MOLECULAR AND CELLULAR BIOLOGY, Oct. 1984, p. 2082–2090
0270-7306/84/102082-09$02.00/0

Vol. 4, No. 10

Inhibition of Protein Synthesis Stabilizes Histone mRNA

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Received 21 May 1984/Accepted 6 July 1984

The inhibition of protein synthesis in exponentially growing S49 cells leads to a specific fivefold increase in histone mRNA in 30 min. The rate of transcription of histone mRNA, measured in intact or digitonin-permeabilized cells, is increased slightly, if at all, by cycloheximide inhibition of protein synthesis. Both approach-to-equilibrium labeling and pulse-chase experiments show that cycloheximide prolongs histone mRNA half-life from approximately 30 min to >2 h. Histone mRNA made before the addition of cycloheximide becomes stable after the inhibition of protein synthesis, whereas removal of the inhibitor is followed by rapid degradation of histone mRNA. This suggests that the increased stability of histone mRNA during inhibition of protein synthesis results not from alteration of the structure of the mRNA, but from the loss of an activity in the cell which regulates histone mRNA turnover.

Maximal rates of histone synthesis and high levels of histone mRNA are usually associated with DNA synthesis in the cell cycle (24, 32, 40, 53, 55). The transcription of histone mRNA increases at the end of G_1 or in early S phase, but the mRNA is most abundant later in S phase, and the mRNA is most stable at the time of maximal DNA synthesis (23, 31, 51). When cellular DNA synthesis is interrupted by treatment with hydroxyurea or cytosine arabinoside, histone mRNA is rapidly degraded (25, 55).

We have previously demonstrated that histone mRNA can be specifically induced in synchronous G_1 HeLa cells by inhibiting protein synthesis (60). We suggested that histone gene expression is not directly coupled to DNA synthesis, but is regulated by a labile protein, and that the histones themselves may be a link between DNA synthesis and the level of histone mRNA.

We have now investigated this effect in exponentially growing S49 cells. These cells have a doubling time of about 17 h, consisting of a short 2-h G_1 phase before a 12-h period of DNA synthesis (S phase), followed by 2.5 h for G_2 plus mitosis (26). Autoradiography with [^{3}H]thymidine shows that about 60% of the population is in S phase (unpublished observation). Histone mRNA accumulates in spite of the inhibition of DNA synthesis after cycloheximide treatment (61). Concurrent treatment with hydroxyurea, an inhibitor of DNA synthesis (20), does not prevent the accumulation.

We find that the accumulation of histone mRNA results not from increased transcription of histone genes, but from decreased turnover of the transcripts. We have measured histone gene transcription and found it to be unaffected by reducing the rate of general protein synthesis. The stability of histone mRNA, however, is greatly increased by cycloheximide inhibition of protein synthesis. The half-life of histone mRNA, which we estimate to be between 20 and 40 min by both approach-to-equilibrium labeling and pulse-chase experiments, is extended to more than 2 h. Preexisting histone mRNA becomes stable when protein synthesis is inhibited. Conversely, the histone mRNA that accumulates in the absence of protein synthesis is degraded when protein synthesis is restored. Histone mRNA made in the presence of cycloheximide has the same size as that made in untreated cells, it is not polyadenylated, and it can be translated in vitro. These findings suggest that the increased stability of histone mRNA during inhibition of protein synthesis results not from alteration of the structure of the mRNA, but from the loss of an activity in the cell which regulates histone mRNA turnover.

MATERIALS AND METHODS

Cells. S49 mouse lymphoma cells were grown in Dulbecco modified Eagle medium with 10% heat-inactivated horse serum. Cells were maintained in suspension culture at densities of 0.1×10^6 to 2.0×10^6 cells per ml.

Inhibitors. Cycloheximide, puromycin, and hydroxyurea were obtained from Sigma Chemical Co. Pactamycin was a gift from The Upjohn Co.

Plasmids. pBR322 containing mouse histone DNA (56) was given to us by W. Marzluff. pMH3.2, which contains the mouse histone H3.2 gene, was used in initial experiments. To increase the hybridization signal of histone mRNA in transcriptional rate measurements, the 1.7-kilobase *Ava*I-*Sal*I fragment from MM221, which contains mouse histone H2b.1-H3.2 genes, was subcloned in pUC7 and designated pH312.

Mouse alpha-actin cDNA (44) was also obtained from W. Marzluff. Chicken beta-actin and chicken tubulin cDNA (15) were obtained from M. Kirschner. Mouse beta-actin cDNA (58) was obtained from B. Spiegelman. Mouse dihydrofolate reductase (1, 12) was obtained from R. Schimke, mouse mammary tumor virus (63) was from K. Yamamoto, and rat alpha-tubulin cDNA (37) was from P. Sharp. Mouse ornithine decarboxylase cDNA (42) was given to us by M. Gupta.

Acid precipitation of cell extracts. Incorporation of radioactive precursors was determined by spotting cells onto Whatman 595 filter papers and washing extensively in large volumes of 5 to 10% trichloroacetic acid.

RNA purification. Total cell RNA was prepared by the method of Chirgwin et al. (13). Cells were lysed in 2.5 ml of 4.0 M guanidinium thiocyanate and centrifuged through 2.0 ml of 5.7 M cesium chloride. The RNA pellet was dissolved in 300 μl of sterile water and precipitated by adding 30 μl of 3 M sodium acetate (pH 7.0) and 1 ml of absolute ethanol. Polyadenylated [poly(A)$^+$] RNA was purified by two cycles of binding to oligothymidylic acid-cellulose (4).

