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**Coordinate regulation of multiple histone mRNAs during the cell cycle in HeLa cells**

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Received 4 January 1983; Revised and Accepted 20 March 1983

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**ABSTRACT**

Core histone gene expression in HeLa S3 cells has been examined as a function of the cell cycle using cloned human histone gene probes. Total cellular histone mRNAs were analyzed by Northern blot analysis, and their relative abundance shown to be temporally coupled to DNA synthesis rates in S phase. The *in vivo* incorporation of <sup>3</sup>H-uridine into at least fifteen heterologous histone mRNAs (in one hour pulse intervals at various times in the cell cycle), was monitored by hybrid selection. Hybridized RNAs were eluted and resolved electrophoretically to give both a quantitative and qualitative assay for multiple mRNA species. Maximal incorporation of <sup>3</sup>H-uridine into histone mRNAs precedes their maximal accumulation, indicating that transcriptional regulation is predominant in early S phase. The turnover of histone mRNAs in late S occurs in the presence of a reduced apparent transcription rate, indicating that post-transcriptional regulation is predominant in late S. All the detected multiple histone mRNAs are coordinately regulated during the HeLa cell cycle.

**INTRODUCTION**

Several lines of evidence suggest that the biosynthesis of the core histone proteins (H2A, H2B, H3 and H4) is temporally coupled with DNA replication in most eukaryotic cells. For example, the histones are found complexed to nuclear DNA in a relatively invariant molar ratio (1), and with the exception of *Xenopus* oocytes (2), no unbound cellular pools of histone proteins have been identified. The synthesis of histone proteins during the DNA synthesis phase of the cell cycle (S-phase) and their rapid sequestration into the nucleus to complex with newly replicated DNA has been observed in HeLa cells (3-5), hamster fibroblast cells (6) and in yeast (7), although there has been one contradictory report (8). Attempts to uncouple DNA synthesis and histone protein translation using inhibitors of DNA replication such as cytosine arabinoside (9) and hydroxyurea (10) have been singularly unsuccessful.

Histone mRNAs have been assayed by *in vitro* translation of polysomal

mRNAs (9,11), hybridization to histone cDNA (12), and by hybridization to cloned genomic histone gene probes (13, 14) in order to determine the mechanism of coupling. These studies indicate that the cellular abundance of histone mRNAs available for translation is the limiting factor in histone protein synthesis, and it is this abundance which is apparently related to DNA replication. Early pulse labelling experiments suggested that histone gene transcription in HeLa cells occurs predominantly during S phase (15); however, recent studies indicate that histone gene transcription may precede DNA replication in yeast (16).

The equimolarity of the four core histone proteins in the cell implies coordinate gene expression, and the notion of polycistronic transcription of clustered histone genes as a possible regulatory mechanism has stimulated the investigation into their genomic organization (17). Although sea urchin histone genes are organized as highly reiterated tandem repeats containing one of each of the five histone genes, transcription of each gene is initiated separately (18). Furthermore, these repeats are only expressed in early development, with a distinct, less reiterated set of histone genes being expressed in "late" embryos (19, 20). In *Drosophila*, on the other hand, only three of five histone genes in the same tandem repeat are transcribed from the same DNA strand (21). *Xenopus* histone genes occur as variant clusters containing several different gene orders (22, 23), a polymorphic organization which has also been observed in yeast (24), chicken (25, 26) and in human (27, 28). The putative selective advantage of conserved tandem gene repeats has therefore not been a major influence in determining histone gene organization in higher eukaryotes, and divergence of coding and noncoding sequences has produced coding sequence heterology, a polymorphic genomic organization and nonallelic variant histone genes (for review see ref. 17). It is now also recognized that although histone amino acid sequences have evolved relatively slowly most eukaryotes have structural variants of the H2A, H2B and H3 proteins, some of which may be differentially synthesized during the cell cycle (29) or differentially expressed during development and differentiation (30, 31).

It is generally accepted that there are approximately 40 copies of each of the four core histone genes per haploid genome in human cells (32), and their polymorphic organization is illustrated by the identification of at least seven distinct human histone gene clusters by two independent laboratories (27, 28). We have described the isolation and characterization of seven  $\lambda$ Ch4A recombinant phages containing human histone gene sequences which

fall into three distinct structural groups based on their restriction maps, and on their ability to protect different HeLa S3 histone H4 mRNAs from S1 nuclease digestion after hybridization in solution (28, 33). We have also resolved at least seven heterologous HeLa histone H4 mRNAs, only three of which are coded by the H4 genes present in our cloned histone gene clusters (33).

In this study we have used cloned human histone gene probes to examine the abundance of histone mRNAs, both total and pulse-labelled, as a function of the cell cycle in HeLa S3 cells. Our basic premise was that the study of histone transcripts required the analysis of expression of approximately 160 genes coding for four distinct proteins and their variants, with multiple mRNAs coding for the same or very similar proteins. The multiplicity of histone mRNAs in HeLa S3 cells permits the analysis of transcripts coded by heterologous genes for any one histone protein, and thus it is possible to determine whether coordinate regulation is operative. We describe an experimental protocol which permits the resolution of more than fifteen different histone gene transcripts, and use the technique to show that the relative abundance of histone mRNAs is coordinately regulated at both the transcriptional and post-transcriptional levels during the cell cycle in HeLa S3 cells.

## MATERIALS AND METHODS

### Materials

Uridine [5,6-<sup>3</sup>H] (40.5 Ci/mmol), [ $\alpha$ -<sup>32</sup>P]-dCTP (300-500 Ci/mmol) and En<sup>3</sup>Hance were purchased from New England Nuclear; [Methyl-<sup>14</sup>C] Thymidine (60mCi/mmol) was from Amersham; polyvinyl sulphonic acid (PVS) and X-ray films were from Eastman Kodak Co., Proteinase K was from E. Merck, and restriction endonucleases were purchased from Bethesda Research Laboratories, Inc. or from New England Biolabs.

### Cell culture, synchronization and pulse labelling

HeLa S3 cells were grown in suspension culture and were synchronized by two successive treatments with 2mM thymidine or by selective mitotic detachment as described (34).

