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LOCALIZATION OF PHOSPHOPROTEINS AND OF PROTEIN KINASES IN CHROMATIN FROM BUTYRATE TREATED HTC CELLS.

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SUMMARY : We have recently shown that phosphoproteins are associated with the chromatin regions accessible to micrococcal nuclease. We have also shown that butyrate treatment modifies the accessibility of chromatin to the nuclease. In this work we have studied the effect of butyrate on the localization of phosphoproteins in chromatin. We observed a strong similarity between the effect of butyrate on the release of DNA fragments and on the release of phosphoproteins by the nuclease, which indicates that in butyrate treated as in control cells the released fragments include phosphoproteins. Butyrate treatment increases strongly chromatin protein kinase specific activity and modifies its localization in the released fragments; it is therefore likely that it modifies its localization in chromatin.

We have recently observed that a mild digestion of chromatin by micrococcal nuclease releases chromatin fragments which include phosphoproteins and protein kinases(1). Since it has been shown that micrococcal nuclease preferentially releases active parts of chromatin (2-7), it may be suggested that phosphoproteins and protein kinases are associated with these parts of chromatin.

Butyrate is able to induce differentiated fonctions in various cell types (8, 9). We have recently shown that sodium butyrate modifies chromatin structure in such a way that a small part of it becomes more accessible to micrococcal nuclease, another small part becomes more resistant to the nuclease attack than in control cells (10). It is therefore suggested that the butyrate induced modifications in gene expression could be related to the modifications in chromatin structure.

One of the purposes of this work was to establish whether the regions of chromatin which become accessible to the nuclease after butyrate treatment are associated with phosphoproteins as they are in control cells.

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MATERIALS AND METHODS

Cell cultures and labelling : HTC cells were grown at 37°C in suspension in Swim's 77 medium supplemented with 10 % new born calf serum as described by Hershko and Tomkins (11). When indicated cells were cultured for 24 h in the presence of 5 mM sodium butyrate. The hyperacetylation of the histones was controlled by polyacrylamide gel electrophoresis performed according to Panyim and Chalkley (12) as modified by Shaw and Huang (13). When the double labelling method was used, cells were first cultured for 4 h in a medium deprived from inorganic phosphate. They were then cultured in a fresh medium at a cell concentration of 10^7 cells per ml and a total amount of 6.10^8cells. Butyrate cells were labelled for 1 h by addition of 83 µCi/ml of [^{32}P] inorganic phosphate (Radiochemical Center, Amersham - U.K.) while the control cells were labelled by addition of 33 µCi/ml of [^{33}P] inorganic phosphate for 1 h (N.E.N.. Boston, Mass. U.S.A.). An equal number of [^{32}P] butyrate treated and [^{33}P] control cells were mixed and the nuclei prepared as previously described (14).

Micrococcal nuclease digestion : Chromatin 100 A_{260} U/ml nuclei was digested with 200 U/ml of micrococcal nuclease (Worthington, N.Y. U.S.A.) according to Shaw et al. (15) for various times. At indicated times aliquots were collected, membranes and undigested chromatin were discarded by 10 min of centrifugation at 3 000 g. The [^{32}P] and [^{33}P] radioactivities were measured on aliquots of the supernatant material after precipitation by 20 % trichloroacetic acid. The acid soluble material obtained from digested DNA was estimated by the EDTA-perchloric acid method.

Sucrose gradient fractionation : The released fragments in 0.3 ml volumes were layered on 5-20 % sucrose linear gradients in 10 mM Tris cacodylate buffer (pH 7.3), 0.7 mM EDTA, 1 mM 2-mercaptoethanol. Centrifugation was performed at 21000 rpm for 16 h in a SW 41 Beckman rotor. Fractions of 0.3 ml were collected. The [^{32}P] and [^{33}P] radioactivities were measured. Absorbance at 260 nm was automatically recorded.

Preparative polyacrylamide gel electrophoresis : Chromatin from sodium butyrate treated and from control cells was digested with micrococcal nuclease until 3 % of DNA were rendered acid soluble. Undigested chromatin was removed by 10 min of centrifugation at 3000 rpm. The released chromatin fragments were fractionated on a 4 % polyacrylamide gel in 0.1 M Tris borate buffer (pH 8.3) as previously described (1,10). The buffer was used for elution. The fragments were fractionated according to their size. Phosvitin kinase was assayed as previously described (16) in each chromatin fraction obtained after electrophoresis.

