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Regulation of c-*myc* mRNA Stability In Vitro by a Labile Destabilizer with an Essential Nucleic Acid Component

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The turnover rates of some mRNAs vary by an order of magnitude or more when cells change their growth pattern or differentiate. To identify regulatory factors that might be responsible for this variability, we investigated how cytosolic fractions affect mRNA decay in an in vitro system. A 130,000 × *g* supernatant (S130) from the cytosol of exponentially growing erythroleukemia cells contains a destabilizer that accelerates the decay of polysome-bound c-*myc* mRNA by eightfold or more compared with reactions lacking S130. The destabilizer is deficient in or absent from the S130 of cycloheximide-treated cells, indicating that it is labile or is repressed when translation is blocked. It is not a generic RNase, because it does not affect the turnover of δ-globin, γ-globin, or histone mRNA and does not destabilize a major portion of polysomal polyadenylated mRNA. The destabilizer accelerates the turnover of the c-*myc* mRNA 3′ region, as well as subsequent 3′-to-5′ degradation of the mRNA body. It is inactivated in vitro by mild heating and by micrococcal nuclease, suggesting that it contains a nucleic acid component. c-*myb* mRNA is also destabilized in S130-supplemented in vitro reactions. These results imply that the stability of some mRNAs is regulated by cytosolic factors that are not associated with polysomes.

mRNA levels can be regulated by changing not only gene transcription rates but also mRNA turnover rates, and the half-lives of some mRNAs can vary by an order of magnitude or more (reviewed in references 55, 56, and 63). Such variations apparently make up part of the normal pleiotropic response to cell proliferation and differentiation factors and usually involve only a subset of mRNAs.

The apparent rationale for regulating mRNA stability is to provide alternate posttranscriptional pathways for controlling the levels of subsets of mRNAs. For example, mRNAs like c-*myc* mRNA, whose products are thought to influence cell replication, are usually relatively unstable, with half-lives of ≤1 h (2, 13). As a result of their intrinsic instability, even modest changes in their turnover rates affect their steady-state levels over a relatively short time. This sort of short-term regulation might ensure that the quantities of these mRNAs per cell are maintained within a very limited range. The necessity for such precise regulation is consistent with the finding that inappropriate expression of these and other mRNAs and their protein products can interfere with cell replication and differentiation (1, 7, 12, 30, 31, 39, 47). mRNAs whose decay rates seem to be regulated include those expressed from proto-oncogenes (13, 15, 20, 35, 41, 72); genes related to cell division, including the histones (reviewed in reference 36); acute-phase response and inflammatory response genes (3, 19); interferon genes (80); heat shock protein genes (65, 71); and developmentally regulated genes (16, 62, 73).

Since the turnover rates of so many mRNAs vary, it is important to identify and characterize putative stability-regulating factors. We and others have developed in vitro mRNA decay systems, three properties of which suggest their usefulness for investigating mRNA stability (46, 48, 57, 58, 67, 68). (i) They apparently mimic the authentic degradation pathways of at least some mRNAs. For example, histone mRNA is degraded in a 3′-to-5′ direction both in cells and in vitro (57, 59). c-*myc* mRNA is degraded in vitro by a pathway involving poly(A) removal followed by degradation of the mRNA body (6), and some polyadenylated mRNAs seem to be degraded by a similar pathway in cells (20, 69, 70, 75). (ii) Structural mutations that affect mRNA stability in cells have similar effects in vitro. Thus, altering the 5′-untranslated region stabilizes c-*myc* mRNA both in cells and in vitro (17, 26, 46, 51, 53), and polyadenylation stabilizes histone mRNA both in cells and in vitro (48). (iii) Most importantly, the in vitro systems seem to be useful for investigating how mRNA stability might be regulated. For example, histone mRNA decay is accelerated in vitro when histone proteins and a postribosomal supernatant fraction (S130) are added to reactions containing polysomes (49). The effect is highly specific for histones and histone mRNA and requires the S130 fraction; histones added alone, without S130, have no effect on histone mRNA decay. This autoregulatory pathway might function to control histone production in cells as they progress through the S and M phases of the cell cycle (reviewed in reference 36).

The experiments described here were designed to identify cytoplasmic components that might regulate the stability of proto-oncogene mRNAs, such as c-*myc* and c-*myb*. We chose to focus on c-*myc* mRNA for three reasons. (i) It is as unstable in vitro as it is in whole cells (6, 13, 72; this paper). (ii) Portions of the c-*myc* mRNA decay pathway in vitro parallel those observed in cells (6, 69, 70). (iii) In some cells, c-*myc* mRNA is stabilized when translation is inhibited and its decay rate varies during the early stages of the mitogen-induced transition from the G_0 to the G_1 phase (13, 27, 32, 72). Therefore, regulatory factors probably control its stability under certain growth conditions (see also reference 44), and we reasoned that such factors might be functional in vitro. Here we present evidence for a labile, nucleic acid-containing activity (or activities) that destabilizes c-*myc* mRNA but not all mRNAs.

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MATERIALS AND METHODS

Cell culture. The human erythroleukemia cell line K562 (14, 33) was maintained as an exponentially growing culture in RPMI 1640 medium with 10% calf serum. Cycloheximide (CHX; Sigma Chemical Co., St. Louis, Mo.) was dissolved in RPMI 1640 and added to the cells at a final concentration of 100 μg/ml for 2 h before harvesting.

Preparation of polysomes and S130. After growing to a concentration of 2×10^5 to 4×10^5 cells per ml in 1-liter spinner bottles, the cells were harvested, washed, concentrated to approximately 6×10^7 cells per ml, and lysed by detergent-free homogenization in buffer A (10 mM Tris hydrochloride [pH 7.6], 1 mM potassium acetate, 1.5 mM magnesium acetate, 2 mM dithiothreitol) (57). Polysomes and S130 were then separated by ultracentrifugation as previously described (57). For some experiments, the S130 fraction was concentrated approximately fourfold by centrifugation at 4°C in a Centricon-10 tube (Amicon Corp., Lexington, Mass.).

In vitro mRNA decay reactions. In all of the experiments reported here, we analyzed the in vitro degradation of polysome-associated mRNAs. All of the mRNA decay reaction mixtures (final volume, 25 μl) contained polysomes and were prepared and incubated as previously described (58). Standard reactions contained 0.7 A_{260} U of polysomes (from $\sim 4 \times 10^6$ cells) and, as noted, variable amounts of S130. One hundred micrograms of S130 protein was derived from approximately 2×10^6 cells. RNA was purified by phenol extraction (57).

Analysis of polysome-associated mRNAs. RNase protection mapping with RNases P1 and T1, deoxyoligonucleotide-directed RNase H cleavage assays (H mapping), and blotting of c-*myc* mRNA were performed as previously described (6). The c-*myc* probes for these assays were derived from cDNA clone pM1-11, kindly provided by Grace Ju and colleagues (42). In some P1-plus-T1 RNase-mapping experiments, the protected fragments were electrophoresed in a polyacrylamide minigel (7 by 9 cm; Bio-Rad Laboratories, Richmond, Calif.). To detect c-*myb* mRNA, a ^{32}P-labeled v-*myb* probe (G. Holland and R. Risser, University of Wisconsin, Madison) was hybridized to the blot for 18 h at 60°C by the method of Church and Gilbert (10). For RNase P1-plus-T1 mapping of the 3′ region of δ-globin mRNA, a portion of the human δ-globin gene from the *Eco*RI site in exon 3 to the *Pst*I site in the 3′-flanking region was subcloned in the antisense orientation downstream of the SP6 promoter in transcription vector pSP65 (Promega Biotec, Madison, Wis.) to construct plasmid pSPδ7. Plasmid DNA was cut with *Eco*RI, and uniformly labeled [^{32}P]RNA was transcribed by SP6 RNA polymerase (Promega Biotec) with [α-^{32}P]UTP as the labeled nucleotide (40). RNA extracted from in vitro decay reactions was hybridized to 10^6 cpm of this probe, treated with RNases P1 and T1, and analyzed by gel electrophoresis as previously described (6). The relative intensities of the protected bands in these experiments were determined by using a soft-laser densitometer, and at least two exposures of the same autoradiogram were analyzed to ensure linearity of the film.

