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Autogenous Regulation of Histone mRNA Decay by Histone Proteins in a Cell-Free System

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We tested the hypothesis that histone mRNA turnover is accelerated in the presence of free histone proteins. In an in vitro mRNA decay system, histone mRNA was degraded four- to sixfold faster in reaction mixtures containing core histones and a cytoplasmic S130 fraction than in reaction mixtures lacking these components. The decay rate did not change significantly when histones or S130 was added separately, suggesting either that the histones were modified and thereby activated by S130 or that additional factors besides histones were required. RecA, SSB (single-stranded binding), and histone proteins all formed complexes with histone mRNA, but only histones induced accelerated histone mRNA turnover. Therefore, the effect was not the result of random RNA-protein interactions. Moreover, histone proteins did not induce increased degradation of gamma globin mRNA, c-*myc* mRNA, or total poly(A)$^-$ or poly(A)$^+$ polysomal mRNAs. This autoregulatory mechanism is consistent with the observed accumulation of cytoplasmic histone proteins in cells after DNA synthesis stops, and it can account, in part, for the rapid disappearance of histone mRNA at the end of S phase.

There are two major classes of histone proteins in cells, one of which is cell cycle regulated (for reviews, see references 45 and 58). Cycle-regulated histones are synthesized exclusively during S phase from labile, nonpolyadenylated mRNAs (1, 27). The mRNAs begin to be transcribed just before the start of S phase, accumulate to high levels during S, and then disappear rapidly after DNA synthesis stops (2, 3, 20, 29, 31; see also reference 28). As a result, there is little, if any, histone mRNA in the G1 cell (10, 21, 32, 33). This pattern of cell cycle restriction ensures that histone proteins are produced only when newly synthesized DNA is being packaged into nucleosomes.

It seems clear that transcriptional regulation is essential for inducing histone gene expression in late G1 and for repressing it in late S (2, 25, 29, 48, 63, 64, 68). The rapid disappearance of histone mRNA after S phase is less well understood. However, the data suggest that histone mRNA is degraded faster at the end of S than during the middle (28, 29; see also reference 2). The goal of the experiments described here was to test a hypothesis that explains how the histone mRNA decay rate increases after DNA synthesis stops.

We suggest that accelerated histone mRNA degradation occurs as a result of an autogenous negative regulatory circuit triggered by the accumulation of free histone proteins in the cytoplasm. During S phase, most newly synthesized histones migrate rapidly to the nucleus, where they bind to newly synthesized DNA. As a result, the cytoplasm of the S-phase cell contains little histone protein (46). At the end of S phase, when histones are no longer required for nucleosome formation, newly synthesized histones accumulate in the cytoplasm until they reach a critical concentration at which they induce accelerated histone mRNA degradation. Faster mRNA turnover, coupled with transcriptional repression in the post-S period, can account for the absence of histone mRNA from the G1 cell. The notion that cytoplasmic histones regulate their own synthesis was proposed in 1973 by Butler and Mueller (12), who suggested that histones affect translation (see also references 67 and 74).

The histone protein autoregulation mechanism is consistent with the fact that histones accumulate in the cytoplasm after DNA synthesis stops (12, 46, 61). It would also explain how DNA and protein synthesis inhibitors affect histone mRNA, as follows. (i) The mRNA is degraded rapidly after exponentially growing cells are exposed to DNA synthesis inhibitors (6, 11, 23, 29). We suggest that excess histone proteins synthesized in these cells function in the same way as those produced post-S phase in uninhibited cells. That is, since DNA synthesis is blocked, histones accumulate in the cytoplasm and induce rapid histone mRNA degradation. (ii) Abundant histone mRNA accumulates in exponentially growing cells treated with translational inhibitors that block either initiation or elongation (6, 65, 68). We suggest that the mRNA level increases because cells completing S phase would not accumulate cytoplasmic histone proteins. As a result, the autoregulation process would not be activated. (iii) Translational inhibitors also stabilize histone mRNA in cells exposed to a DNA synthesis inhibitor (25, 64). Since cytoplasmic histone accumulation is blocked, the autoregulatory mechanism would not function, mRNA turnover would not increase, and the mRNA would accumulate, even without new DNA synthesis.

To test the autoregulation hypothesis, we have asked if core histone proteins induce an increase in histone mRNA turnover in an in vitro system (53). The pathway of histone mRNA degradation in vitro is indistinguishable from that in whole cells (55). In both, the mRNA is degraded 3′ to 5′ by an exonuclease activity, and the first step is the removal of five nucleotides (nt) from the 3′ terminus. This result provides assurance that the in vitro system is a suitable model for investigating factors that regulate mRNA degradation. We found that histone proteins induced accelerated histone mRNA degradation. The effect was histone specific and occurred only in reaction mixtures containing a cytoplasmic supernatant fraction (S130) from high-speed centrifugation. We suggest that S130 either modifies the histones, thereby

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activating their autoregulatory capacity, or supplies one or more additional factors required for autoregulation.

MATERIALS AND METHODS

The preparation of K562 erythroleukemia cell polysomes, the S1 nuclease mapping probes, and the DNA excess S1 mapping procedure were done as described previously (53). The preparation of total cell RNA by extraction with phenol and centrifugation through cesium chloride was done as previously described (52). ^{3}H-labeled polysomes were prepared in the same way as unlabeled polysomes except that the cells were cultured for 18 h in medium containing [^{3}H]uridine (10 μCi/ml). The components of the cell-free mRNA decay reaction mixtures (final volume, 25 μl) were the same as those described previously (54), with the following modifications. (i) Unless otherwise noted, reaction mixtures contained cytoplasmic supernatant S130 (described below). The ratio of S130 to polysomes was 1:1 based on cell equivalents of each. Thus, reactions contained 0.8 A_{260} units of polysomes and 100 μg of S130 protein, each derived from approximately 4×10^6 cells. (ii) All components were mixed together, preincubated at 4°C for 30 min, and then brought to 20°C, usually for 60 min. The rationale for the preincubation step was to allow potential interactions between S130 and histone proteins (complex formation or histone modifications) to occur before the onset of histone mRNA degradation. We do not know if the preincubation step is important or necessary. The purpose of incubating the reaction mixtures at 20 instead of 37°C, as previously described (53, 54), was to facilitate the kinetic experiments involving multiple time points. Although the reaction rate was slower at 20 than at 37°C, the mechanism of degradation (3′-to-5′ exonucleolytic decay) was the same at both temperatures, and histone mRNA decay was accelerated by histone proteins plus S130 to the same extent at both temperatures (data not shown).

Mixtures used for hybridization reactions to detect histone mRNA included a 3′-^{32}P-labeled histone DNA probe at a 10- to 20-fold molar excess, relative to histone mRNA, plus 5 μg of RNA purified from in vitro decay reaction mixtures (53). Since S130 contained an easily detectable amount of RNA but very little histone mRNA (see Fig. 6; data not shown), total RNA from reaction mixtures with polysomes alone had more histone mRNA than an equivalent amount of RNA from reaction mixtures with S130 and polysomes. This explains why the intensity of the S1-protected fragment was slightly greater with RNA from reaction mixtures with polysomes alone than those with polysomes plus S130 (for example, see Fig. 7). S1-resistant DNA fragments were electrophoresed in 15-cm-long 8 or 10% polyacrylamide gels containing 7 M urea for 4 h at 250 V. Autoradiography was performed for different times.

To estimate the rates of histone mRNA degradation in various experiments, gels were autoradiographed without a screen and the amount of undegraded histone mRNA was determined by soft-laser densitometry. Since the band of undegraded mRNA was insufficiently separated from the bands just beneath it (Fig. 1, lane 3), the mRNA band for all experiments was taken to be the DNA migrating between 94 and 104 nt. The four- to sixfold difference in decay rates in reactions with and without histones was determined by averaging the differences in mRNA band intensities observed in 12 separate experiments, some with multiple time points and others with a single time point.

