Vol. 114, No. 1, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 18, 1983 Pages 131-137

INCREASED HISTONE mRNA LEVELS DURING INHIBITION OF PROTEIN SYNTHESIS

Eva Stimac,[1] Vincent E. Groppi,[2] and Philip Coffino[1,3]

Departments of Microbiology and Immunology,[1] Pharmacology,[2] and Medicine[3]
University of California, San Francisco, San Francisco, California 94143

Received May 31, 1983

Inhibition of protein synthesis by cycloheximide or puromycin specifically increases the amount of translatable histone mRNA in exponentially growing and in synchronous G_1 HeLa cells by 5-fold in 3 hours. In this case histone gene expression is uncoupled from DNA replication. We conclude that the level of histone mRNA is regulated by a labile protein and is only indirectly dependent on DNA synthesis.

The major nuclear proteins of eukaryotic cells are the histones. Unlike most proteins (1,2), the histones are not made at a uniform rate throughout the cell cycle. In HeLa tissue culture cells, the bulk of histone synthesis is concurrent with DNA replication (S phase); their increased synthesis begins either late during the gap (G_1 phase) that separates cell division (mitosis) from DNA replication, or at the start of DNA synthesis (S phase) (3). The relative rate of histone protein synthesis is at least 10-fold lower in early G_1 than in S phase cells, and the level of histone mRNA is reduced to a similar extent (4,5).

When DNA synthesis is inhibited in S phase HeLa cells by a drug such as hydroxyurea, both the rate of histone protein synthesis and the level of histone mRNA fall to that found in G_1 cells (6,7). Butler and Mueller found that concurrent treatment of S phase cells with hydroxyurea and with cycloheximide, an inhibitor of protein synthesis, prevents the decline of histone mRNA (8). They suggested that the histones may participate in regulating their own synthesis. If so, by inhibiting protein synthesis, one should be able to fully or partially uncouple histone mRNA from DNA synthesis and thus cause the amount of histone mRNA in G_1 cells to increase. Such an increase was not previously observed, but the experiments were limited to brief treatments of S phase cells (8).

0006-291X/83 $1.50

METHODS

Cell Culture and Synchronization

HeLa S_3 cells were grown in spinner flasks in Swim's 77 medium containing 10% calf serum. G_1 cells were obtained by loading the elutriator rotor (5,9) with exponentially growing cells at 2200 rpm, pump speed 10ml/min, and then increasing the pump speed in 7.5 ml/min increments until the first fraction containing cells free from debris was collected. For autoradiography after incorporation of [3H]thymidine (Amersham), cells fixed on glass slides were coated with Kodak NTB-2 emulsion. Cycloheximide and puromycin were obtained from Sigma.

RNA Analysis

Total cell RNA was prepared by lysing the cells in guanidinium thiocyanide and centrifuging the lysate through 5.7 M CsCl (14). The RNA was spotted directly onto nitrocellulose filter paper (Schleicher & Schuell) (21) in a 1:3 dilution series for each sample, so that the amount of a specific mRNA/µg in each sample could be compared quantitatively. Alternatively, the RNA was fractionated by electrophoresis through a 7M urea 7% acrylamide gel and transferred to diazobenzyloxymethyl paper (Schleicher & Schuell) (17). In both cases the immobilized RNA was hybridized to DNA probes (labelled with [^{32}P] by nick-translation) for 18h in 10% dextran sulphate, 50% formamide, 450 mM NaCl, 45mM Na Citrate, pH7.0, 5x Denhardt's solution at 42°; followed by washing in 15mM NaCl, 1.5mM NaCitrate,0.1% sodium dodecyl sulphate at 50°C. The dried papers were placed under Kodak XAR-5 film with DuPont Lightning Plus intensifying screens at -70°C. The autoradiograms were scanned with a Zeineh Soft Laser Scanning Densitometer to measure the amount of probe bound to each band.

Histone mRNA was also measured by translating total cell RNA using a rabbit reticulocyte lysate containing [^{35}S] methionine (10) and analysing aliquots containing equal amounts of acid- precipitable radioactivity by NEPHGE two dimensional gel electrophoresis (13). The gels were impregnated with Enhance (New England Nuclear), dried , and placed under Kodak XAR-5 film at -70°C. The autoradiograms were scanned with a Joyce-Loebl densitometer to measure the relative intensity of specific spots after each treatment.

