MOLECULAR AND CELLULAR BIOLOGY, June 1989, p. 2734–2737
0270-7306/89/062734-04$02.00/0

Vol. 9, No. 6

A Transfected L-*myc* Gene Can Substitute for c-*myc* in Blocking Murine Erythroleukemia Differentiation

MICHAEL J. BIRRER,[1,2]* LILY RAVEH,[2,3] HIROTOSHI DOSAKA,[1] AND SHOSHANA SEGAL[1,2]

Navy Medical Oncology Branch, National Cancer Institute, Bethesda, Maryland 20892[1]; Israel Institute for Biological Research, 70450 Ness-Ziona, Israel[3]; and Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814[2]

Received 16 November 1988/Accepted 27 February 1989

We investigated the ability of the proto-oncogene L-*myc* to substitute for c-*myc* in blocking murine erythroleukemia differentiation. Murine erythroleukemia cells (line C19) were transfected with recombinant plasmids containing genomic and cDNA fragments of the L-*myc* gene driven by a Moloney murine leukemia virus long terminal repeat. Clones expressing constitutive high levels of L-*myc* failed to differentiate in response to the chemical inducer *N,N'*-hexamethylene bisacetamide (HMBA). The block to differentiation correlated with the level of L-*myc* expression. Furthermore, transfected clones grown in the presence of inducer for an extended period of time showed an increased level of L-*myc* expression. These results suggest that functional domains of the c-*myc* gene involved in differentiation are located in the discrete regions of homology between the c- and L-*myc* genes.

The Friend virus-derived murine erythroleukemia (MEL) cell line C19 is a transformed cell line that still retains the ability to terminally differentiate into erythrocytes under the influence of a number of inducers (8, 17). This differentiation program has been characterized well on both biologic and molecular levels (13, 15). Of particular interest are the proto-oncogene (c-*myc* and c-*myb*) expression patterns during differentiation. Several groups have reported an inverse relationship between c-*myc* and c-*myb* expression levels and the degree of erythroid differentiation (13, 15). Additional evidence for the need to down-regulate these genes to permit differentiation comes from studies in which these genes were transfected into MEL cells under the control of powerful promoters. The results of these experiments showed that a highly expressed c-*myc* gene can block MEL differentiation (5, 7, 18). Likewise, recent data with c-*myb* suggest that constitutive expression will also block differentiation (4, 13a).

We hoped to identify regions in the *myc* gene that are responsible for the apparently critical role of this gene in MEL differentiation. As one approach, we chose to study the effect of constitutive expression of L-*myc* (another member of the *myc* family) on differentiation. The L-*myc* gene has a genomic structure similar to that of c-*myc* and codes for a protein with an overall amino acid sequence 30% homologous with the c-*myc* protein sequence (6, 10). However, this homology is located in several discrete areas of highly conserved amino acid sequence (homology boxes; >90% homology), with major insertions and deletions interspersed between these homology boxes (6, 10). Studies with deletion mutants of c-*myc* have demonstrated that although several of these conserved regions are indispensable for transforming activity, other homology regions are not, which suggests other important functions associated with these areas of the gene (20, 22). Since MEL cells do not express L-*myc*, by examining the effect on differentiation of a transfected L-*myc* gene, we were able to determine whether regions conserved between c- and L-*myc* are important for blocking differentiation.

MEL cells were transfected with the Moloney murine leukemia virus long-terminal-repeat expression vector pVcos-7, containing (i) 1.6 kilobases (kb) of L-*myc* cDNA corresponding to a truncated version of the long-form mRNA (3.9 kb) or (ii) a 10-kb genomic fragment containing the entire L-*myc* gene (10). These plasmid constructs have been shown to be biologically active in two different transformation assays (1; M. Birrer, unpublished data). Transfections were done by $CaPO_4$ precipitation, and G418-resistant clones were isolated and characterized as previously described (7, 21). Analysis of 50 MEL clones expressing L-*myc* mRNA demonstrated inhibition of differentiation, as measured by the decreased formation of a red pellet and benzidine staining after 5 days of induction with *N,N'*-hexamethylene bisacrylamide (HMBA). The degree of inhibition of differentiation varied widely among the transfectants. Seven clones transfected with pVcos-7 alone were analyzed, all of which differentiated normally.

