Binding of Polycyclic Aromatic Hydrocarbons to Transcriptionally Active Nuclear Subfractions of AKR Mouse Embryo Cells

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SUMMARY

The objective of this study was to examine the binding of carcinogenic polycyclic aromatic hydrocarbons in well-characterized nuclear subfractions from transformable cells in culture. A cloned line of AKR mouse embryo cells was exposed to culture medium containing [3H]-3-methyl-cholanthrene (MC) (0.4 μg/ml) (670 Ci/mole). Cellular uptake and nuclear binding were determined after 4 hr of exposure. The addition of unlabeled MC up to 10 μg/ml did not cause reduction of [3H]MC cellular uptake or nuclear binding. From 2 to 5% of the total cellular MC was localized in the nuclei. All nuclear subfractions obtained from mechanically sheared nuclei and separated on sucrose gradients showed some MC binding; however, a high-affinity, high-specific activity binding of MC was associated only with the slower-sedimenting component shown to represent that fraction of nuclear chromatin that is transcriptionally active. Conditions that caused the precipitation of this chromatin also resulted in the precipitation of the radioactive compound, thus suggesting that the MC was physically bound to the chromatin. Unlabeled MC (10 μg/ml) saturated this high-affinity MC binding to the transcriptionally active chromatin but did not saturate the binding to the other nuclear fractions. The binding of another potent carcinogen, [3H]-1,2,5,6-dibenzanthracene, and the "weak" carcinogen, [3H]-1,2,3,4-dibenzanthracene (3,4-DBA), to whole nuclei and nuclear subfractions was also determined. The concentration, specific activity, and time of treatment were identical with those used for MC. The level of binding of [3H]-1,2,5,6-dibenzanthracene was approximately 3-fold greater in whole nuclei on a per mass DNA basis than in those of either the MC or the 3,4-DBA. The binding of MC and 3,4-DBA to whole nuclei was approximately equal. As with MC, the [3H]-1,2,5,6-dibenzanthracene demonstrated a peak of high specific activity binding to the slower-sedimenting fraction of chromatin while the 3,4-DBA displayed considerably less binding to this fraction.

INTRODUCTION

Gene expression is altered both quantitatively and qualitatively in neoplastic cells relative to their nontransformed counterparts (6, 31). Changes in genetic transcription can also be induced by treatment with carcinogenic PAH (2, 20, 21). These compounds also cause chromosome aberrations (15, 16) and activate the expression of viral genes (30). For these reasons the specific binding of PAH resulting in malignant transformation is thought to be within the nucleus to the chromosomal material. There have been numerous studies on binding of PAH to DNA, RNA, and proteins of cells both in vivo (9, 17) and in tissue culture model systems (8, 12, 19). Yet the nuclear binding sites for PAH that are most important to the process of neoplastic transformation are not known.

The present study is concerned with binding of [3H]MC, [3H]-5,6-DBA, and [3H]-3,4-DBA to nuclear subfractions described in the preceding paper (32) from transformable AKR mouse embryo cells.

MATERIALS AND METHODS

Cell Culture. The cell line used in these studies is the 2B subclone of 32C of the AKR mouse embryo cells (26, 30). The subclone has a highly inducible endogenous virus that closely resembles the AKR murine leukemia virus (30). Cells were obtained from Dr. W. P. Rowe (NIH, Bethesda, Md.). These cells are virus negative as determined by electron microscopy, XC cell assay (27), and indirect immunofluorescence using antisera raised in tumor-bearing Fischer rats (obtained from Dr. J. Gruber, National Cancer Institute, Bethesda, Md.). The cells grow rapidly with an epithelioid pattern of growth and display marked density-dependent inhibition of growth (10). They exhibit a nontransformed phenotype, but they can be transformed with a variety of PAH. Transformed cells do show evidence of endogenous C-type virus expression (25).

