Mechanism of Benzo(a)pyrene Induction of Alpha-Human Chorionic Gonadotropin Gene Expression in Human Lung Tumor Cells

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ABSTRACT   Human lung cells (ChaGo) derived from a bronchogenic carcinoma synthesize and secrete in the culture medium the alpha subunit of the glycoprotein hormone, human chorionic gonadotropin (alpha-hCG). The synthesis of alpha-hCG by ChaGo cells could be further stimulated by treatment with sublethal concentrations of the polycyclic aromatic hydrocarbons (PAHs), benzo(a)pyrene (BaP), or dimethylbenzanthracene. The production of alpha-hCG could be correlated to the levels of alpha-hCG–specific mRNA sequences in control and PAH-treated cells. Further analysis of the RNA species (Northern blot) revealed that the level of the mature (~1.0 kb) and the high molecular weight alpha-hCG specific nuclear RNA sequences (~2.2 and 5 kb) were all greater in PAH-treated cells.

Addition of [^{3}H]BaP (0.25 µg/ml) in the culture medium of ChaGo cells led to immediate uptake of the radioactive compound apparently by simple diffusion. SDS PAGE and subsequent fluorography revealed that the radioactive compound interacted and formed covalent complexes with cytoplasmic and nuclear proteins. This covalent interaction of the [^{3}H]BaP molecule with cellular proteins could be significantly inhibited by either inhibiting the activity of the enzyme aryl hydrocarbon hydroxylase with 7,8-benzoflavone or by reducing the cellular concentration of the enzyme by simultaneous incubation with cycloheximide. These results suggested that in ChaGo cells, the observed covalent complexes were formed by the interaction of the BaP metabolites with cellular proteins. The concentrations at which 7,8-benzoflavone or cycloheximide inhibited (a) formation of metabolites from [^{3}H]BaP and (b) their covalent interaction with cell protein did not affect the BaP-induced stimulation of alpha-hCG gene expression. However, the cytotoxic effects of BaP in ChaGo cells seemed to be exerted by the metabolism of the compounds. Results presented in this report suggest that BaP metabolism and the interaction of the metabolites with cell proteins were not essential for the BaP-induced modulation of alpha-hCG gene expression.

Polycyclic aromatic hydrocarbons (PAHs)[1] are currently recognized as one of the major classes of environmental carcinogenic pollutants (1). Of the PAHs, benzo(a)pyrene (BaP) is

[1] *Abbreviations used in this paper:*   AHH, aryl hydrocarbon hydroxylase; BaP benzo(a)pyrene; BF, 7,3-benzoflavone; ChaGo cells, human lung bronchogenic carcinoma cells; DMBA, dimethylbenzanthracene; alpha-hCG, the alpha subunit of human chorionic gonadotropin; alpha-hCG mRNA, alpha-hCG–specific mRNA sequences; PAHs, polycyclic aromatic hydrocarbons; SSC, 3 M NaCl and 0.3 M sodium acetate.

accepted as the prototype (2, 3). Ectopic hormone and inappropriate gene expression are commonly found to be associated with neoplastic transformation of cells. BaP and several other members of the PAHs have been found to induce aryl hydrocarbon hydroxylase (AHH) in different mammalian cell systems (4). These inducers of AHH also enhance the expression of alpha-fetoprotein gene (5); activities of phospholipase A2 (6); cytosolic aldehyde dehydrogenase (7); membrane-bound gamma-glutamyl transpeptidase (8); choline kinase (9); and ethanolamine kinase (9). The exact molecular mechanism

of such BaP-induced gene expression in mammalian cells is not clearly understood as yet. Grover and Sims (10) and Gelboin (11) have independently demonstrated that BaP and other PAHs undergo metabolic activation by microsomal enzymes and the reactive metabolites form covalent complexes with cellular macromolecules. MacLeod et al. (12) have studied the metabolism and identified the several reactive metabolites in hamster embryo cells. One of the common postulates is that it is this covalent interaction of the reactive metabolites of BaP or other PAHs with cellular macromolecules, more specifically with DNA, through which the carcinogenic and cytotoxic effects of these compounds are mediated (13). On the other hand, AHH, which is the first key enzyme in the metabolism of BaP, is induced by the parent compound itself. This is apparently mediated via initial noncovalent interaction of the molecule with a specific receptor protein (4).