Rates of DNA synthesis in synchronized cells were monitored by pulse labelling  $10^6$  cells with 0.2 $\mu$ Ci of <sup>14</sup>C-thymidine for 20 minutes and measuring the incorporation of radioactivity into 10% (w/v) trichloroacetic acid-insoluble material (35).

Total cellular RNA in synchronized HeLa S3 cells was pulse labelled

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by incubating  $2.5 \times 10^6$  cells/ml in the presence of 0.1 mCi of  $^3\text{H}$ -Uridine/ml at  $37^\circ\text{C}$  for one hour. Cells were then washed with ice-cold spinner salts and processed immediately.

### Preparation of Total Cellular RNA

$5 \times 10^7$  cells were lysed in 4.5 mls of a solution containing 1.3mM Tris-HCl (pH 7.4), 0.7mM EDTA,  $1.3\mu\text{gml}^{-1}$  PVS, 2.4% (w/v) SDS and  $0.9\text{mgml}^{-1}$  proteinase K. After a 15 minute incubation at room temperature, and the addition of 0.3 mls of a 5M NaCl solution, the aqueous phase was extracted twice with 2 volumes of buffered phenol: chloroform: isoamyl alcohol (25:24:1, v/v/v), and once with one volume of chloroform: isoamyl alcohol (24:1, v/v). Nucleic acids were precipitated with 3 volumes of ethanol at  $-20^\circ\text{C}$  in the presence of 53mM potassium acetate.

Nucleic acids were recovered by centrifugation, resuspended in 2mls of 10mM Tris-HCl (pH7.4), 2mM  $\text{CaCl}_2$ , 10mM  $\text{MgCl}_2$  and incubated at  $37^\circ\text{C}$  for 20 minutes in the presence of  $0.1\text{mgml}^{-1}$  of DNase I (Sigma, electrophoretically pure) which had been pretreated with proteinase K for 2 hours as described by Tullis and Rubin (36). After addition of 0.05 volumes of 5M NaCl and 0.25 volumes of 10% (w/v) SDS, the RNA solution was extracted with phenol and chloroform, and ethanol precipitated as described above.

### Northern Blot Analysis of Total Cellular RNA

Total cellular RNA (50 $\mu\text{g}$ ) was resolved electrophoretically in 1.5% (w/v) agarose gels containing 6% (w/v) formaldehyde as described (37), except that 6% (w/v) formaldehyde, 50mM boric acid, 5mM sodium borate, 10mM sodium sulphate, 1mM EDTA was used as the electrolyte. RNA was transferred to nitrocellulose filters in 20 x SSC (3M NaCl, 0.3M sodium citrate (pH 7.0)) as recommended by Southern (38), and the filters then baked in vacuo for 2 hours at  $80^\circ\text{C}$ .

Filters were prehybridized at  $45\text{--}50^\circ\text{C}$  for 5-6 hours in hybridization buffer: 50% (w/v) formamide, 5 x SSC,  $100\mu\text{gml}^{-1}$  *E. coli* DNA, and 0.5 x Denhardt's solution (10 x Denhardt's is 0.2% (w/v) ficoll, 0.2% (w/v) polyvinylpyrrolidone). Hybridization was performed at  $45\text{--}50^\circ\text{C}$  for 36-40 hours in hybridization buffer containing  $10^6$  cpm/ml of thermally denatured probe. The probe was prepared by nick-translation with [ $^{32}\text{P}$ ] dCTP as described by Maniatis et al (39).

Prior to autoradiography filters were washed at  $60^\circ\text{C}$  for 30 minutes with 200 mls each of the following: i) 5 x SSC, 1 x Denhardt's; ii) 2 x SSC, 0.1% (w/v) SDS; iii) 1 x SSC, 0.1% (w/v) SDS; and iv) 0.1 x SSC, 0.1% (w/v) SDS. Filters were exposed to preflashed XAR5 or XRPI X-Ray film at  $-70^\circ\text{C}$

for 20-40 hours in cassettes containing Kodak regular intensifying screens. Hybridization was quantitated by densitometry and/or cutting out areas of the nitrocellulose filter containing bands, dissolving them in 1ml of cellosolve, and determining radioactivity by liquid scintillation spectrometry after addition of 10 ml of cellosolve cocktail (35). Differential quenching was corrected by external standardization.

#### Purification of cloned human histone genomic sequences

The isolation and characterization of  $\lambda$ Ch4A recombinants containing human histone genes have been described (28). EcoRI-EcoRI or EcoRI-HindIII restriction fragments of the recombinant DNAs have been subcloned into the plasmid pBR322 (see Fig. 1), transformed into *E. coli* strain HB101, and characterized further by hybrid selection - *in vitro* translation and partial sequencing (unpublished results). All recombinant DNA manipulations were carried out in accordance with the guidelines established by the National Institutes of Health.

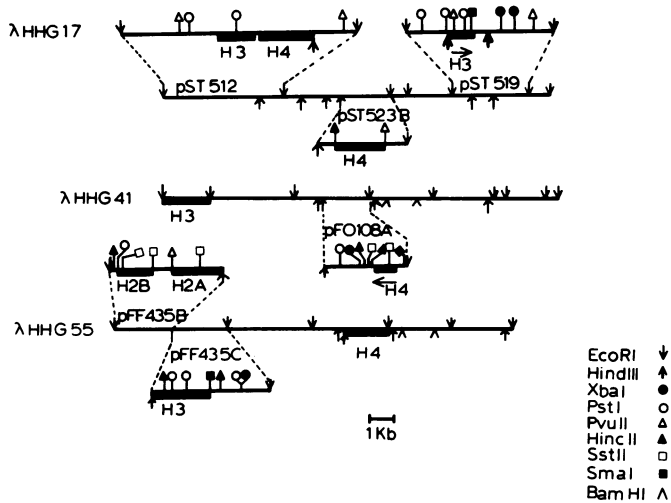
Plasmid DNA was isolated by the sarkosyl lysis procedure and purified further by CsCl buoyant density gradient centrifugation essentially as described by Clewell and Helinski (40). Plasmid DNA was restricted with Eco RI and/or Hind III endonucleases under conditions recommended by the suppliers.