In vitro decay of ^{3}H-labeled poly(A)$^+$ polysomal mRNA. ^{3}H-labeled polysomes were isolated by the method described above, with K562 cells that had been cultured 18 h in medium containing [^{3}H]uridine (10 μCi/ml). In vitro decay reactions (25 μl) contained 0.7 A_{260} U of ^{3}H-labeled polysomes plus 100 μg of bovine serum albumin (BSA) or S130 protein. The reactions were incubated at 37°C for various times, and RNA was extracted. Approximately 5×10^5 cpm of RNA from each time point was separated into poly(A)$^-$ and poly(A)$^+$ fractions by two cycles of chromatography on oligo(dT)-cellulose (Collaborative Research, Inc., Waltham, Mass.). The quantity of trichloroacetic acid-precipitable RNA in each fraction was then measured by liquid scintillation counting. Recoveries of input counts were >90%, and the percentage of bound [poly(A)$^+$] counts was <5% for all samples.

Biochemical characterization of the destabilizer. For proteinase K treatment, 100-μl samples of S130 (500 μg of protein) were incubated at 30°C for 30 min under the following conditions: proteinase K at 1 mg/ml, proteinase K at 1 mg/ml–1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM PMSF, or 1% ethanol (PMSF was dissolved in 100% ethanol and diluted 1:100 into the protease reactions). For micrococcal nuclease treatment, S130 (100 μg of protein) was mixed with $CaCl_2$ and micrococcal nuclease to final concentrations of 1 mM and 1,000 U/ml, respectively. The reaction mixtures were then incubated at 30°C for 10 min, ethyleneglycol-bis(β-aminoethyl ether) *N*,*N*′-tetraacetic acid (EGTA) was added to a final concentration of 2 mM, thereby blocking further nuclease action, and the treated S130 was left on ice for 10 min. In vitro decay reactions with these treated S130s were performed as described above.

RESULTS

Destabilization of c-*myc* and c-*myb* mRNAs by a cytosolic activity (or activities). The rapid fluctuations in the expression of c-*myc* and c-*myb* genes that accompany changes in cell proliferation or protein synthesis rates might result, in part, because the stabilities of their mRNAs are modulated in certain cells (27, 32, 72). Any or all of the following hypotheses could account for the stabilization of the mRNAs that occurs when protein synthesis is inhibited. (i) The mRNAs must be translated to be degraded rapidly. (ii) The mRNAs are degraded by labile nucleases that are destroyed or inactivated soon after translation ceases. (iii) Soluble cytoplasmic components (distinct from mRNA-degrading nucleases) control the stability of these mRNAs and are inactivated when translation is blocked. We used an in vitro mRNA decay system to determihe whether such regulatory components could be assayed in extracts prepared from untreated cells or cells exposed to a translation inhibitor.

The first experiment was performed to ensure that CHX stabilized c-*myc* mRNA in K562 erythroleukemia cells and to estimate the extent of stabilization. Exponentially growing cells were either preincubated for 2 h in medium containing 100 μg of CHX per ml or left untreated. Actinomycin D was then added to both flasks of cells, and samples from each were harvested at various times thereafter. Total cell RNA was prepared, and the quantity of c-*myc* mRNA was determined by RNase P1-plus-T1 mapping (see Materials and Methods; see also the diagram in Fig. 2). CHX treatment clearly stabilized the mRNA (Fig. 1). Its half-life was 20 min in untreated cells, compared with 170 min in treated cells. Therefore, CHX induced seven- to eightfold stabilization of c-*myc* mRNA in K562 cells.

To determine whether the stabilization effect were reproducible in an in vitro mRNA decay system, crude cytoplasmic extracts were prepared from untreated cells and from cells exposed to CHX for 2 h. Each extract was then separated by ultracentrifugation at 130,000 × *g* into polysomes and a postpolysomal supernatant fraction (S130). In the first experiment, the in vitro decay of polysome-associ-

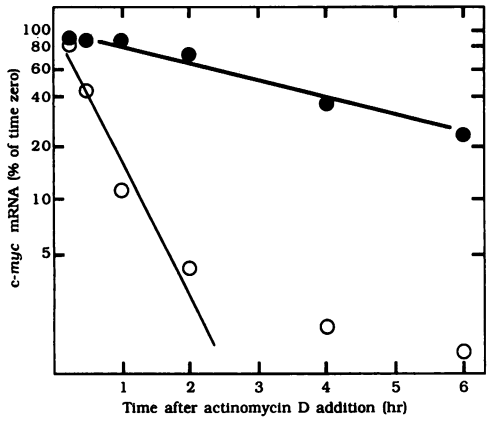

FIG. 1. Half-lives of c-*myc* mRNAs in untreated and CHX-treated K562 cells. Exponentially growing cells were divided between two flasks, one of which was preincubated for 2 h in medium containing CHX at 100 μg/ml. Actinomycin D (5 μg/ml) was then added to both flasks. At various times thereafter, cell samples were harvested and total cell RNA was prepared. The c-*myc* mRNA remaining at each time was determined by hybridizing 5 μg of the RNA with a uniformly labeled RNA probe complementary to the 3′-most 200 nt of the mRNA (see the diagram in Fig. 2). The annealing reactions were incubated with RNases P1 and T1, and protected fragments were electrophoresed and quantitated by scanning densitometry. The percentage of undegraded mRNA was plotted as a function of time after actinomycin addition. Symbols: ○, no CHX; ●, CHX at 100 μg/ml.

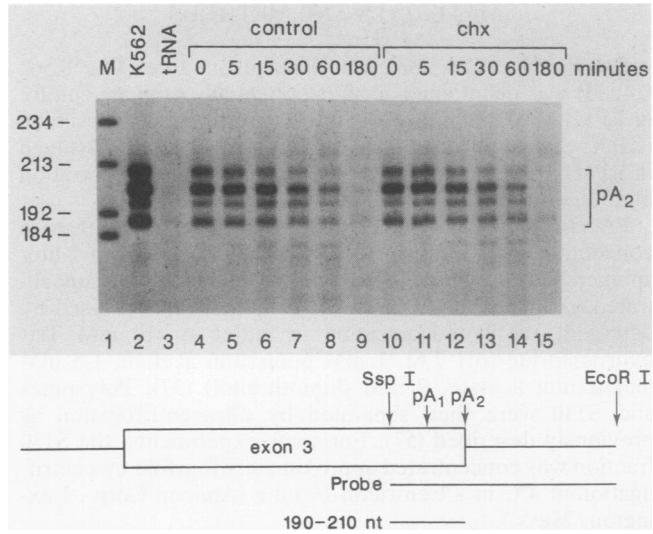

FIG. 2. c-*myc* mRNA decay in reactions containing polysomes from exponentially growing (control) or CHX-treated K562 cells. Standard in vitro decay reactions contained equivalent amounts of polysomes and were incubated for the indicated times. RNA was extracted and annealed with a uniformly labeled, 620-nt [^{32}P]RNA probe (see diagram), and the hybrids were treated with RNases P1 and T1 as previously described (6). Protected fragments were separated in a 7 M urea–6% polyacrylamide gel which was exposed for 2 days with an intensifying screen. Lanes: 1, marker (M) pBR322 ^{32}P-labeled DNA cleaved with *Hae*III (sizes in nucleotides are noted on the left); 2, 15 μg of total RNA from exponentially growing cells; 3, 15 μg of *E. coli* tRNA; 4 to 9, 4 μg of RNA–polysomes from untreated (control) cells; 10 to 15, 2 μg of RNA–polysomes from CHX-treated cells. pA_2 indicates the bands generated by undegraded c-*myc* mRNA; they correspond to four distinct molecules with closely linked poly(A) addition sites located in a region referred to as poly(A) site 2 (6). The diagram shows the [^{32}P]RNA probe from the 3′ region of the human c-*myc* gene. The large open box represents exon 3; straight lines indicate intervening sequence or 3′-flanking DNA. The two poly(A) addition site regions and the *Ssp*I and *Eco*RI sites used to generate the transcription vector are noted. The longer line is the uniformly labeled probe synthesized by transcription of the *Ssp*I-cleaved vector with SP6 RNA polymerase. The shorter line indicates the 190- to 210-nt region of the probe that is protected by c-*myc* mRNAs whose 3′ termini are located at pA_2.

