Vol. 95, No. 3, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 14, 1980 Pages 1253-1260

TIMING OF UBIQUITIN SYNTHESIS AND CONJUGATION INTO PROTEIN A24 DURING THE HELA CELL CYCLE

Ira L. Goldknopf, Swaroop Sudhakar, Faye Rosenbaum and Harris Busch

Department of Pharmacology, Baylor College of Medicine
Houston, Texas 77030

Received June 30,1980

SUMMARY

To determine the relationship between formation of protein A24 and replication of chromatin, incorporation of ^{35}S methionine into ubiquitin, protein A24, histone 4 and high mobility group proteins (HMG) 1 and 2 were compared to DNA synthesis and cell division in HeLa cells synchronized by thymidine block. The biosynthesis of ubiquitin and its coupling into protein A24 occurred in parrallel and were largely interphase events rising to a maximum rate in early G1 phase and declining to a minimum during cell division. The ratio of labeling of protein A24 to histone 4 indicated that a high rate of ubiquitin-histone 2A conjugation occurred, in excess of that accounted for by histone synthesis. This additional conjugation was highest in early and late G1 phase and its distribution during the cell cycle was similar to the biosynthesis of HMG 1 and 2.

INTRODUCTION

Chromatin protein A24 (1) contains two polypeptides, histone 2A and ubiquitin (2-5). The carboxyl terminus of ubiquitin is attached to a glycyglycine bridge in an isopeptide linkage to the ε-NH_2 of lysine 119 of histone 2A (6,7). Approximately 10% of nuclear histone 2A is in the form of protein A24 (1,8) and the conjugate is found in a subset of chromatin nucleosome core octamers (8-10). In the present study, the synthesis of ubiquitin and its conjugation with histone 2A was monitored in synchronized HeLa cells to determine whether these occurred apart from histone synthesis and chromatin replication.

MATERIALS AND METHODS

Synchronization and labeling of cells - In three independent experiments, logarithmically growing HeLa S_3 cells in suspension culture were resuspended at a cell concentration of 4 x 10^5 cells/ml in a total volume of four liters of minimum essential medium containing 10% fetal

0006-291X/80/151253-08$01.00/0

calf serum, 200 mcg/ml streptomycin, 100 u/ml penicillin, 4mM L-glutamine 1mM sodium pyruvate, and 0.1 mM nonessential amino acids. The cells were blocked in S phase by incubating with 2.5 mM thymidine for 24 hours (11-13). They were released from the block and allowed to progress in S phase by washing the cells free of thymidine and resuspending in fresh media. For monitoring the cell cycle 100 ml aliquots were transferred to separate culture flasks and ^{3}H-thymidine (Tdr) was added to 1 μCi/ml. DNA synthesis was measured as the incorporation of ^{3}H-Tdr into 10% trichloracetic acid precipitable material. The number of cells per ml of suspension were monitored in a hemocytometer.

To monitor protein biosynthesis, 1 liter aliquots of cells from 4 segments of the cell cycle were collected by centrifugation for 10 min. at 2,300 x g. The pellets of cells were resuspended in 500 ml of methionine free media containing 1mCi of ^{35}S-methionine and the cells were then incubated in suspension for 3 hours at 37^{o} followed by centrifugation as before. They were washed once in phosphate buffered saline and stored at -80^{o}.

Preparation of labeled protein fractions - To prevent proteolysis, 1mM phenylmethylsulfonylfluoride (14,15) was used throughout and all operations were performed at 4^{o} except where noted below. Cells, labeled with ^{35}S-methionine, were washed twice at 200 mg/ml in 75 mM NaCl, 25 mM EDTA, pH 8, followed by twice in 10 mM Tris, pH 8, and three times in 350 mM NaCl, 10 mM Tris, pH 8. In this manner, whole cell ubiquitin and HMG proteins were obtained by a modification of the method of Goodwin et al (16) in that the supernatants from all the cell washes were combined and the protein obtained by solubilization in 2% trichloroacetic acid followed by acetone precipitation. Protein A24 and the histones were obtained from the preextracted deoxyribonucleoprotein pellet in two extractions with 0.4N H_2SO_4 followed by acetone precipitation (1).

