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Cell Cycle Regulation of Mouse H3 Histone mRNA Metabolism

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The mechanisms responsible for the periodic accumulation and decay of histone mRNA in the mammalian cell cycle were investigated in mouse erythroleukemia cells, using a cloned mouse H3 histone gene probe that hybridizes with most or all H3 transcripts. Exponentially growing cells were fractionated into cell cycle-specific stages by centrifugal elutriation, a method for purifying cells at each stage of the cycle without the use of treatments that arrest growth. Measurements of H3 histone mRNA content throughout the cell cycle show that the mRNA accumulates gradually during S phase, achieving its highest value in mid-S phase when DNA synthesis is maximal. The mRNA content then decreases as cells approach G2. These results demonstrate that the periodic synthesis of histones during S phase is due to changes in the steady-state level of histone mRNA. They are consistent with the conventional view in which histone synthesis is regulated coordinately with DNA synthesis in the cell cycle. The periodic accumulation and decay of H3 histone mRNA appear to be controlled primarily by changes in the rate of appearance of newly synthesized mRNA in the cytoplasm, determined by pulse-labeling whole cells with [^{3}H]uridine. Measurements of H3 mRNA turnover by pulse-chase experiments with cells in S and G2 did not provide evidence for changes in the cytoplasmic stability of the mRNA during the period of its decay in late S and G2. Furthermore, transcription measurements carried out by brief pulse-labeling in vivo and by in vitro transcription in isolated nuclei indicate that the rate of H3 gene transcription changes to a much smaller extent than the steady-state levels of the mRNA or the appearance of newly synthesized mRNA in the cytoplasm. The results suggest that post-transcriptional processes make an important contribution to the periodic accumulation and decay of histone mRNA and that these processes may operate within the nucleus.

The biosynthesis of histones is generally thought to occur periodically in the cell cycle and to be tightly coupled to DNA replication. Early studies utilizing synchronized HeLa cells showed that newly synthesized histones could be found associated with chromatin only in S-phase cells (2, 14, 27). These cells contained an abundance of small polyribosomes with nascent polypeptides that comigrated with histones in gel electrophoresis; these small polysomes were not apparent in G1 cells (2). When cells undergoing DNA replication are treated with inhibitors of DNA synthesis there is a rapid loss of histone production (2, 4). Gallwitz and Mueller (8) showed that histone proteins were not synthesized in vitro on microsomes isolated from such cells, and Borun et al. (1) confirmed these results by a direct mRNA translation assay.

Despite extensive investigations into the mechanisms responsible for the temporal pattern of histone synthesis in the cell cycle, there is still considerable disagreement concerning the level(s) at which histone protein synthesis is regulated. A number of studies support the view that the periodic synthesis of histones is controlled at the level of transcription of histone genes. Histone mRNAs are found in polyribosomes only during S phase (1, 2, 8), and these mRNAs could not be detected in polysomes or other subcellular fractions in G1 cells (1, 4, 7). Using cDNA complementary to H4 histone mRNA, Stein et al. (32) assayed in vitro transcription of histone RNA sequences in isolated HeLa cell nuclei and also in chromatin, using added *Escherichia coli* RNA polymerase. Transcription of histone RNA was detected only with fractions from S-phase cells. However, other studies with HeLa cells suggested that transcription of histone genes occurs throughout the cell cycle. Using cloned sea urchin histone DNA as a hybridization probe, Melli et al. (19) found equal amounts of high-molecular-weight hybridizable RNA in HeLa cells at various times after release from a thymidine block as well as in cells treated with a DNA synthesis inhibitor. Still other, more recent work has suggested that post-translational controls may be important in regulating the deposition of newly synthesized histones in chromatin. Groppi and Coffino (9) reported that the rate of histone protein synthesis in mouse lymphoma and Chinese hamster ovary cells is constant during G1 and S phases, but that newly synthesized histones associate with chromatin only during S phase.

There are several factors that may contribute to the conflicts among the various investigations into histone gene regulation. Considerable variation exists in the methods used to detect histone mRNA; in no case were cloned, homologous histone gene probes used. Various techniques were used to obtain synchronous populations of cells, often involving treatment with drugs or other chemicals that inhibit progression through the cell cycle. It is also possible that different cell lines exhibit variability in the degree to which histone gene expression is regulated in the cell cycle.

The recent isolation of mammalian histone genes (11, 25, 29) has made possible the reexamination of histone gene regulation with cloned probes that can be used to study the metabolism of histone mRNA. Very recent reports (10, 23, 30) utilizing cloned probes confirm the tight coupling between histone mRNA accumulation and DNA synthesis. They also suggest that a combination of transcriptional and post-transcriptional mechanisms regulate histone mRNA

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levels in synchronized cells and during drug-induced inhibition of DNA synthesis. Whereas chemical methods for achieving cell synchrony have proven very useful for investigating the relationship between DNA synthesis and histone production, it is also important to study the regulation of histone mRNA metabolism by methods that do not perturb the cell cycle. The technique of centrifugal elutriation permits the purification of cells at each stage of the cycle from an exponentially growing population. We report here on studies concerning the cell cycle regulation of H3 histone mRNAs in mouse erythroleukemia (MEL) cells that were separated into cell cycle-specific fractions by centrifugal elutriation. By using a cloned mouse H3 gene to assay for H3 histone mRNAs, the fractions were tested for (i) H3 histone mRNA content, (ii) the rate of appearance of newly synthesized H3 mRNA in the cytoplasm, (iii) the turnover of prelabeled H3 mRNA, and (iv) H3 gene transcription in vivo and in vitro in isolated nuclei. These measurements indicate that the periodic accumulation and decay of histone mRNA in the cell cycle is only partly controlled at the level of transcription, suggesting that post-transcriptional events play an important role in the periodic synthesis of histones.

MATERIALS AND METHODS

Cell culture. Clone DS-19 (21) MEL cells were derived from clone 745A obtained from Charlotte Friend. Cells were grown and maintained in suspension culture in Dulbecco modified Eagle medium (GIBCO Laboratories) supplemented with 10% fetal bovine serum (GIBCO).

Centrifugal elutriation. Exponentially growing cells were concentrated by centrifugation, resuspended in growth medium, and separated into size fractions in a Beckman J21C centrifuge equipped with a Beckman JE-6 elutriator rotor as previously described (3). Aliquots of elutriated cells were analyzed for DNA content by flow microfluorimetry, using a Becton-Dickinson Fluorescence-Activated Cell Sorter (FACS II). A second aliquot of cells was labeled to determine the percentage of cells synthesizing DNA. A total of 4×10^6 cells were incubated at 37°C for 15 min in growth medium containing 5 μCi of [^{3}H]thymidine per ml. The cells were pelletted and resuspended in medium without label. Cytocentrifuge slides were prepared, fixed in methanol, dried, and dipped in emulsion (Kodak). The slides were exposed for 7 to 9 days at 4°C, developed, and stained with Giemsa. The percent labeled nuclei was determined.

