MOLECULAR AND CELLULAR BIOLOGY, Jan. 1991, p. 440–444
0270-7306/91/010440-05$02.00/0

Vol. 11, No. 1

Mechanism of Endogenous *myc* Gene Down-Regulation in Eμ-N-*myc* Tumors

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Received 31 May 1990/Accepted 24 September 1990

Transgenic mouse lines carrying the N-*myc* oncogene deregulated by the immunoglobulin heavy-chain enhancer spontaneously develop B-lymphoid tumors (R. Dildrop, A. Ma, K. Zimmerman, E. Hsu, A. Tesfaye, R. DePinho, and F. W. Alt, EMBO J. 8:1121–1128, 1989; H. Rosenbaum, E. Webb, J. M. Adams, S. Cory, and A. W. Harris, EMBO J. 8:749–755). Permanent cell lines derived from these tumors (Eμ-N-*myc* cell lines) express extremely high levels of the N-*myc* transgene but little or no detectable endogenous N-*myc* or c-*myc*. We have employed nuclear run-on assays to show that down-regulation of endogenous N- and c-*myc* expression occurs at the transcriptional level. To determine whether the lack of endogenous *myc* gene transcription is a direct effect of high-level N-*myc* transgene expression, we have generated Abelson murine leukemia virus (A-MuLV)-transformed cell lines from prelymphomatous Eμ-N-*myc* mice (A-MuLV/Eμ-N-*myc* cell lines). Although these A-MuLV/Eμ-N-*myc* lines express very high levels of the N-*myc* transgene, they continue to transcribe the endogenous c-*myc* gene. These findings demonstrate that high-level N-*myc* gene expression alone does not necessarily lead to down-regulation of endogenous *myc* gene expression and suggest that events associated with transformation by N-*myc* may be critical to this process.

The *myc* family of cellular oncogenes includes three well-characterized genes: c-, N-, and L-*myc*. These genes encode structurally related but distinct nuclear phosphoproteins which probably play a role in growth regulation (for reviews, see references 3, 8, and 31). Individual *myc* genes are highly conserved as distinct sequences through evolution, suggesting that they perform unique but related functions. Their divergent expression patterns during murine development reinforce this idea. While c-*myc* is expressed in a wide variety of tissues throughout development, N-*myc* expression and L-*myc* expression are much more restricted (30).

Deregulated c-*myc* expression in tumors such as murine plasmacytomas and human Burkitt's lymphomas is associated with the absence of c-*myc* RNA expression from the normal alleles (1, 18, 28). Different forms of transcriptional repression may be important for silencing the normal allele in different c-*myc* induced tumors (4, 11–13, 19, 22). However, attempts to suppress normal c-*myc* transcription via the deregulated expression of exogenous *myc* genes have yielded conflicting results (e.g., see references 7, 9, 14, 22–24, and 29). Thus, the precise mechanism of c-*myc* suppression in these transformed cells remains unclear. Deregulated N-*myc* expression also is associated with down-regulation of endogenous c-*myc* expression in certain tumors that express extremely high levels of N-*myc* (3, 7, 20, 21). Thus, *myc* family genes may demonstrate cross-regulation as well as autoregulation. Nevertheless, certain normal cells and tumors can coordinately express low levels of N- and c-*myc* simultaneously. It remains unclear whether down-regulation of endogenous c-*myc* genes in high-level N-*myc*-expressing tumors results directly from N-*myc* expression above a certain threshold level (5, 22) or reflects suppression by normal regulatory elements which do not affect the transforming N-*myc* gene. These possibilities have been considered in detail with respect to c-*myc* autoregulation (for a review, see reference 8).

