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Histone mRNA Degradation In Vivo: the First Detectable Step Occurs at or near the 3′ Terminus

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The first detectable step in the degradation of human H4 histone mRNA occurs at the 3′ terminus in a cell-free mRNA decay system (J. Ross and G. Kobs, J. Mol. Biol. 188:579–593, 1986). Most or all of the remainder of the mRNA is then degraded in a 3′-to-5′ direction. The experiments described here were designed to determine whether a similar degradation pathway is followed in whole cells. Two sets of short-lived histone mRNA decay products were detected in logarithmically growing erythroleukemia (K562) cells. These products, designated the −5 and −12 RNAs, were generated by the loss of approximately 4 to 6 and 11 to 13 nucleotides, respectively, from the 3′ terminus of histone mRNA. The same decay products were observed after a brief incubation in vitro. They were in low abundance or absent from cells that were not degrading histone mRNA. In contrast, they were readily detectable in cells that degraded the mRNA at an accelerated rate, i.e., in cells cultured with a DNA synthesis inhibitor, either cytosine arabinoside or hydroxyurea. During the initial stages of the decay process, as the 3′ terminus of the mRNA was being degraded, the 5′-terminal region remained intact. These results indicate that the first detectable step in human H4 histone mRNA decay occurs at the 3′ terminus and that degradation proceeds 3′ to 5′, both in cells and in cell-free reactions.

The expression of many, but not all, histone genes is restricted to the late G1 and S phases in somatic cells (12, 20, 32, 47). Histone mRNA from these genes begins to accumulate at or just prior to the onset of DNA synthesis (5, 15, 32, 33, 48), and histone proteins are produced continuously while DNA is being synthesized (52, 53, 61, 65). After DNA synthesis stops, the histone mRNA level decreases rapidly and, by the time the cell has entered G1, it contains little, if any, histone mRNA (6, 23, 24, 26, 60, 62, 64). Although there is some controversy about the relative importance of transcriptional and posttranscriptional controls on histone gene expression (3, 26, 30, 49), it is clear that the rapid decay of the mRNA plays an essential role in restricting it to one phase of the cell cycle. If it were degraded more slowly, it would persist after S phase. The fact that histone gene expression is rigorously controlled implies that excess production or imbalanced synthesis of histone proteins might in some way harm the cell (43). Therefore, it must be essential for the post-S-phase cell to rid itself of histone mRNA by degrading it rapidly.

In an effort to characterize the enzymes and cofactors that determine the turnover rates of mRNAs, we have set up an in vitro mRNA decay system (54). The reaction mixture includes ATP, GTP, an energy-generating system, divalent and monovalent cations, a nonspecific RNase inhibitor, and polysomes from the human erythroleukemia cell line K562 (19, 41). After incubation at 37°C for various times, the levels of four of the mRNAs that are associated with these polysomes, gamma globin, delta globin, c-*myc*, and H4 histone, are quantitated by S1 mapping. We observe that the relative turnover of these mRNAs in vitro is similar to that in vivo. Gamma globin mRNA is the most stable and histone mRNA the least stable, both in intact cells (56) and in vitro. Delta globin and c-*myc* mRNAs decay at intermediate rates in vitro, consistent with in vivo data (18, 51, 55). We conclude that this system seems to be a valid method for analyzing how mRNAs are degraded.

To this end, we exploited the in vitro system to investigate histone mRNA turnover (54). The initial cleavage step occurs at the 3′ terminus, and the remainder of the mRNA is then degraded in a 3′-to-5′ direction. The first detectable decay product, observed after 5 to 10 min of incubation, is identical to intact H4 histone mRNA except that it lacks approximately 5 to 15 nucleotides from its 3′ terminus. As the reaction continues, smaller decay products are observed. The 5′-terminal region of the mRNA remains intact while the 3′ terminus is being degraded. Therefore, histone mRNA is degraded 3′ to 5′ in vitro, probably by a 3′-to-5′ exonuclease.

The goal of the experiments reported here was to determine whether the mRNA is degraded in a similar fashion in intact mammalian cells. The mechanism of decay of several procaryotic mRNAs has been determined (40, 67), and bacterial mutants have been described that fail to degrade their mRNA properly (21). In contrast, little is known about how or why eucaryotic cells destroy their mRNA, even though mRNA turnover rates apparently play a critical role in determining the level of expression of many genes (14, 16). Therefore, our experiments were performed, in part, to characterize the mechanism of histone mRNA turnover in cells. They were also performed to assess the similarities and differences between mRNA turnover in vitro and in vivo. This comparison should permit us to evaluate the validity of the in vitro system for investigating mRNA decay in cells.

MATERIALS AND METHODS

Cell culture and RNA isolation. K562 human erythroleukemia cells (19) were cultured in suspension in RPMI 1640 medium containing 10% fetal calf serum, 50 U of penicillin per ml, and 50 μg of streptomycin per ml. For these experiments, cells were subcultured into fresh growth medium at 2×10^4 to 3×10^4/ml. They were treated with inhibitors and harvested 44 to 50 h later, at which time they

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were actively growing and had reached a density of 2×10^5 to 4×10^5/ml. The doubling time varied from 13 to 16 h. If S phase lasts for 6 to 8 h, then approximately 40 to 50% of the cells were in S phase. The preparation of polysomes and the in vitro mRNA decay reactions were done as previously described (54).

Inhibitors were dissolved in warm growth medium. At appropriate times after inhibitors were added to the cultures, the cells were harvested by being poured over an equal volume of frozen, crushed F12 (Ham) medium. They were pelleted by centrifugation at 4°C for 4 min, and the medium was removed by suction. For all of the experiments decribed here, the cell pellets were frozen at −70°C. To prepare total cell RNA, 2.4 ml of urea lysis buffer (7 M urea, 2% [wt/vol] sodium dodecyl sulfate, 0.35 M NaCl, 0.001 M EDTA, 0.01 M Tris hydrochloride, pH 8) (34) was added to a still-frozen pellet containing a maximum of 5×10^7 cells. The lysate was shaken vigorously for approximately 3 s, and an equal volume of buffered (pH 7) phenol and one-half volume of chloroform-isoamyl alcohol (CIAA) were added. The lysate was extracted two times with phenol and CIAA in the same proportions and was then extracted two times with one volume of CIAA alone. Solid, ultrapure CsCl (1.1 g; BioRad Laboratories) was added and dissolved. The mixture was layered over a 1.5-ml cushion of 5.7 M CsCl in 0.001 M EDTA–0.01 M Tris hydrochloride, pH 7.6, in an SW60 centrifuge tube. The material was centrifuged at 32,000 rpm, 15°C, SW60 rotor, for 18 to 24 h (25). The RNA pellet was dissolved in 0.3 to 0.5 ml of low-salt buffer (0.001 M EDTA, 0.01 M Tris hydrochloride, pH 7.4), brought to 0.4 M in NaCl, and precipitated by adding 3 volumes of ethanol (−70°C, 15 min). It was then pelleted, dried, and suspended in 0.3 to 0.5 ml of RNA suspension buffer (0.1 M NaCl, 0.001 M EDTA, 0.01 M Tris hydrochloride, pH 7.4). Approximately 14 to 18 pg of RNA was recovered per cell.

