MOLECULAR AND CELLULAR BIOLOGY, Jan. 1989, p. 288–295
0270-7306/89/010288-08$02.00/0

Vol. 9, No. 1

c-*myc* RNA Degradation in Growing and Differentiating Cells: Possible Alternate Pathways

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Received 1 August 1988/Accepted 3 October 1988

Transcripts of the proto-oncogene c-*myc* are composed of a rapidly degraded polyadenylated RNA species and an apparently much more stable, nonadenylated RNA species. In this report, the extended kinetics of c-*myc* RNA turnover have been examined in rapidly growing cells and in cells induced to differentiate. When transcription was blocked with actinomycin D in rapidly growing cells, poly(A)$^+$ c-*myc* was rapidly degraded ($t_{1/2}$ = 12 min). c-*myc* RNA lacking poly(A) initially remained at or near control levels; however, after 80 to 90 min it was degraded with kinetics similar to those of poly(A)$^+$ c-*myc* RNA. These bizarre kinetics are due to the deadenylation of poly(A)$^+$ c-*myc* RNA to form poly(A)$^-$ c-*myc*, thereby initially maintaining the poly(A)$^-$ c-*myc* RNA pool when transcription is blocked. In contrast to growing cells, cells induced to differentiate degraded both poly(A)$^+$ and poly(A)$^-$ c-*myc* RNA rapidly. The rapid disappearance of both RNA species in differentiating cells suggests that a large proportion of the poly(A)$^+$ c-*myc* RNA was directly degraded without first being converted to poly(A)$^-$ c-*myc* RNA. Others have shown that transcriptional elongation of the c-*myc* gene is rapidly blocked in differentiating cells. We therefore hypothesize that in differentiating cells a direct, rapid degradation of poly(A)$^+$ c-*myc* RNA may act as a backup or fail-safe system to ensure that c-*myc* protein is not synthesized. This tandem system of c-*myc* turnoff may also make cells more refractory to mutations which activate constitutive c-*myc* expression.

The cellular proto-oncogene c-*myc* encodes nuclear protein whose function is not known. Hypotheses for c-*myc* protein function range from that of gene (transcriptional) regulator (22) to participant in DNA replication (17, 31) to a component of the RNA-processing machinery (30). c-*myc* gene expression is highest in proliferating cells, and its expression is rapidly turned off when cells are induced to differentiate (18, 25). The importance of regulated c-*myc* expression to normal cellular growth and differentiation has been demonstrated by the participation of the c-*myc* gene in the genesis of several hematopoietic neoplasias. c-*myc* is translocated to the immunoglobulin heavy-chain locus in Burkitt lymphomas, in mouse plasmacytomas, and in rat immunocytomas (28, 31, 33, 35). When the human translocated c-*myc* allele is a transgene in mice, a pre-B-cell hyperplasia results with a greater than 70% progression to lymphoma within 1 year (1, 19). The c-*myc* gene is also translocated to the T-cell receptor locus in T-cell leukemia (14). c-*myc* dysregulation has been implicated in other leukemias, in which a subset of human myeloid and lymphoid leukemias show highly elevated c-*myc* RNA levels (23, 26). Altered expression of the c-*myc* gene has been found in other tumors, such as carcinoma of the breast (20).

A hallmark of the neoplastic phenotype is the disruption of normal cellular differentiation. A dramatic reduction in c-*myc* expression is required for cellular differentiation in several hematopoietic cell lines. Sustained, unregulated expression of introduced c-*myc* genes inhibits cellular differentiation in a dose-dependent manner in mouse erythroleukemia cells (11, 12, 24). Blocking c-*myc* expression in the promyelocytic leukemia cell line HL-60 with antisense oligonucleotides induces differentiation (16). When HL-60 cells are induced to differentiate, c-*myc* mRNA biogenesis is inhibited by a mechanism that blocks transcriptional elongation within the first exon of the c-*myc* gene (3, 5, 13, 21). Several days later, the initiation of transcription is inhibited. These data support the hypothesis that c-*myc* drives proliferation, while its absence allows differentiation.

We have examined the posttranscriptional regulation of c-*myc* expression in growing cells and in cells induced to differentiate in order to determine whether this level of regulation is also utilized to reduce c-*myc* expression. We found evidence that two different c-*myc* RNA degradation pathways are utilized, one in growing cells and one in differentiating cells. This possible posttranscriptional regulation may be a fail-safe mechanism for reducing c-*myc* RNA levels and maintaining low c-*myc* expression in cells which are terminally differentiating. By regulating c-*myc* expression at both posttranscriptional and transcriptional levels, cells may be more resistant to mutations which activate the oncogenic potential of the c-*myc* gene.

MATERIALS AND METHODS

Cell culture, RNA preparation, and Northern (RNA) blot hybridization. HL-60 cells were grown in RPMI 1640 medium plus 15% fetal calf serum. Cells were split 1:10, and experiments were performed 48 h later. Actinomycin D (Sigma Chemical Co., St. Louis, Mo.) was dissolved in RPMI 1640 plus 15% fetal calf serum at a concentration of 200 μg/ml and added to half of the culture to a final concentration of 10 μg/ml (34). Cells were harvested from both the treated and untreated cultures, and whole-cell RNA was prepared by the guanidium-CsCl procedure (9). RNA samples were passed over an oligo(dT)-cellulose column as described by Aviv and Leder (2), and the bound and unbound RNA fractions from the equivalent of 20 or 40 μg of total HL-60 RNA were used for Northern blot analysis. Blots were hybridized and washed by the procedure of Church and Gilbert (10).