ated c-*myc* mRNA was compared in reactions containing polysomes (no S130). mRNA-degrading enzymes are polysome associated in this in vitro system (6, 57). Therefore, c-*myc* mRNA should be degraded more slowly with polysomes derived from inhibitor-treated cells if the mRNases were unstable or if polysome-associated factors alone were responsible for the stabilization effect. c-*myc* mRNA isolated from unincubated polysomes protected four major bands in an RNase P1-plus-T1-mapping assay (Fig. 2, lanes 4 and 10). The bands corresponded to intact c-*myc* mRNA molecules that were polyadenylated at four closely linked sites, referred to collectively as poly(A) site 2 (pA_2) (see reference 6 for a map showing the locations of these sites). The intensities of all four bands diminished at similar rates during incubation, indicating that the mRNA 3′ region was degraded rapidly. However, it was degraded at similar rates with polysomes from control or CHX-treated cells (compare lanes 4 to 9 with lanes 10 to 15). Therefore, the in vitro activity of the polysome-associated mRNase(s) that degrades c-*myc* mRNA was unaffected by CHX treatment. It should be noted that mRNA decay products were generated in these reactions, as previously described (6), but the autoradiogram was exposed for an insufficient time to visualize them clearly.

To determine whether nonpolysomal cytosolic factors affect c-*myc* mRNA turnover in vitro, polysome-containing reactions were supplemented with S130 from control or CHX-treated cells. The S130 fraction is known to contain one or more components that influence histone mRNA turnover (49) and was thus a logical source to search for factors that regulate c-*myc* mRNA stability. Reactions containing polysomes from untreated cells were supplemented with BSA or S130, RNA was extracted and analyzed as described for Fig. 2, and two major observations were made. (i) c-*myc* mRNA was degraded at least eightfold faster with S130 from untreated cells than with BSA (Fig. 3, compare lanes 6 to 10 with lanes 11 to 14; see also Table 1). (ii) The S130 from CHX-treated cells (CHX-S130) did not affect c-*myc* mRNA decay (compare lanes 6 to 10 with lanes 15 to 19; Table 1). In fact, the mRNA was degraded at comparable rates in reactions with BSA, CHX-S130, or no additions (Fig. 3; unpublished data). This experiment, coupled with those presented below, suggested that exponentially growing cells contain a cytosolic factor(s), referred to here as a destabilizer, that accelerates c-*myc* mRNA decay. Since the destabilizer activity was diminished or eliminated when the cells were exposed to CHX for 2 h, continuous protein synthesis is probably required to maintain it at a detectable level.

Several controls supported these conclusions. (i) c-*myc* mRNA was degraded at the same rate in reactions supplemented with control S130 or control S130 and CHX at 100 μg/ml (Table 1). Therefore, residual CHX in CHX-S130 could not account for its low destabilizer activity. This experiment also suggests that the destabilizer functions independently of translation, because the added CHX inhibited any residual in vitro protein synthesis. (ii) None of the

TABLE 1. Approximate mRNA half-lives in in vitro reactions[a]

mRNA and addition to standard decay reaction	Half-life (min)
c-*myc*	
Nothing[b]	35
BSA	40
Control S130	<5
CHX-S130	45
Control S130 + CHX (100 μg/ml)	<5
δ-Globin	
BSA	500
Control S130	1,200
CHX-S130	800
γ-Globin[c] (BSA, control S130, or CHX-S130)	>1,400

[a] Where indicated, reaction mixtures containing polysomes were supplemented with 100 μg of BSA or 100 μg of S130 protein. Undegraded c-*myc* mRNA and globin mRNAs were quantitated by scanning densitometry of several autoradiographic exposures. The average amount of intact mRNA at each time point was plotted as a percentage of the amount at time zero, and the approximate half-life was determined from the plots. The results are averages of at least two experiments.

[b] The reactions contained polysomes but no BSA or S130, as in Fig. 2, lanes 4 to 9.

[c] A maximum of 20% of the γ-globin mRNA was degraded in reactions incubated for 12 h, after which the reaction mixtures were no longer active. Therefore, we did not observe the true half-life of this mRNA in vitro.

S130 preparations contained detectable c-*myc* mRNA (Fig. 3, lanes 4 and 5), indicating that the mRNA analyzed in these experiments was derived from the polysome fraction. (iii) Control S130 did not change the stability of δ- or γ-globin (see below) or H4 histone (49) mRNA. Therefore, the destabilizer is not a general RNase.

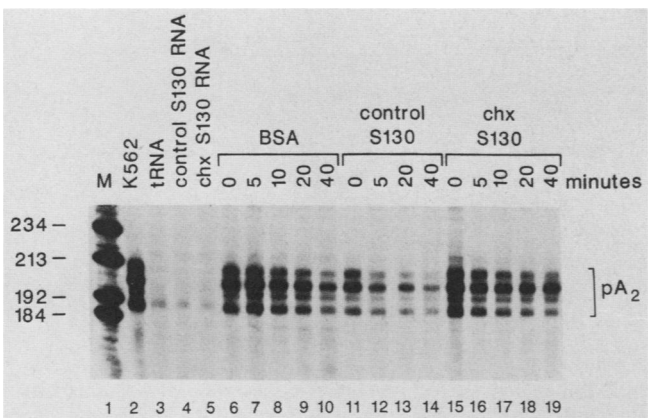

FIG. 3. Effects of K562 cell S130s on in vitro degradation of c-*myc* mRNA. Standard in vitro decay reactions containing equivalent amounts of polysomes from exponentially growing cells were supplemented with 100 μg of BSA, S130 protein from exponentially growing cells (control S130), or S130 protein from cells harvested after 2 h of exposure to CHX at 100 μg/ml (CHX-S130). After incubation in vitro for the indicated times, RNA was extracted and 30 μg from each reaction was analyzed by the RNase P1-plus-T1 protection assay as described in the legend to Fig. 2. The gel was exposed for 2 days with an intensifying screen. Lanes: 1, marker (M) *Hae*III-digested pBR322 DNA prepared as described in the legend to Fig. 2; 2, 30 μg of total RNA from K562 cells; 3, 30 μg of *E. coli* tRNA; 4, RNA extracted from 100 μg of control S130; 5, RNA extracted from 100 μg of CHX-S130; 6 to 10, RNAs from reactions supplemented with BSA; 11 to 14, RNAs from reactions supplemented with control S130; 15 to 19, RNAs from reactions supplemented with CHX-S130.