In vitro translation. Rabbit reticulocyte lysate was prepared by the method of Pelham and Jackson (48), and translation was carried out with [^{35}S]methionine (New En-

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gland Nuclear Corp.). Samples of translation reactions were analyzed on two-dimensional non-equilibrium pH gradient electrophoresis gels (28). The gels were impregnated with En^{3}Hance (New England Nuclear), dried, and placed under Kodak X-AR film at −70°C. A Joyce-Loebl 3CS microdensitometer was used to scan the density of individual polypeptide spots.

RNA dot-blots. RNA was spotted and baked on nitrocellulose filter paper by the method of Thomas (62), prehybridized with 50 μg of salmon sperm DNA (Sigma) per ml in 50% formamide–3× SSC, pH 7.0 (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate)–5× Denhardt solution (1× Denhardt solution is 0.2% bovine serum albumin, 0.2% polyvinyl pyrrolidone, and 0.2% Ficoll 400) for at least 2 h at 42°C, and hybridized in the same buffer plus 10% dextran sulfate for 15 h at 42°C with DNA labeled with [α-^{32}P]dCTP or [α-^{32}P]dATP by nick translation (54). The filters were washed sequentially in 0.1× SSC and 0.1× SSC with 0.1% sodium dodecyl sulfate at 50°C. Du Pont Lightning Plus screens were used with Kodak X-AR film at −70°C. Autoradiographs were scanned with a Zeineh soft laser scanning densitometer. Filters were rehybridized after heating to 68°C in annealing buffer for several hours and then washed as after hybridization.

Electrophoresis and electroblotting of RNA. Samples were denatured in 99% deionized formamide for 2 min at 100°C and then fractionated by electrophoresis through 6% polyacrylamide gels containing 7 M urea in 50 mM Tris, 50 mM borate, and 1 mM EDTA at 20 V/cm for 2.5 to 3.5 h. The gel was stained with ethidium bromide and then destained in several changes of distilled water for 1 h. The RNA was then electroblotted at 0°C to diazobenzyloxymethyl paper (Schleicher & Schuell Co.) in 200 mM sodium acetate (pH 4.0) at 0.5 A for 8 to 12 h. After electroblotting, the diazobenzyloxymethyl paper was inactivated by incubation in 1% glycine at 42°C for at least 12 h. Pre-hybridization in the same solution used for nitrocellulose (above) was for at least 4 h; hybridization and washing were as above. When RNA bound to diazobenzyloxymethyl paper was to be rehybridized, the paper was heated to 68°C in 99% formamide for 2 h and then washed as usual.

Transcription by digitonin-permeabilized cells. Digitonin (Fisher Scientific Co.) was dissolved in ethanol at 10 mg/ml by heating briefly to 68°C and was used to permeabilize S49 cells in the buffers described by Ucker and Yamamoto (64) at a final ratio of 5 μg/10^6 cells. In vitro RNA synthesis was carried out by the method of Ucker and Yamamoto, but RNA was prepared by adding the entire reaction volume (125 μl) to 2.5 ml of guanidinium thiocyanate buffer and proceeding as described above for total cell RNA.

Intact cell labeling of RNA. [^{3}H]uridine (Amersham Corp.) was dried down in a Speed-Vac (Savant), suspended in distilled water at >10 mCi/ml, and 500 μCi/ml was added to cells growing at 37°C in Dulbecco modified Eagle medium at concentrations of 1×10^6 to 2×10^6/ml. The cells were washed in 50 ml of cold phosphate-buffered saline before preparing total RNA as above.

Hybridization of RNA to immobilized DNA. Plasmid DNA was prepared by the method of Holmes and Quigley (33), purified by cesium chloride centrifugation, and bound to 7-mm nitrocellulose filters as described by Palmiter et al. (47), except that 0.5 N sodium hydroxide was used instead of 0.2 N. Hybridization of radioactively labeled RNA to immobilized DNA was by the method of McKnight and Palmiter (43) in 33% formamide at 42°C for 72 h. A total reaction volume of 120 μl contained two filters bearing each plasmid and two blank filters under 200 μl of paraffin. The filters from each reaction were rinsed once with 1 ml of washing solution (0.3 M NaCl, 2 mM EDTA, 10 mM Tris-chloride, pH 7.5) plus 0.1% sodium dodecyl sulfate and then transferred to a 50-ml tube and washed for 2 h in 40 ml of this solution, shaking horizontally in a 45°C water bath. For RNase digestion of the filters, sodium dodecyl sulfate was removed by a 30-min wash at 45°C in washing solution only. Samples (100 μl each) of RNase A and RNase T_1 (Boehringer-Mannheim) were added to 100 ml of washing solution, and 5 to 10 ml was incubated with the filters from each reaction for 30 min at 37°C. The filters were then washed for another 30 min at 37°C in 0.1% sodium dodecyl sulfate washing solution. The radioactivity bound to the filters was counted in 5 ml of Aquassure (New England Nuclear Corp.) after at least 12 h.

Hybridization with increasing amounts of metabolically labeled total cell RNA showed that 10 μg of immobilized pMH3.2 (5 μg per filter) was sufficient for linear binding of the histone mRNA present in up to 20 μg of total RNA from S49 cells. Samples of 20 μg of tubulin or actin cDNA-containing plasmids (10 μg per filter) were used to hybridize up to 10 μg of total cell RNA. The background on filters carrying pBR322 was no greater than on blank filters (about 10^{-5} times the radioactivity included in the reaction), and subsequently only blank filters were used to measure nonspecific binding.

