Journal of Cellular Biochemistry 18:507-513 (1982)
Mechanisms of Chemical Carcinogenesis 299-305

Benzo(a)pyrene-Binding Proteins of Hamster Embryo Cell Nuclei: Comparison of Nuclear Isolation Procedures

Michael C. MacLeod, Betty K. Mansfield, and James K. Selkirk

Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830

Hamster embryo cells metabolize benzo(a)pyrene to derivatives that covalently modify nuclear macromolecules including proteins. Not all proteins are modified to the same extent nor by the same metabolites. In particular, a protein of apparent molecular weight 32,000 is highly modified by derivatives of trans-9,10-dihydro-9,10-dihydroxy B(a)P. This protein is shown here to be preferentially lost from nuclei during purification by centrifugation through high molarity sucrose solutions followed by osmotic shock. It does not appear to be a cytoplasmic contaminant, but shares many properties of an abundant protein from Xenopus laevis oocytes, nucleoplasmin.

Key words: benzo(a)pyrene, macromolecular binding, carcinogen, nuclear proteins, histones, cytoplasmic proteins

Polynuclear aromatic hydrocarbons (PAH), a class of important environmental pollutants, cause damage to susceptible eukaryotic cells that ranges from cytotoxicity to mutation to the induction of malignant transformation [1-5]. Production of this damage requires metabolic activation of PAH to electrophilic intermediates that react with cellular macromolecules [6]. Thus, an understanding of the processes that ultimately lead to malignant transformation requires first an assessment of the types of macromolecules that are targets for activated xenobiotics and, second, elucidation of the functional effects of modification by PAH. For some years it has been known that activated PAH bind covalently to cellular RNA, DNA, and protein [7-11], and in fact the structures of the major adducts formed between nucleic acids and certain PAH have been determined [12]. Despite these elegant and extensive biochemical studies, little is known about the possible relation of these adducts to carcinogenesis.

Much less is known about covalent interactions of PAH with proteins, although proteins are quantitatively the major targets for PAH binding in many situations [10,13]. In microsomal metabolizing systems a subset of available proteins, presumably including the activating enzymes themselves, is subject to modification by benzo(a)pyrene [B(a)P] [14]. In cultured hamster embryo cells (HEC),

Received March 27, 1981; accepted November 30, 1981.

Published 1982 by Alan R. Liss, Inc.

labeling of nuclear proteins by [^{3}H]-B(a)P has been shown to be markedly nonuniform and to involve two distinct metabolic pathways for B(a)P in these cells [10,15a]. One pathway culminates in formation of one or more of the stereoisomers of 7,8-dihydroxy-9,10-oxy-7,8,9,10-tetrahydro-benzo(a)pyrene [B(a)P-7,8-diol-9, 10-epoxide], which covalently binds to RNA, DNA, and two of the nucleosomal core histones, H3 and H2A [15a]. The other pathway is suspected to involve the "reverse" diol-epoxide, 9,10-dihydroxy-7,8-oxy-7,8,9,10-tetrahydrobenzo(a)pyrene [B(a)P-9,10-diol-7,8-epoxide) and results in heavy labeling of a protein or proteins with an apparent M_r on SDS-polyacrylamide-gel electrophoresis of about 32,000 [15a]. We have previously shown that this protein, which we shall call BP32, does not copurify with the very lysine-rich histones nor with the high mobility group proteins during extraction with dilute acid [15b]. However, preliminary experiments using a nuclear purification procedure that was different from our original detergent method [16] appeared to give nuclei with a reduced content of BP32. In the present work we have explored the differences between these two methods for purifying nuclei.