Several independent clones representing MEL transfectants with either the L-*myc* cDNA or genomic constructs were subcloned and used for evaluation. L-*myc* expression in these clones was determined by Northern (RNA) blots and RNase protection assay (Fig. 1), using a probe spanning exon 2 and the 5' portion of intron 2 of L-*myc*. The results revealed a wide range of expression levels. No endogenous L-*myc* mRNA was detected in the parental MEL cell line C19. In the transfectants receiving the cDNA construct, L-*myc* expression varied between the low levels seen in clone CL3 and the much higher levels seen in clones BL-1 and EB12/S6 (Fig. 1). Clone FB10/S2, transfected with the genomic fragment, expressed high levels of normal L-*myc* mRNAs (3.9, 3.6, and 2.2 kb) comparable to those seen in the small-cell lung cancer (SCLC) cell line 510 amplified for the L-*myc* gene (6, 10). The four clones (CL3, BL-1, EB 12/S6, and FB10/S2) and the parental cell line (C19) were subjected to log-phase inductions (three times) with 3 mM HMBA as previously described (7, 21) (Fig. 2). The parental cell line C19 and a pVcos-7 control transfectant (data not shown) accumulated benzidine-positive cells rapidly, with 90% of the culture becoming positive by day 3. In contrast, clone CL3, which expressed low levels of L-*myc* mRNA, was partially delayed in its differentiation profile, whereas

* Corresponding author.

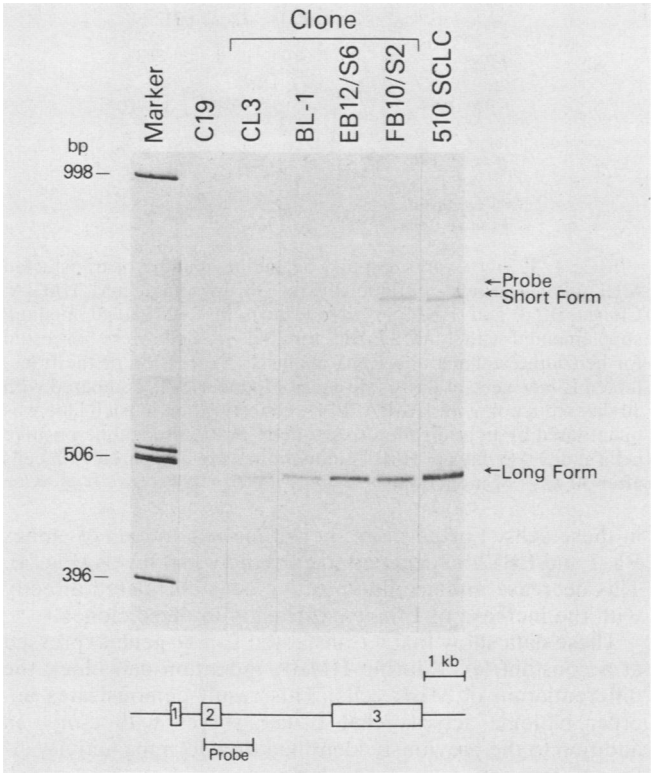

FIG. 1. L-*myc* expression levels in transfected MEL clones as detected by RNase protection assay. The single-stranded ^{32}P-labeled riboprobe was obtained from T7 polymerase transcription of a plasmid containing a 700-base-pair (bp) L-*myc* cDNA fragment spanning exon 2 and the 5′ portion of intron 2 and hybridized to 10 μg of total RNA from MEL transfectants, the parental line C19, and the SCLC cell line 510 overnight at 50°C in 50% foramide. After digestion with RNase, the protected fragments were fractionated on an 8 M urea–5% polyacrylamide gel. Clones CL3, BL-1, and EB12/S6 are L-*myc* cDNA transfectants expressing mRNA corresponding to exons 2 and 3 of the gene and therefore display a protected species of approximately 400 bp (long form) corresponding to the exon 2 coding sequence. Clone FB10/S2 is an L-*myc* genomic transfectant expressing native L-*myc* long-form (3.9 and 3.6 kb) and short-form (2.2 kb) mRNAs. The RNA of this clone gives rise to two protected species: a 400-bp fragment corresponding to exon 2 sequences and a 700-bp fragment corresponding to exon 2 and intron 2 sequences. This pattern is identical to that of the SCLC cell line 510. Arrows indicate positions of the undigested probe and the long-form (exon 2) and short-form (exon 2 and 5′ region of intron 2) protected species. Markers were made by digesting pBR322 with *Eco*RI and *Hin*fI and end labeling with [^{35}S]dATP. 1, 2, and 3 represent L-*myc* exons.

