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Regulation of Histone mRNA Production and Stability in Serum-Stimulated Mouse 3T6 Fibroblasts

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We measured the content and metabolism of histone mRNA in mouse 3T6 fibroblasts during a serum-induced transition from the resting to growing state. The content of several histone H3 and H2b mRNAs was measured by an S1 nuclease procedure. All of these increase in parallel by a factor of about 50 during S phase. However, the rate of H3 gene transcription increased only fivefold during this period, as determined in an in vitro transcription assay. This suggests that histone mRNA content is also controlled at the posttranscriptional level. When resting cells were serum stimulated in the presence of cytosine arabinoside, the rate of H3 gene transcription increased to about the same extent as that in control-stimulated cells. However, cytoplasmic H3 mRNA content increased only five to seven-fold. The half-life of H3 mRNA during S phase was about 4 to 5 h. When cytosine arabinoside was added to cells in the S phase, the half-life of the message decreased to about 15 min. The rapid turnover of H3 mRNA was prevented when the drug was added in the presence of cycloheximide or puromycin. The rate of H3 gene transcription decreased by only 35% after treatment with cytosine arabinoside. These results suggest that H3 gene transcription is not tightly coupled to DNA replication but is controlled temporally during the resting to growing transition. However, there is a correlation between the rate of DNA synthesis and the stability of histone H3 mRNA.

It has been known for many years that histones are incorporated into chromatin primarily during DNA replication (5, 10, 12). There are several classes of histone proteins. The replication variants, whose synthesis is correlated with DNA synthesis, are predominant in cultured cells (29). A second class of histone proteins, the replacement variants, differ in only a few amino acids from the replication histone proteins. These are predominant in nondividing cells in differentiated tissue (8), but are also present in rapidly growing cells at low levels (29).

Detailed analysis of the biochemical mechanisms responsible for controlling histone gene expression have been facilitated by the molecular cloning of the genes for histones from a variety of organisms. These clones have been used as hybridization probes to study the content and metabolism of the various histone mRNA species and the regulation of histone gene transcription. For example, in HeLa cells and in yeasts, the amount and rate of synthesis of histone mRNA increase greatly between G1 phase and S phase (13, 15, 23). However, in S49 mouse lymphoma cells selected by centrifugal elutriation, histones are synthesized at a similar rate in both G1 and S phases (11). In exponentially growing cultured cells, the steady-state levels of mRNAs for the replication variants (but not the replacement variants) are rapidly decreased in response to inhibition of DNA synthesis. This is the result of decreases in both the rate of transcription and the stability of the mRNAs (13, 25).

Cultured mouse 3T6 fibroblasts (26) provide an excellent system for cell cycle studies. They can be arrested for prolonged periods in the G0 state by growth in 0.5% serum and then stimulated to reenter the cell cycle by increasing the serum concentration to 10% (16). DNA synthesis occurs at very low rates until about 10 to 12 h after serum stimulation. This prolonged G0/G1 phase permits a detailed analysis of the biochemical events that are required for reentry into S phase. Previously, we have studied the regulation of a number of enzymes related to DNA replication in this model system, including dihydrofolate reductase (14, 17, 28), thymidylate synthetase (22), and thymidine kinase (18).

Here we report that the levels of the replication variant histone mRNAs are regulated during the transition from the resting to growing state in

mouse 3T6 cells. The steady-state levels of the replication variant histone mRNAs increased 50-fold in parallel with the increase in the rate of DNA synthesis. However, the rate of transcription of the H3 gene, as measured in isolated nuclei, increased only about fivefold. The increase in transcription rate was observed even when DNA synthesis was prevented, although the content of H3 mRNA increased only about five-to-sevenfold. Since there does not appear to be a close correlation between changes in the rate of transcription of the H3 gene and changes in H3 mRNA content under these various conditions, we infer that there is also regulation at a posttranscriptional step, probably at the level of mRNA degradation. Although inhibition of DNA synthesis has only a minor effect on the rate of H3 gene transcription in 3T6 cells, it has a major effect on H3 mRNA stability. The half-life of H3 mRNA is several hours during S phase. When DNA synthesis is blocked during S phase, the half-life of H3 mRNA decreases to about 15 min.

MATERIALS AND METHODS

Cell cultures. Cultures of mouse 3T6 fibroblasts (26) were maintained on plastic petri dishes in the Dulbecco-Vogt modification of Eagle medium (GIBCO Diagnostics) supplemented with 10% calf serum (Colorado Serum). Exponentially growing cells were seeded at low density and fed on the day before use. Cells were about half confluent at the time of the experiment. Resting cells were prepared by seeding cells at a density of 7×10^4 cells per cm^2 in medium containing 0.5% calf serum. Resting cultures were fed on days 2 and 4 after seeding and used for an experiment on day 7. Resting cultures were stimulated to reenter the cell cycle by feeding them with fresh medium containing 10% calf serum (16).

DNA synthesis rate. The rate of DNA synthesis ([^{3}H]-thymidine incorporation) and the percentage of nuclei labeled with [^{3}H]-thymidine were determined as described (C. L. Santiago, M. L. Collins, and L. F. Johnson, J. Cell. Physiol., in press).

