Vol. 203, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 30, 1994 Pages 344-350

INHIBITION OF TRANSCRIPTION SELECTIVELY REDUCES THE LEVEL OF UBIQUITINATED HISTONE H2B IN CHROMATIN

James R. DAVIE and Leigh C. MURPHY

Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Manitoba, 770 Bannatyne Ave., Winnipeg, Manitoba, Canada R3E 0W3

Received June 28, 1994

SUMMARY: The effect of inhibiting transcription and/or replication on the steady state levels of the ubiquitinated histone isoforms was investigated. We show that treatment of chinese hamster ovary (CHO), monkey kidney (COS), human endometrial carcinoma (Hec-50 and Ishikawa) cells with actinomycin D and 5,6-dichloro-1-ß-D-ribofuranosylbenzimidazole, inhibitors of heterogeneous nuclear RNA synthesis, selectively reduced the levels of ubiquitinated (u) H2B, but not uH2A, uH2A.Z, polyubiquitinated H2A or a novel ubiquitinated histone species, in the chromatin of these cells. The level of the ubiquitinated histones was not affected when synthesis of DNA was arrested. These results show that, in general, maintenance of the levels of uH2B in chromatin is dependent upon ongoing transcription.

Ubiquitin is a 76 residue protein that may be attached to proteins via an isopeptide linkage to the ε-amino group of the target protein. Ubiquitin has a central role in mediating the intracellular degradation of selected proteins (1). In the nucleus, histones H2A, H2B and their variant forms are ubiquitinated, but ubiquitination of these proteins does not accelerate their turnover. For histone H2A and H2B, the carboxyl end of ubiquitin is reversibly attached to the ε-amino group of lysine (Lys-119 in H2A and Lys-120 in H2B) (2,3). There is evidence that histone ubiquitination has a role in maintaining the structure of transcriptionally active chromatin. Ubiquitinated histone H2A is complexed to transcriptionally active DNA (4,5), although not all reports agree with this association (6). Ubiquitinated (u) histone H2B is highly enriched in transcriptionally active gene-enriched chromatin fractions (7). Moreover, the transcriptionally active macronucleus, but not the transcriptionally inert micronucleus, of Tetrahymena is associated with uH2B (8). Our observations suggest that uH2B, more so than uH2A, is associated with transcriptionally active DNA.

0006-291X/94 $5.00

Inhibition of transcription does not alter the amount of uH2A in HeLa cell or T-47D-5 human breast cancer cell chromatin (9,10). However, we found that inhibition of transcription, particularly synthesis of hnRNA, leads to a decline in the content of uH2B in T-47D-5 human breast cancer cell chromatin. It was important to find out whether the reduction in uH2B levels following the inhibition of transcription was unique to breast cancer cells or a general phenomenon. Inhibition of transcription, but not replication, resulted in a decrease in uH2B, but not ubiquitinated isoforms of the histone H2A variants, levels in all cell lines tested. These observations show that ubiquitination of histone H2B is dependent on the transcription process, providing support for the idea that uH2B is selectively associated with transcriptionally active DNA.

MATERIALS AND METHODS

Cells and Treatments. T-47D-5 human breast cancer cells, COS-1 monkey kidney cells, and CHO chinese hamster ovary cells (dihyrofolate reductase-deficient mutant DG44) were maintained at 37°C under a humidified atmosphere of 5% CO_2 in 5% fetal bovine serum containing medium as described previously (11). Ishikawa and Hec-50 human endometrial carcinoma cells were grown as described by Gong *et al.* (12). For experiments, cells were plated in 25 ml of medium at 2 X 10^6 cells per 150 mm diameter dish. Three to 4 days later, when the cells were still in the logarithmic phase of growth, the medium was renewed and the cells were subjected to the various treatments described below. Following treatment, the medium was removed, the cells were harvested off the monolayer by scraping with a rubber policeman and placed on ice. After centrifugation at 4°C, the cell pellet was snap frozen and stored below -70°C until further analysis. Different groups of cells underwent the following treatments: a) 0.04 μg/ml actinomycin D for 30 min followed by 75 μM 5,6-dichloro-1-ß-D-ribofuranosylbenzimidazole (DRB) for 40 min (9), b) 40 μM aphidicolin for 70 min (13), c) 40 μM aphidicolin for 70 min followed by 0.04 μg/ml actinomycin D for 30 min and then by 75 μM DRB for 40 min.

