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Analysis of histone gene expression during the cell cycle in HeLa cells by using cloned human histone genes

(S phase/G_1 phase/mRNA/genomic clones)

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ABSTRACT **Although it is generally agreed that histone protein synthesis is restricted to the S phase of the cell cycle—and therefore parallels DNA replication—both transcriptional and posttranscriptional levels of control have been invoked. Using blot hybridization with several cloned genomic human histone sequences representing different histone gene clusters as probes, we have assessed the steady-state level of histone RNAs in the nucleus and cytoplasm of G_1 and S phase HeLa S_3 cells. The representation of histone mRNA sequences of G_1 compared with S phase cells was <1% in the cytoplasm and ≈1% in the nucleus. These data are consistent with transcriptional control, but we cannot completely dismiss the possibility that regulation of histone gene expression is, to some extent, mediated posttranscriptionally. If histone gene transcription does occur in G_1, the RNAs must either be rapidly degraded or be transcribed to a limited extent compared with S phase. An unexpected result was obtained when a blot of cytoplasmic RNA from G_1 and S phase cells was hybridized with λHHG 41 DNA (containing H3 and H4 human genomic histone sequences). Although hybridization with histone mRNAs was observed for RNAs from S phase but not from G_1 cells, hybridization with a nonhistone RNA of ≈330 nucleotides present predominantly in G_1 was also observed.**

In addressing the regulation of histone gene expression during the cell cycle, two basic questions must be considered: When are histone proteins synthesized? When are histone mRNA sequences present in the nucleus and cytoplasm? Central to the issue is whether these events occur throughout the cell cycle or during a restricted period of the cell cycle.

In HeLa S_3 cells it is well documented that histone protein synthesis is restricted to the S phase of the cell cycle (1–3), and the inhibition of histone synthesis that rapidly follows inhibition of DNA replication suggests a functional relationship between these two S phase-specific events (1–3). High resolution, two-dimensional electrophoretic fractionation procedures were used to reevaluate the synthesis of histones during the cell cycle in HeLa S_3 cells (4). In contrast to Groppi and Coffino's observation (5) that histones are synthesized at the same rate throughout the cell cycle in S49 mouse lymphoma cells, we detected newly synthesized histone polypeptides among nuclear and total cellular proteins in HeLa cells only during S phase. The presence of translatable histone mRNAs on the polysomes of S phase but not of G_1 HeLa cells is also consistent with S phase-specific histone protein synthesis (4, 6–11).

Nucleic acid hybridization analysis that uses histone-specific DNAs as probes offers a powerful approach for identification and quantitation of histone mRNA sequences. By RNA excess hybridization with a homologous cDNA complementary to HeLa cell H2A, H2B, H3, and H4 histone mRNAs, we previously observed significant levels of histone mRNA sequences in the nucleus, on the polysomes, and in the postpolysomal cytoplasmic fraction of S phase but not of G_1 cells (11–14). Similar results were obtained by hybridization with ^{3}H-labeled cDNA complementary to one of the HeLa cell H4 histone mRNAs (15). These findings are consistent with control of histone gene expression in HeLa cells being mediated, at least in part, at the transcriptional level. The findings are supported by the results of Parker and Fitschen (16) in mouse 3T6 cells that indicate that by homologous cDNA hybridization the representation of histone mRNAs in G_1 is ≈1% of that observed in S phase. However, histone cDNAs represent only the mRNA coding regions of the histone genes and criteria for purity of the cDNA probes are not unequivocal. Melli *et al.* (17), using cloned sea urchin histone DNA as a probe, reported the presence of a high molecular weight precursor of histone mRNAs in G_1 and S phase HeLa cells, but the limited sequence homology between sea urchin and human histone sequences raised questions relating to interpretation of their results.

To gain more definitive information about regulation of histone gene expression during the cell cycle, we used cloned genomic human histone sequences to reexamine the representation of histone mRNAs in the nucleus and cytoplasm of G_1 and S phase synchronized HeLa S_3 cells. Data presented suggest that, in agreement with our earlier observations (11–15), histone mRNA sequences are present in significant amounts in the nucleus and cytoplasm of HeLa cells only during S phase, when histone protein synthesis occurs.

