An Oligomer Complementary to c-myc mRNA Inhibits Proliferation of HL-60 Promyelocytic Cells and Induces Differentiation

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To study the role of a nuclear proto-oncogene in the regulation of cell growth and differentiation, we inhibited HL-60 c-myc expression with a complementary antisense oligomer. This oligomer was stable in culture and entered cells, forming an intracellular duplex. Incubation of cells with the anti-myc oligomer decreased the steady-state levels of c-myc protein by 50 to 80%, whereas a control oligomer did not significantly affect the c-myc protein concentration. Direct inhibition of c-myc expression with the anti-myc oligomer was associated with a decreased cell growth rate and an induction of myeloid differentiation. Related antisense oligomers with 2 to 12-base-pair mismatches with c-myc mRNA did not influence HL-60 cells. Thus, the effects of the antisense oligomer exhibited sequence specificity, and furthermore, these effects could be reversed by hybridization competition with another complementary oligomer. Antisense inhibition of a nuclear proto-oncogene apparently bypasses cell surface events in affecting cell proliferation and differentiation.

The molecular mechanisms that regulate cellular proliferation and differentiation involve signal transduction from membrane receptors to events that are mediated by nuclear proteins (4, 5). Candidates for such regulatory proteins include the following members of the nuclear proto-oncogene family: c-myc, N-myc, c-myb, p53, c-fos, c-ski (for a review, see reference 54). The evidence that implicates these genes in growth control is the observation that their mutated forms produce dysregulated growth in vitro, such as immortalization, which can be assessed by transformation of primary embryo fibroblasts in cooperation with activated ras oncoproteins (29, 33, 42, 48), and in vivo, such as in tumors in transgenic mice (1, 33, 46, 51) or patients with Burkitt lymphoma (for reviews, see references 28 and 31).

The c-myc gene has a higher level of expression in proliferating cells than in differentiated cells (for reviews, see references 9 and 54). Gene transfer experiments have demonstrated that constitutive expression of c-myc inhibits induced differentiation of cell lines (12, 14, 32, 43, 47), supporting the fact that there is an inverse association between c-myc expression and differentiation, although exceptions have been reported (15, 16, 49). Dysregulated c-myc expression perturbs lymphocyte differentiation during embryonic development in transgenic mice (34) and contributes to tumor development in adult animals (1, 35).

HL-60 promyelocytic leukemia cells (11, 21) represent a useful model system for studying the role of proto-oncogenes in cellular proliferation and differentiation (54). Several mutations in specific proto-oncogenes have been identified in HL-60 cells that may account for the transformed phenotype (39). The c-myc gene is amplified 8- to 30-fold and is highly expressed (10, 13); this is in association with an activated N-ras gene (39) and deletion of another nuclear oncogene, p53 (38). Because both p53 and c-myc transform primary fibroblasts in cooperation with activated ras genes (29, 33, 42), Wolf and Rotter (58) have proposed that the malignant phenotype of HL-60 may be related, in part, to the substitution of an amplified c-myc gene for the combined functions of c-myc and p53. HL-60 cells differentiate along the granulocytic pathway when they are treated with dimethyl sulfoxide (DMSO) (11) or retinoic acid (7) and along the monocytic pathway when treated with phorbol esters (45) or vitamin D analogs (44). A common result of differentiation induction along either pathway is a profound decrease in c-myc expression (18, 24, 56). It is presently unclear, however, whether decreased c-myc expression is a cause or a closely linked consequence of HL-60 differentiation.

The antisense methodology (for reviews, see references 23 and 55) produces inhibition of specific gene products by exploiting base pairing by complementary nucleic acids, producing a block in mRNA processing, transport, or translation (30, 38). Antisense RNA methods have been employed previously to investigate the function of cellular oncogenes; they have demonstrated that c-fos (27, 41) and c-myc expression (R. L. Redner, J. T. Holt, and A. W. Nienhuis, manuscript in preparation) are involved in fibroblast proliferation and that c-src expression contributes to polyomavirus transformation (2, 6). Zamecnik and co-workers (61, 62) have reported antisense inhibition of herpes simplex virus replication (62) and human immunodeficiency virus-encoded protein synthesis (61), in which synthetic deoxyribonucleotides were used. Deoxyribonucleotides that have been modified by use of methylphosphonate linkages (50) or by conjugation to acridine (53) or poly-L-lysine (36) have also been reported to inhibit viral replication or phage protein synthesis.