Isolation of Histones. The cell pellet was resuspended in Buffer A [100 mM KCl, 10 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 10 mM Na butyrate, 1 mM phenylmethanesulfonyl fluoride (PMSF), 0.1 μg/ml aprotinin, and 10 mM iodoacetamide, an inhibitor of isopeptidase (14)] containing 0.1 % NP-40, and homogenized. The nuclei were collected by centrifugation and the pellet was resuspended in Buffer A. Sulphuric acid was added to 0.4 N and the suspension was incubated on ice for 30 min. Acid-soluble proteins were collected following centrifugation (15).

Polyacrylamide Gel Electrophoresis and Immunochemical Detection of Ubiquitinated Histone Species. One-dimensional AUT [acetic acid/6.7 M urea/0.375% (w/v) Triton X-100/15% polyacrylamide gel] and two-dimensional [AUT into SDS (sodium dodecyl sulphate)/15% polyacrylamide gel] gel electrophoresis were done as previously described (7). The gels were stained with Coomassie Blue and, for some gels, also with silver or transferred to nitrocellulose and immunochemically stained for ubiquitin with an anti-ubiquitin IgG as described previously (16).

RESULTS AND DISCUSSION

Histones isolated from CHO, COS, Hec-50 and Ishikawa cell nuclei were electrophoretically resolved on AUT 15% polyacrylamide gels. Fig. 1A shows that the CHO nuclei had the lowest amount of uH2A, with the content of uH2A in COS = Hec-50 > Ishikawa > CHO nuclei. To inhibit transcription, the cell lines were treated with actinomycin D and DRB. Actinomycin D and DRB result in an inhibition of 60 to 75% of the hnRNA synthesis and most of the rRNA synthesis (9,17,18). Treatment of the various cell lines with actinomycin D and DRB did not appear to alter the levels of uH2A as judged by Coomassie Blue staining (Fig. 1A). However, two-dimensional gel electrophoresis (AUT into SDS) of the histones has shown that other proteins comigrate with uH2A (Fig. 2). To find out whether the levels of the ubiquitinated histones were altered by the inhibition of transcription, one dimensional Western blotting experiments using an anti-ubiquitin antibody were done (Fig. 1B). The immunochemical staining shows that the content of uH2A was lower in CHO nuclei than in COS, Hec-50, or Ishikawa nuclei. The nuclear levels of uH2B in the untreated cell lines were also different, with the amount of uH2B in HEC-50 = Ishikawa > CHO > COS nuclei. Fig. 1B also demonstrates that treating cells with

