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Poly(A) Tail Shortening Is the Translation-Dependent Step in c-*myc* mRNA Degradation

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The highly unstable c-*myc* mRNA has been shown to be stabilized in cells treated with protein synthesis inhibitors. We have studied this phenomenon in an effort to gain more insight into the degradation pathway of this mRNA. Our results indicate that the stabilization of c-*myc* mRNA in the absence of translation can be fully explained by the inhibition of translation-dependent poly(A) tail shortening. This view is based on the following observations. First, the normally rapid shortening of the c-*myc* poly(A) tail was slowed down by a translation block. Second, c-*myc* messengers which carry a short poly(A) tail, as a result of prolonged actinomycin D or 3′-deoxyadenosine treatment, were not stabilized by the inhibition of translation. We propose that c-*myc* mRNA degradation proceeds in at least two steps. The first step is the shortening of long poly(A) tails. This step requires ongoing translation and thus is responsible for the delay in mRNA degradation observed in the presence of protein synthesis inhibitors. The second step involves rapid degradation of the body of the mRNA, possibly preceded by the removal of the short remainder of the poly(A) tail. This last step is independent of translation.

A number of proto-oncogenes, including those of the *myc* family, can be activated by events that lead to an increase in the steady-state levels of the gene products. For example, proviral insertion, chromosome translocation, and gene amplification have all been found to be the cause of an increase in c-*myc* mRNA and protein levels in various tumors (5). A strict regulation of the expression of these genes is apparently important for controlled cell growth. The c-*myc* gene appears to be involved in the entrance of quiescent cells into the cell cycle. Stimulation of quiescent cells with growth factors leads to a strong transient induction of c-*myc* gene expression (5). Rapid down regulation of the gene is made possible by the instability of mRNA and protein products. Both c-*myc* mRNA and c-*myc* proteins have unusually short half-lives (about 15 min [6, 13] and about 30 min [11], respectively).

Interest in the regulation of the half-life of c-*myc* mRNA was first aroused when the translocated c-*myc* genes in certain Burkitt lymphomas were found to produce stabilized truncated transcripts lacking exon 1 (19). Shortly thereafter, it was discovered that an A+T-rich sequence in the 3′ untranslated region of the granulocyte-monocyte colony-stimulating factor gene could specifically destabilize β-globin mRNA when inserted into the β-globin gene. A computer search revealed that the A+U-rich sequence was also present in the 3′ ends of many other unstable mRNAs, including that of the c-*myc* gene (23). The study of mRNAs produced by hybrids between the c-*myc* gene and other genes showed that exon 1 did not contain destabilizing sequences but that the 3′ untranslated end of the *myc* gene, containing the A+T-rich region, was required for instability (12). However, the mere presence of the A+U-rich region was not sufficient to render mRNAs unstable. Presumably other factors, such as translatability or secondary and tertiary structure, are also of influence, limiting the use of hybrid genes for this type of study. Therefore, it seemed of interest to turn to a system in which the degradation of the endogenous c-*myc* mRNA can be studied.

It has been reported that treatment of cells with protein synthesis inhibitors causes a substantial stabilization of c-*myc* mRNA (6, 14). Later, this effect was also demonstrated for other growth factor-inducible genes such as c-*myb*, c-*fos*, and c-*jun* (20, 22, 27). Why these mRNAs are stabilized when translation is blocked is not clear. At present, it is believed either that a highly unstable protein is involved in their degradation or that translation of the mRNAs themselves is required for their decay. Recent data from in vivo and in vitro experiments have indicated that c-*myc* mRNA degradation proceeds in at least two steps: shortening of the poly(A) tail and subsequent degradation of the rest of the mRNA (3, 13, 26). Understanding which of these steps is affected by protein synthesis inhibitors may help elucidate the normal route of mRNA decay. Here we show that the stabilization by translation blockers is dependent on the length of the poly(A) tail. Our results suggest a degradation mechanism in which translation-dependent poly(A) tail shortening determines the half-life of c-*myc* mRNA.

MATERIALS AND METHODS

Cell culture and labeling with [^{35}S]methionine. HeLa cells were grown in minimal essential medium supplemented with 4% fetal calf serum and 6% newborn calf serum.

For the labeling experiments, 60-mm-diameter dishes were seeded with 10^6 HeLa cells on the day preceding the experiment. Cells were cultured in medium containing the various drugs for the times indicated in the text. Then the cells were washed twice with phosphate-buffered saline (37°C) containing 0.5 mM Ca^{2+} and Mg^{2+} (PBS), and each dish was incubated with 2 ml of methionine-free minimal essential medium containing 10% dialyzed fetal calf serum, 25 μCi of [^{35}S]methionine per ml, and the appropriate drug

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for 35 min. After two washes with ice-cold PBS, the cells were scraped in 400 μl of 0.5× RIPA lysis buffer (140 mM NaCl, 20 mM triethanolamine [pH 8.0], 0.05% Triton X-100, 0.05% deoxycholate, 0.05% sodium dodecyl sulfate [SDS]) to which protease inhibitors (1 mM phenylmethylsulfonyl fluoride and 0.2 mg of trypsin inhibitor per ml) had been added and were frozen at −80°C. After thawing, the lysates were vortexed for 1 min and centrifuged for 10 min in an Eppendorf centrifuge. For analysis on gel, 20 μl of each lysate was run on a 12.5% polyacrylamide–0.1% SDS gel at 130 V overnight, stained with Coomassie brilliant blue, dried, and exposed to a Kodak XAR film at −80°C. For the trichloroacetic acid precipitations, 50 μl of lysate, to which 50 μl of 20% trichloroacetic acid was added, was incubated at 4°C for 1 h and then centrifuged for 15 min at 4°C. The pellet was washed twice with 96% ethanol, dissolved in 10 μl of 0.1 M NaOH, and counted in 2.5 ml of Packard Scintillation 299 cocktail.