We have previously reported that BaP is able to stimulate the level of expression of an ectopically produced glycoprotein hormone, the alpha subunit of human chorionic gonadotropin (alpha-hCG) in an established human lung bronchogenic carcinoma cell line (ChaGo) under culture conditions (14). A correlation has been established between the increased levels of alpha-hCG–specific mRNA sequences (alpha-hCG mRNA) and BaP-induced hypomethylation of the alpha-hCG gene (14). In this part of the investigation we examined the following parameters: (a) the levels of different species of nuclear and cytoplasmic alpha-hCG mRNA in control and BaP-treated cells; (b) the pattern of BaP metabolism in ChaGo cells; (c) the possible role of the metabolites of BaP on the induction of alpha-hCG gene expression, and finally (d) the cytotoxic effect of BaP metabolites in ChaGo cells. From the results presented in this manuscript we conclude that (a) BaP interaction with cellular macromolecules follows the similar pattern in ChaGo cells as observed in other mammalian cell systems; (b) BaP is metabolized apparently via the similar pathway as described in other cell systems; (c) inhibition of BaP metabolism did not affect BaP-induced alpha-hCG gene expression; and finally (d) BaP-induced cytotoxic effect is mediated via metabolism of the compound.

MATERIALS AND METHODS

Materials: Generally labeled [³H]BaP in toluene (Amersham Corp., Chicago, IL; 25 Ci/mmol) is lyophilized and re-dissolved in dimethyl sulfoxide. Nonradioactive BaP in dimethyl sulfoxide, cycloheximide and flavin mononucleotide in culture medium, and 7,8-benzoflavone (BF) in dimethyl sulfoxide are freshly prepared and all are from Sigma Chemical Co. (St. Louis, MO).

Cell Culture and Labeling: ChaGo cells (15) are grown in Ham's F-10 medium supplemented with 10% fetal calf serum. Cells are grown in 100-mm Falcon tissue culture dishes to a semiconfluent state. For [³H]BaP binding studies, 4 ml of medium is added together with [³H]BaP at 25 μCi/ml (0.25 μg/ml) for the specified durations. Cells in culture are treated with specific inhibitors (at the indicated concentrations) under conditions described in the legend to figures.

Analysis of Cytoplasmic and Nuclear Proteins and Their Interaction with [³H]BaP: Cytoplasmic and nuclear proteins are fractionated according to the procedure of Maniatis et al. (16). All buffers used contained 0.5 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co.). Cytoplasmic and nuclear proteins are analyzed by SDS PAGE on 1.0-mm-thick slab gels of 12.5% total acrylamide concentration according to the method of Laemmli (17). Mass distribution of protein is determined by staining with Coomassie Blue and distribution of radioactive hydrocarbon interaction with cytoplasmic and nuclear proteins is determined by fluorography at −80°C.

Northern Blot Analysis of RNA: RNA is prepared according to the method of Favaloro et al. (18). RNA denaturation and blot transfers are carried out according to the method of Seed (19). RNA is blot-transferred onto a synthetic membrane filter (Zetabind, AMF-Cuno, Meriden, CT). The filter is then washed once for 30 min in 3× SSC, dried, baked for 2 h at 70°C in vacuum, and then prehybridized for 20 h at 42°C (5× SSC, 5× Deinhardt's solution, 50 mM sodium phosphate at pH 6.5, 50% deionized formamide, 1% glycine, 500 μg/ml of single-stranded salmon sperm DNA, and 1% SDS). Hybridization with ³²P-labeled cloned cDNA-hCG probe (1 × 10⁷ cpm of S.A. 2 × 10⁸ cpm/μg DNA per 5 ml hybridization solution) is for 48 h at 42°C (5× SSC, 1× Deinhardt's solution, 10 mM sodium phosphate at pH 6.5, 50% deionized formamide, 100 μg/ml single-stranded salmon sperm DNA, 10% dextran sulphate, and 1% SDS). The filter is then washed five times, 30 min each, at 42°C with the following prewarmed solutions: 5× SSC; 2× SSC; 1× SSC; twice with 0.1× SSC plus 0.1% SDS. It is then air dried and exposed to X-ray film (Kodak XAR5) in the presence of an intensifier (DuPont Lightning Plus) at −80°C.