C3H10T1/2 mouse fibroblasts were grown as monolayers

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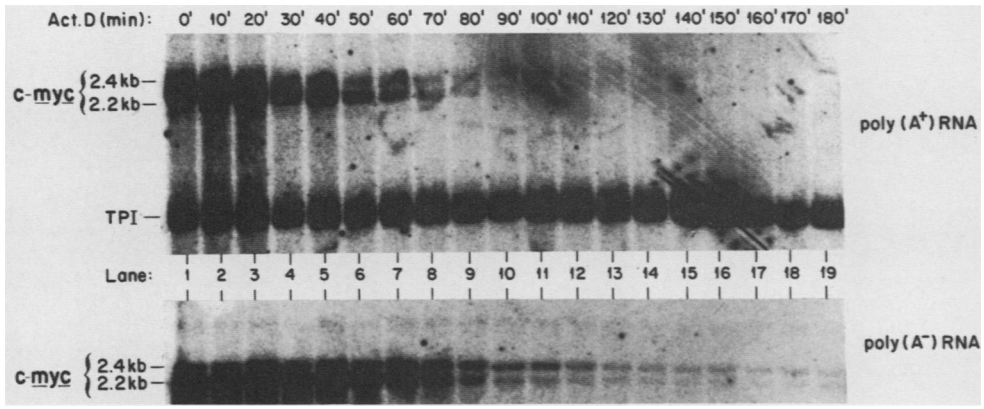

FIG. 1. Northern blots of poly(A)$^+$ and poly(A)$^-$ HL-60 RNA purified from control cells and cells treated with actinomycin D to block trasncription. Cells were cultured and RNA was prepared as described in Materials and Methods. Northern blots were probed with c-*myc* and either TPI cDNA [poly(A)$^+$ RNA] or an 18S RNA probe [poly(A)$^-$ RNA; data not shown]. Lane 1, Control cell RNAs; lanes 2 to 19, RNAs from cells treated for progressive 10-min intervals with actinomycin D as indicated above the lanes.

in Eagle minimal essential medium alpha plus 10% fetal calf serum. Cells were seeded at 10^5 per 150-mm dish. The medium was replaced with fresh medium every third day. When cells became confluent, they were given fresh medium and incubated an additional 48 h before experiments with growth factor were performed.

The c-*myc* probe for c-*myc* RNA sequences was the 1.5-kilobase *Cla*I-*Eco*RI exon 3 fragment of human c-*myc*. This DNA restriction fragment was purified by agarose gel electrophoresis and labeled with [^{32}P]dATP by the random-primer method (15), and 5×10^6 cpm/ml was used for hybridization. The blots were incubated for 16 to 20 h at 68°C and washed as described by Church and Gilbert (10). The blot was exposed to XAR film (Eastman Kodak Co., Rochester, N.Y.) for various times with an intensifying screen. Blots were simultaneously hybridized with triosephosphate isomerase (TPI) probe.

The 18S rRNA probe was a purified mouse *Bam*HI-*Eco*RI 14-kilobase DNA fragment from the plasmid p18S5′ (a gift of Donald Cleveland and Sangram Sisodia, Johns Hopkins University, Baltimore, Md.).

RNA digestions were performed with 10 μg of total RNA and 10-μg equivalents of oligo(dT)-cellulose-fractionated HL-60 cellular RNA as previously described (34).

RESULTS

The extended kinetics of poly(A)$^-$ c-*myc* RNA turnover are biphasic. The primary goal of these experiments was to determine the relationship between c-*myc* RNAs lacking long poly(A) sequences and the poly(A)$^+$ c-*myc* RNAs. We showed previously that there are two major populations of c-*myc* RNAs. One population lacks long poly(A) sequences and is stable for at least 60 min, while the other population is polyadenylated and has a half-life of approximately 12 min (34). We considered and initially rejected the possibility that the poly(A)$^+$ c-*myc* RNA population is a precursor to the poly(A)$^-$ c-*myc* RNA population and is converted by the process of deadenylation (see Discussion in reference 34). However, to investigate this possibility and to extend our previous results, the extended turnover kinetics of both the poly(A)$^-$ and poly(A)$^+$ c-*myc* RNAs were determined.

Rapidly growing HL-60 cells were treated with actinomycin D to inhibit transcription. Whole-cell RNA from each time point was fractionated by oligo(dT)-cellulose chromatography and analyzed by Northern blotting. As an internal reference, the RNA blots were simultaneously hybridized with a TPI cDNA probe and a 1.5-kilobase *Cla*I-*Eco*RI fragment corresponding to the third exon and a 3′-flanking region of the human c-*myc* gene. Subsequently, a probe specific for 18S rRNA was hybridized to these blots (data not shown). After an approximate 10-min lag in decay, poly(A)$^+$ c-*myc* RNAs turned over with rapid kinetics ($t_{1/2}$ = 12 min) as previously determined (Fig. 1). In contrast, the levels of poly(A)$^-$ c-*myc* RNA did not significantly change during the first 70 to 80 min of transcriptional inhibition. After this time, the poly(A)$^-$ c-*myc* RNAs were degraded with kinetics that are similar, although not identical, to those of the poly(A)$^+$ c-*myc* RNAs [poly(A)$^-$ c-*myc* $t_{1/2}$ = 20 to 25 min]. The level of c-*myc* RNA in each lane was quantitated by densitometric scanning and normalized to either TPI [total and poly(A)$^+$ RNA samples] or 18S RNA [poly(A)$^-$ samples]. When these results were plotted for stochastic decay, a unique lag in the degradation of the poly(A)$^-$ c-*myc* RNA was clearly observed (Fig. 2 and Table 1).