Polyacrylamide gel electrophoresis and monitoring of incorporation of ^{35}S-methionine - For estimation of protein, the method of Lowry et al (17) was employed. For two-dimensional polyacrylamide gel electrophoresis, the first dimension contained 10% polyacrylamide 0.9N acetic acid, 4M urea and the second dimension contained 12% polyacrylamide, 6M urea, 0.1M sodium phosphate, 0.1% sodium dodecylsulfate, pH 7.1, as described previously (18). The gels were stained with Coomassie brilliant blue R (Sigma), and the method of Bonner and Laskey (19) was employed for fluororadiographic detection of ^{35}S-methionine labeled proteins. The fluororadiograms were used to locate labeled spots which were then cut out of the dried gels, incubated overnight at 50^{o} in 30% hydrogen peroxide and counted in a packard liquid scintillation counter using a ^{14}C window. Unlabeled areas of the gels were also sampled to obtain background values which were subtracted from the data.

RESULTS

As shown in figure 1, after release from the thymidine block there was sufficient synchrony to distinguish intervals from the middle of S phase (hrs 3-6, MidS), the middle of cell division (hrs 9-12, Mid D), the early part of G_1 phase (hrs 13-16, Early G_1) and the later part of G_1

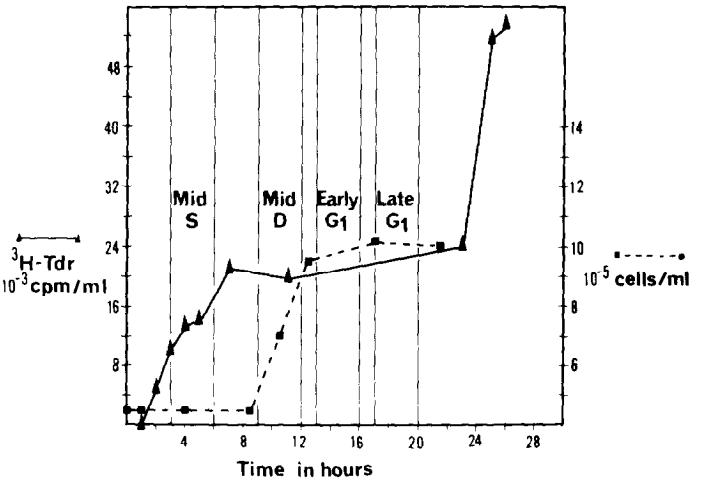

Figure 1 Kinetics of DNA synthesis and cell division after release of HeLa cells from thymidine block during a typical experiment. Shown are the incorporation of ^{3}H-thymidine into TCA precipitable material (▲——▲) and the number of cells per ml of media (■- -■). The areas in between the vertical lines denoted Mid S, Mid D, Early G1 and, late G1 are the three hour intervals during which aliquots of the cell suspension were labeled with ^{35}S-methionine to monitor protein biosynthesis.

phase (hrs 17-20, late G_1). Incorporation of ^{35}S-methionine into protein A24, histone 4, ubiquitin and HMG 1,2 was readily detectable by two-dimensional polyacrylamide gel electrophoresis (Fig. 2a and 3a) followed by fluorography (Fig. 2b and 3b).

The quantitative data obtained from the average of three separate synchronization experiments are shown in figure 4. The data were normalized to 100% in each experiment. As expected (20), the bulk of histone 4 synthesis occurred during mid S phase (Fig. 4a) although, as in previous studies (13,21,22), there were somewhat higher rates of histone 4 synthesis during other periods than would be expected from the rates of DNA replication (Fig. 4b). Although protein A24 contains histone 2A, the biosynthesis of histone 4 was monitored in these experiments because it contains 1 methionine (23,24) and is well resolved in the electrophoresis (25). The biosynthesis of high mobility group proteins 1 and 2 was not coupled to histone 4 synthesis (Fig. 4a) as previously noted by Kuehl (26).

