MOLECULAR AND CELLULAR BIOLOGY, Apr. 1983, p. 539-550
0270-7306/83/040539-12$02.00/0

Vol. 3, No. 4

Regulation of Human Histone Gene Expression: Kinetics of Accumulation and Changes in the Rate of Synthesis and in the Half-Lives of Individual Histone mRNAs During the HeLa Cell Cycle

NATHANIEL HEINTZ,† HAZEL L. SIVE,† AND ROBERT G. ROEDER†*

Departments of Biological Chemistry and Genetics, Division of Biology and Biomedical Sciences, Washington University School of Medicine, St. Louis, Missouri 63110

Received 29 October 1982/Accepted 28 December 1982

We have analyzed the kinetics of accumulation of each of the individual core histone mRNAs throughout the HeLa cell cycle in cells synchronized by sequential thymidine and aphidicolin treatments. These analyses showed that during the S phase there was a 15-fold increase in the levels of histone mRNAs and that this resulted from both an increased rate of synthesis and a lengthening of the half-life of histone mRNAs. A comparison of the kinetics of accumulation of histone mRNA in the total cellular and nuclear RNA populations suggested an increased transcription rate through the S phase. Within 30 min after the inhibition of DNA synthesis in mid-S phase, the steady-state concentration and the rate of synthesis of histone mRNA each declined to their non-S-phase levels. Reactivation of histone mRNA accumulation could occur even after an extended mid-S-phase block in DNA synthesis. These results suggest that the mechanisms responsible for histone mRNA synthesis are not restricted to the G1/S boundary of the HeLa cell cycle, but can operate whenever DNA synthesis is occurring.

The original observation that nuclear DNA synthesis and histone protein synthesis occur at significant rates only during the S phase of cultured HeLa cells (23) has resulted in an extensive literature concerning the coupling of these events in mammalian cells (reviewed in 20). That histone synthesis and DNA synthesis are very tightly coupled is evident from the observation that histone protein synthesis is abruptly terminated when DNA synthesis is inhibited (4, 9, 23). Subsequent studies used a variety of techniques to establish that cell cycle-dependent synthesis of histone proteins reflected a differential accumulation of histone mRNA during the HeLa cell cycle (3, 5, 8, 25). The mechanisms for coupling histone mRNA accumulation and DNA synthesis remain obscure, although it is generally thought that both transcriptional and post-transcriptional processes may be involved (see, for example, 3, 5).

Our approach toward elucidating the detailed mechanisms controlling histone gene expression during the cell cycle is to reconstruct these events in vitro. Prerequisite for such an in vitro approach is a thorough description of the relative contributions of transcriptional or post-transcriptional processes to the accumulation of histone mRNA during the S phase and a complete knowledge of the kinetics of synthesis and accumulation of individual histone mRNAs. The isolation of genomic clones containing several human histone genes (13) provides the necessary tools to describe completely the synthesis and accumulation of individual histone mRNAs. In this report we describe the kinetics of accumulation of individual histone mRNAs during the HeLa cell cycle. Changes in the rate of synthesis and in the stability of histone mRNA are both shown to contribute to the dramatic increase in the steady-state concentrations of histone mRNA during the S phase. Furthermore, it is demonstrated that histone mRNA accumulation can be restored after an extended block in DNA synthesis in the middle of S phase. The mechanisms governing this process are not, therefore, restricted to the G1/S boundary of the HeLa cell cycle but remain operable throughout S phase.

MATERIALS AND METHODS

Suppliers. Aphidicolin was supplied by the Natural Products Branch, Division of Cancer Treatment, National Cancer Institute. Restriction enzymes were purchased from Bethesda Research Laboratories. All radiolabeled precursors were purchased from New England Nuclear. CsCl was obtained from Varlakoid

† Present address: The Rockefeller University, New York, NY 10021.

Chemical Co. Nitrocellulose (BA85) was purchased from Schleicher & Schuell.

Cell culture and synchronization. HeLa cells were grown in suspension culture at 3×10^5 to 6×10^5/ml in Joklik minimal essential medium supplemented with 5% newborn calf serum. Synchronization at the G1/S boundary was achieved with a thymidine block, followed by an aphidicolin block. Cells at approximately 4×10^5/ml were blocked with 2 mM thymidine for 12 h, released from the block by three washes in fresh media minus calf serum, and suspended at 4×10^5/ml in complete media containing 0.24×10^{-4} M thymidine and deoxycytidine. After 9 h, aphidicolin was added to 5 μg/ml for an additional 12 h. The cells were released from the aphidicolin block as described for the thymidine block, but no additional thymidine or deoxycytidine was included in the medium. DNA synthesis was monitored by incorporation of [^{3}H]deoxycytidine, using the procedure of Nilsen and Baglioni (19).

Isolation of HeLa cell RNA. RNA was prepared essentially as described by Glisin et al. (10). Washed whole cells or isolated nuclei were suspended at 10^7/ml in 7.0 M urea–0.35 M NaCl–2% Sarkosyl–0.01 M Tris-hydrochloride (pH 7.5)–1 mM EDTA and homogenized by 15 to 20 strokes in a hand-held glass homogenizer. A 1.0-g portion of solid CsCl was added per 2.5 ml of homogenate, and the mixture was layered over a 1.0-ml 5.7 M CsCl cushion and spun in a Beckman SW60 rotor at 100,000 × *g* for 16 h. The resulting pellet was suspended in sterile double-distilled water and stored as an ethanol precipitate at −20°C. Nuclei were prepared by lysis in hypotonic buffer followed by extensive washing in that same buffer. Nuclear RNA prepared by this procedure represented approximately 8% of the total cellular RNA.

Formaldehyde-agarose gel electrophoresis and northern blot hybridization. Formaldehyde-agarose gel electrophoresis was performed as described by Chen-Kiang et al. (6) except that electrophoresis was carried out at 2.5 V/cm for 14 h in a 1.5% agarose gel. The resolved RNA species were transferred to nitrocellulose, using 10× SSC (SSC = 0.15 M NaCl plus 0.015 M sodium citrate) as described by Southern (24), except that the nitrocellulose filter was baked at 80°C immediately upon completion of transfer. Prehybridization and hybridization were as described by Wahl et al. (27) according to the modifications of Thomas (26). The gels were exposed to Kodak XAR-5 film at −80°C, using Dupont Cronex Lightning Plus intensifying screens. At least two exposures of each gel were quantitated by optical scanning with a Joyce-Loebl recording densitometer.

Several adjacent passes of the autoradiograph through the densitometer ensured that the entire area of each band was measured.

RNA labeling and DNA excess filter hybridization. Continuous labeling of RNA with [^{3}H]uridine was as described in Babich and Nevins (1), except that cold uridine was not included and only 25 μCi of [^{3}H]uridine (~40.5 Ci/mmol) per ml was used in the unsynchronized cell populations. Cytoplasmic RNA was prepared by the CsCl procedure as described above. Five-minute pulse-labels with [^{3}H] uridine (40 to 42 Ci/mmol) were as described in Derman et al. (7), using 0.8 to 1.0 mCi/ml. Incorporation of label into total cellular RNA was approximately equivalent for the cell populations utilized. Hybridization of RNA to excess DNA on nitrocellulose filters was as detailed in Derman et al. (7), using the aqueous hybridization conditions described. Enough plasmid DNA was loaded onto the filter to hybridize approximately 5×10^4 mRNA molecules per cell. Similar studies with twice the amount of plasmid DNA per filter gave identical results (data not shown).