One approach to investigating the mechanisms which regulate cellular processes is to bypass the cell surface-mediated events and directly influence the expression of nuclear proteins. If the nuclear proto-oncogenes represent a final pathway regulating cell proliferation and differentiation, then inhibition of nuclear oncogene expression should influence these processes. We employed an antisense oligomer to inhibit c-myc expression, allowing us to study the role of c-myc in HL-60 growth and differentiation.

MATERIALS AND METHODS

Synthesis and purification of oligomers. Unmodified, 15-base deoxyribonucleotides were synthesized on an automated solid-phase synthesizer (Applied Biosystems Inc.) by using standard phosphoramidite chemistry and were purified.
by two different high-pressure liquid chromatography methods or by gel electrophoresis (20). All oligomers employed in cellular experiments were lyophilized and suspended in HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid)-buffered saline at pH 7.4. Oligomers from the translation initiation region of human c-myc were employed because of a report (E. L. Wickstrom, E. Wickstrom, G. H. Lyman, and D. L. Freeman, Federation of American Societies of Experimental Biology, abstr. no. 1330, 1986) that this region of c-myc mRNA is single stranded and that deoxyribonucleotides are not present in this region to inhibit HL-60 growth. The sequences of synthesized oligomers and their relationship to the organization of the c-myc gene are presented in Fig. 1. Analysis of the homology between the synthesized oligomers and the primate sequences that are present in the GenBank data base revealed no homologies to 13 of 15 or greater (except for c-myc) with any of the synthesized oligomers.

**Determination of oligomer stability and intracellular duplex detection.** Gel-purified, 5'-end-labeled oligomer and sufficient unlabeled carrier oligomer to achieve a concentration of 4 μM were added to logarithmically growing HL-60 cells in improved minimal essential medium supplemented with glutamine and 10% fetal calf serum (heat inactivated at 65°C for 30 min). All experiments were performed with a single lot of fetal calf serum with minimal nuclelease activity, as assayed by the stability of the 5'-end-labeled oligomer. Neither serum-free medium nor HEPES-buffered saline contained detectable nuclelease activity.

In experiments designed to detect intracellular duplex, 5'-end-labeled oligomers were used without added carrier (specific activity 3 × 10^6 to 5 × 10^8 cpm/μg) at a concentration of 4 μM. After incubation for 4 h, the cells were washed three times with HEPES-buffered saline that was prewarmed to 37°C. The cells were then lysed in 100 μl of Nonidet P-40 lysis buffer (10 mM Tris [pH 7.5], 10 mM NaCl, 3 mM MgCl2, 0.05% Nonidet P-40) containing 0.5% sodium dodecyl sulfate, 100 μg of proteinase K per ml, and a 10,000-fold excess of unlabeled oligomer (as the carrier). The amount of carrier added was based on the amount of cell-associated radioactivity remaining on washed cells, which was detected by scintillation counting of cell pellets. Following deproteinization and ethanol precipitation, an S1 nuclelease protection assay was performed at 37°C; the products were analyzed on a denaturing 20% polyacrylamide gel (37). To demonstrate that the duplex was intracellular and not an artifact of RNA isolation, an "add-back" control was performed in which the measured amount of cell-associated radioactivity was added with carrier (excess unlabeled oligomer) to a lysate of cells that were previously unexposed to oligomer. This experiment was performed with duplicate samples on two separate occasions.

**Measurement of c-myc protein.** Immunoblotting was performed by using a modification of standard methods (17, 25), in which affinity-purified rabbit polyclonal antibody directed against human c-myc, which was provided by Stephen Hann (Vanderbilt University, Nashville) or Gerard Evan (Ludwig Institute, United Kingdom), was used. Cells incubated with oligomer (or controls) were lysed and sonicated in buffer containing 10% sodium dodecyl sulfate and 10% β-mercaptoethanol and resolved on a 12% sodium dodecyl sulfate-polyacrylamide gel. Following transfer the blots were probed sequentially with antibody and 125I-labeled Staph protein A (Hoeffer Scientific). The c-myc protein was identified by fluorography after exposure at −70°C. Densitometric scanning provided a quantitative estimate of the relative concentrations of c-myc protein in cell extracts. Filters were subsequently washed with phosphate-buffered saline, blocked with a 5% nonfat dry milk solution, and immunoblotted for actin as described previously (19).