RESULTS

Synchronously growing G_1 HeLa cells were obtained by centrifugal elutriation of an exponentially growing culture (5,9). Synchrony was evaluated by autoradiography after incorporation of [^{3}H]thymidine. The population of smallest cells, those collected in the first fraction, contained less than 4% S phase cells immediately after fractionation. 2-10% of the cells in such elutriated G_1 populations were in S after 4 hours of growth, but 98% had replicated their DNA after 24 hours.

To block protein synthesis, G_1 cells or asynchronous exponentially growing cells were treated for 3.5 hours with cycloheximide or with puromycin. Inhibition of protein synthesis inhibits DNA synthesis (11); this was evident in the reduction of [^{3}H]TdR incorporation seen by autoradiography. It also prevents the progression of G_1 cells into S phase during the treatment (13). Total cell RNA was prepared and translated *in vitro* using a rabbit reticulocyte

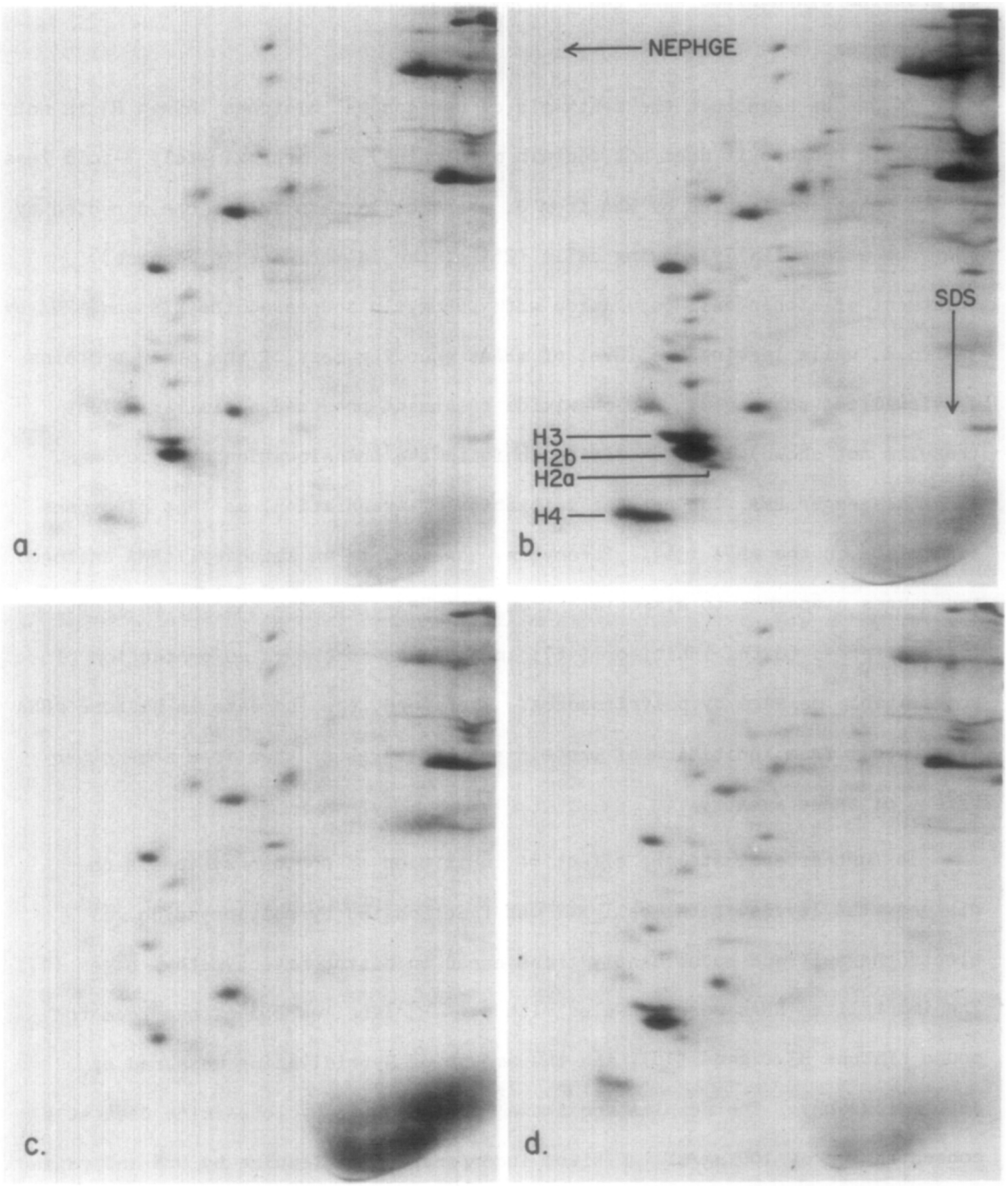

Figure 1. In vitro translation of RNA from a) exponentially growing HeLa cells, b) these cells treated for 3.5 h with 100 µg/ml puromycin, c) synchronously growing G_1 HeLa cells after 3.5 h growth at 37°C, d) G_1 HeLa cells treated for 3.5 h with puromycin. 10 µg of RNA was translated in a rabbit reticulocyte system (10) and 11-20 µl containing 222,000 cpm were analysed by NEPHGE two-dimensional gel electrophoresis (13). Only the basic half of each gel is shown.

system (10) containing [^{35}S]methionine, and the reaction mixture was analyzed by NEPHGE two-dimensional gel electrophoresis (12) (Fig. 1). The relative amounts

of proteins synthesized were quantitated by scanning autoradiograms with a densitometer.

