DIFFERENTIAL ACCESSIBILITY OF (±) *TRANS*-7β,8α-DIHYDROXY-9α,10α-EPOXY-7,8,9,10-TETRAHYDROBENZO[a]PYRENE TO HISTONE PROTEINS

A. KOOTSTRA and T. J. SLAGA

University of Tennessee-Oak Ridge Graduate School of Biomedical Sciences and *Biology Division, Oak Ridge National Laboratory, Oak Ridge, TN 37830, USA*

Received 14 August 1979

1. Introduction

It is now quite evident that at the primary level of organization, chromatin consists of a linear array of nucleosomal core particles [1–4]. Each particle contains ~140 basepairs of DNA which are coiled around an octamer, two of each of the core histones H2A, H2B, H3 and H4. The very lysine-rich histone H1 (and H5 in avian erythrocytes) seems to be associated in part with the linker DNA, which is of variable length depending on the source of chromatin [5]. Evidence is now accumulating that the very lysine-rich histones are essential components in the higher-order packaging of the nucleosomal core particles [6,7].

Although histones are extremely conservative proteins [8,9], postsynthetic modifications such as phosphorylation, acetylation, and methylation of the aminoacyl side chains may provide subtle changes in the histone DNA complex; these modifications have been implicated to play a role in the structural and functional aspects of chromatin [10,11]. Since it has been shown that histone proteins can bind carcinogens in vivo and in vitro [12–14], this type of modification could have important consequences in the structure and function of chromatin.

Analysis of the distribution of benzo[a]pyrene adduct to histone proteins, when ± *trans*-7β,8α-dihydroxy-9α,10α-epoxy,7,8,9,10-tetrahydrobenzo-[a]pyrene (B[a]P diol-epoxide (anti)), the ultimate carcinogenic form of benzo[a]pyrene [15–17], was reacted with chicken erythrocyte chromatin, showed that the carcinogen was mainly associated with H3 and H2B and to a lesser extent with H4 [A.K., T.S., D. E. Olins, submitted). This differential binding may be influenced either by the presence of H5 and/or H1 or by the three-dimensional structure of the nucleosomal core particle itself.

The results reported here suggest that the differential binding of the carcinogen to histone proteins is probably a function of the constraints imposed by the chromosomal DNA, and that the presence of the very lysine rich histones seems to protect histone H2A from interacting with the carcinogen.

2. Materials and methods

2.1. *Isolation of soluble chromatin*

Chicken erythrocyte nuclei were isolated as in [18] except that all solutions contained 0.1 mM phenylmethanesulfonylfluoride (PMSF). The nuclear suspension was diluted with isolation buffer [10 mM Tris (pH 7.0), 10 mM NaCl, 3 mM $MgCl_2$, 0.5% Nonidet P40, 0.1 mM PMSF] to 1.4×10^9 nuclei/ml. The suspension was made 1.0 mM $CaCl_2$, and the digestion by micrococcal nuclease (60 units/ml) at 37°C was stopped after 10 min by addition of ethylenediaminetetraacetic acid (EDTA), 10 mM final conc. The digested nuclear suspension was dialyzed (Spectrapor) for 24 h at 4°C with 3 changes of 0.2 mM EDTA (pH 7.0), 0.1 mM PMSF. The lysed nuclear suspension was centrifuged at 10 000 × *g* for 10 min at 4°C, and the supernatant solution containing solubilized chromatin was concentrated with an Amicon PM10 membrane. Removal of subnucleosomal material, mononucleosomes, and up to penta-

nucleosomes was achieved by centrifugation of the solubilized chromatin through 5–25% sucrose gradients containing 0.2 mM EDTA (pH 7.0), 0.1 mM PMSF, and 20 mM NaCl. The oligonucleosomal fractions (for convenience these oligonucleosomes will be referred to as chromatin) were pooled and dialyzed against 10 mM Tris (pH 7.0), 0.1 mM PMSF at 4°C for 12 h.

2.2. *Dissociation and reaction with carcinogen*

The dialyzed oligonucleosomes were concentrated to 7.3 A_{260} units/ml, and 5 ml aliquots were made 0.65 and 2.0 M in NaCl concentration by slow addition of solid NaCl with gentle stirring. The solutions were kept at 4°C overnight and equilibrated at 37°C for 10 min. The carcinogen, ^{14}C-labeled B[a]P diol-epoxide (anti) (spec. act. 29.4 mCi/mmol), which was dissolved in tetrahydrofuran:triethylamine (19:1, v/v) was added to the chromatin solutions at a DNA: carcinogen ratio of 100:1 (w/w) while the organic solvent concentration was <1.0%. The reaction was carried out at 37°C for 30 min under yellow light, after which the chromatin solutions were dialyzed against 0.2 mM EDTA (pH 7.0) until the radioactive background was constant. The dialyzed, soluble carcinogen-labeled chromatin solutions were isolated and lyophilized. The lyophilized chromatin was solubilized in sodium dodecyl sulfate (SDS) sample application buffer [19] to 1.0 mg/ml with respect to the histone proteins.