AKR-2B cells were grown in McCoy's Medium 5A supplemented with 10% heat-inactivated fetal bovine serum, peni-
cillin G (100 units/ml), and streptomycin (100 μg/ml) under a humidified 5% CO₂-air environment at 36°. Cells were passaged weekly by trypsinization and subsequent seeding at 3 × 10⁵ cells per sq cm. The medium was changed on Day 3 or 4. Cells for nuclear isolation and fractionation were grown in 690-sq cm glass roller bottles which were flushed with 5% CO₂ in air, tightly capped, and incubated in a non-CO₂ incubator. Cells from 5 bottles were harvested by trypsinization for each determination and collected in cold (4°) complete medium with serum. Cells were pelleted at 600 × g at 4° for 5 min and washed once with complete medium containing serum prior to isolation of nuclei.

MC Uptake and Binding. MC (Amersham Searle, Arlington Heights, Ill.; 5000 Ci/mole) was lyophilized and sufficient unlabeled MC (Eastman Organic Chemicals, Rochester, N. Y.) was added to give a specific activity of 670 Ci/mole. Lyophilized material was dissolved in DMSO immediately prior to use, and 50 μl were added to 50 ml of culture medium in the roller bottles to give a final concentration of 1 μCi/ml or a total MC concentration of 0.4 μg/ml. For competition studies, unlabeled MC in a stock solution of DMSO (2 mg/ml) was added to give the appropriate final concentration. Sufficient DMSO was added to cultures with lower concentrations of MC so that the DMSO concentration in all bottles was equal. Cells were consistently treated when in a subconfluent, rapidly proliferating state.

Isolation of Nuclei and Chromatin. Cell pellets were suspended in 20 ml of 1.0 M sucrose in 0.01 M Tris-HCl, (pH 7.5) 0.01 M KCl-0.001 M MgCl₂ buffer, and the cells were disrupted by shearing in a VirTis homogenizer for 30 sec at 10,000 rpm on ice. Aliquots of this whole-cell homogenate were taken for determining whole-cell uptake of labeled compounds as described below. The nuclei were separated by centrifugation as described in the preceding paper (32). The nuclear pellet was either frozen or resuspended in a small volume (500 μl) of 25% glycerol 0.001 M MgCl₂ 0.01 M Tris-HCl (pH 7.9) and stored frozen. Chromatin was isolated from the purified nuclei as previously described (28).

Nuclear Fractionation. Mechanically sheared nuclei were fractionated as described in the preceding paper (32). The degree of mechanical shearing was found to be very important in determining the distribution of DNA and binding of radioactive PAH in the gradients. In order to monitor the extent of shearing, the A₄₅₀ of the suspension of sheared nuclei was determined, and the suspension was then centrifuged at 2000 × g for 15 min at 4°. The A₄₅₀ of the supernatant was then determined. Optimum results were obtained when the A₄₅₀ of the supernatant was 75 to 85% of the A₄₅₀ of the suspension prior to centrifugation. This degree of shearing was generally produced by homogenizing a 4-ml suspension of nuclei in the VirTis homogenizer for 2 min at 50,000 rpm using the microshaft.

Analysis of Fractions. Aliquots (0.5 ml) from each tube collected from the gradients were precipitated with 1.0 ml cold 10% (w/v) TCA containing 1.0% (w/v) Na₂P₂O₇, and the precipitate was collected on 0.45-μm HAMK Millipore filters. The filters were washed 3 times with cold 5% TCA. The filters were air dried and placed in 5 ml toluene base scintillation cocktail, and the radioactivity was quantitated in a liquid scintillation spectrometer. Filters were then removed from the vials and allowed to dry; the DNA was hydrolyzed at 90° for 30 min in 0.5 ml of 0.3 N HClO₄ and quantitated by the diphenylamine reaction (5). Whole cell, intact nuclei, and chromatin samples were analyzed in a similar manner.