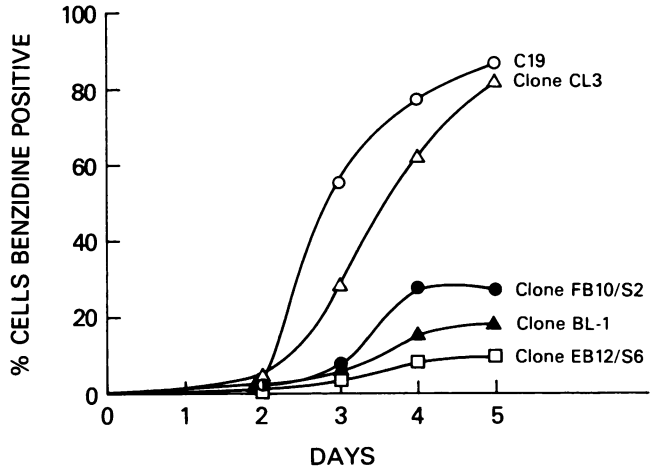

FIG. 2. Benzidine staining of transfected clones and the parental cell line during HMBA induction. Uninduced and logarithmic-phase induced cultures of the parental cell line C19 and clones CL3, BL-1, EB12/S6, and FB10/S2 were assayed for accumulation of benzidine-stained cells (expressed as percentage of total cells in culture). Logarithmic-phase inductions were performed as follows. Logarithmically growing cells were plated at 10^5 cells per ml in growth medium. The next day, 50% of the culture was replaced with fresh growth medium containing 6 mM HMBA (final concentration, 3mM; Sigma Chemical Co., St. Louis. Mo.). This process was repeated each day, and the removed cells were analyzed for the presence of hemoglobin by the color of the pellet or staining of individual cells with benzidine as previously described (7). For both uninduced and induced cultures, 200 cells were scored daily for benzidine reactivity; percentages of positive cells are shown for the indicated time points.

BL-1 and EB12/S6, which expressed higher levels of L-*myc* mRNA (2.5 and 5 times, respectively, the level in CL3, as determined by densitometric analysis) demonstrated a more complete block to differentiation. Therefore, the degree of differentiation seemed to correlate with the level of L-*myc* expression in these clones. On the other hand, clone FB10/S2, although expressing high levels of L-*myc* mRNA (five times the level expressed by CL3), was only partially blocked. This clone was transfected with a genomic fragment of L-*myc* in which transcription of the gene is more complex and the number of mRNA species differs from those seen in L-*myc* cDNA transfectants (Fig. 1).

Steady-state mRNA levels of L-*myc*, c-*myc*, β-globin, and c-*myb* with or without HMBA induction were established for clones EB12/S6, CL3, and C19 (Fig. 3). L-*myc* was expressed with no evidence of down-regulation throughout the induction period in clones EB12/S6 and CL3, whereas no detectable expression was found in the parental cell line C19. Surprisingly, at 1 to 6 h postinduction, we detected a small increase in expression of the L-*myc* gene in all L-*myc*-expressing clones analyzed.

Although there was no evidence for down-regulation of L-*myc* expression during the induction period in the clones, we detected major changes in expression of the endogenous c-*myc* and c-*myb* genes. During the process of differentiation of MEL cells, expression of c-*myc* and c-*myb* displayed a biphasic pattern. The steady-state levels of these two genes dropped dramatically during the first 6 h of induction, which was followed by a transient reexpression during the next 18 h. A second drop occurred after 24 h of induction, which resulted in a two- to three-fold drop in c-*myc* expression, whereas expression of c-*myb* declined more substantially. This pattern was seen for the parental cell line C19 and clone CL3 (Fig. 3). However, whereas clone EB12/S6 did demonstrate the initial drop and recovery in expression of these two genes, it did not display the second drop in expression of the c-*myb* gene. These findings are similar to the previously reported results for MEL cells blocked in differentiation by a constitutively expressed c-*myc* gene (7). This finding is of particular importance given recent data suggesting that the late decline of c-*myc* is required for commitment to terminal differentiation (14, 24).

