Regulation of N-myc Gene Expression: Use of an Adenovirus Vector To Demonstrate Posttranscriptional Control

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We present evidence that differences in the levels of N-myc mRNA among different cell types are the result of posttranscriptional control. First, we noted that while steady-state mouse N-myc mRNA could be detected only in fetal mouse brain, it was transcribed at an equivalent rate in adult brain, liver, spleen, and placenta and in fetal brain. Similarly, the human N-myc gene was transcribed at an equivalent rate in HeLa cells, which do not accumulate this RNA in the cytoplasm, and cell lines G401 (a Wilms tumor-derived cell line) and SKNMc (established from a primitive neuroepithelioma), which do express N-myc RNA. As expected, the N-myc promoter functioned at equivalent rates, as demonstrated by the level of a reporter gene, when introduced into these cell types by using a recombinant adenovirus vector. The suggestion that posttranscriptional mechanisms control the level of this RNA was supported by the observation that sequences in the N-myc third exon specifically decreased the level of E1A mRNA when these sequences were placed downstream of the E1A promoter in a recombinant adenovirus. Finally, we further localized these sequences to a 600-bp fragment of the third exon by introducing various subclones of this sequence downstream of the E1A promoter in both viral and plasmid vectors.

N-myc is a cellular gene that was originally isolated as an amplified sequence in neuroblastoma cells and was found to be structurally related to the c-myc proto-oncogene (17, 18, 24, 25). While c-myc gene expression has been demonstrated in a large number of cell types, expression of N-myc mRNA is restricted in its tissue distribution (9, 13, 31, 32). This property of N-myc is a feature of other genes in the myc gene family, since additional members, including L-myc and R-myc, display restricted tissue cell distributions (21).

The N-myc gene is expressed during mouse development at high levels in neonatal brain, kidney, and intestine (9, 31, 32). Relatively high levels of N-myc mRNA can also be detected in some other neonatal tissues, but only low levels of N-myc mRNA are detectable in most adult tissues. Thus, quantitative and qualitative differences in the levels of N-myc mRNA are demonstrable among different tissues and during development. N-myc mRNA is also detectable in several tumor cell types, including neuroblastoma, retinoblastoma, small-cell lung carcinoma, Wilms tumor, and primitive neuroepithelioma. Most tumor-derived established cell lines, including HeLa cells, have been previously shown not to accumulate N-myc mRNA in their cytoplasm (16, 22).

In this report, we describe studies that address the molecular basis of the differences in the specificity and level of expression of the N-myc gene among different cell types. Since previous studies failed to measure the relative rate of cellular N-myc gene expression in adult tissues from various established cell lines, it was possible that posttranscriptional control mechanisms could contribute to the expression of this mRNA. We have found, in fact, that the mouse N-myc gene is transcribed at equivalent levels in many adult mouse tissues examined and demonstrate that cell-specific differences in the level of N-myc RNA are the result of posttranscriptional control. Additional experiments using cultured tumor cell lines are presented which demonstrate that the DNA sequences necessary for this posttranscriptional control reside in the third exon and likely act to control the stability of N-myc RNA.

MATERIALS AND METHODS

Isolation of recombinant adenoviruses and preparation of virus stock. The viruses used included H5 dI309 (14), which has replicative characteristics in HeLa cells that are indistinguishable from those of wild-type adenovirus type 5; H5 dI313 (14), which has a deletion of the viral DNA sequences (second exon of E1A and the entire EIB gene coding sequences) extending from nucleotides 1338 to 3640; and a series of recombinant adenoviruses containing various regions of the mouse cellular N-myc gene cloned into the left end of the viral genome. The Ad-N-myc3 virus contains the leftmost 194 bp of adenovirus fused to N-myc sequences extending from nucleotides −1800 to +950, relative to the start site of N-myc transcription (11). The N-myc sequences at +950 were then ligated to the adenovirus sequences beginning at nucleotide 3328 (a BglII site) and extend to the right end of the viral genome. The Ad-N-myc3 virus contains the leftmost 1,338 bp of adenovirus (including the E1A gene cap site and 13S and 12S mRNA splice donor and acceptor sites) fused to a region of the mouse N-myc gene extending from nucleotides +4246 to +5726 [exon 3 and sequences 3' to the poly(A) addition site]. The 3' N-myc sequences were then fused to adenovirus sequences at nucleotide 3328, as for the Ad-N-myc1 virus. Both viruses were isolated by in vivo overlap recombination, using H3 dI309 parental viral genomes and human 293 cells to complement the viral E1A and E1B gene defects of the recombinant progeny viruses (14). The methodologies used to construct, isolate, characterize, and prepare high-titer virus stocks have been previously described (11).