Hybridization. To prepare the two S1 mapping probes shown in Fig. 1, the human H4 histone gene clone pHh4A (31) was cleaved with *Sca*I plus *Eco*RI or with *Nco*I plus *Hin*dIII. The appropriate fragments were purified from a low-melting-temperature agarose gel. The 5'-labeled *Sca*I probe was prepared with [γ-^{32}P]ATP and polynucleotide kinase. The 3'-labeled *Nco*I probe was prepared with [α-^{32}P]dATP in a reaction including the Klenow fragment of *Escherichia coli* DNA polymerase I and the other three unlabeled deoxyribonucleoside triphosphates, so that the probe would be blunt ended. The 3'-labeled probe that protects the 3'-terminal region of human γ-globin mRNA has been described (54). The specific activity of all probes was 1×10^7 to 2×10^7 cpm/μg.

^{32}P DNA and unlabeled RNA were mixed, concentrated together by ethanol precipitation, and suspended in 15 to 30 μl (1 μg of RNA per μl) of hybridization buffer (0.4 M NaCl, 0.001 M EDTA, 0.04 M PIPES [piperazine-*N*,*N*'-bis(2-ethanesulfonic acid)], 80% [vol/vol] deionized formamide). The mixture was heated to 70°C for 5 min and was then brought immediately to 45°C. It was annealed at that temperature for 16 to 18 h. Then 0.3 ml of S1 nuclease buffer (0.25 M NaCl, 0.001 M ZnSO4, 0.03 M sodium acetate, pH 4.5) with or without S1 nuclease was added, and the reaction was incubated at 37°C for 60 min. S1-resistant DNA was precipitated with ethanol, suspended in 4 μl of sample buffer (0.015 M EDTA, 0.01 N NaOH, 0.1% xylene cyanol [BDH], 0.1% bromophenol blue [Biorad], 80% deionized formamide), heated to 70°C for 2 min, and electrophoresed at 1,600 V in a 10% polyacrylamide, 40-cm-long sequencing gel. The gel was exposed to Kodak XAR-5 film without a screen.

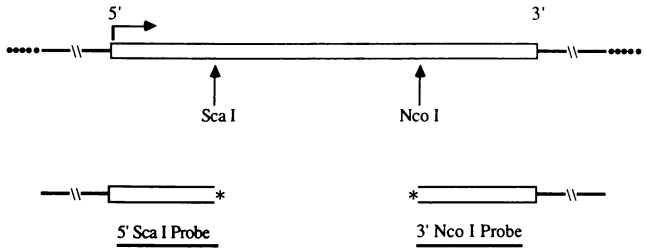

FIG. 1. Diagram of the cloned human H4 histone gene, pHh4A, and the two histone DNA probes. (Top) The human H4 histone structural gene (approximately 386 base pairs [bp]) of pHh4A (31) is shown as the open box, and the human 5'- and 3'-flanking regions are shown as solid lines with slashes to indicate that these regions are not to scale. The transcriptional 5' and 3' termini of the gene are noted. The human DNA insert (structural gene plus flanking regions) is bounded at its 5' and 3' ends by *Eco*RI and *Hin*dIII sites, respectively. Dots indicate vector (pBR322) sequences. (Bottom) Left: 5'-labeled *Sca*I probe. This probe was prepared by cleaving pHh4A with *Sca*I and *Eco*RI and purifying the fragment from an agarose gel. The location of the kinased terminus is noted by the asterisk. This probe protects the 5'-terminal 95 to 97 nucleotides of intact human H4 histone mRNA. Right: 3'-labeled *Nco*I probe. This probe was prepared by cleaving pHh4A with *Nco*I and *Hin*dIII and purifying the relevant fragment from an agarose gel. The location of the terminus labeled with the Klenow fragment of DNA polymerase I is noted by the asterisk. This probe protects the 3'-terminal 102 to 104 nucleotides of intact human H4 histone mRNA.

RESULTS

3'-to-5' degradation of cellular H4 histone mRNA. In an in vitro system, human H4 histone mRNA is degraded in a 3'-to-5' direction (54). The first detectable step is the loss of an estimated 5 to 15 nucleotides from the 3' terminus. The experiments described here were designed to determine whether the mRNA is degraded in the same way in whole cells. The approach was to use S1 nuclease mapping to detect mRNA decay intermediates. If the mRNA is degraded initially at its 3' terminus, we should observe shortened histone RNA molecules (decay products) with a probe that anneals to the 3'-terminal region. No such decay products should be detected with a probe that anneals to the 5' region if the 5' region remains intact while the 3' region is being degraded.

To obtain a population of cells that degrade histone mRNA rapidly and synchronously, cells were treated with DNA synthesis inhibitors. Histone mRNA decays rapidly after DNA synthesis is blocked (see Introduction). For example, the half-life of histone mRNA in cells treated with cytosine arabinoside (CA) is 10 to 20 min. Furthermore, the mRNA should be degraded more or less synchronously in most of the treated cells, facilitating the detection of short-lived intermediates. For comparison, control cells were treated with cycloheximide, a protein synthesis inhibitor that slows the rate of histone mRNA degradation (6, 10, 23, 62, 63). This control was used because histone mRNA turns over with a half-life of 40 min in untreated, nonsynchronized, exponentially growing cells (30), presumably because some percentage of the cells are in the final stages of S phase, when the mRNA is being degraded rapidly. As a result, histone mRNA decay products should exist in untreated cells (see Fig. 4). In contrast, the cycloheximide-treated cells should provide a low background of decay products. Thus, we can search for decay products in cells that are rapidly degrading the mRNA (DNA synthesis inhib-