RESULTS

Levels of Cytoplasmic and Nuclear Alpha-hCG RNA in Control and PAH-treated Cells

Poly(A⁺)-RNA from cytoplasm and total RNA from nuclei are isolated from control, BaP-, or dimethylbenzanthracene (DMBA)-treated cells and are analyzed by Northern blot analysis under denaturing conditions (19). A species of mRNA sequence in the size range of 1.0 kb can be detected in the poly(A⁺)RNA population of cytoplasm (Fig. 1A). This is apparently the mature form of the alpha-hCG mRNA species. Besides this mature form, two other alpha-hCG mRNA species of relatively larger molecular masses can also be detected in the nuclear RNA fraction of both control and carcinogen-treated cells (Fig. 1C). Results presented in Fig. 1 demonstrate that the levels of the mature form of alpha-hCG mRNA species in the cytoplasm (Fig. 1A) and the mature and the high molecular weight alpha-hCG–specific nuclear RNA sequences (Fig. 1, B and C) were greater in BaP or DMBA-treated cells. The results in Fig. 1B demonstrate the difference in the levels of the mature form of alpha-hCG–specific nuclear RNA sequences between the control and PAH-treated cells, whereas results presented in Fig. 1C demonstrate that of high molecular weight alpha-hCG–specific nuclear RNA sequences between control and PAH-treated cells. The observed increased levels of alpha-hCG mRNA sequences in BaP or DMBA-treated ChaGo cells detected by Northern blot analysis not only confirm the previously reported results obtained by dot hybridization analysis (14), but also characterize the molecular sizes of alpha-hCG–specific cytoplasmic and nuclear RNA transcripts. The increased level of alpha-hCG production in PAH-treated cells can thus be correlated to the corresponding elevated levels of different species of cytoplasmic and nuclear alpha-hCG mRNA sequences.

Pattern of [³H]BaP Binding to Cytoplasmic and Nuclear Proteins of ChaGo Cells

The metabolic pathway of [³H]BaP in ChaGo cells is established by binding studies with cytoplasmic and nuclear proteins. Results presented in Fig. 2A show the typical profiles of the Coomassie Blue–stained proteins in the cytoplasm and nuclei of ChaGo cells fractionated by SDS PAGE under conditions described in Materials and Methods. Typical nuclear proteins such as the histones (H4, H2A, H2B, and H3) of the molecular masses, ranging from 12 to 17 kD, could be seen only in the nuclear fraction and not in the cytoplasmic fraction (Fig. 2, A and B). Similarly certain major cytoplasmic
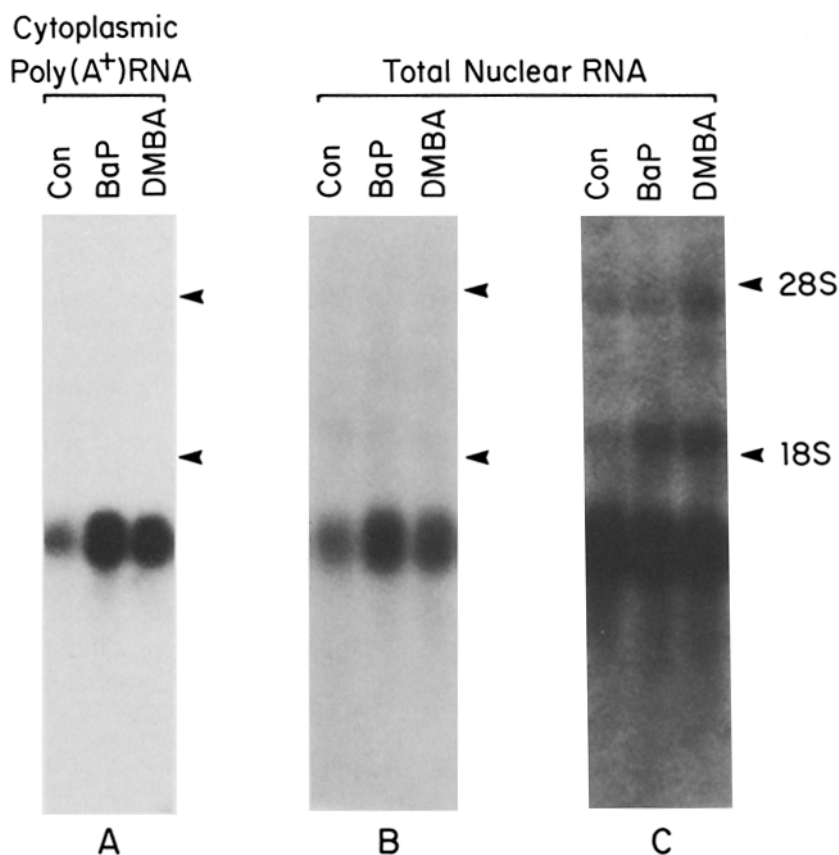
Cytoplasmic Poly(A⁺)RNA