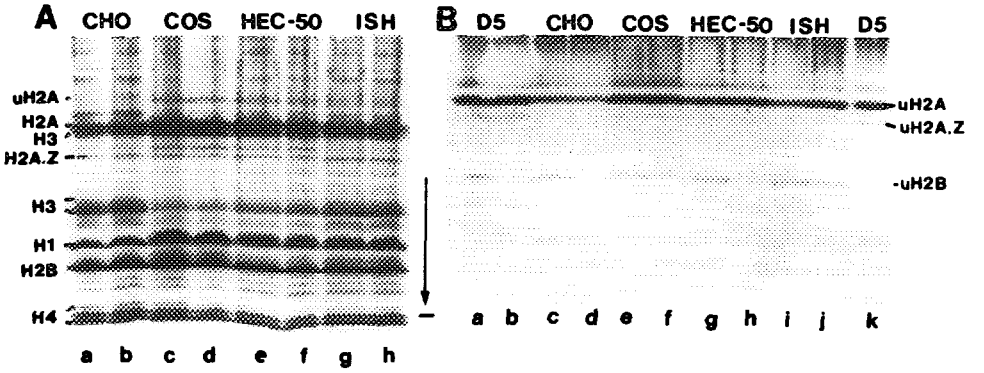

Fig. 1. **Inhibition of transcription reduces the level of uH2B.** Panel A shows Coomassie Blue stained AUT 15% polyacrylamide gel patterns containing acid-soluble nuclear proteins (36 μg) isolated from CHO, COS, HEC-50, and Ishikawa (ISH) cells that were not treated (lanes a, c, e, and g) or incubated with actinomycin D and DRB (lanes b, d, f, and h). Panel B shows the nitrocellulose filter containing the acid-soluble nuclear proteins (36 μg protein for all lanes except lane k which contains 12 μg) that were transferred from an AUT 15% polyacrylamide gels. Lanes a, c, e, g, i, and k contain histones isolated from nontreated cells, while lanes b, d, f, h, and j have histones from cells incubated with actinomycin D and DRB. D5 is T-47D-5 human breast cancer cells. The filter was immunochemically stained for ubiquitin with an anti-ubiquitin IgG and alkaline phosphatase-conjugated goat anti-rabbit antibody.

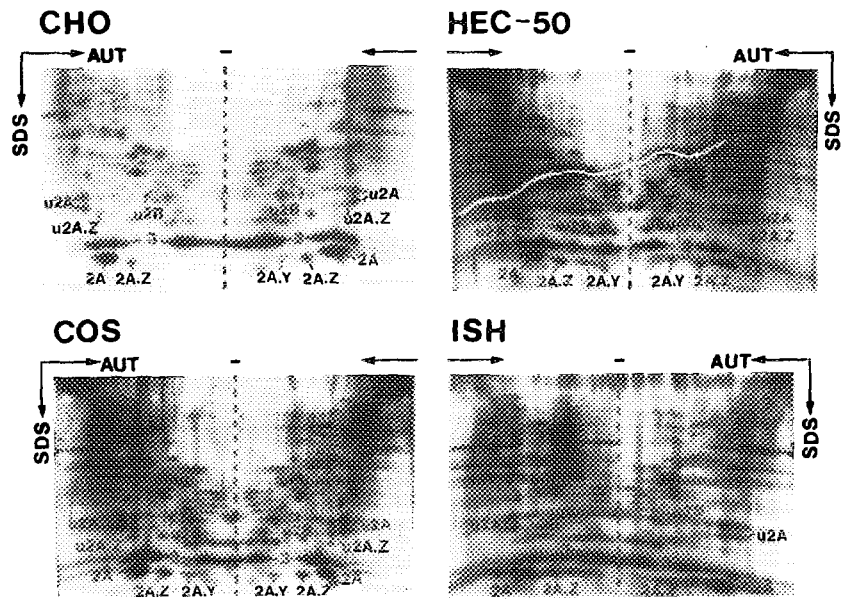

Fig. 2. **Inhibition of transcription does not change the levels of uH2A and uH2A.Z.** Acid-soluble proteins (36 μg) isolated from CHO, COS, HEC-50, and Ishikawa (ISH) cells not treated (left panel) or incubated with actinomycin D and DRB (right panel) were electrophoretically resolved on two dimensional gels (AUT into SDS 15% polyacrylamide gel). The gels were silver stained. Note that the AUT gel containing histones from treated cells was placed onto the second dimension gel in the opposite orientation as the AUT gel containing histones from the untreated cells. Histones H3, H2A, H2A.Z, H2A.Y, and H2B are shown as 3, 2A, 2A.Z, 2A.Y, and 2B, respectively.

actinomycin D and DRB did not affect the level of uH2A in the cell lines. In contrast, the level of uH2B in the nuclei of all cell lines (T-47D-5, CHO, COS, Hec-50, Ishikawa) declined after the cells were incubated with actinomycin D and DRB.