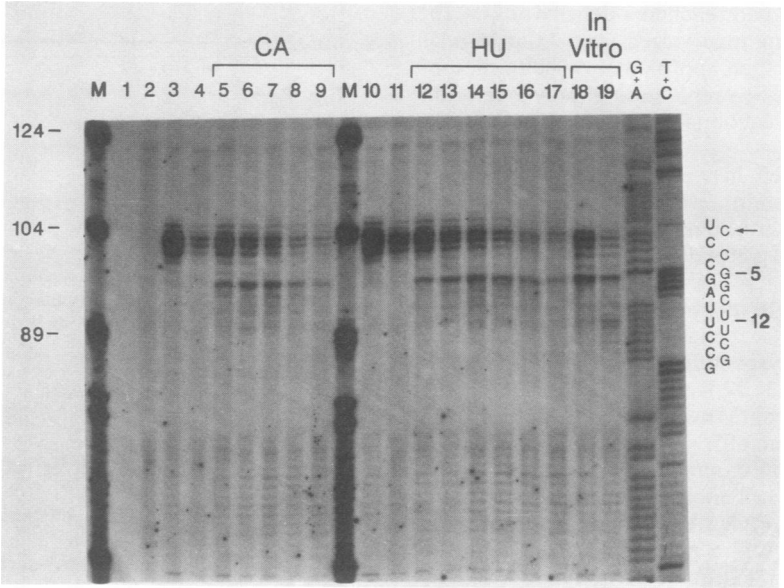

FIG. 2. Analysis of the 3′-terminal region of H4 histone mRNA from whole cells and from cell-free mRNA decay reactions with cycloheximide-treated cells as controls. Three sets of RNAs were analyzed by S1 nuclease mapping with the 3′-end ^{32}P-labeled *Nco*I probe (Fig. 1, bottom right). One was from logarithmically growing K562 cells cultured for 2 h with cycloheximide (100 μg/ml). The second was from growing cells cultured for the indicated times with CA (40 μg/ml) or HU (2 mM). These cultures contained no cycloheximide. The third was from standard in vitro mRNA decay reactions incubated for 5 or 20 min (54). The indicated amounts of RNA were annealed to the probe, the reactions were treated with S1 nuclease, and the S1-resistant DNA fragments were resolved in a 40-cm-long 10% urea–polyacrylamide gel at 1,600 V, run until the xylene cyanol had just exited the gel. The bands were visualized by autoradiography for 3 days without an intensifying screen. The bottom of the figure is the bottom of the gel. Lanes M, Kinased fragments of pBR322 DNA cleaved with *Hae*III. Sizes (in nucleotides) are shown on the left. Lane 1, One-fifth of the 3′-^{32}P-labeled probe used in all the other lanes. No S1 nuclease added. The probe migrated in the upper portion of the gel, which is not shown. Lane 2, 15 μg of *E. coli* tRNA. S1 nuclease added. Lanes 3 and 4, 15 and 3 μg, respectively, of total RNA from K562 cells cultured for 2 h with cycloheximide (100 μg/ml). Lanes 5 through 9, 15 μg of total RNA from cells incubated with CA for 10, 16, 22, 35, and 50 min, respectively. Lanes 10 and 11, As for lanes 3 and 4. Lanes 12 through 17, 15 μg of total RNA from cells incubated with HU for 10, 16, 22, 28, 35, and 50 min, respectively. Lanes 18 and 19, 5 μg of total RNA from in vitro mRNA decay reactions incubated for 5 and 20 min, respectively. The positions of the bands generated by intact histone mRNA (arrow) and by the −5 and −12 RNAs discussed in the text are indicated on the right. Lanes G + A and T + C, Maxam-Gilbert sequencing ladders of the [^{32}P]DNA probe. The sequence of the 3′-terminal portion of the mRNA (derived from the ladder and reference 71) is shown on the right.

ited) and can compare the results with a low-background control (protein synthesis inhibited).

DNA synthesis inhibitors (CA, final concentration 40 μg/ml, or hydroxyurea [HU], final concentration 2 mM) or a protein synthesis inhibitor (cycloheximide) was added to logarithmically growing cells. At various times thereafter, cells were harvested, washed, and frozen. Total-cell RNA was prepared from the frozen cell pellets and analyzed by S1 mapping with two end-labeled DNA probes (Fig. 1). With the 3′-labeled *Nco*I probe, intact histone mRNA from cycloheximide-treated cells protected a set of bands that migrated with or 1 to 2 nucleotides faster than the 104-nucleotide marker (Fig. 2). By comparing these bands with those in the DNA sequencing ladders, the mRNA 3′ terminus was located at a C or U residue just 3′ of the canonical stem-loop (see Fig. 10) (71).

The same bands were detected with RNA from cells treated with DNA synthesis inhibitors. Their intensity decreased during the treatment period, confirming that inhibition of DNA synthesis leads to rapid degradation of the mRNA. Additional, smaller bands were observed with RNAs from DNA synthesis-inhibited cells. The largest and most prominent of these derived from histone RNA lacking approximately 4 to 6 nucleotides from its 3′ terminus. We refer to it as the −5 RNA to indicate that approximately 5 nucleotides have been removed. A fainter set of bands, designated −12, was from histone RNA lacking approximately 10 to 14 nucleotides from its 3′ terminus (see also Fig. 4). The −12 RNA bands were consistently fainter than the −5 band and varied in intensity among different experiments. For example, they were more prominent in Fig. 4 than in Fig. 2.

Several properties of the −5 and −12 RNAs indicate that they are histone mRNA decay products. They were scarce in cycloheximide-treated cells that were not degrading histone mRNA (e.g., Fig. 2, compare lanes 3 and 4 with lanes 5 to 9). They became more abundant during the time interval that histone mRNA was being degraded (see also Fig. 6). They were transient, disappearing after the mRNA had disappeared (Fig. 2 and 4 and unpublished observations). Moreover, they were identical in size to decay products generated in vitro in reactions incubated for 5 and 20 min (compare −5 and −12 bands in lanes 18 and 19 with those in lanes 12 to 17).