#### Hybrid selection of pulse-labelled total cellular RNA

Restricted plasmid DNA (50 $\mu$ g) was denatured in 0.3M NaOH and bound to nitrocellulose filters (Millipore GSWP01300) in 0.15M NaOH, 1M  $\text{NH}_4\text{CH}_3\text{COO}$  as described (41). Filters were pretreated at 43-48°C for 20 hours in hybridization buffer (50%(w/v) formamide, 0.5% (w/v) SDS, 0.5M NaCl, 1mM EDTA, 25mM Hepes (pH7.0)) containing 100 $\mu$ gml<sup>-1</sup> of thermally denatured *E. coli* DNA. Filters were washed and eluted prior to hybridization as described below. After pre-equilibrating with hybridization buffer for 60 minutes, filters were hybridized at 43-45°C for 20 hours with 200 $\mu$ l of hybridization buffer containing 0.25-0.75 $\mu$ g $\mu$ l<sup>-1</sup> of <sup>3</sup>H-labelled total cellular RNA. Filters were then washed with 0.5ml of each of the following solutions for 10 minutes: i) twice with hybridization buffer at 50°C; ii) twice with 0.5M NaCl, 1mM EDTA, 25mM Hepes (pH 7.0), 0.5% (w/v) SDS at room temperature; iii) three times with 0.1 x SSC, 0.5% (w/v) SDS at 60°C; and iv) three times with 10mM Hepes (pH7.0) at room temperature. RNA hybrids were eluted three times with 100 $\mu$ l of 90% (w/v) formamide, 0.5M NaCl, 1mM EDTA, 25mM Hepes (pH 7.0) at 72°C for 5 minutes.

Pooled eluates were quantitated for radioactivity by liquid scintillation

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**Figure 1.** Restriction endonuclease maps of  $\lambda$ Ch4A recombinants containing human histone gene clusters isolated and characterized as described (28), and fine restriction mapping of fragments subcloned into pBR322. Histone coding sequences were identified by hybrid selection-*in vitro* translation, hybridization to homologous and heterologous probes and partial sequencing.

spectrometry in 10mls of triton/toluene cocktail and quantitated by subtracting the radioactivity bound to control filters containing immobilized plasmid pBR322 DNA hybridized in parallel.

Alternatively, the pooled eluates were diluted by adding 1 volume of a solution containing 0.2M NaCl and  $0.34\mu\text{gml}^{-1}$  yeast tRNA, and ethanol precipitated at  $-70^{\circ}\text{C}$ . The RNA was recovered by centrifugation, and electrophoretically resolved on 6% (w/v) polyacrylamide, 50% (w/v) urea gels electrophoresed with a surface temperature of  $50-60^{\circ}\text{C}$  as described by Lichtler et al (33). Gels were soaked in  $\text{En}^3$ Hance for one hour, then in water for one hour, and dried for fluorography with preflashed XAR5 X-ray film at  $-70^{\circ}\text{C}$  for 3-14 days.

Additional quantitation was achieved by densitometry, and by liquid scintillation spectrometry of gel slices in 10mls of triton/toluene scintillation cocktail. Differential quenching was corrected by external standardization.

**RESULTS**

The Abundance of Core Histone mRNA in Synchronized HeLa S3 cells

Given the multiplicity of histone mRNAs, it was imperative to establish

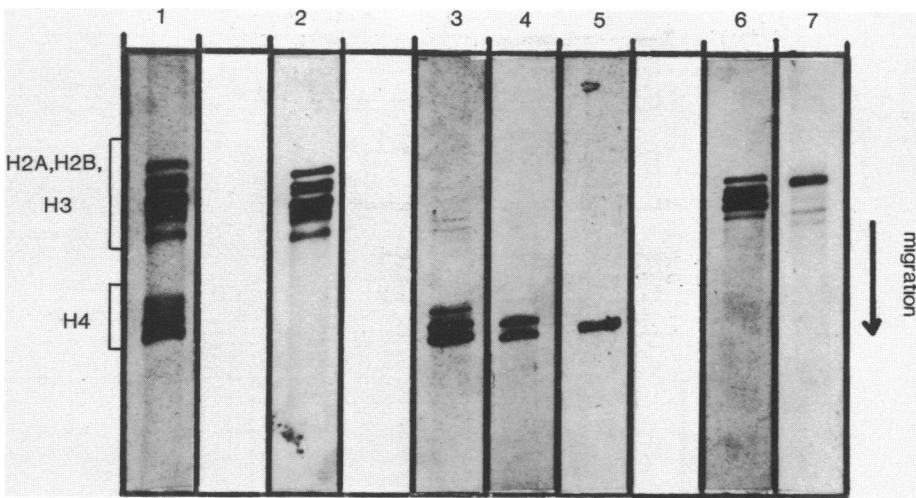


Figure 2. The effect of temperature on hybrid selection of histone mRNA variants. 150 $\mu$ g of *in vivo*  $^3$ H-uridine pulse labelled total cellular RNA was hybridized with 50 $\mu$ g of filter-immobilized cloned human histone sequences. Hybridized RNAs were eluted and resolved by denaturing 6% (w/v) polyacrylamide, 50% (w/v) urea gel electrophoresis and visualized by fluorography. lane 1) pF0108A (H4), pFF435B (H2A + H2B) and pFF435C (H3) hybridized at 43 $^{\circ}$ C; lane 2) pFF435B (H2A + H2B) hybridized at 45 $^{\circ}$ C; lane 3) and 4) and 5) pF0108A (H4) hybridized at 43 $^{\circ}$ C, 45 $^{\circ}$ C and 48 $^{\circ}$ C respectively; and lanes 6) and 7) pFF435C hybridized at 45 $^{\circ}$ C and 48 $^{\circ}$ C respectively.

that, using the available cloned genomic probes (see Fig. 1), we were detecting as many histone mRNA subspecies as possible in synchronized cells. Examples of the variable sequence homology of different histone mRNAs are shown in Fig. 2 where *in vivo*  $^3$ H-uridine pulse-labelled total cellular RNA was hybridized to filter-immobilized plasmid DNAs containing histone gene sequences, the RNAs were then eluted, resolved by denaturing polyacrylamide gel electrophoresis and visualized by fluorography. Several distinct mRNA size classes are selected by each of the three probes used, but as shown for the histone H3 and H4 probes, the complexity of transcripts detected is dependent on the stringency of hybridization (43-48 $^{\circ}$ C hybridization temperature in 50% (w/v) formamide). At an intermediate temperature of hybridization (45 $^{\circ}$ C) we detect over 15 distinct mRNA size classes which code for histone proteins as shown by *in vitro* translation (28), S1 nuclease mapping (33) and by the DNA sequences of the coding regions of the subcloned genomic fragments.