When ^{32}P-labeled cRNA made with the MH3.2 plasmid insert as a template (21) was hybridized with bound MH3.2 plasmid, 40% of the radioactive material remained bound. At first such cRNA was included in each reaction, but since the extent of hybridization varied less than 4% between tubes only one cRNA reaction was later included in each experiment. The extent of the reaction after 72 h was independent of reaction volume between 30 and 120 μl and was unchanged by the addition of extra filters carrying other DNA.

To measure hybridization to actin and tubulin cDNA it was necessary to omit the RNase digestion after hybridization. This increased bound actin mRNA about 10-fold, bound tubulin mRNA 6-fold, and bound histone mRNA about 2-fold (Table 1), and nonspecific binding on the blank filters 2- to 5-fold. We continued to use RNase in all other experiments, because it improved the agreement between duplicate filters and made the background more uniform.

Pulse-chase experiments. S49 cells were labeled under normal growth conditions with 500 μCi of [^{3}H]uridine per ml as above. The pulse was ended by adding the cells to an equal volume of medium containing 20 mM uridine and cytidine plus 30 ml containing 10 mM uridine and cytidine. The cells were spun at room temperature at 200 × *g* for 8 min, washed with 50 ml of medium containing 10 mM uridine and 10 mM cytidine, spun again, and finally suspended at their original concentration in 37°C medium containing 10 mM uridine and 10 mM cytidine. The chase was ended by adding cell samples to 50 ml of ice-cold phosphate-buffered saline, pelleting the cells, and dissolving them in 2.5 ml of guanidinium thiocyanate buffer to prepare RNA as above.

RESULTS

Total cell RNA. Figure 1a shows a dot-blot assay of total cell RNA spotted on nitrocellulose in a series of 1:3 dilutions and hybridized with ^{32}P-labeled cloned genomic DNA for mouse histone H3.2 (56) to measure the amount of histone mRNA. When S49 cells were treated with cycloheximide to inhibit protein synthesis, the level of histone mRNA rose approximately 10-fold (Fig. 1a, lanes 1 and 6). Inhibition of

TABLE 1. Rates of histone and actin mRNA synthesis[a]

Prepn	Histone		Actin	
	ppm hybridized	% of control hybridization	ppm hybridized	% of control hybridization
Intact cells, 10 min of [^{3}H]uridine				
No inhibition	399	100		
20 min of cycloheximide	669	168		
40 min of cycloheximide	575	144		
90 min of cycloheximide	588	147		
No inhibition	763	100		
90 min of hydroxyurea	225	29		
90 min of hydroxyurea and cycloheximide	737	97		
Intact cells, 20 min of [^{3}H]uridine				
No inhibition	1,886	100	2,206	100
20 min of cycloheximide	2,617	139	3,279	149
20 min of hydroxyurea (no RNase digestion)	464	25	2,147	97
Digitonin-permeabilized cells, 12 min of [^{32}P]UTP				
No inhibition	299	100		
20 min of cycloheximide	317	106		
60 min of cycloheximide	319	107		
60 min of hydroxyurea	120	40		
60 min of hydroxyurea and cycloheximide	276	92		

[a] Untreated cells and cells pretreated as shown were labeled with 500 μCi of [^{3}H]uridine per ml (for intact cells) or permeabilized with digitonin and labeled with [α-^{32}P]UTP in the continued presence or absence of inhibitors. Total cell RNA (5×10^5 and 10^6 cpm) from each sample was hybridized with mouse histone H2b.1 and H3.2 DNA (pH312) or chicken beta-actin cDNA (15) bound to nitrocellulose filters. Digitonin treatment, hybridization, and washing are described in the text; RNase digestion was omitted as indicated in experiments with actin cDNA. The parts per million hybridized represent the average of the two reactions with each [^{3}H]RNA sample. Reactions with the same [^{3}H]RNA differed by up to 40%.

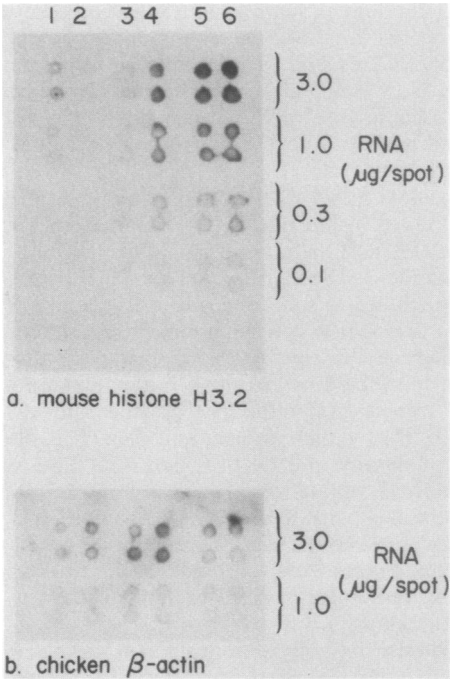

FIG. 1. Histone mRNA after treatment with hydroxyurea and cycloheximide. (a) Total cell RNA from S49 cells was spotted on nitrocellulose in duplicate in a 1:3 dilution series and hybridized with ^{32}P-labeled mouse histone H3.2 DNA as described in the text. Lanes: 1, untreated; 2, 30 min of 1 mM hydroxyurea; 3, 30 min of hydroxyurea followed by 1 h of hydroxyurea plus 100 μg of cycloheximide per ml; 4, 30 min of hydroxyurea followed by 2 h of hydroxyurea plus 100 μg of cycloheximide per ml; 5, 30 min of hydroxyurea followed by 3 h of hydroxyurea plus 100 μg of cycloheximide per ml; 6, 3 h of cycloheximide. (b) The same filter washed as described in the text and rehybridized with chicken beta-actin DNA.