Isolation of cytoplasmic RNA and RNA blot analysis. Cytoplasmic RNA was isolated from cell extracts prepared by lysing cells in 150 mM NaCl–10 mM Tris-hydrochloride (pH 7.4)–1.5 mM $MgCl_2$–0.3% (vol/vol) Nonidet P-40 followed by centrifugation for 10 min at 2,000 rpm. RNA was extracted by the hot-phenol procedure described by Soiero and Darnell (31) with minor modifications as previously described (16). RNA samples, dissolved in 50 mM boric acid–5 mM sodium borate–10 mM sodium sulfate–0.1 mM EDTA–50% formamide–6% formaldehyde, were heated at 65°C for 5 min and fractionated by electrophoresis in 1.2% agarose gels containing 3% formaldehyde. Blot hybridization was performed essentially as described by Thomas (33), using probes nick translated by the method of Rigby et al. (26). The intensity of autoradiographic bands was determined by scanning with a Joyce-Loebl MKIII double-beam recording microdensitometer.

In vivo labeling of cells and isolation of [^{3}H]RNA. Elutriated cell fractions were collected by centrifugation and resuspended in growth medium at 2×10^6 cells per ml. Cells were preincubated in a shaking water bath at 37°C for 10 min and then further incubated with [^{3}H]uridine (approximately 40 Ci/mmol; New England Nuclear) at 0.4 mCi/ml for 5, 15, and 60 min. Incubation was terminated by addition of 5 to 10 volumes of ice-cold lysis buffer (150 mM NaCl, 10 mM Tris-hydrochloride [pH 7.4], 1.5 mM $MgCl_2$), and cells were collected by centrifugation at 1,000 rpm for 5 min in a model PR-2 IEC centrifuge. Cells were washed with 50 ml of lysis buffer and resuspended at about 5×10^6 cells per ml. Cell concentration was determined in a Coulter cell counter. Total cell RNA was isolated by the hot-phenol method. To isolate cytoplasmic RNA, cells were lysed by adding Nonidet P-40 to a final concentration of 0.3% (vol/vol), and nuclei were removed by centrifugation at 2,000 rpm for 10 min. An aliquot of the supernatant was taken to determine the incorporation of [^{3}H]uridine into cytoplasmic RNA. RNA was isolated by proteinase K treatment as previously described (5). Polyadenylated [poly(A^+)] and non-polyadenylated [poly(A^-)] fractions were prepared by chromatography on oligodeoxythymidylate-cellulose (type 2; Collaborative Research) after incubation for 3 min at 65°C in 10 mM Tris-hydrochloride, pH 7.4.

Transcription in isolated nuclei. The cell lysate was prepared as described above. The lysate was layered over 2 volumes of 0.6 M sucrose buffer containing 10 mM Tris-hydrochloride (pH 7.4)–5 mM $MgCl_2$, and the nuclei were pelleted by centrifugation at 2,000 rpm for 10 min. Nuclei were resuspended at about 3×10^8 per ml in 50 mM Tris-hydrochloride (pH 7.9)–25% glycerol–0.1 mM EDTA–10 mM 2-mercaptoethanol–5 mM $MgCl_2$ and either used immediately or after no more than 48 h of storage in liquid nitrogen. The transcription reaction mixture contained the following components in a total volume of 0.15 ml: 1.3×10^7 nuclei per ml, 50 mM Tris-hydrochloride (pH 7.9), 1 mM $MnCl_2$, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM ATP, 0.5 mM GTP, 0.5 mM CTP, 0.15 M KCl, and 2.5 μM [α-^{32}P]UTP (410 Ci/mmol). The mixture was incubated at 25°C for 60 min. The reaction was terminated by the addition of sodium dodecyl sulfate (SDS) to 0.5% and brought to 100 mM NaOAc (pH 5.2)–1 mM EDTA. Nuclear RNA was prepared by the hot-phenol extraction method. RNA was collected by ethanol precipitation in the presence of 50 μg of *E. coli* tRNA per ml. RNA was further purified by chromatography on a Bio-Gel P-6 column (30 by 1 cm; Bio-Rad Laboratories) in 0.5% SDS–0.1 M NaCl–10 mM Tris-hydrochloride (pH 7.5)–1 mM EDTA. The void volume fractions were pooled, brought to 0.2 M NaOAc (pH 5.5), and ethanol precipitated.

DNA filter hybridization. DNA filters were prepared essentially as described by Melli et al. (18). A 100-μg portion of plasmid DNA was bound to each 25-mm-diameter nitrocellulose filter (type HAWP, 0.45-μm pore size; Millipore Corp.). The DNA solution in 10 mM Tris-hydrochloride (pH 7.4)–1 mM EDTA was brought to 0.1 M NaOH and heated for 5 min at 100°C. After quenching on ice, 14 volumes of 2 M NaCl was added. A 100-μg amount of DNA, usually in 4.5 ml, was filtered by gravity, using a Millipore manifold fitted with collection tubes. The eluate was recycled through each filter by gravity. The filters were then washed twice under suction with 10 ml of 6× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate) and baked in vacuo at 80°C for 3 to 4 h. Filters were stored at room temperature under vacuum. Before hybridization, nuclear RNA was reduced in size by incubation at 0°C for 30 min in 0.1 M NaOH–10 mM Tris-hydrochloride (pH 7.4)–10 mM EDTA–0.2% SDS. The samples were neutralized with 2 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) and ethanol precipitated. Alkali-broken RNA was dissolved in 10 mM

TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid] (pH 7.5)–10 mM EDTA–0.2% SDS, heated at 90°C for 2 min, and quenched on ice. DNA filters were cut into four or eight parts so that each reaction contained 25 or 12.5 μg of DNA, respectively. The reaction mixture contained 0.3 M NaCl–10 mM TES (pH 7.4)–10 mM EDTA–0.2% SDS and labeled RNA in a total volume of 0.2 to 0.3 ml. Reaction mixtures were overlaid with 0.3 ml of paraffin oil and incubated at 65°C for 36 to 40 h in small vials. After incubation, the filters were washed six times with hybridization buffer at 65°C for 15 min each and then four times with 2× SSC at room temperature for 15 min each. The filters were treated with pancreatic RNase A (20 μg/ml; Worthington Diagnostics) and RNase T_1 (5 U/ml; Boehringer Mannheim) in 2× SSC at 37°C for 45 min, washed four times with 2× SSC at room temperature, dried under a heat lamp, and counted in a scintillation counter. Hybridization of histone-specific RNA was calculated from the difference between radioactivity bound to filters containing histone DNA and that bound to filters containing plasmid DNA without a histone gene insert, which were included in each reaction. The amount of radioactivity bound to filters of the latter type was <10 ppm for cytoplasmic RNA and 10 to 20 ppm for nuclear and total cell RNA. Duplicate determinations were made on each sample, and the average value was used.