MATERIALS AND METHODS

Cell Culture. HeLa S_3 cells were grown in suspension culture in Joklik-modified Eagle's minimal essential medium supplemented with 7% calf serum. S phase cells were obtained by double thymidine block synchronization, a procedure that yields a population of >98% S phase cells (3). Treatment with 1-β-D-arabinofuranosylcytosine (Ara-C; cytosine arabinoside) at 40 μg/ml was for 1 hr, beginning 1 hr after release from the second thymidine block. G_1 cells were obtained by mitotic selective detachment (3).

Subcellular Fractionation. At appropriate times, cells (5×10^8) were harvested by centrifugation at 800 × *g* for 5 min and washed three times with spinner salt solution (GIBCO). Cells were swollen in 12 ml of reticulocyte standard buffer (RSB) (10 mM NaCl/10 mM Tris·HCl, pH 7.4/1.5 mM $MgCl_2$) and lysed at 4°C with a Wheaton glass homogenizer with a type "A" (tight) pestle. Nuclei were pelleted by centrifugation at 800 × *g* for

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Abbreviations: DBM, diazobenzyloxymethyl; RSB, reticulocyte standard buffer.

3 min and washed three times with 10 mM NaCl/10 mM Tris·HCl, pH 7.4/1.5 mM $MgCl_2$/0.5% Triton X-100. The pooled supernatants constituted the cytoplasmic fraction.

Cytoplasmic RNA Extraction. The cytoplasmic fraction was adjusted to 1% $NaDodSO_4$ at room temperature. Equal volumes of phenol (equilibrated with 10 mM Tris·HCl, pH 7.4/1 mM EDTA) and chloroform/isoamyl alcohol (24:1, vol/vol) were added and extraction was performed for 4 min with vigorous shaking at room temperature. The aqueous phase was reextracted twice with equal volumes of chloroform/isoamyl alcohol and then adjusted to 0.25 M LiCl and precipitated with 2.5 vol of 95% ethanol overnight at −20°C. The RNA pellet was dissolved in distilled water, adjusted to 0.25 M KOAc at pH 5.0, and precipitated with 95% ethanol. RNA samples were stored in water at −20°C.

Nuclear RNA Extraction. Nuclear RNA was isolated by modification of Penman's procedure (18). The nuclear pellet was suspended in 5 ml of high salt buffer (0.5 M NaCl/50 mM $MgCl_2$/10 mM Tris·HCl, pH 7.4/2 mM $CaCl_2$) containing 10 mM vanadyl ribonucleoside complex prepared according to Berger and Birkenmeier (19). DNase I (DN-EP; Sigma) was added to a concentration of 100 μg/ml and the mixture was swirled for 15 min at room temperature. The digestion was stopped by adjusting the solution to 0.5% $NaDodSO_4$ and 0.1 M EDTA at pH 7.4. An equal volume of acidified phenol (equilibrated with 10 mM NaOAc, pH 5.0) was added; after vigorous shaking the lysate was heated to 60°C for 5 min and then was shaken again. This process was repeated after the addition of an equal volume (relative to the aqueous phase) of chloroform/isoamyl alcohol (24:1, vol/vol) and the organic phase was removed by centrifugation at 1000 × *g* for 5 min at room temperature. The aqueous phase was reextracted three times with equal volumes of chloroform/isoamyl alcohol and then was adjusted to 0.25 M LiCl. After addition of 2 vol of 95% ethanol, the sample was stored at −20°C overnight. Nucleic acids were reprecipitated with 0.25 M KOAc at pH 5.0 and 2.5 vol of 95% ethanol and were redissolved in distilled water.

Gel Electrophoresis. RNAs were prepared as described by Bailey and Davidson (20) except that 30–50 mM CH_3HgOH (Alfa, Danvers, MA) was used. Samples were separated by electrophoresis in vertical agarose slab gels (14 cm × 10.5 cm × 0.3 cm) in the presence of 5 mM CH_3HgOH with 50 mM boric acid/5 mM $Na_2B_4O_7$/10 mM Na_2SO_4/1 mM EDTA, pH 8.19, as the buffer. Electrophoresis was carried out at 55 mA (110 V). After electrophoresis, the gel was soaked in a solution of 8.5 mM 2-mercaptoethanol/1 μg of ethidium bromide per ml for 30 min and then was photographed.