2.3. *SDS–polyacrylamide electrophoresis*

15% SDS–slab gel electrophoresis was performed essentially as in [19], and 8 μl of the protein sample (8 μg/slot) were loaded. Electrophoresis was performed at 100 V/gel. The gels were stained for 30 min in 20% acetic acid, 30% isopropanol, 0.01% Coomassie blue [12]; rinsed in water; destained in 10% acetic acid, 20% methanol; then photographed. Densitometer scans of the stained protein bands were obtained with a Zeineh soft laser scanning densitometer.

2.4. *Fluorography of stained slab gels*

For fluorography the slab gels were treated with dimethyl sulfoxide and 2,5-diphenyloxazole (scintillation grade) as in [20]. The films (Kodak X-ray film XRP-5) were exposed at −70°C for 9 weeks. The developed films were photographed and scanned as described above.

3. Results

Figure 1 shows the fractionation of nucleosomal subunits obtained when the solubilized chicken erythrocyte chromatin was centrifuged through a 5–25% sucrose gradient. The presence of 20 mM NaCl greatly facilitated the separation of nucleosomal subunits, since in the absence of NaCl the material sedimented as a broad band. The reaction conditions of oligonucleosomes with the B[a]P diol-epoxide (anti) were strictly controlled with respect to the carcinogen:chromosomal DNA ratio and the final level of organic solvent (tetrahydrofuran:triethylamine) added, since we have observed, by circular dichroism (in preparation), that at >1% final conc. organic solvent the circular dichroism spectrum shows a dramatic change in DNA conformation.

The extraction of chromosomal proteins as in section 2 provided conditions in which the potential loss of proteins was minimized. Acid extraction and organic precipitation were avoided since at this moment no information is available as to the extent of loss of carcinogen-modified histones during such manipulations.

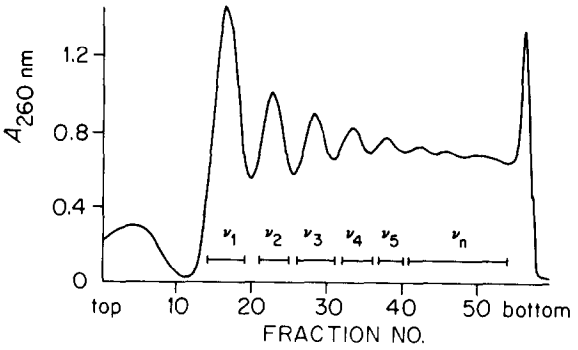

Fig.1. Absorbance profile of sucrose gradient. Chromatin, 2.5 ml (A_{260} = 83.0) was layered on a 5–25% linear sucrose gradient containing 0.2 mM EDTA (pH 7.0), 20 mM NaCl and was centrifuged for 14 h at 23 000 rev./min in an SW 25.2 rotor at 4°C. The gradients were fractionated with an ISCO density gradient collector (Model 640) equipped with an ISCO UV-5 absorbance monitor. Nucleosome monomers, dimers, and oligomers are shown as ν_1, ν_2 and ν_n, respectively.

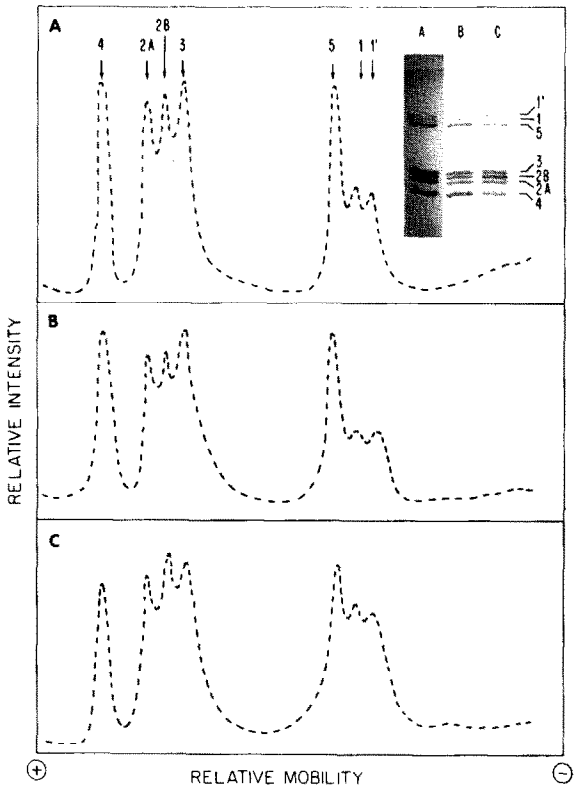

Fig.2. Densitometer tracing of carcinogen-labeled nucleosomal histone proteins after the gels were stained. Histones from oligonucleosomes were labeled (A) in the absence of added NaCl, (B) in the presence of 0.65 M NaCl, or (C) in the presence of 2.0 M NaCl. Inset, stained gel.