Plasmid constructions and DNA transfection assays. Plasmid AdBgl3.8 was isolated by first cloning the adenovirus type 5 Xhol C fragment (nucleotides 0 to 5780) into the plasmid vector pMK2004. Viral sequences between 1338 (an
XbaI site) and 3328 (a BglII site) were deleted to generate a plasmid containing a unique BglII site at this deletion junction. Initially, the mouse N-myc BamHI (−1800) and Clal (+4246)-to-EcoRI (+5726) DNA fragments were cloned into plasmid AdBG3.8 (see Fig. 5C). Subsequently, a series of DNA subclones from the N-myc Clal-EcoRI fragment was cloned into this plasmid vector (9). These subclones included HC (+4246 to +4883), SH (+4883 to +5295), and RS (+5295 to +5726) (see Fig. 5C). For all constructs, the restriction enzyme-generated termini were converted to BamHI sites by using DNA linkers. In most cases, both orientations were obtained. DNAs specific for the three mouse N-myc exons were prepared by subcloning EcoRI-BglII (−1800 to +950), BglII-BglII (+950 to +3464), and BglII-EcoRI (+3464 to +5726) DNAs into the Gemini plasmid vector. An additional plasmid was constructed in which an EcoRI-BamHI restriction fragment extending from −1800 to +180 was subcloned into Gemini. This plasmid was linearized at the unique EcoRI site to generate antisense transcripts capable of scoring the mouse N-myc start site. Probes for the human N-myc gene were generated by subcloning an EcoRI-Xho and a Xho-BamHI fragment from the human N-myc gene into Gemini. The Xho-BamHI fragment contains a portion of the N-myc second exon; the EcoRI-XhoI plasmid is intronic (18).

Human neuroblastoma cell lines SKNMc (27) and G401 (1) and human HeLa cells were maintained as monolayer cultures and grown in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum. All three cell types were transfected at 50% confluence, using the calcium phosphate-DNA coprecipitation technique as previously described (6). The N-myc–adenovirus hybrid plasmids were cotransfected with a simian virus 40 enhancer–β-globin promoter-containing plasmid construct (SVglo), to control for variations in transfection efficiencies, into the three cell lines. After a glycerol shock (10% glycerol in phosphate-buffered saline) at 4 h posttransfection, cytoplasmic RNA was isolated at 36 h posttransfection. RNA was quantitated and mRNA levels were determined as previously described (11).

RNA preparation and analysis. RNAs from mouse tissues or mouse and human cell lines were prepared by the guanidinium thiocyanate technique of Chirgwin et al. (5). Cell pellets or tissues were dissolved in guanidinium thiocyanate, and the RNA was pelleted through a cesium chloride cushion. For quantification of specific RNA species, RNase T2 protection assays were performed in which equivalent amounts of RNA were hybridized to specific uniformly labeled antisense RNA probes and subsequently trimmed with RNase T2 (20). The resulting digestion products were resolved on a denaturing 5% polyacrylamide gel.

