Extreme instability of myc mRNA in normal and transformed human cells

(oncogene/messenger stability/post-transcriptional regulation)

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ABSTRACT To address the possibility that the expression of the *myc* gene might be regulated at a post-transcriptional level, we have investigated the half-life of myc mRNA in various cells. Our survey included normal human embryonic fibroblasts as well as transformed human cells of various origins: cervix carcinoma (HeLa), breast carcinoma (MCF7), Burkitt lymphoma (Daudi), and promyelocytic leukemia (HL60). All these cells revealed an extreme instability of myc mRNA (half-life, ≈10 min), suggesting that the control of myc mRNA degradation might be a general means (although not necessarily exclusive) of regulating both the level and the timing of *myc* gene expression. Inhibition of protein synthesis resulted in a dramatic stabilization of myc mRNA in HeLa, MCF7, and HL60 cells, suggesting that the controlling element might itself be, at least in these cells, a protein of rapid turnover. This finding opens the way to studying the mechanism of myc mRNA inactivation in these different cell types. However, protein synthesis inhibition had no effect on myc mRNA instability in other transformed (Daudi) cell lines as well as normal embryonic human fibroblasts. These different types of behavior suggest that the post-transcriptional control of *myc* gene expression might involve multiple factors that would be differently affected in various cell types.

It is now well recognized that the process that leads from a normal cell to a fully transformed one is a multistep cascade of events (1–3), which are under the control of different classes of oncogenes. Two classes were defined on the basis of their ability to complement each other in the transformation of normal cells, such as rat embryo fibroblasts (for a review, see ref. 3). A first class, referred to as establishment-immortalization genes, includes cellular genes such as *myc*, adenovirus E1a, and polyoma large T. To the second class, referred to as transforming genes, belong the *ras* family of oncogenes and polyoma middle T.

Probably the most efficient and clear-cut example of synergism between oncogenes of these two classes is that evolved by polyoma (1, 4), a small DNA tumor virus that manages its transforming sequences most efficiently by encoding the two functions in the same locus and expressing the immortalizing large T and the transforming middle T by differential splicing. Being only of viral origin, the polyoma oncogenes have no normal nononcogenic cellular counterparts. On the other hand, cellular oncogenes, which are present and expressed in normal cells, have to acquire their oncogenic potential through some activation mechanism. In the case of the Ha-*ras* gene, a single somatic point mutation has been demonstrated to be sufficient to turn the normal gene into a transforming one (5–7). At variance with the situation in the Ha-*ras* gene, enhanced expression of *myc* has been observed in a wide spectrum of neoplasms. This latter result

can be achieved in a variety of ways, two of which have been amply documented. Amplification at the genomic level has been demonstrated in the case of two human cell lines, HL60 derived from a promyelocytic leukemia (8) and APUDoma Colo 320 (9). Translocation of c-*myc* into the immunoglobulin heavy-chain locus has also been observed in a variety of human Burkitt and murine plasmacytomas.

For lack of knowledge about the myc protein and of suitable antibodies against it, studies on the regulation of *myc* gene expression have so far only been concerned with the qualitative and quantitative aspects of the production of its mRNA. Recent studies by Kelly *et al.* (10) have indicated that myc mRNA is induced early after mitogenic stimulation of lymphocytes by lipopolysaccharide or concanavalin A as well as of fibroblasts by platelet-derived-growth factor. Translocation of c-*myc* in Burkitt lymphomas creates a situation in which the expression of the two alleles can be compared. Such studies showed that only the translocated allele is significantly expressed, while the normal one seems inactive (11). This observation supports a model suggesting that *myc* is negatively regulated by a *trans*-acting element (12). Furthermore, Kelly *et al.* (10) recently showed that inhibition of protein synthesis causes a superinduction of *myc* expression, suggesting that it might be under the control of a labile protein. As pointed out by Taub *et al.* (11), this negative control element could act as a repressor of transcription or as a factor accelerating myc mRNA degradation.

To investigate the possibility of such a post-transcriptional control of *myc* expression, we set out to examine the stability of its mRNA in various cells. As an internal reference, the expression of a housekeeping enzyme was monitored in parallel. To serve this purpose, we chose the glyceraldehyde-3-phosphate-dehydrogenase gene (GPDHase), whose cDNA has recently been cloned in our laboratory (unpublished data).