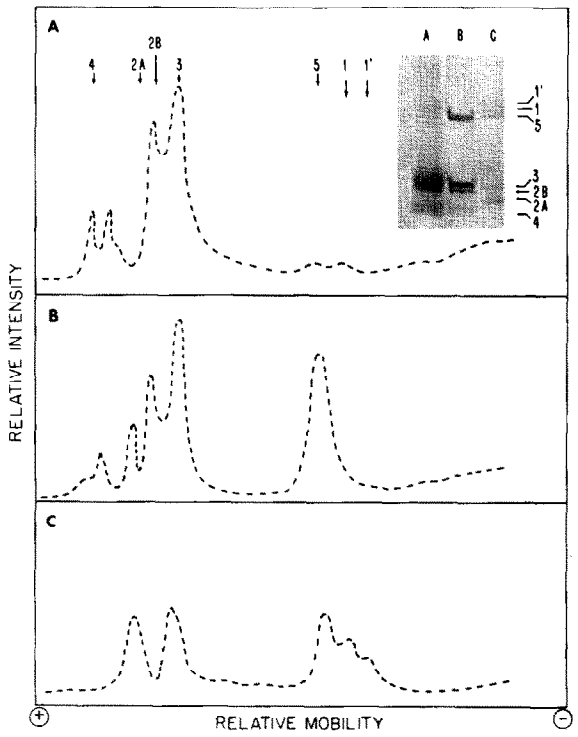

Fig.3. Densitometer tracing of the developed X-ray film of the stained gel shown in fig.2 after treatment for fluorography. A–C refer to the conditions of labeling as described in fig.2. Inset, the developed fluorogram; the apparent increase in mobility of histones H3 and H2A (inset right-hand lane) was caused by a slight deformation of the gel as a consequence of the treatment for fluorography. The developed X-ray film was matched with the stained protein bands in the gel to ensure proper identification.

The stained polyacrylamide gels (fig.2) show that all the histone proteins from the control and from 0.65 and 2.0 M NaCl dissociated and reacted chromatin solutions were present. In contrast, the fluorograms (fig.3) which indicate the relative amount of carcinogen bound to the histone proteins, reveal that in native chromatin, after the reaction with the carcinogen, histones H3 and H2B bound most of the carcinogen whereas histone H4 bound a relatively small amount. The fluorograms also reveal an unidentified labeled molecule which migrated just above histone H4.

When chromatin was labeled under 0.65 M NaCl dissociating conditions, histone H5, but neither H1 molecules, was extensively labeled by the carcinogen. This is in striking contrast to the labeling pattern under non-dissociative conditions. Under these conditions (0.65 M NaCl) histone H2A was now also labeled, although not to the same extent as H3 and H2B.

The labeling pattern of histone proteins when chromatin was dissociated in the presence of 2.0 M NaCl was entirely different: all the lysine rich histones were labeled by the carcinogen, whereas the labeling pattern of the core histones in the soluble chromatin showed that histones H3 and H2A were associated with the carcinogen.

4. Discussion

The experimental evidence presented here shows that B[a]P diol-epoxide (anti), when reacted with chicken erythrocyte chromatin in vitro, has a differential affinity for the histone proteins. Under nondissociating conditions, H3 and H2B were predominantly labeled, whereas H4 bound the carcinogen to a lesser extent. The unidentified carcinogen-labeled molecule that migrated just above H4 could be modified H4 or a low-level nonhistone protein; however, from current experiments it appears that this may be a breakdown product of H3. The relatively low level of binding to H5 and H1 in native chromatin was somewhat surprising, since these histones are thought to be more exposed in the chromatin structure as judged from their rapid disappearance during tryptic digestion [21]. However, since they do become accessible to the carcinogen when chromatin is dissociated in 2.0 M NaCl, the potential reactive groups of the H5 and both H1 molecules appear to be masked by their interaction with DNA. Dissociation of chromatin in 0.65 M NaCl now makes the H5 a target molecule, but neither of the H1 molecules were labeled under these conditions. This suggests that both histone H1 molecules still are complexed with the DNA in a manner that prevents the carcinogen from reacting with the H1 histones, while histone H5 appears to be dissociated from the complex. At the same time histone H2A has become accessible to the carcinogen, under 0.65 M NaCl dissociating conditions. This would suggest that histone H5 when bound to chromatin may be responsible for protecting histone H2A from the attack by the carcinogen. This observation would be in conformation with current evidence suggesting that the very lysine rich histones are in close proximity to histone H2A [22].