The poly(A)$^+$ c-*myc* RNAs in the control sample and early time points were more heterogeneous in size than the poly(A)$^-$ c-*myc* RNAs (Fig. 1 and 3). The poly(A)$^+$ c-*myc* RNA species are then resolved into two bands in samples where transcription had been blocked for longer times (50 to 80 min) (Fig. 1). By a combination of RNA-mapping techniques, we have determined that these two (A)$^+$ c-*myc* RNAs are derived from different promoters, the 2.4-kilobase transcript initiating at the P1 promoter and the smaller 2.2- to 2.3-kb transcription initiating at the P2 promoter (34; data not shown). Likewise, the two poly(A)$^-$ c-*myc* species we observed are initiated at P1 and P2. These data suggest that the poly(A)$^+$ RNAs are rapidly deadenylated, with the resultant RNAs being manifested in the pool of poly(A)$^-$ c-*myc* RNAs. In this precursor-product model, the level of nonadenylated c-*myc* RNAs would remain fairly constant from the influx of deadenylated poly(A)$^+$ c-*myc* RNA until such time as the level of poly(A)$^+$ c-*myc* RNAs was significantly reduced. Alternatively, poly(A)$^-$ c-*myc* may not decay stochastically but may have a predetermined life span. In this age-dependent model, the (A)$^-$ RNA species would be stable for 70 to 80 min and would then be degraded. The apparent first-order kinetics of the eventual (A)$^-$ RNA decay

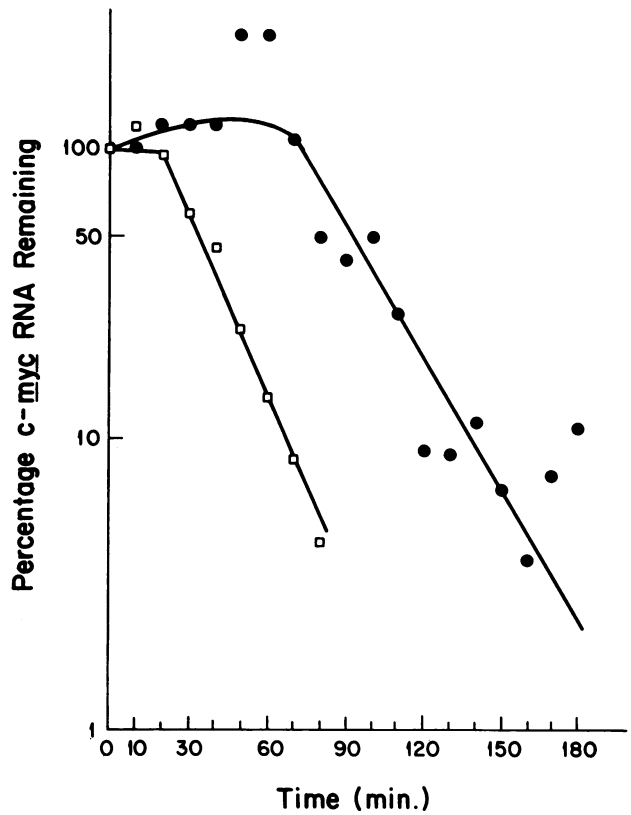

FIG. 2. Kinetic analysis of poly(A)$^+$ and poly(A)$^-$ c-*myc* RNA turnover. Autoradiographs of several different exposure times (one of which is shown in Fig. 1) were densitometrically scanned, and RNA amounts were quantitated. Poly(A)$^+$ c-*myc* RNA levels were normalized to TPI mRNA levels, and poly(A)$^-$ c-*myc* RNA levels were normalized to 18S RNA levels. The logarithm of the percentage of c-*myc* RNA remaining was plotted versus linear time of actinomycin D treatment.

could in this case be due to the heterogeneity in the time of synthesis of these RNAs.

Poly(A)$^+$ c-*myc* RNA undergoes rapid shortening of its poly(A) tail. To choose between these two hypotheses, several additional experiments were performed. First, if poly(A)$^+$ c-*myc* RNA is converted to poly(A)$^-$ c-*myc* RNA by deadenylation, then this deadenylation should be observ-

TABLE 1. c-*myc* RNA half-lives in growing and differentiating HL-60 cells

Cell treatment	RNA type	Half-life (lag time to stochastic decay) (min) of c-myc RNA with promoter(s):		
		P2 + P1	P2	P1
Actinomycin D[a]	Poly (A)$^+$	12 (10)	12 (10)	12 (10)
	Poly (A)$^-$	20 (75)	17 (60)	27 (90)
DMSO[b]	Poly (A)$^+$	15 (10)		
	Poly (A)$^-$	15 (40)		

[a] Quantitated levels of c-*myc* from rapidly growing HL-60 cells in which transcription was inhibited for varying lengths of time as shown in Fig. 1 were plotted on a logarithmic scale as shown in Fig. 2, 5, and 6. After the initial lag in decay, c-*myc* mRNA half-lives were determined.

[b] c-*myc* mRNA half-lives were determined as described above from the graph in Fig. 7 showing the levels of c-*myc* mRNA remaining at various times after HL-60 cells were induced to differentiate with DMSO.