Transcription rates were measured by incubating nuclei isolated from mouse tissues or cell cultures with [32P]UTP and cold GTP, CTP, and ATP to allow previously initiated RNA polymerase molecules to elongate (10). Nuclear RNA was isolated, followed by hybridization of equivalent numbers of counts to filters onto which cloned denatured DNAs had been spotted (10). After hybridization, the filters were treated with RNase T1, RNase A, and proteinase K; washed; and exposed to autoradiography.

RESULTS

The mouse N-myc gene is transcribed in most mouse tissues. Eucaryotic gene expression can be regulated at multiple levels (8). While most tissue-specific genes are transcription-

ally controlled, quantitative differences in the levels of individual mRNAs are often the result of posttranscriptional regulation (2, 10, 12, 23). To consider the level of gene expression relevant in the control of the mammalian N-myc gene, we first measured the transcription rate and steady-state mRNA levels of this gene in a variety of mouse tissues.

The levels of steady-state N-myc mRNAs were measured by use of an RNase T2 protection assay specific for the first exon and transcriptional start site of the mouse N-myc gene. An EcoRI-BamHI fragment was isolated from the mouse N-myc gene which spanned the predominant transcriptional start site (multiple start sites have been identified as a result of the absence of a well-defined TATA element (9)) and 180 bp of the first exon. This was cloned into the Gemini cloning vector. This plasmid was linearized with EcoRI, and an antisense transcript was synthesized by using SP6 RNA polymerase and hybridized to 20 μg of total RNA from fetal brain and from adult brain, liver, spleen, kidney, and placenta (Fig. 1A). A band of ~180 bp was protected in the fetal brain sample. N-myc RNA was not detectable in any other tissues. The levels of actin RNA were similar among these samples except fetal brain, which had an approximately threefold-higher level of actin RNA (23). These data are consistent with prior results documenting the size of the N-myc first exon, the relatively high level of expression in fetal brain, and the low levels of this mRNA in adult tissues (9).

In vitro nuclear run-on assays were performed, using nuclei isolated from these same tissues. An equivalent amount of [32P]UTP-labeled nuclear RNA from each organ (fetal brain, adult brain, liver, spleen, kidney, and placenta) was hybridized to filters onto which were spotted DNAs specific for each of the three N-myc exons as well as actin (10). In each case, similar rates of N-myc transcription were observed among the tissues tested (Fig. 1B). This was determined by quantitating densitometric tracings and correcting signal intensity to that of the actin signal observed. Moreover, the signal intensity, when corrected for insert size, was equivalent for the exon 2 and 3 N-myc probes. With the exception of adult placenta, the exon 1 signal was greater than would have been predicted. Recent studies by Krystal et al. (19) most likely explain our findings. Their studies showed nearly equivalent rates of sense and antisense transcription across exon 1 of the human N-myc gene. Since our probes were double stranded, we were most likely scoring both of these signals with our exon 1 probe, which explains the greater than equimolar signal observed. The inserts from each of these clones hybridized to single bands on Northern (RNA) blots, suggesting that it was the single-copy N-myc sequences that were detected in this assay (data not shown). These findings demonstrate that the N-myc gene is transcribed in most, if not all, tissues at similar rates and that transcription is equimolar (when the exon 1 signal is corrected for the contribution of the antisense signal) across the transcription unit. Therefore, the lack of detectable N-myc mRNA in these mouse organs is likely the result of posttranscriptional regulation. In these experiments, two plasmid DNAs, pBR and Ad3.8, which is a pBR plasmid containing adenoviral sequences, were spotted to control for nonspecific hybridization signals.