Drugs used to block translation, transcription, and poly(A) tail synthesis. Cycloheximide (CH; Sigma) was dissolved in 50% ethanol–50% water to a 10-mg/ml stock solution, stored at −20°C, and used at 10 μg/ml. Puromycin (PU; Sigma) was dissolved in PBS to a 10-mg/ml stock solution, stored at −20°C, and used at 100 μg/ml. Pactamycin (PM), generously provided by The Upjohn Co., was dissolved in dimethyl sulfoxide to a 0.1 mM stock solution, stored at −20°C, and used at 0.1 μM. Actinomycin D (ActD; Calbiochem or Sigma) was dissolved in water to a 1- or 2-mg/ml stock solution, stored at 4°C, and used at 5 μg/ml. 5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole (DRB; Sigma) was dissolved in ethanol to a 3-mg/ml stock solution, stored at 4°C, and used at 30 μg/ml. Cordycepin or 3′-deoxyadenosine (3dA; Sigma) was dissolved in water to a 2.5-mg/ml stock solution, stored at −20°C, and used at 25 μg/ml.

RNA isolation, oligo(dT) selection, and Northern (RNA) blot analysis. On the day before the experiment, cells were seeded on one 15-cm-diameter dish per time point. Cytoplasmic RNA was isolated by quickly washing the cells twice with ice-cold PBS, scraping the cells in PBS, and removing the nuclei after Nonidet P-40 lysis, followed by phenol extraction and ethanol precipitation of the supernatants (16). To separate poly(A)$^+$ and poly(A)$^-$ RNAs, 100 μg of RNA was dissolved in 200 μl of water and heated for 1 min at 65°C. One volume of 2× loading buffer (40 mM Tris hydrochloride [pH 7.5], 1 M NaCl, 2 mM EDTA, 0.1% SDS) was added, and the solution was incubated for 60 min at room temperature with 25 mg of oligo(dT)-cellulose (Boehringer) with gentle agitation. After the cellulose was spun down, the procedure was repeated with the supernatant (poly(A)$^-$ fraction). The pellet was washed with 5 ml of 1× loading buffer (five times for 5 min each time) and then eluted four times with 250 μl of elution buffer (10 mM Tris hydrochloride [pH 7.5], 1 mM EDTA, 0.05% SDS) for 5 min each time. The eluates were phenol extracted, precipitated (20 μg of tRNA was added as carrier), and pooled. After the second incubation of oligo(dT) with the poly(A)$^-$ fraction, 150 μl of water was added to the supernatant, which was phenol extracted and precipitated. Half of the poly(A)$^+$ and poly(A)$^-$ samples (corresponding to 50 μg of cytoplasmic RNA) was used for a Northern blot.

For standard Northern blots, 20 μg of cytoplasmic RNA (unless otherwise stated) was precipitated, washed with 70% ethanol, and dissolved in sample buffer (50% formamide, 2.2 M formaldehyde), denatured at 56°C, and run on a 2.2 M formaldehyde–1% agarose gel. The RNA was transferred to nitrocellulose, and the filters were baked for 2 h at 80°C, washed in 2× SSC (0.3 M NaCl, 0.03 M sodium citrate), cross-linked with UV light for 2 min, and prehybridized at 42°C in 50% formamide–5× SSPE (0.3 M NaCl, 27 mM $NaH_2PO_4 \cdot H_2O$, 2 mM EDTA)–5× Denhardt solution (0.1% bovine serum albumin, 0.1% Ficoll, 0.1% polyvinylpyrrolidone)–1% SDS–100 μg of small denatured salmon sperm DNA per ml for at least 6 h before the ^{32}P-labeled heat-denatured double-stranded DNA probe was added. The filters were washed in 2× SSC–0.1% SDS at 60°C (and sometimes in 1× SSC–0.1% SDS at 60°C) and exposed to Kodak XAR films at −80°C with an intensifier screen. Bands on the autoradiograms were quantitated by using a Bio-Rad densitometer.

Probes. The c-*myc* probe used was a 1.3-kb *Cla*I-*Eco*RI fragment from the 3′ end of the human c-*myc* gene (1). The c-*fos* probe was a 1.65-kb partial human cDNA containing exons 3 and 4, kindly provided by W. Kruijer. The histone 2B probe was a 1-kb *Xho*I-*Bgl*II fragment from the *Xenopus laevis* histone repeat, kindly provided by M. Timmers and D. de Wit. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probe was a 1.3-kb *Pst*I fragment containing the whole rat cDNA (7).

RNase protection analysis. Samples (30 μg) of cytoplasmic RNA from untreated or CH-treated cells were ethanol coprecipitated with 6 μl of ^{32}P-labeled antisense RNA probe corresponding to a 535-bp *Dde*I fragment from the 3′ end of the human c-*myc* gene (13). The probe was made according to the Promega Riboprobe protocol, with minor modifications. We used 0.5 μg of linearized template DNA, with 13 mM [^{32}P]UTP plus 30 mM cold UTP. The probe was kept in a 200-μl ethanol suspension. The RNA pellets were dissolved in hybridization mix [80% formamide, 0.4 M NaCl, 1 mM EDTA, 40 mM piperazine-*N*,*N*′-bis(ethanesulfonic acid) (PIPES) (pH 6.7)] and hybridized overnight at 37°C. Samples were digested for 60 min at 30°C in 300 μl of RNase buffer (10 mM Tris hydrochloride [pH 7.5], 5 mM EDTA, 0.3 M NaCl) containing 1.5 U of RNase T_1 (Promega). After purification and precipitation, the samples were dissolved in 5 μl of 10 mM Tris hydrochloride–1 mM EDTA. Then 1 μl of each sample was added to 8 μl of formamide and 2 μl of layer mix (15% Ficoll and tracking dye), heat denatured, and loaded onto a denaturing 5% polyacrylamide–8 M urea gel. To the remaining 4 μl was added 1 μl of layer mix containing 0.5% SDS, and the samples were loaded onto a nondenaturing 4% polyacrylamide gel. To prevent warming (and denaturation), the native gel was run at 150 V.

