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Transcriptional Inactivation of c-*myc* and the Transferrin Receptor in Dibutyryl Cyclic AMP-Treated HL-60 Cells

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Treatment of HL-60 cells with dibutyryl cyclic AMP induced rapid transcriptional inactivation of c-*myc* and the transferrin receptor. Transcriptional inactivation was followed by loss of c-*myc* and transferrin receptor mRNA and protein. Treated cells completed one round of proliferation, followed by growth arrest, G_1 synchronization, and monocytic differentiation. These data suggest that cyclic AMP-mediated control of growth and differentiation may be achieved, at least in part, by transcriptional regulation of certain growth-associated proto-oncogenes and growth factor receptor genes.

The human promyelocytic cell line HL-60 has been a useful tool for the study of maturational arrest characteristic of myeloid leukemia. HL-60 cells have been induced to monocytic and to granulocytic differentiation by a variety of agents (22). Differentiation is accomplished by inhibition of cell growth, as well as the induction of lineage-specific phenotypic characteristics. Of particular interest in attempting to understand the biochemical and molecular events regulating myeloid cell growth and differentiation is the observation that induction of HL-60 differentiation is correlated with a loss of mRNA expression of the proto-oncogene c-*myc* (22, 26, 41, 46) and loss of cell surface transferrin receptor (TfR) protein (42, 47). Expression of c-*myc* is thought to be required for normal cell growth (3, 14, 19), and deregulated expression of c-*myc* can inhibit differentiation (10, 12, 39). Structural rearrangement or deregulation of this gene (or both) is a frequent concomitant of malignant transformation (2, 4, 11). After the induction of differentiation, c-*myc* transcripts are reduced (22, 26, 41, 46), and 3 days after treatment with dimethyl sulfoxide, c-*myc* transcription is inhibited (16). Surface TfR expression is also a marker of proliferating cells and is required for the growth of both normal and malignant cells (for reviews, see references 32 and 35). Rovera et al. (42) showed that 3 days after the induction of HL-60 differentiation with phorbol ester, surface TfR protein was reduced. Short-term treatment of HL-60 cells with phorbol ester results in a marked decrease in surface TfR levels within 5 min. However, this extremely rapid receptor loss is due to redistribution of surface TfR to a cytoplasmic pool and not by inhibition of TfR synthesis (20, 25).

We have recently shown that treatment of HL-60 cells with the cyclic nucleotide analog dibutyryl cyclic AMP (dbcAMP) induces a marked loss of surface TfR expression (44). Because rapid redistribution of TfR from the cell surface to the cytoplasm can occur without affecting DNA synthesis or cell growth (33), it was of interest to see whether dbcAMP-induced loss of TfR surface expression reflected an effect at the transcriptional level. In this report, we show that dbcAMP induces terminal monocytic differentiation of HL-60 cells. This effect is preceded by transcriptional inactivation of both the c-*myc* and TfR genes and loss of c-*myc* and TfR protein, followed by growth arrest and synchronization in G_1. cAMP has many documented effects on cell growth and maturation (8, 36, 37). The data presented here demonstrate cAMP-mediated transcriptional regulation of two proliferation-associated genes and suggest a new model with which to study hormone-stimulated cyclic nucleotide regulation of growth and differentiation.

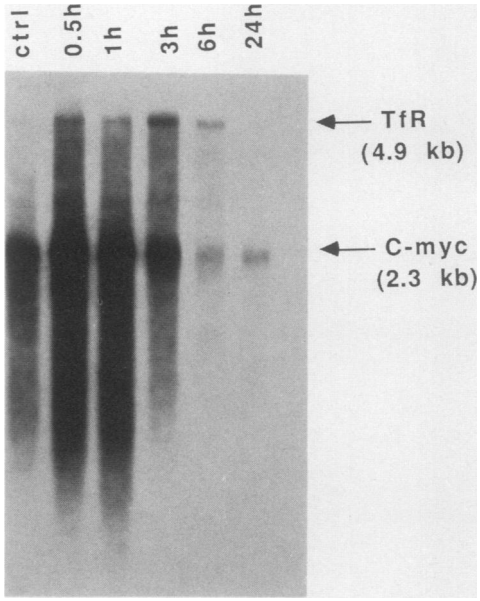

FIG. 1. RNA blot analysis. RNA was isolated by guanidine isothiocyanate extraction and cesium chloride gradient centrifugation (9). Poly(A)$^+$ RNA was isolated by passage over an oligo(dT)-cellulose column, separated on a 1.0% agarose-formaldehyde gel, blotted onto nitrocellulose, hybridized in 50% formamide to ^{32}P-labeled probes, washed, and autoradiographed by standard techniques (23). Full-length human TfR cDNA in the pCD vector was kindly provided by A. McClelland and F. H. Ruddle, Department of Biology, Yale University (27). A 1.7-kilobase *Cla*I-*Eco*RI restriction fragment containing the third exon of human c-*myc* has been described previously (4). Abbreviations: ctrl, control; kb, kilobase.