Bovine core histone proteins were purchased from Sigma Chemical Co. or Boehringer Mannheim Biochemicals. Only the expected core histone bands were observed when these proteins were electrophoresed in sodium dodecyl sulfate-polyacrylamide gels, and we estimate that a contaminant at 1% of the level of any histone would have been detected. Highly purified, individual core histones prepared by high-pressure liquid chromatography were kindly provided by J. Pearson and V. Groppi of The Upjohn Co. Histones from all three sources yielded virtually identical results in different experiments. They were stored desiccated at room temperature, dissolved in water before use, and then kept in small aliquots at −20°C.

To prepare S130, logarithmically growing cells were lysed in low-salt buffer containing 1 mM potassium acetate, as described by Ross and Kobs (53). The lysate was centrifuged at low speed to pellet nuclei, and the supernatant was layered over a 30% sucrose cushion and centrifuged in an SW60 rotor at 4°C for 2.5 h at 36,000 rpm (average, 130,000 × *g*). The pellet was used as a source of polysomes. All of the material above the sucrose cushion was pooled, mixed, and used as a source of S130.

^{32}P-labeled histone RNA was synthesized in vitro with SP6 RNA polymerase, as previously described (42, 53). The 3′ terminus of this approximately 440-nt substrate is located 7 to 8 nt 3′ of the authentic mRNA 3′ terminus. For gel retardation analysis, approximately 1,500 cpm of the [^{32}P] mRNA was incubated in 20-μl reaction mixtures containing 100 mM potassium acetate, 2 mM magnesium acetate, 2 mM dithiothreitol, 3 U of RNasin (Promega Biotec), and 20 mM Tris chloride (pH 7.6) at 20°C for 1 h with different amounts of core histone proteins, SSB (single-stranded binding protein), or RecA. RNA-protein complexes were then electrophoresed in a 5% nondenaturing polyacrylamide gel in 7 mM Tris-acetate–1 mM EDTA (pH 7.5) (22, 24). The [^{32}P]RNA was purified in the presence of excess *Escherichia coli* tRNA. Therefore, binding reaction mixtures contained approximately 0.2 ng of [^{32}P]RNA plus 30 to 60 ng of unlabeled carrier RNA. SSB was purchased from U.S. Biochemicals, and purified RecA protein was kindly provided by S. Moore and M. Cox. Both preparations contained only a single polypeptide, as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

RESULTS

Core histone proteins induce accelerated histone mRNA degradation in a cell-free system supplemented with cytoplasmic supernatant (S130). The cell-free reaction mixtures included polysomes from a human erythroleukemia cell line, K562 (19, 38), ATP, GTP, mono- and divalent cations, and placental RNase inhibitor (54). The mRNA-degrading enzymes were associated with the polysomes, and mRNA degradation was measured by monitoring the loss of intact mRNA and the appearance of mRNA decay products during the incubation. Initial experiments to test the autoregulation hypothesis were performed with reaction mixtures supplemented only with core histone proteins. The histones induced a modest increase in histone mRNA degradation, but the effect was neither striking nor highly reproducible (see Fig. 4). We reasoned that the histones alone were relatively inactive because they needed to be complemented in some way by additional cytoplasmic factors. For this reason, we included S130, the postribosomal supernatant recovered from polysome preparations, in the reaction mixtures.

Under these conditions, histone mRNA was degraded significantly faster in reaction mixtures containing core histone proteins than in those lacking them (Fig. 1 and 2).

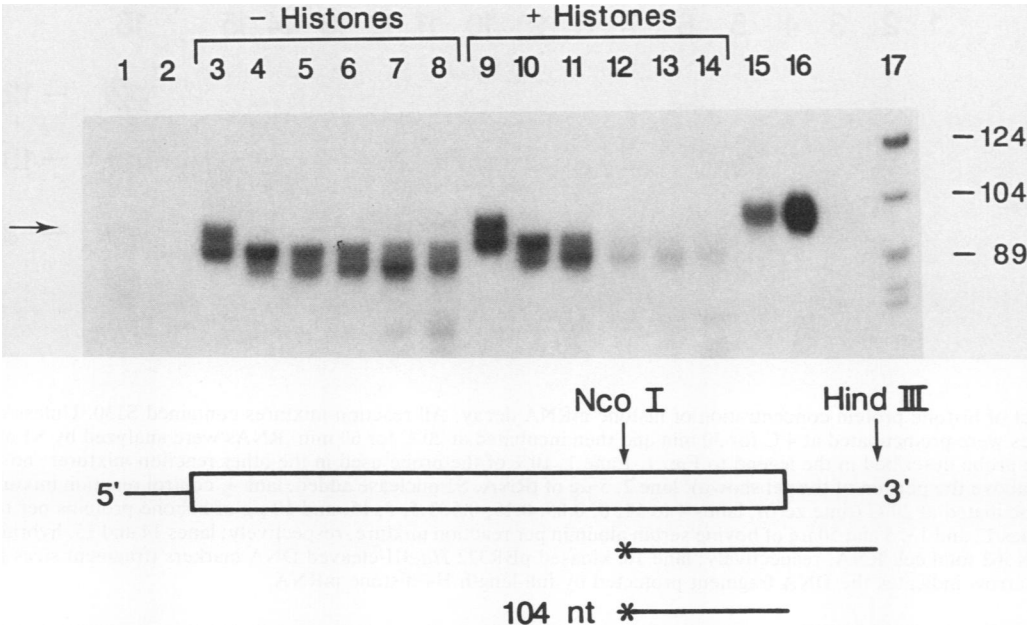

FIG. 1. Effect of core histone proteins on histone mRNA decay in reaction mixtures containing cytoplasmic supernatant (S130). The rate of histone mRNA decay was compared in cell-free reaction mixtures containing or lacking 5 μg of core histone proteins, as described in the text. All reaction mixtures contained S130. RNA was extracted and annealed with a 3'-^{32}P-labeled human H4 histone probe prepared from clone pHh4A DNA cleaved with *Nco*I (bottom panel) (30, 76). S1 nuclease mapping was performed as described in the text. (Top) Lane 1: 10% of the probe used in the other reaction mixtures, no S1 nuclease; the probe migrated above the portion of the gel shown. Lane 2: 5 μg of tRNA, S1 nuclease added. Lanes 3 through 14: 5 μg of RNA from reaction mixtures containing (+) or lacking (−) 5 μg of histone proteins, preincubated at 4°C for 30 min and then incubated at 20°C; for each set of 6 lanes (3 to 8 and 9 to 14), the reaction times at 20°C were 0, 10, 20, 40, 90, and 150 min, respectively. Lanes 15 and 16: hybridization controls; 2 and 10 μg of K562 total cell RNA, respectively. Lane 17: kinased, ^{32}P-labeled pBR322 DNA cleaved with *Hae*III; the sizes (nucleotides) of some of the fragments are indicated on the right. The arrow on the left indicates the DNA fragment protected by full-length H4 histone mRNA. (Bottom) Symbols: ▭, human H4 histone structural gene in pHh4A, approximately 386 base pairs, cloned by Heintz et al. (30); ——, 5' and 3' flanking sequences (not drawn to scale). 5' and 3' indicate the direction of transcription. The 300-base-pair probe and the fragment protected by intact mRNA are indicated by solid lines, with the asterisk representing the 3'-labeled terminus.

Accelerated degradation was observed consistently in 12 separate experiments with different polysome and S130 preparations. It was observed with core histones from two commercial sources and with an equimolar mixture of high-pressure liquid chromatography-purified, separated histones kindly supplied by J. Pearson and V. Groppi, and it occurred in reaction mixtures incubated at 20 (Fig. 1) or 37°C (data not shown). Histone proteins and S130 did not accelerate the degradation of all mRNAs, and S130 plus other basic proteins did not accelerate histone mRNA degradation (see Fig. 9 to 12). As determined by densitometric scanning of S1 nuclease-protected bands, histone proteins plus S130 induced, on average, a four- to sixfold increase in histone mRNA degradation.