Total Nuclear RNA

Con BaP DMBA

Con BaP DMBA

Con BaP DMBA

◄ 28S

◄ 18S

A

B

C

FIGURE 1   Levels of cytoplasmic and nuclear mRNA–alpha-hCG sequences in control and PAH-treated cells. ChaGo cells were grown in the absence or presence of BaP (0.25 μg/ml) or DMBA (0.25 μg/ml) for 48 h under culture conditions described in Materials and Methods. Isolation of cytoplasmic and nuclear RNA, and details of the Northern blot analysis of RNA under denaturing conditions, are also described in Materials and Methods. Equal amounts (3 μg) of cytoplasmic poly(A⁺)RNA and 50 μg of total nuclear RNA, from control, BaP-, or DMBA-treated cells were denatured (19) and applied in lanes designated Con, BaP, or DMBA respectively (A, cytoplasmic poly(A⁺)RNA; B and C, total nuclear RNA). Arrows show the mobility of 28S and 18S RNA electrophoresed under identical conditions. Hybridization was carried out in the presence of 1 × 10⁷ cpm of ³²P-labeled (35) cloned cDNA-alpha-hCG probe (specific activity, 1 × 10⁸/μg DNA) in 5 ml hybridization mixture, as described in Materials and Methods. Autoradiography was for 48 h for A and B, at −80°C in the presence of an intensifier (DuPont Co., Lightning Plus). To detect the high molecular weight nuclear alpha-hCG mRNA sequences, filter containing the nuclear RNA sequences was exposed to X-Ray film (Kodak, XAR5) for 6 d (C).
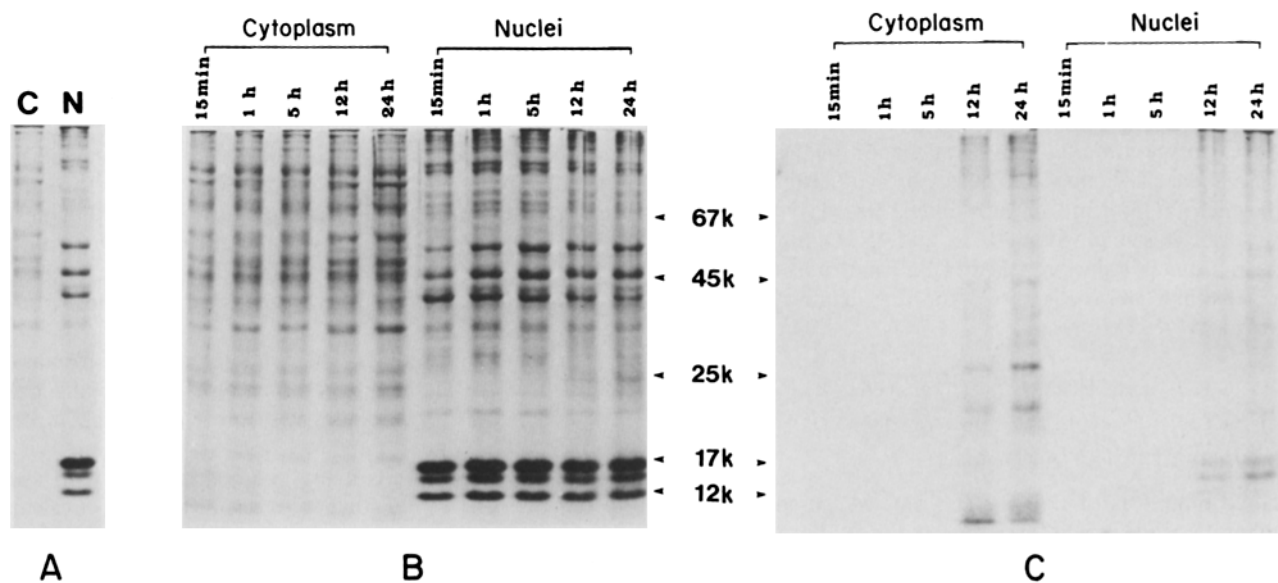