RESULTS

HeLa cell synchronization with aphidicolin. To obtain large numbers of synchronized cells for subsequent in vitro studies and to obtain cells synchronized at the G1/S boundary, we have chosen to investigate cell cycle-dependent histone gene expression in HeLa cells grown in spinner culture. Previous studies (8) have indicated that cells synchronized by double thymidine blockage are not sufficiently well synchronized for this purpose. However, Pedrali-Noy et al. (21) showed that HeLa cells can be effectively and reversibly blocked at the G1/S boundary of the cell cycle by using aphidicolin. Furthermore, they reported that this drug does not affect S-phase duration, cell viability, or the synthesis of deoxynucleotide triphosphates and DNA polymerases. In this study, synchronized HeLa cell populations were obtained by a double-block protocol in which thymidine and aphidicolin were used in the first and second blocks, respectively (see Materials and Methods). Figure 1 shows the rate of [^{3}H]deoxycytidine incorporation into DNA as a function of time after release from the aphidicolin treatment.

It is evident from these data that the incorporation of [^{3}H]deoxycytidine into HeLa cell DNA during S phase is biphasic. This result is also obtained with [^{3}H]thymidine as the labeled precursor and has been shown to reflect the behavior of a single population of cells by autoradiography of labeled nuclei isolated at 2 and 6 h post-release (data not shown). Also, the trough in incorporation of deoxynucleotide triphosphates into HeLa cell DNA reproducibly occurs 5 h after release into S phase. It is not known whether the rate of incorporation of these precursors into DNA accurately reflects the actual rate of DNA synthesis, or whether changing deoxynucleotide triphosphate pool sizes are responsible for these changes in [^{3}H]deoxycytidine incorporation. In either case, the extremely rapid increase in the incorporation of DNA precursors after release from the block and the biphasic nature of the S phase indicate that the cell population progresses through S phase in unison. Less precise synchrony would mask the apparent biphasic nature of S phase.

HeLa cells synchronized by this procedure proceed through the cell cycle in a significantly shorter period (13 to 14 h) than the same cells before treatment (20 to 24 h). This is probably

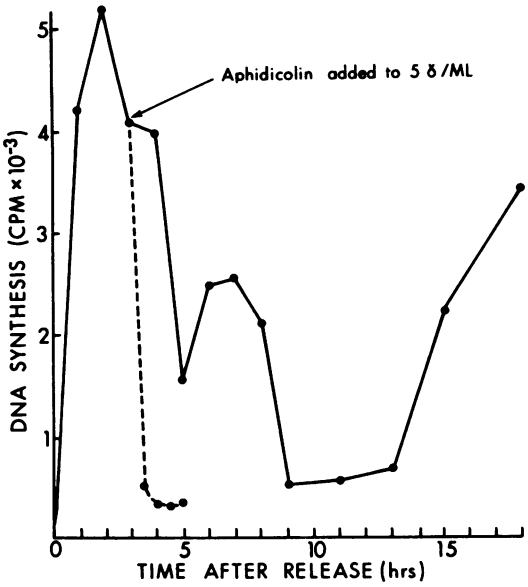

FIG. 1. Kinetics of DNA synthesis in HeLa cells synchronized by sequential blocks of thymidine and aphidicolin. The rate of DNA synthesis was monitored in HeLa cells before release and for 18 h thereafter by incorporation of [^{3}H]deoxycytidine (●—●), as described in the text. Aphidicolin was added 3 h after release from the block, and the decline in DNA synthesis was monitored at 30-min intervals thereafter (●—●). γ, Milligrams.

because aphidicolin, a specific inhibitor of DNA polymerase α (for a review, see 16), does not interfere with the normal metabolism of the cell. Those products necessary for traversal of the cell cycle may, therefore, accumulate during the aphidicolin treatment and permit the cells to proceed rapidly from the initial S phase after release to the next S phase. Although this results in a severely shortened G1 period, it has proven advantageous here because it results in a more synchronous population as the cells enter the second S phase, allowing us to monitor the accumulation of histone mRNAs in cells that have been free from drug treatment for an entire cell cycle.

Human histone gene probes. Our original report concerning the isolation of human histone genes and their preliminary characterization (13) described a variety of genomic DNA fragments containing the H3, H4, H2a, and H2b histone genes. For the present studies, we wished to prepare genomic DNA probes containing sequences hybridizing solely to histone RNAs. Therefore, we screened the subcloned genomic fragments containing histone genes for regions encoding nonhistone genes. This was done by two means: first, Northern hybridization analysis of HeLa cell RNA (as detailed in Materials and Methods) with these fragments as probes was used to detect transcripts (histone and nonhistone) present in HeLa cells; second, the presence of RNA polymerase III transcription units was assayed by in vitro transcription of these fragments in soluble extracts (28). Although a complete description of the results will be presented elsewhere, three points bear mention. First, those clones containing sequences transcribed by RNA polymerase III in vitro contain sequences which hybridize to a large number of HeLa cell RNAs, and could not be used as specific probes for histone mRNA. Second, several of the subclones hybridize to a small HeLa cell transcript (385 bases) which is present throughout the HeLa cell cycle (data not shown). Third, Northern blot analysis did not reveal the presence of a histone H1 gene on these clones.

With the exception of pHh4A, those subclones chosen as specific probes in this study (Fig. 2) contained a single human histone gene and no additional transcribed sequences. pHh4A, on the other hand, hybridizes to the small (385-nucleotide) transcript mentioned above; therefore, the 0.7-kilobase *Eco*RI/*Hin*dIII fragment containing the H2b gene was isolated and used in the Northern hybridization analysis. Information regarding the expression of these particular human histone genes has not yet been obtained. In these studies, we measured that population of histone mRNA which hybridizes to these probes at the hybridization stringency used in each experiment. Since nucleotide sequence analysis has revealed no gross aberration in the coding regions of these genes

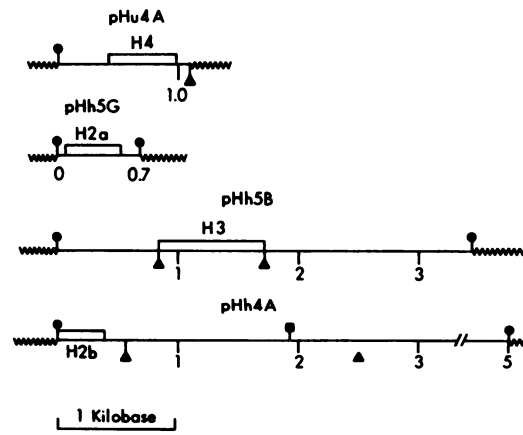

FIG. 2. Human histone gene probes. Restriction maps of subcloned human histone gene probes, known to contain a single human histone gene (13), which are used in this study. Blocked area indicates location of the gene within each subclone. Symbols: ●, *Eco*RI; ▲, *Hin*dIII; ■, *Xba*I.

(R. Zhong, unpublished data), it is probable that their use in the experiments presented here allows accurate assessment of the biogenesis of the major HeLa histone mRNA species. As discussed below, however, it is possible that the minor differences in the results presented for the H2a, H2b, H3, and H4 mRNAs may reflect varying efficiencies of hybridization with constitutively expressed histone mRNAs.