Treatment of the various cell lines with aphidicolin, a potent inhibitor of DNA polymerase α and DNA replication (19,20), did not alter the level of the ubiquitinated histones, in agreement with our previous observations using the T-47D-5 human breast cancer cells (10). The uH2B levels in the nuclei of these cells incubated with aphidicolin, actinomycin D and DRB or with actinomycin and DRB were similar (data not shown).

Histones from the cell lines were resolved by two-dimensional gel electrophoresis, and the gels were silver stained (Fig. 2). Alternatively, the histones were transferred to nitrocellulose which was immunochemically stained with the anti-ubiquitin antibody (Fig. 3). A distinctive feature of the ubiquitinated histones is that they often migrate as doublets in the second dimension SDS gel (7). The silver

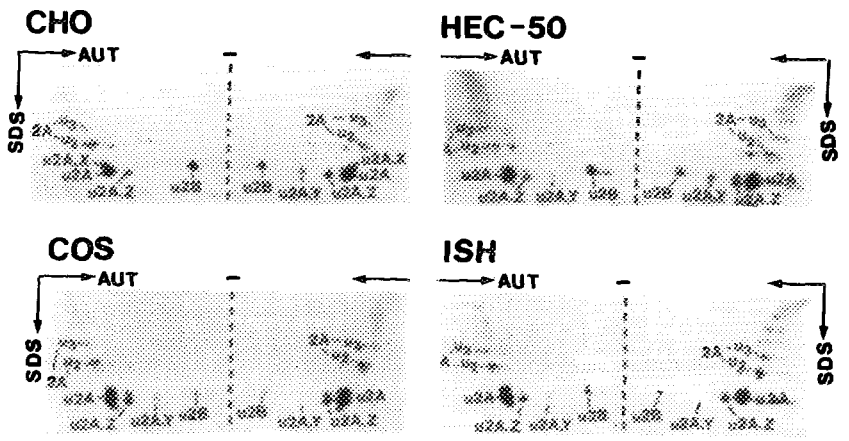

Fig. 3. **Inhibition of transcription reduces the level of uH2B, but not uH2A, uH2A.Z or uH2A.Y.** Acid-soluble proteins (36 μg) resolved on two-dimensional gels as shown in Fig. 2 were transferred to nitrocellulose filters which were immunochemically stained for ubiquitin as described in Fig. 1. The left panel contains histones from untreated cells, and the right panel has histones from cells treated with actinoycin D and DRB. The ubiquitinated isoforms of histones H2A, H2A.Z, H2A.X, H2A.Y, and H2B are shown as u2A, u2A.Z, u2A.X, u2A.Y, and u2B, respectively. The di- and triubiquitinated isoforms of histone H2A are shown as $u_2$2A and $u_3$2A, respectively.

stained two-dimensional gel patterns show that the nuclear contents of both uH2A and uH2A.Z were higher in Hec-50 and COS cells than in CHO and Ishikawa cells (Fig. 2). Fig. 2 and 3 demonstrate that treatment of the various cell lines with actinomycin D and DRB significantly reduced the amount of uH2B, but not uH2A, uH2A.Z or uH2A.X. The levels of the di- and triubquitinated isoforms of H2A were similarly not affected. Frequently, we observed a fourth ubiquitinated histone species (tentatively named uH2A.Y) which to our knowledge has not been previously reported. Based upon the ubiquitinated histones electrophoretic mobility in the two-dimensional gel, the ubiquitinated histone variant, uH2A.Y, may be a member of the histone H2A family (21). The possible unmodified form of this novel histone variant (H2A.Y) is shown in Fig. 2. We have observed this ubiquitinated histone variant in all of the cell lines used in this study and in T-47D-5 human breast cancer cells. Because of its low abundance, uH2A.Y was not always detected on the Western

blots. This ubiquitinated histone variant was not found in chicken erythrocyte chromatin. As with the other ubiquitinated isoforms of histone H2A variants, the levels of this novel ubiquitinated histone (uH2A.Y) species were not affected by the various treatments.