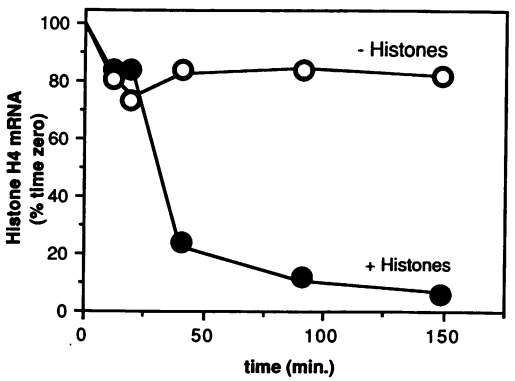

FIG. 2. Quantitation of the autoradiogram shown in Fig. 1. The 94 to 104-nt region of the gel, which included the full-length protected DNA fragment and the first two degradation products, was scanned with a soft laser densitometer (see the text). It was necessary to scan in this manner because it was not possible to resolve adequately the full-length band from the two largest degradation products. The quantity of the S1 nuclease-protected fragments remaining at each time point relative to that at time zero is plotted for reaction mixtures lacking (○) and containing (●) histone proteins. All reaction mixtures contained S130.

Several additional features of Fig. 1 and 2 should be noted. First, two bands were observed with RNA from reaction mixtures preincubated at 4°C but not then incubated at 20°C (Fig. 1, lanes 3 and 9; see Materials and Methods). The upper band was derived from undegraded histone mRNA. We have shown that it contains 104 nt, as determined by electrophoresis in high-voltage sequencing gels, but because of a stem-loop structure at its 5' terminus, it migrated faster than the 104-nt marker in the low-voltage gels shown here (see reference 55). The lower band was the initial histone mRNA decay product, lacking 5 nt from the mRNA 3' terminus (55). It was generated because the histone mRNA exonuclease retains some activity at 4°C. Its intensity in preincubated reaction mixtures varied somewhat from experiment to experiment. Second, in reaction mixtures supplemented with S130 and histones, there was a lag period before the decay rate increased (Fig. 2). The reason for the lag is not known. Perhaps it corresponds to the time required to activate the histones (see Discussion). Third, histone mRNA

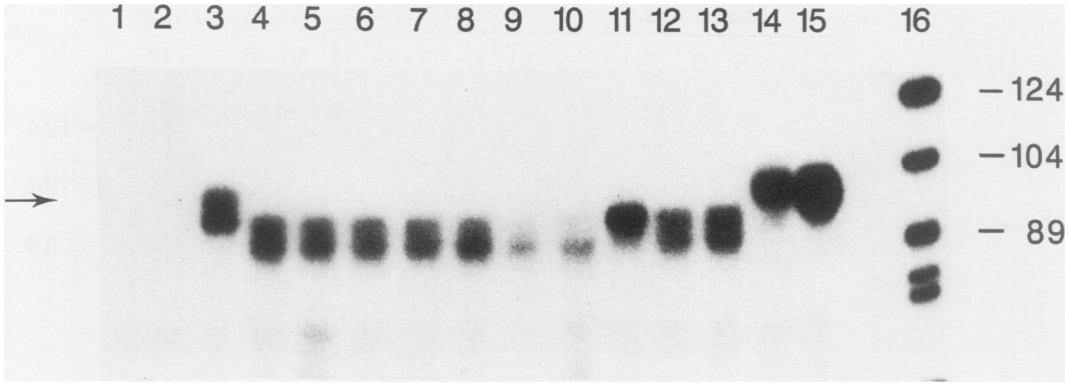

FIG. 3. Effect of histone protein concentration of histone mRNA decay. All reaction mixtures contained S130. Unless otherwise noted, reaction mixtures were preincubated at 4°C for 30 min and then incubated at 20°C for 60 min. RNAs were analyzed by S1 nuclease mapping with the histone probe described in the legend to Fig. 1. Lane 1, 10% of the probe used in the other reaction mixtures, no S1 nuclease (the probe migrated above the portion of the gel shown); lane 2, 5 μg of tRNA, S1 nuclease added; lane 3, control reaction mixture, preincubated at 4°C but not incubated at 20°C (time zero); lanes 4 to 11, 0, 0.05, 0.15, 0.5, 1.5, 5, 15, and 50 μg of histone proteins per reaction mixture, respectively; lanes 12 and 13, 5 and 50 μg of bovine serum albumin per reaction mixture, respectively; lanes 14 and 15, hybridization controls, 2 and 10 μg of K562 total cell RNA, respectively; lane 16, kinased pBR322 *Hae*III-cleaved DNA markers (fragment sizes [nucleotides] are indicated). The arrow indicates the DNA fragment protected by full-length H4 histone mRNA.

is eventually degraded in the absence of S130 and histones, as shown by analyzing reaction mixtures incubated for longer than 150 min (unpublished observations; see Fig. 7). Therefore, histones plus S130 accelerate the rate, not the extent, of degradation.

To determine how much histone protein was required to induce accelerated degradation, reaction mixtures containing an S130/polysome ratio of 1.0 were incubated with different amounts of core histones. Although degradation increased slightly with 0.5 to 1.5 μg of histones, a narrow optimum was observed at 5 to 15 μg per reaction mixture (Fig. 3, lanes 9 and 10). Assuming an average molecular weight of 14,000 for the core histones (34), this amount corresponds approximately to 1.5×10^{-5} to 4.5×10^{-5} M. For unknown reasons, a still larger amount slowed the degradation process. Serum albumin did not affect the decay rate (lanes 12 and 13).

To determine whether the S130 was necessary and to assess the optimum level required, different amounts were added to reaction mixtures containing a fixed amount of polysomes, with or without histones (Fig. 4). In reaction mixtures lacking S130, histones induced only a modest increase in degradation, confirming that histones alone do not significantly affect histone mRNA turnover (Fig. 4, no S130 added, open versus closed circles). S130 alone also had little or no effect. In contrast, the degradation rate was accelerated in reaction mixtures containing both components. The optimum S130/polysome ratio was 0.5 to 1.5 on a per-cell basis. Higher ratios were not tested.

S130 is probably required either to modify the histones covalently or to provide some additional, complementing factor(s). To determine whether the S130 component was thermolabile, mRNA degradation was assayed in histone-containing reaction mixtures supplemented with heated (65°C for 30 min) or unheated S130. The decay rate was significantly faster with control than with heated S130 (Fig. 5). In fact, the mRNA was degraded at the same rate in reaction mixtures with histones plus heated S130 as in those with histones alone (data not shown). Although this experiment does not prove that the thermolabile S130 factor is an enzyme, it does exclude the possibility that a thermostable histone-binding protein similar to nucleoplasmin, which leaks from oocyte nuclei during cell lysis (37), was by itself the required component.

Absence of RNase in the histones. Control experiments were performed to exclude trivial explanations for histone-induced autoregulation, to determine whether S130 and histones change the 3′-to-5′ degradation pathway, and to gauge the specificity of the effect with respect both to histone proteins and to histone mRNA. To assay for the presence of RNase activity in the histone protein preparations, deproteinized K562 total cell RNA was incubated in modified reaction mixtures containing histones but lacking polysomes

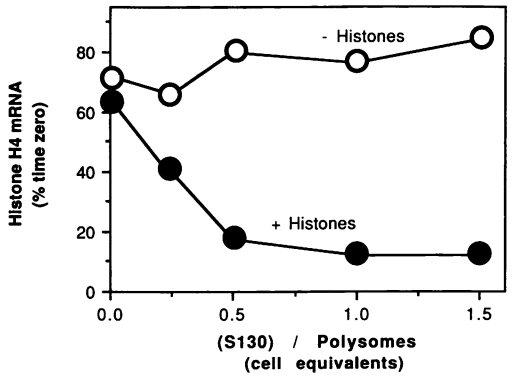

FIG. 4. Histone mRNA decay in the presence or absence of histone proteins: the effect of different amounts of S130. Each reaction mixture contained 0.8 A_{260} units of polysomes isolated from approximately 4×10^6 cells and, when indicated, 5 μg of core histones plus different amounts of S130. The S130 levels are expressed as the S130/polysome ratio, based on cell equivalents per reaction mixture. At a 1/1 ratio, reaction mixtures contained 100 μg of S130 protein. The reaction mixtures were preincubated as usual for 30 min at 4°C and then incubated for 60 min at 20°C. RNA was hybridized with the 3′-labeled *Nco*I probe (Fig. 1) and treated with S1 nuclease. Nuclease-resistant fragments were electrophoresed, and the amount of undegraded mRNA was estimated by scanning densitometry (see the text). Symbols: ○, no histones; ●, 5 μg of histones per reaction mixture.