METHODS

The sources of all materials and methods of cell culture, handling of B(a)P and incubation of [^{3}H]-B(a)P with cells have been described, as have the methods used in electrophoretic analysis [10, 15a–18]. All buffers contained 0.5 mM phenyl methyl sulfonyl fluoride and all preparative steps were carried out at 0-4°C. Preparation of detergent-treated nuclei has been described [16]. Briefly, washed cells were homogenized in buffer [10 mM NaCl, 10 mM Tris (pH 7.0), 1.5 mM $MgCl_2$] containing 0.5% Triton X-100 and 0.5% sodium deoxycholate, and nuclei pelleted by centrifugation; this step was repeated. Sucrose nuclei were prepared by a modification of the method of Berezney and Coffey [19]. Cell lysis was obtained by Dounce homogenization ("B" pestle, 15 strokes) in 10 mM Tris-HCl (pH 7.4), and crude nuclei were pelleted. This step was repeated, and the final pellet was suspended in TM buffer [50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$] containing 2.2 M sucrose. Nuclei were collected by a 90 min centrifugation at 21,000 rpm (40,000g) in a Beckman 50 Ti rotor. Purified nuclei were washed twice with TM buffer containing 0.25 M sucrose. Aliquots of each nuclear preparation were subsequently purified further by the alternate procedure. Nuclear preparations were dissolved in 6 M guanidine-HCl, 10 mM EDTA (pH 7.0) and extracted three times with 2.5 vol ethyl acetate; cytoplasmic extracts were adjusted to 6 M guanidine-HCl before extraction. All preparations were dialyzed extensively against H_2O and concentrated either by lyophilization or by precipitation with methanol (2 vol, 24 hr at 4°C) before analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis [17].

RESULTS

In Figure 1, we have compared the effects of detergent treatment during purification with the effects of hydrodynamic shear produced by centrifugation through 2.2M sucrose. The distribution of protein and protein binding in detergent-treated nuclei (lane D) was similar to previously published data [15a]. Two major differ-