RESULTS AND DISCUSSION

Effect of sodium butyrate on the localization of phosphoproteins in the chromatin fragments released by micrococcal nuclease digestion.

An equal number of [^{32}P] butyrate treated cells and of [^{33}P] control cells were mixed and the chromatin was digested by micrococcal nuclease for various times under such conditions that 0.5 - 7 % of the DNA were rendered acid soluble. The [^{32}P] and the [^{33}P] radioactivities were measured in

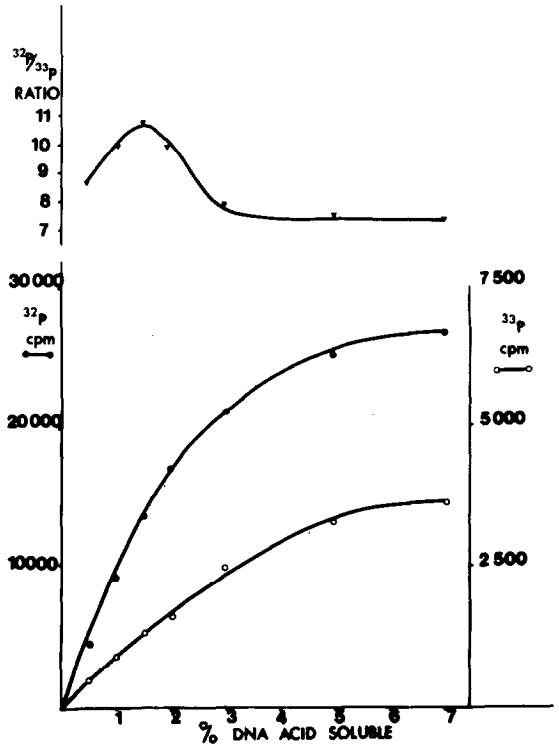

Figure 1 : Kinetics of phosphoprotein release from chromatin of butyrate treated and control of HTC cells by micrococcal nuclease. Butyrate treated cells were cultured for 1 h in the presence of [^{32}P] phosphate and control cells in the presence of [^{33}P] phosphate. The cells were mixed and 100 A_{260} U/ml nuclei were digested by 200 U/ml of micrococcal nuclease for various times at 37°C. The released material was precipitated with trichloroacetic acid. [^{32}P] radioactivity (●——●), [^{33}P] radioactivity (o——o) and the [^{32}P/^{33}P] radioactivity ratios (▼——▼) were measured.

aliquots of the released fragments after precipitation by trichloroacetic acid. Fig. 1 shows a fast release of phosphoproteins until approximately 3 % of the DNA were rendered acid soluble, much less phosphoproteins were released afterwards and a plateau was reached when 7 % of the DNA, corresponding to 25 % of chromatin, were rendered acid soluble. However the kinetics of the release were not identical from the butyrate treated and from the control cells as shown by the measure of the [^{32}P/^{33}P] ratio. This ratio was 7.5 in intact chromatin. The ratio increased in the released material with the time of digestion and was much higher than in intact

chromatin until 1.5 % of DNA was acid soluble, indicating that the released phosphoproteins preferentially originated from butyrate treated cells. When digestion proceeded the ratio decreased until 3 % of the DNA were rendered acid soluble, showing that during the second part of the nuclease attack, chromatin from control cells was predominantly digested. After longer digestions the radioactivity ratio remained constant around 7.5 indicating that both chromatins were equally digested.

These results were confirmed and extended by the use of sucrose gradient fractionation which allows the separation of the released fragments according to their size. A_{260} was automatically recorded and produced the familiar pattern of chromatin fragments seen in digestion of various types of chromatin and each peak was identified by the electrophoretic determination of the DNA lengths as previously described (1). The [$^{32}P/^{33}P$] ratio was measured in each fraction after trichloroacetic precipitation (Fig. 2 and 3). In samples corresponding to 0.5, 1 and 1.5 - 2 % of DNA rendered acid soluble the ratios increased with the digestion time, especially in the small fragments including mono-, di- and trimeres of nucleosomes and became much higher than 7.5 which indicates that phosphoproteins are associated with the small fragments preferentially released from the butyrate treated cells. When digestion proceeded, until 3 % of the DNA were rendered acid soluble, we observed a strong decrease in the radioactivity ratio, which was then 8 - 9, indicating that phosphoproteins were present in the chromatin fragments which were then released from control cells. After longer digestion times the radioactivity ratio was around 7, showing that phosphoprotein containing fragments were equally released from butyrate treated and from control cells.