It can be seen that the synthesis of nucleosomal histones (human H1 is not detectable because it does not contain methionine) was approximately 3-fold less in the reaction directed by RNA from G_1 phase cells than in the one directed by RNA from exponentially growing cells (25% of the latter were in S phase). Treatment of either cell population with puromycin increased the histone mRNA by 3-4 fold, while leaving the level of mRNAs encoding most of the other proteins we visualized unchanged. Cycloheximide treatment produced a similar effect (results not shown). Cycloheximide inhibits the translocation of ribosomes along messenger RNA, but not the initiation of translation, so that ribosomes accumulate on the mRNA (15). Puromycin, however, is an aminoacyl tRNA analogue and causes ribosomes to dissociate from the mRNA after the nascent polypeptide is transferred to the inhibitor (16); in this case there is no protection of translatable message by polyribosomes. Therefore, the increase in histone mRNA must result from inhibition of protein synthesis rather than from some other effect of these agents.

To further evaluate the effect of inhibition of protein synthesis on histone mRNA levels, total cell RNA was fractionated by polyacryamide gel electrophoresis and subsequently transferred to diazobenzyloxymethyl paper (17). The immobilized RNAs were annealed with a [^{32}P] labelled DNA cloned from the mouse histone H3.2 gene (18), and the amount of hybridization measured by autoradiography. The results are shown in Figure 2. Cycloheximide used at a concentration of 100 µg/ml inhibited incorporation of leucine by 96% and raised histone mRNA levels 4-fold within 3 h. Less complete inhibition of protein synthesis was less effective, as was cycloheximide treatment for shorter periods.

Inhibiting protein synthesis has the secondary effect of inhibiting both the synthesis and processing of ribosomal RNA in the nucleolus (19,20). We found that incorporation of uridine declined by 30-50% after 3h treatment with 100 µg/ml cycloheximide. To rule out the possibility of a non-specific