protein synthesis results in rapid inhibition of DNA synthesis (see Fig. 4). It is clear that under these conditions the level of histone mRNA is no longer coupled to the rate of DNA synthesis. However, when DNA synthesis was inhibited by hydroxyurea, histone mRNA fell 10-fold (Fig. 1a, lane 2). If cycloheximide was added after the loss of histone mRNA in the continued presence of hydroxyurea, histone mRNA gradually returned to control levels (after 1 to 2 h; Fig. 1a, lanes 3 and 4) and then increased to levels about 10-fold higher (after 3 h; Fig. 1a, lane 5), approximately the same as in cells treated the whole time with cycloheximide alone (Fig. 1a, lane 6). We have rehybridized such dot-blots with actin (Fig. 1b), tubulin, dihydrofolate reductase, ornithine decarboxylase cDNA, and mouse mammary tumor virus DNA (data not shown; the plasmids are described above) and found no change in the amounts of these mRNAs after inhibition of protein or DNA synthesis.

The extra histone mRNA that accumulates during the inhibition of protein synthesis can be translated in vitro by a rabbit reticulocyte system, and analysis of the translation products by two-dimensional gel electrophoresis reveals no generalized effect on the levels of mRNA for other proteins resolved on these gels. The translational efficiency of total cell RNA from cycloheximide-treated and untreated cells was similar, and samples containing equal acid-precipitable radioactivity were loaded on each gel. The autoradiographs of the gels were scanned with a densitometer, and the results were normalized by using reference spots on each film. RNA from cells treated with cycloheximide stimulated fivefold more histone synthesis than that from controls (Fig. 2).

The extent of the enrichment in histone mRNA and the time course of the induction both correlate with the inhibition of protein synthesis, measured by incorporation of labeled amino acids into acid-insoluble molecules (Fig. 3). We have used several inhibitors to exclude drug-peculiar effects such as the "shielding" of mRNA on polysomes that has been observed with cycloheximide and other inhibitors of translocation (59). Puromycin (Fig. 3a), a chain-terminating tRNA analog (50), and pactamycin (data not shown), which inhibits both initiation and elongation of the polypeptide chain (39), both increase histone mRNA much as cycloheximide does.

To compare histone mRNA from cycloheximide-inhibited and uninhibited cells, total cell mRNA was fractionated according to poly(A) content and analyzed by acrylamide-urea gel electrophoresis. Figure 4a shows an autoradiograph of this RNA electroblotted to diazobenzyloxymethyl paper and hybridized with ^{32}P-labeled mouse histone H2b.1 and H3.2 DNA (pH312, described above). The autoradiograph was scanned with a densitometer to quantitate hybridization. The cycloheximide-treated cells contained 10-fold more histone mRNA apparently identical in size to that in untreated cells. Poly(A)$^-$ RNA showed sixfold more histone mRNA after cycloheximide inhibition. Poly(A)$^+$ RNA gave no visi-

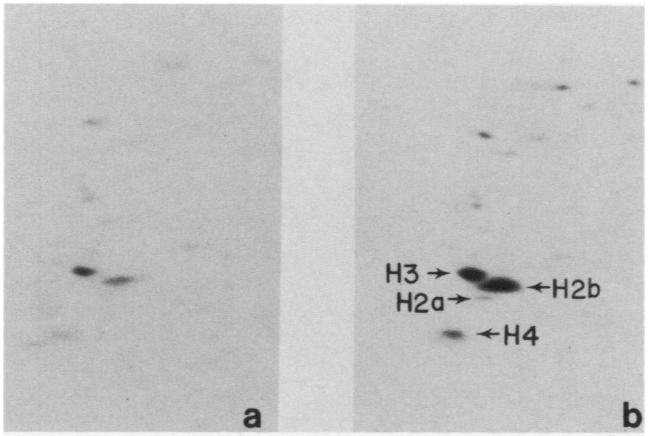

FIG. 2. In vitro translation of RNA from cells inhibited by cycloheximide. Total RNA (20 μg) from (a) untreated cells or (b) cells treated for 3 h with 20 μg of cycloheximide per ml was added to the rabbit reticulocyte lysate described in the text. A 15-μl sample containing approximately 50,000 cpm from each reaction was analyzed by two-dimensional non-equilibrium pH gradient electrophoresis. The autoradiographs were scanned with a Joyce-Loebl microdensitometer.

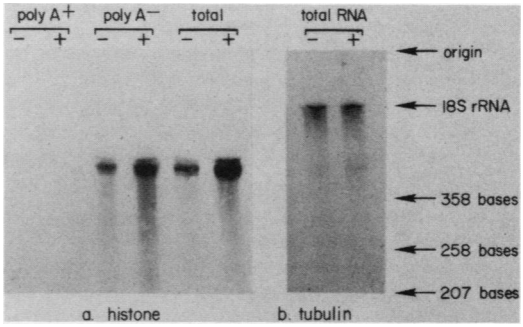

FIG. 4. Polyacrylamide-urea gel electrophoresis of histone mRNA after treatment with cycloheximide. Total cell RNA (5 μg per lane), poly(A)$^+$ RNA (0.5 μg per lane), and poly(A)$^-$ RNA (5 μg per lane) from growing cells and from cells inhibited for 1 h with 20 μg of cycloheximide per ml were analyzed by gel electrophoresis and electroblotted to diazobenzyloxymethyl paper as described in the text. The bound RNA was hybridized with ^{32}P-labeled mouse histone H2b.1 and H3.2 DNA (pH312) (a). The diazobenzyloxymethyl was washed and rehybridized with rat alpha-tubulin cDNA (37) (b); some bound histone DNA is still visible on this autoradiograph. The positions of 18S rRNA and of pBR322 fragments in the same gel are shown. The autoradiograph was scanned with a Zeineh scanning densitometer.

ble hybridization to histone DNA. Control and cycloheximide-treated cells contained equal amounts of tubulin mRNA (Fig. 4b).