Immunoprecipitation was performed by using the same rabbit anti-c-myc antibodies described above. Cells were incubated with oligomer (or controls) for 4 or 24 h, washed twice with HEPES-buffered saline, and then labeled for 90 min in methionine-free medium (containing 10% heat-inactivated dialyzed serum and 4 μM oligomer, when indicated) in the presence of 300 μCi of [35S]methionine (New England Nuclear Corp., Boston, Mass.). Sample preparation and immunoprecipitation were performed as described previously (17, 25). The amount of label added to each sample was determined by trichloroacetic acid precipitation.

**Determination of growth rate and DNA content analysis.** All studies for the determination of growth rate and DNA content analysis were performed with HL-60 cells that were passaged early and that were passaged and analyzed at cell concentrations of 2 × 10^5 and 1.2 × 10^6 cells per ml. The HL-60 cell line used in this study had a doubling time of approximately 21 h and a low rate of spontaneous differentiation (less than 5%), as determined by Nitro Blue Tetrazolium (NBT) reduction, chloroacetate esterase staining, and alpha naphthyl acetate esterase staining. In addition, the cells differentiated appropriately with either DMSO or phorbol ester induction. Subsequent studies with other HL-60 lines showed less-dramatic phenotypic effects after anti-myc oligomer addition.

Initial oligomer studies were performed by adding a single dose of oligomer to cells that had been growing in the same medium for 5 days. The cell concentration was adjusted at a density of 800,000/ml by centrifuging and then suspending 2 × 10^5 cells in the supernatant (this ensured the fact that the cells were continuously exposed to the same media but minimized cell density effects). Cell counts were performed with trypan blue (to ensure viability) by using a hemacytometer.

Subsequent oligomer addition studies were performed by using daily medium changes. In these studies, the cell concentration was adjusted daily, but cells were suspended in fresh medium containing 4 μM oligomer. Cells that were induced with 1.25% DMSO were not exposed to fresh DMSO-containing medium daily, because results of initial experiments demonstrated that this was toxic.

The DNA content of cells incubated with sense or antisense oligomer (by using daily medium changes) was determined by flow cytometry (EPICS; Coulter Electronics, Inc., Hialeah, Fla.) (22) along with simultaneous measurements of growth rate. This analysis was calibrated with fluorescent microspheres. At least 10^4 cells were counted for each analysis. The percentage of cells in each cell cycle phase was determined by replicate analysis, with a standard error of 5%.

**Measurement of differentiation markers.** Morphology was assessed by cytocytophotography followed by methanol fixation and Wright-Giemsa staining. NBT reduction was performed by a modification of standard methods by using 1 μg of phorbol 12-myristate 13-acetate per ml (3). Cytochemical reactions for naphthol AS-D chloroacetate esterase and alpha naphthyl acetate esterase were performed by standard methods (59). The percentage of positive cells was assessed by counting 200 cells. Purified peripheral blood granulocytes or mononuclear cells were employed as positive controls for cytochemical assays.

**RESULTS**

Oligomers enter cells and form a duplex, reducing the c-myc protein concentration. The sequences of the oligomers em...
ANTI-myc OLIGOMER INDUCES HL-60 DIFFERENTIATION

**FIG. 1.** Oligomer sequences, their relationship to c-myc mRNA, and their effect on the doubling time of HL-60 cells. The drawing illustrates the genomic organization of the human c-myc proto-oncogene on chromosome 8. Boxes represent exons (transcribed regions), and lines represent flanking and intervening sequences (introns). Arrows labeled P1 and P2 show the cap sites, which represent the start of transcription from the two major c-myc promoters. The AUG coincident with the ATG of the sense oligomer is the translation initiation codon for the major c-myc protein (9). Mismatched nucleotides of the MUC sequences are indicated with asterisks. Note that the antisense and MUC oligomers are listed 3' to 5' from left to right to facilitate comparison with the c-myc mRNA sequences. Doubling times were determined from the 5-day growth curves on duplicate samples (performed on at least two occasions).