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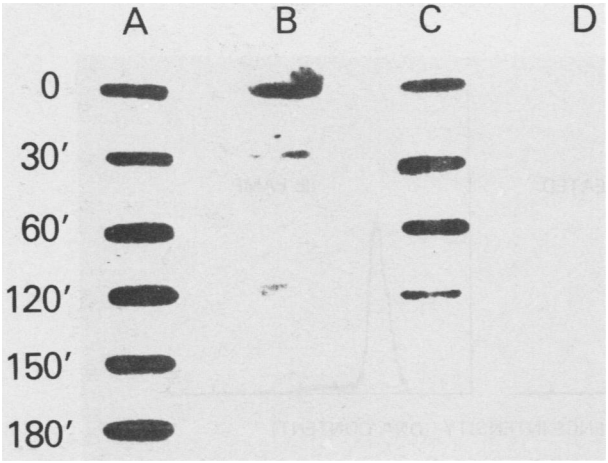

FIG. 2. Nuclear runoff transcription assay. Nuclear runoff transcription assays were done as described by Groudine et al. (17). HL-60 cells (10^8 cells per condition) were treated for various times (30 min to 24 h) with 1 mM dbcAMP and compared with untreated, log-phase cells. DNA probes (10 μg per slot) were blotted onto nitrocellulose by using a slot blotter (Schleicher & Schuell, Inc., Keene, N.H.). The c-*myc* and TfR probes are described in the legend to Fig. 1. Beta-2-microglobulin cDNA in pBR322 was a kind gift of J. G. Seidman, Department of Genetics, Harvard Medical School. After overnight prehybridization, the slots were hybridized with ^{32}P-labeled RNA (generated from 20×10^6 nuclei per condition) for 3 days at 40°C, washed, air dried, and autoradiographed. Lanes: A, beta-2-microglobulin; B, c-*myc*; C, TfR; D, pBR322.

Effect of dbcAMP on c-*myc* and TfR gene transcription. We studied the transcription of c-*myc* and the TfR gene after incubation of HL-60 cells with 1 mM dbcAMP. dbcAMP treatment significantly reduced the steady-state level of c-*myc* mRNA by 6 h and reduced that of TfR mRNA by between 6 and 24 h (Fig. 1). In view of reports of the posttranscriptional regulation of c-*myc* mRNA accumulation (6, 13, 21), the effect of dbcAMP on the transcriptional activity of the c-*myc* and TfR genes was examined. Nuclear runoff transcriptional assays are shown in Fig. 2. HL-60 c-*myc* transcription was reduced to undetectable levels within 30 min of treatment with dbcAMP, whereas transcription of the TfR gene was nearly undetectable by 2 h. In contrast, transcription of the beta-2-microglobulin gene remained unaffected throughout the course of the experiment. A pBR322 control hybridized simultaneously gave no detectable signal. Therefore, the levels of steady-state mRNA accurately reflect transcriptional inactivation of the c-*myc* and TfR genes. Although c-*myc* expression has been shown to be regulated at both transcriptional (5, 14, 16) and posttranscriptional levels (6, 13, 21), in the studies presented here the regulation by dbcAMP of c-*myc* gene expression would appear to be purely at the transcriptional level. However, since the transcriptional assay was done by using a third exon c-*myc* probe, it is possible that the dbcAMP-induced loss of c-*myc* transcripts may reflect a block of elongation (5).