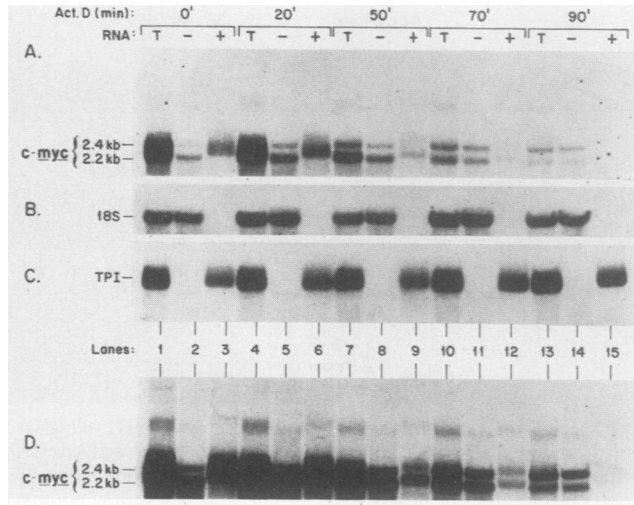

FIG. 3. Northern blot analysis of poly(A)$^+$ c-*myc* RNA shortening with increasing time of actinomycin D treatment. Selected HL-60 RNA samples from Fig. 1 were rerun along with their cognate total cellular RNA sample in contiguous lanes (0, 20, 50, 70, and 90 min of actinomycin D treatment, as indicated at the top). The blot was initially probed with a human c-*myc* exon 3 probe, and an 18-h exposure (panel A) and a 5-day exposure (panel D) are shown. An 18S rRNA probe or a TPI cDNA probe were then annealed to the blot (panels B and C, respectively). Lanes: 1, 4, 7, 10, and 13, total cellular RNA; 2, 5, 8, 11, and 14, poly(A)$^-$ RNA; 3, 6, 9, 12, and 15, poly(A)$^+$ RNA from HL-60 cells.

able. For example, Brewer and Ross (6) have shown that the poly(A) stretch of c-*myc* RNA is progressively shortened in an in vitro RNA degradation system. If deadenylation proceeds by a progressive shortening, as by exonucleolytic attack, then poly(A) shortening, and therefore shortening of poly(A)$^+$ c-*myc* RNA, should be observed. We have examined RNAs of selected time points from our initial turnover experiment to support this notion. Total poly(A)$^-$ and poly(A)$^+$ RNA from each of these samples was electrophoresed in contiguous lanes (Fig. 3A and D). To unequivocally show the separation of poly(A)$^+$ and poly(A)$^-$ RNA, the blot was rehybridized to an 18S ribosomal RNA probe (nonadenylated) and a triosephosphate isomerase probe (a long-lived mRNA that is >99% adenylated) (Fig. 3B and C). The results clearly show that the poly(A)$^+$ c-*myc* RNA is initially heterogeneous in size and both shortens and resolves into two discrete sizes with increasing time of transcription inhibition. Both the P1- and P2-initiated poly(A)$^+$ c-*myc* RNA species were slightly larger than their nonadenylated c-*myc* RNA counterparts (Fig. 3A and D). That these results were not due to contaminating nonadenylated RNA was shown by the quantitative separation of 18S RNA and TPI mRNA by the method employed [oligo(dT)-cellulose chromatography] (Fig. 3B and C). Below, we show that this shortening of the poly(A)$^+$ c-*myc* RNA occurred within the poly(A) tail.

Poly(A)$^+$ c-*myc* RNA is most likely a precursor to poly(A)$^-$ c-*myc* RNA. We have put forth two explanations for the lag in the disappearance of poly(A)$^-$ c-*myc* RNA. To choose between the age-dependent degradation hypothesis and the precursor-product hypothesis, a system in which c-*myc* transcription can be briefly turned on and then blocked is needed. This would provide a pulse of c-*myc* RNA which could be chased by blocking transcription so that precursor-product relationships could be examined. Therefore, the

appearance of poly(A)$^+$ and poly(A)$^-$ c-*myc* RNA was examined in mitogenically stimulated mouse fibroblasts. The cell line employed was the C3H10T1/2 cell line. These cells were allowed to reach confluency. c-*myc* RNA is undetectable in these quiescent fibroblasts but rapidly appears when growth is stimulated. We have used this system because HL-60 cells do not readily quiesce when serum starved, nor do c-*myc* RNA levels decrease after serum starvation (A. J. Kinniburgh, unpublished data). If poly(A)$^-$ c-*myc* RNA has age-dependent degradation kinetics, then poly(A)$^-$ and poly(A)$^+$ c-*myc* RNA should appear simultaneously, but poly(A)$^-$ c-*myc* RNA should show a lag in its degradation when actinomycin D is added to block transcription. When transcription is blocked, neither poly(A)$^+$ nor poly(A)$^-$ c-*myc* RNA levels should increase. On the other hand, if poly(A)$^+$ c-*myc* is the obligate precursor to poly(A)$^-$ c-*myc* RNA, then the adenylated c-*myc* RNA species should appear before the nonadenylated species, since the precursor must appear before the product. If transcription is blocked at a point where only the (A)$^+$ c-*myc* species has appeared, the poly(A)$^-$ species should still arise due to deadenylation. Since little or no (A)$^-$ c-*myc* RNA exists before transcription is blocked, no significant lag in the disappearance of (A)$^-$ c-*myc* RNA should be observed.