The absorbance curves from the autoradiograms of the native gel were obtained by scanning the gels on an LKB 2222-020 UltroScan XL laser densitometer coupled to a line printer.

RESULTS

Incomplete stabilization of c-*myc* mRNA by translation blockers. Three different drugs known to act through different mechanisms (18, 28) were used to block translation in HeLa cells. CH, which is an elongation blocker, locks the ribosomes onto the mRNA, causing the formation of stabilized polysomes. PU, which also blocks elongation, is an analog of the incoming aminoacyl-tRNA and causes premature release of the peptide chain and the ribosomes, resulting in polysome-free mRNAs. PM is an initiation inhibitor that acts by stabilizing the 40S–Met-tRNA initiation complex and preventing the 60S ribosomal subunit from binding. All three drugs blocked translation in HeLa cells (CH and PM by 95% and PU by more than 99%, as measured by [^{35}S]methionine

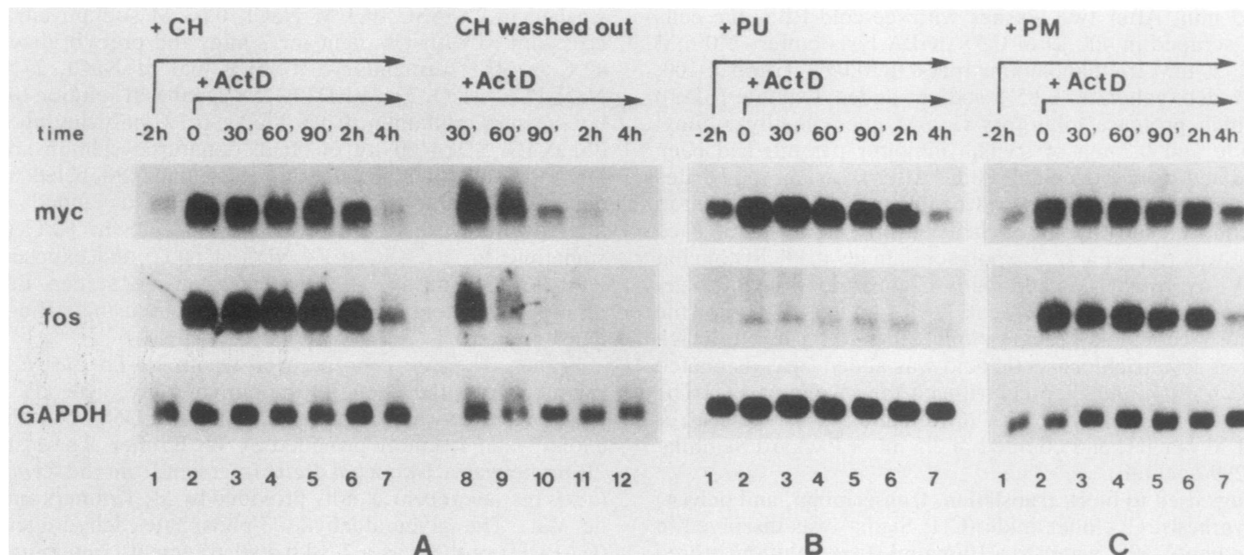

FIG. 1. Northern blot analysis of cytoplasmic RNA from HeLa cells that had been treated for 2 h with CH (A), PU (B), or PM (C) and to which ActD was added at $t = 0$. In lanes 8 to 12, the CH was washed out just before ActD addition. The times indicated refer to the times of addition of ActD. The apostrophe stands for minutes. Because the levels of *myc* and *fos* mRNAs in the cells were not equal after 2 h of treatment with the drugs, the films were exposed to the blots for different lengths of time. Exposures were chosen in which the decay pattern of the mRNAs is clearly visible. Exposure times: (A) *myc*, 6 h; *fos*, 10 days; GAPDH, 3 h; (B) *myc*, 10 h; *fos*, 10 days; GAPDH, 6 h; (C) *myc*, 6 h; *fos*, 16 h; GAPDH, 3 h.

incorporation) and caused an increase in c-*myc* and c-*fos* mRNA (Fig. 1A to C, lanes 1 and 2). For c-*fos*, increased levels of mRNA after treatment of cells with translation blockers have been shown to be caused by both an increase in transcription initiation and a stabilization of the mRNA (8). The levels of c-*fos* mRNA after treatment with the drugs were lower than the induced c-*myc* mRNA levels and varied considerably, depending on which drug was used. To determine the effect of these drugs on the half-life of c-*myc* mRNA, HeLa cells were incubated with each drug for 2 h, whereupon ActD was added to block transcription, and mRNA was isolated at various time intervals. The amount of c-*myc* mRNA present at each time point was determined by Northern blotting (Fig. 1). At the same time, an experiment was performed in which CH was washed out before addition of ActD. After a short lag time presumably due to incomplete removal of CH, rapid decay of c-*myc* mRNA resumed, with the normal half-life of 15 min (Fig. 1A). The results are presented graphically in Fig. 2, in which the intensity of the *myc* bands is plotted logarithmically against time. Variations in mRNA content of the lanes were corrected by comparison with the control GAPDH bands. GAPDH mRNA has a long half-life and is not visibly influenced by drugs that block translation (14, 19). c-*myc* mRNA was consistently stabilized by all three drugs. However, this stabilization was not complete; during the first 2 h after ActD addition, the mRNA decayed slowly, with an apparent half-life of more than 2 h. Around that time, c-*myc* mRNA started disappearing more rapidly. A similar effect was observed for c-*fos* (Fig. 1).