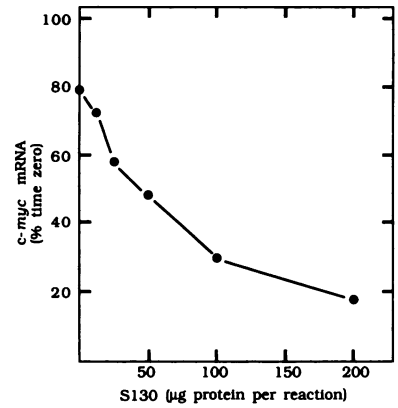

FIG. 4. Effect of control S130 concentration on c-*myc* mRNA decay in vitro. Each 25-μl reaction mixture contained 0.7 A_{260} U of polysomes (from 4 × 10^6 cells) plus different amounts of S130 protein from exponentially growing cells (100 μg corresponds to 2 × 10^6 cells). Reactions were incubated at 37°C for 10 min, and RNA was extracted and analyzed by the RNase P1-plus-T1 mapping procedure as described in the legend to Fig. 2. The percentage of undegraded c-*myc* mRNA was quantitated by scanning densitometry of the four protected bands in autoradiograms exposed without an intensifying screen.

The extent of destabilization increased approximately in proportion to the quantity of control S130 protein added, up to 50 to 100 μg per reaction (Fig. 4). The mRNA was degraded most rapidly with 100 to 200 μg per reaction, corresponding to an S130-polysome ratio of 1:2 to 1:1, respectively, on a per-cell basis. Higher ratios were not tested. Since the destabilizer was slowly inactivated at 37°C (see Fig. 10), we were unable to determine from these kinetics whether it acted stoichiometrically or catalytically or whether more than one component was required for the destabilization effect.

c-*myb* mRNA is at least as unstable as c-*myc* mRNA in cells (72) and was also destabilized in reactions with control S130 (Fig. 5, compare lanes 1 to 6 with lanes 7 to 12). It was at least eightfold less stable in reactions supplemented with S130 than in those with BSA. Moreover, it was degraded at the same rate in reactions with CHX-S130 as in those with

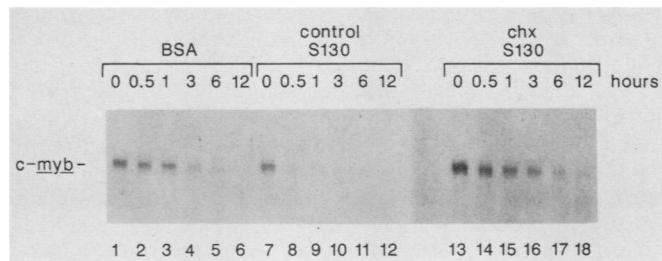

FIG. 5. Effects of K562 cell S130s on in vitro degradation of c-*myb* mRNA. Standard in vitro decay reactions containing equivalent amounts of polysomes from exponentially growing cells were supplemented with 100 μg of BSA, S130 protein from exponentially growing cells (control S130), or S130 protein from cells harvested after 2 h of exposure to CHX at 100 μg/ml (CHX-S130). After incubation in vitro for the indicated times, RNA was extracted, and 5 μg from each reaction was electrophoresed and transferred. The blot was hybridized with a ^{32}P-labeled v-*myb* probe (Materials and Methods). Lanes: 1 to 6, RNAs from reactions supplemented with BSA; 7 to 12, RNAs from reactions supplemented with control S130; 13 to 18, RNAs from reactions supplemented with CHX-S130.

BSA (Fig. 5, compare lanes 1 to 6 with lanes 13 to 18). Therefore, c-*myc* and c-*myb* mRNAs were destabilized by control S130 to approximately the same extent but were not destabilized with CHX-S130. In spite of these similarities, we do not know whether they were affected by the same factor(s). However, these results do indicate that other mRNAs besides c-*myc* are destabilized in vitro by labile regulatory factors.

Acceleration of c-*myc* mRNA 3′-end degradation by S130 from exponentially growing cells. In the experiments described below, we focused on characterizing how control S130 affected c-*myc* mRNA stability. Poly(A) removal seems to be the first step in the decay of c-*myc* mRNA in reactions lacking S130; the mRNA body is degraded only after its poly(A) is removed (6). The following experiment was performed to determine whether both of these steps were accelerated by the destabilizer. Polysomes were incubated under standard conditions in reactions supplemented with BSA or with S130 from exponentially growing cells. RNA extracted from each reaction was then analyzed by the H-mapping technique, which permits visualization of the poly(A) removal process (6; Materials and Methods; Fig. 6B). c-*myc* mRNA from unincubated reactions generated the expected heterogeneous fragments of 400 to 600 nucleotides (nt) (Fig. 6A, top, lanes 1, 5, and 9, bracket at left). In contrast, c-*myc* mRNA that was deadenylated generated a single, discrete band (lane 13, top), indicating that the heterogeneity resulted from mRNA molecules with poly(A) tracts of various sizes. Poly(A) shortening was observed within the first 5 min of incubation in reactions containing BSA (lanes 1 to 4, top), and a band comigrating with deadenylated mRNA appeared after 5 to 20 min (lanes 2 and 3, top). This result was consistent with the previously reported stepwise pathway involving (i) deadenylation, (ii) a transient delay or holdup period during which the deadenylated product was not degraded, and (iii) degradation of the deadenylated product.

The decay process in reactions with S130 differed in several ways from that in the BSA-containing reactions. (i) The heterogeneous fragments disappeared more rapidly, especially with 100 μg of S130 per reaction (Fig. 6A, top, lanes 9 to 12). As determined by scanning densitometry, these fragments disappeared seven times faster in reactions with S130 than in those with BSA. In contrast, S130 failed to affect γ-globin mRNA decay (Fig. 6A, bottom). (ii) The intensity of the band corresponding to the deadenylated product was less prominent in S130-containing reactions than in those with BSA.

These data indicate that degradation of the mRNA 3′ region was accelerated by S130, and it appeared as though poly(A) removal and degradation of the deadenylated product were both affected. However, the decay process occurred so rapidly in S130-supplemented reactions that a clear precursor-product degradation pathway was not readily apparent. Therefore, several models could account for these findings. (i) S130 accelerates the stepwise decay pathway for most or all of the mRNA molecules. (ii) Some mRNA molecules are degraded by the stepwise pathway, which would account for the small quantity of deadenylated molecules observed in S130-supplemented reactions (Fig. 6A, top, lanes 8 and 12). However, other molecules are degraded by a separate pathway, presumably activated by the destabilizer and perhaps involving endonucleolytic cleavages.

To determine whether the destabilizer affected the 3′-to-5′ directionality of c-*myc* mRNA degradation, RNA was extracted from in vitro decay reactions supplemented with