Inhibition of protein synthesis by cycloheximide is reversible when cells are washed and suspended in fresh medium (67). In S49 cells protein synthesis (measured by incorporation of [^{35}S]methionine) recovered to 80% of the control rate within an hour; DNA synthesis (incorporation of [^{3}H]thymidine) recovered only 20% in that time. Figure 5 shows that the extra histone mRNA that accumulated during the cycloheximide inhibition was rapidly degraded when the inhibitor was removed. The autoradiograph was scanned with a densitometer to determine that cycloheximide-inhibited cells contained ninefold more histone mRNA than uninhibited cells, but that the amount of histone mRNA 1 h after the removal of the inhibitor was approximately equal to that in untreated cells. The apparent half-life of the mRNA after reversal of cycloheximide inhibition was less than 15 min.

Transcriptional rates. The rate of transcription of histone genes was measured by hybridization of radioactive RNA to cloned DNA bound to nitrocellulose under conditions of DNA excess. To increase the hybridization signal of histone mRNA, we constructed a plasmid (pH312, described above) that contains both the histone H3.2 and H2b.1 genes. Specifically bound counts were approximately eightfold higher than with the pMH3.2 plasmid, but the qualitative results were the same.

In intact cells, cycloheximide had little, if any, effect on the rate of transcription of histone genes (Table 1). The apparent 40 to 70% increase was similar to the 50% increase in actin transcription, whether the rate was measured after

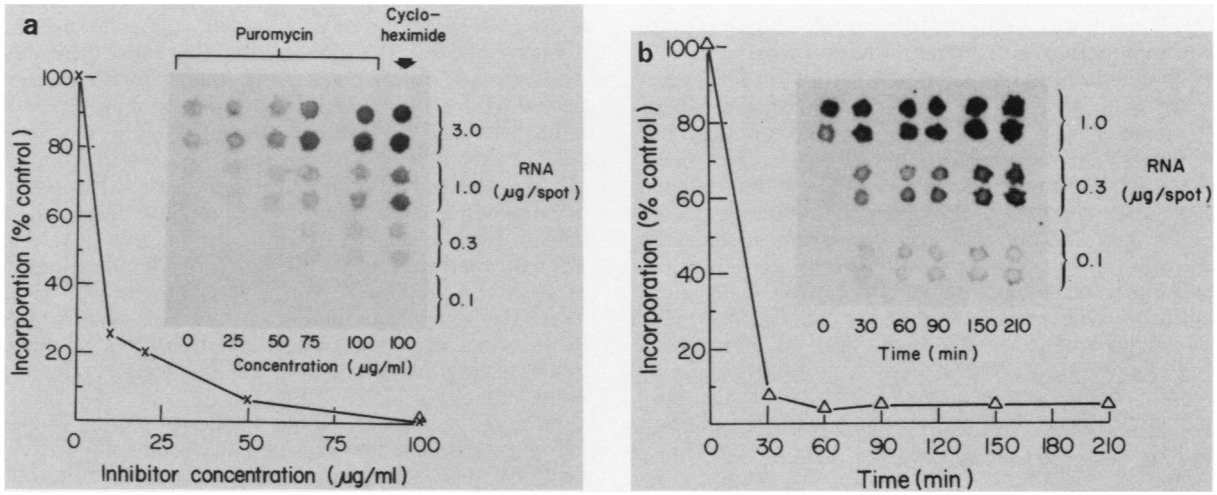

FIG. 3. Response of histone mRNA to increasing inhibition of protein synthesis. RNA was prepared, immobilized on nitrocellulose, and hybridized with MH3.2 DNA as in Fig. 1. The acid-precipitable incorporation of amino acid was determined as described in the text. (a) RNA from cells treated with increasing concentrations of puromycin (×, lanes 2 through 5) or 100 μg of cycloheximide per ml (△, lane 6) for 3 h. Cells were labeled with [^{35}S]methionine during the last 10 min of treatment. (b) RNA from cells treated for increasing times with 100 μg of cycloheximide per ml. A sample of cells was labeled with [^{14}C]leucine for the last 10 min of each period.

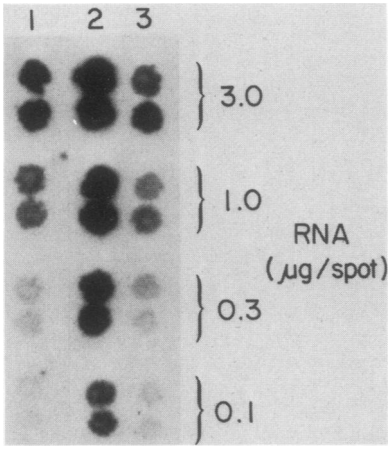

FIG. 5. Histone mRNA after recovery of protein synthesis. Cells treated for 3 h with 100 μg of cycloheximide per ml were pelleted, washed, and suspended at their original density in medium without inhibitor. Total cell RNA was prepared before washing and 60 min after suspending the cells. The RNA was spotted on nitrocellulose in 1:3 dilutions and hybridized with ^{32}P-labeled mouse histone DNA (pH312). The autoradiograph was scanned with a Zeineh densitometer to measure the amount of hybridization to histone mRNA. Lanes 1, untreated; 2, 3 h of 100 μg of cycloheximide per ml; 3, 3 h of cycloheximide, 1 h of fresh medium without inhibitor.