In order to compensate for the variable sequence homology between

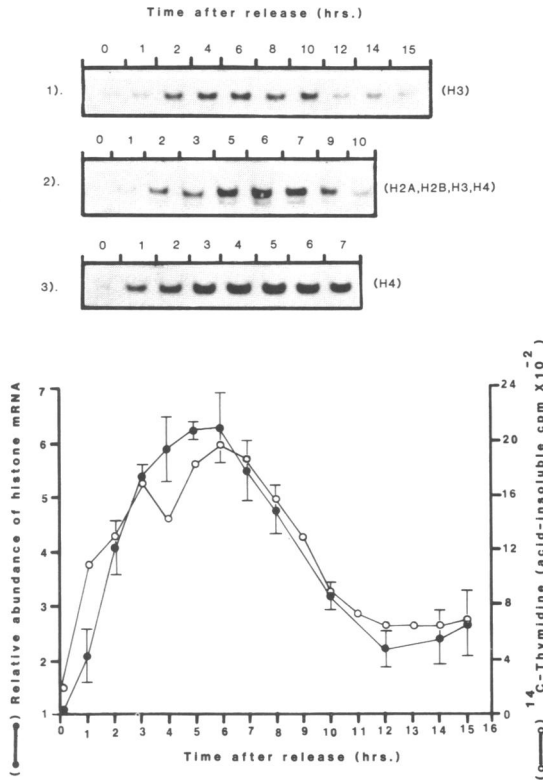


Figure 3. a) Northern blot analysis of total cellular histone mRNA in HeLa cells after synchronization by two cycles of 2mM thymidine treatment. At various times after release from the second thymidine block, total cellular RNA was isolated, 50µg of RNA from each preparation was resolved by 1.5% (w/v) agarose, 6% (w/v) formaldehyde gel electrophoresis, and the RNAs were transferred to nitrocellulose. Filters were hybridized at 48°C in 50% (w/v) formamide to one of three nick-translated probes. 1) pFF435C (H3); 2) pFF435(B+C)(H2A, H2B and H3) and p2.6H (chicken H3+H4); 3) pF0108A (H4) and hybrids visualized by autoradiography. b) The relative abundance of histone mRNAs (●—●, ±S.E.M.) in cells released into S phase after synchronization by two cycles of thymidine treatment, and assayed by Northern blot analysis. Hybrids were quantitated for 9 Northern blots by densitometry and/or liquid scintillation spectrometry, after hybridization with the five histone gene probes as described above, and in the text. Values represent the mean relative abundances of histone H4, H3 or H2A+H2B mRNAs. The rate of DNA synthesis (○—○) was monitored by pulse labelling 10<sup>6</sup> cells with 0.2µCi of <sup>14</sup>C-thymidine for 20 minutes and measuring the incorporation of radiolabel into acid-precipitable material.



variant transcripts, we adopted an essentially statistical approach to the analysis of total histone mRNA levels during the cell cycle. HeLa cells were synchronized at the G1-S phase boundary by exposure to two cycles of 2mM thymidine treatment, and total cellular RNA was isolated at various times as the cells progressed through S phase after release. Northern blot analysis of this RNA was then performed with four different cloned human histone gene probes, and a heterologous chicken histone gene fragment (p2.6H) kindly provided by J. Wells (25). Moreover, the stringency of hybridization was varied in order to vary the complexity of multiple transcripts detected with any given probe. Three northern blot analyses are shown in Fig. 3a, and the data for nine such blots from three independent cell synchronization experiments are quantitatively summarized in Fig. 3b. The consensus pattern for the relative abundance of histone mRNAs varied only within the limits of experimental error, indicating that the relative cellular levels of the detected core histone mRNAs are coordinately regulated. The rates of DNA synthesis were monitored in parallel by pulse-labelling with  $^{14}\text{C}$ -thymidine, and as shown in Fig. 3b, the relative abundance of histone mRNAs, that is, their total levels, are temporally coupled with the relative rates of DNA synthesis.

To eliminate the possibility that the temporal coupling was an artifact of the synchronization technique, an analogous experiment was performed with cells synchronized by selective mitotic detachment (Fig. 4a). The relative abundance of histone mRNAs in cells progressing from G1 to S after mitosis, and detected by Northern blot analysis, exhibits an equivalent dependence on the rates of DNA synthesis (Fig. 4b), indicating that the temporal coupling of histone mRNA levels and DNA synthesis rates is independent of the method of synchronization. Similar results have been observed following the stimulation of quiescent WI38 human fibroblasts to proliferate (42), and in yeast cells progressing into S phase after synchronization in G1 with the yeast mating pheromone (13), suggesting that the temporal coupling is neither a characteristic of the HeLa genotype, nor a characteristic of transformed human cells grown in culture.