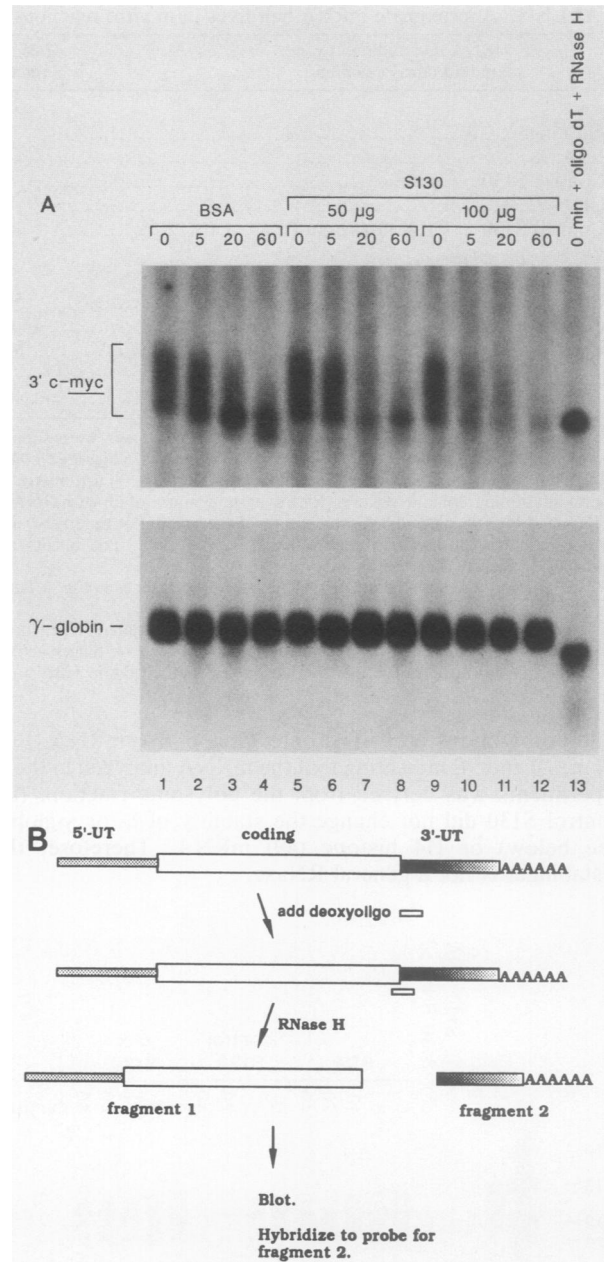

FIG. 6. H-mapping analysis of rapid degradation of the c-*myc* mRNA 3′ region in reactions containing control S130. (A) Standard in vitro mRNA decay reactions were supplemented with 100 μg of either BSA or S130 from exponentially growing cells. RNA (5 μg) was extracted from each reaction and annealed with a 21-nt single-stranded DNA that was complementary to a sequence located 400 nt 5′ of c-*myc* pA_2 (see the panel B and reference 6). The hybrids were treated with RNase H, and the resulting fragments were separated by electrophoresis and transferred to a Zeta-Probe membrane (Bio-Rad). The blot was then hybridized separately to ^{32}P-labeled probes complementary to either the 3′-terminal 400 nt of c-*myc* mRNA (top) or the coding and 3′-untranslated regions of human γ-globin mRNA (74) (bottom). The blot was autoradiographed with an intensifying screen for 3 days for c-*myc* or 1 day for globin. Reaction times (minutes) are shown above the lanes. Lanes: 1 to 4, reactions supplemented with 100 μg of BSA; 5 to 8, reactions supplemented with 50 μg of S130 protein; 9 to 12, reactions supplemented with 100 μg of S130 protein; 13, 5 μg of total RNA from K562 cells annealed with both the c-*myc* 21-mer and oligo$(dT)_{12-18}$ and then treated with RNase H. (B) Diagram of the H-mapping technique. UT, Untranslated.

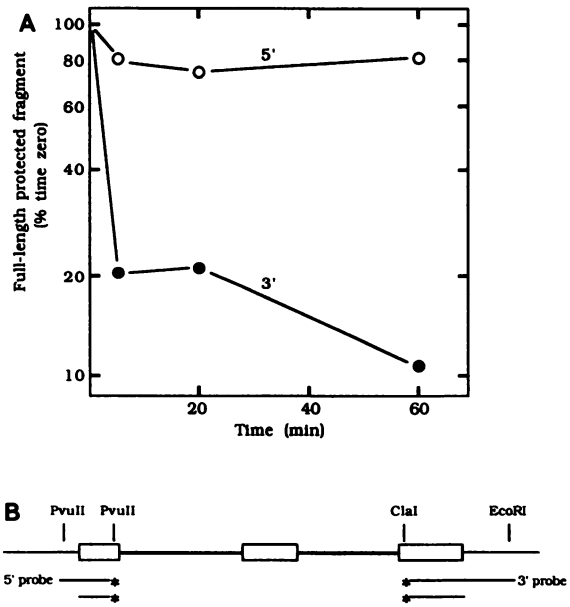

FIG. 7. 3'-to-5' directionality of c-*myc* mRNA decay in reactions containing control S130. (A) RNAs extracted from reactions containing 100 μg of S130 protein from the experiment shown in Fig. 6A (lanes 9 to 12) were analyzed by S1 nuclease mapping with 3'- and 5'-^{32}P-labeled c-*myc* DNA probes (see diagram). The amount of undegraded c-*myc* mRNA was quantitated by scanning densitometry of the full-length protected bands in the autoradiograms. (B) Diagram showing the end-labeled S1 nuclease mapping probes derived from human c-*myc* genomic DNA. The open boxes denote exons, the thick lines are intervening sequences, and the thin lines are 5'- and 3'-flanking DNAs. The probes are indicated below the gene, and their labeled termini are indicated by asterisks. The protected fragments scored as full-length fragments in the graph above are shown beneath the probes.

S130 and was analyzed by S1 nuclease mapping with 5'- and 3'-end-labeled DNA probes. The results were similar to those of unsupplemented reactions, except that S130 accelerated the 3' region decay rate by approximately sevenfold (Fig. 7; data not shown). Therefore, the overall directionality of c-*myc* mRNA decay was unaffected by the destabilizer.

Specificity of S130 destabilizer activity. The destabilizer affected c-*myc* and c-*myb* RNAs but not γ-globin or H4 histone mRNA (Fig. 5 and 6; reference 49). Therefore, it displayed considerable specificity. However, it could be that neither γ-globin nor histone mRNA is an entirely appropriate substrate to serve as a control, because γ-globin is extremely stable, whereas histone is not polyadenylated. For this reason, additional experiments were performed to determine whether the destabilizer affected the decay of either δ-globin mRNA or a large percentage of polysome-associated polyadenylated mRNA. δ-Globin mRNA was a reasonable control, because it is polyadenylated and is degraded more rapidly than γ-globin mRNA but more slowly than c-*myc* mRNA, both in vitro and in cells (57, 60). Although δ-globin mRNA was degraded in these reactions as well, it was not destabilized in reactions containing control S130 compared with CHX-S130 or BSA (Table 1). If anything, it was partially stabilized by control S130. Therefore, the destabilizer activity had no detectable effect on δ-globin mRNA turnover.

To determine whether the S130 fraction affected a large percentage of the total polysome-associated, poly(A)$^+$ mRNA population, cells were incubated for 18 h in medium containing [^{3}H]uridine and their polysomes were isolated and incubated in reactions with or without S130. RNA was extracted and fractionated on oligo(dT)-cellulose, and the amounts of polyadenylated RNA at each time point were measured. The total acid-precipitable radioactivity remaining after 60 min of incubation was approximately 90% of the amount at time zero, indicating that most of the labeled RNA (which was ribosomal) was not hydrolyzed into small fragments. In contrast, 10 to 20% of the poly(A)$^+$ mRNA was degraded (i.e., no longer bound to the column) within the first 20 min, and additional mRNA was degraded at a slower rate with longer incubation times, consistent with differential decay of various mRNA classes (2, 50, 52; Fig. 8). However, no significant differences in total mRNA turnover were observed among reactions with control S130, CHX-S130, or BSA. c-*myc* mRNA was degraded rapidly with these polysomes, ensuring that mRNA turnover processes with the labeled polysomes were not unusual (data not shown). Together with the experiments described above, these data indicate that the destabilizer affected a subset of mRNAs.

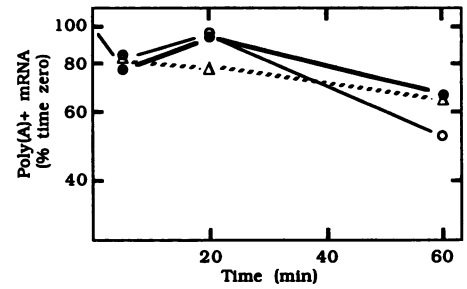

FIG. 8. Lack of effect of S130s on the degradation of most poly(A)$^+$ polysomal mRNAs. Exponentially growing K562 cells were cultured for 18 h in medium containing [^{3}H]uridine (10 μCi/ml). The polysomes isolated from these cells were then incubated in standard reaction mixtures with 100 μg of BSA, S130 protein from exponentially growing cells (control), or S130 protein from CHX-treated cells. RNA was extracted and separated into poly(A)$^-$ and poly(A)$^+$ fractions by two cycles of chromatography on an oligo(dT)-cellulose column. A portion of each fraction was counted to determine the percentage remaining compared with time zero. Recovery of total acid-precipitable radioactivity was 95% or more for each oligo(dT)-cellulose cycle. Symbols: △, BSA; ○, control S130; ●, CHX-S130.