Isolation of cytoplasmic RNA. Cells were washed with isotonic saline, lysed with Nonidet P-40, and subjected to low-speed centrifugation to remove nuclei and cellular debris, as described previously (16). RNA was isolated from the cytoplasmic compartment by phenol-chloroform extraction at room temperature essentially as described previously (16). Polyadenylic acid-containing RNA species were separated from those that lacked polyadenylic acid by chromatography on oligodeoxythymidylic acid cellulose (2, 3).

Determination of histone mRNA content. The steady-state levels of histone mRNA were measured with specific probes prepared from the histone gene cluster MM 221 (24). The coding region of each gene was cut with a restriction enzyme, and the fragment was labeled with polynucleotide kinase and [δ-^{32}P]ATP. Labeled DNA (10 to 70 fmol) was hybridized with cytoplasmic RNA (0.5 to 10 μg) by the procedure of Weaver and Weissman (27). Hybridization was performed in 5 μl of 0.4 M NaCl, 1 mM EDTA, 50 mM PIPES (piperazine-*N*-*N*'-bis[2-ethanesulfonic acid]) (pH 6.4), and 80% formamide for 3 h at 58°C, as described previously (25). The fragments are described in the figure legends. Each gene has been completely sequenced (D. B. Sittman, R. A. Graves, and W. F. Marzluff, unpublished data).

After hybridization, the samples were diluted with 10 volumes of 30 mM sodium acetate (pH 4.5)–0.25 M NaCl–2 mM $ZnSO_4$–20 μg of denatured *Escherichia coli* DNA. The hybrids were then treated with 1,000 U of S1 nuclease per ml at 25°C for 1 h. The S1-resistant material was recovered by precipitation with ethanol in the presence of carrier tRNA. The hybrids were dissolved in 0.1 M NaOH–95% formamide and analyzed by electrophoresis on a 6% polyacrylamide gel in 7 M urea. The size standard was pBR322 digested with *Hin*fI and end labeled with polynucleotide kinase. The amount of protected DNA, which was proportional to input RNA, was quantitated by scanning the autoradiogram with a densitometer.

Isolation of nuclei. Nuclei were prepared and transcribed in vitro as described previously (Santiago et al., in press). Briefly, cultures of cells on 100-mm petri dishes were washed extensively with ice-cold phosphate-buffered saline. Transcription buffer (50 mM Tris-hydrochloride [pH 7.8], 5 mM $MgCl_2$, 6 mM KCl, 75 mM $(NH_4)_2SO_4$, 0.2 M sucrose) containing 0.5% Nonidet P-40 was added, and the cells were scraped off the dishes with a rubber policeman. Cells were homogenized with a Teflon homogenizer, layered on a 5-ml sucrose cushion (0.88 M sucrose in transcription buffer), and centrifuged at $1,000 \times g$ for 5 min. The nuclear pellet was suspended in 0.46 ml of transcription buffer containing 25% glycerol, quickly frozen in liquid nitrogen, and stored at −80°C.

In vitro transcription and RNA isolation. Nuclei were incubated in a 0.5-ml volume containing 50 μCi of [^{3}H]UTP (ICN; 40 Ci/mmol), 5 mM ATP, 0.2 mM CTP, 0.2 mM GTP, 2.5 mM $MnCl_2$, 1 mM dithiothreitol and 150 μg of heparin per ml at 25°C. Heparin was included to inhibit nucleases and to prevent the reinitiation of transcription (7). The reaction was stopped by the addition of 0.5 ml of ice-cold 0.5 M NaCl–0.05 M $MgCl_2$–0.01 M Tris-hydrochloride (pH 7.4). The mixture was treated with 20 μg of DNase I (Worthington Diagnostics) at 37°C for 30 s to fragment the DNA; 0.25 mg of tRNA and 2 volumes of 95% ethanol were added, and the mixture was kept at −20°C for at least 1 h. The precipitate was then centrifuged at $10,000 \times g$ in a Sorvall HS-4 rotor for 30 min at −20°C. The pellet was resuspended in 1.5 ml of sodium dodecyl sulfate buffer (0.1 M NaCl, 0.01 M Tris-hydrochloride [pH 7.4], 0.001 M EDTA, 0.5% sodium dodecyl sulfate) and treated with 0.1 mg of pronase per ml for 1 h at 37°C. The volume was brought to 2.5 ml with sodium dodecyl sulfate buffer, and the mixture was phenol-chloroform extracted and ethanol precipitated as described above. After centrifugation, the pellet was dissolved in 0.25 ml of 10 mM Tris-hydrochloride (pH 7.4)–1 mM EDTA and fractionated on a Sephadex G-100 column. Fractions (1 ml each) were collected, and those containing high-molecular-weight RNA were pooled, brought to 0.2 M NaCl, and precipitated with 2 volumes of ethanol. After centrifugation, the RNA was dissolved in 0.3 M NaCl–0.03 M sodium citrate (pH 7.4) for hybridization.

Control experiments showed that the relative

amount of histone RNA synthesized in isolated nuclei was the same (within experimental error) when incubation times ranging from 10 to 55 min were used. To maximize the amount of incorporation into histone RNA, a 55-min transcription period was used in all subsequent experiments.