RESULTS

Isolation of a DNA hybridization probe specific for mouse H3 histone mRNAs. The isolation of mouse genomic DNA segments containing histone genes has been described previously (29). MM221 was isolated from a library of mouse DNA in Charon 4A and shown to contain two H3 genes, an H2B gene, and a portion of an H2A gene (Fig. 1). The phage DNA was cut with *Eco*RI and *Sal*I, and a 0.8-kilobase fragment, containing a portion of the H3-2 gene originating from codon 57 and including 3′-flanking DNA, was purified and cloned into pBR322 from which the *Eco*RI-*Sal*I fragment had been removed. When this plasmid, pH3-2, was hybridized to RNA blots of MEL cell cytoplasmic RNA that had been fractionated on oligodeoxythymidylate-cellulose, a strongly hybridizing band was observed in the poly(A^-) fraction at the position expected for 9S histone mRNA (Fig. 2A). However, in poly(A^+) RNA samples, hybridization was observed to heterodisperse RNA sequences migrating more rapidly than 9S histone RNA. It may be that the DNA region 3′ to the H3-2 gene contains repetitive sequences with partial homology to other cellular RNA transcripts.

To obtain a hybridization probe that is completely specific for H3 mRNA sequences, pH3-2 was cut with *Sal*I and *Pvu*II and the DNA fragment corresponding to codons 57 to 125 of the H3-2 gene was inserted into *Sma*I/*Sal*I-digested plasmid pLL10, a derivative of pBR322 containing a *Sma*I site (28; Fig. 1). The resulting plasmid, designated pRAH3-2, hybridized exclusively to poly(A^-) 9S histone mRNA (Fig. 2B). Very little hybridization was observed to poly(A^+) RNA, and this occurred only at the 9S position.

Cell cycle analysis by centrifugal elutriation. A variety of methods are available for obtaining synchronous cell populations. Many of these involve drug or other chemical treatments or deprivation of essential growth factors that lead to arrest of cells at specific positions in the cell cycle (24). Upon reversal of the inhibitory conditions cells resume cycling in a more or less synchronous manner. Cell synchrony can sometimes be achieved without perturbing the cell cycle, as, for example, in the method of selective mitotic detachment, but the yield of synchronous cells in this procedure is low, often limiting biochemical analysis.

An alternative technique, which avoids the use of cell cycle inhibitors and produces large numbers of synchronous cells, is the separation of cells by centrifugal elutriation. In this procedure cells suspended in growth medium are continuously fractionated by size in a specially designed centrifuge rotor. In the experiment shown in Fig. 3, exponentially growing MEL cells were separated by centrifugal elutriation into 14 fractions of increasing cell size. Figure 3 shows the DNA content, determined by flow microfluorimetric analy-

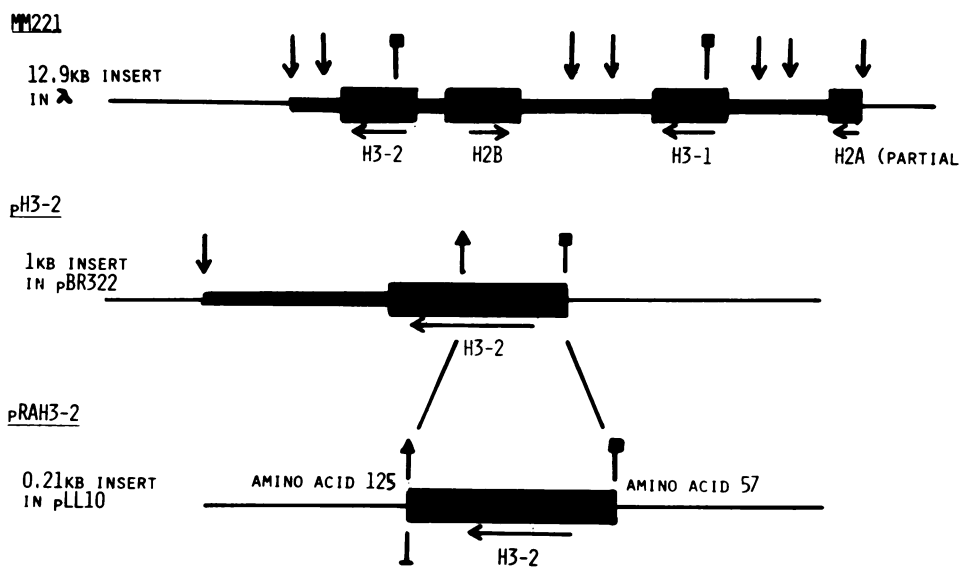

FIG. 1. Construction of recombinant clones containing mouse histone genes. See text for details. The histone gene sequences are indicated by the thick black boxes; flanking mouse genomic DNA, by the narrower black boxes; and plasmid and phage DNA, by lines. The direction of transcription (5′ to 3′) shown by the horizontal arrows was determined by sequencing portions of each gene (Sittman, personal communication). The cleavage sites for different restriction enzymes were determined by Sittman et al. (29): ↓, *Eco*RI; ⍑, *Sal*I; ↑, *Pvu*II; ⊥, *Sma*I. KB, Kilobases.

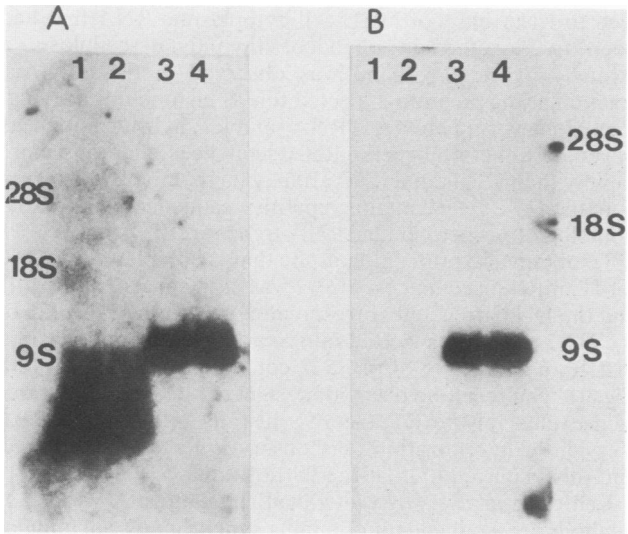

FIG. 2. Blot hybridization analysis of RNA with pH3-2 and pRAH3-2 probes. Poly(A^+) (lanes 1 and 2) and poly(A^-) (lanes 3 and 4) fractions from 20 μg of total cytoplasmic RNA were prepared as described in the text and fractionated on denaturing agarose gels. RNA blots were prepared and hybridized with recombinant plasmid DNAs made radioactive by nick translation. The positions of 28S and 18S rRNAs determined by ethidium bromide staining of the gels are indicated. (A) Autoradiogram of blot hybridized with pH3-2 containing H3-coding sequences and 3'-flanking mouse genomic DNA; (B) autoradiogram of blot hybridized with pRAH3-2 containing exclusively H3-coding sequences.