Transfer of RNA to Diazobenzyloxymethyl (DBM)-Paper. DBM-cellulose sheets were prepared according to Alwine *et al.* (21, 22). The gel was incubated in 10 mM iodoacetic acid for 30 min and then was soaked in 200 mM KH_2PO_4 at pH 5.0, followed by soaking in 50 mM KH_2PO_4. RNAs were electrophoretically transferred from the gel according to the procedure of Stellwag and Dahlberg (22) by using an electrophoretic transfer system (EC Apparatus, St. Petersburg, FL). The voltage potential was 3.6 V/cm for 4 hr. Prechilled 50 mM KH_2PO_4 at pH 5.0 (4°C) was used as the transfer buffer.

Preparation of Phage and Plasmid DNA. λ Charon 4A phages containing human genomic histone sequences were grown and phage DNA was isolated by modification (unpublished data) of the procedure of Blattner *et al.* (23). Plasmids containing human histone sequences were prepared according to McMaster *et al.* (24) or by the cleared lysate procedure (25).

Filter Hybridization and Autoradiography. RNA blots were prehybridized by using a modification of the procedure of Alwine *et al.* (21). Prehybridization was carried out for 6–24 hr at 50°C in 50% formamide (vol/vol)/5× Denhardt's solution (minus bovine serum albumin) (26)/1% glycine/0.7–1 mg of yeast RNA per ml/0.75 M NaCl/0.075 M Na Cit. Hybridization was for 72 hr at 50°C in an identical solution except that glycine was omitted and 0.1% $NaDodSO_4$ and denatured [α-^{32}P]dCTP-labeled probe (50–75 ng/ml, 1–5 × 10^6 cpm/ml) were added. After hybridization the filters were washed by using increasingly stringent conditions with a final wash of 15 mM NaCl/1.5 mM Na Cit/0.1% $NaDodSO_4$ at 65°C for 30 min. The filters were then blotted dry and analyzed autoradiographically with XAR-5 film (Kodak) with a Cronex Lightning Plus intensifying screen (Du Pont) at −70°C.

RESULTS

Representation of HeLa cell histone mRNAs was assayed by hybridization to cloned genomic human H4, H3, H2B, and H1 histone DNA sequences (27–29). To standardize conditions for hybridization, we took advantage of the long-standing observation that inhibition of DNA replication results in a rapid loss of histone mRNAs from the polysomes of S phase HeLa cells (1–4). Under the hybridization conditions employed in our

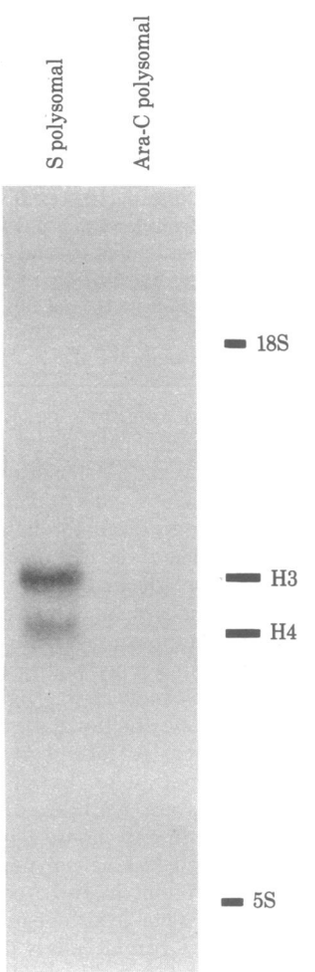

FIG. 1. Hybridization of electrophoretically fractionated polysomal RNA from S phase and Ara-C-treated S phase HeLa cells with ^{32}P-labeled human genomic H3 and H4 histone sequences. Total HeLa cell polysomal RNAs (100 μg) were electrophoretically fractionated in a 2% agarose gel containing 5 mM CH_3HgOH, electrophoretically transferred to DBM-paper, and hybridized with ^{32}P-labeled (nick-translated) plasmid DNA containing human genomic H3 and H4 histone sequences (pHu 2.6H).

studies, ^{32}P-labeled plasmid DNAs containing genomic human histone sequences annealed with S phase HeLa cell polysomal RNAs fractionated electrophoretically in CH_3HgCl agarose gels and transferred electrophoretically to diazotized cellulose (Fig. 1). Consistent with the anticipated loss of histone mRNA sequences from the polysomes after inhibition of DNA synthesis, hybridization of the same histone-containing plasmid DNAs to filter-immobilized polysomal RNAs was inhibited by 95% (comparable to DNA synthesis inhibition) in Ara-C-treated compared with control S phase cells. The sensitivity of our hybridization assay is such that we can detect <500 pg of histone mRNA.