As a control for the behavior of a nonhistone gene during the cell cycle, we have examined the changes in levels of RNA hybridizing to plasmid pCGα, a cDNA clone containing sequences which correspond to the mRNA encoding the α subunit of human chorionic gonadotropin (2). The αhCG protein is synthesized in a variety of placentally and nonplacentally derived tumors, including HeLa cells, and there is some indication that secretion of this protein is stimulated in cells which are not synthesizing DNA (for a review, see 17). The αhCG mRNA in HeLa cells has recently been shown to be similar in size to the mRNA species seen in the human placenta (1a).

Kinetics of accumulation of histone mRNAs during the HeLa cell cycle. To monitor the accumulation of specific histone mRNAs during the HeLa cell cycle, total cellular RNA was prepared from cells before release from the aphidicolin treatment and at 1-h intervals for 16 to 18 h post-release. Samples, 30 μg, from each preparation were analyzed for the presence of individual histone mRNAs by Northern hybridization, and the relative amount of histone mRNA present was quantitated densitometrically (Fig. 3). Results are expressed as the relative increase over prerelease mRNA levels or prerelease rates of incorporation of [^{3}H]deoxycytidine so that data from different experiments can be directly compared.

It is immediately apparent from the autoradiographs shown in Fig. 3 that there is a dramatic increase in the steady-state level of each of the histone mRNAs as the cells progress through S phase. For example, H2a mRNA begins to accumulate in the cell immediately after release from the aphidicolin block, reaching its maximal steady-state level 5 h post-release (Fig. 3A). At this time there is approximately 17-fold more H2a mRNA than is present before release or after completion of S phase (at 10 h after release). Furthermore, H2a mRNA concentration declines between the peak of its accumulation (at 5 h post-release) and the completion of S phase, with an apparent half-life of 90 min. As the cells begin to enter a second S phase, 12 to 13 h after release from the aphidicolin block, H2a mRNA again begins to accumulate. Both the rate of incorporation of [^{3}H]deoxycytidine into DNA and histone mRNA accumulation are less rapid at the onset of the second S phase, presumably reflecting a loss of synchrony in the cell population. To demonstrate the reproducibility of this type of analysis, RNA prepared before release and 3 h into S phase in a separate experiment are included in the autoradiograph shown in Fig. 3A. The level of induction of H2a mRNA at 3 h post-release is between 10- and 11-fold in each of these experiments.

The kinetics of accumulation of H2b and H3 mRNAs (Fig. 3C and 3D) are similar to those described for H2a message and show maximally 13- and 15-fold increases in respective mRNA levels. Whereas H4 mRNA shows a similar (13-fold) increase in concentration, its kinetics of accumulation appear slightly different (Fig. 3B) from those of the other histone mRNAs. Thus, the steady-state level of H4 mRNA increases more slowly and reaches its maximal steady-state level slightly later than the other histone mRNAs. Whether this difference reflects a biologically interesting phenomenon or whether it results from the methodology used is not known. However, this result has been reproducibly obtained when nitrocellulose filters used in Northern hybridization analysis (as described in Materials and Methods) were rehybridized to an H2a, H2b, or H3 probe. A possible explanation for this is that this particular H4 probe can cross-hybridize to constitutively expressed histone mRNA (see 29) to a greater extent than can the other histone gene probes we have used.

To determine whether the kinetics of histone mRNA accumulation reflect the behavior of the entire mRNA population, we have analyzed the steady-state levels of αhCG mRNA during the HeLa cell cycle. αhCG mRNA is present at high concentrations in cells blocked with aphidicolin and, after a slight increase immediately after release, decreases to a low level during S phase (Fig. 3D). As the cells traverse G2, M, and G1, αhCG mRNA levels increase approximately fivefold and again decrease with the onset of the second S phase. The accumulation of αhCG mRNA is, therefore, regulated in a manner different from that of histone mRNA during the HeLa cell cycle.

Rate of histone mRNA synthesis during the HeLa cell S phase. To determine the contribution of an increased rate of histone mRNA synthesis to its accumulation during the DNA synthetic period of the HeLa cell cycle, we have measured the relative rate of histone H3 and H2a mRNA synthesis in cells blocked at the G1/S boundary of the cell cycle, in S-phase cells at 2.5 h post-release (when the rate of histone mRNA accumulation is maximal), and in S-phase cells in which DNA synthesis was blocked with aphidicolin 30 min before the pulse. To minimize possible error due to the turnover of histone