The data presented here would, therefore, suggest that the differential affinity of B[a]P diol epoxide (anti) for chicken erythrocyte chromosomal histone proteins in vitro appears to be a function of the three-dimensional arrangement of the nucleosomal subunit structure, and that the presence of the very lysine-rich histones prevents the carcinogen from interacting with histone H2A. These histone modifications may well play an important part in changing the conformational and functional relationship of chromatin, and by doing so, initiate the carcinogenic events. Therefore, these protein modifications could be related to the epigenetic changes that have been postulated to occur [23], in the process of carcinogenesis.

Acknowledgements

We thank Drs A. L. Olins, D. E. Olins, and A. P. Butler for discussions during this investigation. We are indebted to the Carcinogen Repository of the National Cancer Institute for the radioactive B[a]P diol-epoxide (anti). This research was sponsored by NIH Grant CA20076 and by the Office of Health and Environmental Research, US Department of Energy, under contract W-7405-eng-26 with the Union Carbide Corporation.

References

[1] Olins, A. L. and Olins, D. E. (1974) Science 183, 330–332.
[2] Oudet, P., Gross-Bellard, M. and Chambon, P. (1975) Cell 4, 281–300.
[3] Woodcock, C. (1973) J. Cell Biol. 59, 368a.
[4] Kornberg, R. D. (1977) Annu. Rev. Biochem. 46, 931–954.
[5] Compton, J. L., Bellard, M. and Chambon, P. (1976) Proc. Natl. Acad. Sci. USA 73, 4382–4386.
[6] Renz, M., Nehls, P. and Hozier, J. (1977) Proc. Natl. Acad. Sci. USA 74, 1879–1883.
[7] Strätling, W. H. (1979) Biochemistry 18, 596–603.
[8] Elgin, S. C. R. and Weintraub, H. (1975) Annu. Rev. Biochem. 44, 726–774.
[9] Kootstra, A. and Bailey, G. S. (1978) Biochemistry 171, 2504–2510.
[10] Allfrey, V. G. (1975) in: Chromatin and Chromosome Structure (Li, J. J. and Eckhardt, R. eds) pp. 167–191, Academic Press, New York.
[11] Bradbury, E. M., Inglis, R. J. and Matthews, H. R. (1974) Nature 247, 257–260.
[12] Kootstra, A., Slaga, T. J. and Olins, D. E. (1979) in: Polynuclear Aromatic Hydrocarbons (Jones, P. W. and Leber, P. eds) pp. 819–834, Ann Arbor Science, in press.
[13] Kuroki, T. and Heidelberger, C. (1971) Cancer Res. 31, 2168–2175.
[14] Bresneck, E., Vaught, J. B., Chuang, A. H. L., Stroming, T. A., Bockman, D. and Mukhtor, H. (1977) Arch. Biochem. Biophys. 171, 257–269.
[15] Slaga, T. J., Bracken, W. M., Viaje, A., Levin, W., Yagi, H., Jerina, D. M. and Conney, A. H. (1977) Cancer Res. 37, 4130–4133.

[16] Huberman, E., Sachs, L., Yang, S. K. and Gelboin, H. V. (1976) Proc. Natl. Acad. Sci. USA 73, 607–612.
[17] Kapitulnik, J., Wislocki, P. G., Levin, W., Yagi, H., Jerina, D. M. and Conney, A. H. (1978) Cancer Res. 38, 354–358.
[18] Olins, A. L., Carlson, R. D., Wright, E. G. and Olins, D. E. (1976) Nucleic Acids Res. 4, 3271–3291.
[19] Laemmli, U. K. (1970) Nature 227, 680–685.
[20] Laskey, R. A. and Mills, A. D. (1975) Eur. J. Biochem. 56, 335–343.
[21] Weintraub, H. and Van Lente, F. (1974) Proc. Natl. Acad. Sci. USA 71, 4249–4253.
[22] Bonner, W. M. and Stedman, J. D. (1979) Proc. Natl. Acad. Sci. USA 76, 2190–2194.
[23] Trosko, J. E., Chang, C. C. and Glover, T. (1977) Colloq. Int. Cent. Natl. Rech. Sci. 256, 353–388.