Mixing experiments with S130s from control and CHX-treated cells. To confirm that S130 from untreated cells contained a destabilizer, the decay of c-*myc* mRNA was compared in reactions supplemented with 100 μg of control S130 protein, 100 μg of CHX-S130 protein, or a mixture containing 100 μg of each (total, 200 μg). If CHX treatment inactivated a destabilizer, the mixture should display decay kinetics similar to those of control S130 alone. As determined by scanning densitometry of the full-length bands, c-*myc* mRNA was degraded at approximately equivalent rates in reactions supplemented with control S130 alone or with the mixture (Fig. 9, compare lanes 8 to 11 or 12 to 15 with lanes 4 to 7). However, it was degraded approximately sixfold faster with the mixture compared with CHX-S130 alone (compare lanes 12 to 15 with lanes 16 to 19). These data indicate that growing cells contain a destabilizer that requires continuous protein synthesis to maintain high levels of activity. The results are also consistent with the finding that c-*myc* mRNA was degraded at similar rates in reactions supplemented with CHX-S130, BSA, or no additions (Table 1).

Partial characterization of the destabilizer. The destabilizer

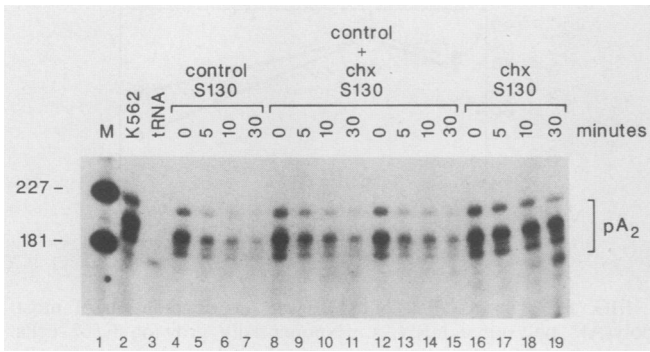

FIG. 9. Effect of mixing control S130 and CHX-S130 on c-*myc* mRNA decay—evidence for a destabilizer in control S130. Standard in vitro decay reactions containing polysomes from untreated cells were supplemented with 100 μg of control S130 (from exponentially growing cells), 100 μg of control S130 plus 100 μg of S130 from CHX-treated cells, or 100 μg of CHX-S130. The reactions were incubated for the indicated times, RNA was extracted, and 15 μg of RNA was analyzed by the RNase P1-plus-T1-mapping procedure (Fig. 2). The gel was exposed for 7 h with an intensifying screen. Lanes: 1, marker (M) pBR322 ^{32}P-labeled DNA cleaved with *Hae*II (sizes in nucleotides are noted on the left); 2, 15 μg of total RNA from K562 cells; 3, 15 μg of *E. coli* tRNA; 4 to 7, control S130; 8 to 15, two separate decay reactions containing the 1:1 mixture of control S130 and CHX-S130; 16 to 19, CHX-S130.

was heat labile in vitro; most of its activity was lost by preincubation at 45°C for 15 min (Fig. 10, lane 6). Surprisingly, it was not inactivated by preincubation with proteinase K (Fig. 11A, compare lane 6 with lanes 2 and 4), although most of the S130 proteins that were visible in a stained gel were degraded by this treatment (Fig. 11B, lane 5). Experiments are in progress to determine whether the destabilizer lacks protein components or whether it contains a proteinase-resistant protein(s), as described for the scrapie prion protein and other proteins (reviewed in reference 38).

To determine whether a nucleic acid component was associated with the destabilizer, S130 was preincubated with

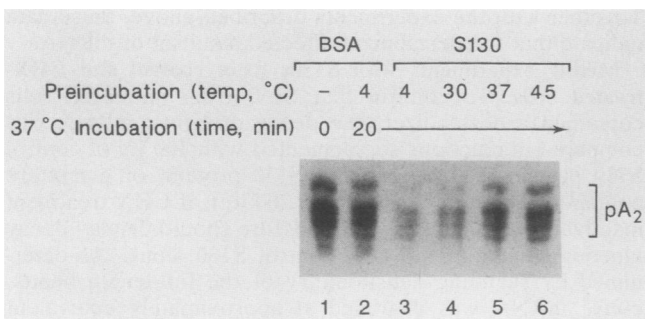

FIG. 10. In vitro thermolability of S130 destabilizer activity. BSA or control S130 was preincubated in separate tubes at the indicated temperatures for 15 min. The tubes were then placed in ice water, and 100 μg of protein from each preincubation was added to 25-μl mRNA decay reaction mixes which were incubated at 37°C for either 0 min (lane 1) or 20 min (lanes 2 to 6). c-*myc* mRNA was analyzed by RNase P1-plus-T1 mapping, and protected fragments were separated in a polyacrylamide minigel which was autoradiographed for 7 h with an intensifying screen. Lanes: 1, BSA—time zero; 2, BSA—mRNA decay reaction incubated for 20 min at 37°C after BSA preincubation at 4°C; 3 to 6, S130—mRNA decay reactions incubated for 20 min at 37°C after S130 preincubations at the indicated temperatures.