The resumption of a more rapid mRNA degradation could be explained in several ways. One possibility is that prolonged incubation of the cells with the drugs causes stress and cell death. GAPDH mRNA, however, was completely unaffected by the drugs (Fig. 1), and increased cell death was not observed. Another possibility is that somehow translation can resume during prolonged incubation. It did not seem likely that all three drugs were being inactivated or degraded. However, to exclude this possibility, we added a second dose of CH to the cells 2 h after addition of ActD. No change in the pattern of *myc* mRNA degradation was observed (not shown). To test whether the cells were still sensitive to the drugs after prolonged treatment, we measured the [^{35}S]methionine incorporation in cells that had been treated with translation blockers for 4 h. Protein synthesis was still blocked by 95% or more. Thus, it seemed that the observed decay pattern was truly an effect of the translation block. We then performed an experiment similar to the one described above, but instead of preincubating the cells with CH, we added CH and ActD to the cells simultaneously. The effect of CH on c-*myc* mRNA degradation was extremely rapid (Fig. 3). The pattern of c-*myc* mRNA disappearance was similar to that observed when cells were pretreated with CH for 2 h before addition of ActD. Comparable results were obtained with PU and PM (not shown).

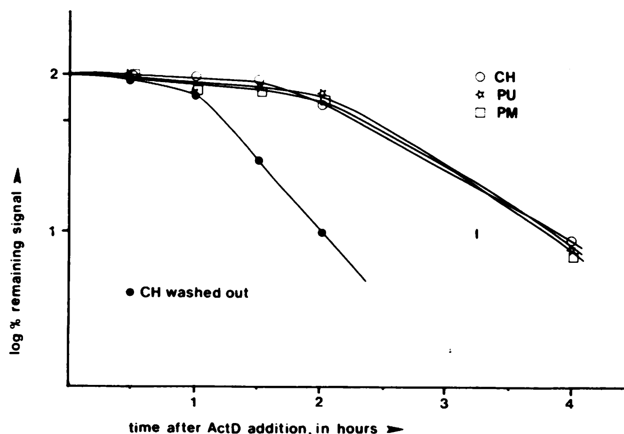

FIG. 2. Graphic representation of the results presented in Fig. 1. The intensity of the c-*myc* bands was corrected for RNA content in each lane by comparison with the GAPDH bands. The percentage of remaining signal was plotted logarithmically against time.

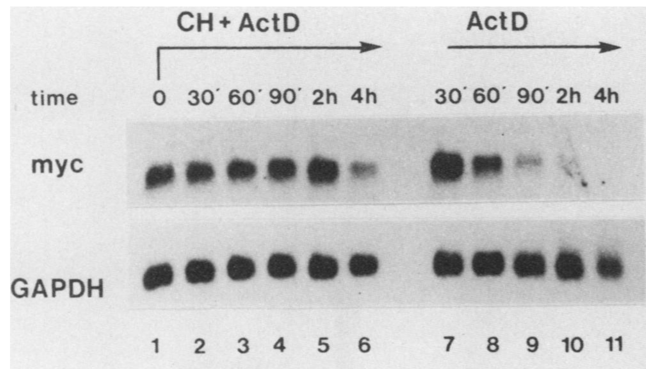

FIG. 3. Northern blot of cytoplasmic RNA from HeLa cells treated with CH and ActD or ActD alone. The times indicated refer to the times of addition of the drugs. The apostrophe stands for minutes. Exposure times: *myc*, 2 days; GAPDH, 3 h.

Poly(A) tail shortening is slowed by drugs that block translation. In an in vitro system used to study mRNA decay, Brewer and Ross (3) have observed that the poly(A) tail of c-*myc* mRNA is shortened, followed by cuts in the 3′ end of the mRNA body. Swartwout and Kinniburgh (26) have shown that *myc* mRNA with a shortened or no poly(A) tail arises by deadenylation of poly(A)$^+$ precursors. Using an RNase protection analysis, we have previously shown that the c-*myc* poly(A) tail is rapidly shortened (13). These experiments indicated that the degradation of c-*myc* mRNA proceeds in at least two steps: shortening of the poly(A) tail, followed by degradation of the rest of the mRNA. Blocking translation may affect either one or both of these steps. Inhibition of poly(A) tail shortening would lead to a stabilized population of c-*myc* mRNAs with long poly(A) tails, whereas inhibition of the degradation of the mRNA body would lead to an accumulation of stabilized poly(A)$^-$ *myc* mRNA. To investigate whether the inhibition of translation affects poly(A) tail shortening, HeLa cells were incubated in medium containing either ActD alone or ActD and CH. RNA was isolated at several time points and an RNase protection experiment was performed with a probe from the 3′-most end of the c-*myc* gene. This probe gave rise to two protected fragments on a denaturing gel (Fig. 4A), corresponding to c-*myc* mRNA ending at either of the two polyadenylation sites (pA1 or pA2). The majority of the messengers end at the downstream (pA2) site (13). The samples were treated with RNase T_1, which cuts exclusively 3′ to G residues and therefore does not degrade the poly(A) tail. In a nondenaturing gel, the migration of the bands will depend on the length of the poly(A) tail (13). The major pA2 band is then visible as a smear extending upwards of 370 nucleotides (the position of the protected band from nonpolyadenylated pA2 mRNA). The smear derived from the pA1 band is too weak to be visible.

In the experiment shown in Fig. 4A, the level of c-*myc* mRNA initially increased (t = 30 min) when CH was added together with ActD. This phenomenon was observed several times and is presumably due to the fact that CH, which enters the cell very quickly (20), stimulates transcription before transcription can be blocked by ActD. From the denaturing gel (Fig. 4A), it is clear that c-*myc* mRNA disappeared much less quickly in the presence of CH. Whether this was due to accumulation of mRNAs with short or long poly(A) tails can be deduced from the native gel, where one can see that a long smear remained present for up to 2 h. In the absence of CH, there was a rapid decrease in poly(A) tail length and in mRNA levels. The changes within the smears are more obvious in the densitometric scans of the autoradiogram of the native gel (Fig. 4B to D). In the presence of CH (Fig. 4B), there was a slow decrease in signal, with a slow loss of mRNA with long poly(A) tails (top of the smear). In the absence of CH (Fig. 4C), there was a rapid loss of mRNA with long poly(A) tails. That the observed shortening of the poly(A) tail smear seen on the autoradiograph is not due to lack of signal in the lanes containing RNA from later time points is illustrated by lanes A to C in Fig. 4A. Though these lanes contained comparable amounts of signal (lane C is a longer exposure of the 4-h time point in the presence of CH), the distributions of the signals were not alike, as visualized by the scans shown in Fig. 4D. Whereas the signal was strongest in the top of the smear at time point 0, most of the signal was in the middle after 90 min of CH and ActD treatment and at the bottom after 4 h. It therefore appears that the patterns of degradation in the presence or absence of CH were similar but that the process of degradation was substantially slowed. We did not see an accumulation of stabilized deadenylated mRNA, which one would expect if poly(A) tail shortening occurred at the normal pace. Therefore, we conclude that shortening of the c-*myc* RNA poly(A) tail is slowed when translation is blocked.