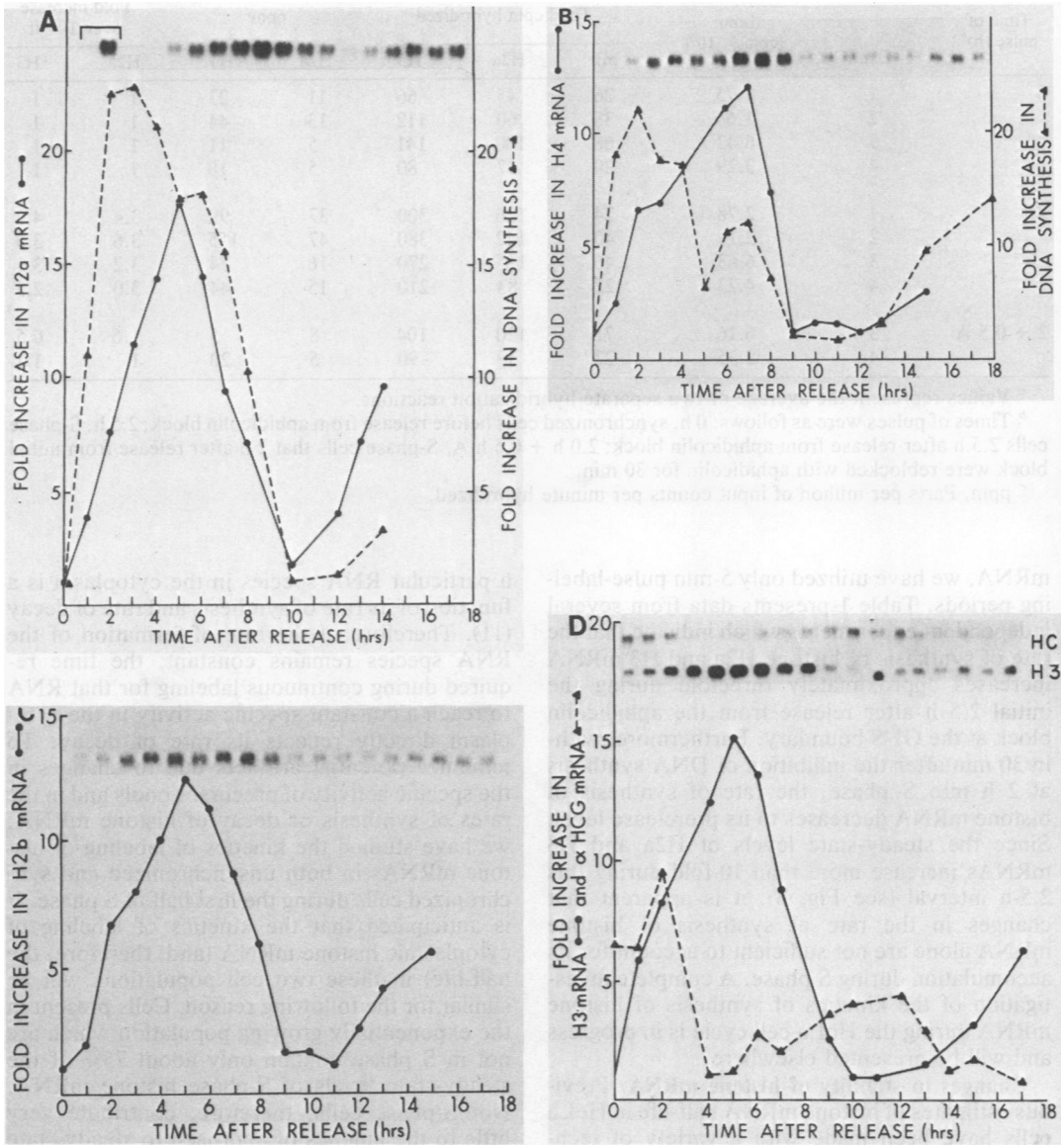

FIG. 3. Kinetics of accumulation of individual histone mRNAs during the HeLa cell cycle. Samples, 30 μg, of total cellular RNA prepared at various times during the cell cycle were resolved on 1.5% formaldehyde-agarose gels and assayed for specific sequences by Northern blot hybridization, using specific gene probes. Densitometric quantitation of the autoradiograms shown in each panel is also presented. (A) Symbols: ●, H2a mRNA; ▲, [^{3}H]deoxycytidine incorporation. The leftmost two autoradiographic lanes (bracketed) correspond to prerelease and 3 h post-release RNA samples from a separate experiment. Remaining lanes correspond to RNA samples prepared before release and at hourly intervals after release from aphidicolin block. (B) Symbols: ●, H4 mRNA; ▲, [^{3}H]deoxycytidine incorporation. (C) Symbol: ●, H2b mRNA. (D) Symbols: ●, H3 mRNA; ▲, mRNA encoding the α-subunit of human chorinic gonadotropin. (B, C, and D) Autoradiographic lanes correspond to RNA samples prepared before release and at hourly intervals post-release until 16 h; final lane is 18-h post-release sample. These panels are derived from a single experiment and, therefore, the kinetics of DNA synthesis for (C) and (D) have not been replotted. A few individual points which are obviously aberrant as judged by similar analysis in other blots from these RNA preparations, or corresponding preparations from separate experiments, have been omitted on the basis of experiments repeated at least three times.

TABLE 1. Rate of histone mRNA synthesis[a]

Time of pulse (h)[b]	Expt	Input (cpm × 10^6)	Total cpm hybridized			ppm[c]		Fold increase over T = 0	
			pBr	H2a	H3	H2a	H3	H2a	H3
0	1	1.75	26	45	66	11	23	1	1
	2	1.66	38	60	112	13	44	1	1
	3	6.42	68	100	141	5	11	1	1
	4	3.29	20	37	80	5	18	1	1
2.5	1	2.78	24	126	300	37	99	3.4	4.3
	2	2.19	40	142	380	47	155	3.6	3.5
	3	6.63	46	155	270	16	34	3.2	3.1
	4	4.23	22	83	210	15	44	3.0	2.5
2 + 0.5 A	3	6.16	71	120	104	8	5	1.6	0.5
	4	3.35	22	39	90	5	20	1	1.1

[a] Values represent the average of two separate hybridization reactions.

[b] Times of pulses were as follows: 0 h, synchronized cells before release from aphidicolin block; 2.5 h, S-phase cells 2.5 h after release from aphidicolin block; 2.0 h + 0.5 h A, S-phase cells that 2 h after release from initial block were reblocked with aphidicolin for 30 min.

[c] ppm, Parts per million of input counts per minute hybridized.

mRNA, we have utilized only 5-min pulse-labeling periods. Table 1 presents data from several independent experiments which indicate that the rate of synthesis of histone H2a and H3 mRNA increases approximately threefold during the initial 2.5 h after release from the aphidicolin block at the G1/S boundary. Furthermore, within 30 min after the inhibition of DNA synthesis at 2 h into S phase, the rate of synthesis of histone mRNA decreases to its prerelease level. Since the steady-state levels of H2a and H3 mRNAs increase more than 10-fold during this 2.5-h interval (see Fig. 3), it is apparent that changes in the rate of synthesis of histone mRNA alone are not sufficient to account for its accumulation during S phase. A complete investigation of the kinetics of synthesis of histone mRNA during the HeLa cell cycle is in progress and will be presented elsewhere.

Changes in stability of histone mRNA. Previous estimates of histone mRNA half-life in HeLa cells have been made with a variety of techniques and have resulted in a wide range of estimates (4, 9, 18), from 60 min to several hours. Although it was suggested in these early studies that the half-life of histone mRNA may change during the HeLa cell cycle, there is very little experimental support for this idea. To explain the discrepancy between our results concerning the magnitude of changes in the steady-state levels (Fig. 3) and rates of synthesis (Table 1) of histone mRNA during S phase, we have measured the half-life of histone mRNA during and in the absence of DNA synthesis.

The derivation of RNA half-life from the kinetics of labeling of cytoplasmic RNA is based on the fact that the steady-state concentration of a particular RNA species in the cytoplasm is a function of its rate of synthesis and rate of decay (11). Therefore, if the rate of formation of the RNA species remains constant, the time required during continuous labeling for that RNA to reach a constant specific activity in the cytoplasm directly reflects its rate of decay. To minimize potential artifacts due to changes in the specific activity of precursor pools and in the rates of synthesis or decay of histone mRNA, we have studied the kinetics of labeling of histone mRNAs in both unsynchronized and synchronized cells during the first half of S phase. It is anticipated that the kinetics of labeling of cytoplasmic histone mRNA (and, therefore, the half-life) in these two cell populations will be similar for the following reason. Cells present in the exponentially growing population which are not in S phase contain only about 75% of the steady-state levels of S-phase histone mRNA. Non-S-phase cells, therefore, contribute very little to the kinetics of approach to steady-state labeling in the unsynchronized cell population; rather, these kinetics are determined principally by the S-phase cell population.

In the experiment shown in Fig. 4, the half-life of histone mRNA in exponentially growing HeLa cells was determined essentially as described by Babich and Nevins (1), except that no cold uridine was included in the media. Under these conditions, the exogenously added uridine was only 0.675×10^{-6} M. Incorporation of [^{3}H]uridine into total cytoplasmic RNA remained linear during the initial 3 h of the experiment (Fig. 4A), and significant amounts of labeled RNA could be detected in the cytoplasm within 30 min after introduction of the label. It is