sis of cells present in alternate fractions. The first fractions from the elutriation contain cells with a 2C DNA content characteristic of G1 cells. As cells of increasing size are elutriated, the average DNA content per cell increases, approaching the 4C value characteristic of cells in late S, G2, and M. To determine quantitatively the degree of contamination of cells synthesizing DNA in the G1 and G2 populations, aliquots from the fractions were labeled with [^{3}H]thymidine for 15 min and the percent labeled nuclei was determined after autoradiography. This analysis showed that the earliest fractions (G1) were contaminated 10 to 20% with early S-phase cells, whereas the latest fractions (G2) contained about 20 to 30% cells synthesizing DNA (data not shown).

Analysis of histone mRNA content in the cell cycle. The level of H3 histone mRNA in elutriated cells at various positions in the cell cycle was determined by quantitative RNA blot experiments. Total cytoplasmic RNA, extracted from elutriated cells, was fractionated by electrophoresis in denaturing formaldehyde-agarose gels, blotted onto nitrocellulose, and hybridized with ^{32}P-labeled pRAH3-2 (Fig. 4). Marked differences were observed in the amount of hybridizable histone mRNA present in cell cycle-specific fractions. Small amounts of histone mRNA were detected in G1 cells (lane 1), probably due to the low level of contamination of these fractions with S-phase cells. Increasing amounts of histone mRNA were found in fractions containing cells with higher average C values, reaching a maximum in the middle of S phase (lanes 4 and 5). Histone mRNA content decreased dramatically thereafter as cells approached G2. Densitometric analysis of the results presented in Fig. 4. showed that the maximum histone mRNA content observed with cells in mid-S phase is 8 times that of cells in the G1 fraction and 12 times that of cells in the G2 fraction. Similar quantitative results were obtained in RNA dot blot experiments (data not shown). Several experiments of the type shown in Fig. 4 were performed. In most experiments the H3 histone mRNA content of cells in mid-S phase was about 10 times that of cells in the G1 and G2 fractions. Occasionally smaller differences were observed between mid-S and G2 fractions. In these instances the G2 fractions contained larger numbers of S-phase cells.

The periodic accumulation and decay of histone mRNA content in the cell cycle were compared with those of another abundant mRNA. RNA blots of total cytoplasmic RNA from several fractions obtained from a separate elutriation were hybridized simultaneously with pRAH3-2 and pL10, a cDNA clone homologous with the mRNA for mouse ribosomal protein L10 (20). The L10 mRNA migrates at about 900 nucleotides on denaturing gels behind the smaller H3 mRNA (Fig. 5A). Densitometric analysis (Fig. 5B) of the autoradiogram presented in Fig. 5A shows that, in contrast

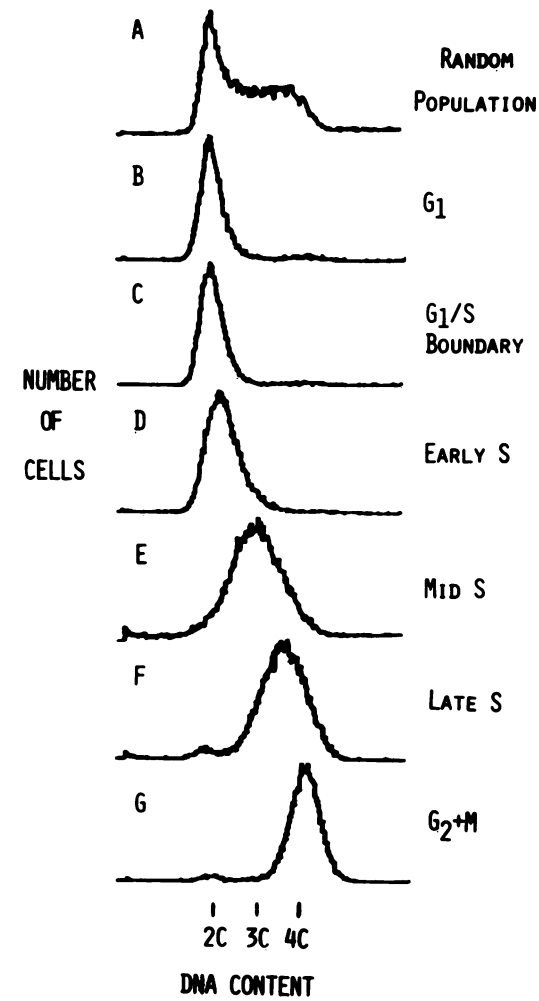

FIG. 3. DNA content of elutriated cell cycle fractions. Exponentially growing MEL cells were separated by centrifugal elutriation into 14 fractions of increasing cell size. The average DNA content of cells from each alternate fraction was determined by flow microfluorimetric analysis after staining with propidium iodide. The results for alternate fractions are shown in (B) through (G). The ordinate is the number of cells having the DNA content given on the abscissa. (C) is the haploid DNA content of MEL cells in G1. The flow microfluorimetric profile in (A) represents the exponential culture before elutriation.