Effect of dbcAMP on intracellular c-*myc* protein level. Expression of the proto-oncogene c-*myc*, like TfR, has been proposed to be essential for cell growth and particularly for traversal of the G_1 phase of the cell cycle (3, 14, 19). We examined c-*myc* protein levels in untreated HL-60 cells and in HL-60 cells treated with 1 mM dbcAMP. In all cases, the protein was localized to the nucleus, and antibody staining was blocked by prior incubation with purified recombinant c-*myc* protein. A decrease in c-*myc* protein expression was first seen between 2 and 4 h after treatment (data not shown), and at 8 h c-*myc* protein was completely undetectable (Fig. 3A). Protein expression remained undetectable 72 h after dbcAMP treatment, as determined both by immunocytochemistry (data not shown) and by flow cytometry (Fig. 3B).

Surface TfR expression after dbcAMP treatment. When HL-60 cells were incubated with 1 mM dbcAMP, there was a large decrease in surface TfR expression. By 72 h, surface TfR were no longer detectable on the dbcAMP-treated cells (Fig. 3C). The kinetics of the response showed a lag period of 5 h before a measurable decrease in surface TfR expression after treatment with dbcAMP (data not shown). Sodium

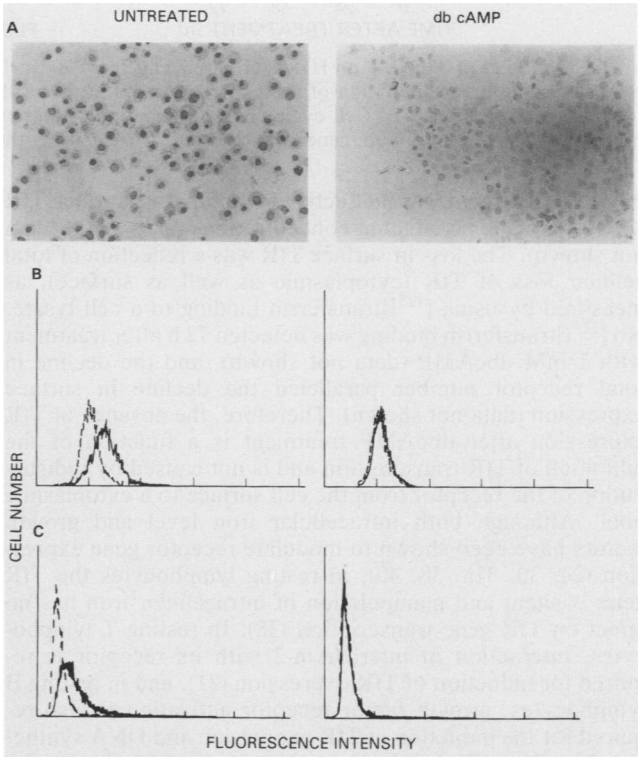

FIG. 3. Visualization of c-*myc* and TfR protein. (A) HL-60 cells, untreated (left) or exposed to 1 mM dbcAMP for 8 h (right), were pelleted onto glass slides in a cytocentrifuge and fixed immediately in 5% paraformaldehyde. After 30 min in paraformaldehyde, the cells underwent three 5-min washes in Tris-buffered saline containing 1% normal goat serum and were stained with either affinity-purified rabbit anti-human c-*myc* immunoglobulin (45) (5 μg per slide) or normal rabbit immunoglobulin. The slides were then processed as described by Hsu et al. (18). Antibody staining was visualized by using peroxidase-coupled goat anti-rabbit immunoglobulin (Tago, Burlingame, Calif.). (B) Intracellular c-*myc* protein levels were determined by flow cytometry in untreated log-phase cells (left) and in cells treated for 3 days with 1 mM dbcAMP (right). Cells were fixed as described above, washed, and incubated with anti-c-*myc* antibody (0.5 μg/500,000 cells) in PBS containing 1% bovine serum albumin. After 1 h at 4°C, cells were washed twice with PBS containing 1% goat serum and reacted with fluorescein-labeled goat anti-mouse immunoglobulin for 30 min at 4°C. After two washes in PBS with goat serum, cells were analyzed by flow microfluorometry on a FACS II (Becton Dickinson FACS Systems Inc., Mountain View, Calif.) by using the 488 nm band of an argon laser (Spectra Physics, Nutley, N.J.) at 500 mW. (C) Surface TfR expression was detected by using labeled monoclonal anti-TfR antibody (OKT9; Ortho Diagnostics, Inc., Raritan, N.J.), followed by fluoresceinated goat anti-mouse immunoglobulin.