We and others have analyzed the transforming potential of an N-*myc* transgene (Eμ-N-*myc*) that was deregulated with an immunoglobulin heavy-chain enhancer element (10, 25). Lymphoid tumors (Eμ-N-*myc* tumors), which arose stochastically in transgenic mice, all express extremely high levels of the N-*myc* transgene and undetectable levels of c-*myc* and endogenous N-*myc*. We now have employed nuclear run-on transcription analyses to determine whether down-regulation of endogenous *myc* gene expression occurs at the transcriptional level. Furthermore, we have tested whether decreased expression of endogenous *myc* genes in Eμ-N-*myc* lines is a direct effect of high-level N-*myc* expression by comparing *myc* gene expression levels in Eμ-N-*myc* tumors with those found in Abelson murine leukemia virus (A-MuLV)-transformed cell lines from Eμ-N-*myc* and normal mice.

MATERIALS AND METHODS

Generation of Eμ-N-*myc* and A-MuLV/Eμ-N-*myc* cell lines. Eμ-N-*myc* cell lines were derived from spontaneously arising Eμ-N-*myc* tumors as described before (10). A2, B3, and C1 cell lines were subcloned by limiting dilution from the parental lines 84.27, 1810.15, and 171.732, respectively. A2 and B3 are surface immunoglobulin-positive cell lines representing the B-cell stage of development, while C1 has rearranged endogenous heavy-chain but not light-chain genes, expresses cytoplasmic heavy chain, and thus represents the pre-B-cell stage of development. A-MuLV cell lines were generated by infecting the bone marrow cells of 4-week-old Eμ-N-*myc* mice and normal littermates (for details, see reference 26). The frequency of A-MuLV transformants from several independent infections was scored, and independent clones were expanded for analysis.

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Nuclear run-on analysis. Isolation of nuclei, labeling of nascent mRNA, and hybridization to immobilized *myc* gene probes were performed essentially as described before, with the modification that membranes were treated with 10 μg of RNase per ml for 1 h at 37°C before probe was washed off (17). Autoradiograms were analyzed with a Molecular Dynamics 300A computing densitometer (Columbia University Cancer Center). After subtraction of background, each *myc* gene fragment signal was quantitated and normalized to the glyceraldehyde phosphate dehydrogenase (GAPDH) signal from the same filter.

S1 protection analysis. For S1 analyses of endogenous N-*myc* expression, a 660-bp *Sac*I-*Bam*HI genomic fragment spanning the initiation of exon I of murine N-*myc* was subcloned into an M13 phage vector and uniformly labeled with [^{32}P]dATP by a previously described technique (6). A single-stranded DNA probe was hybridized to RNA from various cell types, digested with S1 nuclease, and analyzed on denaturing polyacrylamide gels as described before (16).

RESULTS

Lack of transcriptional initiation of c-*myc* in Eμ-N-*myc* cell lines. To determine the level at which c-*myc* down-regulation occurs in Eμ-N-*myc* lines, nuclear run-on assays were used to quantitate N- and c-*myc* transcription in Eμ-N-*myc* and control lines. N-*myc* and c-*myc* plasmids were first digested with appropriate enzymes to release fragments distinguishing the various exons of the N- and c-*myc* genes (Fig. 1C) and then the fragments were separated on 1% agarose gels and transferred to nitrocellulose filters. An insert corresponding to a GAPDH cDNA was included as a control. Figure 1A and B show a photograph and sketch of a gel containing the various *myc* gene fragments. Nuclei were prepared from Eμ-N-*myc* pre-B-cell (C1) and B-cell (A2, B3) tumor lines and from A-MuLV-transformed pre-B-cell lines (300-19P, ED20) from normal mice. Nascent mRNA strands in these nuclei were metabolically labeled with [^{32}P]UTP, isolated, and used to probe the Southern blots (Fig. 2; only results from the C1 and 300-19P lines are shown).