DNA-excess filter hybridization. The recombinant DNA plasmid pMH3.1 was constructed by cloning a 2.4-kilobase *Eco*RI fragment containing the entire mouse histone H3.1 gene and flanking sequences into pACYC184 (24, 25). The plasmid lacks repetitive mouse DNA. Large quantities of plasmid were prepared by using an alkaline-sodium dodecylsulfate procedure (4) which we modified for large-scale preparations. The conditions for DNA-excess filter hybridization have been described in detail previously (14, 28). A control filter containing 5 μg of pBR322 was included in each hybridization. In some experiments, a second control filter with 5 μg of a plasmid containing mouse *a*-actin cDNA (21) was also included in the hybridization vial. All work with recombinant DNA plasmids was conducted according to National Institutes of Health guidelines for recombinant DNA research in effect at the time of the experiments.

RESULTS

DNA synthesis and histone mRNA level in serum-stimulated cells. Figure 1 shows that when resting mouse 3T6 cells were serum stimulated to reenter the cell cycle, the rate of DNA synthesis began to increase at about 10 h, and reached a maximum about 18 h after serum addition. About 60% (and in other experiments, as many as 80%) of the cells were in S phase between 15 and 18 h after stimulation. In continuous labeling experiments, we usually observed that about 90% of the nuclei were labeled with [^{3}H]thymidine by 24 h after stimulation.

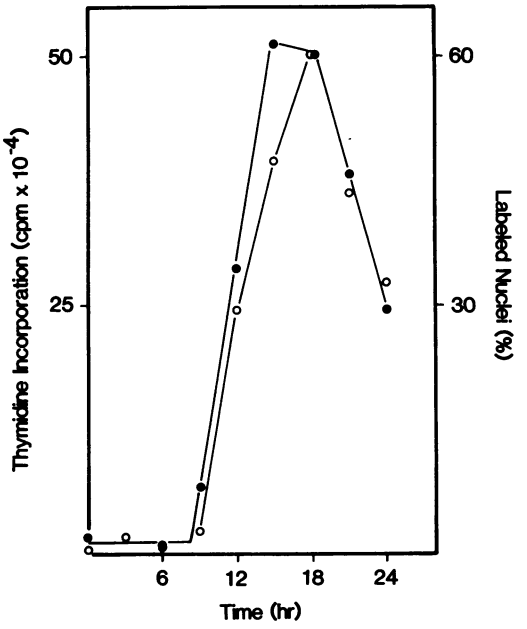

FIG. 1. Rate of DNA synthesis in serum-stimulated 3T6 cells. Cultures of resting 3T6 cells were serum stimulated at time zero. At the indicated times, cultures were exposed to [^{3}H]thymidine for 30 min. The rate of incorporation into trichloroacetic acid-insoluble material (○) (indicating the rate of DNA synthesis) or the percentage of labeled nuclei (●) (indicating the percentage of cells in S phase) were determined.

The content of several histone H3 and H2B mRNA species relative to total cellular RNA was determined at various times after stimulation by an S1 nuclease procedure. This procedure allows the simultaneous and unambiguous quantitation of several closely related messages in a single determination. Typical autoradiograms are shown in Fig. 2. The data were quantitated by densitometry, and the results are plotted in Fig. 3. We found that the content of all of the replication variant histone mRNAs began to increase at essentially the same time as the cells entered S phase and increased in parallel by a factor of about 50 by 18 h after stimulation. The amount of these messages decreased at later times as the cells completed S phase.

In vitro transcription of nuclei from serum-stimulated cells. Our subsequent studies focused on the expression of the histone H3 genes. The increase in the content of H3 mRNA could be controlled at the transcriptional or at the post-transcriptional level or both. To study the rate of transcription of the histone H3 gene, we measured the rate of synthesis of H3 RNA relative to total RNA in nuclei isolated from cells at various times after serum stimulation. Labeled H3 RNA was quantitated by DNA excess filter hybridization. This was normalized to the total amount of labeled RNA in the hybridization reaction to give the relative rate of transcription of the H3 gene. A typical result is shown in Table 1. Control experiments show that the filter hybridization assay is specific for the class of replication-variant histone H3 RNAs; the replacement-variant H3 RNAs do not hybridize under these conditions (W. F. Marzluff, unpublished data). The relative rate of transcription of the H3 gene increases about fivefold between 3 and 18 h after stimulation (Fig. 4). The increase begins about 10 h after stimulation, the same time that we observed the increase in H3 mRNA content.