We have stimulated quiescent fibroblasts with epidermal growth factor (EGF) and subsequently blocked transcription with actinomycin D for various times. The RNA from these cells was analyzed on a Northern blot. The results strongly support a precursor-product relationship between poly(A)$^+$ c-*myc* RNA and poly(A)$^-$ c-*myc* RNA (Fig. 4). The poly(A)$^+$ c-*myc* RNA appeared and reached a maximum level after 30 min of EGF treatment. The poly(A)$^-$ c-*myc* RNA was present at a low level after 30 min of EGF treatment (approximately 12% of the maximal level). The poly(A)$^+$ c-*myc* RNA nearly quantitatively disappeared within the next 15 min of actinomycin D treatment (transcription inhibition). On the other hand, poly(A)$^-$ c-*myc* RNA reached maximal levels after transcription was inhibited. Therefore, a significant amount of the poly(A)$^+$ c-*myc* RNA must be deadenylated to give rise to the poly(A)$^-$ c-*myc* RNA after transcription has been blocked (Fig. 4). It is clear that a significant fraction of c-*myc* RNA flows from the poly(A)$^+$ to the poly(A)$^-$ pool (densitometric quantitation estimates of this fraction were approximately 35%). The quantitative conversion of poly(A)$^+$ c-*myc* RNA to poly(A)$^-$ c-*myc* RNA was not expected, since turnover of the poly(A)$^-$ c-*myc* RNA occurred simultaneously with the influx of RNA from the poly(A)$^+$ c-*myc* RNA pool. The poly(A)$^-$ c-*myc* RNA level was rapidly reduced after continued incubation of the cells in actinomycin D. The (A)$^-$ c-*myc* RNA was reduced by 75% within this 15-min period (30 min total time of incubation with actinomycin D) (Fig. 4). This would indicate an approximate half-life of 7 to 10 min for poly(A)$^-$ c-*myc* RNA in these mouse fibroblasts. This is similar to, yet shorter than, the $t_{1/2}$ of poly(A)$^-$ c-*myc* RNA in HL-60 cells after the initial lag in disappearance ($t_{1/2}$ = 20 min). The decay of the poly(A)$^+$ c-*myc* RNA pool was too fast to accurately measure the $t_{1/2}$.

These data agree with the data of Brewer and Ross (6), who showed that poly(A) shortening and removal precede c-*myc* degradation in an in vitro mRNA degradation system. We have previously argued against the notion of poly(A)$^+$ c-*myc* RNA being a precursor to poly(A)$^-$ c-*myc* RNA (34). However, with the more detailed and extended analyses presented in this report, we believe there is a precursor-product relationship between polyadenylated and nonadenylated c-*myc* in growing cells.

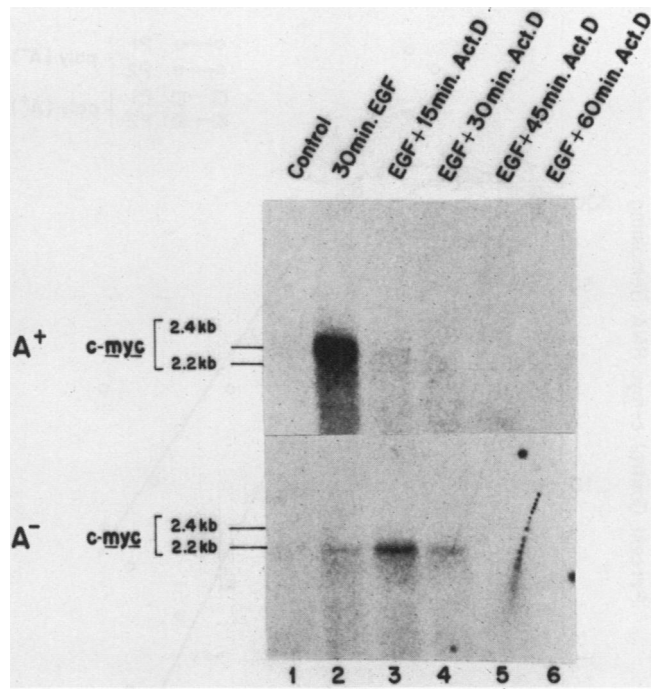

FIG. 4. Northern blot analysis of c-*myc* RNA precursor-product relationship in C3H10T1/2 fibroblasts. Fibroblasts were grown to confluency. EGF was then added to a final concentration of 10 ng/ml. After 30 min of incubation at 37°C, actinomycin D was added to a final concentration of 5 μg/ml. Poly(A)$^+$ and poly(A)$^-$ c-*myc* RNA was separated, electrophoresed, and blotted. The Northern blot was hybridized to a c-*myc* exon 3 probe and subsequently to TPI and 18S RNA probes (data not shown). Lanes: 1, control RNAs; 2, 30-min EGF treatment; 3, 45-min EGF treatment plus 15-min actinomycin D treatment; 4, 60-min EGF treatment plus 30-min actinomycin D treatment; 5, 75-min EGF treatment plus 45-min actinomycin D treatment; 6, 90-min EGF treatment plus 60-min actinomycin D treatment. Top panel, Poly(A)$^+$ RNA; bottom panel, poly(A)$^-$ RNA.

Transcripts from different c-*myc* promoters are deadenylated at the same rate but turned over with different kinetics. One of our initial observations on c-*myc* RNA turnover was that the P1-initiated c-*myc* RNAs appeared to be degraded with slower kinetics than were the P2-initiated c-*myc* RNAs. This could be seen readily in poly(A)$^-$ c-*myc* RNAs but could not be easily discerned for the poly(A)$^+$ species (Fig. 1 and 3). We therefore questioned whether the observed difference could be due to a difference in the rate at which the P1 and P2 transcripts are deadenylated. For example, if the P2-initiated poly(A)$^+$ c-*myc* RNA were deadenylated more rapidly than the P1-initiated RNAs, the P1-initiated poly(A)$^-$ RNA pool would be sustained longer than the P2-initiated poly(A)$^-$ c-*myc* RNAs. On the other hand, if deadenylation of P1- and P2-initiated transcripts occurs at the same rate, then the half-life of the P1-initiated (A)$^-$ c-*myc* RNA pool must be longer than that of the P2-initiated (A)$^-$ c-*myc* RNA pool. To address this question, poly(A)$^+$ RNA was isolated from HL-60 cells treated with actinomycin D for 10-min intervals from 0 through 120 min. The samples used are from the experiment shown in Fig. 1. The (A)$^+$ RNAs were deadenylated in vitro by hybridizing the RNAs with oligo(dT) and digesting the poly(A) tail from the