Nascent RNA from 300-19P hybridized to all three exons of both N- and c-*myc* genes (Fig. 2A). This corresponds to the coordinate low-level steady-state expression of these genes seen by Northern (RNA) analysis (Fig. 3, 300-19P lane). In contrast (Fig. 2B), nascent RNA from the C1 Eμ-N-*myc* line hybridized only to N-*myc* exons II and III (derived from the truncated N-*myc* transgene; see below) and not to any c-*myc* exons. To compare relative *myc* gene transcription in different lines, c- and N-*myc* signals from each autoradiogram (e.g., Fig 2) were quantitated by densitometry and normalized relative to the GAPDH signal for that cell line (Table 1). These results clearly indicate that transcription across the c-*myc* gene is markedly reduced in Eμ-N-*myc* pre-B-cell (C1) and B-cell (A2, B3) lines compared with normal A-MuLV transformed pre-B-cell lines. Transcriptional attenuation of c-*myc* transcription in the first intron has been shown to be an important mechanism by which c-*myc* expression is regulated (for a review, see reference 31). Thus, the lack of hybridization to the c-*myc* exon I probe in Eμ-N-*myc* lines indicates that c-*myc* down-regulation occurs because of the lack of transcriptional initiation rather than transcriptional attenuation. Therefore differences in transcriptional initiation correlate with differences in steady-state c-*myc* message levels observed by Northern blot analyses. Furthermore, since B3 represents a B-cell line while C1 represents a pre-B-cell line, the down-