All of our measurements of H3 gene transcription are expressed as relative rather than absolute rates. Consequently, a fivefold increase in the relative rate of H3 gene transcription could be due to a fivefold decrease in the rate of total gene transcription rather than a fivefold increase in H3 gene transcription. This possibility is unlikely and is not supported by previous studies which measured the rate of synthesis of RNA in nuclei isolated from serum-stimulated 3T6

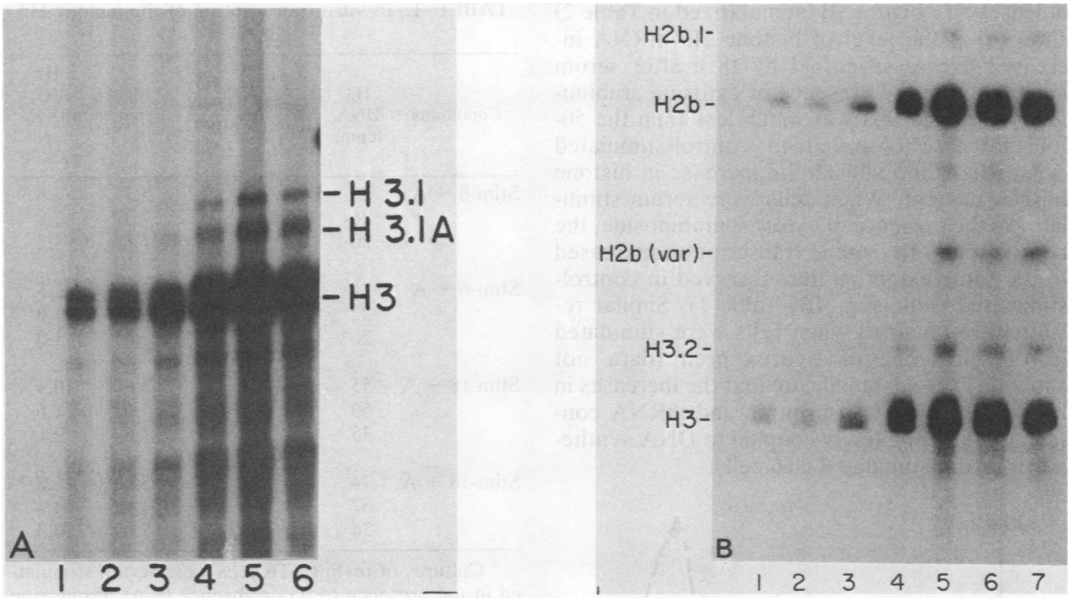

FIG. 2. Analysis of histone mRNA content. (A) Cytoplasmic RNA was prepared from cells which had been arrested by serum limitation and at various times after serum stimulation. The level of H3.1 mRNA was measured by S1 nuclease mapping with a mouse H3.1 gene labeled at the *Sal*I site at amino acid 58 as a probe. This was hybridized to 10 μg of RNA from each sample. The protected DNA fragments were resolved by polyacrylamide gel electrophoresis. The following bands were identified: H3, protection of the probe to the AUG codon by a family of mRNAs homologous to this gene; H3.1, protection to the start of the mRNA derived from this gene; and H3.1A, an RNA found so far only in mouse fibroblasts (unpublished data). Lanes: 1, serum-limited cells; 2, 4 h after serum stimulation; 3, 8 h after serum stimulation; 4, 12 h after serum stimulation; 5, 18 h after serum stimulation; 6, 24 h after serum stimulation. (B) The same RNA samples (10 μg) were hybridized with an *Ava*I-*Sal*I fragment containing the 5′ portion of both an H2b and an H3 gene. The H2b gene was labeled at the *Ava*I site at amino acid 92, and the H3 gene was labeled at the *Sal*I site at amino acid 58. The DNA fragments protected by S1 nuclease digestion are as follows: H3, the DNA protected by a family of H3 mRNAs up to the AUG codon; H3.2, DNA protected by the mRNA derived from this gene; H2b (var), DNA protected up to an amino acid substitution at amino acid 18 in this gene (D. B. Sittman, R. A. Graves, and W. F. Marzluff, submitted for publication); H2b, DNA protected by a family of H2b mRNAs up to the AUG codon; and H2b.1, DNA protected by the mRNA derived from this gene. Lanes 1 through 6 are as described in the legend to Fig. 2A. Lane 7 is RNA from exponentially growing cells.

cells. Mauck and Green (20) showed that the rate of synthesis of total heterogeneous nuclear RNA is constant for the first 12 h after serum stimulation and increases at later times in parallel with DNA content. When 3T6 cells are serum stimulated in the presence of cytosine arabinoside, the amount of DNA and the rate of synthesis of heterogeneous nuclear RNA remain constant for 40 h after stimulation (20). Therefore, it is likely that changes in the relative rate of H3 gene transcription are due primarily to changes in the rate of transcription of the H3 gene itself. Our results are consistent with the idea that an increase in the rate of histone H3 gene transcription is one of the major factors responsible for the increase in H3 mRNA content in serum-stimulated cells. However, since H3 mRNA content increases to a much greater extent than the rate of H3 gene transcription, it is likely that this is not the only level of regulation.