The human N-myc gene is transcribed in many cultured cell types. To examine the regulatory level at which the N-myc gene is controlled in cultured cells, the transcription rates and steady-state levels of N-myc mRNA were measured in subconfluent monolayers of several human cell lines, including one neuroblastoma, IMR (29); a Wilms tumor, G401 (1);
FIG. 1. Analysis of N-myc gene transcription and cytoplasmic mRNA accumulation in fetal and adult mouse tissues. Fetal mouse brains (FB) and adult mouse brains (AB), spleens (AS), livers (AL), kidneys (AK), and placentas (AP) were isolated, and total organ RNA was prepared (5). After purification through a cesium chloride cushion (see Materials and Methods), RNA from each tissue [20 µg of poly(A)-selected RNA] was hybridized to a uniformly 32P-labeled SP6-generated antisense RNA probe (7.5 × 10^6 cpm) specific for the mouse N-myc 5' end or the human γ-actin mRNA (20) (A). After hybridization and RNase T1 digestion, the T1-resistant hybrids were electrophoresed; autoradiographic exposure was for 48 h. The length of the protected RNA probe was determined by comparison with labeled DNA markers; these lengths are shown at the bottom of and on each side of panel A. To analyze the rates of N-myc gene expression among the various mouse organs (B), nuclei were isolated and labeled nuclear RNA was extracted after nascent-chain elongation in the presence of [32P]UTP (10). Labeled RNA (10 × 10^6 cpm) was hybridized to dots of DNA (7 µg per dot) on nitrocellulose filters. Unpaired RNA was digested by RNase, and hybrids were detected by autoradiography. (C) Positions of the various DNAs. These DNAs include the mouse N-myc exon 1 (ex 1; -1800 to +950), exon 2 (ex 2; +950 to +3464), and exon 3 (ex 3; +3464 to +5726) (9), chicken β-actin, pBR322, and AdBG3.8. The latter two DNAs were used as controls for nonspecific hybridization signals.

two primitive neuroepithelioma tumors; SKNMc (27) and SKNDw (27); and HeLa cells. The IMR neuroblastoma cell line has been shown to express high levels of N-myc mRNA as a result of amplification of the endogenous N-myc gene sequences; the Wilms tumor cells have been known to express high levels of N-myc RNA but have not amplified the N-myc gene; primitive neuroepithelioma tumors are reported to express low levels of the N-myc mRNA from a single copy of the N-myc gene; and HeLa cells do not synthesize steady-state N-myc RNA (22). Analysis of the levels of steady-state N-myc RNA are shown in Fig. 2A, in which a subclone of the human N-myc second exon was used as the template for the synthesis of [32P]UTP-labeled antisense poly(A)-selected RNA and then hybridized at 59°C to RNA from each of these cell lines (24). An RNase T1-protected RNA of 577 nucleotides was detected in all of the neuronal cell types. Consistent with prior reports, IMR expressed 100-fold higher levels of N-myc RNA than did G401 and SKNMc cells. In this experiment, SKNMc cells had higher levels of N-myc RNA than did G401 cells. The signal present in the HeLa cell lane of Fig. 2A is full-length probe which was improperly digested by RNase. HeLa cells failed to accumulate any N-myc mRNA, which is consistent with previous studies (22).

The rate of cellular N-myc gene transcription was measured in these cell lines by isolating nuclei from subconfluent monolayers and labeling previously initiated RNA chains with [32P]UTP. Labeled RNA was isolated and hybridized to filters onto which had been spotted two subclones of the human N-myc gene that were derived from a 1-kb BamHI-EcoRI fragment that includes a portion of intron 1 and exon 2. Subclones of this 1-kb fragment were ligated into Blue-script as a 600-bp Bam-Xho fragment that is intronic and a 500-bp Xho-EcoRI fragment that includes a small amount of intronic sequence and 400 bp of exon 2. Neither of these clones contains repetitive DNA, since both hybridize to single bands on Southern blots (data not shown). The Xho-EcoRI clone hybridized to a single RNA species on a Northern blot, and the Bam-Xho clone did not give a detectable signal when hybridized to RNA derived from these cell lines (data not shown).

Similar rates of transcription were seen among HeLa, SKNMc, and G401 cell lines even though HeLa cells do not accumulate cytoplasmic N-myc mRNA. The transcription
rate in the cells with amplified N-myc sequences (IMR), however, was 10-fold higher than that in cell lines with a single copy of the N-myc gene. This increased transcription rate of the N-myc gene in IMR cells did not completely account for the 100-fold difference in the level of steady-state mRNA in these cells. Thus, posttranscriptional control may account for qualitative and quantitative differences in the levels of N-myc RNA among cell lines with a single copy of the endogenous N-myc gene.