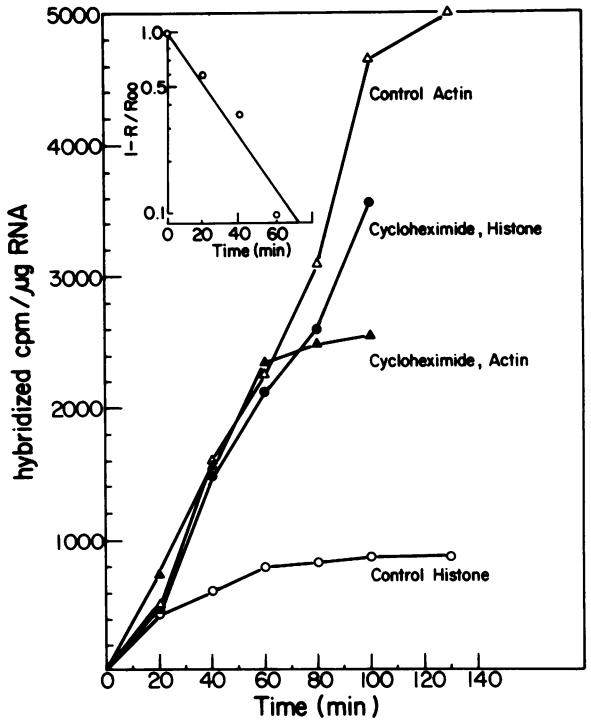

FIG. 6. Approach-to-equilibrium labeling of histone and actin mRNA. Untreated cells and cells pretreated for 15 min with 20 μg of cycloheximide per ml were labeled with 500 μCi of [^{3}H]uridine per ml for the times shown in the continued presence or absence of inhibitor. Total cell RNA (5×10^5 and 10^6 cpm) from each time point was hybridized with mouse histone DNA (pH312) or mouse alpha-actin cDNA (44) on nitrocellulose filters as described in the text. The filters were not digested with RNase during the washing procedure. (Inset) Data from this figure and from hybridization of the same RNA to histone H3.2 and H2b DNA (pH312) with the RNase digestion included in the washing procedure by the method of Perry and Kelley (49) are shown. $R\infty$ is the equilibrium value for hybridization of histone mRNA, and R is the value at the indicated time.

20, 40, or 90 min of inhibition of protein synthesis. Hydroxyurea reduced histone gene transcription to about 30% of the control rate, and this was prevented by the simultaneous addition of cycloheximide, as has been observed by others (23, 57); it had no effect on actin transcription.

These measurements of transcription would be prejudiced if inhibition of protein and DNA synthesis reduced the incorporation of labeled uridine into RNA. Ribosomal RNA synthesis is known to shut off several hours after the inhibition of protein synthesis (67); this limited our experiments to approximately 2 h of treatment when the acid-precipitable incorporation of [^{3}H]uridine per cell and the specific activity of total cell RNA (data not shown) were unaffected by cycloheximide.

The UTP pool was analyzed directly by high-pressure liquid chromatography (19). After 90 min of treatment with cycloheximide and 15 min of labeling with [^{3}H]uridine at 2×10^{-5} M, the concentration used in our transcription experiments, the size of the UTP pool approximately doubled, but the specific activity remained 78% of that in untreated cells. Hydroxyurea had similar effects, but puromycin reduced UTP specific activity to 35%, possibly by inhibiting uridine uptake (29), and was not used in experiments to measure transcription.

To exclude uptake and pool effects we also measured transcriptional rates in digitonin-permeabilized S49 cells. Such cells incorporate [α-^{32}P]UTP into RNA at a rate that is linear for 15 min at 25°C, unaffected by cycloheximide or hydroxyurea (Table 1; data not shown). Again, the only effect of cycloheximide treatment on the rate of transcription of histone genes was to prevent the 60% reduction caused by hydroxyurea.

Approach-to-equilibrium labeling. It followed by exclusion that cycloheximide might increase the concentration of histone mRNA by stabilizing the mRNA. This could be tested by measuring the kinetics of approach to steady-state labeling with [^{3}H]uridine in intact cells (27). As described above, the experiment's duration was limited by the partial inhibition of uridine incorporation, which occurs after several hours of inhibition of protein synthesis (data not shown).

Figure 6 shows that in untreated S49 cells, the radioactivity in histone mRNA per μg of total cell RNA reached a plateau after 60 to 80 min. Replotting the data (Fig. 6, inset) indicates a half-life of about 30 min (49). (This is a maximum half-life, since it includes the time required for equilibration of the UTP and CTP pools.) Cells pretreated for 20 min with cycloheximide continued to accumulate [^{3}H]uridine into labeled histone mRNA at an approximately linear rate for 100 min, the length of the experiment. This stabilizing effect of cycloheximide was specific for histone mRNA; labeled actin (Fig. 6) and tubulin (data not shown) mRNA increased in the same way both in untreated and in cycloheximide-inhibited cells, with half-lives too long to affect the accumulation of label in RNA within 2 h.

Pulse-chase. We carried out a series of pulse-chase experiments to confirm the half-life measurements that we had made by approach-to-equilibrium labeling and by following the loss of total histone mRNA after removal of cycloheximide. This procedure also allowed us to distinguish the fate of histone mRNA made before the inhibition of protein synthesis from that made after the addition of cycloheximide.