If the initial degradation step occurs at the 3′ terminus, then the 5′ region of the mRNA should remain intact while the 3′ region is being destroyed. To test this notion, total-cell RNAs were anlayzed with a 5′-labeled *Sca*I probe (Fig. 1, bottom left). Intact histone mRNA from cycloheximide-treated cells protected a prominent 96-nucleotide fragment (Fig. 3, lanes 3, 4, 11, and 12; arrow). The same band was observed with RNAs from CA- and HU-treated cells (lanes 5 to 10 and 13 to 18) and from an in vitro reaction (lane 19). Additional bands some 13 to 21 nucleotides larger presumably arose from histone mRNAs whose cap sites are located 5′ to the major cap site, as described for a different H4

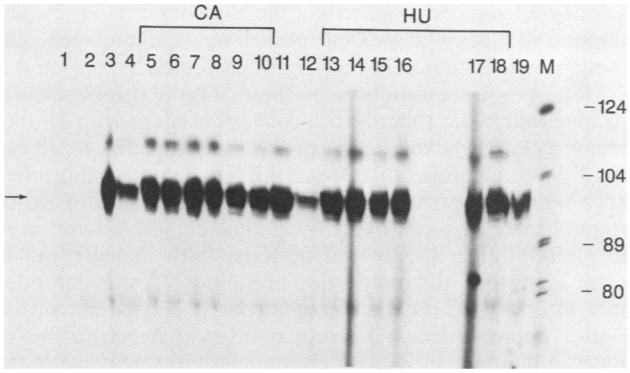

FIG. 3. Analysis of the 5'-terminal region of H4 histone mRNA from whole cells and from a cell-free mRNA decay reaction, with cycloheximide-treated cells as controls. RNA samples were analyzed by S1 nuclease mapping with the 5'-^{32}P-labeled *Sca*I probe (Fig. 1, bottom left). S1-resistant DNA fragments were electrophoresed as described in the legend to Fig. 2 and visualized by autoradiography for 3 days without an intensifying screen. Lane 1, One-fifth of the 5'-^{32}P-labeled probe used in all the other lanes. No S1 nuclease added. The probe migrated in the upper portion of the gel, which is not shown. Lane 2, 30 μg of *E. coli* tRNA. S1 nuclease added. Lanes 3 and 4, 30 and 3 μg, respectively, of total RNA from K562 cells cultured for 2 h with cycloheximide (100 μg/ml). Lanes 5 through 10, 30 μg of total RNA from cells incubated with CA for 10, 16, 22, 28, 35, and 50 min, respectively. Lanes 11 and 12, As for lanes 3 and 4. Lanes 13 through 18, 30 μg of total RNA from cells incubated with HU for 10, 16, 22, 28, 35, and 50 min, respectively. Lane 19, 5 μg of total RNA from an in vitro mRNA decay reaction incubated for 20 min. Lane M, Markers: pBR322 DNA cleaved with *Hae*III and 5'-^{32}P-labeled with T4 polynucleotide kinase. The sizes (in nucleotides) of the fragments are noted on the right. The position of the band generated by intact histone mRNA (major cap site transcript) is indicated by the arrow on the left.

histone gene (27). They were more prominent in the gel shown in Fig. 5. The intensity of the major cap site band decreased with time, consistent with turnover of the mRNA (see Fig. 7). The important observation is that even though the autoradiogram was overexposed, smaller degradation products were not observed in DNA synthesis-inhibited cells or in cell-free reactions. If the mRNA was being degraded at or near its 5' terminus, bands smaller than 96 nucleotides should appear. We conclude that histone mRNA is not degraded by an enzyme that initially attacks the 5' terminus.

The experiment shown in Fig. 2 and 3 was repeated, but the control cells were not treated with cycloheximide. S1 mapping analysis with the 3' probe (Fig. 1, bottom right) revealed that untreated cells, DNA synthesis-inhibited cells, and in vitro mRNA decay reactions all contained intact histone mRNA as well as the −5 and −12 RNAs (Fig. 4, top panel). In this experiment the −12 RNAs included three to five bands, corresponding to RNAs that lacked approximately 10 to 14 nucleotides from the 3' terminus. The quantities of −5 and −12 RNAs, relative to intact mRNA, increased with time of exposure to the DNA synthesis inhibitors (lanes 3 to 8 and 11 to 16; see also Fig. 6), indicating that these RNAs were decay products. These products were identical in size to those generated in vitro (lanes 17 and 18).

This experiment confirmed the prediction that nonsynchronized, growing cells contain histone mRNA decay products (lanes 1, 2, 9, and 10). This result is significant, because it indicates that the −5 and −12 RNAs are not aberrant transcripts generated by inhibitor treatment (see also Fig. 8 and 9). It also emphasizes the correlation between histone mRNA decay and the shortened RNAs. That is, these RNAs were relatively abundant in growing cells that were degrading the mRNA (Fig. 4, lanes 1, 2, 9, and 10) but not in