The maximal levels of histone mRNAs in S phase represent a 6-7 fold increase compared with those found in cells immediately before release from the second thymidine block (Fig. 3). As this increase was measured relative to the mRNA levels found in cells blocked at the G1-S boundary by 2mM thymidine, it was of interest to determine whether the mRNA in cells at the G1-S boundary represented a basal pool of histone mRNAs whose

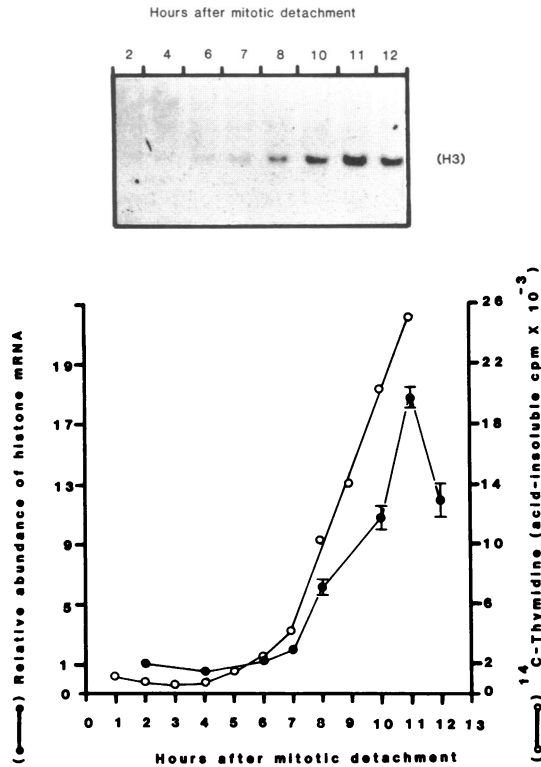
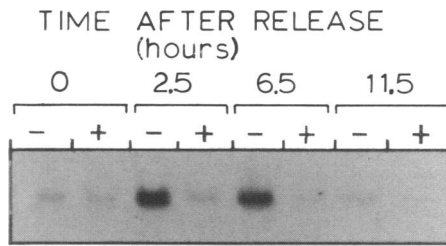


Figure 4. a) Northern blot analysis of total cellular histone mRNA in HeLa cells synchronized by selective mitotic detachment. At various times after mitotic detachment, total cellular RNA was isolated, 50µg of each was resolved by 1.5% (w/v) agarose, 6% formaldehyde gel electrophoresis, and the RNAs transferred to nitrocellulose. The filter was hybridized to nick-translated pST519 (H3) probe, and visualized by autoradiography.

b) Histone mRNA accumulation (●—●, ± S.E.M.) assayed for three Northern blots probed with pST519 (H3), pF0108A (H4), or pFF435B (H2A+H2B), quantitated as described in Fig. 3b. DNA synthesis rates (○—○) were monitored by measuring the incorporation of <sup>14</sup>C-thymidine into acid-precipitable material in a 20 minute pulse.

abundance is not regulated during the cell cycle, or whether this represented a "leaky" or inefficient arrest of DNA synthesis in cells at the G1-S boundary in the presence of 2mM thymidine. Wu and Bonner (29) have recently defined the "basal" level of histone protein synthesis in G1 as that which is relatively insensitive to the DNA synthesis inhibitor hydroxyurea. We therefore applied the same criterion to distinguish between basal levels of histone mRNAs and those which are due to the inefficient arrest of cells at the G1-S



**Figure 5.** Northern blot analysis of total cellular histone mRNA in HeLa cells synchronized by two cycles of 2mM thymidine treatment and further exposed at various times in S phase to 1mM hydroxyurea for 30 minutes. At 0, 2, 6 and 11 hours after release from thymidine cell aliquots were treated with 1mM hydroxyurea for 30 minutes, and total cellular RNA isolated from treated (+) and untreated (-) cells. 50 $\mu$ g of RNA from each preparation was resolved by 1.5% (w/v) agarose, 6% (w/v) formaldehyde gel electrophoresis, transferred to nitrocellulose, hybridized to nick-translated pF0108A (H4) at 48°C in 50% (w/v) formamide, and hybrids visualized by autoradiography. 1mM hydroxyurea inhibited the incorporation of  $^{14}$ C-thymidine into acid-precipitable material by more than 95% within 30 minutes of exposure.

boundary after synchronization. Synchronized cells at 0, 2, 6, or 11 hours after release from the second thymidine block were exposed to 1mM hydroxyurea for 30 minutes, total cellular RNA was isolated, and histone mRNA levels were determined by Northern blot analysis (43). By comparison with control cells not treated with hydroxyurea, we found that the relative abundance of histone mRNAs decreased to 30-60% of the levels found just prior to release from the second thymidine block (Fig. 5), irrespective of the levels observed in control cells. Thus, only some 50% of the total histone mRNAs found in cells arrested at the G1-S boundary by two cycles of thymidine treatment represents a basal pool which, by these criteria, is not S phase specific. Extrapolation indicates that cellular histone mRNA levels increase some 14-20 fold above "basal levels" during maximal rates of DNA synthesis in S phase, a stimulation observed in cells progressing from G1 to S after synchronization by selective mitotic detachment (Fig. 4).

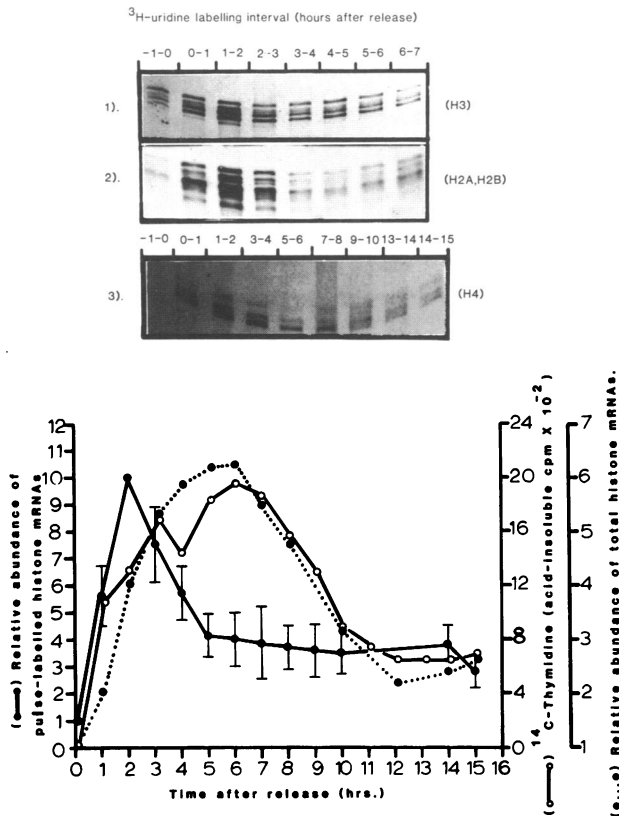
#### Histone mRNA Synthesis during the Cell Cycle