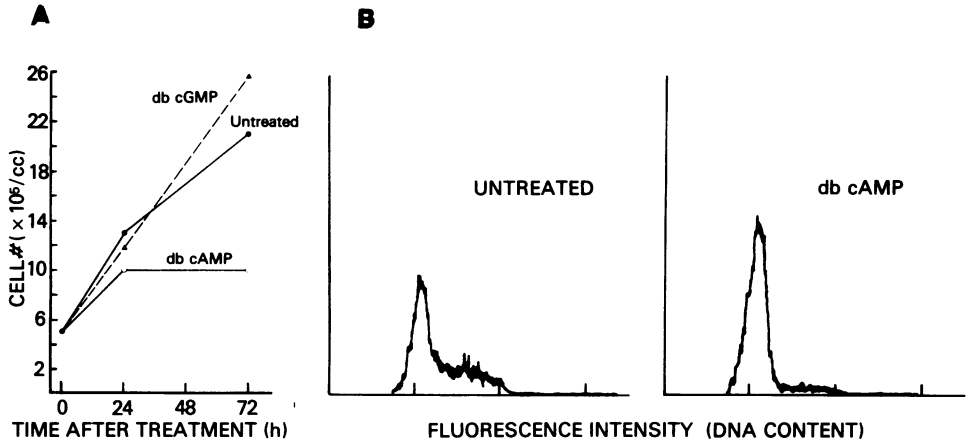

FIG. 4. Effect of dbcAMP on HL-60 growth and cell cycle distribution. (A) Cells were split to a concentration of 3×10^5 to 5×10^5 cells per ml the day before initiation of the experiment and again on the day agents were added. dbcAMP and dbcGMP were used at a final concentration of 1 mM. (B) Cell cycle phase distribution of untreated log-phase cells (left) and cells treated for 24 h with 1 mM dbcAMP (right). DNA histograms were generated on a FACS II by using the DNA intercalating dye propidium iodide (7).

butyrate treatment was ineffective in modulating surface TfR levels, showing no effect at concentrations up to 1 mM (data not shown). The loss in surface TfR was a reflection of total cellular loss of TfR (cytoplasmic as well as surface), as measured by using [^{125}I]transferrin binding to a cell lysate. No [^{125}I]transferrin binding was detected 72 h after treatment with 1 mM dbcAMP (data not shown), and the decline in total receptor number paralleled the decline in surface expression (data not shown). Therefore, the absence of TfR expression after dbcAMP treatment is a function of the inhibition of TfR transcription and is not caused by redistribution of the receptor from the cell surface to a cytoplasmic pool. Although both intracellular iron level and growth factors have been shown to modulate receptor gene expression (24, 30, 31a, 38, 40), in resting lymphocytes the TfR gene is silent and manipulation of intracellular iron has no effect on TfR gene transcription (38). In resting T lymphocytes, interaction of interleukin-2 with its receptor is required for induction of TfR expression (31), and in resting B lymphocytes, growth factor receptor activation is also required for the induction of TfR expression and DNA synthesis (34). The biochemical transductions which lead from growth factor receptor activation to TfR induction and cell growth are unknown. Since manipulation of intracellular iron level has been shown neither to initiate transcriptional activity of the TfR gene (38) nor to cause an absolute cessation of this activity (40), fluctuating intracellular iron levels may serve to modulate TfR gene activity, whereas other mechanisms may regulate initiation of transcription of this gene. The data presented here demonstrating transcriptional regulation of the TfR gene by an agent other than intracellular iron suggest that under certain circumstances cyclic nucleotides can play a critical role in TfR transcriptional control.

dbcAMP inhibits HL-60 growth and induces G_1 synchronization. We tested the effect of dbcAMP and dbcGMP on HL-60 cell growth. Dose-response studies have shown that 1 mM dbcAMP causes a maximal reduction of surface TfR without affecting cell viability and that at this dose dbcGMP does not affect viability and has minimal effect on surface TfR expression (data not shown). Fig. 4A shows that the number of dbcAMP-treated cells doubled at 24 h, after which cell growth was completely inhibited. dbcGMP did not inhibit HL-60 cell proliferation, demonstrating that the inhibition of cell growth observed after treatment with dbcAMP was not caused by the butyrate moiety.