MATERIALS AND METHODS

Cells. HeLa (S3) cells were grown in suspension as described (13). Daudi (obtained from B. Lebleu) and HL60 cells (obtained from J. Caraux) were grown in RPMI 1640 medium containing 10% fetal calf serum. MRC5 human embryonic fibroblasts (obtained from Biomérieux) and MCF7 cells (obtained from H. Rochefort) were grown in Dulbecco's modified minimal essential medium containing 10% newborn calf serum and 0.6 μg of insulin per ml for these latter cells.

RNA Isolation. Cold total cellular RNA was extracted by the urea/LiCl procedure of Auffray and Rougeon (14). Poly(A)$^+$ RNA was prepared by oligo(dT)-cellulose chromatography according to Bantle *et al.* (15).

RNA Electrophoresis and Transfer. RNA was electropho-

Abbreviation: GPDHase, glyceraldehyde-3-phosphate-dehydrogenase.
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resed on 1.2% (wt/vol) agarose (Sigma type II) gels containing 1 M formaldehyde and transferred onto nitrocellulose as described by Thomas (16). The sizes of myc RNA species were estimated by comparison with those of 18S and 28S rRNA and of GPDHase mRNA (unpublished observations). Hybridization with ^{32}P-labeled cloned probes (5000 cpm per cm^2 of filter) was for 48 hr in the presence of 10% dextran sulfate, according to Wahl *et al.* (17). Autoradiography was on Kodak X-O-Mat S film, using DuPont Cronex Quanta 3 intensifying screens at −70°C for the indicated times.

Preparation of Labeled Probes. GPDHase probe was a partial cDNA clone (pHGAPDH-4) from HeLa cells prepared in our laboratory by insertion into the *Pst* I site of pBR322 plasmid. The human c-*myc* gene was screened out of Maniatis' library by Leprince *et al.* (18). After excision with *Eco*RI and *Xba*, an *Eco*RI/*Xba* fragment containing exons 2 and 3, intron 2, and part of intron 1 was subcloned into the pKH47 plasmid along with another small *Xba*/*Eco*RI located upstream and used as a linker (for a map of the human *myc* gene, see ref. 19). This latter construct was kindly given to us by D. Stehelin. To reduce the hybridization background probably due to the presence of the oligo(dA) and oligo(dT) tails in the pKH47 plasmid vector (20), RNA blots were probed with a small fragment carrying only exon 3 excised by *Cla* I and purified on a low-melting agarose gel. The entire pHGPDHase-4 plasmid or *myc Cla* I fragment was labeled by nick-translation (21).

All other experimental procedures are described in the figure legends.

RESULTS

myc mRNA Is Unstable in Transformed As Well As Normal Cells. Poly(A)$^+$ RNA was extracted from HeLa cells before and at various times after treatment with high doses of actinomycin D (5 μg/ml) to block essentially all transcriptional activity. Each sample was then electrophoresed under denaturing conditions, transferred to nitrocellulose (16), and analyzed for its content of myc as well as GPDHase mRNA, the latter one serving as a reference for a maintenance gene expressed in all tissues. As a matter of fact, HeLa cells contain high amounts (2%–3% of total soluble proteins) of GPDHase (22, 23) and of its mRNA (1%) (unpublished observations). The experiment of Fig. 1 (*Lower Left*) confirms the abundance of GPDHase mRNA in untreated HeLa cells and also shows that they express myc mRNA at an easily detectable level (*Upper Left*), in agreement with a report by Marcu *et al.* (24). The doublet of bands at 2.2–2.4 kilobases indicates that the two *myc* promoters (25) are being used. Most strikingly, after a 2-hr chase in the presence of actinomycin, myc mRNA had completely disappeared (*Upper Left*), while the level of GPDHase mRNA was nearly unchanged (*Lower Left*), in agreement with a previously determined half-life of ≈8 hr (35). A more detailed kinetic analysis over a 45-min chase followed by quantitative densitometry of RNA blots allowed the determination of a half-life of ≈15 min for myc mRNA (Fig. 2). This experiment, therefore, reveals a dramatic difference in the stability of GPDHase and myc mRNAs after an actinomycin chase. It should be noted that transcripts from both *myc* promoters appear to be comparably unstable (Fig. 1 *Upper Left*).