RESULTS

Cellular Uptake and Nuclear Binding. The total cell uptake and nuclear and chromatin binding of [³H]MC was examined from 30 min to 24 hr after treatment (Chart 1). A 0.4-μg/ml dose of [³H]MC (specific activity, 670 Ci/mole) was utilized because it is a transforming dose with little cytotoxic effect. After an early plateau between 2 and 4 hr, there is an approximately 2-fold increase in cell uptake and nuclear and chromatin binding at the 8-hr time point followed by a gradual decrease to 24 hr (Chart 1). During the time period from 4 to 24 hr, between 2 and 5% of the whole-cell radioactivity was present in the nucleus when yields were corrected to cpm/mg DNA. Also during this interval, 80 to 90% of the nuclear tritium activity was found in isolated chromatin. Prior to the 4-hr point a lower percentage of the nuclear activity was found in isolated chromatin. On the basis of these data the 4 hr time point was chosen for the studies on early MC binding.

The effects of varying concentrations of MC in the culture medium on cell uptake and nuclear binding were determined by adding varying amounts of unlabeled MC to the culture medium (from 0.5 to 10 μg/ml) in addition to the 0.4-μg/ml dose of [³H]MC (Chart 2). Higher concentrations of unlabeled MC were not used because of problems with solubility of the MC in the tissue culture medium and cytotoxicity. Unexpectedly, the total uptake and nuclear binding of [³H]MC did not decrease even with a 25-fold dilution of the ³H-labeled compound with unlabeled MC, 10 μg/ml. Since the cpm/mg DNA at each time point was approximately the same, there was a progressive increase in the number of MC molecules bound in the whole cell and nucleus (Chart 2). The isolated chromatin binding closely paralleled nuclear binding, with the chromatin specific activity...
MC Binding to Nuclear Subfractions

Effect of Dose on MC Binding to Nuclear Subfractions. This same slow-sedimenting peak of [3H]MC binding on sucrose gradients of sheared nuclei was observed with concentrations in the culture medium as low as 0.1 μg/ml which was the lowest concentration tested. Higher doses resulted in a linear increase in total MC binding in Subfraction I up to 5 μg/ml (Chart 5). The Subfraction I binding at 10 μg/ml was the same as that observed with 5 μg/ml, demonstrating that MC, 5 μg/ml, in the culture medium causes saturation of the Region I binding sites (Chart 5). In a similar experiment, the saturation of the other 2 subfractions was also attempted. The relative percentage of the total radioactivity in the gradients bound to the 3 nuclear subfractions after exposure of cells to varying concentrations (0.1 to 10.0 μg/ml) of [3H]MC is shown in Chart 6. Subfraction I shows the highest relative binding with the lower concentrations of [3H]MC, indicating that this site has the highest affinity for [3H]MC. Higher concentrations resulted in a decrease in the percentage of radioactivity in Subfraction I with a pronounced increase in the percentage of counts in Subfraction III demonstrating that, while

(cpm/mg DNA) being 80 to 90% of the nuclear for all doses (data not shown).

Binding of [3H]MC to Nuclear Subfractions. After 4 hr of exposure to [3H]MC, 4 μg/ml, nuclei were isolated, mechanically sheared, and fractionated on sucrose gradients as described in the preceding paper (32). The distribution of [3H]MC radioactivity and DNA in a typical gradient is shown in Chart 3. Fractions from specific regions of the gradients were pooled to form the 3 nuclear subfractions as illustrated in Chart 3. These subfractions correspond with the 3 nuclear subfractions described in the preceding paper (32). Subfraction I consisted of the slower sedimenting area which showed the major rapid uptake of [3H]uridine into nascent RNA. Subfraction II represents the lower part of the gradient containing the more rapidly sedimenting material relatively inactive in RNA synthesis and Subfraction III represents the pelleted material which shows marked template restriction relative to Region I. A peak of [3H]MC binding was observed in Region I (Subfraction I) (Chart 3). The peak of [3H]MC binding was generally present in 4 to 5 fractions while the [3H]uridine incorporation was in 10 to 12 fractions of a 30-fraction gradient (32). Table 1 shows the percentage of distribution of radioactivity in each of the 3 gradient regions in addition to the sample. While 45% of the [3H]MC cpm were in Subfraction I of the gradient, only 15% of the total DNA was present in this region. Therefore, the specific activity of [3H]MC binding expressed as cpm/mg DNA was very high in Region I and low in the remainder of the gradient (Chart 4).