Coupling of histone gene expression and DNA replication. The increase in histone H3 mRNA content and gene transcription occur at approximately the same time as the cells enter S phase. It is possible that these may be tightly coupled to the rate of DNA synthesis. Alternatively, it is possible that these increase as a result of a timed program of gene expression during the G0 to S phase transition and that there is no obligatory coupling between histone H3 mRNA levels and the rate of DNA synthesis. To examine this, cells were stimulated with serum in the presence of 5 μg of cytosine arabinoside per ml. This concentration of drug inhibits DNA synthesis by

at least 95%. Figure 5B (summarized in Table 2) shows that the level of histone H3 mRNA increased five to sevenfold by 18 h after serum stimulation in the presence of cytosine arabinoside. Although this was much less than the 50-fold increase observed in control-stimulated cells, it is still a substantial increase in histone mRNA content. When cells were serum stimulated in the presence of cytosine arabinoside, the relative rate of H3 gene transcription increased to the same extent as that observed in control-stimulated cells (Fig. 4B, Table 1). Similar results were obtained when cells were stimulated in the presence of hydroxyurea (data not shown). These data indicate that the increases in histone H3 gene transcription and mRNA content are not completely coupled to DNA synthesis in serum-stimulated 3T6 cells.

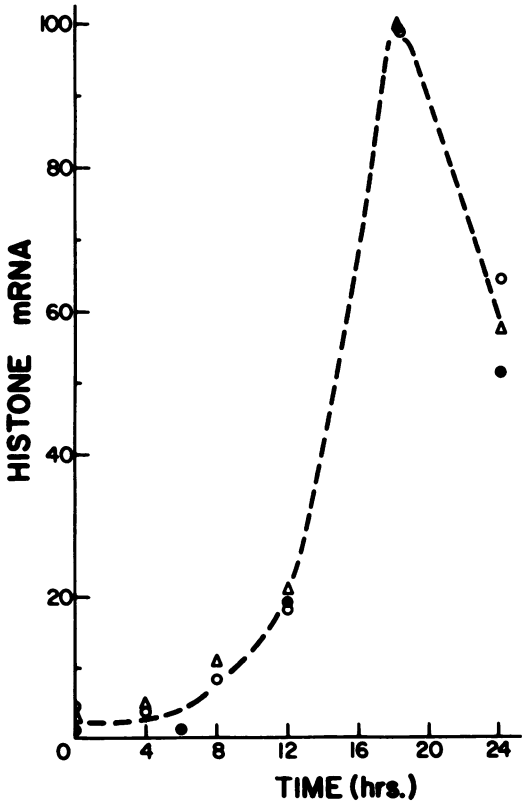

FIG. 3. Quantitation of histone mRNA levels after serum stimulation. The amount of histone mRNA was quantitated by densitometry after S1 nuclease mapping as shown in Fig. 2. To achieve a similar magnitude of signal from each RNA preparation, various amounts of RNA were used (10 μg from cells in G1 to 0.5 μg for cells in S phase). Results are expressed in arbitrary units relative to the 18 h value which was set at 100. Each point represents the average of three closely agreeing independent determinations on each RNA sample. Symbols: ○, H3.2 mRNA; ●, H3.1 mRNA; △, H2b.1 mRNA.

TABLE 1. In vitro transcription of the histone H3 gene[a]

Conditions	H3 DNA (cpm)	pBR322 (cpm)	H3 DNA-pBR322 (cpm)	Input (cpm × 10^{-5})[b]	H3 DNA-pBR322/Input (×10^5)[c]
Stim-6 − A	25	4	21	5.77	3.6
	26	5	21	7.39	2.8
	20	5	15	4.66	3.2
Stim-6 + A	24	6	18	7.08	2.5
	22	2	20	6.68	3.0
	26	3	23	7.67	3.0
Stim-18 − A	55	3	52	5.00	10.4
	60	1	59	5.07	11.6
	35	2	33	3.64	9.1
Stim-18 + A	74	3	71	5.53	12.8
	67	3	64	4.43	14.4
	74	1	73	5.09	14.3

[a] Cultures of resting 3T6 cells were serum stimulated in the presence (+A) or absence (−A) of cytosine arabinoside. Cultures were harvested at 6 h (stim-6) and 18 h (stim-18), and nuclei were prepared for in vitro transcription. The transcription product was hybridized with a filter containing histone H3 DNA or pBR322. The results of the filter hybridization are shown in the table. Background (18 cpm) has been subtracted from the numbers shown in the H3 DNA and pBR322 columns.

[b] Input refers to the total amount of labeled RNA added to the hybridization vial.

[c] H3 DNA-pBR322/Input is the relative transcription rate of the H3 gene.

The increase in histone H3 mRNA content in cells serum stimulated in the presence of cytosine arabinoside is much lower than that normally observed. The results of previous studies suggest that this may be due to an increase in the rate of turnover of histone mRNA under these conditions (6, 13, 25). Therefore, we determined the effect of cytosine arabinoside on histone mRNA stability in S-phase 3T6 cells. Cultures were stimulated with serum for 16 h and then cytosine arabinoside was added. The content of histone H3 mRNA decreased to 15% of that in the control cells within 1 h of treatment and then remained constant in the presence of the drug (Fig. 5A, summarized in Table 2).

Treatment of S-phase cells with cytosine arabinoside also resulted in a decrease in the relative rate of histone H3 gene transcription to about 65% of the rate in control cells (Fig. 6). Thus, inhibition of DNA synthesis during S phase resulted in an 85% reduction in the steady-state level of histone H3 mRNA but only a 35% reduction in the rate of transcription of the H3 gene.