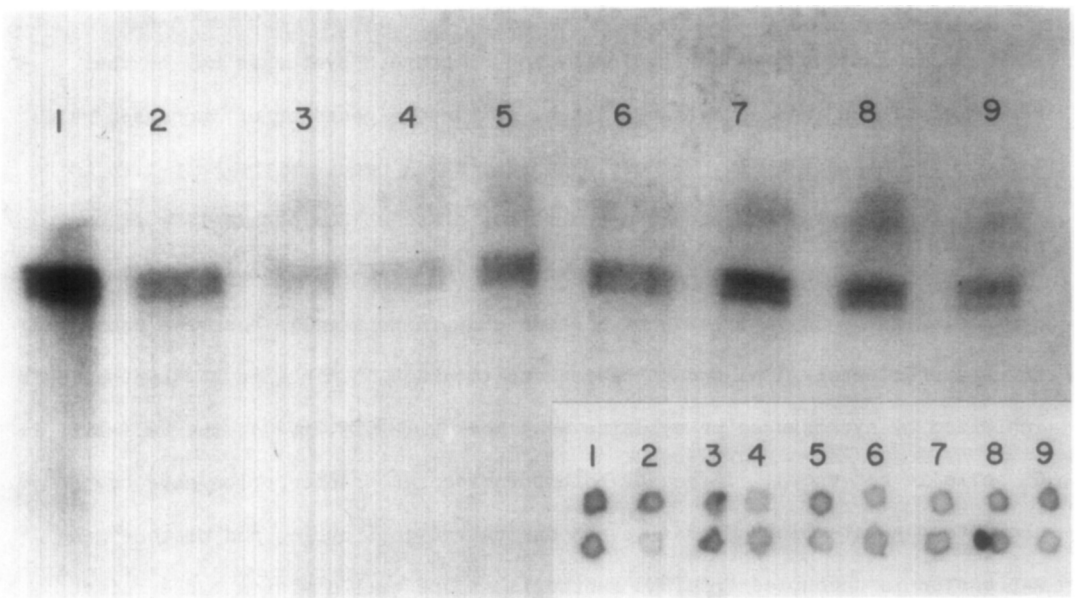

Figure 2. Histone mRNA after cycloheximide treatment. 10 µg RNA were fractionated on a 7M urea 7% acrylamide electrophoresis gel, electroblotted to diazobenzyloxymethyl paper (17), and hybridized with 10^7 cpm [^{32}P] labelled mouse histone MH3.2 gene (18). Inhibition of overall protein synthesis was determined by labeling an aliquot of cells with 5 µCi/ml [^{14}C]leucine for the last 30 min of each treatment and measuring acid-precipitable radioactivity.
Inset: 3 µg of RNA were spotted on nitrocellulose paper (21) and hybridized with [^{32}P] labelled chick actin cDNA probe (22).

Lane 1: Exponentially growing HeLa cells + 100 µg/ml cycloheximide for 180 min. 95% inhibition of protein synthesis.
Lane 2: Exponentially growing untreated cells
Lane 3: G_1 cells, untreated
Lane 4: G_1 cells + 0.2 µg/ml cycloheximide for 180 min, 54% inhibition.
Lane 5: G_1 cells + 2.0 µg/ml cycloheximide for 180 min, 82% inhibition.
Lane 6: G_1 cells + 20 µg/ml cycloheximide for 180 min, 91% inhibition.
Lane 7: G_1 cells + 100 µg/ml cycloheximide for 180 min, 96% inhibition.
Lane 8: G_1 cells + 100 µg/ml cycloheximide for 120 min, 96% inhibition.
Lane 9: G_1 cells + 100 µg/ml cycloheximide for 60 min, 97% inhibition.

enrichment for hybridizable mRNA rather than a specific increase in histone mRNA in our experiments, we reprobed total RNA from the previous experiments with probes homologous to chick actin (Figure 2, inset) and tubulin genes (22). In contrast to the observations made on histone mRNA, the level of actin or tubulin mRNA was found to be essentially the same in G_1 and S phase cells and to be little changed by inhibition of protein synthesis.

DISCUSSION

We conclude that histone synthesis is regulated through one or more proteins whose synthesis is inhibited in our experiments. As Butler and Mueller

suggested (8), the histones themselves might be the target proteins. The increase in histone mRNA during inhibition of protein synthesis may be the result of either increased transcription of histone genes or of increased half-life of the histone mRNA. Transcriptional control requires the half-life to be short. For example, kinetic models must postulate a 12 min half-life if the increase we observe in 3 h is to be explained by a 10-fold increase in rate of synthesis (23). The half-life of histone mRNA in HeLa cells has been estimated to be approximately 1 h, and to be reduced to about 15 min when DNA synthesis is inhibited by hydroxyurea or cytosine arabinoside (24,25,26,27); the half-life in G_1 cells is not known. In yeast, both turnover and transcription have been shown to regulate histone mRNA levels during the cell cycle, and histone gene expression is uncoupled from DNA synthesis in one cell division cycle mutant blocked in G_1 (28). Further investigation will be required to identify the protein(s) that control histone mRNA levels, the molecular mechanisms by which that occurs, and the relationship of the pharmacological effect demonstrated here to the modulation of histone gene expression in cycling cells.