Analysis of β-globin mRNA levels during HMBA induction revealed that whereas C19 and the low-L-*myc*-expressing clone CL3 were induced to differentiate and produced high levels of globin mRNA, clone EB12/S6 was

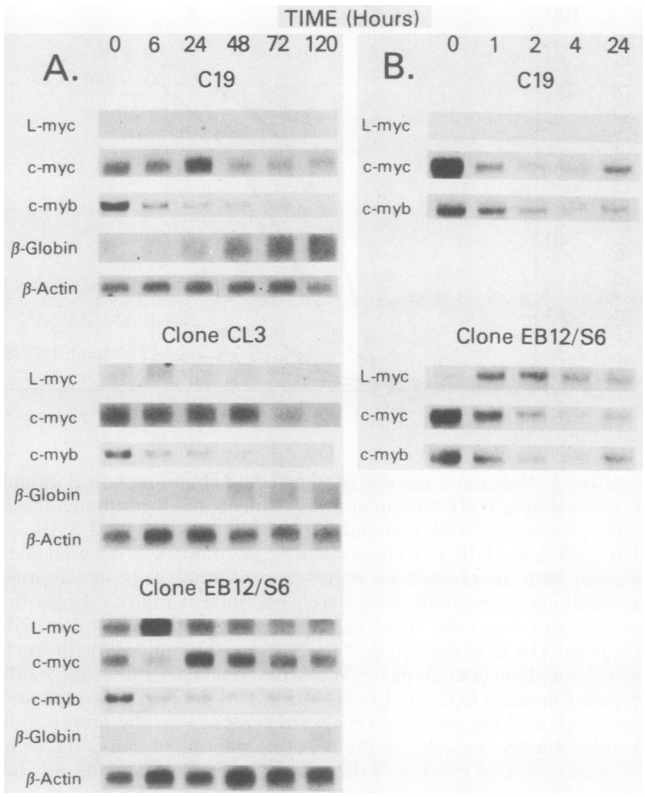

FIG. 3. (A) Data on 120-h time course expression of L-*myc*, c-*myc*, c-*myb*, β-actin, and β-globin in transfected clones and the parental cell line during logarithmic-phase induction with HMBA. Transfected clones CL3 and EB12/S6 and the parental cell line C19 were grown logarithmically. L-*myc*, c-*myc*, c-*myb*, and β-globin mRNA expression levels were analyzed by electrophoretically separating 10 μg of total RNA from each clone on 1% agarose gels by the technique of Lehrach et al. (16) and blotting by transfer to nitrocellulose filters. The nitrocellulose filters were then hybridized to the following ^{32}P-labeled nick-translated probes (19): for L-*myc*, a 700-bp cDNA fragment; for c-*myc*, a 1.5-kb murine cDNA fragment containing exons 2 and 3; for β-globin, a 4.4-kb *Pst*I fragment of the human gene; and for c-*myb*, a 2.4-kb cDNA fragment of the mouse gene. The blots were sequentially hybridized to these probes without stripping. Hybridization with β-actin (2.0-kb *Bam*HI fragment) was performed last to ensure and quantitate the presence of intact RNA in each lane. Filters were exposed to XAR-5 or XRP film at −70°C with intensifying screens for variable times. (B) Data on 24-h time course expression of L-*myc*, c-*myc*, and c-*myb* during HMBA induction. Total RNA (10 μg each) from clone EB12/S6 and parental cell line C19 was electrophoresed on 1% agarose gels and blotted. The Northern blots were hybridized to different probes as described above. For these early time points, no hybridization to β-globin was done, since minimal levels are transcribed. The amounts of RNA in all lanes were equal, as determined by ethidium bromide staining.

significantly suppressed in expression of globin RNA message (Fig. 3A), which reflected its block to differentiation.

To further explore the function of L-*myc* in MEL clones BL-1 and EB12/S6, we grew these clones in the presence of HMBA for 30 days and then examined L-*myc* expression by Northern blot analysis (Fig. 4). Survival of the clones under these conditions depended on their inability to differentiate. It is clear that a significant increase in L-*myc* expression occurred during growth in the presence of inducer, consistent with a critical L-*myc*-dependent block to differentiation