Cytoplasm   Nuclei

C N   15min 1 h 5 h 12 h 24 h   15min 1 h 5h 12 h 24 h

◄ 67k ►

◄ 45k ►

◄ 25k ►

◄ 17k ►
◄ 12k ►

Cytoplasm   Nuclei

15min 1h 5h 12h 24h   15min 1h 5h 12h 24h

A

B

C

FIGURE 2   Kinetics of [³H]BaP binding to cytoplasmic and nuclear proteins of ChaGo cells. ChaGo cells were grown in 100-mm tissue culture dishes. [³H]BaP was added to the culture media (0.25 μg/ml, 25 μCi/ml, specific activity, 25 Ci/mmol), and cells were incubated for specified periods of time (15 min, or 1, 5, 12, and 24 h). Medium was then removed and cells were washed four times with Hanks' buffered solution. Preparation of cytoplasmic and nuclear proteins, fractionation in SDS PAGE system, and the fluorography are all described in detail in Materials and Methods. 100 μg of protein from cytoplasm and nuclear fractions of each sample were denatured and applied to the designated lanes. A shows the fractionation profile of cytoplasmic (C) and nuclear (N) proteins of ChaGo cells following Coomassie Blue staining after electrophoresis. B shows that of cytoplasmic and nuclear proteins of [³H]BaP-treated ChaGo cells. C shows the fluorogram of the dried gel (exposed for 2 wk). Periods indicated on each lane designate the duration of incubation of [³H]BaP exposure of the cells, from which cytoplasmic and nuclear fractions were prepared and subjected to SDS PAGE. Numbers indicated by the arrows show the electrophoretic mobility of the marker proteins of known sizes electrophoresed under identical conditions.

proteins were absent in the nuclear fraction (Fig. 2, A and B). These results suggested that within the sensitivity range of Coomassie Blue staining, the cross-contaminations between the two protein fractions were minimal.

Administration of [³H]BaP (0.25 µg and 25 µCi/ml) to ChaGo cells for different durations revealed no apparent alteration in the pattern of cytoplasmic and nuclear proteins (Fig. 2 B). This pattern was very similar to that observed in untreated ChaGo cells (Fig. 2 A). The corresponding fluorogram (Fig. 2 C) revealed the pattern of binding (under denaturing conditions) of [³H]BaP and/or metabolites, to several but not all of the cytoplasmic and nuclear (Fig. 2 C) macromolecules. Radioactivity from the covalent complex could be completely solubilized by treatment of the samples with proteolytic enzyme (Proteinase K, E. Merck-Dramstadt, Germany) (data not shown), whereas treatment of nuclear and cytoplasmic extracts with RNase (pancreatic A and T1) and DNaseI (pancreatic) did not abolish the radioactive complex formation (results not shown). It may be concluded from these results that the cellular macromolecules to which [³H]BaP and/or its metabolites were covalently bound are mostly proteins in nature.