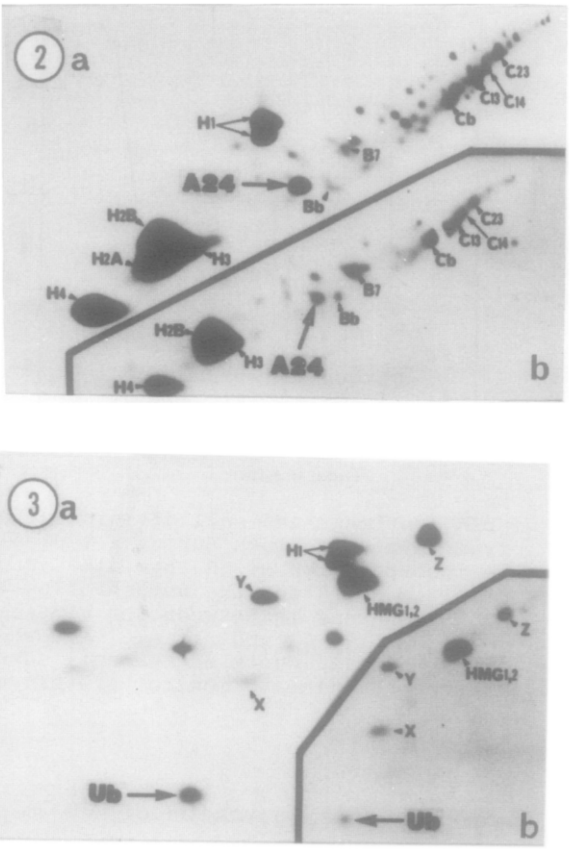

Figure 2 Two-dimensional polyacrylamide gel electrophoretic and fluororadiographic analysis of 0.4 N H_2SO_4 soluble proteins from chromatin preextracted with saline-EDTA, Tris and 0.35M Sodium Chloride as in methods. Proteins were labeled with ^{35}S-methionine during mid S phase. As estimated by Lowry (17) analysis, 250 µg of protein were subjected to electrophoresis from right to left in the urea - acetic acid first dimension and from top to bottom in the sodium dodecyl sulfate-urea second dimension (18). The stained gel pattern is in the upper portion (a) and the inset (b) is the fluororadiogram. The fluororadiograms contain all the spots visualized due to incorporation of ^{35}S-methionine.

Figure 3 Two-dimensional polyacrylamide gel electrophoresis and fluorography of high mobility group (HMG) proteins labeled during mid S phase. Data displayed as in figure 2.

The biosynthesis of ubiquitin and its conjugation into protein A24 were parallel through the cell cycle, rising to a maximum in early G_1 phase, continuing throughout interphase and declining to a minimum during cell division (Fig. 4a). Histones 4 and 2A are synthesized in parallel (13) and in chromatin the molar ratio of histones 4:2A: proteir

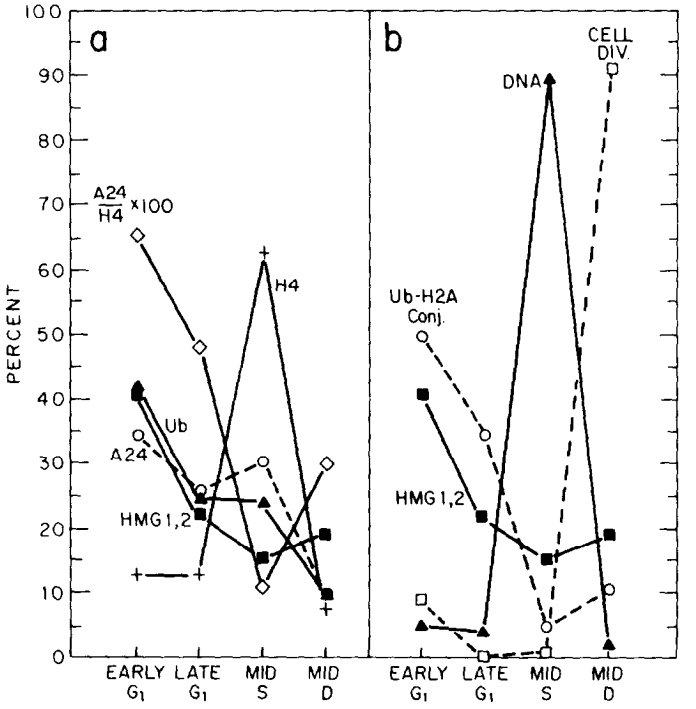

Figure 4 a. The pattern of incorporation of ^{35}S-methionine into protein during the 4 3 hr intervals designated in fig. 1. The data from three separate synchronization experiemnts were normalized to 100% = total cpm = cpm Early G1 + cpm Late G1 + cpm Mid S + cpm Mid D: +—+, H4 (100% = 3115); ▪——▪ , HMG1,2 (combined, 100%=2734); ▲——▲ , ubiquitin (100%=610); 0---0, A24 (100%=721). The ratio of incorporation of ^{35}S-methionine into A24 vs H4 is also the average of 3 experiments and is expressed as a percent. ◇——◇ .