There is then a good correlation between the pattern of release of phosphoproteins and the pattern of release of DNA fragments observed in the previous work (10). It could mean either that butyrate induced changes in phosphoprotein localization in such a way that the parts of chromatin which

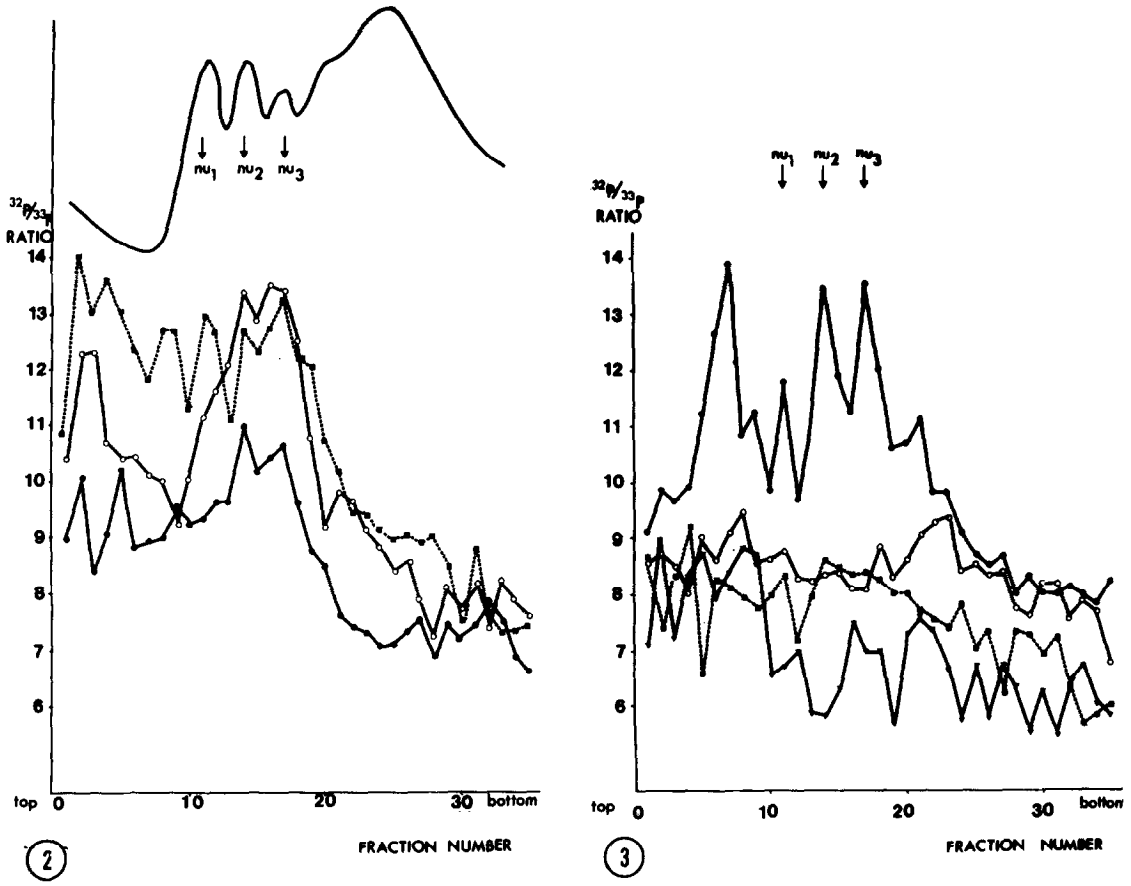

Figure 2 : Sucrose gradient fractionation of chromatin fragments released by short micrococcal nuclease digestion.
Nuclei, 100 A_{260} U/ml, were digested by 200 U/ml of micrococcal nuclease for various times at 37°C. The released material was analyzed by centrifugation on 5 - 20 % sucrose linear gradient for 16 h at 21 000 rpm in a SW 41 Beckman rotor. The [$^{32}P/^{33}P$] ratio was measured in 0.3 ml fractions after precipitation with trichloroacetic acid.
Sample corresponding to 0.5 % (●——●), 1 % (o——o), 1.5 % (■.....■) of DNA rendered acid soluble.
A_{260} was automatically recorded (———). The size of the fragments was established by the electrophoretic determination of DNA length.