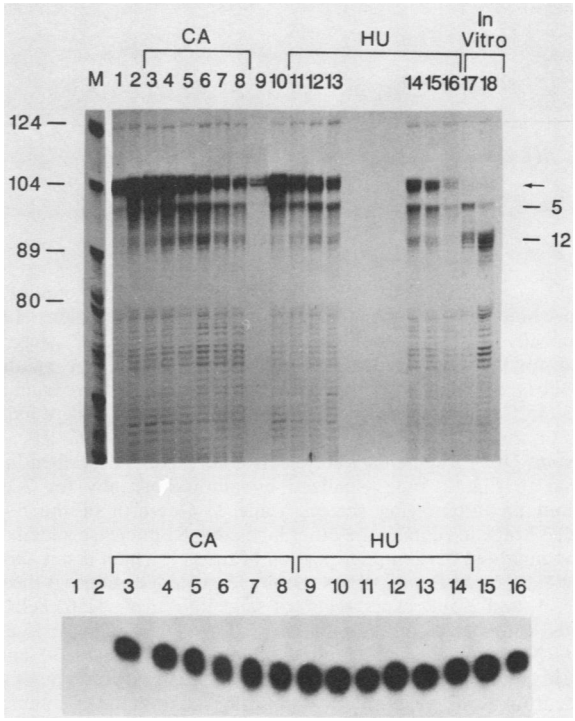

FIG. 4. Analysis of the 3'-terminal region of H4 histone mRNA from whole cells and from cell-free mRNA decay reactions. Logarithmically growing human erythroleukemia cells (K562) were cultured for the indicated times with or without DNA synthesis inhibitors, CA (40 μg/ml), or HU (2 mM). Standard in vitro mRNA decay reactions were incubated for 5 or 20 min. (Top) The indicated amounts of total cellular RNA or of RNA from in vitro mRNA decay reactions were hybridized to the 3'-^{32}P-labeled *Nco*I probe (Fig. 1, bottom right). All reactions were treated with S1 nuclease and analyzed as described in the Fig. 2 legend. The bands were visualized by autoradiography for 8 days without an intensifying screen. The bottom of the figure is 2 cm above the bottom of the gel. Lane M, Kinased fragments of pBR322 cleaved with *Hae*III. Sizes (in nucleotides) are shown on the left. Lanes 1 and 2, 3 and 15 μg, respectively, of total RNA from K562 cells not treated with DNA synthesis inhibitors. Lanes 3 through 8, 15 μg of total RNA from cells incubated with CA for 6, 12, 18, 24, 30, and 40 min, respectively. Lanes 9 and 10, 1 and 15 μg, respectively, of total RNA from K562 cells not treated with DNA synthesis inhibitors. Lanes 11 through 16, 15 μg of total RNA from cells incubated with HU for 15, 20, 25, 30, 35, and 40 min, respectively. Lanes 17 and 18, 5 μg of total RNA from in vitro mRNA decay reactions incubated for 5 and 20 min, respectively. The positions of the bands generated by intact histone mRNA (arrow) and by the −5 and −12 RNAs discussed in the text are indicated on the right. (Bottom) 1 μg of total cellular RNA was incubated with a 3'-^{32}P-labeled probe that anneals to the 3'-terminal 167 nucleotides of human gamma globin mRNA. S1-resistant DNA was electrophoresed in a 4% polyacrylamide, 15-cm-long gel and was visualized by autoradiography for 1 day without a screen. Lane 1, One-fifth of the probe used in all the other lanes. No S1 nuclease added. The probe migrated in the upper portion of the gel, which is not shown. Lane 2, 1 μg of *E. coli* tRNA. S1 nuclease added. Lanes 3 through 8, cells incubated with CA for the same times as for lanes 3 through 8 above. Lanes 9 through 14, Cells incubated with HU for the same times as for lanes 11 through 16 above. Lane 15, Cells incubated for 2 h with cycloheximide (100 μg/ml). Lane 16, Untreated cells.

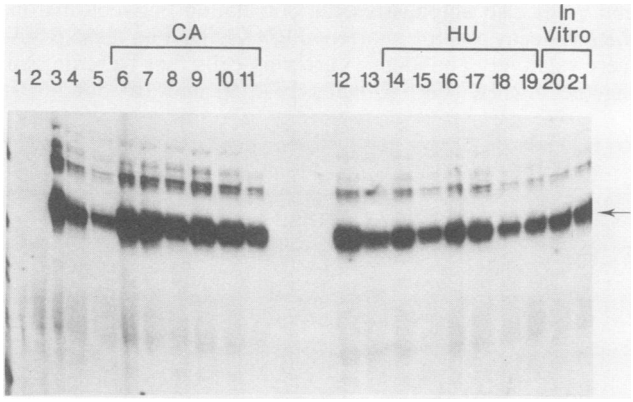

FIG. 5. Analysis of the 5'-terminal region of H4 histone mRNA from whole cells and from cell-free mRNA decay reactions. Logarithmically growing human erythroleukemia cells (K562) were cultured for the indicated times with or without DNA synthesis inhibitors. Standard in vitro mRNA decay reactions were incubated for 5 or 20 min. The indicated amounts of total RNA were hybridized to the 5'-^{32}P-labeled *Sca*I probe (Fig. 1, bottom left). S1-resistant DNA fragments were electrophoresed as described in the legend to Fig. 2 and visualized by autoradiography for 7 days without an intensifying screen. Lane 1, One-fifth of the 5'-^{32}P-labeled probe used in all the other lanes. No S1 nuclease added. The probe migrated in the upper portion of the gel, which is not shown. Lane 2, 15 μg of *E. coli* tRNA. S1 nuclease added. Lanes 3 through 5, 15, 5, and 2 μg, respectively, of total RNA from K562 cells not treated with DNA synthesis inhibitors. Lanes 6 through 11, 15 μg of total RNA from cells incubated with CA for 6, 12, 18, 24, 30, and 40 min, respectively. Lanes 12 and 13, 5 and 2 μg, respectively, of total RNA from K562 cells not treated with DNA synthesis inhibitors. Lanes 14 through 19, 15 μg of total RNA from cells incubated with HU for 15, 20, 25, 30, 35, and 40 min, respectively. Lanes 20 and 21, 5 μg of total RNA from in vitro mRNA decay reactions incubated for 5 and 20 min, respectively. The arrow on the right indicates the fragment protected by full-length, undegraded histone mRNA (major cap site).

growing cells in which the mRNA was more stable (Fig. 2, lanes 3, 4, 10, and 11). We also noted that the quantity of histone mRNA in cycloheximide-treated cells was two- to fourfold greater than that in untreated cells, because the mRNA was not degraded when protein synthesis was inhibited (6, 23, 60, 62, 64).

As an additional control, the cell RNAs were annealed to a 3'-labeled probe for gamma globin mRNA, an RNA that should not be affected by brief treatment with DNA synthesis inhibitors. As predicted, this mRNA was stable in treated cells (Fig. 4, bottom). Moreover, the 5'-labeled *Sca*I probe again failed to detect discrete histone mRNA decay fragments, both in cells and in vitro (Fig. 5).

Five observations indicate that the first detectable step in cellular histone mRNA decay occurs at the 3' terminus and that degradation is 3' to 5'. (i) Shortened RNAs (−5 and −12) were observed with a 3'- but not with a 5'-specific probe (Fig. 2 through 5). (ii) Identical decay products were generated in vitro. (iii) Compared with intact mRNA, the quantity of −5 RNA increased during the course of inhibitor treatment. Gels were exposed to X-ray film without a screen for various times, and the relative intensities of the full-length mRNA and −5 RNA bands were determined. In each experiment, the −5 RNA level increased with time (Fig. 6). Thus, the −5 RNA accumulated and then disappeared, a result consistent with its being a transient decay product. (iv) In four of four experiments, the 3' terminus of the mRNA was degraded more rapidly than the 5' terminus (Fig. 7). The kinetics of decay were determined by scanning gels and comparing the amount of full-length protected 3' *Nco*I and 5' *Sca*I probes as a function of time. The 3' probe clearly disappeared faster than the 5' probe, consistent with 3'-to-5' decay. (v) A 3'-labeled *Sca*I probe that annealed to all but the 5' 96 nucleotides of histone mRNA detected shortened RNAs similar to those observed with the 3' *Nco*I probe (unpublished observations). Still-shorter RNAs appeared only after longer incubation periods, both in cells and in vitro. Therefore, the midportion of the mRNA was degraded only after the 3' region was destroyed, consistent with a 3'-to-5' decay process. There was no evidence with this or other S1 mapping probes for endonucleolytic cleavage of the mRNA.