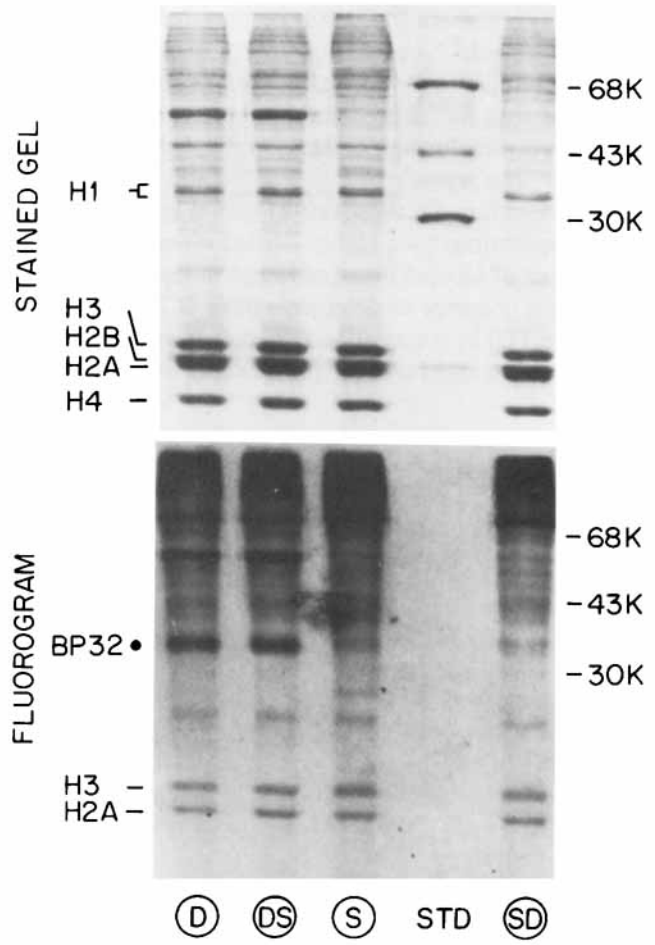

Fig. 1. Distribution of labeled proteins in different nuclear preparations. Confluent tertiary cultures of hamster embryo cells were incubated for 24 hr in the presence of 4 μM [^{3}H]B(a)P (6 Ci/mmole). The same preparation of labeled cells was used in Figures 1, 2, and 3. Nuclei were prepared as described in Methods by the detergent technique (lane D) or by the sucrose technique (lane S). An aliquot of sucrose nuclei was further purified by treatment with detergent (lane SD) and an aliquot of detergent nuclei was further purified using the sucrose technique (lane DS). All nuclear preparations were extracted with ethyl acetate, prepared for electrophoresis, and analyzed on 15%polyacrylamide gels by the method of Laemmli [17]. The upper panel represents the Coomassie blue stained proteins. The stained gel was then subjected to fluorography (lower panel, reference 18) to determine the distribution of hydrocarbon among the proteins. Marker proteins (lane STD) were bovine serum albumin (68K), ovalbumin (43K), and carbonic anhydrase (30K).

ences were found when nuclei were prepared by the sucrose technique (lane S). An abundant protein in the 50,000–55,000 apparent molecular weight range, which is highly labeled by B(a)P, was virtually absent from sucrose purified nuclei. Secondly, BP32 was absent or much reduced in the sucrose-purified nuclei. When sucrose-purified nuclei were subsequently treated with detergent (lane SD), we detected no increase in labeling in the 32,000 molecular weight region of the gel. This suggested that BP32 did not appear as a result of detergent-induced breakdown or aggregation

of other proteins normally present in nuclear preparations. Conversely, further purification of detergent nuclei by centrifugation through 2.2 M sucrose did not remove labeled BP32 (lane DS). We conclude from these studies that there are physical differences between nuclei prepared by these two techniques such that BP32 is easily removed from nuclei during purification through sucrose if it has not been previously treated with detergents.

One possible explanation for these results would be a differential contamination of the nuclear preparations by a highly labeled cytoplasmic protein. In Figure 2, we compared patterns of labeled proteins found in the cytoplasmic extracts of cells homogenized in the presence of detergents (lane DC) with those homogenized without detergent (lane TC) in preparation for sucrose purification of nuclei. For comparison, the detergent-treated nuclear preparation was electrophoresed in lane

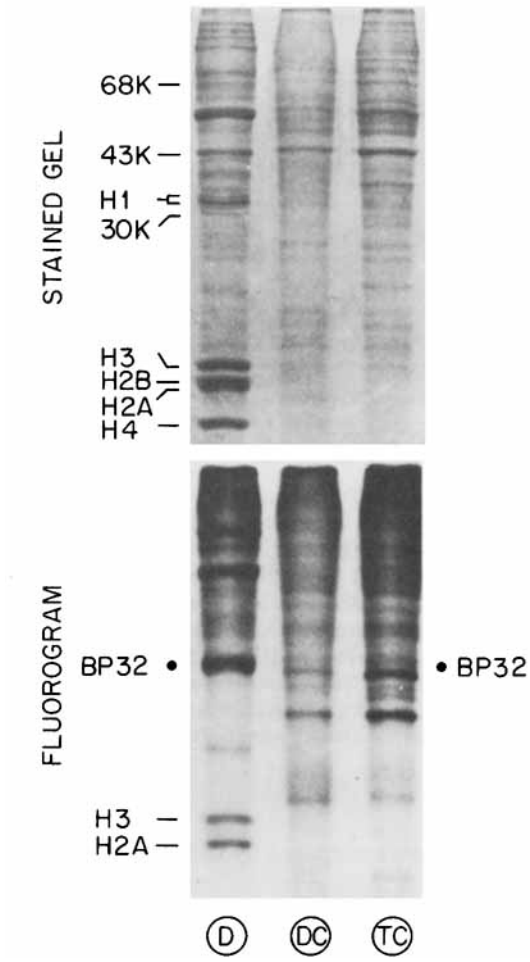

Fig. 2. Distribution of labeled proteins in cytoplasmic preparations. The cytoplasmic extracts of cells homogenized in buffer without detergent (lane TC) or homogenized with detergent (lane DC) were analyzed. Detergent-treated nuclei were also analyzed to mark the position of BP 32 (lane D).