The mouse N-myc promoter functions in cultured human cell lines regardless of the endogenous levels of N-myc mRNAs. These results predict that an exogenous N-myc promoter should function at equivalent rates in all cells regardless of whether endogenous steady state N-myc RNA is present. To monitor the activity of the mouse-N-myc promoter in cultured cells, we made use of a recombinant adenovirus expression vector. This vector was previously used to demonstrate cell-specific expression of the rat albumin and mouse immunoglobulin promoters (11). A 2.75-kbp EcoRI-BglII fragment of the mouse N-myc gene which extends from nucleotides −1800 to +950 was subcloned into the BglII site of the parent plasmid, AdBgl6, using BglII linkers. AdBgl6 is a modified plasmid that includes adenoviral sequences from 0 to 15.5 map units (11). In this plasmid, adenoviral DNA sequences between bp 194 and 3320 have been deleted and replaced with the BglII linker. In the resulting plasmid, the E1A gene as well as the E1B promoter and first exon have been removed and replaced by the N-myc promoter and first exon. In this construct, the N-myc promoter directs the synthesis of a fusion mRNA in which the mouse N-myc first exon is spliced to the adenoviral E1B second exon. These sequences were then introduced into a full-length adenovirus genome by in vivo overlap recombination, and the resulting recombinant virus was named Ad-N-myc1 (Fig. 3A). Both of the alternative leader sequences of the N-myc first exon were included in this construct (28).

In our initial experiments, sub confluent monolayers of each of the cultured cell lines shown in Fig. 2 were infected at 20 PFU per cell with Ad-N-myc1 (Fig. 3A). Cytoplasmic RNA was prepared from infected cells at 8 h postinfection. RNase protection assays were performed by hybridizing antisense 32P-labeled RNA probes corresponding to the viral E1B gene (Fig. 3B), N-myc 5′ end (Fig. 3C), viral E2A gene (Fig. 3D), and human β-actin gene (data not shown) to RNA (20 μg) from infected G401, SKNMc, SKNDw, and HeLa cells (11). Steady-state levels of N-myc-transcribed E1B mRNA were present in the G401, SKNMc, and HeLa cell lines; in fact, higher levels of E1B were demonstrable in HeLa cells (which do not accumulate endogenous N-myc mRNA) (Fig. 3B). To control for variations in virus uptake by these cell lines, the level of another viral gene, E2A, was measured by using an E2A riboprobe. We could score an E2A mRNA signal in all of the cell lines (Fig. 3D), suggesting that all of the cell types could be infected. An analysis of cellular actin mRNA levels from the different cell lines revealed similar levels, suggesting that the integrity and quantitation of the RNAs were correct (data not shown). To demonstrate that the correct N-myc initiation sites were used on the viral templates, the infected-cell RNA was hybridized to the probe depicted in Fig. 1 and corresponding...
FIG. 3. Demonstration that expression of the mouse N-myc gene from an adenoviral vector results in N-myc exon 1–E1B hybrid mRNA accumulation in HeLa cells. The construction, isolation, and genotypic characterization of the Ad-N-myc1 virus are described in Materials and Methods. Mouse N-myc gene sequences extending from nucleotides −1800 to +950 (including the N-myc transcriptional start site and first exon) were inserted into the left end of the adenovirus genome, resulting in a deletion of the viral E1A gene and 5′ portion of the E1B gene (A). Symbols: \( \square \), N-myc exonic sequences; \( \square \), adenovirus E1B exons. Cytoplasmic RNA was isolated from HeLa, SKNDw, SKNMc, and G401 cells infected with Ad-N-myc1 virus (20 PFU per cell) at 8 h postinfection, and 20 μg was hybridized to \(^{32}\)P-labeled antisense RNA probes (7.5 \( \times \) 10⁵ cpm) representing the viral E1B (B) and E2A (D) genes and mouse N-myc 5′ cap site and exon 1 (C). After RNase T₁ digestion, the T₁-resistant hybrids were analyzed on 5% denaturing acrylamide gels. The lengths (in nucleotides) of the protected RNA probes for each transcription unit are indicated in the line drawings below the panels. The SP6 riboprobe used to detect N-myc mRNA is described in the legend to Fig. 1. Cell lines are indicated above the lanes; lane M contained labeled DNA markers (sizes are indicated in nucleotides).