To determine whether the temporal coupling between histone mRNA levels and rates of DNA synthesis occurred at the transcriptional or post-transcriptional level, we measured the incorporation of  $^3$ H-uridine into histone mRNAs in one hour pulses at various times during the HeLa cell cycle. In principle, shorter pulse times should give a more definitive

indication of transcription rates; however, the one hour pulse-label yielded total cellular RNA preparations of a sufficiently high specific activity for the detection of  $^3\text{H}$ -uridine incorporation into individual histone mRNA variant size classes, and thus we might detect the differential regulation of specific members of the polymorphic histone gene family.

Pulse-labelled total cellular RNA was hybridized to filter-immobilized plasmid DNAs containing cloned human histone gene sequences and the eluted RNAs were resolved by denaturing polyacrylamide gel electrophoresis. This technique gives both a qualitative and quantitative assay for  $^3\text{H}$ -uridine incorporation into specific histone mRNA size classes, and also permits a direct evaluation of the specificity of hybridization. To maximize the complexity of mRNA variants hybridizing to any given immobilized probe, the temperature of hybridization was kept at 45°C in 50% (w/v) formamide (see Fig. 2) in order to compensate for variable mRNA sequence homology. Under these conditions we have observed a small amount of cross hybridization with ribosomal RNAs, attributed to the high G:C content of both ribosomal RNA and histone mRNAs. This cross hybridization can be eliminated by increasing the stringency of hybridization to above 48°C in 50% (w/v) formamide, but as shown in Fig. 2, this is at the expense of a population of variant histone mRNA size classes. For quantitation therefore, either RNA was hybridized at a higher stringency and the bound radioactivity monitored after the subtraction of radioactivity bound to control filter-immobilized pBR322 DNA treated identically; or, radioactivity was monitored in specific bands after electrophoretic resolution and fluorography of RNAs hybridized at the lower temperature.

To follow the incorporation of  $^3\text{H}$ -uridine into histone mRNAs, HeLa cells were synchronized by double thymidine blocks, pulse-labelled for one hour at various times after release into S phase, and total cellular RNA was then hybridized with cloned histone DNAs. The hybrid-selected RNAs were electrophoretically resolved as shown in Fig. 6a. There are no obvious qualitative differences at any time during S phase in the histone mRNA size classes selected by these three probes (pF0108A (H4), pFF435B (H2A + H2B) and pFF435C (H3), see Fig. 1), and the abundance of any one mRNA size class relative to another in the same sample does not change appreciably as cells traverse S phase. In total, five probes (pST512, pST519, pF0108A, pFF435B and pFF435C, see Fig. 1) were used to hybrid select pulse-labelled total cellular RNA from three synchronization experiments, and this same pattern was observed at two hybridization



**Figure 6.** Electrophoretic resolution of histone mRNAs pulse-labelled *in vivo* for one hour (for the times indicated) after synchronization of HeLa cells by double thymidine blocks.  $150\mu\text{g}$  of one hour  $^3\text{H}$ -uridine pulse labelled total cellular RNA was hybrid selected at  $45^\circ\text{C}$  with  $50\mu\text{g}$  of filter-immobilized plasmid DNA. Eluted RNAs were electrophoretically resolved on denaturing 6% (w/v) polyacrylamide, 50% urea gels, and visualized by fluorography. Histone mRNAs selected by 1) pFF435C (H3), 2) by pFF435B (H2A+H2B) and 3) by pF0108A (H4).

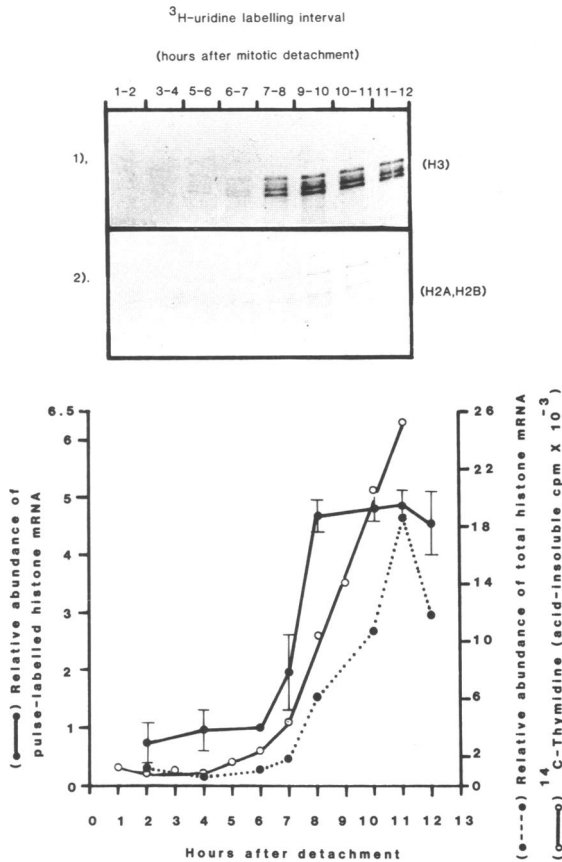
b) (●—●,  $\pm$  S.E.M.) Quantitation for 9 hybrid selections using five different histone gene probes as described in the text, and estimated as described in experimental procedures. Values are plotted at the termination of the pulse period, and represent the mean relative abundances of pulse-labelled histone H4, H3, or H2A+H2B mRNAs. DNA synthesis rates (○—○) and the relative abundance of total cellular histone mRNA (●.....●) are reproduced from Fig. 3b.

temperatures ( $45^\circ\text{C}$  and  $48^\circ\text{C}$ ), and over a three-fold concentration of cellular RNA ( $50\text{--}150\mu\text{g}$  of total cellular RNA/ $50\mu\text{g}$  of filter-immobilized plasmid DNA).