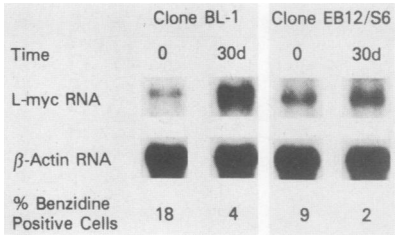

FIG. 4. L-*myc* expression and benzidine staining of transfected MEL clones grown continuously for 30 days in 3 mM HMBA. Clones BL-1 and EB12/S6 were grown in RPMI 1640 medium supplemented with 3 mM HMBA for 30 days. Cells were harvested for benzidine staining and RNA analysis. Expression of the transfected L-*myc* gene at day 0 (absence of inducer) was compared with 30-day induction with HMBA. RNA concentration in each lane was quantitated by hybridization to a β-actin probe. Benzidine-positive cells at day 0 (5 days of HMBA induction) were compared with cells after 30 days of induction.

in these cells. Furthermore, benzidine reactivities of clones BL-1 and EB12/S6 declined to extremely low levels (Fig. 4). This decrease in benzidine-positive cells correlated directly with the increase of L-*myc* expression in these clones.

These data show that a transfected L-*myc* gene expressed at a constant level during HMBA induction can block the differentiation of MEL cells. This result demonstrates another biologic activity that L-*myc* shares with c-*myc* in addition to the previously identified transforming activity (1, 6). Of interest, L-*myc* has been reported to have weak transforming activity in comparison with c-*myc*, but, as seen in the data presented here, it seems to function efficiently in blocking the differentiation of MEL cells (1). L-*myc* is frequently amplified and overexpressed in SCLC cell lines (10). It is interesting to hypothesize whether one of its functions in these cells is to maintain them in a partially undifferentiated state.

Multiple lines of evidence suggest that c-*myc* plays a critical role in the balance between cell growth and differentiation. High or constitutive levels of c-*myc* expression are often associated with some form of growth deregulation (11, 12). Conversely, during terminal differentiation, c-*myc* mRNA decrease in a number of cell lines (2, 15, 23). In addition, constitutive expression of a transfected c-*myc* gene inhibits or blocks differentiation in several of these systems (5, 7, 18, 21). Our findings for the structurally related L-*myc* gene support the important role of c-*myc* in the differentiation of MEL cells. Furthermore, since L-*myc* is not normally expressed in these cells, its effectiveness in blocking differentiation presumably stems from structurally similar domains shared by these two genes. These domains, known as homology boxes, are discrete regions of 70 to 90% predicted amino acid sequence homology located in exons 2 and 3 (6, 10). Interestingly, while some of these regions are indispensable for rat embryo cell transformation activity, two regions (c-*myc* amino acids 1 to 104 and 144 to 320) have been shown to tolerate some or even extensive mutations, with little effect on transformation. Since sequence conservation suggests functional selection, these areas of the gene may serve as targets for functions other than transformation. It is possible that these regions are more critical to c-*myc* in its physiologic role during differentiation than during transformation. Further dissection of the *myc* gene will be required to test this hypothesis.

We thank Michael Kuehl, James Battey, Richard Rosenberg, and Marion Nau for reviewing the manuscript, Gail Gray for secretarial

assistance, and W. Lee and Lanny Kirsch for providing recombinant probes. In addition, we acknowledge John Minna for his constant help and support.