The stabilizing effect of translation blockers is dependent on the length of the poly(A) tail. If the stabilizing effect of translation-blocking drugs works via a slowing of poly(A) tail removal, one could assume that mRNAs with shortened or no poly(A) tails would be less susceptible to stabilization by these drugs. We tested this hypothesis in two ways. First, we incubated HeLa cells in medium containing ActD and added CH either directly or after 45, 60, or 90 min. At the moment that ActD is added, the c-*myc* mRNA population is a mixture of RNAs with different poly(A) tail lengths, reflecting the situation in exponentially growing cells. However, after transcription has been blocked for 45 min or longer, the average poly(A) tail length of c-*myc* mRNA will have decreased substantially (13). The stabilizing effect of CH decreased as the preincubation time with ActD was lengthened (Fig. 5). When CH was added after 45 min, the period of stabilization was shorter than when CH was added together with ActD. When CH was added after 60 min, it barely had any effect. After 90 min, c-*myc* mRNA was no longer visible (not shown). Thus, it seems that the period during which stabilization occurs is dependent on the length of the poly(A) tail.

To confirm this result, we performed another set of experiments by using the adenosine analog 3dA or cordycepin. Even though 3dA has an inhibitory effect on transcription, it mainly interferes with the formation of the poly(A) tail by preventing chain elongation (24, 31). mRNA with shortened poly(A) tails due to 3dA treatment has been shown to be spliced correctly and is still capable of being transported into the nucleus and entering the polysomes (31).

In exponentially growing HeLa cells, most of the c-*myc* mRNA had a long poly(A) tail, leading to its retention upon oligo(dT) selection (Fig. 6, lane 2). A minority of the RNA had a tail so short that it was not bound (lane 3). Treatment of the cells with 3dA for 2 h resulted in the formation of mRNAs that did not bind to oligo(dT)-cellulose (lanes 4 to 6). Most of the original poly(A)$^+$ c-*myc* mRNA had been degraded by that time. Because 3dA inhibits poly(A) tail formation by blocking its elongation, it seems likely that these mRNAs are not completely poly(A)$^-$ but have short