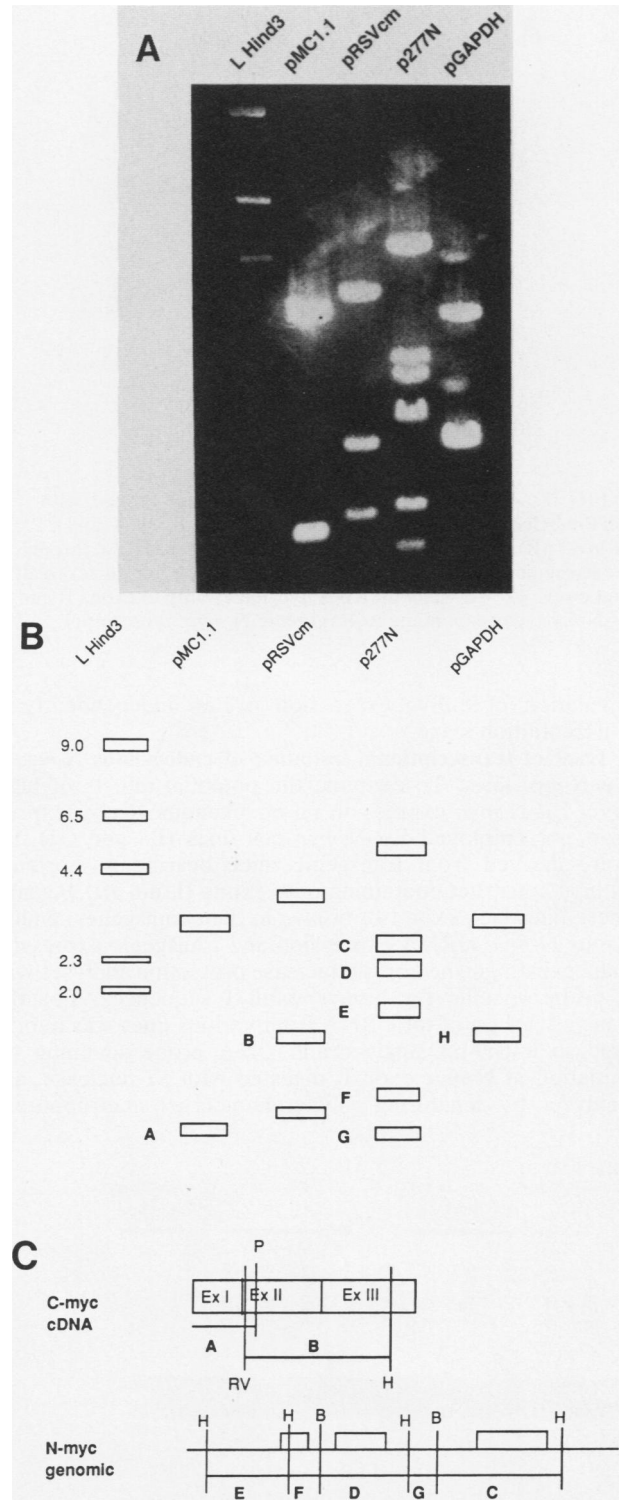

FIG. 1. (A) Photograph of 1% agarose gel. Lanes (from left to right) show lambda phage *Hin*dIII markers, *Pst*I digest of pMC1.1 releasing 680-bp *Pst-Pst* cDNA fragment corresponding to exon I and 120 bp of exon II c-*myc*, *Eco*RV-*Hin*dIII digest of pRSVcm releasing 1.6 kb of coding sequences of exons II and III, *Bgl*II-*Hin*dIII digest of p277N releasing five genomic fragments spanning N-*myc*, and *Pst*I digest of pGAPDH releasing a 1.5-kb cDNA fragment. (B) Sketch indicating gel bands of N- and c-*myc* genes. (C) Sequences of N- and c-*myc* genes. H, *Hin*dIII; B, *Bgl*II; P, *Pst*I; RV, *Eco*RV; A through G, gene segments corresponding to bands in panels A and B.

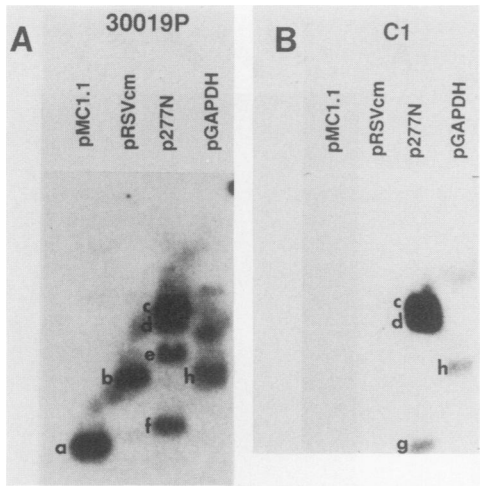

FIG. 2. Autoradiographs of a Southern blot probed with [^{32}P] UTP-labeled nascent RNA from 300-19P (A-MuLV) (A) and C1 (Eμ-N-*myc*) (B) cell lines (see Materials and Methods). a through h, Gene segments indicated in Fig. 1. 300-19P RNA hybridizes to all N- and c-*myc* exons, while C1 RNA hybridizes only to exons II and III of N-*myc*, corresponding to transgenic N-*myc* transcription.

TABLE 1. Transcription of *myc* genes in A-MuLV and Eμ-N-*myc* cell lines

Oncogene and exon(s)	Relative transcription in[a]:			
	A-MuLV cell line 300-19P	Eμ-N-*myc* cell line		
		A2	B3	C1
N-*myc*				
I	1.35	26.1	0.05	0.04
II and III	2.94	29.0	6.7	19.6
c-*myc*				
I	1.89	<0.1	<0.1	<0.1
II and III	1.46	<0.01	<0.01	<0.01

[a] Values are band intensities of run-on analyses determined by densitometry and normalized to GAPDH signal, which was taken to be 1.

regulation of c-*myc* expression occurs independently of differentiation stage.

Lack of transcriptional initiation of endogenous N-*myc* in Eμ-N-*myc* lines. To examine the potential affects of high-level Eμ-N-*myc* expression on endogenous N-*myc* expression, we employed Eμ-N-*myc* cell lines (B3 and C1) that were derived from transgenic mice bearing a shortened N-*myc* construct containing only exons II and III. Because these lines lack exon I of N-*myc* in their transgenes, endogenous N-*myc* mRNA expression and transgenic expression can be distinguished by S1 nuclease protection analyses with a probe specific for N-*myc* exon I sequences. For this analysis, 20 μg of total RNA from various lines was hybridized to a 660-bp single-strand DNA probe spanning the initiation of N-*myc* exon I, digested with S1 nuclease, and analyzed by denaturing polyacrylamide gel electrophoresis (Fig. 4). As observed with other Eμ-N-*myc* lines (25), steady-state endogenous N-*myc* levels, reflected by a 180-bp protected signal, are clearly reduced in Eμ-N-*myc* lines (10.9, 171, and 1810) compared with levels in the control lines 300-19P (A-MuLV line) and NBA.2 (murine neuroblastoma cell line containing a single copy of N-*myc*). Analysis