The above experiment is subject to two kinds of reservation. First, the observed situation might be a nonphysiological artifact induced by actinomycin treatment, although this drug was successfully used for measuring the half-lives of adenovirus E1a and E1b (26). Second, it might be argued that the observed turnover is that of the poly(A) tail rather than of the mRNA itself. These two reservations were overcome by analyzing the kinetics of accumulation (in the absence of actinomycin) of myc and GPDHase mRNAs in both
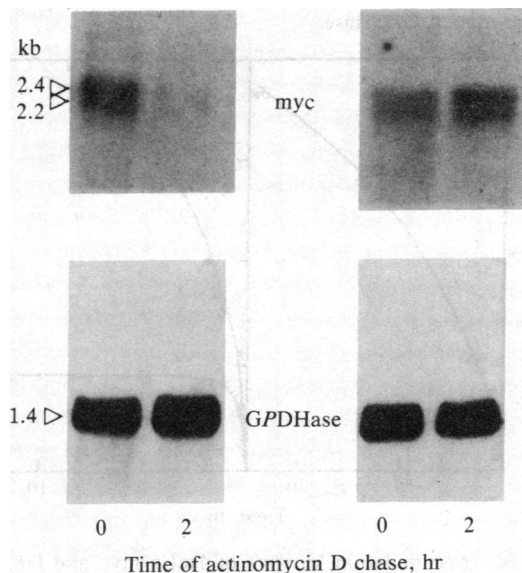

FIG. 1. RNA blot analysis of HeLa myc and GPDHase mRNAs during an actinomycin chase in the absence or presence of emetine. HeLa cells were grown for 2 hr in the presence of 5 μg of actinomycin D per ml without (*Left*) or with (*Right*) 0.1 mM emetine. In the latter case, emetine was added 15 min before actinomycin. Poly(A)$^+$ RNA (10 μg) was electrophoresed under denaturing conditions, transferred to nitrocellulose, and hybridized with nick-translated pHGAPDH-4 and *myc* exon 3 probes. Autoradiography was overnight. kb, Kilobases.

poly(A)$^+$ and poly(A)$^-$ forms. HeLa cells were continuously exposed to [^{3}H]uridine, and the flow of label into these two messengers was measured by hybridizing cytoplasmic poly(A)$^+$ and poly(A)$^-$ RNA extracted after various labeling times to filter-bound DNA of the corresponding clones. As a control that the reaction had gone to completion, hybridization mixtures were taken at the end of the experiment and shown to be depleted in these two mRNAs by rechallenging them with fresh DNA-containing filters (data not shown). The results of Fig. 3 show quite different kinetics of accumulation of these two mRNAs in the poly(A)$^+$ compartment. GPDHase mRNA steadily accumulates for at least 48 hr (for best comparison with myc, only the first 12 hr are shown in