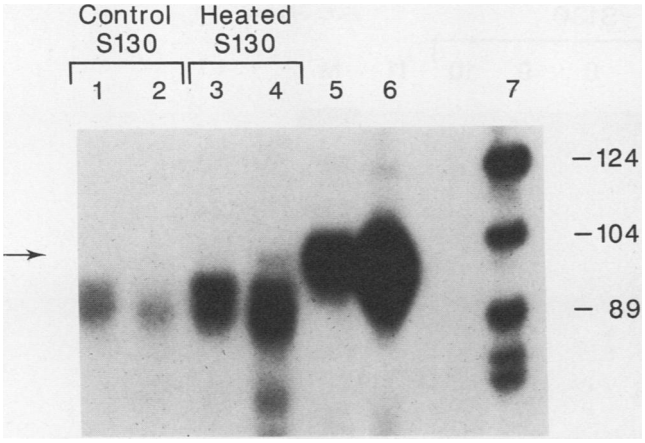

FIG. 5. Thermolability of S130. In vitro reaction mixtures containing 5 μg of histone proteins were supplemented with untreated S130 (control) or S130 that had been heated to 65°C for 30 min. The reaction mixtures were preincubated at 4°C for 30 min and then incubated at 20°C for the times indicated below. RNAs were analyzed by S1 nuclease mapping with the histone probe described in the legend to Fig. 1. Lanes 1 and 3, 40-min incubation; lanes 2 and 4, 80-min incubation (RNAs from control reaction mixtures lacking S130 generated bands that were indistinguishable from those in lanes 3 and 4 [data not shown]); lanes 5 and 6, hybridization controls, 2 and 10 μg of K562 total cell RNA, respectively; lane 7, markers, as indicated in the legend to Fig. 1. The arrow indicates the DNA fragment protected by undegraded H4 histone mRNA.

and S130. As determined by agarose gel electrophoresis and ethidium bromide staining, neither the total recovery nor the structure of the rRNA was affected by histone proteins (Fig. 6A). Histone mRNA also was not degraded by histone proteins (Fig. 6B). Similar results were obtained with all of the histone protein preparations used in these experiments. Therefore, the histones were not contaminated with detectable RNase activity.

S130 does not contain a significant amount of H4 histone mRNA. One trivial explanation for the accelerated histone mRNA degradation would be that the S130 itself contained histone mRNA that was degraded rapidly in the presence of histone proteins. To assess this possibility, approximately 3 × 10^6 cell equivalents of S130 were extracted with phenol to purify nucleic acid. This material, as well as total RNA from a comparable number of cells, was then analyzed by S1 nuclease mapping with the histone probe. No histone mRNA was detected in the S130 (Fig. 6C, lane 5).

S130 alone does not induce accelerated histone mRNA degradation. Two sets of reaction mixtures, one containing and one lacking S130 and both lacking histones, were incubated at 20°C for different times. S130 alone had no significant effect on the decay rate (Fig. 7). Similar results were observed with reaction mixtures incubated at 37°C (data not shown). Therefore, S130 does not contain RNases that selectively degrade histone mRNA.

The pathway of histone mRNA degradation is not changed by histone proteins plus S130. The 3′-to-5′ directionality of histone mRNA degradation in vitro was first observed by comparing via S1 nuclease mapping the kinetics of turnover of the 5′ and 3′ regions (53). Using a 3′-labeled *Nco*I probe,

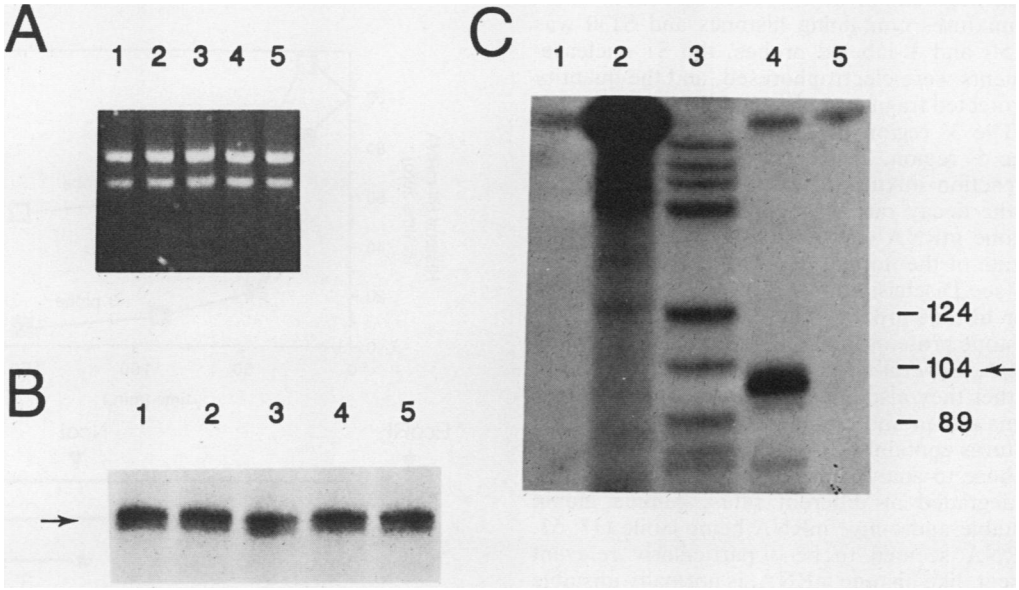

FIG. 6. Absence of RNase activity in histone proteins and absence of histone mRNA in S130. (A and B) Absence of RNase from histone proteins. All reaction components except polysomes and S130 were added to 10 μg of deproteinized K562 total cell RNA. Histone proteins were added, and the reaction mixtures were incubated (without polysomes) for 30 min at 4°C and then for 1 h at 20°C. Total RNA was purified as described in the text. Recovery from incubated reaction mixtures was 85 to 100% of that from the unincubated control. For panel A, 0.3 μg of RNA was electrophoresed in a 1% agarose gel and visualized by ethidium bromide staining and UV light. For panel B, 5 μg of the same RNA was analyzed by S1 nuclease mapping with the histone probe described in the legend to Fig. 1. Lanes 1, unincubated control (time zero); lanes 2 to 5, 0, 0.05, 0.5, and 5 μg of histone proteins per reaction mixture, respectively. The arrow to the left of panel B indicates the DNA fragment protected by full-length H4 histone mRNA. (C) Lack of histone mRNA in S130. Total RNA was prepared separately from K562 cells and from S130 corresponding to the same number of cells. The RNA was then hybridized and S1 nuclease mapped with the histone probe described in the legend to Fig. 1. Lane 1, 5 μg tRNA; lane 2, [^{32}P]DNA probe, no S1 nuclease added; lane 3, pBR322 DNA cleaved with *Hae*II and kinased (the sizes [nucleotides] of some of the bands are indicated on the right); lane 4, total RNA from 3 × 10^6 cells; lane 5, RNA from S130 recovered from 3 × 10^6 cells. The arrow is defined in the legend to panel B.