Figure 3 : Sucrose gradient fractionation of chromatin fragments released by longer micrococcal nuclease digestion.
The experimental conditions were as described under Fig. 2.
Samples corresponding to 2 % (●——●), 3 % (o——o), 5 % (■.....■), 7 % (▼——▼) of DNA rendered acid soluble.

became accessible to nuclease digestion became associated with these phosphoproteins or that butyrate induced increase in accessibility concerned chromatin regions which include phosphoproteins.

Effect of sodium butyrate on the localization of protein kinases in the chromatin fragments released by micrococcal nuclease digestion.

A 24 h treatment of HTC cells with sodium butyrate induced a 60 % increase in the protein kinase specific activity in total chromatin. A 3.4 fold increase in specific activity was observed when the assays were carried out in the fragments released by micrococcal nuclease digestion performed under such conditions that 3 % of DNA were rendered acid soluble. This increase results from a specific effect of butyrate since it was expected from observations made in several cell types that the inhibition of cell growth would result in a decrease in chromatin protein kinase specific activity (17 - 21).

We have compared the localization of protein kinase in the chromatin fragments released by nuclease digestion of butyrate treated and control cells. Both chromatins were digested until 3 % of the DNA were rendered acid soluble. After removal of the undigested material by a short centrifugation, the release material was fractionated by preparative electrophoresis. Six fractions were obtained from mono- to hexameres of nucleosomes. Protein kinase was assayed on a sample of each fraction, using phosvitin as substrate (Fig. 4). Whereas in control cells, protein kinase activity was found associated with dimeres and to a lesser extent to trimeres of nucleosomes, in butyrate treated cells the enzyme activity was found in all fractions from mono to pentameres of nucleosomes, the highest activity being associated with mono and dinucleosomes.

Butyrate has then induced a modification in the localization of protein kinase in chromatin. The question remains open whether the increase in specific activity is due to an activation of the enzyme activity, to a synthesis of new enzyme molecules, to the removal of an inhibitor, or to the translocation into the nucleus of a cytoplasmic enzyme.

Since butyrate acts in a dose dependent and reversible manner (9, 22), which could partly be due to an inhibition of histone deacetylase (23 - 25),