To address the level at which regulation of histone gene expression occurs during the cell cycle in HeLa S_3 cells, we assayed hybridization of ^{32}P-labeled (nick-translated) histone DNAs with filter-immobilized RNA from G_1 and S phase cells. The rationale was that because histone synthesis is confined to S phase, hybridization of the histone DNA probe to RNAs from S phase but not from G_1 cells would be consistent with nuclear or transcriptional level control (or both). However, hybridization of the histone DNA to both G_1 and S phase RNAs would suggest that regulation of histone gene expression resides at a posttranscriptional step.

Hybridization between λHHG 55 DNA—a recombinant Charon 4A phage containing human genomic H3 and H4 histone sequences (27, 29)—and G_1 nuclear and cytoplasmic RNAs was barely detectable, whereas hybridization of λHHG 55 DNA with both nuclear and cytoplasmic RNAs of S phase HeLa cells was apparent (Fig. 2). In these experiments, G_1 cells were obtained by mitotic selective detachment, a procedure that yields a G_1 population containing <0.5% S phase cells. G_1 and S phase RNA (100 μg each) were fractionated electrophoretically, and ethidium bromide staining indicated that similar amounts of 18S and 28S RNAs from G_1 and S phase cells were fractionated in the gels and transferred to diazotized cellulose (>90% transfer was obtained). Because there is an increase in the amount of ribosomal RNA per cell in S phase compared with G_1 cells, the hybridization observed was probably an underestimation of the amount of histone mRNA in S phase cells. On longer exposure of the blot, some hybridization of the H3–H4 probe with G_1 RNA became apparent (≈1% of the level observed with S phase RNA); this amount of annealing was within the limits that can be expected from the number of S phase cells in a G_1 population—the biological limits of the system.

When the blot of G_1 and S phase HeLa cell RNAs shown in Fig. 2 was rehybridized with ^{32}P-labeled plasmid DNA (designated pHu 2.6H) containing H3 and H4 histone-coding sequences from another histone gene cluster (28), S phase-specific hybridization was also observed (Fig. 3*A*). Similarly, when these blots were hybridized with a plasmid (designated pHu 4.8E) containing H2B and H1 histone coding sequences derived from the same human histone gene cluster as pHu 2.6H, S phase-specific hybridization was also observed (Fig. 3*B*).

An unexpected result (Fig. 4) was obtained when a blot of cytoplasmic RNA from G_1 and S phase HeLa cells was hybridized with ^{32}P-labeled DNA from λHHG 41, a recombinant