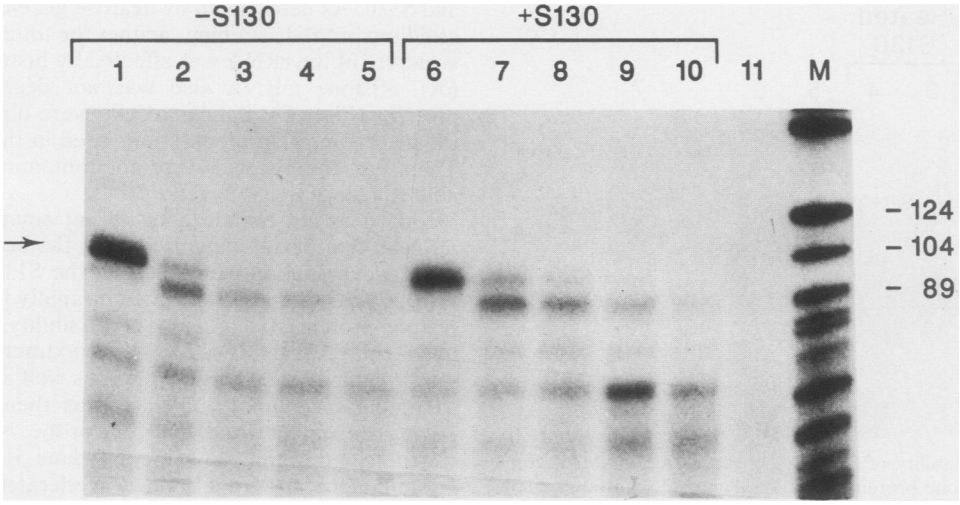

FIG. 7. Effect of S130 on histone mRNA decay rate in reaction mixtures lacking histone proteins. In vitro mRNA decay reaction mixtures were preincubated at 4°C for 30 min and then incubated for different times at 20°C without added histones and with (+) or without (−) S130. Total RNA was extracted and analyzed by S1 nuclease mapping with the histone mRNA probe as described in the legend to Fig. 1. Lanes 1 to 10, For each set of five lanes, the incubation times at 20°C were 0, 1, 2, 3, and 5 h, respectively; lane 11, probe hybridized with 5 μg of tRNA and treated with S1 nuclease; lane M, pBR322 DNA cleaved with *Hae*III and kinased (the sizes [nucleotides] of some of the bands are indicated on the right). The arrow indicates the band protected by undegraded histone mRNA.

we observed that the 3′ terminus was rapidly destroyed; using a 5′-labeled *Nco*I probe, we observed that the 5′ region was not degraded until much later. Therefore, histone mRNA is degraded 3′ to 5′. A similar approach was used to determine whether histones and S130 induce accelerated turnover by changing the degradation mechanism, for example, by inducing degradation in a 5′-to-3′ direction. RNA from reaction mixtures containing histones and S130 was annealed with 5′- and 3′-labeled probes, the S1 nuclease-protected fragments were electrophoresed, and the quantity of full-length protected fragments was measured by scanning densitometry. The 3′ region (Fig. 8) was destroyed more rapidly than the 5′ region. This result is the same as that observed for reaction mixtures lacking histones and S130 (53), although the decay rate was slower at 20 than 37°C. Therefore, histone mRNA degradation is increased by accelerating the rate of the normal pathway not by activating new pathways (see Discussion).

Specificity for histone mRNA. The data presented above indicate that histone proteins induce accelerated degradation of histone mRNA. Several experiments were performed to determine whether they also affect the degradation of other mRNAs. Gamma globin and c-*myc* mRNAs were analyzed in reaction mixtures containing S130 with or without histone proteins. We chose to analyze these mRNAs because they are normally degraded at different rates, gamma globin mRNA being stable and c-*myc* mRNA being labile (17, 53, 56). c-*myc* mRNA seemed to be a particularly relevant control, because it, like histone mRNA, is normally unstable but is stabilized by translation inhibitors (17). Moreover, a portion of K562 cell c-*myc* mRNA molecules are poly(A) deficient, permitting us to determine whether histones plus S130 affect another nonpolyadenylated mRNA (see below). Gamma globin mRNA was stable in reaction mixtures containing histones plus S130 (Fig. 9A), and c-*myc* mRNA was degraded at approximately the same rate with or without histones plus S130 (Fig. 9B). The RNAs used to obtain the results shown in Fig. 9B were from the same reaction mixtures analyzed for Fig. 1. Therefore, in the same set of reactions, c-*myc* mRNA degradation was essentially unaffected by histones and S130, whereas histone mRNA degradation was significantly accelerated. We conclude that S130 and histone proteins do not induce accelerated degradation of all mRNAs.

Since mammalian cells contain a significant amount of functional mRNAs that fail to bind to poly(U) or oligo(dT)

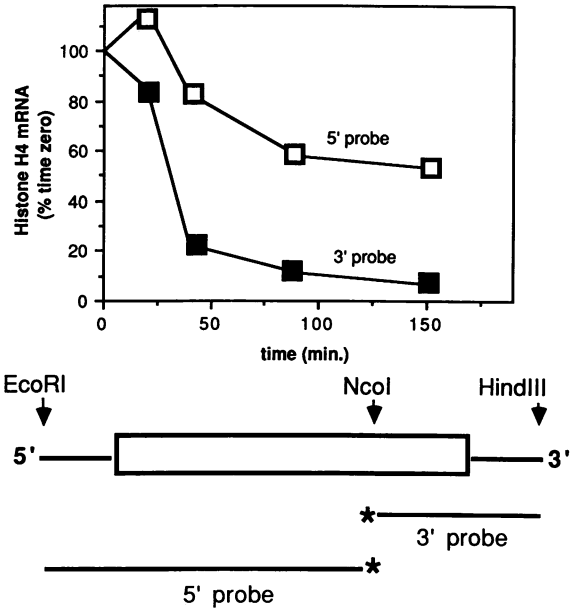

FIG. 8. Histone mRNA is degraded 3′ to 5′ in reaction mixtures containing histone proteins plus S130. Reaction mixtures containing histones and S130 were preincubated at 4°C and incubated at 20°C for different times. RNA was isolated and analyzed by S1 nuclease mapping with both 3′- and 5′-labeled *Nco*I probes. The amount of undegraded mRNA remaining at each time point was determined by scanning densitometry (see the text). At the bottom is a diagram of the histone gene and the two probes (see the legend to Fig. 1 for details).