to the first exon of the mouse N-myc gene. A 180-nucleotide RNA (although multiple RNA species are observed as a result of multiple start sites) characteristic of the correctly initiated mouse N-myc gene was scored in all of the virus-infected cell lines, and the amount of this protected RNA was proportional to the levels of E1B mRNA observed in each cell type. This result was expected, since both assays scored the same mRNAs. This probe was shown not to cross-react with the endogenous human N-myc RNAs expressed in the SK and G401 cell lines (data not shown). We can also conclude that viral DNA replication had not occurred in any of the virus-infected cell lines, since the activity of the viral pIX promoter located in the E1B gene sequences could not be detected (11).

The results depicted in Fig. 3 demonstrate that a correctly initiated N-myc fusion RNA is synthesized in these four cell lines. Thus, both exogenous and endogenous N-myc promoters appear to function at equivalent rates among three cell lines that differ in the accumulation of steady-state levels of the endogenous human N-myc mRNA.

Sequences 5′ to the N-myc poly(A) addition site confer cell-specific expression. After examining the sequence of the N-myc gene, we noted an A+T-rich sequence in the third exon that resembled the sequence seen in other genes that are controlled at the level of RNA stability. Furthermore, the Ad-N-myc1 virus studies suggested that the N-myc sequences in the first exon were not responsible for the cell-specific stability of the N-myc mRNAs. We next tested the possibility that defined sequences were important in N-myc gene control by again using an adenovirus vector. First, a plasmid was constructed in which the viral E1A gene second exon and poly(A) site were deleted at an XbaI site at bp 1338 and were replaced by a Clal EcoRI fragment (nucleotides +4246 to +5726) of the mouse N-myc gene which contained the N-myc third exon and poly(A) site (Fig. 4) (11). This plasmid, in which the E1A promoter directs the synthesis of the N-myc 3′ end, was incorporated into an intact adenovirus by homologous recombination. The genomic organization of the Ad-N-myc3 virus is shown in Fig. 4. Two additional viruses were used in these experiments as a control: H5 dl309, which contains the wild-type E1A gene, and H5 dl313, in which the E1A second exon and poly(A) site were deleted and replaced by the E1B second exon and poly(A) site (Fig. 4A) (14). These viruses were then used to infect G401, HeLa, IMR, and SKNMc cells at a multiplicity of 20 PFU per cell. Whole-cell RNA was prepared 8 h postinfection and hybridized to a \(^{32}\)P-labeled antisense E1A probe as well as to an actin probe.

The effect of the N-myc third exon on RNA stability was monitored by comparing the level of E1A mRNA synthe-
spliced RNA species). In SKNMc and G401 cells, the N-myc third exon reduced the level of E1A RNA two- to threefold relative to the control level. For all studies, the levels of actin mRNA were quantitated to control for variations in RNA. These results demonstrate that RNA sequences in the N-myc third exon can posttranscriptionally regulate the levels of these hybrid mRNA species in a cell-specific fashion and that these sequences are also functional in a recombinant adenovirus. The activity of this element appears to account for at least part of the qualitative and quantitative differences in the expression of N-myc RNA. Nevertheless, these experiments do not distinguish whether the differences in the levels of E1A RNA synthesized from the Ad-N-myc3 virus are the result of differential RNA stability or effects on the efficiency of polyadenylation or nuclear cytoplasmic mRNA transport.