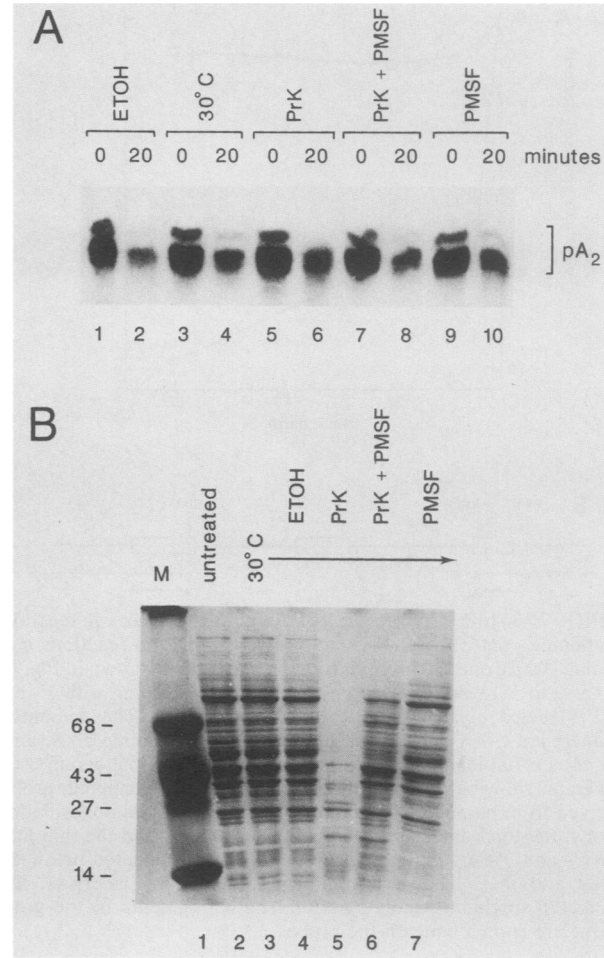

FIG. 11. Resistance of S130 destabilizer activity to digestion by proteinase K (PrK). (A) mRNA decay reactions. Samples (100 μl) of concentrated S130 from exponentially growing cells were preincubated at 30°C for 30 min as indicated. These S130s were then chilled in ice water for 5 min, and 100 μg of protein from each was added to mRNA decay reactions which were incubated at 37°C for either 0 min (lanes 1, 3, 5, 7, and 9) or 20 min (lanes 2, 4, 6, 8, and 10). c-*myc* mRNA was analyzed by the RNase P1-plus-T1-mapping procedure as described in the legend to Fig. 2, and the minigel was autoradiographed for 14 h with an intensifying screen. Lanes: 1 and 2, 1% ethanol (ETOH); 3 and 4, no additives; 5 and 6, proteinase K at 1 mg/ml; 7 and 8, proteinase K at 1 mg/ml plus 1 mM PMSF; 9 and 10, 1 mM PMSF. (B) Effect of proteinase K on S130 proteins. Samples of preincubated S130s from the experiment described in panel A (each initially containing approximately 30 μg of protein) were electrophoresed in a sodium dodecyl sulfate–10% polyacrylamide minigel and stained by Coomassie blue. Lanes: 1, Prestained markers (M; Bethesda Research Laboratories, Inc., Gaithersburg, Md.) (sizes are indicated in kilodaltons on the left); 2 to 7, S130s preincubated as for the experiment shown in panel A.

calcium and micrococcal nuclease at 30°C for 10 min. Further nuclease action was blocked by adding excess EGTA, and destabilizer activity was assayed in mRNA decay reactions incubated for 20 min. Nuclease pretreatment inactivated the activity, because c-*myc* mRNA was degraded at comparable rates in reactions with nuclease-treated S130 or BSA (Fig. 12A and B, compare lanes 2, 3, and 5). This result was confirmed in four independent experiments with different S130 preparations (Fig. 12B; data not shown). Destabilizer activity was inhibited to a lesser extent when

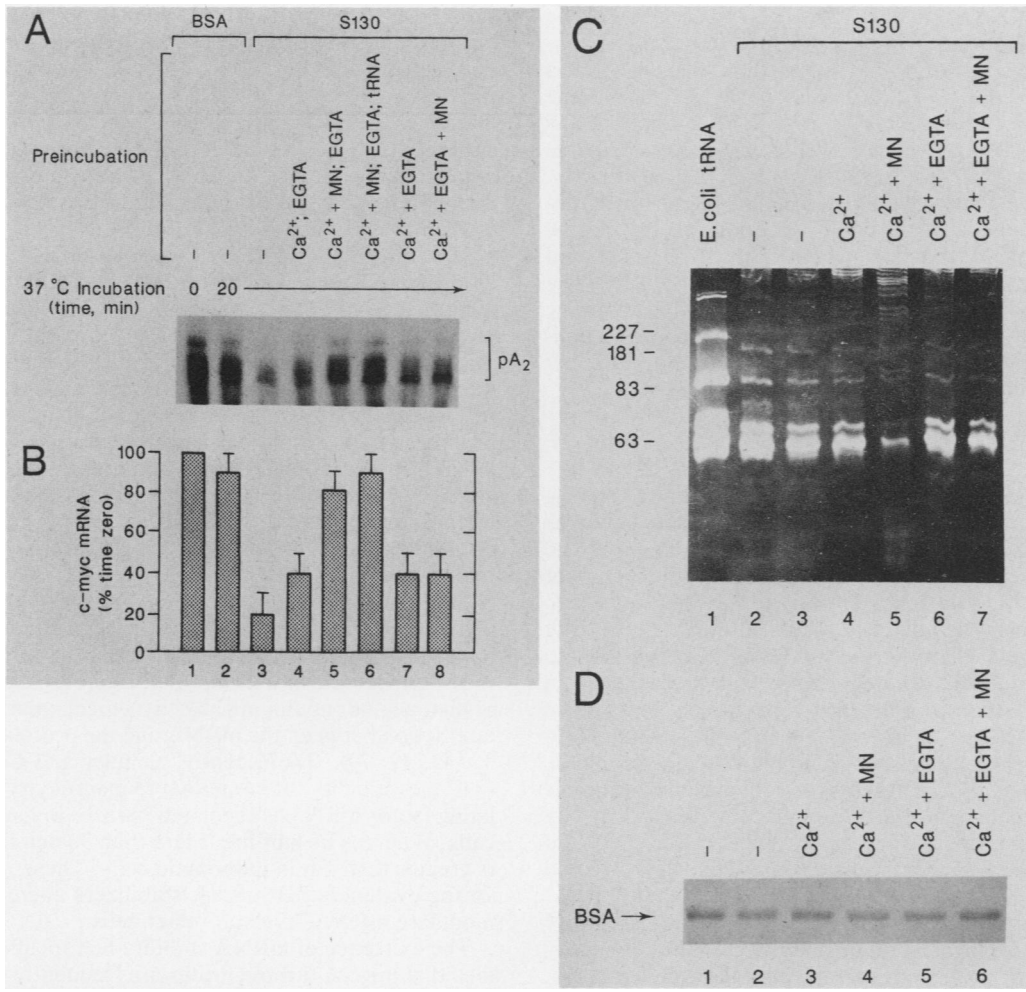

FIG. 12. Sensitivity of the S130 destabilizer activity to digestion by micrococcal nuclease (MN). (A) mRNA decay reactions with preincubated S130s. Samples of S130 from exponentially growing cells were preincubated at 30°C for 10 min as indicated in Materials and Methods. Plus signs indicate that the reagents were added together in the indicated order, and semicolons indicate sequential additions. Thus, for lane 4, Ca^{2+} was added, the reactions were preincubated, and then EGTA was added; for lane 8, Ca^{2+}, EGTA, and micrococcal nuclease were added together in that order. Following preincubation, each reaction mixture was chilled in ice water for 10 min, and 100 μg of the indicated protein was added to in vitro decay reactions. Following incubation at 37°C for either 0 min (lane 1) or 20 min (lanes 2 to 8), RNA was extracted and analyzed by the RNase P1-plus-T1-mapping assay with the c-*myc* probe (Fig. 2); the minigel was exposed for 14 h with an intensifying screen. (B) Quantitation of the effect of micrococcal nuclease on the destabilizer in S130. Autoradiograms from the experiment shown in panel A and from three similar experiments were scanned to estimate the relative decay of c-*myc* mRNA. The results are plotted as percentages of undegraded mRNA after 20 min of incubation compared with time zero. The line above each bar denotes the standard deviation. (C) Nucleic acid digestion by micrococcal nuclease. S130 was incubated for 0 min (lane 2) or 10 min (lanes 3 to 7) at 30°C as indicated. For lane 7, Ca^{2+} and EGTA were added before the micrococcal nuclease. Nucleic acid extracted from 10 μl of each reaction was electrophoresed in a 7 M urea–8% polyacrylamide minigel which was stained with ethidium bromide. The numbers on the left indicate the positions of DNA marker fragments from *Hae*II-digested pBR322. (D) Absence of detectable protease activity in micrococcal nuclease. BSA (2 μg) was incubated in 20-μl reaction mixtures for 0 min (lane 1) or 10 min (lanes 2 to 6) at 30°C as indicated. A 0.5-μg sample of protein from each reaction was electrophoresed in a sodium dodecyl sulfate–8% polyacrylamide minigel and stained by Coomassie blue.

S130 was preincubated without nuclease but with Ca^{2+} or Ca^{2+} plus excess EGTA, suggesting that calcium-responsive factors affect mRNA stability (Fig. 12A and B, compare lanes 3, 4, and 5). However, Ca^{2+} plus micrococcal nuclease was the only treatment that completely inactivated the destabilizer. We did not investigate the calcium or calcium-EGTA effects further, but the findings are consistent with the fact that calcium fluxes affect mRNA abundance in cells (43, 54).