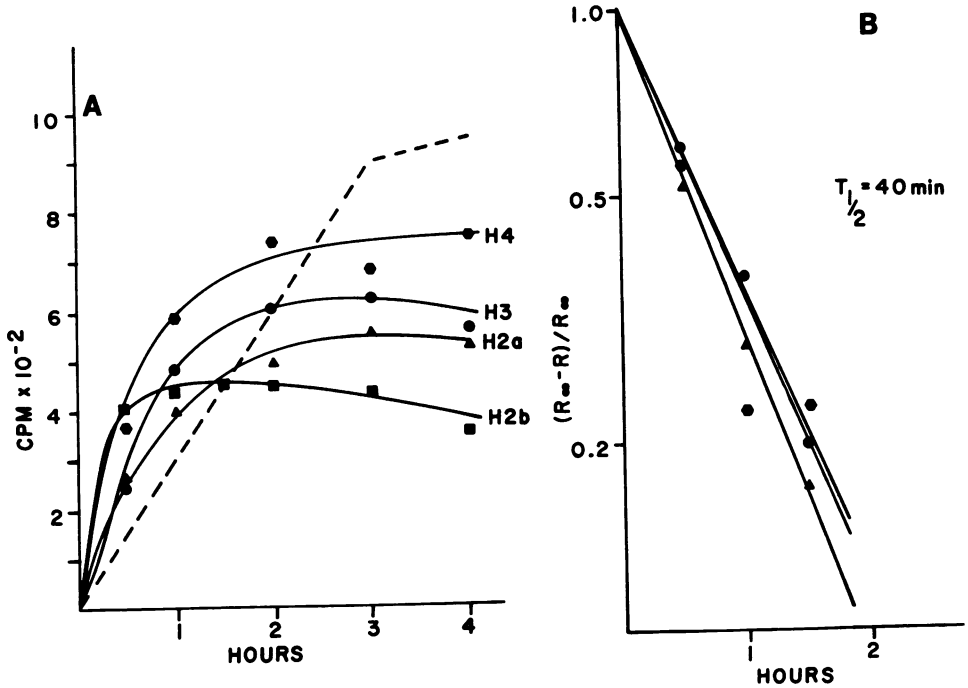

FIG. 4. Kinetics of accumulation and decay rates of histone mRNAs in exponentially growing HeLa cells. Exponentially growing HeLa cells were concentrated to a density of 2×10^6 cells per ml in fresh media, and [^{3}H]uridine was added to 25 μCi/ml. At the times indicated, cytoplasmic RNA was prepared from 2×10^7 cells and hybridized to filters bearing the histone plasmids (see text). (A) (---) [^{3}H]uridine incorporation into total cytoplasmic RNA; accumulation of [^{3}H]uridine into H4(●), H3 (●), H2a (▲), and (■) RNAs hybridizing to the plasmid pHh4A-containing the H2b gene. (B) Decay curves for individual, histone mRNA species according to the method of Greenberg (11) and plotted as in Babich and Nevins (1). R_{00} = average cpm present in RNA at steady state; R = cpm present in mRNA at a given time of labeling.

clear from the data presented (Fig. 4A) that the specific activities of cytoplasmic histone H3, H4, and H2a mRNAs each plateau at approximately 2 h. The half-life of each of these mRNAs was determined by means of a reciprocal plot of the data from the accumulation curves (1) and indicate that histone H3, H4, and H2a mRNAs decay with a half-life of approximately 40 min in exponentially growing HeLa cells (Fig. 4B). However, the specific activity of the RNA hybridizing to nitrocellulose filters containing pHh4A (containing an H2b gene) DNA had reached a steady state by the initial time point. We infer from these latter data that pHh4A hybridizes to an additional RNA species which is either extremely abundant (>50,000 molecules per cell) or has a very short half-life. We also argue from this early plateau that the RNA precursor pools must have equilibrated within 30 min.

The half-life of histone mRNA during the HeLa S phase was measured by the kinetics of approach to steady-state labeling of H2a, H3, and H4 mRNAs in synchronized cells from the second to fifth hours of S phase (Fig. 5). In this experiment 14 μM cold uridine was included in the media with the introduction of the labeled uridine. This resulted in a significant lag in the appearance of labeled RNA in the cytoplasm, but ensured linear incorporation during the course of the experiment (Fig. 5A). As expected from the results of a similar experiment in unsynchronized cells (see above), the half-life of histone mRNA during S phase is approximately 35 to 40 min (Fig. 5B). This must be regarded as a maximal estimate because of the time required for equilibration of the ribonucleotide triphosphate pools.

To account for differences in the rates of accumulation and synthesis of histone mRNA during S phase, the half-life of histone mRNA must be significantly longer in S-phase cells than it is in non-S-phase cells. Since the maximal half-life of histone mRNA during S phase is 40 min, the turnover of histone mRNA must be extremely rapid in the absence of DNA synthesis. Measurement of such a brief half-life by the kinetics of labeling of that RNA is inappropriate due to pool equilibration problems. In fact, our attempts to measure histone mRNA turnover in

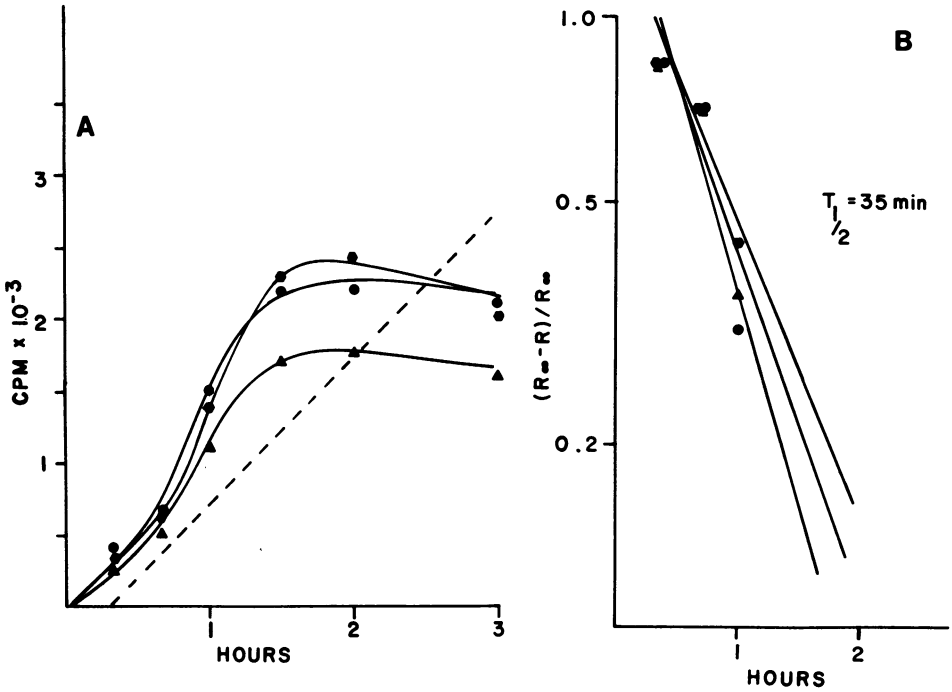

FIG. 5. Kinetics of accumulation and decay rates of histone mRNAs in synchronized HeLa cells. HeLa cells were synchronized and released into S phase as described in the text. Two hours after release from the block, cells were concentrated to 2×10^6 cells per ml and 50 μCi of [^{3}H]uridine per ml was added (total uridine concentration, 14 μM). At the times indicated, cytoplasmic RNA was prepared from 2×10^7 cells and hybridized to filters bearing the histone plasmids. (A) (---) [^{3}H]uridine incorporation into total cytoplasmic RNA; accumulation of [^{3}H]uridine into H4(●), H3 (●), and H2a (▲) histone mRNAs. (B) Decay curves for the individual histone mRNA species derived as in the legend to Fig. 4.

non-S-phase cells by this method have been unsuccessful. We therefore measured the rate of decay of histone mRNA after a block in DNA synthesis during S phase (Fig. 6).

In this experiment, DNA synthesis was inhibited with aphidicolin and total cellular RNA was assayed for the presence of histone mRNA at 10-min intervals thereafter. The apparent half-life of histone mRNA under these conditions is about 8 min (Fig. 6). This is somewhat shorter than the original estimate of the half-life of histone mRNA after hydroxyurea treatment (13 min [9]) and probably reflects differences in both the methods of analysis and DNA synthesis inhibitor used. This must also be regarded as a maximal estimate since, although the rate of synthesis of histone mRNA is minimal by 30 min after the block (Table 1), it is possible that histone mRNA continues to be synthesized at a high rate for several minutes after addition of the aphidicolin.