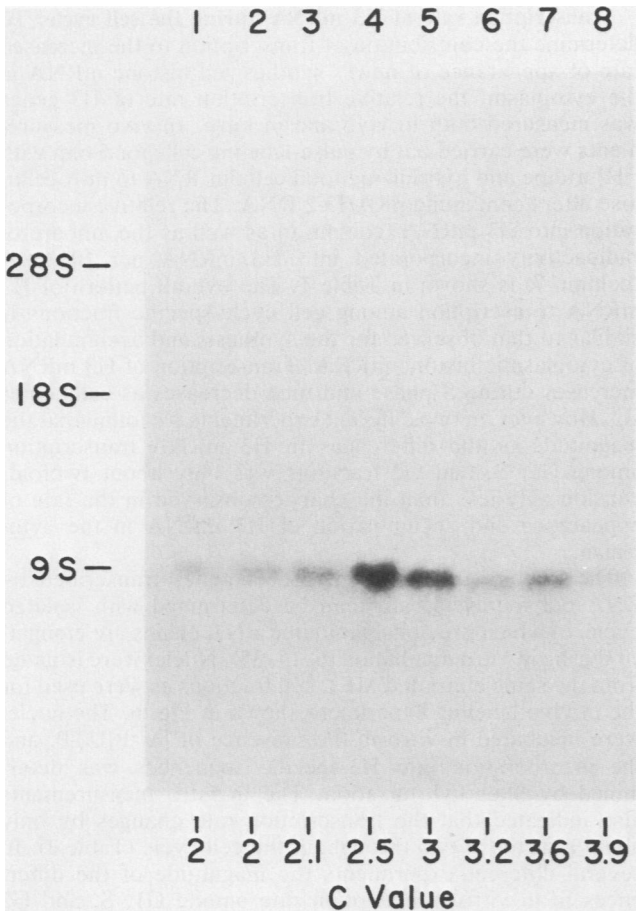

FIG. 4. Periodic accumulation and decay of H3 histone mRNA content in the cell cycle. A 20-μg portion of total cytoplasmic RNA from eight elutriated cell fractions was analyzed by blot hybridization as in Fig. 2B, using labeled pRAH3-2 DNA as a probe. The average DNA content of cells in each fraction is indicated at the bottom.

to the marked changes observed in H3 mRNA content, the amount of L10 mRNA is almost constant throughout the cell cycle.

Rate of appearance of newly synthesized histone mRNA in the cytoplasm. The steady-state level of an mRNA in the cytoplasm is determined by several parameters, including the rate of transcription, the efficiency of nuclear post-transcriptional processes and transport, and the rate of degradation. The rate of appearance of newly synthesized mRNA in the cytoplasm can be measured by carrying out pulse-labeling experiments, providing that the duration of the pulse is short compared with the mRNA half-life. Our measurements presented below indicate that H3 mRNA turns over with a half-life of no less than 30 min.

To determine whether the periodic accumulation of H3 histone mRNA was paralleled by changes in mRNA synthesis, elutriated MEL cell fractions were labeled for 15 and 60 min with [^{3}H]uridine. Total cytoplasmic RNA was isolated and separated into poly(A$^+$) and poly(A$^-$) fractions. The proportion of radioactivity incorporated into H3 mRNA sequences was determined by hybridization with nitrocellulose filters containing pRAH3-2 DNA. No hybridization above nonspecific binding was observed with poly(A$^+$) RNAs. The relative incorporation of [^{3}H]uridine into H3 mRNA was corrected for changes in the total radioactivity incorporated into cytoplasmic RNA among the various cell cycle-specific fractions (Fig. 6B). As expected from previous work (22), there is a gradual increase in the rate of total cytoplasmic RNA synthesis as cells duplicate their DNA during S phase. That the actual rate of RNA synthesis is correctly represented by the incorporation of radioactivity was shown by measuring the UTP pool specific activity (S. Ganguly and A. I. Skoultchi, manuscript in preparation). These measurements indicated that the rate of labeling of the UTP pool is relatively constant throughout the cell cycle (data not shown).

The amount of radioactivity incorporated into H3 mRNA per 10^6 cells was found to exhibit a temporal pattern very similar to that seen in steady-state measurements (Fig. 6A).

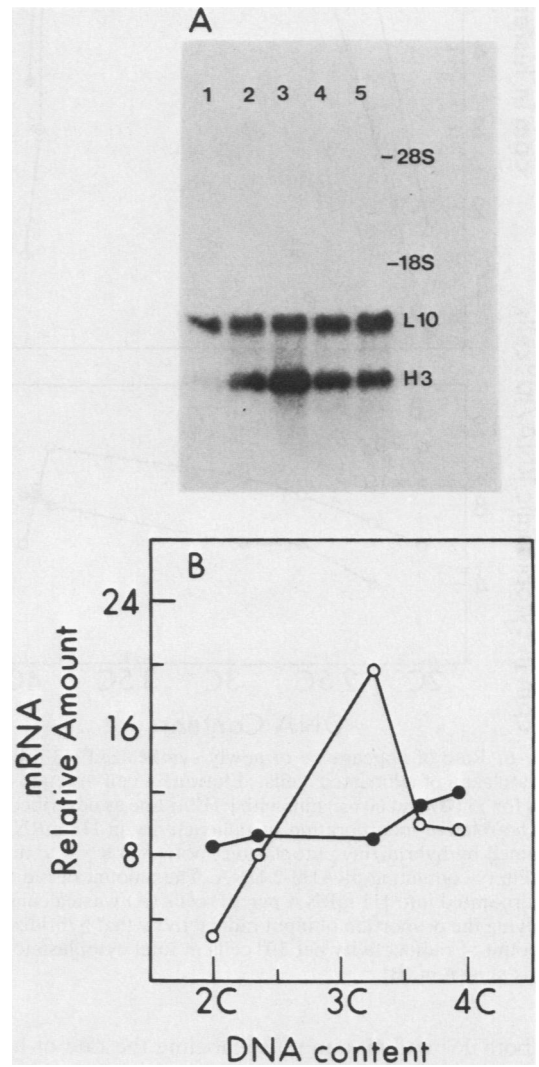

FIG. 5. Comparison of H3 and ribosomal protein L10 mRNA levels in elutriated cells. A 10-μg portion of total cytoplasmic RNA from elutriated cell fractions was analyzed by blot hybridization. (A) Autoradiogram of a blot hybridized simultaneously with a mixture of radioactive pRAH3-2 and pL10 DNAs. (B) The autoradiogram in (A) was quantitated by densitometry to determine the relative levels of H3 (○) and L10 (●) mRNAs. In this experiment the G2 fraction contained about 40% cells synthesizing DNA, accounting for the higher level of H3 mRNA in this fraction compared with other experiments.