In spite of difficulties in efficiently chasing radioactively labeled uridine from the UTP pool in some cell types (66; unpublished observations), simply washing and suspending cells in medium containing high concentrations of uridine and cytidine sometimes achieves an effective dilution (5, 36). When S49 cells labeled for 30 min with 500 μCi of [^{3}H]uridine per ml were washed and suspended in medium containing 10 mM uridine and cytidine, the incorporation of [^{3}H]uridine into acid-soluble molecules ceased after the washes. The specific activity of total acid-precipitable cell RNA declined slowly over the next 2 h independent of the inhibition of protein synthesis, implying that the efficiency of the chase was unaffected by the inhibition. The incorporation of [^{35}S]methionine was unchanged by the addition of 10 mM uridine and cytidine for 2 h; viability began to decline after 6 h (data not shown).

The experiments described in Fig. 7a measured the decay in the presence or absence of cycloheximide of histone mRNA labeled in either cycloheximide-treated or untreated cells. The half-life of histone mRNA in control cells was approximately 30 min; it seemed slightly shorter in cells labeled in the presence of cycloheximide and then chased after the drug was washed out. Cycloheximide protected all histone mRNA from degradation, whether it was made before or during the inhibition of protein synthesis. Rat tubulin cDNA (37) was included in several experiments to confirm that the effect was specific for histone mRNA. Tubulin mRNA was lost with a half-life of approximately 50 min (Fig. 7b) and was not made stable by cycloheximide. Tubulin mRNA labeled in the presence of cycloheximide showed the same half-life whether or not the chase included the inhibitor (data not shown).

DISCUSSION

We conclude that the coupling of histone and DNA synthesis is mediated by one or more labile proteins whose synthesis is inhibited in our experiments. The inhibition of protein synthesis prevents the accumulation of such proteins and stabilizes histone mRNA even in the absence of DNA synthesis.

Previous work has shown that synchronous G_1 HeLa cells accumulate histone mRNA during inhibition of protein synthesis (61). The experiments described in this paper were done with exponentially growing S49 cells, about 60% of which are making DNA at any time. The 5- to 10-fold magnitude of the response to cycloheximide makes it unlikely that only early G_1 cells, which contain approximately 5-fold less histone mRNA than do S-phase cells (18), are involved. It follows that the level of histone mRNA in S-phase cells is not maximal, but increases when protein synthesis is inhibited.

Cycloheximide increases histone mRNA by stabilizing the mRNA. We and others (23, 57) have shown that histone transcriptional rates show no significant change. Histone mRNA in cycloheximide-treated cells is stable for over 2 h. Its actual half-life in the presence of cycloheximide could not be determined by equilibrium labeling because the incorporation of [^{3}H]uridine declines after several hours of inhibition of protein synthesis, probably as ribosomal RNA synthesis is inhibited (67); nor could it be measured by pulse-chase experiments, since 10 mM uridine and cytidine becomes harmful to S49 cells after 4 h by at least one criterion, the reduced incorporation of [^{35}S]methionine (unpublished observation).

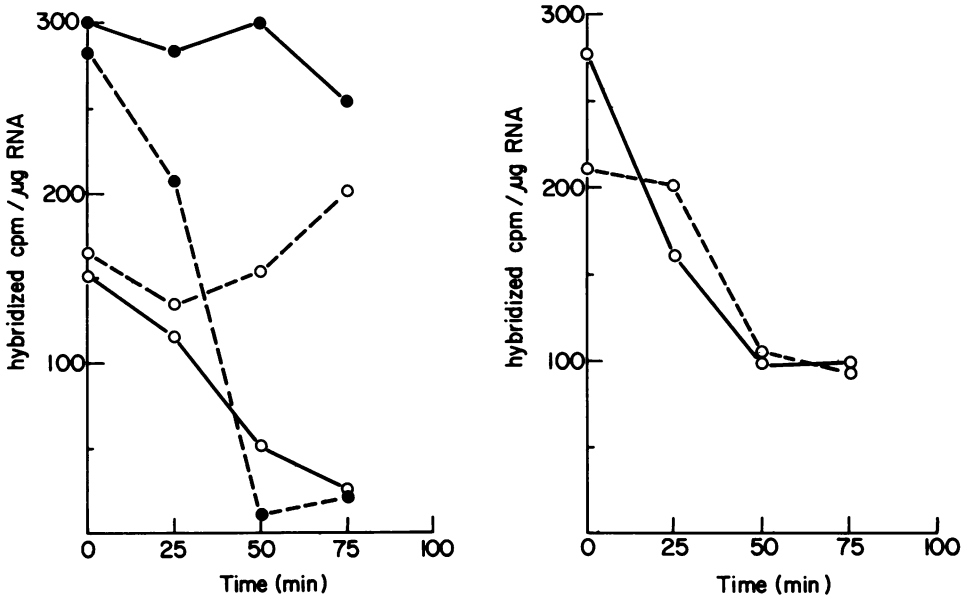

FIG. 7. Stabilization of histone mRNA by cycloheximide. Control cells or cells pretreated for 10 min with 20 μg of cycloheximide per ml were labeled for 20 min with [^{3}H]uridine, washed, and suspended in medium containing 10 mM uridine and 10 mM cytidine. Total cell RNA was prepared at the times shown after suspending the cells. Samples of approximately 5 and 10 μg of RNA (2×10^5 to 3×10^5 cpm/μg) from each sample were hybridized to DNA immobilized on nitrocellulose. Two filters bearing each plasmid and two blank filters were included in each reaction. Symbols: (●———●) pretreatment and pulse with cycloheximide, wash and chase with cycloheximide; (●----●) pretreatment and pulse with cycloheximide, wash and chase without cycloheximide; (○———○) pretreatment and pulse without cycloheximide, wash and chase without cycloheximide; (○----○) pretreatment and pulse without cycloheximide, wash and chase with cycloheximide. (a) Hybridization to mouse histone H3.2 and H2b DNA (pH312). The data are averages of one experiment including RNase digestion and one without. (b) Hybridization to mouse alpha-tubulin cDNA (pIL alpha T1). No RNase was used in washing these filters.