b. The pattern of excess ubiquitin histone 2A conjugation, HMG1,2 biosynthesis, DNA synthesis and cell division during the 4 3 hr intervals designated in figure 1. Data normalized to 100% as in a: 0---0, ubiquitin-histone 2A conjugation, due to incorporation of ^{35}S-methionine into A24/H4 > 0.1 (100% = 431); ▪——▪ ,HMG1, 2 (same as in a); ▲——▲ , incorporation of ^{3}H thymidine into acid precipitable material, average of 3 experiments performed as in Fig. 1 (100% = 8200 cpm/ml); □----□ , increase in number of cells per ml, average of 3 experiments performed as in Fig. 1 (100% = 5.8 x 10^5 cells/ml).

A24 is approximately 10:9:1 (1,8). Protein A24 also contains 1 methionine, located at the NH_2-terminus of ubiquitin (1,4,5). Thus, when the ratio of incorporation of ^{35}S-methionine into protein A24 to that of histone 4 (Fig. 4a) exceeded 0.1, the conjugation of ubiquitin to histone 2A was greater than expected solely on the basis of histone synthesis. In

these experiments 59.8% of the ubiquitin-histone 2A conjugation measure was of this type and the pattern of its distribution during the cell cycle was similar to that of the biosynthesis of HMG proteins 1 and 2 (Fig. 4b).

DISCUSSION

Cleavage of the ubiquitin-histone 2A bond of protein A24 has been correlated with both transcription and repression of chromatin. Loss of protein A24 was demonstrated during hyperinduction of rRNA synthesis in rat liver nucleoli due to thioacetamide administration (27) and partial heptectomy (28). Recently, an enzymatic activity that converts protein A24 into histone 2A and ubiquitin in vitro has been demonstrated in such nucleoli (29). Reduced quantities of protein A24 and the presence of free ubiquitin was noted in transcriptionally active rat liver nuclear chromatin fractions (30,31). Cleavage of the ubiquitin-histone 2A bond and loss of ubiquitin has been shown to accompany transcriptional shutdown during chicken erythropoiesis (32). Decreased protein A24 and an increased content of histone 2A in metaphase chromosomes has been reported (33). Those studies indicated that subsequent formation of protein A24 was rapid and did not require ubiquitin synthesis.

The present study showed that biosynthesis of ubiquitin and the formation of protein A24 occurred at high rates, not only during histone synthesis and chromatin replication but throughout interphase. The possibility that the continued biosynthesis of protein A24 reflects shifting patterns of chromatin structure and template activity requires further investigation. In addition, this result is of increased interest in view of recent studies showing that conjugation with ubiquitin preceeds proteolytic degradation of cytoplasmic proteins (34-35).

ACKNOWLEDGMENTS

These studies were supported by the Research Program Project Grant CA10893, awarded by the National Cancer Institute, DHEW.