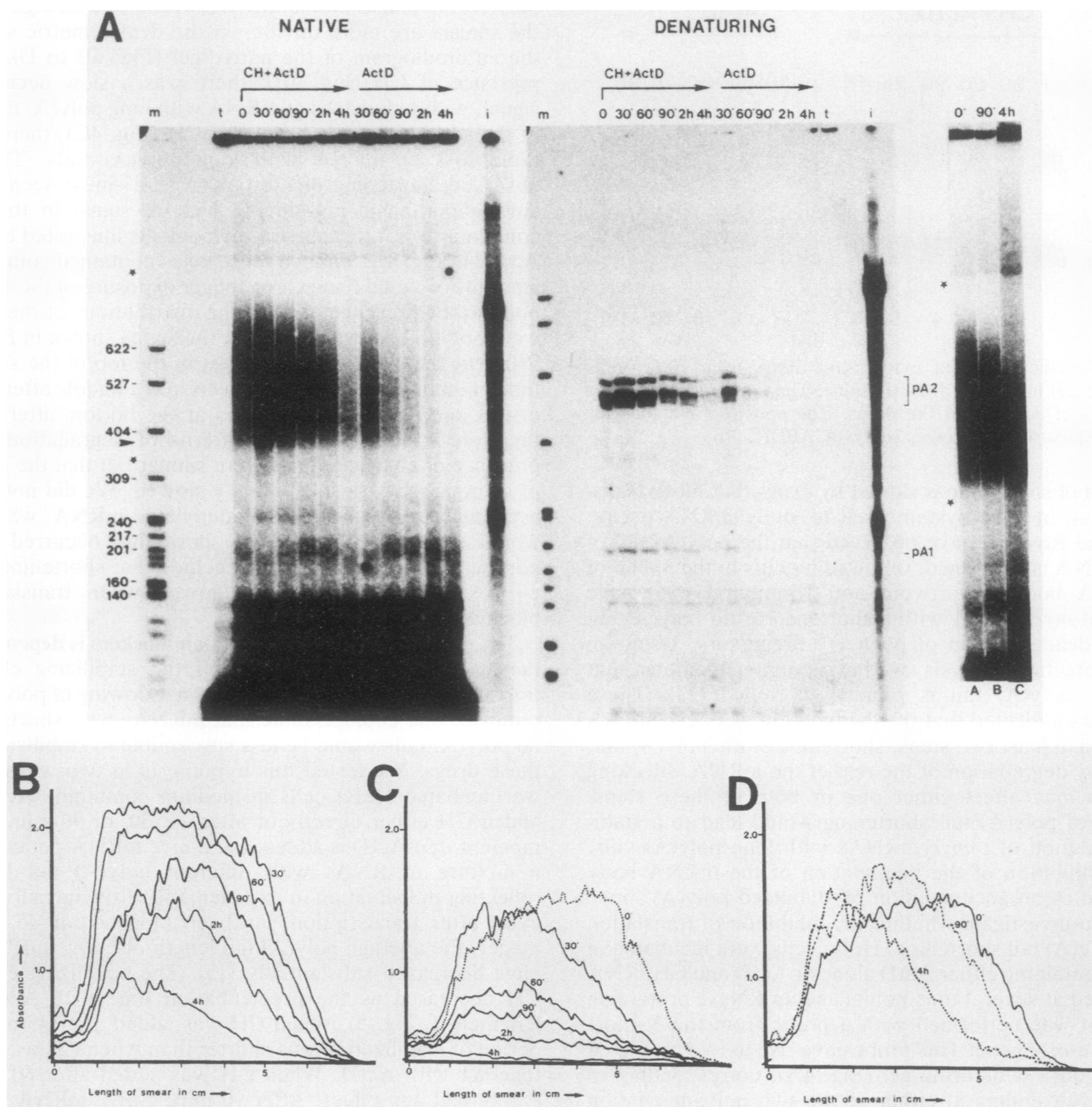

FIG. 4. (A) RNase protection analysis of cytoplasmic RNA from HeLa cells treated with CH and ActD or ActD alone. RNA (30 μg) was hybridized to a 3′ c-*myc* RNA probe as described in Materials and Methods. The samples were digested with RNase T_1, purified, and precipitated. Then 20% of each sample was denatured and loaded onto a denaturing polyacrylamide gel. The rest of each sample was loaded onto a native gel. Numbers at the top of the lanes indicate incubation times with CH plus ActD or with ActD alone. The apostrophe stands for minutes. The letters m, t, and i refer to marker, tRNA control sample (an equivalent amount of *Escherichia coli* tRNA was used), and input probe (5 to 10 cps of probe was loaded), respectively. Numbers alongside the gel indicate sizes of the molecular weight marker (pBR322 digested with *Hpa*II) in nucleotides. pA2 and pA1 refer to the positions of the protected bands of mRNAs terminating at either poly(A) site in the denaturing gel. The arrowhead alongside the native gel denotes the expected position of poly(A)$^-$ *myc* mRNA ending at pA2. The region between the asterisks was scanned densitometrically (see panels B to D). The native and denaturing gels were exposed for 16 h. Lanes A, B, and C at the right are three lanes from the native gel (samples plus CH) that have been aligned to facilitate comparison of the poly(A) tail smear. Lanes A and B are 16-h exposures; lane C is a 2-day exposure. The intensities of the signal are comparable in all three lanes. (B to D). Densitometric scans of autoradiograms from the native gel. (B) Disappearance of signal in the presence of CH. Because the *myc* mRNA level increased between t = 0 and t = 30 min, the t = 0 curve has been omitted. (C) Disappearance of signal in the absence of CH. t = 0 is represented by a dotted line. (D) Scans of lanes A (0′), B (90′), and C (4 h). For the lengths of the smears, a relative scale in centimeters was used.

runs of adenylate residues at their 3′ ends. By comparing the stability of c-*myc* mRNA in cells treated with 3dA in the presence or absence of CH, one can establish the effect of a translation block on mRNAs with a short poly(A) tail (Fig. 7). The half-life of c-*myc* mRNA from 3dA-treated cells was similar to that of normal *myc* mRNA. Addition of CH to 3dA-treated cells (though initially stimulating transcription before it could be blocked by ActD) did not visibly stabilize c-*myc* mRNA compared with incubations in the absence of CH. Histone 2B mRNA, however, was stabilized by CH in

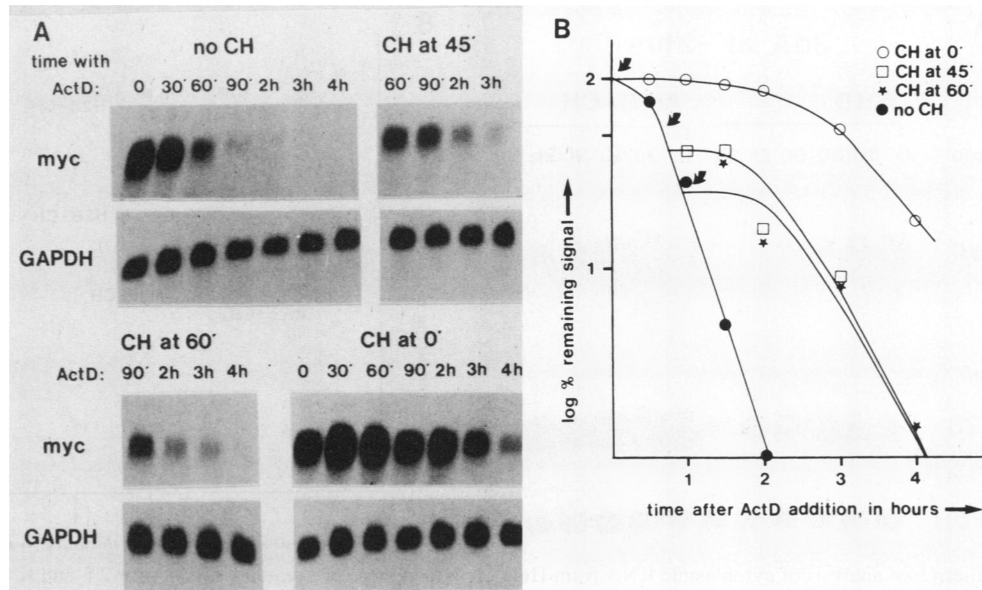

FIG. 5. (A) Northern blot analysis of cytoplasmic RNA from HeLa cells treated with ActD for the times given, either without CH or with the addition of CH at the time specified. The apostrophe stands for minutes. Exposure times: *myc*, 2 days; GAPDH, 6 h. (B) Graphic representation of the data shown in panel A. The intensity of the *myc* bands was corrected for RNA content of the lanes by comparison with the intensity of the GAPDH control bands. Arrows indicate the addition of CH. Multiple experiments have produced similar results.