A 600-bp sequence in the N-myc third exon is likely responsible for N-myc RNA stability. The results from the Ad-N-myc3 virus studies suggested that N-myc gene expression is controlled to a large extent by sequences in the third exon. To further delineate the DNA sequences involved in the regulation of this gene, a series of constructs was made in which a number of subgenomic fragments of the N-myc third exon were introduced into the second exon of the E1A gene in a plasmid containing the wild-type E1A promoter and E1B poly(A) addition sites (Fig. 5C). In initial experiments, restriction fragments of the N-myc gene were ligated into the adenovirus gene sequences between nucleotides 1338 (in the E1A gene second exon) and 3328 in plasmid pE1A, using XbaI linkers (11). Plasmid pBE fused the E1A second exon to a BamHI site in the N-myc first exon (+185) and included all three N-myc exons and the poly(A) addition site (+5726). In plasmid pCE, the E1A second exon was ligated to a ClaI site in the N-myc third exon (+4246) and included only the third exon and poly(A) addition site (+5726). pBE therefore contains nearly the entire N-myc coding sequence, and pCE contains only the N-myc third exon. Both plasmids as well as two control plasmids, wild-type E1A plasmid (Ad) and a plasmid in which E1A mRNA utilizes the E1B poly(A) site (AdΔa), were introduced separately into HeLa and SKNMc cells by CaPO4 transfection. After 36 h, cytoplasmic RNA was prepared and assayed for the level of E1A RNAs, using an RNase T2 protection assay. A control plasmid, SVglo, which contains the simian virus 40 enhancer upstream of the mouse β-globin promoter and directs the synthesis of E1B RNA, was cotransfected in each case as an internal control for transfection efficiency (6).

The level of E1A RNA synthesized from pBE and pCE was compared with that synthesized from the control plasmids. In this experiment, the levels of the E1A–N-myc hybrid RNA synthesized from pBE and pCE were considerably lower in HeLa cells than in SKNMc cells after correction for transfection efficiency by using the SVglo control plasmid (Fig. 5A). These data confirm the results for the recombinant adenovirus and also suggest that sequences between the Bam site (+185) in the first exon of the N-myc gene and the Cla site (+4246) in the third exon do not further diminish the level of RNA synthesized in HeLa cells.

To further elucidate which exon 3 sequences were responsible for the diminished level of mouse N-myc expression in HeLa cells, restriction fragments from the N-myc third exon were subcloned into the pE1A plasmid in the sense and antisense orientations. These plasmids were cotransfected into HeLa cells along with the SVglo plasmid control. The level of E1A expression was scored for each of these plasmids and compared with the wild-type E1A level. In this
experiment (Fig. 5B), the levels of E1A mRNA synthesized from the wild-type plasmid 36 h posttransfection were equivalent among the wild-type E1A control and all plasmids except pHC+ (nucleotides +4246 to +4883). Steady-state RNA produced from this plasmid was reduced by a factor of 10 in HeLa cells. However, the plasmid that contained a copy of this sequence in the sense orientation functioned at a rate equivalent to the wild-type E1A rate in G401 and SKNMc cells (data not shown). The restriction fragment that conferred this effect did not include the poly(A) addition site. These data demonstrated that the posttranscriptional control of the N-myc gene is conferred by the activity of DNA sequences in the 3′ untranslated region of this gene.

DISCUSSION

In this study, we investigated the mechanism by which the level of N-myc RNA is regulated among different cell types in vivo and in cultured cells. Our results suggest that the cell-specific regulation of the N-myc gene is effected primarily at the posttranscriptional level. The supporting evidence for this conclusion includes the following: (i) the equivalent
rates of transcription of the N-myc gene, obtained by using in vitro nuclei, among all cell types tested irrespective of the levels of steady-state endogenous RNA (also, there is no evidence to suggest that premature termination is relevant in the control of the N-myc gene, as has been reported for c-myc [3], since transcription is equimolar across the transcription unit in brain, placenta, liver, kidney, and spleen); (ii) the equivalence of the activity of the N-myc promoter in directing the synthesis of a reporter gene even in HeLa cells, which do not accumulate cytoplasmic N-myc mRNA; and (iii) the demonstration that specific sequences in the N-myc third exon can confer cell-specific N-myc mRNA stability when inserted into viral and plasmid constructs which are transcribed at equivalent rates among HeLa, SKNMe, and G401 cells.