LITERATURE CITED

1. **Birrer, M. J., S. Segal, J. S. DeGreve, F. Kaye, E. A. Sausville, and J. D. Minna.** 1988. L-*myc* cooperates with *ras* to transform primary rat embryo fibroblasts. Mol. Cell. Biol. **8**:2668–2673.
2. **Campisi, J., H. Gray, A. Pardee, M. Dean, and G. Sonenshein.** 1984. Cell-cycle control of c-myc but not c-ras expression is lost following chemical transformation. Cell **36**:241–247.
3. **Chirgwin, J. M., A. E. Prybyla, R. J. MacDonald, and W. J. Rutter.** 1974. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. J. Biochem. **18**:5294–5299.
4. **Clarke, M. F., J. F. Kukowska-Latallo, E. Westin, M. Smith, and E. V. Prochownik.** 1988. Constitutive expression of a c-*myb* cDNA blocks Friend murine erythroleukemic cell differentiation. Mol. Cell. Biol. **8**:884–892.
5. **Coppola, J. A., and M. D. Cole.** 1986. Constitutive c-myc oncogene expression blocks mouse erythroleukemia cell differentiation but not commitment. Nature (London) **320**:760–763.
6. **Dephino, R. A., K. S. Hatton, A. Tesfaye, G. D. Yancopoulus, and F. W. Alt.** 1987. The human myc gene family: structure and activity of L-myc and a L-myc pseudogene. Genes Dev. **1**: 1311–1326.
7. **Dmitrovsky, E., W. M. Kuehl, G. Hollis, I. R. Kirsch, T. P. Bender, and S. Segal.** 1986. Expression of a transfected human c-myc oncogene inhibits differentiation of a mouse erythroleukemia cell line. Nature (London) **322**:748–750.
8. **Friend, C., W. Scher, J. G. Holland, and T. Sato.** 1971. Hemoglobin synthesis in murine virus-induced leukemic cell in vitro: stimulation of erythroid differentiation by dimethyl sulfoxide. Proc. Natl. Acad. Sci. USA **68**:378–382.
9. **Graham, F. L., and A. J. Van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus DNA. Virology **52**:456–467.
10. **Kaye, F., J. Battey, M. Nau, B. Brooks, E. Seifter, J. De Greve, M. Birrer, E. Sausville, and J. Minna.** 1988. Structure and expression of the human L-*myc* gene reveal a complex pattern of alternative mRNA processing. Mol. Cell. Biol. **8**:186–195.
11. **Keath, E. J., P. G. Caimi, and M. D. Cole.** 1984. Fibroblast lines expressing activated c-myc oncogene are tumorigenic in nude mice and syngenic animals. Cell **39**:339–348.
12. **Kelekar, A., and M. D. Cole.** 1986. Tumorigenicity of fibroblast lines expressing the adenovirus E1a, cellular P53, or normal c-*myc* genes. Mol. Cell. Biol. **6**:7–14.
13. **Kirsch, I. R., V. Bertness, J. Silver, and G. F. Hollis.** 1986. Regulated expression of the c-myb and c-myc oncogenes during erythroid differentiation. J. Cell. Biochem. **32**:11–21.

13a. **Kuehl, W. M., T. P. Bender, J. Stafford, D. McClinton, S. Segal, and E. Dmitrovsky.** 1989. Expression and function of the c-*myb* oncogene during hematopoietic differentiation. Curr. Top. Microbiol. Immun. **141**:318–323.

14. **Kume, T. U., S. Takada, and M. Obinata.** 1988. Probability that the commitment of murine erythroleukemia cells differentiation is determined by the c-myc level. J. Mol. Biol. **202**:779–786.
15. **Lachman, H. M., G. Cheng, and A. I. Skoultchi.** 1984. Expression of c-myc changes during differentiation of mouse erythroylekemia cells. Nature (London) **310**:592–594.
16. **Lehrach, H., D. Diamond, J. Wozney, and H. Boedtker.** 1977. RNA molecular weight determination by gel electrophoresis under denaturing conditions, a critical reexamination. Biochemistry **16**:4743–4751.
17. **Marks, P. A., and R. A. Rifkind.** 1978. Erythroleukemic differentiation. Annu. Rev. Biochem. **47**:419–448.
18. **Prochownik, E. V., and J. Kukowska.** 1986. Deregulated expression of c-myc by murine erythroleukemia cells prevents differentiation. Nature (London) **322**:848–850.
19. **Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg.** 1977. Labeling of DNA to high specific activity in vitro by nick translation with DNA polymerase one. J. Mol. Biol. **113**: 237–251.
20. **Sarid, J., T. D. Malazonetis, W. Murphy, and P. Leder.** 1987. Evolutionary conserved regions of the human c-myc protein can be uncoupled from transforming activity. Proc. Natl. Acad. Sci. USA **84**:170–173.
21. **Segal, S., E. Dmitrovsky, and M. P. de la Cedena.** 1988. The effect of a transfected c-myc proto-oncogene on cellular differentiation. Mol. Immunol. **25**:1129–1132.
22. **Stone, J., T. DeLange, G. Ramsay, E. Jakobovits, J. M. Bishop, H. Varmus, and W. Lee.** 1987. Definition of regions in human c-*myc* involved in transformation and nuclear localization. Mol. Cell. Biol. **7**:1697–1709.
23. **Westin, E. H., R. C. Gallo, S. K. Arya, A. Eva, L. M. Souza, M. A. Baluda, S. A. Aaronson, and F. Wong-Staal.** 1982. Differential expression of the AMV gene in human hematopoietic cells. Proc. Natl. Acad. Sci. USA **79**:2194–2198.
24. **Wingrove, T. G., R. Watt, P. King, and I. G. Macara.** 1988. Stabilization of myc proto-oncogene proteins during Friend murine erythroleukemia cell differentiation. J. Biol. Chem. **263**:8918–8924.