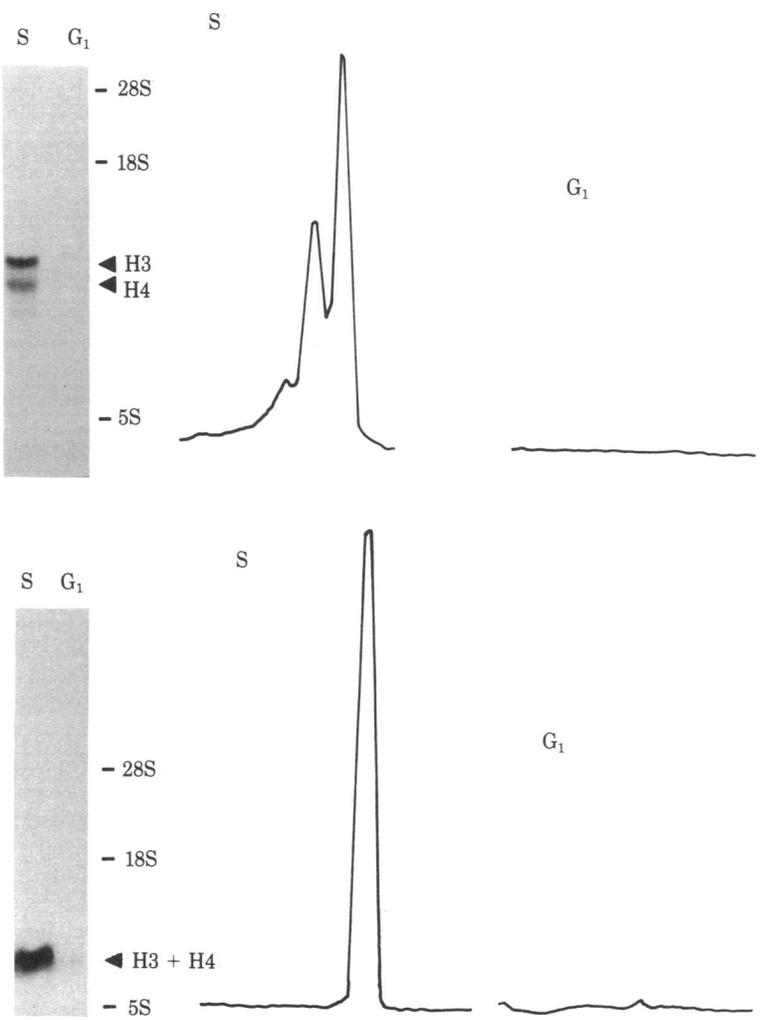

FIG. 2. Hybridization of human genomic recombinant phage λHHG 55 DNA containing H3 and H4 histone sequences with cytoplasmic (*Upper*) and nuclear (*Lower*) RNA from G_1 and S phase HeLa cells. S phase cells were obtained 1 hr after release from two cycles of 2 mM thymidine block whereas the G_1 population was obtained by mitotic selective detachment. Total G_1 and S phase cytoplasmic RNAs (100 μg) were fractionated in a 2% agarose/5 mM CH_3HgOH gel and equal amounts (100 μg) of G_1 and S phase nuclear RNA were fractionated on a gel containing 1% agarose/5 mM CH_3HgOH. The positions of 28S, 18S, and 5S markers were determined optically after staining the gel with ethidium bromide. RNAs were electrophoretically transferred to DBM-paper and hybridized with ^{32}P-labeled (nick-translated) λHHG 55 DNA. After hybridization and washing, the blots were analyzed autoradiographically at −70°C with preflashed XAR-5 film (Kodak) with a Cronex Lightning Plus intensifying screen. Autoradiograms were scanned with a Joyce–Loebl densitometer and quantitated by planimetric analysis.

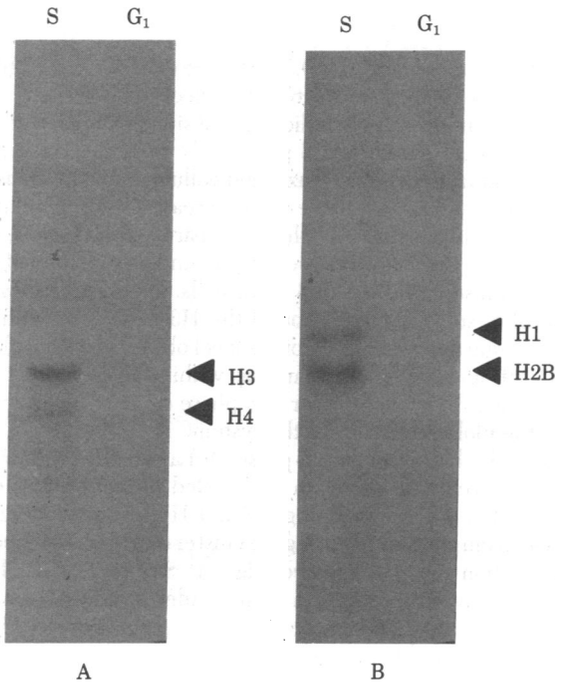

FIG. 3. Autoradiographic analysis of G_1 and S phase cytoplasmic RNAs hybridized with cloned genomic human H3, H4, H2B, and H1 histone sequences. G_1 and S phase HeLa cell cytoplasmic RNAs (50 μg) were fractionated electrophoretically in a 2% agarose/5 mM CH_3HgOH gel, transferred electrophoretically to DBM-paper, and hybridized with (*A*) ^{32}P-labeled (nick-translated) pHu 2.6H plasmid DNA containing H3 and H4 histone sequences or (*B*) ^{32}P-labeled pHu 4.8E plasmid DNA containing H2B and H1 histone sequences.

Charon 4A phage containing H3 and H4 coding sequences derived from yet another human histone gene cluster (27, 29). Although hybridization was observed with histone mRNAs present in S phase but not in G_1 phase cytoplasmic RNA, hybridization was also observed with a nonhistone-coding RNA of ≈330 nucleotides present predominantly in G_1 cells. The intensity of the hybridization of this RNA in G_1 cells is approximately equivalent to that of histone mRNAs in S phase cells.