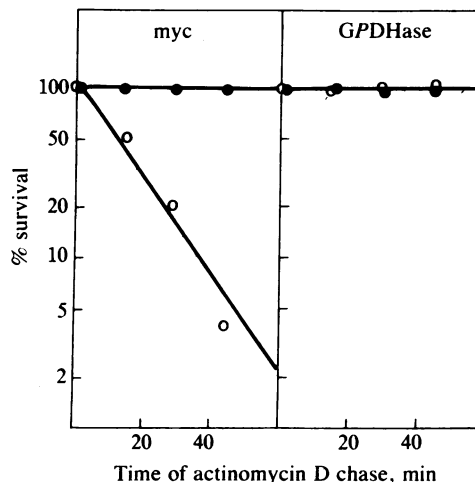FIG. 2. Half-lives of HeLa myc and GPDHase mRNAs in the absence (○) or presence (●) of emetine. Same experiment as in Fig. 1 except that various lengths of actinomycin chase were analyzed. mRNAs were quantitated by densitometry scanning of the autoradiographs, using the zero time of actinomycin treatment as a reference for 100% survival.
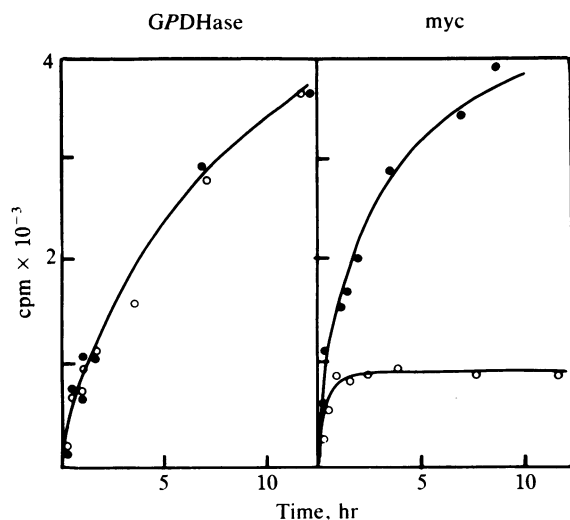
FIG. 3. Accumulation kinetics of HeLa myc and GPDHase mRNAs in the absence (○) or presence (●) of cycloheximide. Samples of HeLa cells continuously labeled with [³H]uridine were taken at indicated times and poly(A)⁺ RNA isolated from the cytoplasmic fraction was hybridized to filter-bound plasmid DNA carrying an excess of either GPDHase cDNA or *myc* genomic DNA (plasmid containing the entire c-*myc* gene) as described by Wilson *et al.* (27). After hybridization, filters were treated with pancreatic RNase and washed as described by Nevins (28). For experiments in the presence of cycloheximide, the drug was added at 20 μg/ml 45 min before addition of [³H]uridine. It was verified that the rate of label incorporation into total RNA remained constant throughout the experiment.
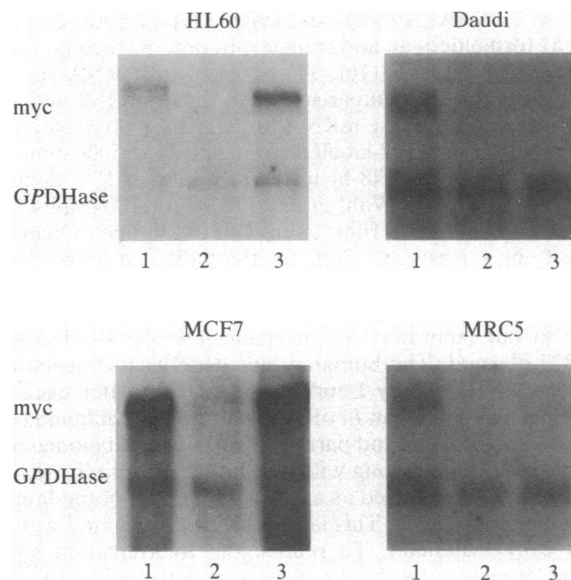

FIG. 4. myc mRNA instability in the absence and presence of emetine in various human cells. Experimental conditions were as described in Fig. 1 except that GPDHase and *myc* (exon 3) probes were used simultaneously. Lanes 1, control with no actinomycin and no emetine; lanes 2, actinomycin chase; lanes 3, actinomycin chase in the presence of emetine. Comparing the intensities of bands in the different cells is not relevant, as the four blots shown did not come from the same transfer, nor were the autoradiographs exposed for the same time (12 hr for HL60, 48 hr for MCF7, 56 hr for Daudi and MRC5).

Fig. 3), while the myc mRNA level has already reached a plateau in <1 hr. When treated according to Greenberg's equation (29), these data also indicate a short half-life for myc mRNA, although it cannot be accurately measured because of the 20 min required for the equilibration of [³H]uridine into the UTP pool (30, 31). These results are, therefore, in full agreement with those obtained using actinomycin and thus validate the use of this drug for these experiments. Moreover, in neither case was a significant hybridization observed with the poly(A)⁻ fraction (data not shown), ruling out the alternative interpretation of the actinomycin experiment in terms of poly(A) turnover. From the plateau values of the fraction of input RNA that hybridized to filter-bound cloned cDNAs (after correction for the incompleteness of the GPDHase insert), one can calculate that steady-state GPDHase and myc mRNAs represent, respectively, ≈1% and ≈0.08% of poly(A)⁺ RNA.

To assess the generality of myc mRNA unstability, we have carried out the same actinomycin chase experiment in various other transformed cell lines (HL60, Daudi, and MCF7) as well as in human embryonic fibroblasts (MRC5). RNA blots of poly(A)⁺ RNA extracted from all these cells before or after a 2-hr actinomycin chase were monitored for myc and GPDHase mRNA by using a mixture of the two probes in the same experiment. In all cases, RNA blots revealed the same 2.2- to 2.4-kilobase myc mRNA species before actinomycin treatment, which disappeared after a 2-hr chase, while GPDHase mRNA levels remained essentially unchanged (Fig. 4). They confirm the high instability of myc as opposed to GPDHase mRNA in all cells tested. The four blots presented are not intended to serve as a basis for comparing the levels of myc and GPDHase mRNAs in these cells, because they do not come from the same transfer.