We have measured a 20- to 40-min half-life for histone H2b.1 and H3.2 mRNA in S49 mouse cells by two techniques, approach-to-equilibrium and pulse-chase labeling. This is somewhat less than the 30- to 60-min measurements of histone mRNA half-life made by approach-to-equilibrium labeling of exponentially growing HeLa cells by Heintz et al. (30) and the 1-h half-life of labeled histone mRNA on polyribosomes observed after actinomycin D treatment of HeLa cells (7, 8). Alterman et al. (2) found two components with half-lives of 25 and 92 min in pulse-chase labeling of histone mRNA in mouse erythroleukemia cells.

Others have observed the half-life of histone mRNA to be closer to the half-life of the S phase itself. Early experiments with mouse L cells by Perry and Kelley (49) showed a half-life of approximately 6 h by approach-to-equilibrium labeling. Recently, DeLisle et al. (23) used a pulse-chase procedure in 3T6 cells and observed the loss of labeled histone mRNA with a half-life of 4.5 h. Whether these longer estimates are due to differences in experimental design or to variation between cell types remains to be determined.

We included tubulin as a control in several experiments. The free tubulin monomer concentration in mammalian cells regulates the abundance of tubulin mRNA (6, 16). The tubulin transcriptional rate in runoff nuclei is unchanged by increasing free tubulin (14), implying that mRNA stability may be regulated. The effect of inhibiting protein synthesis on tubulin monomer concentration is unknown, but we observed no effect on tubulin mRNA concentration in S49 cells, nor did we see any effect on tubulin mRNA transcription or stability. The half-life of tubulin mRNA in actinomycin D-treated 3T6 cells is approximately 2 h (6). Since hybridized RNA was not digested with RNase in our experiments with tubulin cDNA, at least a part of the turnover we measured may reflect RNA processing; the apparent half-life of tubulin mRNA was approximately 50 min. This half-life is comparable to the 30-min half-life we observe for histone mRNA, but tubulin mRNA is not stabilized by cycloheximide whether the inhibitor is included in the pulse, chase, or both.

There are other instances where inhibition of protein synthesis enhances specific mRNAs (11, 17, 34, 35, 41, 68). There are also a few precedents for the regulation of gene expression through mRNA stability (9, 22, 38, 65, 69). In early *Drosophila* development, the half-life of histone mRNA decreases approximately sevenfold when histone production becomes coupled to DNA synthesis. This could be because maternal histone mRNA is replaced by less stable newly synthesized histone mRNA, or because all histone mRNA becomes less stable with a change in the concentration of a regulator of histone mRNA turnover (3). Similarly, the stabilization of histone mRNA and its uncoupling from DNA synthesis by the inhibition of protein synthesis could be due to one of two mechanisms; the histone mRNA made in the absence of protein synthesis could be structurally different from normal, "coupled" histone mRNA, or the stability of all histone mRNA might be regulated by a labile protein. Our ability to translate in vitro histone mRNA made during the inhibition of protein synthesis as well as the apparently normal size of such mRNA in gel electrophoresis exclude a gross structural difference. The fact that inhibition of protein synthesis stabilizes preexisting histone mRNA argues against such a structural difference, although if a large part of histone mRNA exists in an unprocessed form the post-transcriptional rescue of this population would be possible (52). The inhibition of protein synthesis does not cause polyadenylation of histone mRNA. We have not excluded the possibility that all poly(A)$^-$ RNA becomes stable under these conditions, but the potential utility of such a mechanism for coordinate regulation of this class of RNA is not obvious.

Our experiments support the concept of a protein regulator of histone mRNA. Such a protein could be a histone-specific RNase or a protein that induces or activates such an RNase or binds to histone mRNA to signal its degradation. Butler and Mueller (10) proposed that the histones themselves couple DNA synthesis and histone mRNA stability. This would be consistent with our results, with the small free histone pool in mammalian cells (45), and with the histones' nucleic acid-binding properties, which might extend specifically to their own mRNA. Yeast histones show dosage compensation, as would be expected from autogenous regulation: when an extra pair of histone genes is inserted into the haploid genome, total histone gene transcription increases proportionally, but the normal level of histone mRNA is maintained by the increased turnover of histone transcripts (46).

Coupling of histone and DNA synthesis results from, at the least, regulation of both histone gene transcription and histone mRNA stability. Understanding the molecular basis for this coupling will require careful study of both processes and may be useful for elucidating and manipulating the stability of other mRNAs.

ACKNOWLEDGMENTS

We are most grateful to Marvin Cohen for measuring the specific activity of UTP pools under our labeling conditions.

This work was supported by Public Health Service grant CA29048 from the National Institutes of Health and by National Science Foundation grant PCM 78-07382. E.S. was supported by an Anna Fuller postdoctoral fellowship and subsequently by National Research Service award CA09043.

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