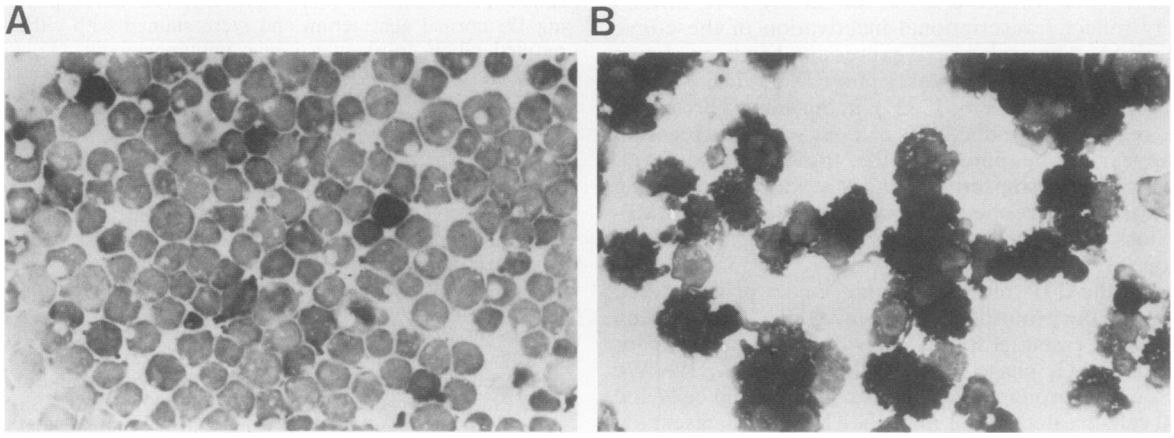

FIG. 5. α-Naphthyl butyrate esterase activity determined on cytocentrifuge slide preparations of untreated log-phase cells (A) and cells treated for 3 days with 1 mM dbcAMP (B).

We examined the effect of dbcAMP on HL-60 cell cycle distribution to determine whether the growth-arrested HL-60 cells were accumulating in one phase of the cell cycle. Cells were incubated for 3 days with 1 mM dbcAMP and compared with untreated, exponentially growing controls. DNA histograms were generated by using flow microfluorometry and the DNA intercalating dye propidium iodide. dbcAMP treatment induced over 90% synchronization in G_1 (Fig. 4B). These data combined with those from Fig. 4A show that dbcAMP-treated cells go through one round of division before growth arrest associated with G_1 synchronization.

dbcAMP induces monocytic differentiation. As the DNA histograms and cell growth data presented in Fig. 4 were consistent with terminal differentiation, we tested three cytochemical markers of myeloid differentiation on log-phase control and 3-day dbcAMP-treated cells. Figure 5 shows a striking increase in α-naphthyl butyrate esterase activity, a marker for monocytic differentiation (29), after treatment with dbcAMP. Untreated HL-60 cells were negative for this enzyme marker, as has been reported previously (43). A time course of the induction of esterase activity revealed that esterase positivity was seen at 24 h but was not detectable at 8 h after the addition of dbcAMP (data not shown). The monocytic marker α-naphthyl acetate esterase followed the same pattern as seen with α-naphthyl butyrate esterase, whereas myeloperoxidase, a marker of granulocytic differentiation, was negative in untreated and dbcAMP-treated cells (data not shown).

cAMP is known to directly affect the transcription of several eucaryotic genes, presumably via cAMP-dependent protein kinase (28). Control experiments in this study established that the effects seen with dbcAMP were not elicited by dbcGMP or sodium butyrate and, therefore, probably represent an effect of altered intracellular cAMP levels. Elias and Stewart have recently suggested that intranuclear cAMP-dependent protein kinase may play a role in induction of differentiation in HL-60 cells (15). Three potential mechanisms could explain the observations presented here. cAMP may activate a cytoplasmic or nuclear cAMP-dependent protein kinase to remove the block to maturation characteristic of HL-60, with a resultant decrease in c-*myc* and TfR gene activities. Alternatively, a cytoplasmic or nuclear kinase could directly decrease the expression of c-*myc*, TfR, and other growth-promoting influences with the emergence of monocytic differentiation after withdrawal of these influences. Finally, there may be a direct novel, non-kinase-mediated effect on the transcriptional or differentiation-specific apparatus, analogous to the known ability of cAMP to affect transcriptional activity in procaryotic systems (1). Although further experiments will be needed to distinguish among these possibilities, the model system described here could be of value in exploring transcriptional regulation during differentiation of hematopoietic cells and maturation arrest in hematopoietic neoplasms.

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