Though radioactivity could be associated with several cytoplasmic and nuclear proteins, the binding pattern of the radioactive compound was independent of the relative abundance of the specific proteins. These results suggest that binding affinity of [³H]BaP and/or its metabolites was different for different proteins and that there is a certain degree of specificity of this covalent interaction.

The kinetics of binding to cytoplasmic and nuclear proteins demonstrate that the radioactive [³H]BaP and/or its metabolites formed covalent complexes with cytoplasmic and nuclear proteins, and the covalent complex formation increased with time. The radioactive complex formation could be detected as early as 12 h after exposure of the cells to [³H]BaP. In other experiments, the fluorographic analysis showed that incubation of cells with [³H]BaP also led to such radioactive covalent complex formation after 5 h of incubation of the cells with [³H]BaP (data not shown). The major cytoplasmic proteins with which [³H]BaP and/or metabolites formed covalent complexes were in size range of 20, 25, and 45 kD. Similarly, the nuclear proteins with which the [³H]BaP and/or its derivatives interacted in ChaGo cells were of the sizes ranging 12, 15, 17, 21, 62, and 74 kD.

Covalent Interaction of [³H]BaP with Cytoplasmic and Nuclear Proteins in the Presence of Specific Inhibitors of BaP-Metabolism

Three inhibitors of BaP metabolism were used to verify whether it was the parent ³H-compound or the ³H-metabolites that were responsible for covalent interaction with the cellular proteins. It was also observed in other cell systems that AHH catalyzes the initial reaction, which converts the parent PAHs to their hydroxylated derivatives. BF is a strong inhibitor of the catalytic activity of the enzyme AHH (20, 21). Cycloheximide being an inhibitor of general protein synthesis inhibits the BaP-mediated induction of AHH and thus lowers the effective level of the enzyme in the cell. Flavin mononucleotide inhibits the interaction of the metabolite with nucleic acids (22, 23).

Results presented in Fig. 3 A show the protein profile (SG) and the pattern of covalent interaction of the radioactive

compounds (F) with cytoplasmic (CYT) and nuclear (NUC) proteins in control (−) and in BF-treated (+) cells. It is evident from these results that no significant difference either in the relative concentrations of specific proteins or in the protein profile between control and BF-treated cells could be detected. However, the fluorographic results (lanes F) clearly demonstrate that treatment of the cells with BF abolished completely the covalent interaction between the radioactive compound and the cytoplasmic and nuclear proteins.

These results established the metabolism of the parent compound, [³H]BaP, in ChaGo cells. This seems to be similar to that observed in other cell systems. If the formation of the metabolites were inhibited by treatment of the cells with BF, which is a potent inhibitor of the enzyme AHH, the formation of covalent complexes could be inhibited.

It is believed that treatment of cells with PAH leads to the induction of the enzyme AHH which catalyzes the conversion of the parent PAH to reactive metabolites (11). Cycloheximide which is a potent inhibitor of general protein synthesis, when added to ChaGo cells together with [³H]BaP, also inhibited significantly the formation of the covalent complexes between the [³H]BaP and cytoplasmic (Fig. 3 B [CYT]) and nuclear (Fig. 3 B [NUC]) proteins. These results again suggest that the formation of the covalent complexes of [³H]BaP and cellular proteins were affected significantly by the inhibition of metabolism of the BaP either because of reduction in the level of the enzyme (by cycloheximide) or by inhibition of the activity of the enzyme (by BF) responsible for the metabolism of the carcinogen. It is apparent from the protein profile of the cytoplasmic and nuclear fraction following longer (24 h) treatment of the cells with cycloheximide, that the levels of the specific proteins to which the [³H]BaP or metabolites were bound were affected to a certain extent. Thus, the reduced radioactive covalent complex formation in the presence of cycloheximide may be due either to the lower levels of the BaP-metabolizing enzyme or due to the lower levels of specific proteins with which BaP interacted, or due to both reasons. However, it seems that the level of the BaP-metabolizing enzyme is more important in the covalent complex formation between [³H]BaP and cellular proteins. Because, though the levels of the certain proteins were not affected, cycloheximide treatment did indeed affect the radioactive complex formation with the cellular proteins after treatment with the drug for the same period of time.