It is evident from these experiments that the half-life of histone mRNA is very brief during all stages of the HeLa cell cycle. Furthermore, these measurements suggest that there may be a significant change in the half-life of histone mRNA during the S phase and that this change in the stability of histone mRNA may contribute to its accumulation during DNA synthesis.

Effect of inhibition of DNA synthesis on levels of histone mRNA. It is well documented that histone protein synthesis (23) and polysomal histone mRNA (3, 8, 9) disappear from HeLa cells after the inhibition of DNA synthesis, but it is not clear whether transcription per se is blocked or whether this phenomenon results solely from increased mRNA degradation. It was of interest, therefore, to determine whether individual histone mRNAs disappear from the total cellular RNA population under these conditions, or whether compartmentalization or modification of these mRNAs might explain these results. Furthermore, it has been proposed that histone gene transcription is "activated" by DNA replication or some process associated with it (5). Consistent with this suggestion are models in which histone genes are activated either by (events leading to) generalized total nuclear DNA synthesis or by the replication of the histone genes themselves. A simple proposal

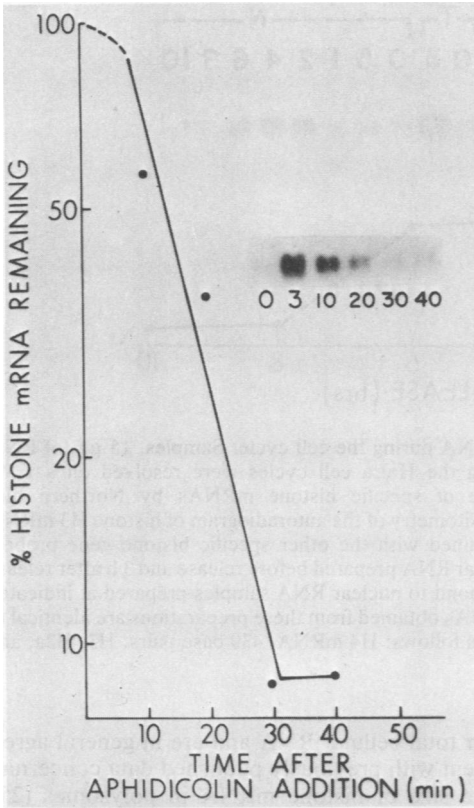

FIG. 6. Decay of histone mRNA after inhibition of DNA synthesis. Autoradiogram of histone H2a mRNA prepared at 10-min intervals after the aphidicolin block and semilogarithmic plot of quantity of H2a mRNA remaining at the times indicated. Autoradiographic lanes labeled 0 and 3 indicate RNA samples prepared prerelease and 3 h post-release (time at which the secondary aphidicolin block was made); lanes labeled 10 to 40 correspond to minutes after aphidicolin addition.

encompassing both of the above models is that initial activation of the histone genes alters their conformation such that they are made accessible to the transcription apparatus and that, when generalized DNA synthesis is interrupted, they return to this repressed, preactivated state. According to this model, reactivation of nonexpressed histone genes may require that cells again traverse the G1/S boundary. It was of interest, therefore, to determine whether cells blocked in DNA synthesis during the middle of S phase for an extended period could retain their ability to accumulate histone mRNA upon release from the block and resumption of DNA synthesis.

In the experiments shown in Fig. 7, synchronized HeLa cells were released into S phase for 3 h, blocked with aphidicolin for 3 h, and then rereleased to complete S phase. The rate of [^{3}H]deoxycytidine incorporation into DNA was reduced to its prerelease value upon the addition of aphidicolin at 3 h and, after rerelease into fresh media after 3 h in the presence of the drug, rapidly increased to high levels (Fig. 7). If one monitors DNA synthesis for several additional hours, the cells complete S phase in approximately 11 to 12 h. It appears, therefore, that the 3-h interruption of DNA synthesis during its peak in early S phase does not grossly alter the metabolism of the cells. Rather, one might consider this treatment as reversibly "freezing" the cells at any point during the DNA synthetic period of the cell cycle.

The steady-state level of histone H2a mRNA decreases to its prerelease level within 30 min after the aphidicolin treatment 3 h into S phase (Fig. 7). Furthermore, H2a mRNA levels remain low during the aphidicolin treatment (maintained for a total of 3 h) and, upon rerelease from the aphidicolin block, histone H2a mRNA again accumulates with the same kinetics seen after release at the G1/S boundary. It is apparent,

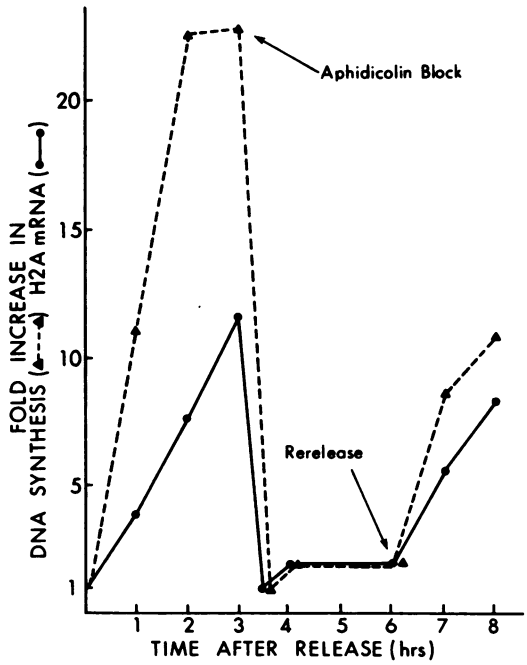

FIG. 7. Effects of aphidicolin treatment on histone mRNA levels during S phase. HeLa cells were synchronized and released into S phase as described in the text. Aphidicolin was added into the culture 3 h after release, the cells were incubated with the drug for 3 h, and the aphidicolin was again removed. Total cellular RNA was prepared and quantitated at various times during the experiment as described for Fig. 3. Symbols: ●, H2a mRNA; ▲, [^{3}H]deoxycytidine incorporation.

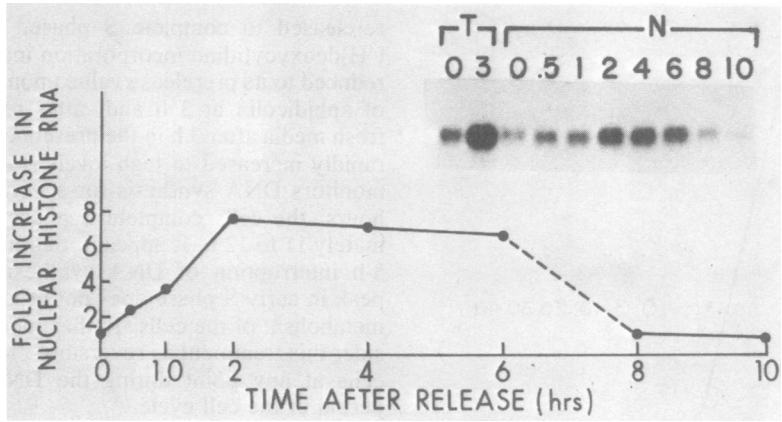

FIG. 8. Kinetics of accumulation of nuclear histone mRNA during the cell cycle. Samples, 15 μg, of RNA prepared from the nuclei of cells at various times during the HeLa cell cycles were resolved on a 1.5% formaldehyde-agarose gel and assayed for the presence of specific histone mRNAs by Northern blot hybridization. The quantitation plotted was obtained by densitometry of the autoradiogram of histone H3 mRNA as described in the text and is very similar to those obtained with the other specific histone gene probes. Autoradiographic lanes labeled T:0 and 3 indicate total cellular RNA prepared before release and 3 h after release from the aphidicolin block. Lanes labeled N:0 to 10 correspond to nuclear RNA samples prepared at indicated hours after release from block. The sizes of the histone mRNAs obtained from these preparations are identical to those derived from analysis of total cellular RNA and are as follows: H4 mRNA, 430 base pairs; H3, H2a, and H2b mRNA, 560 base pairs.

therefore, that those mechanisms operating to effect the accumulation of histone mRNA during S phase are not restricted to the G1/S boundary of the cell cycle, but remain active throughout S phase. Although this experiment does not indicate the relative contribution of alterations in transcription rate and in mRNA stability to the reaccumulation of histone mRNA, the facts that histone mRNA synthesis decreases to its prerelease rate within 30 min after the aphidicolin treatment and that the kinetics of accumulation parallel those seen at the G1/S boundary strongly suggest that the mechanisms effecting accumulation of histone mRNA during these two time periods are identical.