3dA-treated cells. The half-life of nonpolyadenylated histone mRNAs is dependent both on a hairpin structure at the 3′ end of the mRNA and on complete translation of the coding region (10), leading to stabilization of histone mRNA in cells treated with CH (25). Since histone messengers lack a poly(A) tail, one does not expect 3dA treatment to affect the stabilization of this mRNA by CH.

DISCUSSION

Treatment of cells with drugs that inhibit protein synthesis leads to a stabilization of c-*myc* and c-*fos* mRNAs, as measured by ActD chase experiments. ActD blocks transcription by intercalating into DNA and might interfere with mRNA degradation by intercalating into double-stranded RNA regions. However, the half-life of c-*myc* mRNA as calculated from the approach to steady-state labeling (19) is similar to the value derived from ActD chase experiments. The half-life of c-*fos* mRNA calculated from the rate of disappearance of the mRNA after growth factor (GF) induction is also comparable to measurements from ActD experiments (29). This result indicates that ActD can be used to obtain a representative value for the half-lives of these mRNAs. We have also used the transcription blocker DRB, which is a ribonucleotide analog that interacts with the RNA polymerase II transcription complex (15, 30), to measure the half-life of c-*myc* RNA. Values similar to those from our ActD chase experiments were obtained (not shown). When DRB was used to block transcription in CH-treated cells, we observed a stabilization of c-*myc* mRNA (not shown), as we did for CH in combination with ActD. Thus, the kind of drug used to block translation (CH, PU, or PM) or transcription (ActD or DRB) did not affect the results obtained in our experiments.

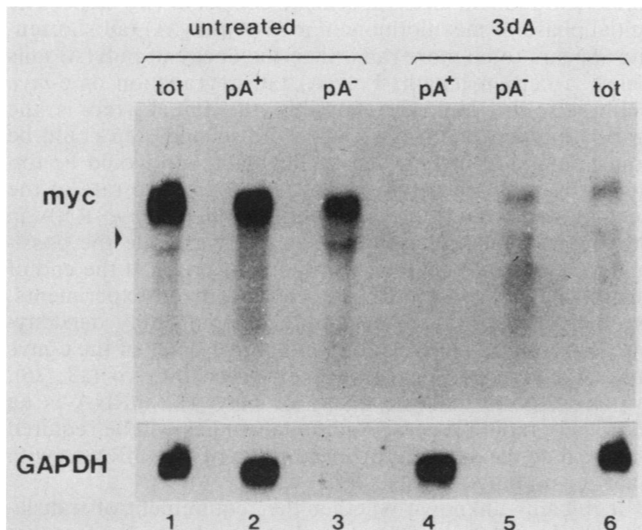

FIG. 6. Northern blot analysis of total cytoplasmic RNA (tot), poly(A)$^+$ RNA (pA$^+$), and poly(A)$^-$ RNA (pA$^-$) from untreated HeLa cells or cells treated with 3dA for 2 h. The arrow denotes the position of the 18S rRNA band. This band interferes with the signal obtained from *myc* mRNA with short poly(A) tails because of the similar sizes of these RNAs. Exposure times: *myc*, 3 days; GAPDH, 7 h.

The stabilization of c-*myc* mRNA in cells treated with translation-blocking drugs was not complete. There was a small but reproducible decrease in c-*myc* RNA during the first 2 h after the addition of ActD. At later time points, the disappearance of the mRNA seemed to accelerate. This pattern of decay was independent of the preincubation time of the cells with the translation-blocking drugs and was always seen after a block of transcription. Therefore, it seems that this pattern can be observed only when no new *myc* mRNA is synthesized. This conclusion is in agreement with the fact that older c-*myc* mRNAs with shorter poly(A) tails are not stabilized to the same extent as newly synthesized ones (see below). When the production of new mRNAs is blocked, the poly(A) tails will gradually become shorter until a stage is reached in which only mRNAs with short poly(A) tails are left. Since the degradation of these mRNAs

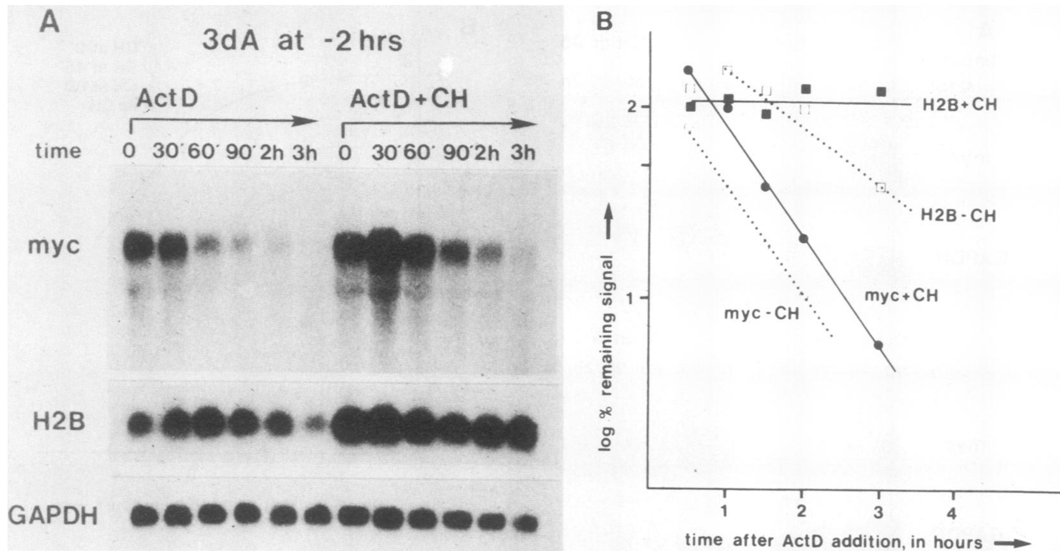

FIG. 7. (A) Northern blot analysis of cytoplasmic RNA from HeLa cells that were pretreated with 3dA for 2 h and to which either ActD alone or ActD and CH were added. Times given at the top of the lanes correspond to the times of addition of ActD and CH. The apostrophe stands for minutes. Exposure times: *myc*, 4 days; histone 2B (H2B), 16 h; GAPDH, 1.5 h. (B) Graphic representation of the results given in panel A. The intensities of the *myc* and histone 2B bands were corrected for the amount of RNA loaded in each lane by comparison with the bands of the GAPDH control. The percentage of remaining signal was plotted logarithmically against time.

does not require translation, these mRNAs decay more rapidly.