The results of nine such hybridizations are quantitatively summarized

in Fig. 6b, along with the rates of DNA synthesis monitored by pulse labelling with  $^{14}\text{C}$ -thymidine and the total histone mRNA levels detected by Northern blot analysis. The maximal relative abundance of pulse-labelled histone mRNAs of all size classes occurs in the second hour after release into S phase. As histone mRNA levels and DNA synthesis rates have only reached 50-60% of maximal levels at this time, we conclude that transcription is the predominant regulatory mechanism modulating the cellular abundance of core histone mRNAs in the first 6 hours of S phase. We have observed a 2-3 fold increase in  $^3\text{H}$ -uridine incorporation into total RNA in the first three hours after release from the thymidine block, but as this is 3-4 fold less than the apparent increase in  $^3\text{H}$ -uridine incorporation into histone mRNAs observed at that time (Fig. 6b), we conclude that there is a preferential stimulation of histone gene transcription at the onset of S phase. At five to six hours after release from the thymidine block (Fig. 6), a transition point is reached at which DNA synthesis rates and total histone mRNA levels are maximal; but the abundance of pulse-labelled mRNAs is reduced to approximately 30% of maximum. Between 6 and 11 hours after release, histone mRNA levels decrease almost linearly with an apparent half-life of about two hours, but the concentration of pulse-labelled histone mRNAs remains constant. We infer that both the rate of transcription and the mRNA turnover rate are approximately constant in late S phase, but the turnover rate exceeds the rate of transcription. In mid- to late S phase, therefore, the cellular abundance of histone mRNAs is predominantly modulated at the post-transcriptional level.

It is unclear whether the concentration of pulse-labelled histone mRNAs 6 hours after release represents an S phase specific "basal level" of transcription, or whether it reflects synchrony decay in mid- to late- S phase (Fig. 6). It was therefore of interest to examine  $^3\text{H}$ -uridine incorporation into histone mRNAs over a one hour pulse in cells progressing from G1 to S after synchronization by selective mitotic detachment. HeLa cells were pulse-labelled at various times after mitotic detachment, and total cellular RNA was hybrid-selected for histone mRNAs, which were then electrophoretically resolved as shown in Fig. 7a. We observed no obvious qualitative differences in the histone mRNA size classes detected as cells progress from G1 to S phase, nor was there a significant change in the abundance of one mRNA size class relative to another in the same sample. As these histone mRNAs are qualitatively equivalent to those described for cells synchronized at the G1-S boundary by double thymidine



**Figure 7.** a) Electrophoretic resolution of histone mRNAs pulse labelled *in vivo* for one hour ( at the times indicated) after synchronization of HeLa cells by selective mitotic detachment. 150µg of <sup>3</sup>H-uridine pulse labelled total cellular RNA was hybridized at 45°C with 50µg of filter-immobilized plasmid DNA. Bound RNAs were eluted and electrophoretically resolved on denaturing 6% (w/v) polyacrylamide, 50% urea gels, and visualized by fluorography. RNA selected by 1) pFF435C (H3) and 2) pFF435B (H2A+H2B). b) (●—●, ± S.E.M.) Quantitation for 3 hybrid selections using three probes (pFF435B (H2A+H2B), pFF435C (H3) and pF0108A (H4)), estimated as described in experimental procedures. Values are plotted at the end of the hour pulse label, and represent the mean relative abundances of pulse-labelled histone H4, H3, or H2A+H2B mRNAs. The DNA synthesis rates (○—○) and the relative abundance of total histone mRNAs (●.....●) are reproduced from Fig. 4b.

blocks (Fig. 6), we conclude that the histone mRNA size classes we detect are coordinately regulated, both at the transcriptional and post-transcriptional levels, in the HeLa cell cycle.

A consensus pattern for the incorporation of  $^3\text{H}$ -uridine into histone mRNAs of cells progressing from G1 to S is presented in Fig. 7b, with the rates of DNA synthesis and relative histone mRNA steady state levels (see Fig. 4) reproduced for comparison. Pulse-labelled histone mRNAs are detected in G1 (0-7 hours after mitosis) but these are significantly less abundant than those found 8-12 hours after mitosis (Fig. 7). There is therefore a detectable but much reduced apparent level of transcription in cells 0-7 hours after mitotic detachment, but it is not clear whether this represents a G1 basal level of transcription, or whether it reflects contamination with a small proportion of S phase cells after mitotic shake-off. As there is not a substantial accumulation of histone mRNAs until 8 hours after mitosis (Fig. 7), we infer that the reduced apparent transcription in G1 is insufficient to permit accumulation above the G1-specific "basal" histone mRNAs translated in the presence of hydroxyurea as described by Wu and Bonner (29).

There is a sharp increase in the relative abundance of pulse-labelled histone mRNAs 7-8 hours after mitosis, and this level does not change significantly over the next four hours (Fig. 7b). As maximal histone mRNA levels are not reached until 11 hours after mitosis, we conclude that these data are compatible with maximal histone mRNA synthesis in early S phase as was observed in cells synchronized by double thymidine treatment (Fig. 6). The relatively constant abundance of pulse-labelled histone mRNAs in early- and mid- S phase after selective mitotic detachment is attributed to the synchrony decay observed as cells in G1 enter S phase with a given probability (44). These cells therefore probably represent a semisynchronous population which initiates DNA synthesis over a 3-4 hour period.