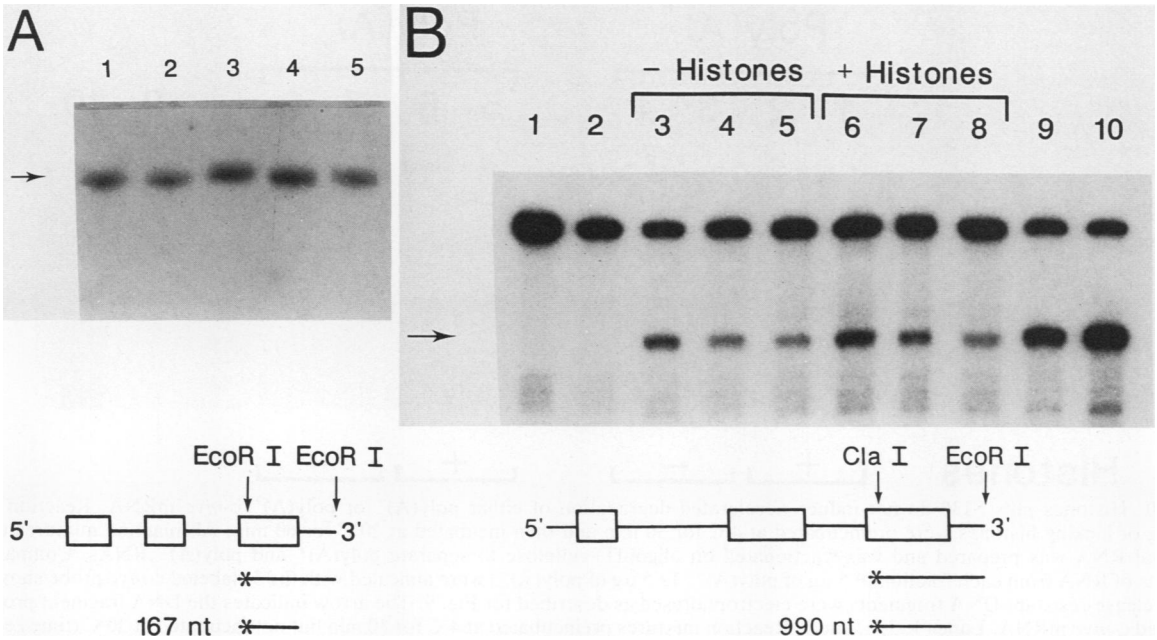

FIG. 9. Failure of histone proteins to affect the degradation of gamma globin and c-*myc* RNAs. In vitro mRNA decay reaction mixtures containing S130 were incubated with or without histone proteins as described below. Total RNA was analyzed by S1 nuclease mapping with the indicated probes. (A) Gamma globin mRNA. Reaction mixtures containing S130 and different amounts of histones were preincubated at 4°C for 30 min and then incubated at 20°C for 60 min. RNA (0.5 μg) from each reaction mixture was hybridized with a 3′-labeled probe derived from a subclone of the human G gamma globin gene cleaved with *Eco*RI. This probe protects the 3′-terminal 167 nucleotides of gamma globin mRNA. The S1 mapping procedure was essentially as described in the text, but the S1 nuclease-resistant DNA was electrophoresed in a 5% urea–polyacrylamide gel. Lane 1, unincubated control (time zero); lanes 2 to 5, 0, 0.05, 0.5, and 5 μg of histone proteins added, respectively. The arrow indicates the position of the DNA fragment protected by undegraded gamma globin mRNA. (B) c-*myc* mRNA. In vitro reaction mixtures were preincubated at 4°C for 30 min and then incubated at 20°C with S130 and with (+) or without (−) 5 μg of histone proteins. The reaction mixtures were from the experiment for which results are shown in Fig. 1. Total RNA (5 μg) from some of the time points was annealed with a 3′-labeled *Cla*I probe for human c-*myc* mRNA. This probe anneals to an approximately 990-nt fragment from the c-*myc* mRNA 3′ region. The DNA was electrophoresed in a 3% urea–polyacrylamide gel. Lane 1: 10% of the probe used in the other reaction mixtures; no S1 nuclease. Lane 2: 5 μg of tRNA; S1 nuclease added. Lanes 3 to 8: for each set of three lanes, the incubation times were 0, 40, and 150 min, respectively; the amounts of undegraded c-*myc* mRNA at each time point were determined by scanning densitometry and calculated as percentages of that at time zero; the values for lanes 4, 5, 7, and 8 were 65, 55, 75 and 60%, respectively. Lanes 9 and 10: hybridization controls; 5 and 15 μg of K562 total cell RNA, respectively. The arrow indicates the position of the DNA fragment protected by undegraded c-*myc* mRNA (second polyadenylation site). Symbols in diagrams in panels A and B: ▭, exons; ——, intervening and flanking sequences. Transcriptional 5′ and 3′ orientations are noted. The probes, protected fragments, and labeling sites (asterisks) are shown beneath the diagrams.

and are thus poly(A) deficient (35), it seemed important to determine whether histone proteins and S130 induce accelerated degradation of any poly(A)$^-$ mRNA. Two experiments were performed to address this possibility. First, we exploited the observation that approximately 50% of K562 cell c-*myc* mRNA is poly(A) deficient (70; G. Brewer, unpublished observations). If histones induce accelerated degradation of all poly(A)$^-$ mRNAs, poly(A)$^-$ c-*myc* mRNA should be degraded faster in the presence than in the absence of histones plus S130. RNA from in vitro reactions was fractionated by oligo(dT)-cellulose chromatography, and the separated poly(A)$^-$ and poly(A)$^+$ samples were annealed to 3′-labeled histone or c-*myc* probes. As expected, histones plus S130 accelerated histone mRNA degradation (data not shown). In contrast, they had no appreciable effect on the degradation of either class of c-*myc* mRNA (Fig. 10; see legend for quantitation). This result is consistent with the results of the experiment with unfractionated c-*myc* mRNA (Fig. 9B). A significant difference in degradation rates would have been evident with total c-*myc* mRNA because 50% of it is poly(A) deficient.

The second experiment was done to determine whether total poly(A)$^+$ and poly(A)$^-$ mRNA degradation rates were affected by histones plus S130. [^{3}H]uridine-labeled polysomes were incubated for 8 h in cell-free mRNA decay reaction mixtures. RNA was extracted and fractionated on oligo(dT)-cellulose, the bound [poly(A)$^+$] and unbound [poly(A)$^-$] RNAs were electrophoresed in formaldehyde-agarose gels, and the radioactivity in the gel slices was counted. The percentages of total acid-insoluble material recovered from reaction mixtures without and with histones were 69 and 72%, respectively (data not shown). Furthermore, the extent of poly(A)$^+$ or poly(A)$^-$ RNA degradation was not significantly affected by histones plus S130 (Fig. 11). Degradation was not limited to a restricted size class of RNA, and no differences in turnover rates were observed in reaction mixtures incubated for 1 h with or without histones plus S130 (data not shown). We conclude that histones plus S130 affect histone mRNA degradation in a highly specific manner.

Specificity for histone proteins. Since histones are basic proteins, it seemed possible that any cationic protein capable of binding to histone mRNA might accelerate histone mRNA turnover. To investigate the specificity of histone proteins

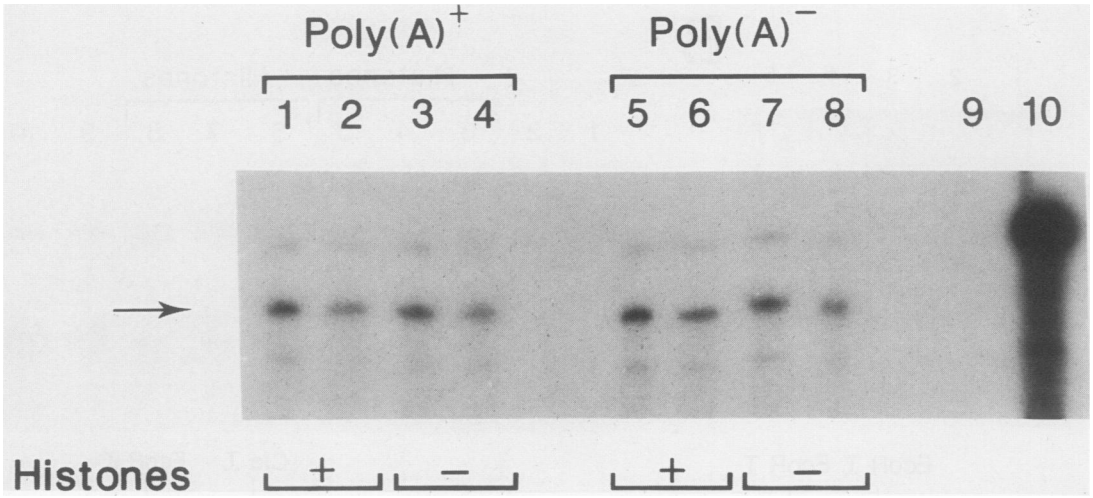

FIG. 10. Histones plus S130 do not induce accelerated degradation of either poly(A)$^-$ or poly(A)$^+$ c-*myc* mRNA. Reaction mixtures containing or lacking histones were preincubated at 4°C for 30 min and then incubated at 20°C for 60 min. All reaction mixtures contained S130. Total RNA was prepared and was fractionated on oligo(dT)-cellulose to separate poly(A)$^+$ and poly(A)$^-$ RNAs. Comparable cell equivalents of RNA from each fraction [0.5 μg of poly(A)$^+$, 12.5 μg of poly(A)$^-$] were annealed with the 3′-labeled c-*myc* probe shown in Fig. 9B. S1 nuclease-resistant DNA fragments were electrophoresed as described for Fig. 9. The arrow indicates the DNA fragment protected by undegraded c-*myc* mRNA. Lanes 1, 3, 5, and 7, Reaction mixtures preincubated at 4°C for 30 min but not incubated at 20°C (time zero); lanes 2, 4, 6, and 8, reaction mixtures preincubated at 4°C for 30 min and then incubated at 20°C for 60 min. Lanes 1, 2, 5, and 6 contained histones (+) and S130; lanes 3, 4, 7, and 8 contained S130 but not histones (−). As determined by scanning densitometry, the intensities of the bands in the 60-min-incubation lanes, as compared with those at time zero, were as follows: lane 2 (+histones, +S130), 71%; lane 4 (−histones, +S130), 64%; lane 6 (+histones, +S130), 81%; lane 8 (−histones, +S130), 75%.