Control of myc mRNA Instability. A recent paper by Kelly *et al.* (10) reported the superinduction of myc mRNA by mitogens in the presence of cycloheximide, an inhibitor of protein synthesis. According to these authors, these data sug-

gest that the myc mRNA level is regulated by a labile protein that could either act as a repressor or in some way interfere with myc mRNA stability. The above two types of experiments were therefore reproduced on HeLa and various other cells in the presence of cycloheximide or emetine, an even more potent inhibitor of protein synthesis (32). Cycloheximide and emetine were added 45 and 15 min, respectively, before initiating chase or accumulation experiments to allow the establishment of protein synthesis inhibition. These two inhibitors had the same effect, and examples of both are presented.

Fig. 1 (*Right*) shows that addition of emetine 15 min before a 2-hr actinomycin chase has a profound stabilizing effect on HeLa myc mRNA without affecting the normally stable GPDHase mRNA. The detailed kinetics of myc mRNA decay over a 45-min actinomycin chase was also determined in the presence of emetine (Fig. 2). The same results were obtained with cycloheximide in place of emetine (not shown). No obvious differential effect was detected between myc transcripts initiated from the two promoters (25), in agreement with observations by Kelly *et al.* (10). The effect of cycloheximide (added at −45 min) on the kinetics of accumulation of myc and GPDHase mRNA in HeLa cells has also been investigated (Fig. 3). In perfect agreement with the conclusions of the actinomycin experiment, inhibition of protein synthesis results in a dramatic change in the accumulation of myc mRNA, which now continuously increases similar to GPDHase mRNA without affecting the latter.

All the above experiments were exclusively concerned with cytoplasmic messengers and overlooked possible nuclear effects of protein synthesis inhibitors. To rule out a direct effect on myc transcription, it had to be shown that these inhibitors had no effect on nuclear RNA. To this aim, we have attempted to repeat the accumulation experiment with nuclear RNA. Although the results (not shown) seemed to confirm the absence of a significant effect of cycloheximide, this experiment had to rely on too low radioactive counts to
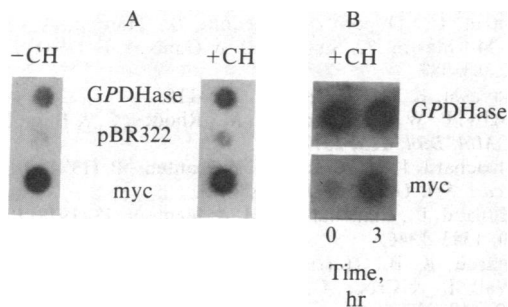
FIG. 5. Effect of cycloheximide on GPDHase and *myc* gene transcription in isolated nuclei (*A*) and on messenger accumulation in total RNA (*B*). (*A*) Nuclei from HeLa cells either untreated or incubated *in vivo* for 3 hr in the presence of cycloheximide (CH) at 20 μg/ml were treated with pancreatic RNase and incubated with [^{32}P]UTP under the conditions described by Schibler *et al.* (33) for elongation of nascent RNA chains. Deproteinized RNA (10^6 counts) was then hybridized to a nitrocellulose filter (Schleicher & Schüll, BA 85) carrying dot spots of heat-denatured DNAs from pBR322, pHGPDHase-4, and *myc* plasmids (5 μg per dot). Conditions for hybridization were as specified by Schibler *et al.* (33). Exposure time was 6 hr. Absolute intensities of GPDHase and *myc* signals should not be compared, as the lengths of the fragments used for hybridization were quite different. (*B*) Poly(A)$^+$ RNA was extracted from the same batch of cells as in *A* before and after a 3-hr exposure to cycloheximide, dot-spotted, and hybridized as described by Thomas (16). The same filter was successively hybridized with *myc* exon 3 and GPDHase probes. Exposure time was 16 hr.

provide definitive evidence. We therefore measured the *in vitro* transcriptional activity of isolated nuclei from HeLa cells under the conditions described by Schibler *et al.* (33). The results of such an experiment clearly show that pretreatment of cells with cycloheximide for an even longer time than in the above experiments (3 hr) did not affect the *in vitro* transcriptional activity of either *myc* or GPDHase genes in isolated nuclei (Fig. 5*A*). As a control of the *in vivo* effect of cycloheximide (Fig. 3) on the same batch of HeLa cells used for nuclei preparation, dot blots of poly(A)$^+$ RNA were also hybridized to both probes (Fig. 5*B*). As expected, this control reveals a dramatic increase of *myc* mRNA accumulation as a result of protein synthesis inhibition.