**FIG. 2.** Autoradiograph demonstrating the stability of 5'-end-labeled sense and antisense oligomers following incubation with HL-60 cells. Gel-purified oligomers were incubated with logarithmically growing cells for the indicated number of hours. Labeled molecular weight markers (not shown) demonstrated that the observed pattern corresponds to integral single-base decrements over time. The doublet present at time zero for the antisense oligomer was an effect of the secondary structure.
Detection of an S1 nuclease-resistant duplex (Fig. 3) following incubation with 5'-end-labeled oligomer demonstrated that the oligomer entered the cells and formed a 15-base-pair (bp) double-stranded duplex. The quantity of duplex detected was approximately 70 copies per cell (calculated by comparing the intensities of autoradiographic signals with known standards and adjusting for the specific activity). We previously determined that there are approximately 200 copies of c-myc mRNA in these cells, suggesting that 30% of the c-myc mRNA is duplexed with the antisense oligomer after 4 h of incubation. This percentage is roughly comparable to the observed 50 to 80% inhibition of steady-state c-myc protein levels. The absence of a duplex in the add-back control indicated that the duplex formed intracellularly and not during RNA isolation. A faint signal representing approximately 500-fold less duplex was observed when cells were incubated with 5'-end-labeled sense oligomer.

HL-60 cells grown in 4 μM antisense oligomer contained 50 to 90% less c-myc protein than did either control HL-60 cells or cells grown in 4 μM sense oligomer, but contained more c-myc protein that did DMSO-induced cells (Fig. 4A). Immunoblots demonstrated a selective decrease in the steady-state level of c-myc protein as early as 4 h after incubation with antisense oligomer, with no apparent decrease in the steady-state levels of actin (Fig. 4B). No further reduction occurred over the 72 h following administration of a single dose. These immunoblotting experiments were performed in this study are presented in Fig. 1. The approximate half-life of the full-length (5'-end-labeled), 15-base oligomer was estimated to be 24 to 48 h, based on densitometric analysis of autoradiographs (Fig. 2). The degradation which occurred was a 3' or 5' exonuclease activity. No significant endonuclease activity was detected in cell culture experiments. In several experiments it was demonstrated that full-length oligomer was present 5 days after it was added to the medium (data not shown).
Repeated to confirm the results. Immunoprecipitation studies also demonstrated a selective decrease of c-myc protein synthesis following either 4 or 24 h of incubation with the antisense oligomer (Fig. 5). The control sense oligomer did not produce a significant decrease in either the c-myc protein level (Fig. 4) or the rate of synthesis of c-myc protein (Fig. 5).

Decreased c-myc expression is associated with a decreased rate of cellular proliferation. Characterization of the parameters of HL-60 growth was performed to allow manipulation of media to ensure logarithmic growth. Daily replacement of medium allowed HL-60 cells to grow logarithmically up to $5 \times 10^5$ cells per ml. Results of further studies demonstrated that pelleting and suspension in fresh medium does not adversely affect the growth rate of HL-60 cells, and it actually increased the growth rate slightly (compare Fig. 6A and B). Growth of cells in heat-inactivated (65°C) medium had no effect on the growth rate (data not shown).

The addition of either a single dose or daily doses (with medium change) of 4 μM antisense oligomer to HL-60 cells produced a similar 50% decrease in the growth rate over a 5-day period (Fig. 6A and B). The logarithmic plot of the data (Fig. 6A and B, insets) demonstrated that the degree of growth inhibition increased with time. The inhibition of cell growth by the antisense oligomer was less extensive than that produced by 1.25% DMSO. The growth of cells was not influenced by the addition of 4 μM sense oligomer. A total of 10 separate purified lots of antisense oligomer and 12 separate lots of sense oligomer were used. All lots of antisense oligomer inhibited HL-60 cell growth (regardless of the method of purification), and none of the lots of sense oligomer inhibited HL-60 cell growth at a concentration of 4 μM. Inhibition of cell growth associated with antisense oligomer was dose dependent over the range of 1 to 8 μM (Fig. 7). Analysis of the DNA content of the cells that were incubated with 4 μM antisense oligomer (medium changed daily) showed a generalized prolongation of the cell cycle, but no significant accumulation of cells in any one phase, whereas cells treated with DMSO were arrested in the G₀ and G₁ phases within 48 h (Fig. 8).