The rapid decrease in histone H3 mRNA

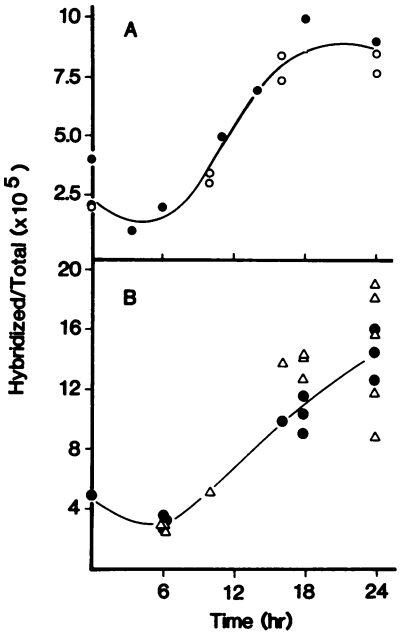

FIG. 4. Transcription of the histone H3 gene in isolated nuclei. (A) Nuclei were purified from cultures of cells at the indicated times after serum stimulation. The nuclear preparations were incubated in an in vitro transcription mixture, and the amount of incorporation into histone H3 RNA relative to total RNA was determined by DNA excess filter hybridization as in Table 1. Open and filled symbols represent the results from two separate experiments. (B) Same as in panel A, except that cytosine arabinoside (5 μg/ml) was added to some cultures (△) at the time of stimulation. Control cultures (●) were stimulated in the absence of drug.

content after the addition of cytosine arabinoside was prevented when the drug was added in the presence of the protein synthesis inhibitors cycloheximide or puromycin (Fig. 5A and Table 3). In fact, H3 mRNA content increased somewhat when protein synthesis was inhibited. This suggests that protein synthesis may be necessary for the rapid turnover of H3 mRNA.

To study the turnover of H3 mRNA in more detail, a pulse-chase experiment was conducted. Histone H3 mRNA was labeled for 1 h with [^{3}H]uridine early in S phase. A large excess of unlabeled uridine and cytidine was then added to the cultures (1). After a 4-h chase period (to reduce the UTP pool specific activity), cytosine arabinoside was added to some of the cultures. Control cultures were incubated in the absence of the drug. The half-life of H3 mRNA during S phase is about 4 to 5 h (Fig. 7). The amount of labeled actin mRNA, a stable mRNA species (19), remained constant during the chase period. However, immediately after the inhibition of DNA synthesis, the half-life of histone H3 mRNA decreased to about 15 min. The turnover of actin mRNA as well as total polyadenylic acid-containing mRNA (data not shown) were not affected by the addition of cytosine arabinoside, indicating that a general destabilization of all mRNA species had not occurred.

DISCUSSION

In our experiments, we studied the mechanisms for regulating the expression of the class of genes coding for the replication variant histone H3. The increase in H3 mRNA content that occurs during S phase is partly due to an increase in the rate of transcription of the H3 gene. The fact that H3 mRNA content increases to a greater extent than the rate of transcription indicates that the cell also regulates H3 gene expression at the posttranscriptional level. Previous studies have shown that the stability of histone mRNA is regulated over a wide range under various conditions and that this plays an important role in controlling histone mRNA content in yeast, mouse, and HeLa cells (9, 13, 15, 25). We found that the half-life of histone H3 mRNA is about 4 to 5 h during S phase. If the half-life of the message were substantially shorter than 4 to 5 h during the first 10 h after serum stimulation, this could account for the discrepancy between the increases in the content and the rate of transcription of the histone H3 gene in serum-stimulated cells. Unfortunately, it would be extremely difficult to measure the stability of a labile histone H3 mRNA species during the first 10 h after serum stimulation since it is difficult to obtain a sufficient amount of incorporation into the message or to achieve a rapid chase under these conditions.

The 4 to 5 h half-life that we estimated for histone H3 mRNA during S phase is much greater than the 40 min half-life estimated in HeLa cells (13). However, when the half-life of histone H3 mRNA was estimated for S phase 3T3 cells by measuring the approach to steady-state labeling (as was used for the HeLa determination), it was found to be greater than 2.5 h (W. F. Marzluff, unpublished data). In a similar experiment, the half-life of the message was found to be about 1 h in mouse myeloma cells (unpublished data). Therefore, it appears that there are significant differences between different cell lines in the stability of this message. It is interesting that the message is less stable in cell lines derived from tumors.

Coupling of histone gene expression with DNA replication. It has been known for many years that the synthesis of histones and the content of histone mRNA are tightly coupled with DNA replication. We found that the rate of transcrip-