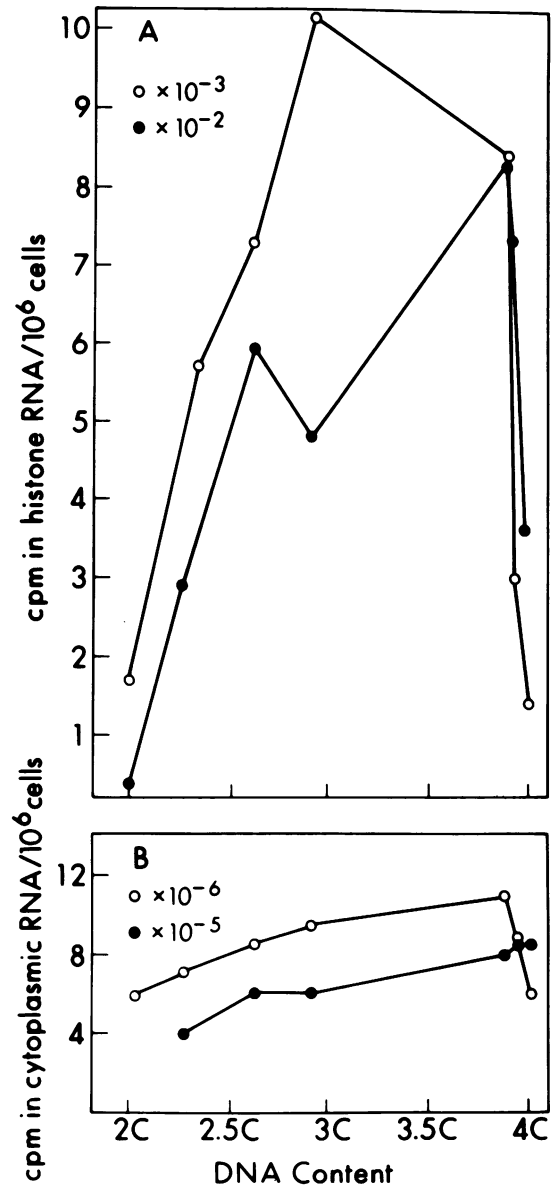

FIG. 6. Rate of appearance of newly synthesized H3 mRNA in the cytoplasm of elutriated cells. Elutriated cell fractions were labeled for 15 (●) and 60 (○) min with [^{3}H]uridine as described in the text. The relative incorporation of radioactivity in H3 mRNA was determined by hybridizing cytoplasmic, poly(A$^-$) RNA to nitrocellulose filters containing pRAH3-2 DNA. The amount of radioactivity incorporated into H3 mRNA per 10^6 cells (A) was calculated by multiplying the proportion of input radioactivity that hybridized and the amount of radioactivity per 10^6 cells in total cytoplasmic RNA, which is shown in (B).

With both 15- and 60-min pulse-labeling the rate of histone mRNA synthesis was seen to rise sharply during the transition from G1 to S phase and then fall as cells approached G2. These results are consistent with the view that the periodic accumulation and decay of histone mRNA during S phase are controlled primarily by the rate with which newly synthesized histone mRNA transcripts appear in the cytoplasm. However, they do not exclude the possibility that histone mRNA is synthesized throughout the cell cycle and very rapidly degraded upon entering the cytoplasm of cells not engaged in DNA synthesis.

Transcription rate of H3 mRNA during the cell cycle. To determine the contribution of transcription to the increased rate of appearance of newly synthesized histone mRNA in the cytoplasm, the relative transcription rate of H3 genes was measured both in vivo and in vitro. In vivo measurements were carried out by pulse-labeling cells for 5 min with [^{3}H]uridine and hybridizing total cellular RNA to nitrocellulose filters containing pRAH3-2 DNA. The relative incorporation into H3 mRNA (column 6) as well as the amount of radioactivity incorporated into H3 mRNA per 10^6 cells (column 7) is shown in Table 1. The overall pattern of H3 mRNA transcription among cell cycle-specific fractions is similar to that observed for the synthesis and accumulation of cytoplasmic histone mRNA. Transcription of H3 mRNA increases during S phase and then decreases as cells enter G2. However, in two different experiments we found that the magnitude of the differences in H3 mRNA transcription among G1, S, and G2 fractions was only about twofold, considerably less than the changes observed in the rate of appearance and accumulation of H3 mRNA in the cytoplasm.

The relative transcription rate of genes transcribed by RNA polymerase II also can be determined with isolated nuclei in which previously initiated RNA chains are elongated during in vitro incubation (6, 15, 35). Nuclei were isolated from the same elutriated MEL cell fractions as were used for the in vivo labeling experiment shown in Fig. 6. The nuclei were incubated in vitro in the presence of [α^{32}P]UTP, and the incorporation into H3-specific sequences was determined by filter hybridization. The in vitro measurements also indicated that the transcription rate changes by only about a factor of two throughout the cell cycle (Table 2). In several different experiments the magnitude of the differences in in vitro transcription rate among G1, S, and G2 fractions was only 1.5- to 2.5-fold. Together the two types of transcription measurements suggest that differences in transcription rate do not fully account for the periodic accumulation and decay of histone mRNA in the cytoplasm.

Turnover of H3 histone mRNA in the cell cycle. The results presented above suggest that differential transcription makes only a partial contribution to the regulation of histone mRNA content in the cell cycle. We sought to determine whether the accumulation and decay of histone mRNA in the cytoplasm might also be controlled by changes in mRNA stability. H3 histone mRNA turnover was measured by pulse-chase experiments in which cells were labeled for 1 h with [^{3}H]uridine and then chased with a great excess of unlabeled uridine and cytidine. The effectiveness of the chase procedure was evaluated by measuring the total incorporated radioactivity per cell. These values did not increase above the 1-h incorporation level at early times in the chase. In contrast, continuously labeled cultures exhibited linearly increasing incorporation for several hours (data not shown).

In one experiment (Fig. 7A) early S-phase cells were labeled for 1 h. At the time when the chase was begun these cells had progressed nearly to mid-S phase (C value = 2.8). Thus, in this experiment the measurements of histone mRNA turnover were initiated in cells with a maximal histone mRNA content and were followed to the point where the cells had reduced their level of histone mRNA in G2. In a second experiment (Fig. 7B) cells in late S phase were labeled for 1 h and the chase was begun as the cells entered G2. These cells maintained a 4C value throughout the chase period, indicating a failure to undergo cell division. We believe that the inhibition of cell division is due to exposure

to high concentrations of [^{3}H]uridine or unlabeled uridine and cytidine, because in the absence of this treatment these cells undergo synchronous cell division. Thus, in this second experiment the turnover of histone mRNA synthesized in late S phase was measured in cells primarily in G2. Although histone mRNA turnover was measured in cells that had ceased DNA synthesis, we cannot be certain that these cells exhibit all properties of a normal G2 period because they did not divide.

The results presented in Fig. 7 indicate that in both S and G2 phases the turnover of prelabeled H3 histone mRNA is best represented by a two-component decay curve. About 50% of the H3 mRNA synthesized in early S phase decays during S with a half-life of 25 min, whereas the more stable component has a calculated half-life of 92 min. Similar values of 32 and 108 min, respectively, were obtained for the two components in G2 cells. Interestingly, the less stable component appears to constitute a greater proportion, at least 80%, of the H3 histone mRNA in G2 cells. Although the significance of the two components observed in both types of cells remains to be determined, the results do not provide evidence for substantial differences in the half-lives of H3 mRNA between cells in the S and G2 phases of the cycle.