Decreased c-myc expression is associated with induction of myeloid differentiation. Incubation of HL-60 cells with 4 μM antisense oligomer for 5 days produced significant changes in the morphology of 60 to 80% of the cells (Fig. 9B). These cells developed a decreased nuclear:cytoplasmic ratio, looser chromatin, less prominent nucleoli, segmented nuclei, and less basophilic cytoplasm than did the HL-60 cells incubated in the control oligomer (Fig. 9A). Many more cells incubated with antisense oligomer showed NBT reduction (Fig. 10B) than did cells incubated with the sense oligomer (Fig. 10A) or untreated HL-60 cells. Results of multiple determinations with different preparations demonstrated that 17 to 32% of the cells were positive with the 4 μM anti-c-myc oligomer on days 3 and 5, whereas fewer than 5% of the control cells (4 μM sense treated or untreated HL-60 cells) were positive. The number of cells that reduced NBT was similar, regardless of the frequency of medium changes.

The antisense oligomer produced fewer cells that were positive than the 68 to 95% positive cells observed with DMSO induction. Incubation with antisense oligomer produced 10 to 20% chloroacetate esterase-positive cells, compared with fewer than 5% positive cells with the control sense oligomer.

Oligomer inhibition of cell growth and induction of differentiation exhibits sequence specificity. Control oligomers with base compositions identical to those of the antisense oligomer, but with shuffled sequences, produced no apparent inhibition of cell growth or induction of differentiation. These were tested at 4 μM for 5 days with a daily medium change. The sequences of five mismatched oligomers and their effect on HL-60 doubling time are shown in Fig. 1. NBT reduction assays on days 3 and 5 following the addition of

![FIG. 5 Autoradiograph demonstrating the rate of c-myc protein synthesis. Equal numbers of trichloroacetic acid-precipitable counts were employed for the immunoprecipitation experiment (lanes 1 to 6). Lanes 7 to 12 represent equal numbers of counts that were loaded directly onto the gel (without immunoprecipitation) as a control for the specificity of the antisense oligomer effects on c-myc protein synthesis. Lane 1 and 7, Untreated HL-60 cells; lanes 2 and 8, HL-60 cells incubated in antisense oligomer for 24 h; lanes 3 and 9, HL-60 cells incubated in sense oligomer for 4 h; lanes 4 and 10, HL-60 cells incubated in antisense oligomer for 4 h; lanes 5 and 11, HL-60 cells incubated in sense oligomer for 24 h; and lanes 6 and 12, HL-60 cells incubated in 1.25% DMSO for 24 h. Kd, Kilodaltons.](http://mcb.asm.org/)

![FIG. 4. Autoradiograph demonstrating steady-state levels of c-myc protein following incubation with antisense oligomer. Immunoblot of cell lysates from HL-60 cells probed with an anti-c-myc antibody (A) and an antiaitin antibody (B). See the text for technical details. Lanes 1 to 6 represent cells incubated with 4 μM antisense oligomer for 4 h (lanes 1), 8 h (lanes 2), 12 h (lanes 3), 24 h (lanes 4), 48 h (lanes 5), and 72 h (lanes 6). Lanes 7, 8-h incubation with sense oligomer; lanes 8, 24-h incubation with sense oligomer; lanes 9, 72-h incubation with sense oligomer; lanes 10, sample after 72 h of exposure to 1.25% DMSO. Molecular mass markers were as follows: myosin H chain, 200,000 kilodaltons (Kd); phosphorylase b, 97,400 kilodaltons; bovine serum albumin, 68,000 kilodaltons; ovalbumin 43,000 kilodaltons. The actin protein (B) is represented by the intense band at 42 kilodaltons; the higher-molecular-mass bands represent nonspecific binding of antibody to abundant cellular proteins.](http://mcb.asm.org/)
FIG. 6. Growth curves of HL-60 cells incubated with 4 µM oligomers. A logarithmic transformation of the linear growth curve is presented in the insets on the upper left of both panels. Points and bars represent the means ± standard errors. (A) Cells were incubated in the same medium for 5 days. Results represent pooled data from seven separate experiments, with two replicate cultures for each point. (B) Cells were incubated in medium that was changed daily (with fresh oligomer added). Results represent pooled data from two experiments, with two replicate cultures for each point. Symbols: ○, HL-60 cells; □, sense oligomer addition; △, anti-myc addition; ○, DMSO treatment.

the mismatched oligomers showed fewer than 5% positive cells for each mismatched oligomer.