Controls for DNA synthesis inhibitor and S1 mapping artifacts. Two additional control experiments were performed to confirm these conclusions. The first was to confirm the data shown in Fig. 4, which indicated that the degradation products were not generated by the DNA synthesis inhibitors. If they are authentic decay products, they should be scarce in cells that are not degrading histone mRNA, even if DNA synthesis is blocked. To test this prediction, we exploited the observation that cells exposed to a DNA synthesis inhibitor do not degrade their histone mRNA if they are treated simultaneously with a protein synthesis inhibitor (13, 23, 60, 62–64). Two flasks of growing cells were incubated for 22 min with either CA or HU. Two separate flasks were preincubated with cycloheximide for 60 min and then with CA or HU for 22 min. Total-cell RNA was

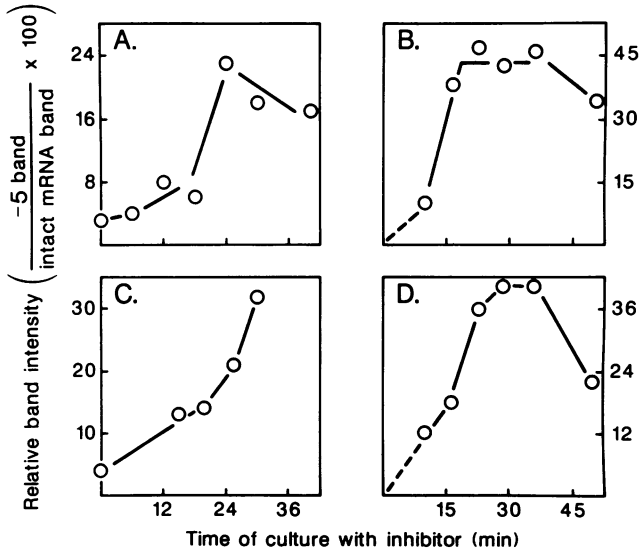

FIG. 6. Ratio of undegraded histone mRNA to the −5 decay product in cells treated with CA (A and B) or HU (C and D). RNAs from inhibitor-treated cells were analyzed by S1 mapping with the 3'-labeled *Nco*I probe, essentially as described in the legends to Fig. 2 and 4. The gels were exposed for various times to x-ray film without a screen. The two relevant bands were scanned in a densitometer to determine the relative amounts of each. The data are plotted as the quantity of −5 RNA divided by the quantity of intact mRNA times 100. (B and D) The time-zero control cells were cultured with cycloheximide (see text). Since there was little, if any, −5 decay product in these cells, the value for time zero could not be determined. In logarithmically growing cells the level of −5 RNA was 3 to 5% of that of the intact mRNA (time zero of panels A and C; unpublished observations).

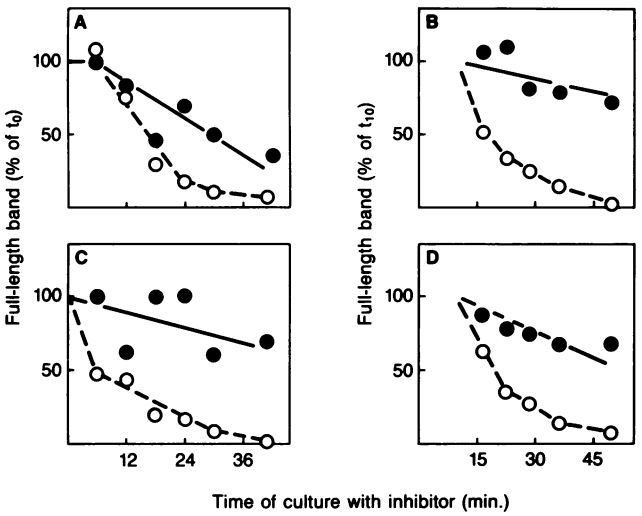

FIG. 7. Kinetics of degradation of the 5' and 3' regions of H4 histone mRNA. Gels from S1 mapping experiments with the 5'- and 3'-labeled probes were exposed for various times to x-ray film without a screen. Care was taken to ensure that the band intensities were proportional to exposure time. The relative amounts of full-length band detected with each probe were determined for each time point. Cells were treated with CA (A and B) or HU (C and D). (B and D) The time-zero control cells were cultured with cycloheximide, while the experimental (DNA synthesis-inhibited) samples contained no cycloheximide. Therefore, to compare the relative degradation rates of the 5' and 3' termini in the experimental samples, the levels at the 16- through 50-min time points were compared with those at 10 min; that is, the levels at 10 min are taken as 100%. Symbols: ●, 5' region of the mRNA measured with the 5'-labeled *Sca*I probe (Fig. 1, bottom left); ○, 3' region of the mRNA measured with the 3'-labeled *Nco*I probe (Fig. 1, bottom right).

annealed with the 3' probe. Histone mRNA decay products were detected in cells treated with DNA synthesis inhibitors (Fig. 8, lanes 6 and 8) but not in cells treated simultaneously with a DNA synthesis inhibitor and cycloheximide (lanes 7 and 9). The −12 RNAs appeared to be less distinct in this than in other experiments, for unknown reasons, but the difference in the level of decay products among lanes 6 through 9 was clear. Therefore, the existence of decay products correlates with the degradation of histone mRNA.

The second control was performed to determine the effects of the S1 nuclease reaction conditions on the mRNA decay bands. RNA from cells treated with CA or cycloheximide was annealed with the 3'-labeled *Nco*I probe, and the nuclease reactions were performed either at different temperatures or with increasing amounts of enzyme at a constant temperature. With RNA from CA-treated cells, the −5 band was observed at temperatures from 13°C below to 8°C above the standard reaction temperature of 37°C (Fig. 9, lanes 3, 5, 7, and 9). It was absent or faint with RNA from cycloheximide-treated cells (lanes 4, 6, 8, and 10). Therefore, within this range, this band did not arise from aberrant S1 nuclease cleavages. In the S1 titration curve, the lowest amount of enzyme was clearly insufficient to generate discrete fragments (Fig. 9, lanes 11 and 12). However, with higher amounts, the −5 band was observed with the expected RNA samples, even when the S1 level was threefold below that used in standard reactions (lane 13). These data support the contention that the −5 and −12 bands are authentic histone mRNA degradation products. Additional evidence against the possibility of S1 nuclease artifacts is summarized as follows. (i) The ratio of intact mRNA to −5 RNA increased during the period that histone mRNA was being degraded (Fig. 2, 4, and 6). Since identical S1 reaction conditions were used for each time point in these experiments, artifacts could not account for the changing ratios. (ii) Histone mRNA was relatively stable in cycloheximide-treated cells, which was consistent with the paucity of decay products. If the decay product bands were generated by nuclease artifacts, they should be as prominent with RNA from cycloheximide-treated cells as they were in CA- and HU-treated cells. (iii) Identical decay product bands were observed with RNA from cells and from in vitro reactions. The decay products were absent or scarce in unincubated reactions, appeared at early times, increased in intensity, and then disappeared at later times (Fig. 2 and 4) (54). These are properties expected of transient decay products, not of nuclease artifacts.