D. Allowing for differences in the total amount of protein actually loaded in the two lanes, there were no major differences between the patterns of labeled proteins present in the cytoplasmic extracts. A few highly labeled proteins, which were absent from nuclear preparations, were seen in both preparations. Significantly, the highly labeled BP32 did not appear to be a major component of the cytoplasmic extracts. Thus, cytoplasmic contamination is not a likely explanation for the presence of BP32 in detergent-treated nuclei.

We next asked at what step in the sucrose purification procedure was BP32 removed from the nuclei. Proteins present in the 2.2 M sucrose wash and in the subsequent wash with 0.25 M sucrose were analyzed in Figure 3 (lanes 2.2 W and 0.25 W, respectively). Each lane represented approximately 1/5 of the total material present in the extract. The major portion of the radioactive protein that was released in these washes was recovered in the low molarity sucrose wash rather than in

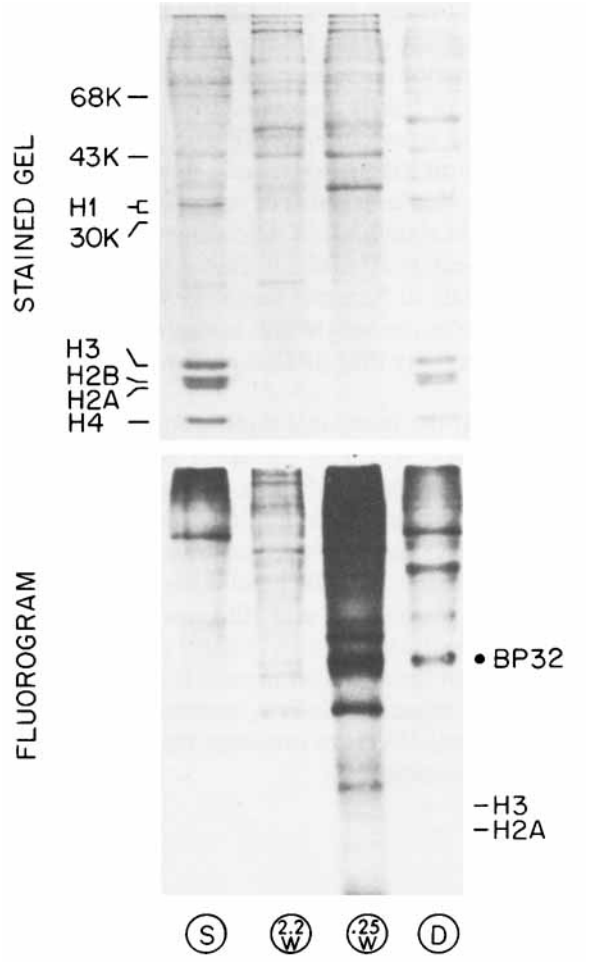

Fig. 3. Distribution of labeled protein in nuclear wash fractions. Proteins removed during preparation of sucrose nuclei (lane S) by centrifugation through 2.2 M sucrose (lane 2.2W) or by washing with 0.25 M sucrose (lane .25 W) were analyzed. Lane D is the detergent nuclei marker preparation.

the initial 2.2 M sucrose centrifugation step. In particular, a very strong band with the same electrophoretic mobility as BP32 was found in the 0.25 M sucrose extract. Although a similar band is seen in the 2.2 M extract, densitometry of a fluorogram, which was exposed for a shorter period of time than the one shown in Figure 3, demonstrated that more than 90% of the BP32 in these washes was recovered in the 0.25 M sucrose extract.