for histone mRNA autoregulation, reaction mixtures containing S130 were supplemented with histones, *E. coli* single-stranded binding protein (SSB), or the *E. coli recA* gene product (RecA) (36, 41). All of these proteins bind to single-stranded nucleic acid, and a preliminary gel retardation experiment was performed to determine their relative affinities for histone mRNA. Each protein was incubated individually with deproteinized, ^{32}P-labeled histone mRNA. RNA-protein binding was then analyzed directly by electrophoresis in a nondenaturing gel. Under appropriate conditions, nucleoprotein complexes migrate more slowly than free nucleic acid in these gels (22, 24). As expected, each protein retarded RNA migration, indicating the formation of RNA-protein complexes (Fig. 12A). Histones seemed to bind most efficiently, with complete binding occurring at 1.5 $\times$ 10^{-6} M (lane 12). Maximum binding with SSB and RecA occurred at approximately 1.5 $\times$ 10^{-5} M (lanes 5 and 9, respectively). Therefore, the effects of SSB and RecA on mRNA turnover were determined at concentrations of 10^{-5} M and greater.

As expected, histone proteins induced accelerated histone mRNA degradation (Fig. 12B, cf. lane 2 with lanes 3 and 4). In contrast, SSB and RecA had no significant effect (cf. lane 2 with lanes 5 to 8). Therefore, the binding of basic proteins to histone mRNA is not by itself sufficient to accelerate histone mRNA degradation. Rather, there must be a significant degree of specificity, perhaps involving interacting or complementary structural features that are unique to histone messenger ribonucleoprotein and to the histone core proteins.

DISCUSSION

These results with an in vitro system indicate that histone mRNA degradation is accelerated in the presence of histone proteins and S130. There is a significant degree of specificity, because histones and S130 had little or no effect on the degradation of gamma globin mRNA, c-*myc* mRNA, or total poly(A)$^+$ and poly(A)$^-$ polysomal mRNAs. Moreover, other nucleic-acid-binding proteins, SSB and RecA, did not increase histone mRNA degradation.

Autoregulation can be explained in terms of the fate of newly synthesized histones during versus after S phase. Since histones produced during S phase migrate rapidly into the nucleus, the pool of free cytoplasmic histones is low (9, 12, 46, 73). In contrast, at the end of S or when DNA synthesis is inhibited, the extranuclear histone pool increases, thereby activating the autoregulatory process (12, 28, 46, 61, 62). Based on the recovery of total histone proteins from cultured mammalian cells (46), the intracellular histone protein concentration (in interphase) is 10^{-3} to 10^{-4} M, depending on the cell volume. Assuming that histones are synthesized at a constant rate during S phase (6 to 8 h) and that they are translated at the same rate post-S, then the cytoplasmic histone concentration would reach 10^{-5} M soon after DNA synthesis slows or stops. This concentration is close to the minimum level of histones required for accelerated histone mRNA degradation (Fig. 3) and is at least 10-fold lower than the total histone content of the cell. These calculations, coupled with the fact that histone proteins accumulate in the cytoplasm after S phase, support the idea that histone mRNA degradation is autoregulated in vivo. Moreover, our estimates of a four- to sixfold acceleration are similar to those made by others who have compared histone mRNA turnover in synchronized *Saccharomyces cerevisiae* and mammalian cells in different cell cycle phases; the decay rate during late S or after exposure to DNA synthesis inhibitors was approximately threefold faster than that during mid-S (29; see also reference 40).

The autoregulatory mechanism could also account for the effects of DNA and protein synthesis inhibitors on histone mRNA degradation, because both classes of inhibitors would be expected to change the concentration of histone proteins in the cytoplasm (see above). In fact, we predict

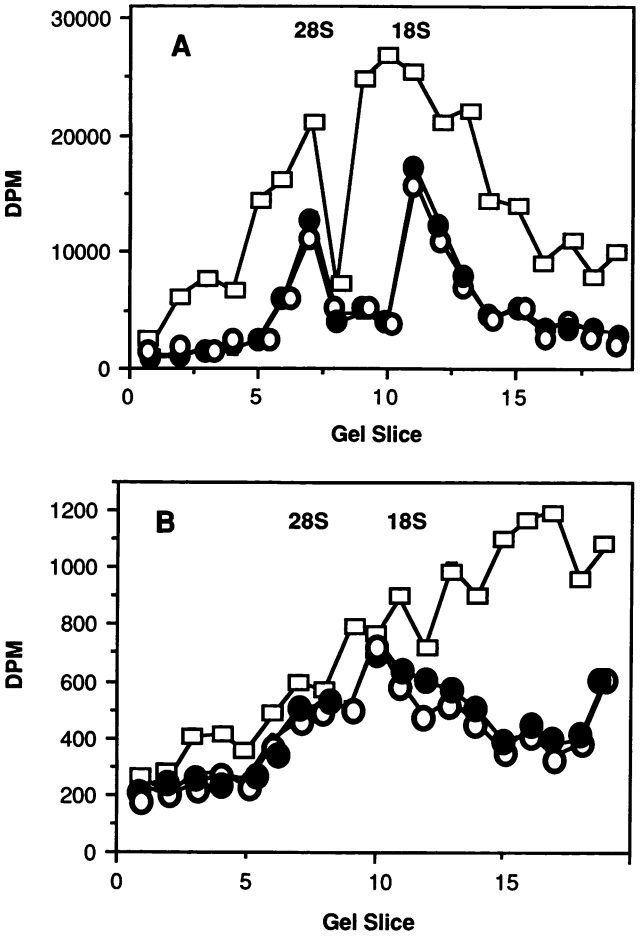

FIG. 11. Histone proteins plus S130 do not accelerate the degradation of total poly(A)$^+$ and poly(A)$^-$ polysomal mRNAs. Exponentially growing K562 cells were cultured for 18 h in medium containing [^{3}H]uridine (10 μCi/ml). Polysomes were isolated as usual and incubated in standard reaction mixtures for 30 min at 4°C and then for 8 h at 20°C. Control reaction mixtures (time zero) were preincubated at 4°C but were then harvested immediately. RNA was extracted and separated on oligo(dT)-cellulose into poly(A)$^+$ and poly(A)$^-$ fractions, and 2.5 × 10^4 and 5 × 10^5 cpm, respectively, were electrophoresed in a 1% formaldehyde-containing agarose gel at 80 V for 5 h. The gel was divided into 0.5-cm slices which were placed in scintillation vials with 1 ml of H_2O. The vials were microwaved briefly to melt the agarose, and the radioactivity of fractions containing 4S to 50S RNA was counted in ScintiVerse II fluid. (A) Poly(A)$^-$ mRNA; (B) poly(A)$^+$ mRNA. □, time zero; ○, S130 alone; ●, S130 plus histones.

that any change in the ratio of free DNA to free histone protein would affect histone mRNA turnover. Thus, if cells in S phase produced histones in excess of the free DNA, histone mRNA would be destabilized (48). Conversely, it would be stabilized, even at the end of S phase, if free DNA was introduced into the cells at a level sufficient to titrate the cytoplasmic histones.