DISCUSSION

The results reported here are consistent with the conventional view that histones are synthesized periodically in the mammalian cell cycle during the time of DNA replication. Using a cloned mouse H3 histone gene probe to assay histone mRNA content in elutriated cell cycle-specific fractions, we found that the steady-state level of H3 mRNA gradually increases as cells begin DNA synthesis, reaching a maximum value in the middle of S period, and then decreases as cells approach G2. The periodic accumulation and decay of H3 mRNA was contrasted with the behavior of the mRNA for the ribosomal protein L10, which was found to be present at a relatively constant level throughout the cell cycle. In several different experiments the maximum histone mRNA content observed with cells in mid-S phase was about 10 times that of G1 and G2 fractions. This estimate is likely to be a minimum value because the G1 and G2 fractions obtained by centrifugal elutriation do contain some cells that are synthesizing DNA as judged by autoradiography after [^{3}H]thymidine labeling. Most of the contaminating cells probably lie near the G1-S and S-G2 boundaries, respectively. It is likely that the G1 and G2 cells contain only very small amounts of H3 histone mRNA. In fact, a low level of histone mRNA is expected to be present in G1 and G2 phases because cells in these stages have been shown to synthesize small amounts of specific histone variants that constitute basal histone production persisting throughout the cell cycle (35). Our results agree with recent reports (10, 23, 25) showing much higher levels of histone mRNA in S-phase HeLa cells than in G1 cells. They are not consistent with the findings of Groppi and Coffino (9), who reported equivalent rates of histone protein synthesis in G1 and S phases of two other rodent cell lines. At the present time we cannot explain the discrepancy between these two sets of experiments. It may be that different cultured cell lines regulate histone gene expression in the cell cycle to varying extents.

Having established that S-phase MEL cells contain much larger amounts of H3 histone mRNA than G1 or G2 cells, we investigated how this periodic accumulation and decay is controlled by studying the synthesis of histone mRNA in whole cells and in isolated nuclei. To do this we isolated an H3 gene probe (pRAH3-2) that contains coding sequences exclusively. This was necessary because we found that a histone gene clone including DNA sequences 3′ to the H3 gene (Fig. 1) also hybridized with heterodisperse, low-molecular-weight RNA transcripts that chromatographed with poly(A$^+$) RNA on oligodeoxythymidylate-cellulose. This clone also hybridized about twice as much pulse-labeled cytoplasmic RNA as that obtained with pRAH3-2, suggesting that the small RNA transcripts are as abundant as H3 mRNA in newly synthesized RNA. These properties are reminiscent of other cellular RNAs that have been described as having partial homology to *Alu*-like repetitive DNA sequences (17). Since the multiple H3 genes known to exist in the mouse genome differ very little in their coding sequences (D. Sittman et al., personal communication), the pRAH3-2 probe used in our studies is expected to hybridize with most or all H3 gene transcripts. Thus, the metabolism of H3 mRNA reported here is an average of these multiple H3 gene products.

The rate of appearance of newly synthesized H3 mRNA in the cytoplasm, determined by pulse-labeling whole cells with [^{3}H]uridine, was found to follow closely the pattern observed for histone mRNA content among cells at various stages of the cycle. The maximum rate of histone mRNA synthesis, which occurred in the middle of the S period, was

TABLE 1. Relative in vivo transcription rate of H3 histone mRNA[a]

Cell cycle fraction (C value)	cpm (×10^6) in total RNA/10^6 cells	Hybridization input (cpm × 10^6)	cpm bound[b]		H3 mRNA hybridized (avg ppm)[c]	Avg cpm in H3 mRNA/10^6 cells[d]
			pLL10	pRAH3-2		
2.1	2.1	3.0	48	201	47	99
		6.0	100	355		
2.2	2.3	3.0	42	237	58	134
		6.0	98	410		
2.3	2.1	3.0	45	214	64	134
		6.0	116	541		
2.5	2.9	3.0	42	252	68	196
		6.0	94	481		
3.3	3.1	3.0	50	168	38	118
		6.0	74	304		
4.0	3.5	3.0	30	119	30	105
		6.0	64	245		

[a] Elutriated cell fractions were labeled for 5 min with [^{3}H]uridine, and total cell RNA was isolated by the hot-phenol method.
[b] Values represent the average of two separate hybridization reactions.
[c] Parts per million of input radioactivity hybridized.
[d] Calculated by multiplying the value in column 2 by that in column 6.

TABLE 2. Relative in vitro transcription of H3 genes[a]

Cell cycle fraction (C value)	cpm ($\times 10^6$) in total RNA/10^6 nuclei	Hybridization input (cpm $\times$ 10^6)	cpm bound[b]		H3 mRNA hybridized (avg ppm)[c]	Avg cpm in H3 mRNA/10^6 nuclei[d]
			pLL10	pRAH3-2		
2.0	0.71	1.1	24	193	144	102
		2.2	27	321		
2.2	0.99	1.1	19	124	99	98
		2.2	20	245		
2.6	0.68	1.1	19	236	201	137
		2.2	24	478		
2.9	1.2	1.1	20	258	190	228
		2.2	20	382		
3.8	1.2	1.2	18	263	194	233
		2.4	24	465		
3.9	1.1	1.1	23	186	151	166
		2.2	34	372		
4.0	1.1	1.2	20	128	93	102
		2.4	25	256		

[a] Nuclei isolated from elutriated cell fractions were labeled for 60 min with [α-^{32}P]UTP, and nuclear RNA was isolated by the hot-phenol method.

[b,c,d] See footnotes *b*, *c*, and *d*, Table 1.

achieved by a gradual increase during early S and decreased precipitously as cells approached G2. A similar pattern also was seen by measuring transcription with a 5-min [^{3}H]uridine pulse of whole cells and by in vitro transcription in isolated nuclei. However, in several experiments with both methods, we consistently found that the maximum rate of H3 mRNA transcription in S phase was only 1.5- to 2.5-fold greater than in G1 or G2. Taken together, the results indicate that transcription of H3 genes occurs periodically in the cell cycle, but that differences in transcription rate do not fully account for the periodic accumulation and decay of histone mRNA in the cytoplasm. The results suggest that post-transcriptional processes also make a major contribution to the control of histone mRNA levels in cycling cells.