The above results suggest that *myc* mRNA instability is controlled in HeLa cells by a protein whose effect is short-lived enough to have completely disappeared during the 15-min preincubation with emetine (Fig. 2). However, at variance with *myc* mRNA instability, which held for all cells surveyed, the situation with respect to the effect of protein synthesis inhibitors is not quite as general (Fig. 4). Indeed, emetine (or cycloheximide) did not restore the stability of *myc* mRNA either in normal MRC5 fibroblasts or in Daudi cells. To rule out possible differences in the sensitivity of these other cells to the drug, it was verified that emetine did actually block at least 95% of protein synthesis *in vivo* (not shown). On the other hand, MCF7 and HL60 behaved similar to HeLa as far as this stabilizing effect on *myc* mRNA is concerned. Most interestingly, the RNA blots of Figs. 1 and 4 provide a preliminary indication as to possible differences in the behavior of transcripts initiated from the two *myc* promoters: while both *myc* mRNA species are stabilized in HeLa and MCF7, only the 2.2-kilobase species initiated from the proximal promoter appears to be stabilized in HL60 cells. This observation lends further support to the existence of a specific regulatory mechanism, as opposed to a general stabilizing effect related to the stalling of translating ribosomes.

DISCUSSION

The purpose of the present paper was to investigate the possibility that *myc* expression might be regulated at a post-tran-

scriptional level by controlling the stability of its mRNA. A recent series of papers from Leder's group suggested that *myc* expression is under negative control by a *trans*-acting element (11, 12). The observation that inhibition of protein synthesis superinduces *myc* expression led Kelly *et al.* (10) to infer a control by a labile protein, which could be either a repressor or a factor affecting *myc* mRNA degradation (10, 11).

The results presented here clearly establish the extreme instability of *myc* mRNA in all human cells tested. Our survey included both normal human embryonic fibroblasts and transformed cells of various origins: cervix carcinoma (HeLa), mammary carcinoma (MCF7), Burkitt lymphoma (Daudi), and promyelocytic leukemia (HL60). We, therefore, tend to believe that *myc* mRNA instability may be a general phenomenon. It should be noted that some degree of instability is to be expected from an mRNA expressing a cycle-dependent function, as was proposed for *myc* (10), although cycle-dependence seems to be an attribute of normal but not transformed cells (34). Furthermore, the short half-life of *myc* mRNA (≈15 min) suggests that a fine temporal tuning of *myc* expression is required for normal cell function. Whatever its physiological significance, this observation establishes that *myc* expression is regulated at least in part by post-transcriptional events affecting the degradation of its messenger but allows no inference on and therefore by no means excludes the possibility of transcriptional control. In any case, it is striking to note that the adenovirus-encoded *E1a* gene, which belongs to the same class of immortalizing oncogenes as *myc* (3), is also regulated at the level of mRNA stability (27).

On the other hand, the way in which the various cells tested enforce their control of *myc* mRNA degradation is certainly not straightforward. Only in HeLa, MCF7, and HL60 is *myc* mRNA stabilized by protein synthesis inhibitors, a situation consistent with the hypothesis of control by a labile protein based on the superinduction of stimulated lymphocytes by this drug (10). However, neither Daudi cells nor normal human embryonic fibroblasts exhibited this type of behavior, suggesting that the post-transcriptional control of *myc* gene expression might involve multiple factors that would be differently affected in these various cells.

A quite interesting additional feature of HL60 cells is the preliminary observation of a differential stabilizing effect of cycloheximide on the 2.2-kilobase but not the 2.4-kilobase *myc* mRNA species. Should this observation be confirmed by more accurate S1 nuclease mapping experiments, it would strongly suggest that the 5' noncoding region of these mRNAs might be an important regulatory target for their degradation. In addition to providing adequate material for cloning individual *myc* mRNA species under superinduction conditions, these two cell lines also appear to be well suited to studying the regulation of *myc* mRNA degradation. Along this line, it would be interesting to investigate the stability of various forms of *myc* mRNA (for example, v-*myc* transcripts lacking exon 1) in these different cellular contexts.

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