Effect of Dose on MC Binding to Nuclear Subfractions. AKR-2B cells were incubated in the presence of [3H]MC, 0.4 μg/ml (670 Ci/mole), in addition to varying concentrations of unlabeled MC (from 0.5 to 10 μg/ml) for 4 hr. The cells were then harvested by trypsinization and washed with culture medium. Nuclei were isolated as described in "Materials and Methods" and the molecules of MC bound per cell were determined for whole cells and nuclei.

The cpm per fraction (x) and DNA per fraction (•) were determined as described "Materials and Methods." The concentration of sucrose per fraction was estimated with a refractometer. Regions I, II, and III designate the nuclear subfractions described in the preceding paper (32). The binding of [3H]MC was generally present in 4 to 5 fractions while the [3H]uridine incorporation was in 10 to 12 fractions of a 30-fraction gradient (32). Table 1 shows the percentage of distribution of radioactivity in each of the 3 gradient regions in addition to the sample. While 45% of the [3H]MC cpm were in Region I of the gradient, only 15% of the total DNA was present in this region. Therefore, the specific activity of [3H]MC binding expressed as cpm/mg DNA was very high in Region I and low in the remainder of the gradient (Chart 4).

Experiment 1. Competition of unlabeled MC for [3H]MC-binding sites in nuclear subfractions. AKR-2B cells were incubated in the presence of [3H]MC, 0.4 μg/ml, in addition to unlabeled MC, 10 μg/ml, for 4 hr. Cells were harvested and processed as described under Chart 3. The binding of [3H]MC in the presence of unlabeled MC, 10 μg/ml, is expressed as cpm/mg DNA (•). The data from Chart 3 showing binding of [3H]MC without competition from unlabeled MC are plotted as cpm/mg DNA (×).
H. L. Moses et al.

Dient indicating that dissociation of radioactivity from the chromatin was unlikely.

**Comparative Binding of MC, 5,6-DBA, and 3,4-DBA to Nuclear Subfractions.** In order to determine the specificity of the Subfraction I binding and its possible relationship to carcinogenic activity, the binding of the strong carcinogen, 5,6-DBA, and its weak carcinogen analog, 3,4-DBA, to the nuclear subfractions was determined. Cells were incubated in the presence of [3H]-5,6-DBA, 0.4 µg/ml (specific activity, 670 Ci/mole), or [3H]-3,4-DBA, 0.4 µg/ml (specific activity, 670 Ci/mole), for 4 hr; and the nuclei were isolated, sheared, and fractionated as with the MC experiments. The total nuclear binding per mass DNA was about 3 times higher with 5,6-DBA than with 3,4-DBA or MC. The total binding of the latter 2 compounds was roughly equal. However, the distribution of binding of the 3 PAH on the gradient was distinctly different (Chart 8). A higher peak of binding in Region I was observed with MC and 5,6-DBA than with 3,4-DBA. The relative distribution in the 3 parts of the gradient for each PAH and/or its metabolite is shown in Table 1. Cells incubated with [3H]-3,4-DBA displayed 23% of the total radioactivity in Subfraction I while cells incubated with [3H]-MC or [3H]-5,6-DBA exhibited 45 and 36% in Subfraction I, respectively. A greater portion of the radioactivity in cells incubated with [3H]-3,4-DBA was found in Subtraction II (Chart 7).