Additional controls supported the notion that the destabilizer required a nucleic acid component. (i) Its activity was only partially repressed when S130 was preincubated with micrococcal nuclease, Ca^{2+}, and EGTA together. Therefore, complete inactivation did not result from a calcium-independent contaminant in the nuclease (Fig. 12A and B, lane 8). (ii) Another control was related to a previously described artifact, whereby micrococcal nuclease inhibited in vitro polyadenylation reactions by masking the RNA substrate and blocking access to it (23, 37). The artifact was eliminated by adding competitor RNA following the nuclease treatment. To determine whether micrococcal nuclease inhibits destabilizer activity in a similar way, S130 was preincubated with the nuclease and Ca^{2+}, EGTA was added, the mixture was placed on ice for 10 min, and 1 μg (or 5 μg

[data not shown]) of *Escherichia coli* tRNA was added as the competitor. The destabilizer was inhibited under these conditions as well, suggesting that nucleolytic cleavage, not a masking artifact, destroyed its activity (Fig. 12A and B, lane 6). (iii) Most of the nucleic acid in the S130 fraction was cleaved by micrococcal nuclease (Fig. 12C, lane 5), while significantly less of it was degraded by the other treatments. (The residual nucleolytic activity in S130 might be significant, however, because it could account for the partial inactivation of the destabilizer when S130 was preincubated with calcium or calcium-EGTA [Fig. 12A and B].) (iv) The micrococcal nuclease was not contaminated with an active Ca^{2+}-dependent protease (Fig. 12D). These controls indicated that micrococcal nuclease inactivated the destabilizer by destroying an essential nucleic acid component.

DISCUSSION

These data indicate that S130 from exponentially growing cells contains a labile activity (or activities) that destabilizes polysome-associated c-*myc* and c-*myb* mRNAs in vitro. The significance of this finding is highlighted by the apparent correlation between destabilizer activity in vitro and c-*myc* mRNA turnover in intact cells (Fig. 1). Furthermore, the steady-state levels of c-*myc* and c-*myb* mRNAs are two- to fourfold higher in CHX-treated cells, which are deficient in the destabilizer, than in untreated cells, which contain the destabilizer (13, 72). The destabilizer in S130 affects only a subset of mRNAs, indicating that it is not an indiscriminate RNase. We suggest that its expression probably requires continuous protein synthesis to replace what is lost, by whatever mechanism, during its normal turnover. We did not determine how rapidly it disappears from CHX-treated cells, but its half-life must be significantly less than 2 h.

Mixing experiments confirmed that the c-*myc* regulatory factor is a destabilizer, not a stabilizer, and preliminary characterization suggested that it contains nucleic acid, presumably RNA. It is also resistant to digestion by proteinase K. However, protein-free nucleic acid isolated from control S130 by phenol extraction lacks destabilizer activity (data not shown), suggesting that the destabilizer is a ribonucleoprotein. If so, it would be important to know whether it has enzymatic activity, as reported for other ribonucleoproteins (4, 18, 21, 25, 28, 76), and whether its putative RNA component is complementary to any portion of the mRNA(s) it destabilizes (11). Until we characterize it further, we cannot explain the apparent paradox that the activity diminishes in CHX-treated cells but is not inactivated (in vitro) by proteinase K. We also do not know whether the same factor(s) affects both c-*myc* and c-*myb* mRNAs. However, the v-*myc* and v-*myb* proteins share several properties, and the c-*myc* and c-*myb* genes are regulated in a similar fashion in response to cell growth factors (22, 34, 66, 72). In view of these common features, it seems reasonable to suggest that the two mRNAs respond to some of the same regulatory components, including some that influence their stability.

The basis for the specificity of the destabilizer is unknown, but it presumably interacts with mRNAs that share some common sequences and/or secondary structure features. We favor the notion that it recognizes a specific conformational feature of certain mRNAs and increases their susceptibility to nuclease attack by modifying that conformation. For example, the 3′ region of c-*myc* mRNA contains several interspersed AU islands, with sections of 8 to 25 nt consisting exclusively of A and U (8, 64). Since these islands are interspersed, they have the capacity to generate alternative stem-loop structures; one AU island can base pair with two or more other such islands. Perhaps the destabilizer binds to one or more AU-rich stem-loops, unwinds them, and thereby permits new ones to form. As a result, the conformation of the mRNA is changed, rendering the mRNA more susceptible to nuclease digestion. Conformational switching of this sort accounts for some instances of transcriptional attenuation and mRNA stabilization in procaryotes (reviewed in references 61 and 78).

The following findings suggest that mRNA stability-regulating factors play an important role in the expression of many genes. (i) Various hormones trigger dramatic (10-fold or more), reversible changes in the turnover of specific mRNAs, and at least some of these changes might be influenced by stabilizer or destabilizer factors (reviewed in reference 63). Experiments by Gordon et al. (24) suggest that prolonged exposure of chickens to estradiol induces a destabilization activity that affects apolipoprotein II and vitellogenin II mRNAs. The activity becomes apparent following estrogen withdrawal. (ii) Translation inhibitors induce the accumulation of various mRNAs, a phenomenon frequently referred to as superinduction (3, 13, 20, 35, 41, 71–73, 80). Although the mechanism of superinduction is incompletely understood, our in vitro experiments suggest that, in some instances, it might involve inactivation of destabilizer activities. (iii) Cytoplasmic components autoregulate the stability of histone and tubulin mRNAs by a mechanism that involves interaction between the mRNA and the protein it encodes (9, 36, 45, 49, 79). (iv) Recently, Schuler and Cole (62) found that the stability of granulocyte-macrocyte colony-stimulating factor mRNA is regulated in *trans* in monocytic tumor cells. Whereas its half-life is less than 30 min in most cells, it is greater than 2 h in monocytic cells. These results provide strong evidence that mRNA stabilizers and/or destabilizers modulate mRNA levels in intact cells.

The existence of mRNA stability factors also supports the idea that mRNA turnover rates are frequently determined by multiple interactions. In the simplest case, an mRNA whose stability was regulated might be affected by two sorts of interactions. (i) Some sequences would determine the intrinsic or unregulated half-life independently of regulatory factors (reviewed in references 55, 56, and 63). These sequences might influence the susceptibility of the mRNA to RNases. (ii) Some sequences would interact with regulatory factors that modify the intrinsic half-life. Mutational analyses, coupled with in vitro assays of regulatory components, should distinguish whether the sequences that determine intrinsic half-life differ from those that respond to regulatory factors (26, 64, 75). It will also be important to assess whether intrinsic half-life or regulated half-life plays a more prominent role in determining the overall stability of an mRNA.

We suggest that the regulation of mRNA stability provides two major advantages for the cell. (i) It permits the cell to respond rapidly to changes in its environment; changing the rate of gene transcription and mRNA turnover would permit a faster response than simply changing one or the other, particularly for mRNAs that are intrinsically unstable (see below). (ii) Subsets of gene products could be coordinately regulated in various ways. For example, by mixing and matching at the mRNA decay level, specific mRNAs encoded by genes whose transcription had been induced in response to unrelated stimuli and by unrelated factors could be controlled coordinately by a single cytoplasmic regulator. The rationale for stabilizing certain mRNAs in response to stresses like translation inhibition seems straightforward; the

cell might need the stabilized mRNAs for essential proteins once the translation block is relieved. The more interesting question seems to be why destabilizers (and stabilizers?) would be used to regulate mRNAs, like histone, c-*myc*, and c-*myb* mRNAs, that are already relatively unstable. Perhaps their protein products need to be maintained within specific levels, above or below which they interfere with essential cell functions (12, 29, 39, 77). This idea is consistent with the rapid turnover rates of c-*myc* and c-*myb* proteins (reviewed in reference 34) and with the fact that additional control mechanisms are exploited to regulate the expression of their genes (5).

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