Accumulation of histone RNA in HeLa cell nuclei. It is clear from the analysis of total cellular RNA present in Fig. 3 that cell cycle-dependent histone synthesis is directly controlled by changes in the abundance of total cellular histone mRNA. To reveal any gross changes in the transport of histone RNA from the nucleus, and as an assay for potential histone mRNA precursors, we have analyzed nuclear histone RNA during the cell cycle by Northern hybridization. The kinetics of accumulation of histone RNA in the nucleus of HeLa cells during S phase did not parallel those observed for total cellular RNA (Fig. 8). Rather, nuclear histone RNA reached its maximal level by the second hour of S and remained at that level until the end of S phase. The sizes of histone RNAs detected in the nucleus were identical to those observed for total cellular RNA and are in general agreement with previously published data concerning the size of histone mRNA in polysomes (25). Finally, quantitation of the gel shown in Fig. 8 allowed us to calculate that approximately 1 to 2% of the total cellular histone RNA was present in the nucleus at the peak of accumulation during S phase.

DISCUSSION

The isolation and characterization of human histone genes have provided specific hybridization probes which have allowed us to use direct and quantitative methodology to confirm the general result that HeLa cell histone mRNA accumulation is coupled to DNA synthesis (3, 5, 8, 25), and to extend these results to reveal important aspects of cell cycle-dependent histone gene regulation.

The increase in steady-state concentrations of histone mRNA during the HeLa cell S phase is approximately 15-fold. Cells blocked at the G1/S boundary of the cell cycle, cells blocked within S phase, and growing cells not engaged in DNA synthesis all contain low but significant levels of histone H2a, H2b, H3, and H4 mRNA. Several possible explanations, including an incomplete block of DNA synthesis in the presence of aphidicolin, can account for this result. However, we note that there may exist a subpopulation of human histone genes which are not regulated according to the cell cycle and that are responsible for the low level of non-S-phase histone

mRNA (29). Direct nucleotide sequence analysis of non-S- and S-phase histone mRNA populations may result in the identification of such constitutively expressed histone sequences.

To determine the degree to which transcriptional and post-transcriptional processes are responsible for histone mRNA accumulation, we have measured both the rates of synthesis and the half-life of histone mRNA during or in the absence of DNA synthesis. Quantitation of nascent histone mRNA synthesized during a 5-min pulse-labeling in vivo suggests that the rate of histone mRNA synthesis is three- to fourfold higher in cells at the point of maximal rate of accumulation of histone mRNA (2.5 into S phase) than in cells blocked at the G1/S boundary. Although careful kinetic studies of the rate of histone mRNA synthesis during the cell cycle must be done to ensure that this is the maximal increase in the rate of transcription of these genes, it is evident that transcriptional induction cannot fully explain the accumulation of histone mRNA. Furthermore, our studies of the half-life of histone mRNA after a block in DNA synthesis (8 min) or during S phase (40 min) suggest that the stability of histone mRNA may be increased by as much as fivefold during DNA synthesis. Since changes in the rate of synthesis of histone mRNA during the beginning of S phase or the time required for equilibration of nucleoside triphosphate pools could both result in a lengthened approach to equilibrium during continuous labeling, this change in the half-life of histone mRNA must be regarded as maximal.

The kinetics of accumulation of both cytoplasmic and nuclear histone mRNA suggest that histone gene expression remains elevated throughout S phase. This conclusion is further supported by the observations that cytoplasmic histone mRNA sequences continue to accumulate until at least 5 h after entry into S phase and that the apparent half-life (90 min) of histone mRNA during its disappearance in the latter half of S phase is greater than the maximal measured half-life of histone mRNA (40 min). Moreover, if the transit time of histone mRNA from the nucleus to the cytoplasm is constant throughout the cell cycle, then the kinetics of synthesis of histone mRNA should parallel nuclear histone mRNA accumulation. On the basis of this assumption, the kinetics of accumulation of nuclear histone mRNA support the conclusion that histone mRNA synthesis is maximal by the second hour of S phase and continues at that rate until the end of S phase.

As an integral part of this study, we wished to examine the temporal coordination of changes in DNA synthesis and histone mRNA concentration. Our results suggest that the relationship between the rate of [^{3}H]deoxycytidine incorporation into DNA and histone mRNA accumulation is not simple. Comparison of the kinetics of these processes reveal that histone mRNA accumulation continues for several hours beyond the peak of [^{3}H]deoxycytidine incorporation and that the biphasic nature of the latter is not reflected by the changing steady-state concentrations of histone mRNAs. It is clear, therefore, that if the rate of cellular DNA synthesis is directly correlated with histone mRNA accumulation, then the incorporation of [^{3}H]deoxynucleotide triphosphates into DNA does not adequately assay this rate. A disparity between the peak of DNA synthesis and maximal accumulation of yeast histone mRNA has been reported by Hereford et al. (15).

It is significant that histone mRNA accumulation can occur immediately after release from an extended block in DNA synthesis during the middle of S phase. The similarity of the kinetics of accumulation of histone mRNA seen after release from such a block to those seen after release from a block at the G1/S boundary, and the decline of histone gene transcription rate to its pre-S-phase value during a secondary DNA synthetic block, suggest that in both instances equivalent transcriptional and post-transcriptional mechanisms govern changes in histone mRNA concentration. As described in Results, this is an important consideration with respect to models proposing the coupling of histone gene transcription and DNA replication (5), since it suggests either that histone genes retain some component of activation, which is required but is not sufficient for histone gene transcription, or that the genes may become transcriptionally "reactivated" after release from the mid-S-phase block. Clearly, an analysis of the temporal relationship between the transcriptional competence and replication of individual histone genes will facilitate our understanding of the mechanism(s) responsible for cell cycle-dependent expression of this gene family.

In summary, our results indicate that cell cycle-dependent histone gene expression is mediated at both the transcriptional and the post-transcriptional levels. Formulation of specific models to explain the mechanisms for these processes is complicated by the fact that human histone genes are reiterated in the genome and that the expression of individual members of this gene family may be regulated in different ways. Recently, Hereford et al. (14, 15) have examined the yeast cell cycle with respect to the contributions of transcriptional and post-transcriptional events in moderating histone mRNA levels. Changes in histone mRNA stability are clearly operative during both the HeLa and the yeast (15) cell cycles. However, in contrast to our conclusions, there is indication (14) that in-

creased transcription of the yeast histone genes may precede the onset of DNA synthesis. Whereas it is possible that the transcriptional activation of a specific subpopulation of HeLa histone genes is regulated in this manner, we believe that the simplest interpretation of our work is that human histone gene transcription is reversibly induced severalfold by DNA synthesis per se, and that post-transcriptional processes ensure that histone mRNA abundance is regulated to provide for the changing requirements for histone protein during S phase.