**DISCUSSION**

Most studies on PAH binding within the cell have been concerned with covalent binding to DNA (13, 22). A good correlation has been reported (4, 7), between the capability...
of a compound to bind covalently to DNA and its carcinogenic activity, although evidence to the contrary does exist (11, 19). This evidence and the demonstration that many of the carcinogenic agents are mutagenic have been often cited in support of the somatic mutation theory of chemical carcinogenesis (1). However, an alternative mechanism of chemical carcinogenesis is through induction of "permanent" aberrations in cell differentiation (33). Sex steroid hormone-induced cell differentiation has been shown to be mediated through noncovalent binding of the hormones to receptor proteins and a subsequent interaction of the hormone-receptor complex with chromatin and its proteins (24). Chemical carcinogens could act in a similar manner through a noncovalent binding to cellular macromolecules other than DNA. Since the DNA within the cell is mostly covered with proteins and RNA, binding of carcinogens to these latter macromolecules is highly probable. Several carcinogens are reported to react with cellular proteins or RNA's to a greater extent than with DNA (9, 13). PAH have been shown to bind to whole chromatin as well as to nuclear histone and acidic proteins (3, 14, 18, 23). These chromatin proteins are suspected to be involved in gene regulation (29). Unfortunately, none of the many carcinogen-binding sites in cells has been shown to be the specific site necessary for malignant transformation.

In the present study we have shown that the whole cell uptake as well as nuclear and chromatin binding by MC in AKR-2B cells is not saturable even with a dose of MC 25-fold greater than that required for transformation. We have demonstrated through the fractionation of sheared nuclei on sucrose gradients a previously unreported saturable binding of MC to a specific nuclear subtraction. This subtraction exhibits a high affinity for MC and displays a marked concentration of the MC relative to the other nuclear subfractions. Approximately 45% of the total nuclear MC binding is present in this subtraction which contains approximately 15% of the nuclear DNA. It is of considerable interest that the strong carcinogen, 5,6-DBA, also binds with high specific activity to this same nuclear subtraction, whereas the weak carcinogen, 3,4-DBA, exhibits minimal binding.

The fact that the PAH move into the gradient and are TCA precipitable indicates binding to a macromolecule; however, the specific macromolecule to which they are bound has not been identified as yet. The slower-sedimenting region of the nuclear gradients (designated Subtraction I) which binds PAH is the region showing rapid incorporation of [3H]uridine and a high content of RNA and acidic protein relative to the other gradient fractions (32). The distribution of the rapidly labeled nascent RNA in Region 1 is different from the MC binding distribution which has a sharper peak distributed over a smaller area of the gradient than the [3H]uridine incorporation. The effect of calcium on the distribution of the [3H]MC in the gradients suggests that the binding is chromatin associated; however, the only statement that can be made on the basis of present evidence is that the MC binding cosediments with the chromatin active in RNA synthesis. Electron microscopic studies of the nuclear subfractions indicate that the MC binding is probably not to the nuclear envelope, since abundant membranes were observed in Subtraction II of the nuclear gradients (32) that showed a very low level of MC binding.

If this high-affinity MC binding to a specific nuclear subtraction has a functional significance, it could be related either to induction of drug-metabolizing enzymes or to malignant transformation. 3,4-DBA shows relatively less binding to this subnuclear site than do the more potent carcinogens, MC and 5,6-DBA. Studies on the binding of nontransforming, aryl hydrocarbon hydroxylase-inducing PAH may provide some insight into this problem. An additional interest in these nuclear fractionation studies is that they could represent an initial step in the purification of a nuclear macromolecule that specifically and saturably binds carcinogenic PAH with high affinity.

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REFERENCES

4. Table 1
   Comparison of MC, 5,6-DBA, and 3,4-DBA binding to nuclear subfractions

   | Sample | I | II | III |
   | MC     | 4.5 ± 0.3* | 45.0 ± 0.1 | 12.8 ± 2.4 | 37.7 ± 2.6 |
   | 5,6-DBA| 8.6 ± 1.0  | 35.6 ± 0.8  | 23.9 ± 1.3  | 32.0 ± 1.4  |
   | 3,4-DBA| 3.9 ± 0.4  | 23.3 ± 1.4  | 33.1 ± 1.3  | 39.0 ± 0.5  |

   * Mean of percentage of total cpm in gradient from 2 separate experiments ± variation.
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