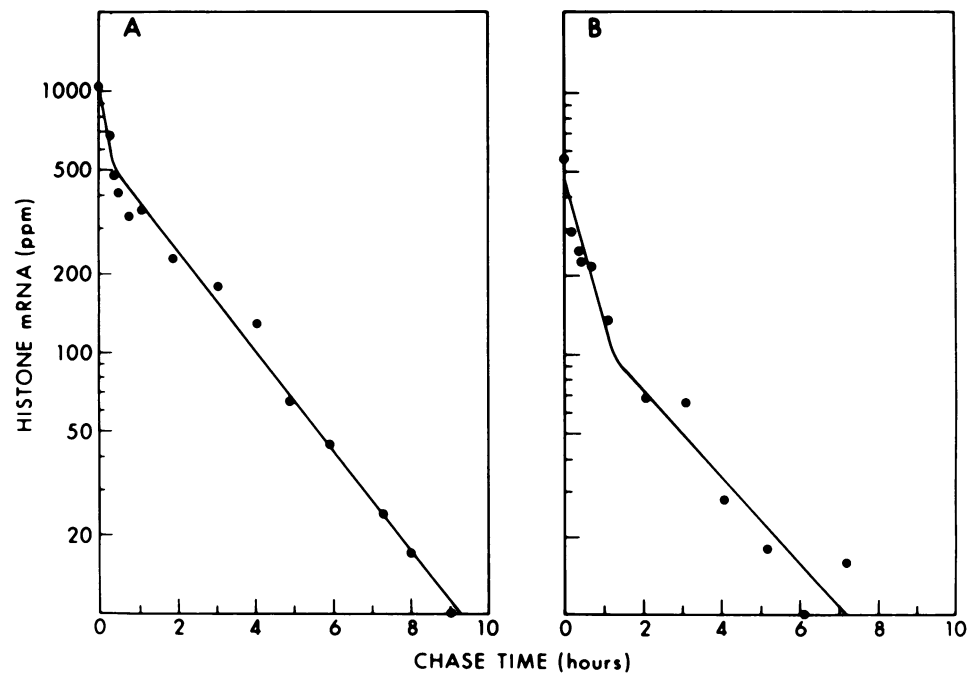

FIG. 7. Turnover of prelabeled H3 mRNA in S and G2 phases. Early S-phase (A) and late S-phase (B) cells with average DNA contents of 2.1 and 3.2C, respectively, were collected by centrifugal elutriation. The cells were labeled for 1 h with [^{3}H]uridine (10 μM) as described, washed, resuspended in 30% conditioned growth medium supplemented with 5 mM unlabeled uridine and 2 mM unlabeled cytidine according to Volloch and Housman (34), and further incubated at 37°C. At the indicated times, aliquots were removed from the cultures and the total incorporated radioactivity per cell was determined. Total cytoplasmic RNA was prepared as described in the text, and the proportion of radioactivity in H3 mRNA was measured by nitrocellulose filter hybridization. These values were plotted on a logarithmic scale as a function of chase time. In each graph a smooth curve was drawn based upon two straight lines determined by separate linear regressions of the data points before and after 1 h. The calculated half-lives described by these two lines were 25 and 92 (A) and 32 and 108 (B) min. At the beginning of the chase period, cells in the experiment shown in (A) had an average DNA content of 2.8C. By 7 to 8 h of incubation they achieved a 4C DNA value. Cells in the experiment shown in (B) had a 4C DNA content at the beginning of the chase and this value remained constant throughout the chase period.

Post-transcriptional control of histone synthesis in mammalian cells was suggested much earlier from studies in which DNA synthesis in S phase was interrupted by treatment with hydroxyurea or cytosine arabinoside (2, 4). Two recent studies in which cloned histone genes were used to directly measure histone mRNA levels showed that treatment with these inhibitors (30) or aphidicolin (10) leads to a very rapid loss of histone mRNA, at a rate much greater than the normal decay time. Similar results were obtained by using a yeast mutant that is temperature sensitive in DNA chain elongation (13). We attempted to determine whether changes in cytoplasmic H3 mRNA half-life might be responsible for the decreased levels of H3 mRNA in late S phase and G2. This was an attractive possibility because of the relatively high rate of H3 RNA transcription at these times. Pulse-chase experiments indicated the presence of two decay components in the H3 mRNA population. The more rapidly decaying component had a half-life of about 30 min, similar to that measured recently in HeLa cells by a different method (10). The second component decayed with a half-life of about 100 min. At present we do not understand the significance of these two components. We do know that H3 mRNAs are produced by transcription of multiple H3 genes; there are at least 10 to 20 H3 gene copies in the mouse genome and some of these certainly differ in their noncoding sequences (Sittman et al., personal communication). Therefore, it is possible that the two decay components represent two or more different H3 gene products that cross-react in the filter hybridization assay. Regardless of the interpretation, both components were observed in mid-S phase and in G2. Furthermore, the half-lives of H3 mRNA in G2 were not substantially different from that in mid-S phase. Thus, these pulse-chase studies failed to provide any evidence for changes in decay rates of cytoplasmic histone mRNA during the normal decrease in DNA synthetic rate that occurs as cells approach and enter G2. It may be that such changes occur only during periods of abrupt change in the rate of DNA synthesis or are due to a pleiotropic effect on another metabolic process also affected by the chemicals used to inhibit DNA synthesis. Recently, Sittman et al. (30) showed that histone mRNA levels are rapidly reduced by treatment with hydroxyurea even in G1 lymphoma cells that are not synthesizing DNA. This result suggests that this inhibitor can indeed affect histone mRNA metabolism via a mechanism that is independent of DNA synthesis.

The cell cycle regulation of histone gene expression has been most thoroughly studied in yeasts by Hereford and colleagues. By studying the in vivo synthesis and accumulation of H2A and H2B mRNAs in yeast strains that carry cell cycle mutations, they concluded that histone gene expresion is regulated by a combination of transcriptional and post-transcriptional mechanisms (12, 13). They suggest that histone gene transcription is activated in G1 and ceases early in S. The argument is based in part on finding that the maximal rate of histone mRNA synthesis occurs earlier in the cell cycle than the time of maximal mRNA accumulation and DNA synthetic rate. The data further suggest that the periodic accumulation and decay of histone mRNA are also regulated by changes in its half-life. Although we have studied the synthesis of a different histone mRNA, it seems likely that the regulation of core histones should be similar. Our results support the notion that both periodic transcription and a post-transcriptional mechanism are involved. However, we did not observe a temporal separation in the peaks of histone mRNA synthesis and accumulation and DNA synthesis, which all appear to occur in mid-S phase. Moreover, the in vivo and in vitro transcription measurements reported here indicate that histone transcription in mammalian cells occurs in G1 and G2 as well as throughout S. The data suggest that histone transcription is modulated quantitatively during the time of DNA replication, rather than undergoing distinct activation and termination events early in the cell cycle as suggested in yeasts. Finally, in pulse-chase experiments we failed to observe a difference in cytoplasmic H3 mRNA half-lives between cells in S and G2. This suggests that the post-transcriptional processes that appear to be necessary for regulating histone mRNA levels during the mammalian cell cycle operate within the nucleus rather than in the cytoplasm. Because histone mRNAs do not undergo RNA splicing or polyadenylation, the control mechanism may involve nuclear mRNA turnover or transport.

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