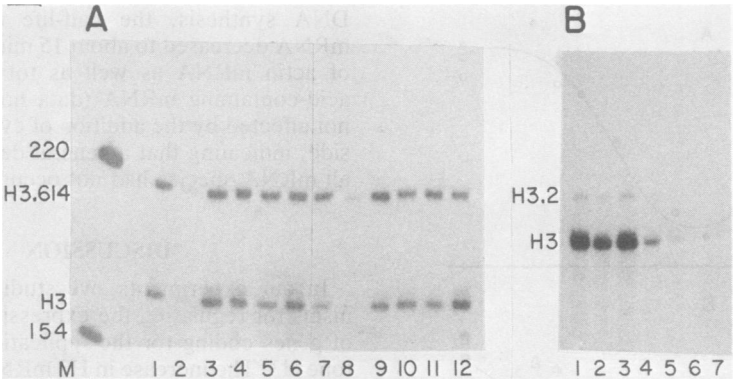

FIG. 5. Effect of cytosine arabinoside on histone mRNA levels. (A) S phase cells (18 h after serum stimulation) were treated with various inhibitors for 1 h (lanes 1 through 6) or 3 h (lanes 7 through 12). The levels of H3 mRNA were analyzed by S1 nuclease mapping. The H3 gene used, H3.614, codes for an H3.2 protein. This gene codes for about 50% of the H3 RNA expressed in cultured mouse cells (R. A. Graves and W. F. Marzluff, unpublished data). The bands are as follows: H3, protection of H3 mRNAs to the AUG codon and H3.614, protection of the mRNA derived from this gene. Lane M shows the marker fragments (154 and 220 nucleotides) from a *Hin*fI digest of pBR322. Lanes: 1 and 7, no treatment; 2 and 8, cytosine arabinoside; 3 and 9, cytosine arabinoside plus cycloheximide; 4 and 10, cytosine arabinoside plus puromycin; 5 and 11, cycloheximide; 6 and 12, puromycin. (B) Cells were stimulated with serum in the presence or absence of cytosine arabinoside. Cytoplasmic RNA was prepared at the indicated time and analyzed for histone H3 mRNA by S1 nuclease mapping. The probe used was from the gene for the H3.2 mRNA. The protected bands are labeled as in the legend to Fig. 2B. Lanes: 1, 24-h stimulation; 2, 24-h stimulation plus cytosine arabinoside; 3, 18-h stimulation; 4, 18-h stimulation plus cytosine arabinoside; 5, 6-h stimulation; 6, 6-h stimulation plus cytosine arabinoside; 7, resting cells.

tion of the H3 genes begins increasing at about the same time the cells enter S phase. Similar results have been obtained with yeast and HeLa cells (13, 15). We were surprised to see that the rate of H3 gene transcription increases to about the same extent when the cells are stimulated in the presence of cytosine arabinoside. Furthermore, when S-phase cells are treated with cytosine arabinoside, the rate of transcription of the histone H3 genes decreases by only about 35%. These observations suggest that although the transcription of the histone H3 genes occurs at the same time as the cells begin DNA replication, there is no tight coupling between the rate of transcription of the H3 gene and the rate of DNA replication. This is very similar to our observations of the regulation of dihydrofolate reductase gene transcription (Santiago et al., in press). It is possible that these two genes (as well as those for other histones and S-phase proteins) are activated by common transcriptional control factors.

When DNA synthesis is inhibited in mouse myeloma cells, the rate of H3 gene transcription decreases about 80%, and the content of H3 and H2b mRNA decrease about 90% (25). A 90% decrease in H2a mRNA content was also observed in HeLa cells (13). These decreases are about twice as great as those observed in 3T6 cells after the inhibition of DNA synthesis. The reason for these differences is not known, although they reinforce the idea that there are subtle (or even major) differences in the control of histone gene expression in different cell lines (11).

When DNA replication is blocked in S-phase cells by the addition of cytosine arabinoside, H3

TABLE 2. Summary of the effects of cytosine arabinoside on histone H3 mRNA content and gene transcription[a]

Treatment	Histone H3 mRNA level[b]	Histone H3 gene transcription[c]
Serum, 6 h	3	20
Serum, 18 h	100	100
Serum plus ara C, 6 h	2	20
Serum plus ara C, 18 h	15	130
Serum, 16 h, then ara C for 1 h[d]	15	65
Serum, 16 h, then ara C for 3 h[d]	18	65

[a] Resting cultures were serum stimulated in the presence or absence of 5 μg of cytosine arabinoside (ara C) per ml at time zero.

[b] Data from Fig. 3 and 5. Each value has been normalized to the value observed for 18-h stimulated cells, which was arbitrarily set at 100.

[c] Data from Fig. 4 and 6. Each value normalized as described in footnote *b*.

[d] Cytosine arabinoside was added at 16 h after stimulation.

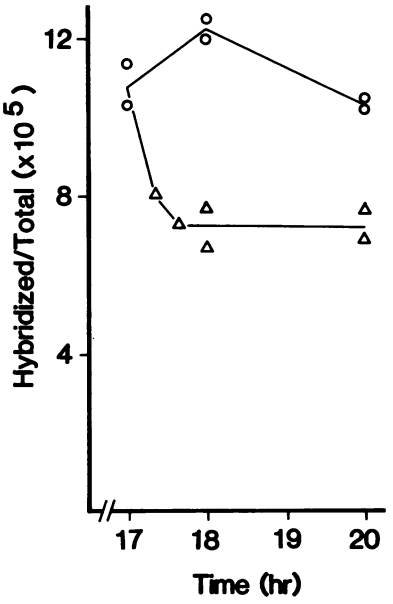

FIG. 6. Effect of cytosine arabinoside on histone H3 gene transcription. Cultures of 3T6 cells were serum stimulated for 17 h. Cytosine arabinoside was then added to some of the cultures (△). Control cultures (○) were not treated with the drug. At the indicated times, cultures were harvested, and nuclei were prepared for in vitro transcription. RNA was isolated from the transcription reaction, and the amount of labeled histone H3 RNA relative to total labeled RNA was determined.