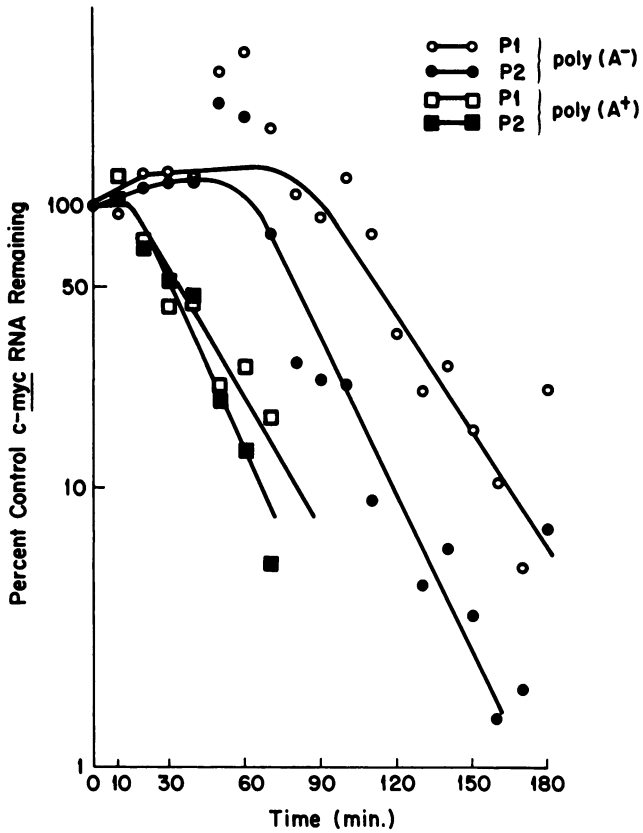

FIG. 5. Kinetic analysis of P1- and P2-initiated poly(A)$^+$ and poly(A)$^-$ c-*myc* RNA. Several exposures of autoradiographs of the Northern blots shown in Fig. 1 [poly(A)$^-$] and in others (not shown) [poly(A)$^+$] were densitometrically scanned and quantitated. Special attention was taken to resolve the P1- and P2-initiated transcripts. The amounts of each RNA species were plotted as log percentages of c-*myc* RNA remaining (with untreated cell RNAs as controls) versus linear time of actinomycin D treatment.

resulting poly(A · dT) hybrid with RNase H. By removing the size heterogeneity inherent in the poly(A) tail, the P1- and P2-initiated c-*myc* RNAs of the poly(A)$^+$ pool could be observed and quantitated (data not shown). The P1- and P2-initiated c-*myc* RNAs disappeared with similar rates from the poly(A)$^+$ RNA pool (Fig. 5). These results also support our conclusion that poly(A) shortening occurs after transcription is blocked with actinomycin D, since in vitro deadenylated poly(A)$^+$ c-*myc* RNA was identical in size at each time point, whereas a gradual decrease in the size of the adenylated c-*myc* RNA occurred with time (Fig. 1 and 3). When the disappearance of both poly(A)$^+$ RNA species was compared with the rate at which the P1- and P2-initiated poly(A)$^-$ c-*myc* RNA pool decayed (from the experiment shown in Fig. 1), an interesting observation was made (Fig. 5). The P1-initiated poly(A)$^-$ c-*myc* RNA pool decayed more slowly than did the P2-initiated poly(A)$^-$ c-*myc* RNA pool. From the time the P1- and P2-initiated poly(A)$^-$ c-*myc* RNAs began to decay rapidly (90 and 60 min, respectively), the half-lives were estimated to be approximately 17 and 26 min, respectively (Table 1). These results are in contrast to the report of Broome et al. (7), in which both transcripts decayed with a similar half-life. However, there are two major differences in their study: total cellular RNA was analyzed, and the rate at which the two transcripts decayed was not determined later than 60 min after transcription was inhibited. Our results indicate that the conclusions of these investigators mainly reflect the decay of the poly(A)$^+$ c-*myc* RNA component.

In summary, in vitro deadenylation of poly(A)$^+$ c-*myc* RNA revealed that both P1- and P2-initiated poly(A)$^+$ RNA species disappeared (were deadenylated) at similar rates. Furthermore, the intrinsic stability of both c-*myc* RNAs species appeared to be more accurately determined by the rate at which the poly(A)$^-$ c-*myc* RNAs turned over rather than by the rate at which these RNAs were deadenylated.

c-*myc* RNA degradation in HL-60 cells induced to differentiate. c-*myc* mRNA production is rapidly extinguished in HL-60 cells which have been induced to differentiate (25). This is accomplished by a block in transcriptional elongation, which occurs near the 3′ end of exon 1 and the 5′ end of intron 1 (3, 5, 13, 21). These events occur quite rapidly (within minutes when dimethyl sulfoxide [DMSO] is the inducing agent) and are initially reversible. After several days transcriptional initiation is inhibited, coincident with the commitment of these cells to terminally differentiate (29). Terminally differentiated cells lose their ability to proliferate. If c-*myc* expression is maintained in cells signalled to differentiate, these cells will fail to differentiate and continue to proliferate (11, 12, 24). The turnoff of c-*myc* expression is therefore intimately involved with the proliferation of HL-60 cells. Since the initial down-regulation of c-*myc* is reversible and since escape from this mechansim could result in uncontrolled cellular growth, we investigated whether a second, posttranscriptional mechanism might operate to reduce c-*myc* gene expression when cells are induced to differentiate.

