor-coupled adenylate cyclase complex, may prove to serve as an important second messenger intermediary between extracellular signals and nuclear regulation of oncogene expression.

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Note added in proof. Since this work was completed, Vaulont et al. (1986. *J. Biol. Chem.* 261:7621–7625) have reported that another mammalian gene, L-type pyruvate kinase, is transcriptionally down-regulated by dibcAMP in rat hepatic cells.

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