DISCUSSION

Since proteins account for greater than 70% of B(a)P binding to nuclear macromolecules in HEC [10], we have been interested in defining the major target proteins and in understanding their role in carcinogenesis. Previous studies [15] have shown that histones H3 and H2A are targets, but have argued against the possibility that the very lysine-rich H1 histones were major targets, leaving the identity of BP32 unknown. The present results show that BP32 is preferentially lost from nuclei purified by a modification of the Chauveau method [19]. The data of Figure 2 show that BP32 is not a major component of the cytoplasmic extracts, suggesting that it is either intranuclear or tightly associated with the nucleus. This suggestion is supported by the experiment shown in Figure 3. The major loss of BP32 from nuclei occurs not as a result of the hydrodynamic shearing produced by centrifugation through 2.2 M sucrose, but as a result of osmotic shock when the 2.2 M sucrose pellet is resuspended in a solution of much lower osmolarity (0.25 M sucrose). This tendency of BP32 to leak from nuclei is similar to the properties of nucleoplasmin [20], an abundant protein in Xenopus laevis oocytes. These proteins also have approximately the same molecular weight and insolubility in dilute acid, and we are currently exploring the possibility that BP32 is a mammalian counterpart of nucleoplasmin.

The larger question of the functional significance of binding of PAH to nuclear proteins remains open. It is clear that some but not all major structural proteins of the cell can be modified by B(a)P. The strongly stained bands at 43,000 daltons and about 52,000 daltons seen in both nuclear and cytoplasmic preparations probably represent actin (43K) and a mixture of tubulin and proteins of the intermediate filament family (52K); the 52K band is highly labeled by B(a)P, while the 43K band is not. Similarly, histones H3 and H2A are modified while H2B, H4, and H1 are not. Binding of bulky hydrocarbons to these proteins is likely to perturb the cellular architecture of which the modified protein is a part, and thereby produce a variety of effects, including structural defects, increased turnover, and altered function. The relationship of these effects to processes that lead to malignant transformation remains for future investigation.

ACKNOWLEDGMENTS

This research was sponsored by the Office of Health and Environmental Research, US Department of Energy, under contract W-7405-eng-26 with the Union Carbide Corporation. We thank Nancy Trent for excellent secretarial assistance.

REFERENCES

1. Heidelberger C: Annu Rev Biochem 44:79, 1975.
2. Berwald Y, Sachs L: J Nat Cancer Inst 35:641, 1965.
3. Diamond L, Defendi V, Brookes P: Cancer Res 27:890, 1967.
4. Huberman E, Sachs L: Int J Cancer 13:326, 1974.
5. Gelboin HV: Adv Cancer Res 10:1, 1967.
6. Miller JA: Cancer Res 30:559, 1970.
7. Miller EC: Cancer Res 11:100, 1951.
8. Wiest WG, Heidelberger C: Cancer Res 13:250, 1953.
9. Kuroki T, Heidelberger C: Cancer Res 31:2168, 1971.
10. MacLeod MC, Cohen GM, Selkirk JK: Cancer Res 39:3463, 1979.
11. Ivanovic V, Geacintov NE, Yamasaki H, Weinstein IB: Biochemistry 17:1597, 1978.
12. Jeffrey AM, Weinstein IB, Jennette KW, Grzeskowiak K, Nakanishi K, Harvey RG, Autrup H, Harris C: Nature (London) 269:348, 1977.
13. Huberman E, Sachs L: Int J Cancer 19:122, 1977.
14. Tunek A, Schelin C, Jergil B: Chem Biol Interact 27:133, 1979.

15a. MacLeod MC, Kootstra A, Mansfield BK, Slaga TJ, Selkirk JK: Proc Nat Acad Sci USA 77:6396, 1980.

15b. MacLeod MC, Kootstra A, Mansfield BK, Slaga TJ, Selkirk JK: Cancer Res 41:4080, 1981.

16. MacLeod MC, Mansfield BK, Huff A, Selkirk JK: Anal Biochem 97:410, 1979.
17. Laemmli U: Nature (London) 227:680, 1970.
18. Laskey RA, Mills AD: Eur J Biochem 56:335, 1975.
19. Berezney R, Coffey DS: J Cell Biol 73:616, 1977.
20. Mills AD, Laskey RA, Black P, de Robertis EM: J Mol Biol 139:561, 1980.