Since the detailed mechanism of autoregulation is unknown, a number of questions about its function remain unanswered. How many histone proteins are required? Is a single histone sufficient or are histone-histone complexes required? In the appropriate salts and at the appropriate pH, the association constant for the formation of some histone dimers is 10^{-6} M (18, 34), which is close to the minimum histone concentration required to induce accelerated degradation in vitro (Fig. 3). Experiments are in progress in which highly purified histone proteins are added to the in vitro system, either individually or in various combinations.

Why are both histones and S130 required? One possibility is that histones interact in some way with S130 component(s) but are not modified. Another is that histones are covalently modified by the S130. Histones isolated from chromatin can be modified with acetyl and methyl groups, phosphate, poly(ADP-ribose), and ubiquitin (34). Cytoplasmic modifications necessary for autoregulation might be distinct from those that affect nuclear histones. For example, autoregulation might be activated by histone peptides generated by proteolytic processing.

How do histone proteins interact with the mRNA decay system? The simplest possibility is that they activate the exonuclease that degrades histone mRNA. In this case, any exonuclease substrate would be degraded rapidly in the presence of sufficient activated histone protein. We consider this hypothesis unlikely for the following reason. In vitro experiments indicate that the exonuclease can degrade other nonpolyadenylated mRNAs besides histone mRNA, albeit at different rates (49). Yet, histone autoregulation is highly specific for histone mRNA. If histones plus S130 activated the exonuclease directly, we should have observed accelerated degradation of other nonpolyadenylated mRNAs besides histone mRNA (Fig. 10 and 11). Therefore, a more likely possibility is that histones interact in some specific way with histone messenger ribonucleoprotein, perhaps at its 3′ terminus. The 3′-terminal regions of virtually all cell-cycle-regulated histone mRNAs contain a stem-loop structure that plays an essential role in 3′-end formation (for reviews, see references 8 and 72). Cytoplasmic histones might interact with this region, perhaps analogous to the interactions of *E. coli* ribosomal proteins and phage T4 gene 32 protein with their mRNAs (16, 44, 71). The histone protein-histone mRNA interaction might in some way cause the mRNA to become highly susceptible to exonuclease attack.

At what stage(s) in the degradation pathway do the histones function? Histone mRNA is degraded stepwise: 5 nt are removed in step 1, and an additional five to seven are removed in step 2 (55). In some experiments it appeared that step 1 occurred at the same rate with or without histones, suggesting that they function after step 1 (Fig. 1). However, additional experiments are necessary to confirm this suggestion. The steps that are most affected might be identified more readily by preincubating histones and S130, isolating the premodified histones, and adding them directly to the cell-free system without S130.

What roles do translation and polysome structure play in autoregulation? Translational inhibitors stabilize histone mRNA, even when DNA synthesis is blocked simultaneously (6, 11, 23, 29, 65, 68). We suggest that stabilization results, in part, because newly synthesized histone proteins do not accumulate and the autoregulatory process is not activated. Transfection experiments indicate that histone mRNA turnover (and perhaps autoregulation as well) is affected by mutations that change the overall structure of histone mRNA-containing polysomes. Nonsense mutations within the histone mRNA coding region interfere with histone mRNA degradation, perhaps because the ribosomes are unable to translate downstream to within a minimal distance from the 3′-terminal stem and loop (26). Mutations that disrupt the normal termination site, so that translation continues to the end of the mRNA, also affect histone cell

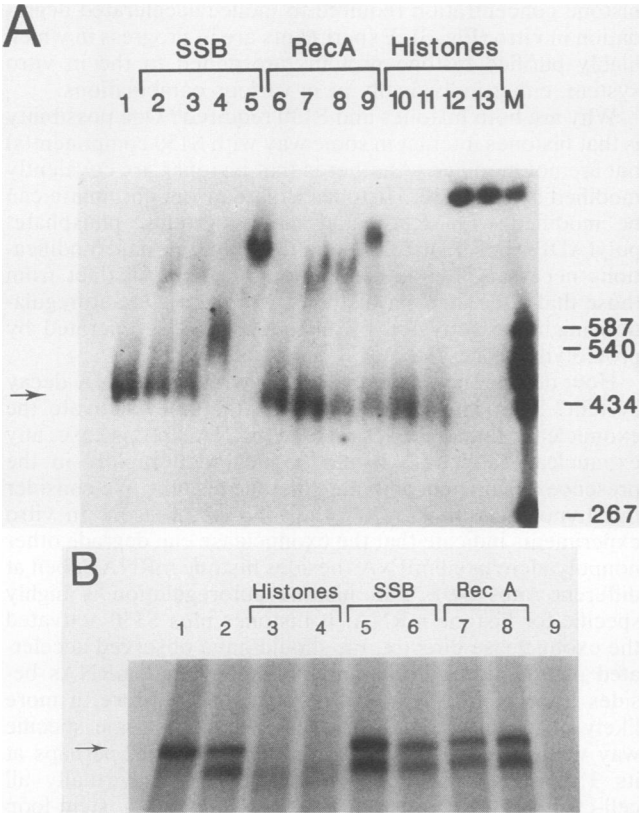

FIG. 12. Effects of histones, SSB, and RecA on histone mRNA decay. (A) Gel retardation assay. ^{32}P-labeled histone RNA (1,500 cpm; 0.1 to 0.3 ng; approximately 10^{-10} M) prepared by using SP6 polymerase was incubated at 20°C for 1 h with the indicated amounts of protein. RNA-protein complexes were electrophoresed in a 5% nondenaturing gel (see the text). Lane 1, no protein added; lanes 2 to 13, the concentrations of each protein added to each group of four reaction mixtures were 1.5×10^{-8} M, 1.5×10^{-7} M, 1.5×10^{-6} M, and 1.5×10^{-5} M, left to right, respectively; lane M, pBR322 DNA cleaved with *Hae*III and kinased (the sizes [nucleotides] of fragments are indicated on the right). The arrow indicates the uncomplexed ^{32}P-labeled-histone RNA substrate. (B) Histone mRNA decay. In vitro mRNA decay reaction mixtures containing S130 were incubated with or without the indicated proteins at 4°C for 30 min and then at 20°C for 1 h. Total RNA was extracted and analyzed by S1 nuclease mapping with the histone probe shown in Fig. 1. Lane 1, Unincubated control (time zero); lane 2, no protein added; lanes 3 and 4, histone core proteins, 5×10^{-6} and 1.5×10^{-5} M, respectively; lanes 5 and 6, SSB protein, 8.5×10^{-6} and 4.4×10^{-5} M, respectively; lanes 7 and 8, RecA protein. 9×10^{-6} and 4.5×10^{-5} M, respectively; lane 9, 5 μg of tRNA. The arrow indicates the DNA fragment protected by undegraded histone mRNA.

cycle regulation (13). Although we know that puromycin and cycloheximide have no effect on the acceleration of histone mRNA degradation induced by S130 and histone proteins (S.W.P., unpublished observations), we do not know how translation rates and polysome structure affect autoregulation. Since histone mRNA metabolism tends to be affected by most mutations that alter its 5′ or 3′ sequences or its intracellular location (4, 7, 13, 26, 39, 43, 59, 60, 66, 75), we suggest that normal regulation of histone mRNA turnover is strictly dependent not only on the stem and loop but also on additional histone-specific sequences that specify how and where it is translated. The constraints placed on normal histone mRNA regulation are consistent with our observation that autoregulation by histones and S130 is histone mRNA specific.

Is autoregulation of mRNA degradation a common mechanism for controlling the levels of other cell-cycle-restricted mRNAs? For example, the level of tubulin mRNA is inversely proportional to the tubulin monomer concentration in growing cells (15). The monomers might exert their effect at the posttranscriptional level by increasing tubulin mRNA degradation (14, 50; see also references 5 and 57).

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