Antisense oligomer effects are reversed by hybridization competition. The reversal of both growth inhibition and differentiation induction when a 10-fold excess of complementary sense oligomer was added with 4 µM antisense oligomer is shown in Table 1. The addition of equal amounts of antisense and sense oligomers produced intermediate degrees of growth inhibition and NBT positivity.

DISCUSSION

Incubation of HL-60 cells with the antisense oligomer resulted in decreased c-myc expression, growth inhibition, and differentiation. The degree of growth inhibition was concentration dependent. The apparent mechanism that was responsible for the phenotypic effects was duplex formation, as demonstrated by an S1 nuclease protection assay, analysis of mismatched oligomers, and reversal of effects through
competition experiments. Duplex-mediated inhibition of c-myc expression was associated with growth inhibition and differentiation in a significant proportion of the cells, supporting a role for c-myc in these cellular processes. The presence of the remaining undifferentiated cells (following antisense oligomer incubation) may reflect a heterogeneous cellular response to the antisense oligomer.

Because the antisense oligomer was stable in culture and formed a duplex that was associated with decreased c-myc expression, we were able to test the role of c-myc expression in HL-60 cells. End-labeled oligomers were present for several days in the appropriate media, extending the observation that oligomers are stable for at least 2 h (57). An intracellular duplex was identified that contained labeled oligomer, providing direct evidence that the 15-base oligomer entered the cell. The small quantity of duplex that was protected with the control sense oligomer may represent hybridization to a previously described endogenous antisense c-myc RNA (40) or the nonspecific background inherent in the assay. Estimates that 30% of HL-60 c-myc mRNA was associated with the duplex are roughly comparable to the observed 50 to 90% reduction of steady-state c-myc protein levels. Zamecnik et al. (61) have reported similar levels of inhibition of human immunodeficiency virus protein synthesis following incubation with antisense oligomers. Demonstration of both the antisense duplex and decreased c-myc protein after incubation for 4 h supports our interpretation that the duplex directly inhibits c-myc gene expression.

Because results of the initial experiments suggested that the antisense oligomer affects growth and differentiation by hybridization with c-myc mRNA, we tested this hypothesis directly. Two distinct approaches were employed: (i) synthesis of oligomers with sufficient sequence homology with c-myc mRNA to prohibit duplex formation, and (ii) addition of a second complementary oligomer which should have reversed the antisense oligomer effects by hybridization competition. The lack of phenotypic effects of the mismatched oligomers supports the fact that there is considerable sequence specificity in antisense oligomer inhibition. For example, an oligomer with only two mismatches (MUC 4) produces no apparent inhibition of growth or induction of differentiation. The theoretical melting temperature for the perfectly matched duplex between c-myc mRNA and antisense oligomer is 56°C in physiological saline, and that for a duplex between c-myc mRNA and MUC 4 (2-bp mismatch) is 41°C. These melting temperature estimates support the observed result because DNA-RNA hybrids are most stable at 10 to 20°C below the melting temperature (8), although hybridization within the cell may involve factors that are not included in the calculation of the theoretical melting temperature. As a further test for oligomer specificity, competition experiments were performed which showed that the phenotypic effects induced by the antisense oligomer may be reversed by the addition of another complementary oligomer. Results of these studies provide strong support for a hybridization-based mechanism of antisense oligomer effects.

In a proportion of cells the oligomer entered and formed a sufficient amount of duplex to inhibit c-myc expression below a critical level, thereby triggering differentiation. HL-60 cells are leukemia cells that are arrested at the promyelocytic stage of myeloid development with a prolif-

FIG. 7. Dose-response curve of antisense oligomer. Percent inhibition was calculated by comparison with the number of cells present in the control cultures incubated with the sense oligomer. Each point represents the mean of two replicate cultures from a representative experiment. The experiment was repeated, and similar results were obtained.

FIG. 8. DNA content histogram of HL-60 cells incubated for 5 days with 4 μM oligomers or controls. The scale for panel D (DMSO) is one-half of the scale for the other samples. The x axis indicates fluorescence intensity (DNA content), and the y axis indicates cell number.