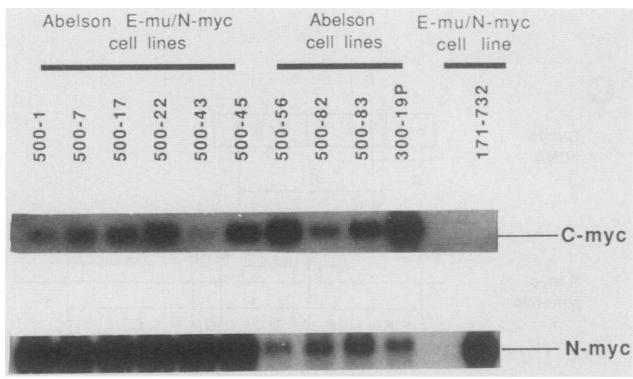

FIG. 3. Autoradiographs of duplicate Northern blots of 10 μg of total RNA from A-MuLV/Eμ-N-*myc* (500-1 through 500-45), A-MuLV (500-56 through 300-19P), and Eμ-N-*myc* (171.732) cell lines hybridized with nick-translated N- and c-*myc* probes. Steady-state N-*myc* expression is seen at high levels in all A-MuLV/Eμ-N-*myc* lines, at slightly lower levels in the Eμ-N-*myc* line (171.732), and at low levels in normal A-MuLV lines. Steady-state c-*myc* expression is seen in all cell lines except 171.732.

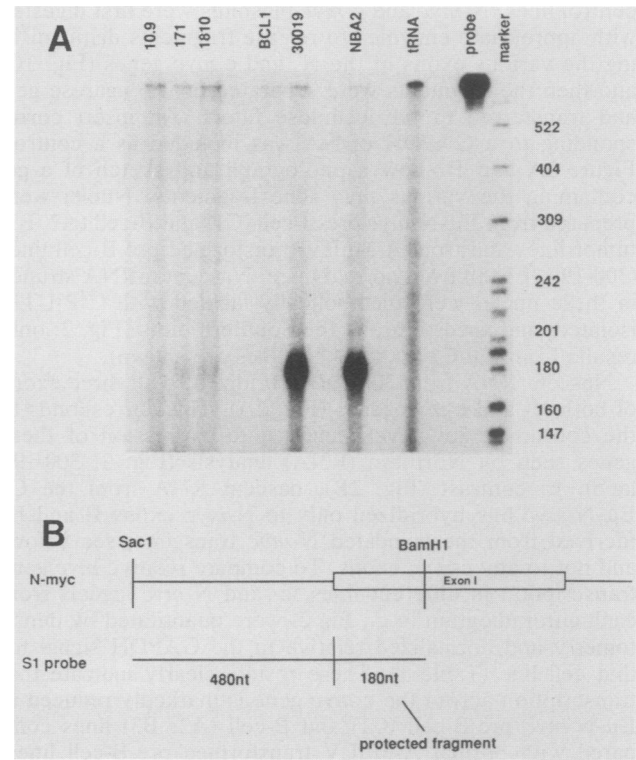

FIG. 4. S1 protection analysis of various cell lines using uniformly labeled, M13 single-stranded DNA probe (see Materials and Methods). (A) A 660-bp *Sac*I-*Bam*HI genomic fragment was subcloned into M13, uniformly labeled with [^{32}P]dATP, and isolated. The resulting single-stranded probe was hybridized to 20 μg of total RNA from the following cell lines, in lanes from left to right: 10.9, 171.732, and 1818.15 (Eμ-N-*myc* cell lines from transgenes containing only exons II and III [10]); BCL1 (B-cell line which does not express N-*myc*); 300-19P (A-MuLV pre-B-cell line); and NBA.2 (single copy neuroblastoma cell line). (B) Predicted protected fragment for N-*myc* transcripts originating at normal exon I initiation is 180 bp. nt, Nucleotides.