### DISCUSSION

We describe here a sensitive hybridization procedure which enabled us to study the in vivo incorporation of  $^3\text{H}$ -uridine into multiple histone mRNA size classes in synchronized HeLa S3 cells. The multiplicity of core histone gene transcripts has been described for HeLa H4 mRNAs (33) and for sea urchin histone mRNAs (45), and is inferred from both the polymorphic organization of human histone genes (27, 28) and the presence of variant histone amino acid sequences within the same cell (1, 29). By analyzing



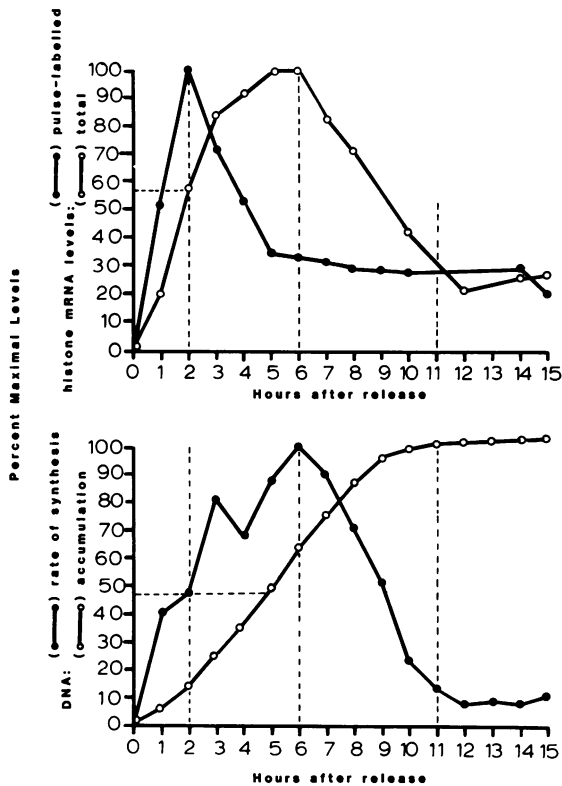


Figure 8. Summaries for the data obtained for cells synchronized by double thymidine blocks shown in figures 3 and 5.

Data are expressed as the percentage maximal levels detected above those observed in cells just before release from the second thymidine block.

- a): (○—○) The relative abundance of total histone mRNA.  
 : (●—●) The relative incorporation of  $^3\text{H}$ -uridine into histone mRNA in one hour labelling intervals, plotted at the end of the pulse.
- b): (●—●) Relative rates of DNA synthesis monitored by pulse labelling with  $^{14}\text{C}$ -thymidine.  
 : (○—○) Accumulation of DNA calculated from the rates of DNA synthesis.

multiple transcripts as a function of the cell cycle, we have shown that more than fifteen different histone gene transcripts are coordinately regulated.

Histone mRNA levels are temporally coupled with DNA synthesis rates in HeLa S3 cells, an observation which is consistent with data obtained for WI38 fibroblasts (42) and yeast cells (13). As these mRNA levels closely parallel both the histone protein synthesis rates *in vivo* (3-7) and the *in vitro* translatability of polysomal histone mRNAs (9, 11), it

is clear that the control of histone gene expression is regulated by the cellular abundance of histone mRNAs available for translation. We have shown that maximal incorporation of  $^3\text{H}$ -uridine into pulse-labelled histone mRNAs precedes maximal total levels, indicating that the regulation of cellular histone mRNA abundance in early S phase is predominantly at the transcriptional level (Fig. 8). A similar observation has been made for the periodic transcription of two nonallelic yeast variant H2B genes (16). Our results cannot distinguish whether histone gene transcription is initiated before the initiation of DNA synthesis, as is apparently the case in yeast (16), or concomitant with the initiation of DNA synthesis, although our data would suggest that the two occur in HeLa cells within a 30 minute period ( $\sim 6\%$  of the HeLa cell S phase).

In late S phase, the histone mRNA levels are modulated primarily at the post-transcriptional level as indicated by the apparent kinetics of turnover of histone mRNAs, and by the relatively invariant incorporation of  $^3\text{H}$ -uridine detected in pulse-labelled histone mRNAs (Fig. 8). Although we have not directly compared the turnover of histone mRNAs synthesized at various times during S phase, the data are consistent with an approximately constant turnover rate in late S phase, and transcription is also approximately constant (but reduced compared with early S phase) but is insufficient to prevent the turnover of histone mRNAs accumulated in early S phase. Studies with yeast (16) and ours with HeLa cells suggest that transcriptional rates vary in S phase, and it is now of interest to determine whether the turnover of histone mRNAs varies during S phase, in order to distinguish whether the modulation of both transcriptional and post-transcriptional events, or, just the transcriptional ones, are responsible for regulating the availability of histone mRNAs for translation in S phase. Perry and Kelley (46), using direct measurements of turnover in exponentially growing mouse L cells, showed a mean lifetime of polysomal histone mRNAs equal to the length of S phase. Alternatively, histone mRNA half-life measurements performed after perturbing cells with metabolic inhibitors such as hydroxyurea (10, 47), cytosine arabinoside (15), cycloheximide (10), or actinomycin D (15) have produced estimates for histone mRNA half-lives varying from 10-15 minutes (10, 15, 47) to one hour (15). These apparent half-lives are difficult to interpret although it is clear that histone mRNAs are preferentially destabilized by DNA synthesis inhibitors. The simplest postulated mechanism for the regulation of cellular histone mRNA abundance in vivo is one for which only transcription is modulated in the

presence of a constant, concentration - independent turnover rate, a postulate consistent with our observation that maximal transcription and accumulation of histone mRNAs occur predominantly in early S phase (70% accumulation in 2-3 hours, Fig. 8) and that the mean lifetime of polysomal histone mRNAs is approximately that of S phase (46).

As a working model, we propose that the regulation of histone gene expression may occur in early S phase when an elevated transcription rate permits the accumulation of histone mRNAs, and histone mRNA levels decrease in late S after the reduction of transcription in the presence of a constant turnover rate. Furthermore, the abundance of pulse-labelled histone mRNAs and the kinetics of accumulation of histone mRNAs in early S phase (Fig. 8) are suggestive of a feedback regulatory mechanism. We are currently investigating the possibility that HeLa S3 histone genes are replicated in early S phase, a concept consistent with the identification of putative origins of replication in close proximity to the histone gene clusters in yeast (16).

#### ACKNOWLEDGMENT

We thank Drs. H. Gray and F. Marashi for their helpful discussion and criticism. This work was supported by National Science Foundation grants numbers PCM80-18075 and PCM81-18951, and the March of Dimes Birth Defects Foundation Grant 1-813.

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