HL-60 cells were treated with DMSO (1.6%, vol/vol) to block c-*myc* transcriptional elongation and initiate differentiation. The turnover of c-*myc* RNA was then examined by Northern blotting (Fig. 6). In cells induced to differentiate, poly(A)$^+$ and poly(A)$^-$ c-*myc* RNA were both rapidly degraded, although the poly(A)$^-$ c-*myc* pool had a longer lag before its disappearance compared with poly(A)$^+$ c-*myc* RNA (Fig. 6 and 7). Furthermore, the intrinsic degradation rate of the poly(A)$^-$ c-*myc* RNA species was not significantly different in either actinomycin D-treated or DMSO-treated HL-60 cells. Rather, there was a difference in the length of time that the poly(A)$^-$ c-*myc* RNA pools were maintained before stochastic turnover was observed. The half-lives of poly(A)$^-$ c-*myc* RNA in growing cells (actinomycin D-treated) and in DMSO-treated cells were quite similar (Table 1). The slight destabilization of the poly(A)$^-$ c-*myc* RNA pool in DMSO-treated HL-60 cells ($t_{1/2}$ = 15 min [versus $t_{1/2}$ = 20 min in untreated cells]) is insufficient to account for the decreased lag before stochastic decay of the poly(A)$^-$ c-*myc* RNA pool in these cells. We have performed the c-*myc* RNA decay experiment with DMSO several times with similar results. If actinomycin D and DMSO were added simultaneously to HL-60 cells, similar results were again obtained (unpublished data). Also, DMSO and actinomycin D treatment, individually, must act within the cell to block transcription with similar pharmacokinetics, since the disappearance of the poly(A)$^+$ c-*myc* RNA pools was quite similar with either treatment (within experimental variation). Therefore, the different lag times observed for the poly(A)$^-$ RNA pools in DMSO- and actinomycin D-treated cells cannot be accounted for by a difference in the rates at which DMSO and actinomycin D act to block transcription. One hypothesis which explains these data well is that poly(A)$^+$ c-*myc* RNA is directly degraded when cells are

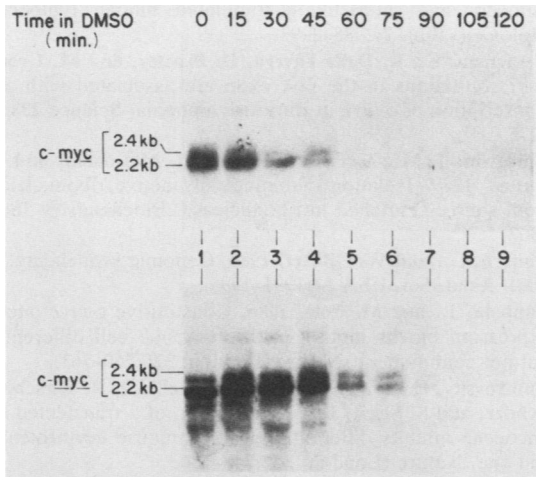

FIG. 6. Northern blot of poly(A)$^+$ and poly(A)$^-$ c-*myc* RNA from HL-60 cells induced to differentiate with DMSO. HL-60 cells were grown to mid-log phase. DMSO was added to a final concentration of 1.6% (vol/vol). Poly(A)$^+$ and poly(A)$^-$ RNA were prepared, electrophoresed, blotted, and hybridized with a c-*myc* probe and subsequently with a TPI and an 18S rRNA probe (data not shown). Top panel, poly(A)$^+$ RNA; bottom panel, poly(A)$^-$ RNA. Lanes: 1 , control cell RNAs; 2 to 9, RNA from cells treated for various times with DMSO (15, 30, 45, 60, 75, 90, 105, and 120 min, respectively, as indicated above lanes).

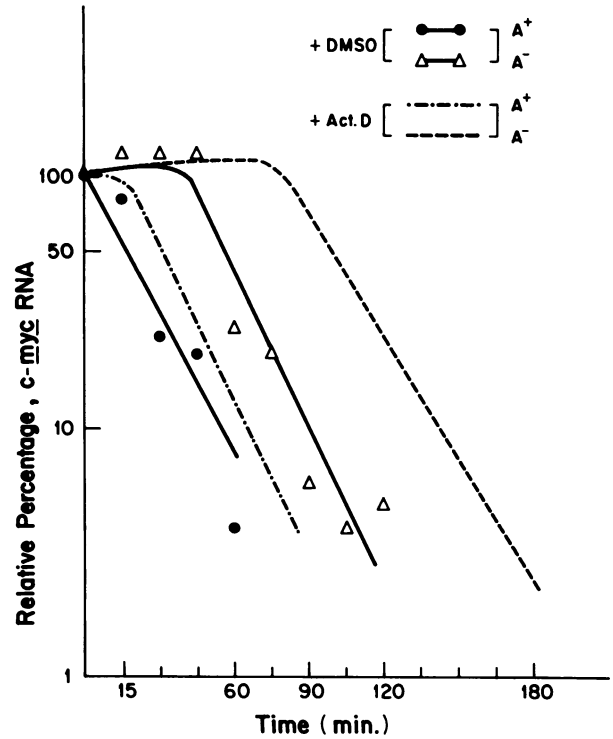

FIG. 7. Kinetics of poly(A)$^+$ and poly(A)$^-$ c-*myc* RNA disappearance in growing HL-60 cells and in HL-60 cells induced to differentiate. Autoradiographs of the Northern blot shown in Fig. 7 were scanned densitometrically and the amount of c-*myc* RNA was quantitated. The log relative percentage of c-*myc* RNA was plotted versus linear time of treatment with DMSO or actinomycin D. The data from Fig. 2 (actinomycin D-treated cells) was plotted without actual data points for comparison.

induced to differentiate, with a small amount of poly(A)$^+$ RNA being deadenylated to sustain the poly(A)$^-$ c-*myc* pool. The short lag in poly(A)$^-$ c-*myc* RNA degradation in this case could be accounted for by this small flow of deadenylated poly(A)$^+$ c-*myc* RNA to the poly(A)$^-$ c-*myc* RNA pool. This may simply be the time necessary for the activation of the differentiation turnover pathway. Alternatively, both putative pathways may be used in cells induced to differentiate. At present we know of no way to block the removal of the poly(A) tract from mRNA and therefore cannot test this model directly. Results with other cell lines (S49 mouse lymphoma) which down-regulate c-*myc* levels when stimulated to growth arrest are similar to those reported here for HL-60 cells (A. J. Kinniburgh and V. Groppi, unpublished data).