DISCUSSION

The steady-state level of histone mRNAs was examined during the cell cycle of HeLa S_3 cells by using cloned human histone DNAs from three different histone gene clusters. The >100-fold increase in the representation of both nuclear and cytoplasmic histone mRNAs in S phase compared with G_1 phase cells suggests that histone genes are preferentially expressed during a restricted period of the cell cycle—when DNA replication occurs. Because synthesis of histone proteins in HeLa cells is also confined to S phase (1–4), it is reasonable to postulate that nuclear or transcriptional level control (or both) is operative. Transcriptional regulation of histone gene expression during the cell cycle of HeLa S_3 cells is consistent with earlier observations that histone cDNA hybridized preferentially with nuclear and cytoplasmic RNAs, *in vitro* nuclear transcripts, and *in vitro* chromatin transcripts of S phase compared with G_1 phase cells (11–15).

However, an unequivocal demonstration that regulation of histone gene expression during the HeLa cell cycle resides solely at the transcriptional level requires establishing that (*i*) the very limited presence of histone mRNA sequences in G_1 cells obtained by mitotic selective detachment is attributable

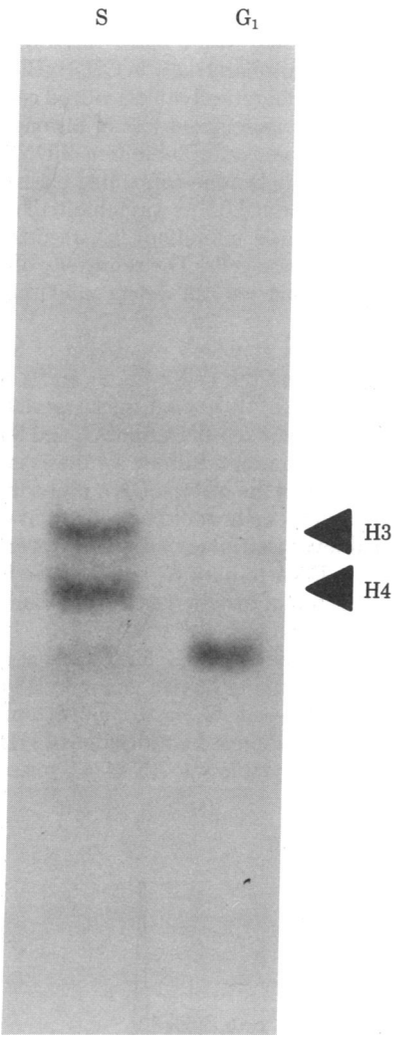

FIG. 4. Autoradiographic analysis of G_1 and S phase RNA hybridized with DNA from λHHG 41, a recombinant phage containing H3 and H4 human histone sequences. The DBM-paper blot shown in Fig. 2 *Upper* was incubated in sterile water at 100°C to remove ^{32}P-labeled λHHG 55 probe. After confirming that the λHHG 55 probe was no longer detectable, the filters containing fractionated cytoplasmic RNAs were hybridized with ^{32}P-labeled λHHG 41 DNA.

to those few S phase cells in the G_1 population (<0.5%) and (*ii*) histone gene transcription is initiated only during S phase.

Although Melli *et al.* (17) reported the presence of approximately equivalent amounts of histone gene transcripts in G_1 and S phase HeLa cells, these results should be cautiously interpreted because histone sequences were assayed by hybridization under nonstringent conditions with a heterologous probe (sea urchin histone DNA sequences cloned in λ); also, cells were synchronized by double thymidine block, yielding a G_1 population containing ≈25% S phase cells.

We cannot completely dismiss the possibility that regulation of histone gene expression during the HeLa cell cycle may to some extent be mediated posttranscriptionally. However, the low steady-state level of histone mRNA sequences we observed in the nuclei of G_1 cells would indicate that if histone sequences are transcribed at the same rate throughout the cell cycle they must be rapidly degraded in G_1 cells. Alternatively, it is possible that histone sequences are transcribed at a much lower level outside of S phase. Such low level transcription might be functional or might reflect "leaky" transcription of some or all of the

histone genes. Within this context the persistence of a limited amount of histone transcripts from the previous S phase into the subsequent G_1 phase should be considered.

A definitive explanation for the observed hybridization of ^{32}P-labeled λHHG 41 DNA (containing H3 and H4 human histone genes) with a 330-nucleotide, nonhistone cytoplasmic RNA species present predominantly in G_1 cells is not yet available. However, because the G_1 RNA species is encoded in a genomic sequence in close proximity to histone coding sequences, a possible regulatory role for this G_1 RNA can be considered. Further analysis of the RNA and the genomic DNA sequences in which it is encoded is under way.