ACKNOWLEDGEMENTS

E.S. was supported by an Anna Fuller post-doctoral fellowship, and this work was supported by NSF grant PCM 78-07382.

REFERENCES

1. Milcarek, C. and Zahn, K. (1978) J. Cell Biol. 79, 833-838.
2. Bravo, R., and Celis, J.E. (1980) J. Cell Biol. 84, 795-802.
3. Marashi, F., Baumbach, L., Rickles, R., Sierra, F., Stein, J.L., and Stein, G. (1982) Science 215, 683-685.
4. Rickles, R., Marashi, F., Sierra, F., Clark, S., Wells, J., Stein, J., and Stein, G. (1982) Proc. Natl. Acad. Sci. USA 79, 749-753.
5. Coffino, P., Stimac, E., Groppi, V.E., and Bieber, D. (1983) in Histone Genes and Histone Gene Expresion, Stein, G.S., Stein, J.L., and Marzluff, W.F., eds. (in press).
6. Wu, R.S., and Bonner, W.M. (1981) Cell 27, 321-330.
7. Breindl, M., and Gallwitz, D. (1974) Eur. J. Biochem. 45, 91-97.
8. Butler, W.B., and Mueller, G.C. (1973) Biochim. Biophys. Acta 294, 481-496.
9. Meistrich, M.L., Meyn, R.E., and Barlogie, B. (1977). Exp. Cell. Res. 105, 169-177.
10. Pelham, H.R.B., and Jackson, R.J.B. (1976) Eur. J. Biochem. 67, 247-256.
11. Stimac, E., Housman, D., and Huberman. J. A. (1977) J. Mol. Biol.115, 485-511.
12. Brooks, R.F. (1977) Cell 12, 311-317.
13. Sanders, M.M., Groppi, V.E., Jr., and Browning, E.T. (1980) Anal. Biochem. 103, 157-165.
14. Chirgwin, J.M., Przbyla, A.E., MacDonald, R.J., and Rutter, W.J. (1979) Biochem. 18, 5294-5299.
15. Lodish, H.F., Housman, D., and Jacobsen, M. (1979) Biochem. 10, 2348-2356.

16. Nathans, D. (1964) Proc. Natl. Acad. Sci. USA 51, 585-592.
17. Stellwag, E.J., Dahlberg, A.E. (1980) Nucleic Acid Res. 8, 299-317.
18. Sittman, D.S., Chin, I., Pan, C., Cohn, R.H., Kedes, L.H., and Marzluff, W.F. (198 Proc. Natl. Acad. Sci. USA 78, 4078-4082.
19. Willems, M., Penman, M., and Penman, S. (1969) J. Cell. Biol. 41, 177-187.
20. Grummt, I., Smith, V.A., and Grummt, F. (1976) Cell 7, 439-445.
21. Thomas, P.S. (1980) Proc. Natl. Acad. Sci. USA 77, 5201-5205.
22. Cleveland, D.W., Lopata, M.A., MacDonald, R.J., Cowan, N.J., Rutter, W.J., and Kirschner, M.W. (1980) Cell 20, 95-105.
23. Berlin, C.M., and Schimke, R.T. (1965) Mol. Pharmacol. 1, 149-156.
24. Borun, T.W., Scharff, M.D., and Robbins, E. (1967) Proc. Natl. Acad. Sci. USA 58, 1977-1983.
25. Gallwitz, D. (1975) Nature 257, 247-248.
26. Pederson, T. (1976) in Protein Synthesis, vol. 2., McConkey, E.H., ed. (Marcel Dekker, N.Y.) p. 69-123.
27. Heinz, N., Sive, H.L., and Roeder, R.G. (1983) Mol. Cell. Biol. 3, 539-550.
28. Hereford, L.M., Bromley, S., and Osley. M.A. (1982) Cell 30, 305-310.