TABLE 1. Reversal of growth inhibition and differentiation induction by hybridization competition

<table>
<thead>
<tr>
<th>Anti-myc oligomer</th>
<th>Sense oligomer</th>
<th>Doubling time (h)</th>
<th>% NBT positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 μM</td>
<td>0 μM</td>
<td>29</td>
<td>20-32</td>
</tr>
<tr>
<td>4 μM</td>
<td>4 μM</td>
<td>27</td>
<td>11-17</td>
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<td>4 μM</td>
<td>40 μM</td>
<td>21</td>
<td>4-6</td>
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<tr>
<td>0 μM</td>
<td>40 μM</td>
<td>21</td>
<td>1-5</td>
</tr>
<tr>
<td>0 μM</td>
<td>0 μM</td>
<td>21</td>
<td>1-2</td>
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* When HL-60 cells were incubated with 1.25% DMSO, the doubling time was 42 h, and the percentage of cells that were NBT positive was 68 to 69%. * Doubling time and NBT reduction results were those obtained after 3 days of incubation with a single dose of the oligomers.
erative capacity but an inability to differentiate in the absence of inducing agents (11, 21). The number of differentiated cells observed following antisense oligomer incubation probably represents a minimum value, because differentiated cells are presumably lost from culture and have limited proliferative capacity. Previously (18, 60), in studies with HL-60 cells, it has been demonstrated that growth-arrested (serum-starved) cells do not automatically differentiate. This suggests that the induction of differentiation which we observed was not necessarily a direct result of growth inhibition. The inhibition of growth and induction of differentiation observed in HL-60 cells suggests that down regulation of

FIG. 9. Photomicrograph showing morphology of Wright-Giemsa-stained cytocentrifuge preparations of HL-60 cells incubated with 4 μM sense (A) and 4 μM antisense (B) oligomers for 5 days. Magnification, ×330.
FIG. 10. Photomicrograph showing morphology of HL-60 cells following the NBT reduction assay. (A) Cells were incubated with 4 μM sense oligomer for 5 days. (B) Cells were incubated with 4 μM antisense oligomer for 5 days. Magnification, ×220.
c-myc expression in these promyelocytic leukemia cells eliminates their inherent block to maturation, permitting differentiation in the absence of inducing agents. Although c-myc expression is influenced by extracellular factors, results of this study indicate that inhibition of nuclear proteins may bypass surface events that directly influence cellular processes.

Antisense oligomer induced differentiation in a significant proportion of the HL-60 cells, but not in all of them. Although a majority of cells exhibited altered morphology, fewer reduced NBT. We tested the possibility that a longer exposure time to full-length oligomer might increase the proportion of cells that reduced NBT. No significant change in the proportion of cells that reduced NBT or in growth rate (compare Fig. 6A and B) was observed, regardless of the frequency of medium changes. Cells that did not reduce NBT may represent those in a heterogeneous population with submaximal responses to antisense oligomer resulting from (i) nonuniform uptake of oligomer; (ii) variable amounts of c-myc mRNA, duplex mRNA, or c-myc protein; or (iii) the presence of cellular factors that influence the effect of the c-myc protein concentration on differentiation induction. The relative importance of each of these factors might be addressed by in situ determinations of the proliferative index and the c-myc protein.

DNA content analysis demonstrated that incubation with antisense oligomer results in a proportional prolongation of all phases of the cell cycle, with no relative accumulation in any one phase. These results are in contrast to DMSO-induced differentiation which produces a drastic G$_0$ and G$_1$ phase arrest (18, 60) (Fig. 8). Growth curves revealed no evidence that an uninhibited population emerged (which might skew this analysis), as the slope of the logarithmetic plot remained relatively constant with time (Fig. 6B). Although the heterogeneity of the population complicates interpretation of the cell cycle analysis, the data suggest that c-myc expression is required throughout the cell cycle. This interpretation is supported by the equal expression of c-myc in all cell cycle phases (26, 52).

In this study we employed oligomers to inhibit a specific cellular gene (c-myc) by using the antisense method. In previous studies it has been demonstrated that complementary, unmodified oligomers inhibit viral infection in chicken embryo fibroblasts (62) and HeLa cells (61). In this study we extended these observations to include inhibition of expression of an endogenous cellular gene in a nonadherent cell line, HL-60. Further studies are necessary to determine the range of cell types and genes that may be inhibited by this approach. The use of antisense methods to bypass cell surface events and to manipulate the expression of nuclear proteins represents an important tool for the study of nuclear proto-oncogenes.

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ADDITION IN PROOF


LITERATURE CITED