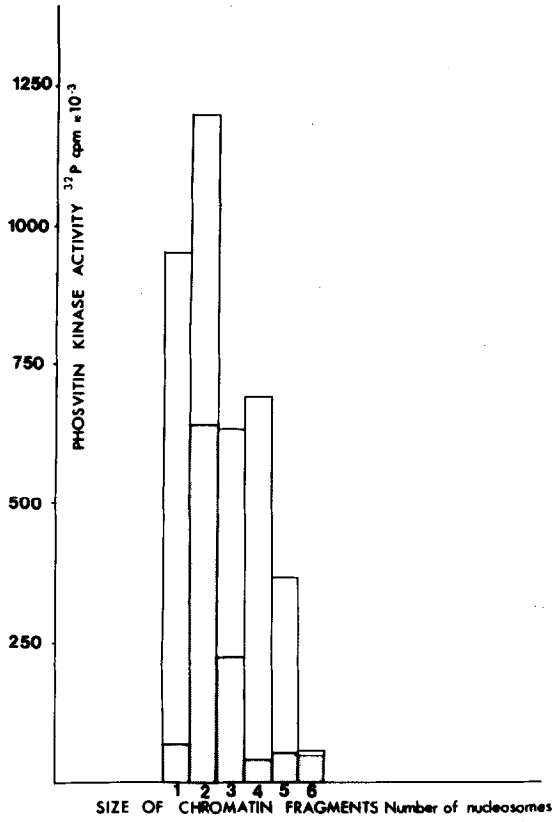

Figure 4 : Phosvitin kinase activity in chromatin fragments separated by preparative polyacrylamide gel electrophoresis.
Nuclei, 100 A_{260} U/ml, were digested by micrococcal nuclease until 3% of DNA were rendered acid soluble. After removal of the undigested chromatin by centrifugation, the released material was fractionated by preparative electrophoresis on 4 % polyacrylamide gel. Six fractions were eluted, corresponding to mono- to hexameres of nucleosomes. Phosvitin kinase activity was estimated in each fraction from butyrate treated (white column) and control (dark column) cells by incubation of 50 µg of proteins from each fraction with 100 µg of substrate and 2.10^6 c.p.m of (γ -[^{32}P]) ATP for 30 mn at 37°.

these observations and observations made by others suggest that acetylation and phosphorylation could both be involved in the control of gene expression. The role of histone acetylation has recently been emphasized by the observation of Reeves and Candido (26) who found that HMG 14 and 17, non histone proteins associated with active genes, inhibit histone deacetylase as does butyrate.

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REFERENCES

1. Kitzis, A., Tichonicky, L., Defer, N., and Kruh, J., Eur. J. Biochem. In Press.
2. Bellard, M., Gannon, F. and Chambon, P. (1978) Cold Spring Harbor Symp. Quant. Biol. 42, 779-791.
3. Reeves, R. (1978) Cold Spring Harbor Symp. Quant. Biol.42, 709-722.
4. Mathis, D.J. and Gorowski, M.A. (1978) Cold Spring Harbor Symp. Quant. Biol·42, 773-778.
5. Senior, M.B., and Frankel, F.R. (1978) Cell 14, 857-863.
6. Bloom, K.S. and Anderson, J.N. (1978) Cell 15, 141-150.
7. Levy-Wilson, B., Connor, W. and Dixon, G.H. (1979) J. Biol. Chem. 254, 609-620.
8. Prasad, K.N. and Sinha, P.K. (I976) In Vitro 12, 125-132.
9. Leder, A. and Leder, P. (1975) Cell 5, 319-322.
10. Kitzis, A., Tichonicky, L., Defer, N. and Kruh, J. (1980) Biochem. Biophys. Res. Commun. 93, 833-841.
11. Hershko, A. and Tomkins, G.M. (1971) J. Biol. Chem. 246, 710-714.
12. Panyim, S. and Chalkley, R. (1969) Biochemistry 8, 3972-3979.
13. Shaw, L.M. and Huang, R.L. (1970) Biochemistry 9, 4530-4542.
14. Befort, J.J., Befort, N., Beck, J.P., Beck, G., Defer, N. and Kruh, J. (1976) Cell Differentiation 5, 255-262.
15. Shaw, B.R., Corden, J.L., Sahasrabuddhe, C.G. and Van Holde, K.E.(1974) Biochem. Biophys. Res. Commun. 61, 1193-1198.
16. Dastugue, B., Tichonicky, L. and Kruh, J. (1974) Biochimie 56, 491-500.
17. Siebert, G., Ord, M.G. and Stocken, L.A. (1971) Biochem. J. 122, 721-725.
18. Brade, W.P., Thomson, J.A., Chiu, J.F. and Hnilica, L.S. (1974) Expl Cell Res. 84, 183-190.
19. Ishida, H. and Ahmed, K. (1974) Expl Cell Res. 84, 127-136.
20. Leibovitch, M.P., Tichonicky, L. and Kruh, J. (1978) Biochem. Biophys. Res. Commun. 81, 623-629.
21. Defer, N., Tichonicky, L., Kruh, J., Zoutewelle, G. and Van Wijk, R. (1979) Biochimie 61, 855-859.
22. Fiszman, M.Y., Montaras, D., Wright, W. and Gros, F. (1980) Expl Cell Research 126, 31-37.
23. Riggs, M.G., Whittaker, R.G., Neumann, J.R. and Ingram, V.M. (1977) Nature 268, 462-464.
24. Vidali, G., Boffa, L.C., Bradbury, E.M. and Allfrey, V.G. (1978) Proc. Natl. Acad. Sci. USA 75, 2239-2243.
25. Sealy, L. and Chalkley, R. (1978) Cell 14, 115-121.
26. Reeves, R. and Candido, E.P.M. (1980) Nucleic Acids Res. 8, 1947-1964.