Our results show that, in general, inhibition of transcription, but not replication (data not shown, see 10), selectively decreases the steady state level of uH2B. Histone H2B ubiquitination is the only histone modification that is dependent upon ongoing transcription. We postulate that transcription results in the dissolution of the nucleosome, presenting the otherwise sequestered C-terminal tail of histone H2B to the enzymes catalyzing the addition of ubiquitin. Inhibition of transcription blocks the addition of ubiquitin but does not prevent removal of the ubiquitin by isopeptidase (10). The net effect is a reduction in the level of uH2B. Histone H2A does not have its C-terminal tail buried in the nucleosome, and thus ubiquitination of histone H2A and its variants is not dependent on processes that alter nucleosome structure. Once the nucleosome is initially opened by transcription and histone H2B ubiquitinated, uH2B may impede nucleosome refolding (22,23).

ACKNOWLEDGMENTS

This project was supported by a grant from the Medical Research Council of Canada (MT-9186, JRD; MT-10345, LCM) and the National Cancer Institute of Canada (to LCM). LCM is an NCI Scientist and JRD is an MRC Scientist.

REFERENCES

1. Hershko, A., and Ciechanover, A. (1992) Annu. Rev. Biochem. 61, 761-807.
2. Goldknopf, I.L., and Busch, H. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 864-868.
3. Thorne, A.W., Sautiere, P., Briand, G., and Crane Robinson, C. (1987) EMBO J. 6, 1005-1010.
4. Levinger, L., and Varshavsky, A. (1982) Cell 28, 375-385.
5. Barsoum, J., and Varshavsky, A. (1985) J. Biol. Chem. 260, 7688-7697.
6. Huang, S.-Y., Barnard, M.B., Xu, M., Matsui, S.-I., Rose, S.M., and Garrard, W.T. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 3738-3742.
7. Nickel, B.E., Allis, C.D., and Davie, J.R. (1989) Biochemistry 28, 958-963.
8. Davie, J.R., Lin, R., and Allis, C.D. (1991) Biochem. Cell Biol. 69, 66-71.
9. Ericsson, C., Goldknopf, I.L., and Daneholt, B. (1986) Exp. Cell Res. 167, 127-134.
10. Davie, J.R., and Murphy, L.C. (1990) Biochemistry 29, 4752-4757.
11. Murphy, L.C., and Dotzlaw, H. (1989) Cancer Res. 49, 599-604.
12. Gong, Y., Anzai, Y., Murphy, L.C., Ballejo, G., Holinka, C.F., Gurpide, E., and Murphy, L.J. (1991) Cancer Res. 51, 5476-5481.

13. Sariban, E., Wu, R.S., Erickson, L.C., and Bonner, W.M. (1985) Mol. Cell Biol. 5, 1279-1286.
14. Matsui, S.-I., Sandberg, A.A., Negoro, S., Seon, B.K., and Goldstein, G. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 1535-1539.
15. Davie, J.R., and Nickel, B.E. (1987) Biochim. Biophys. Acta 909, 183-189.
16. Delcuve, G.P., and Davie, J.R. (1992) Anal. Biochem. 200, 339-341.
17. Perry, R.P. (1963) Exp. Cell Res. 29, 400-406.
18. Seghal, P.B., Darnell, J.E., and Tamm, I. (1976) Cell 9, 473-480.
19. Pedrali-Noy, G., and Spadari, S. (1979) Biochem. Biophys. Res. Commun. 88, 1194-1202.
20. Arabshahi, L., Brown, N., Khan, N., and Wright, G. (1988) Nucleic. Acids. Res. 16, 5107-5113.
21. West, M.H.P., and Bonner, W.M. (1980) Biochemistry 19, 3238-3245.
22. Van Holde, K.E., Lohr, D.E., and Robert, C. (1992) J. Biol. Chem. 267, 2837-2840.
23. Morse, R.H. (1992) Trends. Biochem. Sci. 17, 23-26.