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1. Spalding, J., Kajiwara, K. & Mueller, G. (1966) *Proc. Natl. Acad. Sci. USA* **58**, 1535–1542.
2. Robbins, E. & Borun, T. W. (1967) *Proc. Natl. Acad. Sci. USA* **57**, 409–416.
3. Stein, G. S. & Borun, T. W. (1972) *J. Cell Biol.* **52**, 292–307.
4. Marashi, F., Decker, L., Rickles, R., Sierra, F., Stein, J. & Stein, G., *Science*, in press.
5. Groppi, V. E. & Coffino, P. (1980) *Cell* **21**, 195–204.
6. Gallwitz, D. & Mueller, G. (1969) *J. Biol. Chem.* **244**, 5947–5952.
7. Borun, T. W., Scharff, M. D. & Robbins, E. (1967) *Proc. Natl. Acad. Sci. USA* **58**, 1977–1983.
8. Gallwitz, D. & Breindl, M. (1972) *Biochem. Biophys. Res. Commun.* **47**, 1106–1111.
9. Borun, T. W., Gabrielli, F., Ajiro, K., Zweidler, A. & Baglioni, C. (1975) *Cell* **4**, 59–67.
10. Jacobs-Lorena, M., Baglioni, C. & Borun, T. W. (1972) *Proc. Natl. Acad. Sci. USA* **69**, 2095–2099.
11. Stein, J. L., Thrall, C. L., Park, W. D., Mans, R. J. & Stein, G. S. (1975) *Science* **189**, 557–558.
12. Stein, G. S., Stein, J. L., Park, W. D., Detke, S., Lichtler, A. C., Shephard, E. A., Jansing, R. L. & Phillips, I. R. (1978) *Cold Spring Harbor Symp. Quant. Biol.* **42**, 1107–1120.
13. Detke, S., Stein, J. L. & Stein, G. S. (1978) *Nucleic Acids Res.* **5**, 1515–1528.
14. Stein, G. S., Park, W. D., Thrall, C. L., Mans, R. J. & Stein, J. L. (1975) *Nature (London)* **257**, 764–767.
15. Detke, S., Lichtler, A., Phillips, I., Stein, J. & Stein, G. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 4995–4999.
16. Parker, M. I. & Fitschen, W. (1980) *Cell Differ.* **9**, 23–30.
17. Melli, M., Spinelli, G. & Arnold, E. (1977) *Cell* **12**, 167–174.
18. Penman, S. (1966) *J. Mol. Biol.* **17**, 117–130.
19. Berger, S. L. & Birkenmeier, L. S. (1979) *Biochemistry* **18**, 5143–5149.
20. Bailey, J. M. & Davidson, N. (1976) *Anal. Biochem.* **70**, 75–85.
21. Alwine, J. C., Kemp, D. J. & Stark, G. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5350–5354.
22. Stellwag, E. J. & Dahlberg, A. E. (1980) *Nucleic Acids Res.* **8**, 299–317.
23. Blattner, F. R., Williams, B. G., Blechl, A. E., Denniston-Thompson, K., Faber, H. E., Furlong, L.-A., Grunwald, D. J., Kieter, D. O., Moore, D. D., Schuman, J. W., Sheldon, E. L. & Smithies, O. (1977) *Science* **196**, 161–169.
24. McMaster, G. K., Samulski, R. J., Stein, J. L. & Stein, G. S. (1980) *Anal. Biochem.* **109**, 47–54.
25. Clewell, D. B. & Helinski, D. R. (1970) *Biochemistry* **9**, 4428–4440.
26. Denhardt, D. T. (1966) *Biochem. Biophys. Res. Commun.* **23**, 641.
27. Stein, G. S., Stein, J. L., Marashi, F., Parker, M. I. & Sierra, L. F. (1980) *Cell Biophys.* **2**, 291–314.
28. Clark, S. J., Krieg, P. A. & Wells, J. R. E. (1981) *Nucleic Acids Res.* **9**, 1583–1590.
29. Sierra, F., Lichtler, A., Marashi, F., Rickles, R., Van Dyke, T., Clark, S., Wells, J., Stein, G. & Stein, J., *Proc. Natl. Acad. Sci. USA*, in press.