We have demonstrated that the normally rapid poly(A) tail shortening of c-*myc* mRNA is delayed in the absence of translation. A similar phenomenon has been described by Wilson and Treisman for c-*fos* (29). They were able to show a clear shortening of the poly(A) tail of c-*fos* mRNA synthesized after GF induction. In the presence of CH, this shortening was significantly slowed but not completely blocked. The residual poly(A) tail shortening observed for c-*myc* and c-*fos* can explain why these mRNAs are not stabilized completely. This residual decay could be explained by the low level of translation still taking place in the cell or by the fact that the coupling between translation and poly(A) tail degradation is not 100%. Because the pattern of decay of c-*myc* was independent of which drug was used to block translation, while only PU blocked protein synthesis for more than 99%, we favor the latter possibility.

We have used two types of experiments to study the effect of protein synthesis inhibition on the decay of mRNAs with short poly(A) tails. By adding CH to cells at different times after ActD addition, we show that the stabilizing effect of CH on c-*myc* mRNA decreases with the age of the mRNA. Similar results were obtained by Rhamsdorf and co-workers (20), who studied the effect of CH on c-*fos* mRNA decay after GF induction. When CH was added together with GF, a strong stabilization was observed. However, addition of CH 60 min after GF induction resulted in only a very slight stabilization. These findings can be explained by assuming that CH most strongly affects the degradation of newly synthesized c-*fos* mRNAs, which have long poly(A) tails. At 60 min after GF induction, a large part of the synthesized c-*fos* messengers has already been degraded, and the remaining c-*fos* mRNA has a very short poly(A) tail (29) and thus will be stabilized very little by CH addition.

The effect of a translation block on the decay of newly synthesized mRNA with short poly(A) tails was studied by using the drug 3dA. We have shown that c-*myc* mRNA from HeLa cells, treated with 3dA for 2 h, decays with the same speed in the presence or absence of CH. In contrast, histone 2B mRNA, which is not polyadenylated and therefore is not affected by 3dA treatment, is stabilized by CH addition. This result shows that c-*myc* mRNA can be degraded independently of translation when it lacks a long poly(A) tail.

In view of our findings, we propose that the degradation of c-*myc* mRNA proceeds in at least two steps. The first is a translation-dependent poly(A) tail shortening, and the second is a translation-independent decay of the rest of the mRNA. Studies of poly(A) tail shortening have indicated that this does not necessarily proceed with the same rate for poly(A) tails of different lengths (17). Poly(A) tail shortening of metallothionein mRNA seems to consist of two steps. The initial phase of metallothionein mRNA poly(A) tail shortening appears to be more rapid than the decay of poly(A) tails below a certain length. Poly(A) tail degradation of c-*myc* could also be biphasic. Possibly the initial step is the translation-dependent one, while the second step could be translation-independent decay. This last step would be followed by rapid decay of the mRNA body. The rate of the second step would determine the half-life of *myc* RNA in 3dA-treated cells. However, we cannot exclude the possibility that the presence of a 3′ hydroxyl group at the end of the tail may influence mRNA decay. From our experiments, we cannot determine the half-life of completely deadenylated c-*myc* mRNA. Degradation intermediates of the c-*myc* mRNA body have never been observed in vivo (13, 26), which suggests that the decay of poly(A)$^-$ mRNA is an extremely rapid process. Additional studies will be required to elucidate the degradation mechanism of *myc* mRNAs with short or no poly(A) tails.

It remains unknown whether the requirement of translation for degradation of the poly(A) tail is due to the fact that an unstable protein is involved or that the translation of the unstable messengers themselves is needed for their degradation. From the fact that CH stabilizes c-*myc* almost immediately, it appears that a putative unstable protein involved in mRNA degradation would have an extremely short half-life of just a few minutes. Brewer and Ross (4) have reported

evidence for an unstable protein from their in vitro experiments. Addition of a certain cellular fraction to their decay reactions accelerated c-*myc* mRNA degradation, while addition of this fraction isolated from cells treated for 3 h with CH did not affect decay rates. However, they did not make extracts from cells treated with CH for shorter periods of time. Moreover, the influence of translation in their in vitro system is still unclear. There are at least two precedents for the coupling of translation of an mRNA to its proper degradation. Translation is involved in the degradation both of β-tubulin mRNA and of nonpolyadenylated histone messengers. The coupling of tubulin mRNA degradation to protein synthesis is a result of the fact that in the presence of an excess of free tubulin subunits, the nascent peptide somehow activates a mechanism that destroys the mRNA (9). For the proper decay of histone mRNA, it must contain a hairpin structure at its 3′ end and must be translated until the normal stop codon. Insertion of nonsense codons upstream of the normal stop codon or increasing the distance between the normal stop codon and the 3′ end of the mRNA disrupts the regular decay mechanism (10). Indeed, CH treatment leads to stabilization of histone mRNAs (25; Fig. 7). A polysome-associated nuclease involved in histone mRNA degradation has been reported (21). Possibly such a polysome-associated nuclease is also involved in poly(A) tail removal of c-*myc* mRNA. Recently, a nuclease activity that is tightly associated with mRNA has been reported (2). The authors report that the nuclease responsible for this activity is not ribosome bound, but that movement of the ribosomes along the mRNA may be important for its function. Our experiments with the different translation blockers also suggest that the presence of ribosomes on the RNA is not sufficient for rapid decay, but that ribosome movement may be important. To address this question, we are in the process of analyzing mutated *myc* genes containing nonsense codons upstream of the normal stop codon.

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