of relative hybridization to the N-*myc* exon I fragment in the nuclear run-on experiments (Fig. 1; Table 1) reveals that the absence of steady-state endogenous N-*myc* mRNA in B3 and C1 is correlated with the lack of N-*myc* exon I transcription in the B3 and C1 lines. Exon I transcription in the A2 cell line is presumably derived from the full-length transgene in this line. Since B-cell lines generally lack N-*myc* expression, the absence of N-*myc* transcription in the B-cell-stage line B3 can be due to multiple factors. However, pre-B-cell lines coordinately express both N- and c-*myc*. Therefore, the down-regulation of endogenous N-*myc*, like that of c-*myc*, occurs at the level of transcriptional initiation in the pre-B-cell-stage line C1.

High level N-*myc* expression is not sufficient to down-regulate c-*myc*. To study whether high-level N-*myc* expression per se is sufficient to explain down-regulation of c-*myc* expression in Eμ-N-*myc* lymphomas, A-MuLV-transformed pre-B-cell lines (26) were generated by infecting bone marrow cells from 4- to 5-week-old transgenic Eμ-N-*myc* mice and normal littermates. In several experiments, no reproducible differences in the numbers of A-MuLV transformants obtained from Eμ-N-*myc* and normal mice were observed, suggesting that transformation of these lines was not influenced by the Eμ-N-*myc* transgene (data not shown). Multiple cell lines were established from these transformations; assays of their genomic DNA for hybridization to an *abl* probe demonstrated that all were clonal and arose from independent events (data not shown). Various analyses, including characterization of immunoglobulin heavy- and light-chain rearrangements and λ-5 expression, demonstrated that the A-MuLV/Eμ-N-*myc* lines resembled normal A-MuLV pre-B-cell lines with respect to commonly used staging assays (data not shown) (27).

Steady-state levels of N- and c-*myc* RNAs from various normal A-MuLV transformants, A-MuLV transformants from Eμ-N-*myc* mice, and Eμ-N-*myc* cell lines were quantitated by Northern blot analysis. A-MuLV/Eμ-N-*myc* lines all expressed high levels of N-*myc*, compared with normal A-MuLV lines and the Eμ-N-*myc* line, 171.732 (Fig. 3). Western blot (immunoblot) analyses of nuclear lysates from Eμ-N-*myc* and A-MuLV/Eμ-N-*myc* lines revealed N-*myc* protein levels proportional to RNA expression levels (data not shown). Nevertheless, c-*myc* RNA is expressed at significant levels in all A-MuLV/Eμ-N-*myc* lines with a range of levels comparable to those observed in normal A-MuLV lines (Fig. 3). This contrasts with the lack of detectable c-*myc* expression in 171.732 (Fig. 3) and in 13 other independent Eμ-N-*myc* cell lines studied previously (10). Nuclear run-on assays of one the A-MuLV/Eμ-N-*myc* lines confirmed detectable c-*myc* transcriptional initiation in proportion to c-*myc* steady-state RNA levels (data not shown). Therefore, high-level N-*myc* expression is insufficient to dramatically down-regulate c-*myc* transcription.

DISCUSSION

As a transforming gene in Eμ-N-*myc* tumors, N-*myc* appears to replace c-*myc* in supporting cell growth. Deregulated N-*myc* expression may also function analogously to deregulated c-*myc* expression in causing suppression of endogenous gene transcription in these tumors. We do not detect transcription across the endogenous c- and N-*myc* genes, and thus, we conclude that the absence of transcriptional initiation accounts for the absence of steady-state endogenous *myc* gene expression. The finding that Eμ-N-*myc* pre-B- and B-cell tumors lack both endogenous c-*myc* transcription and endogenous N-*myc* transcription suggests that a common mechanism affects both *myc* genes during the genesis of these tumors. Indeed, the findings that c-*myc* down-regulation in c-*myc* and v-*myc* induced tumors occurs at the level of transcriptional initiation (7, 22) suggest that a similar general mechanism may account for both auto- and cross-regulation of *myc* genes.