Finally, it has very recently been reported that the levels of nuclear and cytoplasmic histone RNA in S-phase HeLa S_3 cells is approximately 100-fold greater than that present in G1 cells (22). The explanations for the discrepancies between these results and those presented herein remain obscure. It is possible, however, that the levels of histone RNA in non-S-phase cells differ in the cell populations used because of the methods of synchrony, particular cell type, or details of the methods of analysis of the various RNA populations.

ACKNOWLEDGMENTS

We are grateful to members of this laboratory for helpful discussion and criticism. We thank Irving Boime for providing plasmid pCGα and Robert Darnell for discussion of and assistance with the cell cycle synchronization experiments.

N.H. is the recipient of a Public Health Service postdoctoral fellowship from the National Institutes of Health and was a Damon Runyon-Walter Winchell Cancer Fund postdoctoral fellow. This work was supported by Public Health Service research grants CA23615 and CA16640 from the National Cancer Institute and by a Henry Dreyfus Teacher-Scholar Award to R.G.R.

LITERATURE CITED

1. **Babich, A., and J. R. Nevins.** 1981. The stability of early adenovirus mRNA is controlled by the viral 72 Kd binding protein. Cell **26**:371–379.
1a. **Boime, I., M. Boothby, M. Hoshina, S. Daniels-McQueen, and R. B. Darnell.** 1982. Expression and structure of human placental hormone genes as a function of placental development. Biol. Reprod. **26**:73–91.
2. **Boothby, M., R. Luddon, C. Anderson, D. McWilliams, and I. Boime.** 1981. Single gonadotropin α subunit gene in normal tissue and tumor-derived cell lines. J. Biol. Chem. **256**:5121–5127.
3. **Borun, T. W., F. Gabrielli, K. Asiro, A. Zwiedler, and C. Baglioni.** 1975. Further evidence of transcriptional and translational control of histone messenger RNA during the HeLa S3 cycle. Cell **4**:59–67.
4. **Borun, T. W., M. D. Scharff, and E. Robbins.** 1967. Rapidly labeled polyribosome associated RNA having the properties of histone messenger. Proc. Natl. Acad. Sci. U.S.A. **58**:1977–1983.
5. **Butler, W. B., and G. C. Mueller.** 1973. Control of histone synthesis in HeLa cells. Biochim. Biophys. Acta **294**:481–496.
6. **Chen-Kiang, S., A. J. Wolgemuth, M.-T. Hsu, and J. E. Darnell.** 1982. Transcription and accurate polyadenylation *in vitro* of RNA from the major late adenovirus 2 transcription unit. Cell **28**:575–584.
7. **Derman, E., K. Krauter, L. Walling, C. Weinberger, M. Ray, and J. E. Darnell.** 1981. Transcriptional control in the production of liver-specific mRNAs. Cell **23**:731–739.
8. **Detke, S., A. Lichtler, I. Hillips, J. Stein, and G. Stein.** 1979. Reassessment of histone gene expression during cell cycle in human cells using homologous H4 histone cDNA. Proc. Natl. Acad. Sci. U.S.A. **76**:4995–4999.
9. **Gallwitz, A., and G. C. Mueller.** 1969. Histone synthesis *in vitro* on HeLa cell microsomes. J. Biol. Chem. **244**:5947–5952.
10. **Glisin, V., R. Crkvenjakov, and C. Byus.** 1974. Ribonucleic acid isolated by cesium chloride centrifugation. Biochemistry **13**:2633–2637.
11. **Greenberg, J. R.** 1972. High stability of messenger RNA in growing cultured cells. Nature (London) **240**:102–104.
12. **Harpold, M. M., R. M. Evans, M. Solditt-Georgieff, and J. E. Darnell.** 1979. Production of mRNA in Chinese hamster cells: relationship of the rate of synthesis to the cytoplasmic concentration of nine specific mRNA sequences. Cell **17**:1025–1035.
13. **Heintz, N., M. Zernik, and R. G. Roeder.** 1981. The structure of the human histone genes: clustered but not tandemly repeated. Cell **24**:661–668.
14. **Hereford, L., S. Bromley, and M. A. Osley.** 1982. Periodic transcription of yeast histone genes. Cell **30**:305–310.
15. **Hereford, L., M. A. Osley, J. R. Ludwig II, and C. S. McLaughlin.** 1981. Cell cycle regulation of yeast histone mRNA. Cell **24**:367–375.
16. **Huberman, J.** 1981. New views of the biochemistry of eukaryotic DNA replication revealed by aphidicolin, an unusual inhibitor of DNA polymerase α. Cell **23**:647–648.
17. **Hussa, R. O.** 1980. Biosynthesis of human chorionic gonadotropin. Endocr. Rev. **1**:268–294.
18. **Liberti, P., A. Festucci, and C. Baglioni.** 1973. Minimum estimate of the lifetime of histone messenger RNA of HeLa cells. Mol. Biol. Rep. **1**:61–67.
19. **Nilsen, T., and C. Baglioni.** 1979. Unusual base-pairing of newly synthesized DNA in HeLa cells. J. Mol. Biol. **133**:319–338.
20. **Pederson, T.** 1976. Cellular aspects of histone synthesis, p. 69–123. *In* E. H. McConkey (ed.), Protein synthesis. Marcel Dekker, New York.
21. **Pedrali-Noy, G., S. Spadari, A. Miller-Faures, A. O. A. Miller, J. Kruppa, and G. Koch.** 1980. Synchronization of HeLa by inhibition of DNA polymerase α with aphidicolin. Nucleic Acids Res. **8**:377–387.
22. **Rickles, R., F. Marashi, F. Sierra, S. Clark, J. Wells, J. Stein, and G. Stein.** 1982. Analysis of histone gene expression during the cell cycle in HeLa cells by using cloned human histone genes. Proc. Natl. Acad. Sci. U.S.A. **79**:749–753.
23. **Robbins, E., and T. W. Borun.** 1967. The cytoplasmic synthesis of histones in HeLa cells and its temporal relationship to DNA replication. Proc. Natl. Acad. Sci. U.S.A. **58**:1977–1983.
24. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
25. **Stephens, R. E., C. J. Pan, K. Asiro, T. W. Dolby, and T. W. Borun.** 1977. Studies on human histone messenger RNA. J. Biol. Chem. **252**:166–172.
26. **Thomas, P. S.** 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. U.S.A. **77**:5201–5205.
27. **Wahl, G. S., M. Stern, and G. M. Stark.** 1979. Efficient transfer of large DNA fragments from agarose gels to diazobenzylmethyl-paper and rapid hybridization by using dextran sulfate. Proc. Natl. Acad. Sci. U.S.A. **76**:3683–3687.
28. **Weil, P. A., J. Segall, B. Harris, S. Ng, and R. G. Roeder.** 1979. Faithful transcription of eukaryotic genes by RNA polymerase III in systems reconstituted with purified DNA templates. J. Biol. Chem. **254**:6163–6173.
29. **Wu, R. S., and W. M. Bonner.** 1981. Separation of basal histone synthesis from S-phase histone synthesis in dividing cells. Cell **27**:321–330.