These results are similar in some respects to those of Zimmerman et al. (31), who demonstrated that appropriate expression of the transfected mouse N-myc gene is not observed in cultured cells, since cells which failed to express human N-myc RNA still expressed the mouse N-myc RNA. However, these studies showed that sequences from the third exon of the N-myc gene did not alter the level of expression of the reporter gene (31), as we have reported. Of further interest is the observation that N-myc sequences that lack the third exon (which we have shown to destabilize the mRNA in cultured cells) still act to direct cell-specific expression of N-myc in transgenic animals (32). However, it is not entirely clear from these studies whether the expression of the transgene is the result of transcriptional or posttranscriptional control. The reason for this discrepancy is not entirely clear, although the choice of promoter and efficiency of mRNA translation may influence the effect of N-myc exon 3 sequences on mRNA levels. Wilson and Treisman (30) have suggested that the half-life of the cellular fos mRNA is determined by the translation efficiency of the mRNA itself. In our studies, the E1A-N-myc fusion mRNAs may be more efficiently translated than the endogenous N-myc mRNAs and consequently may turn over rapidly as a result of the exon 3 sequences.

In additional experiments, we have localized the sequences responsible for the regulated expression of N-myc RNA to a 600-bp fragment of the N-myc third exon. Cell-specific expression of the gene does not appear to require any additional sequences outside the third exon, since a construct which includes all three exons and extends from a BamHI site in the first exon to the EcoRI site downstream of the third exon is expressed at a rate equivalent to that of constructs which include only sequences from the third exon. Thus, it appears that sequences from the N-myc third exon are both necessary and sufficient for the regulated and cell-specific expression of this RNA.

Previous reports have demonstrated, however, that the regulated expression of some RNAs, particularly those with a short half-life, can be the result of differences in RNA stability (2, 4, 7, 15, 26). These reports have noted that mRNAs which are regulated by differences in RNA stability often have A+U-rich sequences in the 3' untranslated region of the RNA. mRNAs in this class have included certain lymphokines, cytokines, and proto-oncogenes and often have a characteristic TTATTTATT consensus sequence in the 3' untranslated region (4). The mouse N-myc gene appears to be yet another member of this group. The DNA sequences of the N-myc third exon do indeed contain several A+T-rich regions, although the TTATTTATT consensus sequence was not noted in the sequences which are responsible for differences in the stability of the N-myc RNA. c-myc RNA is also regulated by differences in RNA stability, and this mRNA also does not contain a TTATTTATT sequence. The c-myc 3' untranslated region does include an uninterrupted stretch of 11 U residues (15). Similarly, the N-myc RNA has an uninterrupted stretch of 14 U residues (15). It is not clear whether this sequence represents a new motif that regulates RNA stability. A more refined deletion analysis of the sequences in this region that includes the testing of linker-scanning mutants should help localize further the RNA sequences that are required for this effect and determine whether a poly(U) tract can function to destabilize specific RNAs.

In conclusion, we have demonstrated that cell-specific expression of N-myc RNA is likely the result of posttranscriptional control. The sequences that confer this regulation are present in the third exon and include a poly(U) tract, in common with sequences of the c-myc gene that control RNA stability. Finally, we have demonstrated that posttranscriptional gene control can be demonstrated in an adenovirus vector. The use of pulse-chase and approach-to-steady-state methods in cells infected with these viruses may make it possible to study the kinetics of this process.

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LITERATURE CITED