REFERENCES

1. Goldknopf, I. L., Taylor. C. W., Baum, R. M., Yeoman, L. C. Olson, M. O. J., Prestayko, A. W., and Busch, H. (1975) J. Biol. Chem. 250, 7182-7187.
2. Goldknopf, I. L, and Busch, H. (1975) Biochem. Biophys. Res. Commun. 65, 951-960.
3. Hunt, L. T., and Dayoff, M. O. (1977) Biochem. Biophys. Res. Commun. 74, 650-655.
4. Olson, M. O. J., Goldknopf, I. L., Guetzow, K. A., James, G. T., Hawkins, T. C., Mays-Rothberg, C. J., and Busch, H. (1976) J. Biol Chem. 251, 5901-5903.
5. Schlessinger, D. H., Goldstein, G., and Nial, H. D. (1975) Biochemistry 14, 2214-2218.
6. Goldknopf, I. L., and Busch, H. (1977) Proc. Natl. Acad. Sci. USA 74, 864-868.
7. Goldknopf, I. L., and Busch, H. (1978) The Cell Nucleus (H. Busch, Ed.), Vol. VI, pp. 149-180, Academic Press, New York.
8. Albright, S. C., Nelson, P. P., and Garrard, W. T. (1979) J. Biol. Chem. 254, 1065-1073.
9. Goldknopf, I. L., French, M. F., Musso, R., and Busch, H. (1977) Proc. Natl. Acad. Sci. USA 74, 5492-5495.
10. Martinson, H. G., True, R., Burch, J. B. E., and Kunkel, G. (1979) Proc. Natl. Acad. Sci. USA 76, 1030-1034.
11. Xeros, N. (1962) Nature 194, 682-683.
12. Peterson, D. F., and Anderson, E. C. (1964) Nature 203, 542-643.
13. Gurley, L. R., and Hardin, J. M. (1968) Arch. Biochem. Biophys. 128, 285-292.
14. Ballal, N. R., Goldberg, D. A., and Busch, H. (1975) Biochem. Biophys. Res. Commun. 62, 972-982.
15. Chae, C.-B., and Morris, H. P. (1975) Biochem. J. 146, 281-281.
16. Goodwin, G. H., Sanders, C., and Johns, E. W. (1973) Eur. J. Biochem. 38, 14-19.
17. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randel, R. J., (1951) J. Biol. Chem. 193, 265-275.
18. Orrick, L. R., Olson, M. O. J., and Busch, H. (1973) Proc. Natl. Sci. USA 70, 1316-1320.
19. Bonner, W. M., and Laskey, R. A. (1974) Eur. J. Biochem. 46, 83-88.
20. Borun, T. W., Scharff, M. D., and Robbins, E. (1967) Proc. Natl. Acad. Sci. USA 58, 1977-1983.
21. Gurley, L. R., Walter, R. A., and Tobey, R. A. (1972) Arch. Biochem. Biophys. 148, 633-641.
22. Detke, D., Lichter, A., Phillips, I., Stein, J., and Stein, G., (1979) J. Cell Biol. 83, 421a.
23. DeLange, R. J., Fambrough, D. M., Smith, E. L., and Bonner, J. (1969) J. Biol. Chem. 244, 319-334.
24. Ogawa, Y., Quagliarotti, G., Jordan, J., Taylor, C. W., Starbuck, W. C., and Busch, H. (1969) J. Biol. Chem. 244, 4387-4392.
25. Goldknopf, I. L., and Busch, H. (1973) Physiol. Chem. Phys. 5, 131-140.
26. Kuehl, L., (1979), J. Biol. Chem. 254, 7276-7281.
27. Ballal, N. R., Goldknopf, I. L., Goldberg, D. A., and Busch, H. (1974) Life Sci. 14, 1835-1845.
28. Ballal, N. R., Kang., Y. J., Olson, M. O. J., and Busch, H. (1975) J. Biol. Chem. 250, 5921-5925.
29. Anderson, M. W., Ballal, N. R., Goldknopf, I. L., Wilson, G., and Busch, H. (1980) J. Cell Biol. in press.

30. Goldknopf, I. L., French, M. F., Daskal, Y., and Busch, H. (1978) Biochem. Biophys. Res. Commun. 84, 786-793.
31. Watson, D. C., Levy, W., B. and Dixon, G. H. (1978) Nature 276, 196-198.
32. Goldknopf, I. L., Wilson, G., Rosenbaum, F., Ballal, N. R., and Busch, H. (1980) Fed. Proc. 31, 1650.
33. Matsui, S.-I., Seon, B. K., and Sandberg, A. (1979) Proc. Natl. Acad. Sci. USA 76, 6386-6390.
34. Wilkinson, K. D., Urban, M. K. and Haas, A. L. (1980) J. Biol. Chem. in press.
35. Hershko, A., Ciechanover, A., Heller, H., Haas, A. L., and Rose, I. A. (1980) Proc. Natl. Acad. Sci. USA 77, 1783-1786.