DISCUSSION

Comparison of H4 histone mRNA decay in vitro and in vivo. The first detectable step in H4 histone mRNA decay occurs at the 3' terminus. The remainder of the mRNA is then degraded in a 3'-to-5' direction. The largest and most prominent decay product (−5) results from the loss of approximately 4 to 6 nucleotides from the 3' end. The next smaller products (−12) lack an additional 5 to 9 nucleotides. We do not know whether the −12 RNAs are generated from the −5 product or arise by a different pathway. By comparing the

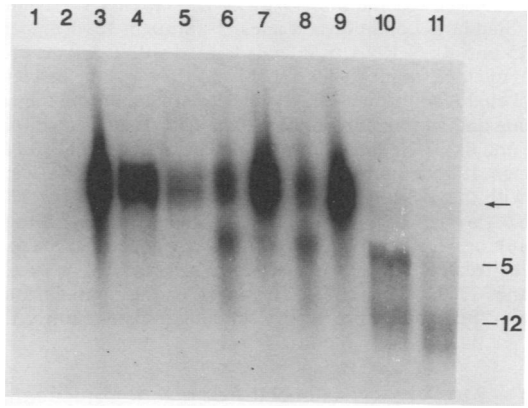

FIG. 8. Correlation of the appearance of histone mRNA decay products with the degradation of histone mRNA. Four flasks of logarithmically growing K562 cells were treated as follows. Two were cultured for 1 h with cycloheximide (100 μg/ml). One of these then received CA (40 μg/ml), the other HU (2 mM). Both were cultured for an additional 22 min and were then harvested, pelleted, and frozen (see Materials and Methods). The other two flasks received CA or HU but no cycloheximide, and these were also cultured for 22 min before harvesting and freezing. Total-cell RNA was prepared, and 30 μg was analyzed by S1 mapping with the 3'-^{32}P-labeled *Nco*I probe (Fig. 1, bottom right), as described in the legend to Fig. 2. The gel was exposed to X-ray film for 24 h without a screen. Lane 1, One-fifth of the 5'-^{32}P-labeled probe used in all the other lanes. No S1 nuclease added. The probe migrated in the upper portion of the gel, which is not shown. Lane 2, 30 μg of *E. coli* tRNA. S1 nuclease added. Lanes 3 through 5, 30, 10, and 3 μg, respectively, of total RNA from K562 cells cultured for 2 h with cycloheximide (100 μg/ml). Lane 6, CA alone. Lane 7, Cycloheximide plus CA. Lane 8, HU alone. Lane 9, Cycloheximide plus HU. Lanes 10 and 11, 15 μg of total RNA from in vitro mRNA decay reactions incubated for 5 and 20 min, respectively. The bands protected by intact histone mRNA (arrow), the −5 RNA decay product, and the −12 decay product are indicated on the right.

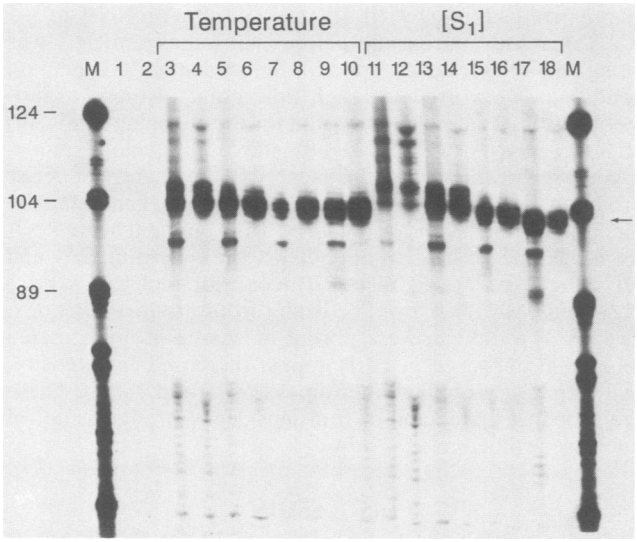

FIG. 9. S1 nuclease mapping controls. RNAs from cells treated with either cycloheximide for 2 h or CA for 22 min were annealed with the 3′-labeled *Nco*I probe and analyzed under different S1 reaction conditions. The S1 nuclease was prepared in our laboratory by the method of Vogt (66). Standard conditions: Vogt buffer containing 0.25 M NaCl and 1 μl (approximately 2 to 6 Sigma units) of S1, 37°C, 1 h. Lanes M, Kinased fragments of pBR322 DNA cleaved with *Hae*III. Sizes (in nucleotides) are shown on the left. Lane 1, One-fifth of the DNA probe used in all the other lanes. No S1 nuclease added. The probe migrated in the upper part of the gel, which is not shown. Lane 2, 15 μg of *E. coli* tRNA. S1 nuclease added. Standard conditions. Lanes 3 through 10, Temperature curve: 15 or 5 μg of RNA from cells treated with CA (lanes 3, 5, 7, and 9) or cycloheximide (lanes 4, 6, 8, and 10) was annealed with the 3′-^{32}P-labeled *Nco*I probe. The S1 nuclease reactions contained 1 μl of enzyme and were incubated for 1 h at 24°C (lanes 3 and 4), 30°C (lanes 5 and 6), 37°C (lanes 7 and 8), or 45°C (lanes 9 and 10). Lanes 11 through 18, S1 nuclease concentration curve: RNAs were annealed with the 3′-^{32}P-labeled *Nco*I probe and analyzed at 37°C for 1 h with 0.1 μl (lanes 11 and 12), 0.3 μl (lanes 13 and 14), 1 μl (lanes 15 and 16), or 4 μl (lanes 17 and 18) of S1 nuclease. The RNAs were from cells treated with CA (lanes 11, 13, 15, and 17) or cycloheximide (lanes 12, 14, 16, and 18). The arrow on the right indicates the location of the bands protected by full-length histone mRNA.

ratio of −5 to −12 RNAs in cell-free reactions incubated for 5 or 20 min, it appears as though the −12 RNAs derive from the −5 RNA (Fig. 2, 4, and 8). Several observations indicate that these RNAs are mRNA decay products. First, they accumulated transiently and were prominent only in cells that were degrading the mRNA. Second, apparently identical products were generated in whole cells and in vitro. This result seems particularly significant, because it provides confidence that mRNA turnover in vitro mimics that in intact cells.