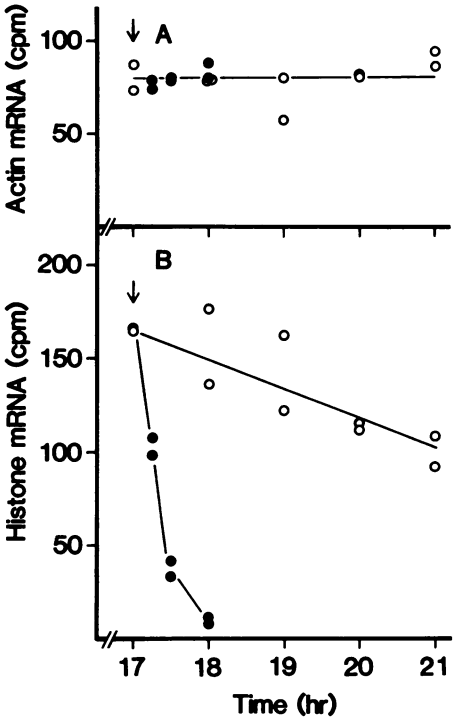

FIG. 7. Rapid turnover of histone H3 mRNA after inhibition of DNA synthesis. Cultures of cells were labeled for 60 min with [^{3}H]uridine at 12 h after serum stimulation. The cultures were then incubated in the presence of unlabeled uridine (5 mM) and cytidine (2.5 mM) to reduce the pool specific activity as quickly as possible (1). At 17 h (arrow), cytosine arabinoside was added to some cultures (●). Control cultures (○) were incubated in the absence of drug. At the indicated times, cultures were harvested, and the amount of labeled α-actin mRNA (A) or labeled histone H3 mRNA (B) was determined.

mRNA turns over with a half-life of about 15 min. Similar observations have been made in other cell systems (5, 9, 13, 25). Therefore, the coupling between histone H3 mRNA content and DNA replication appears to occur primarily at the posttranscriptional rather than the transcriptional level in serum-stimulated 3T6 cells.

The mechanism responsible for the rapid turnover of histone H3 mRNA is not known; however, several observations relevant to this mechanism have been made during this investigation. The rapid turnover of histone H3 mRNA is prevented if protein synthesis is blocked before the inhibition of DNA synthesis. This has also been observed previously (6, 25). The stabilization of the message is probably not due to protection of the message by ribosomes, since puromycin is just as effective as cycloheximide in preventing the turnover of the message. It is not likely that the rapid turnover of histone mRNA is caused by the de novo synthesis of a nuclease after inhibition of DNA synthesis, since the turnover of histone H3 mRNA is initiated without any detectable lag after the addition of cytosine arabinoside. It is more likely that histone mRNA is made more vulnerable to a preexisting nuclease or that a preexisting nuclease is activated after the inhibition of DNA synthesis.

TABLE 3. Effect of DNA and protein synthesis inhibitors on histone H3 mRNA content[a]

Length of treatment (h)	Histone H3 mRNA content with:					
	Control	Ara C	Ara C + cyclo	Ara C + puro	Cyclo	Puro
1	100	15	140	95	110	120
3	100	18	140	110	115	125

[a] Cells which were in S phase (16 h after serum stimulation) were treated with cytosine arabinoside (Ara C), cycloheximide (cyclo), or puromycin (puro). RNA was prepared 1 or 3 h later, and the amount of H3 mRNA was determined by S1 nuclease mapping (see Fig. 5A), followed by densitometry as shown in Fig. 3. The untreated cells were assigned a value of 100.

The rapid turnover of histone H3 mRNA is not the result of the general destabilization of all mRNA molecules, since there was no detectable effect on the rate of turnover of total mRNA or actin mRNA after inhibition of DNA synthesis. Furthermore, under these conditions, the mRNA for the replacement histone H3 is not affected (25; unpublished data). Since both the replication and the replacement types of histone H3 mRNA lack polyadenylic acid, this does not appear to be the signal that is responsible for identifying one type of histone message for rapid turnover. The signal may reside in the 5′ or 3′ untranslated regions, since these are quite dissimilar in the two types of mRNA (unpublished data).

An interesting possibility is that histones regulate the translation of their own mRNAs (6). Inhibition of DNA synthesis would result in the accumulation of histones in the cytoplasm. If histone H3 was able to bind specifically to its mRNA (perhaps in the 5′ untranslated region), this might prevent translation as well as activate nonspecific nucleases that would destroy specific messages. Since the untranslated regions in the replacement variants are different, this would account for the destruction of the mRNA for the replication but not the replacement histone H3. Another possibility is that inhibition of DNA synthesis leads to the accumulation of DNA precursors which activate either a preexisting nuclease or translational control factors that are specific for mRNAs coding for the replication variant histones.

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