Extremely high levels of N-*myc* expression have been observed in nearly all tumors that down-regulate c-*myc* expression. N-*myc* expression levels above a threshold from an exogenous expression vector down-regulate major histocompatibility complex class I gene expression in some cell lines (5). In addition, exogenous c-*myc* protein levels have been inversely correlated with endogenous c-*myc* transcription in cell lines (22). However, our analyses clearly demonstrate that c-*myc* down-regulation occurs in Eμ-N-*myc* lines but not A-MuLV/Eμ-N-*myc* lines despite similar levels of N-*myc* expression. Furthermore, we have used an anti-N-*myc* antiserum to demonstrate comparable levels of N-*myc* protein in A-MuLV/Eμ-N-*myc* and Eμ-N-*myc* cell lines. Thus, the high level of N-*myc* expression in Eμ-N-*myc* lines is not sufficient to explain c-*myc* down-regulation.

We propose that differences in the mechanism of tumor formation between Eμ-N-*myc* and A-MuLV-transformed Eμ-N-*myc* B-lineage tumors are critical factors leading to down-regulation of endogenous *myc* genes. Since the number of A-MuLV lines derived from Eμ-N-*myc* mice is similar to the number of A-MuLV lines derived from normal littermates, the N-*myc* transgene does not contribute in a primary way to the development of A-MuLV/Eμ-N-*myc* lines. By contrast, the N-*myc* transgene is clearly involved in the development of Eμ-N-*myc* tumors. Thus, factors related to the participation of N-*myc* in the transformation of Eμ-N-*myc* lines are probably critical to the suppression of endogenous N- and c-*myc* genes. Our results are in accord with recent findings of down-regulation of the endogenous c-*myc* gene in Eμ-c-*myc* pre-B lines but not in A-MuLV-transformed Eμ-c-*myc* pre-B lines (2).

A general picture of endogenous *myc* gene suppression in *myc*-induced tumors emerges from the consideration that virtually all known tumors express c-*myc* except those in which a deregulated *myc* gene plays a role in the transformation process. c-*myc* is known to be important in the proliferation of normal cells, and its expression is probably necessary for the proliferation of most tumors (for a review, see reference 8). Constitutive normal c-*myc* expression in tumor cells can be explained if an activated oncogene in the tumor exerts its influence directly or indirectly through c-*myc* (15). We consider *myc* expression in the A-MuLV transformants to represent such a case; an alternative way of stating this possibility is that expression of the *abl* product in such cells induces normal endogenous *myc* expression (2). The only tumors not expected to require endogenous *myc* expression would be those with directly deregulated *myc* expression, i.e., *myc*-induced tumors. In such tumors, negative feedback upon endogenous *myc* genes may be effected by normal upstream growth regulation signals.

According to this model, deregulated N-*myc* expression could replace c-*myc* function in tumor cells, leading to down-regulation of endogenous *myc* genes. N-*myc* deregulation is seen in fewer tumors and its expression levels are generally more substantially increased than those of c-*myc* in corresponding tumors in which deregulation of the genes has been respectively implicated. Therefore, it is possible that additional genetic alterations associated with the role of N-*myc* in the transformation process are required for N-*myc*

to fully replace c-*myc*. These could include activation of other oncogenes and/or additional modifications of the N-*myc* gene leading to altered gene products.

ACKNOWLEDGMENTS

This work was supported by the Howard Hughes Medical Organization and by NIH grants CA42335, AI20047 (F.W.A.), CA23767 (F.W.A.), and CA 24220 and CA 33771 (N.R.). A.M. was supported by a Physician-Scientist Award.

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