There was one apparent difference between the in vitro and in vivo systems, namely, the cell-free reactions seemed to begin more synchronously. After 5 min of in vitro incubation, most of the RNA that hybridized to the histone probe was partially degraded (for example, Fig. 2, lane 18, and Fig. 4, lane 17). In contrast, only a portion of the cellular histone mRNA molecules was degraded at any time point (Fig. 2, lanes 5 to 9 and 12 to 17); the remainder was intact. This apparent asynchrony is not surprising, because the DNA synthesis block probably begins at different times in different cells. Moreover, the quantity of exonuclease might be limiting in cells but not in vitro. The enzyme is bound to ribosomes in cell lysates (54), but it might exist independently in cells. In other words, it might bind to and be concentrated on ribosomes while they are being prepared. The concentration effect would increase the enzyme-to-substrate ratio, thereby accelerating the onset of the in vitro reaction.

We have also noted that a small fraction (1 to 5%) of cellular histone mRNA remained undegraded after 40 to 50 min of exposure to a DNA synthesis inhibitor. This accounts for the decreasing ratio of −5 RNA to intact mRNA at the later time points (Fig. 6B to D). Perhaps a small fraction of the cells takes up or activates the inhibitors more slowly than the others. Alternatively, histone mRNA synthesis might begin in the late G1 phase, i.e., before DNA replication starts (32). If so, then the mRNA present in late-G1-phase cells might be unaffected by DNA synthesis inhibition.

Properties of histone mRNA that account for its lability. As noted in the Introduction, cell cycle-regulated histone mRNAs must be degraded rapidly at the end of S phase. Therefore, it is important to know what structural features determine their turnover kinetics. Since the mRNA is first attacked at its 3′ terminus, this region probably contains the degradation signals. Although H4 histone mRNA is smaller than the average mRNA, the most striking difference between it and other mRNAs is that it lacks poly(A) (2, 28, 31), which might account, at least in part, for its lability. This conclusion is supported by several observations. (i) The 3′ terminus is the primary site of exonucleolytic attack (Fig. 2 to 5 and 7) (54). (ii) Preliminary in vitro experiments indicate that H4 histone mRNA to which poly(A) has been added is at least 20-fold more stable than nonpolyadenylated histone mRNA (S.W.P. and J.R., data not shown). (iii) Polyadenylated histone mRNA is more stable than unmodified histone mRNA in oocytes (35, 70) and mammalian cells (4, 10, 42). (iv) Some of the so-called variant or basal histone genes generate mRNAs that are polyadenylated, are more stable than the core histone mRNAs, and are not restricted to S phase (17, 45, 68; see also reference 10).

The poly(A) protection effect might account for the manner in which histone mRNA is metabolized in amphibian and echinoderm oocytes and embryos. Oocytes are capable of degrading some nonpolyadenylated RNAs (35, 70). We suggest that they do not rapidly degrade their histone mRNA because it is polyadenylated (39, 57). It is deadenylated after fertilization and is then degraded during early embryogenesis (58, 69). Thus, there appears to be a direct correlation between deadenylation and histone mRNA decay (see also reference 50).

On the other hand, the absence of poly(A) cannot be the only feature that determines the histone mRNA turnover rate. rRNAs and tRNAs lack poly(A) but are very stable, both in growing cells (1, 59) and in vitro (J.R. and G.K., unpublished observations). Some polyadenylated mRNAs turn over as rapidly as histone mRNA (18, 29, 36). One subclass of H4 histone mRNA in myoblasts in polyadenylated, but its half-life (on polysomes) is almost as short as that of nonadenylated H4 histone mRNA (10). These data imply that several properties of histone mRNA determine its turnover rate. Perhaps conserved structural features in the 3′-terminal region are as important for degrading the mRNA as they are for 3′-end formation (8, 9; reviewed in reference 11).

Properties of the enzyme that degrades histone mRNA. The exonuclease is probably located in the cytoplasm, because we detected it in cytoplasmic extracts (54), and several of its properties differ from those of the well-characterized nuclear

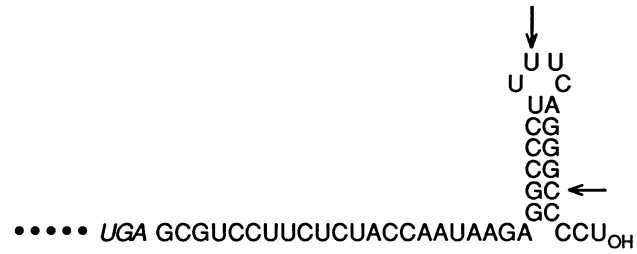

FIG. 10. Secondary structure and cleavage sites or holdup points in the 3'-terminal region of human H4 histone mRNA. The sequence of the 3' untranslated region with its stem-loop (8, 9) is shown. The UGA terminator is italicized. Arrows indicate two regions that are putative cleavage sites or exonucleolytic pause sites that would account for the −5 and −12 RNA decay products (see Discussion).

and nucleolar exonucleases (22, 37, 38). The enzyme sediments with polysomes, because polysomes are the only source of histone mRNA-degrading activity in the in vitro system. We do not know whether the enzyme is associated with polysomes in cells or exists free in the cytoplasm and binds to ribosomes after the cells are lysed. It seems to attack 3' termini exclusively, because 5' exonucleolytic cleavage products were not observed in vitro or in vivo, even when the gels were overexposed (Fig. 3 and 5) (54). The existence of discrete 3'-terminal decay products implies that the enzyme either excises oligonucleotides or removes mononucleotides but pauses or stops at specific holdup points. The putative cleavage or holdup regions are noted by arrows in Fig. 10, which shows the 3'-terminal structure of this mRNA and the canonical stem-loop. The decay of some procaryotic mRNAs by 3' exonucleases is retarded at sites containing significant secondary structures (7, 46). However, this model would not account for the histone decay products, because the first cleavage or pause site is within a stem. We suggest that the enzyme pauses longer at the first holdup point (−5) than at the second (−12), because the −5 decay product was consistently more abundant than the −12 (for example, compare the intensities of −5 and −12 bands in Fig. 4).

Perhaps the most important unanswered questions about the exonuclease relate to its function during the cell cycle and to its potential role in degrading other mRNAs. Does it degrade mRNAs whose poly(A) has been shortened below some critical length (44)? Is it active throughout S phase, when the steady-state level of histone mRNA remains relatively constant, or is it activated near the end of S phase, when the level declines rapidly? We have suggested that free histone protein might somehow activate the exonuclease (54). At completion of S phase, free DNA is not available for binding newly synthesized histones. As a result, unbound histone proteins accumulate (13). They might activate the exonuclease, thereby accelerating histone mRNA decay. This autoregulatory mechanism would explain why inhibition of protein synthesis leads to stabilization of histone mRNA, i.e., by reducing the level of free histone protein.

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