DISCUSSION

We have elucidated differences in c-*myc* RNA degradation between growing cells and cells induced to differentiate. In growing cells, poly(A)$^-$ c-*myc* RNA levels initially remained constant (for 80 min) when transcription was blocked. Subsequently, the poly(A)$^-$ c-*myc* RNA pool rapidly disappeared. The lag in poly(A)$^-$ c-*myc* RNA disappearance observed in growing cells was due to the conversion of poly(A)$^+$ c-*myc* RNA to poly(A)$^-$ c-*myc* RNA by deadenylation (Fig. 4). Since both species were ultimately labile (Table 1), the flow of poly(A)$^+$ species to the poly(A)$^-$ pool initially sustained poly(A)$^-$ c-*myc* RNA levels even though poly(A)$^-$ c-*myc* RNA molecules were being degraded during this period. This maintenance of the poly(A)$^-$ c-*myc* RNA level was progressively less effective as the poly(A)$^+$ pool was depleted (Fig. 1 and 2). When cells were induced to differentiate, both poly(A)$^+$ and poly(A)$^-$ c-*myc* RNA turned over rapidly, although poly(A)$^-$ c-*myc* RNA had a short lag in its turnover, indicating that perhaps some poly(A)$^-$ c-*myc* RNA was being derived by deadenylation of poly(A)$^+$ c-*myc* RNA (Fig. 3). The proportion may be as high as 30 to 50% (as estimated from the ratio of lag times). This may simply reflect the switch between the deadenylation pathway (in growing cells) and a direct pathway (in differentiating cells) of poly(A)$^+$ degradation. Alternatively, both pathways may be active in differentiating cells. Preliminary evidence indicates that poly(A) shortening is not as pronounced in cells induced to differentiate (unpublished data). These data are consistent with the proposed switch in c-*myc* RNA degradation modes. We found that both c-*myc* RNA species were present on the polyribosomes of cells (unpublished data). Therefore, the rapid reduction of both c-*myc* RNA species in cells induced to differentiate should have a rapid impact on the levels of the labile c-*myc* protein.

The maintenance of a transcriptional elongation block in differentiating cells would seem to obviate the need for a posttranscriptional mechanism which maintains low c-*myc* levels. However, if the transcriptional attenuation mechanism is leaky, then a backup or fail-safe mechanism may be important to maintain reduced levels of c-*myc* expression so that proliferation does not continue or cannot be reinitiated. If this dual system is active, it may also make the cell more resistant to mutations which disrupt one of these regulatory mechanisms. For example, several mutations may need to accumulate in exon 1 for the transcriptional elongation block to be disrupted (8, 36).

It might be expected that disruptions in the orderly degradation of c-*myc* RNA would have drastic effects on c-*myc* RNA levels. For example, in bone marrow cells of certain

patients with acute myeloid leukemia, highly elevated levels of poly(A)$^-$ c-*myc* RNA (20-fold) were observed without an increase in poly(A)$^+$ c-*myc* RNA levels compared with normal bone marrow cells (A. J. Kinniburgh and H. Preisler, unpublished). This defect may arise by a nucleotide sequence change(s) which increases the stability of the poly(A)$^-$ c-*myc* RNA species without affecting the rate of poly(A)$^+$ c-*myc* RNA deadenylation.

c-*myc* RNA is one of a family of labile mRNAs that includes messengers for lymphokines and other proto-oncogenes. Shaw and Kamen (27) have demonstrated that an AU-rich sequence, AU_3A, can confer extreme lability to normally stable mRNAs. Both c-*myc* RNA species contain multiple sequences related to the AU_3A motif. The effects of these sequences, however, can be modified by other sequences. For example, c-*myc* RNAs initiated at a minor, upstream promoter (P1) are more stable than were c-*myc* RNAs initiated at the major promoter (P2) (P1 $t_{1/2}$ = 27 min; P2 $t_{1/2}$ = 17 min). This was only observed in the poly(A)$^-$ c-*myc* RNA species, since deadenylation of these species occurs at nearly identical rates (Fig. 5). Therefore, the transcribed sequences which differ between these two promoters must act to stabilize the P1-initiated RNAs. The P0-initiated transcripts (P0 is the most distal c-*myc* promoter) are even more stable than P1- and P2-initiated c-*myc* RNAs (4; unpublished results). The cellular need for a differential stability based on promoter usage is not clear at present.

In conclusion, the data presented here support the idea that direct degradation of poly(A)$^+$ c-*myc* RNA is activated in differentiating cells, whereas deadenylation to a poly(A)$^-$ c-*myc* RNA species occurs in growing cells. In growing cells, the poly(A)$^-$ c-*myc* RNA is the ultimately degraded c-*myc* RNA species.

ACKNOWLEDGMENTS

We thank Jeff Ross, Gary Brewer, Arthur Skoultchi, and Efraim Racker for their comments on an earlier version of the manuscript. We also thank Bill Held, Charles Wenner, Maartin Linskens, Steven Pruitt, and K. Gross for helpful comments and Nancy C. Frame for secretarial assistance.

This work was supported by Public Health Service grants CA 43661 and CA 41285 from the National Institutes of Health to A.J.K.

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