Flavin mononucleotide, which did not affect the formation of the reactive metabolites of BaP also did not affect the interaction of ³H-metabolites with proteins in ChaGo cells (Fig. 3 C).

From all these results it may be concluded that it is the metabolites of [³H]BaP that were responsible for covalently interacting with certain cytoplasmic and nuclear proteins of ChaGo cells. The parent compound, [³H]BaP, was not involved in such covalent interactions with cellular proteins. These results established the metabolic pathway of [³H]BaP in ChaGo cells. It is to be noted that the fluorograms (F) shown in Fig. 3 have protein bands that are more intensely labeled than those in Fig. 2 C. The apparent reason for this is that the fluorograms in Fig. 3 were exposed for 4 wk whereas that in Fig. 2 C was exposed for 2 wk only.

BaP metabolism in ChaGo cells is studied by exposure of cells to [³H]BaP for different periods of time followed by measurement of radioactive metabolites in the alkaline–ethanol phase of the hexane-extracted cell extract according
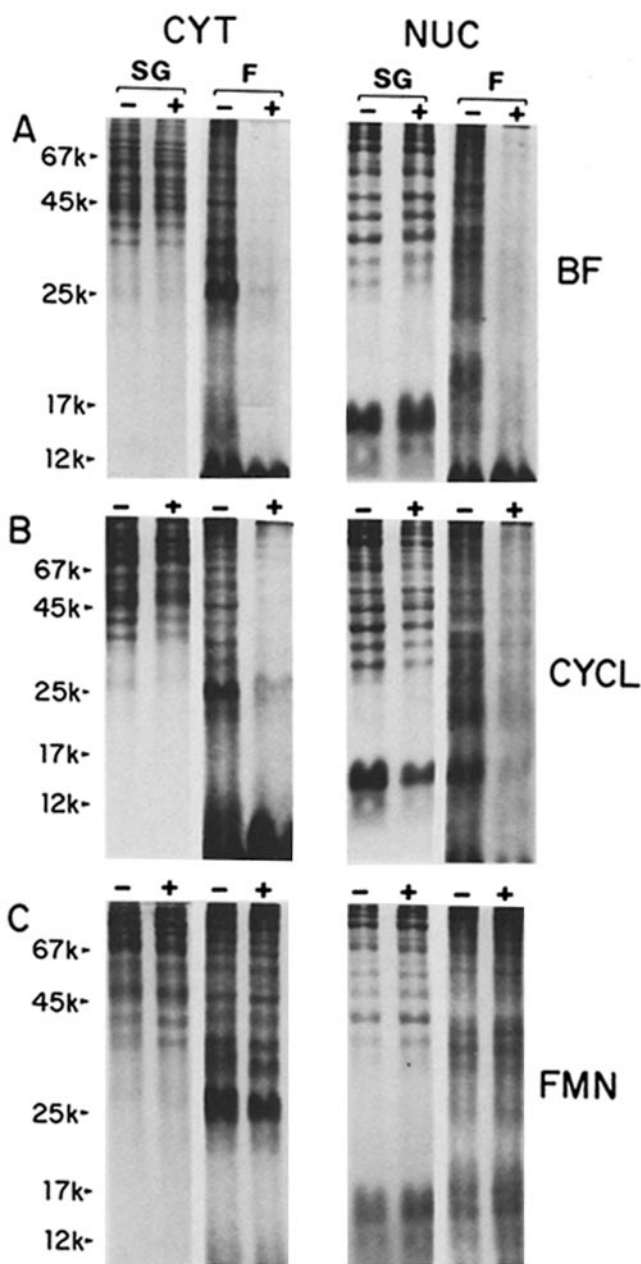
CYT NUC

SG F SG F

A

BF

B

CYCL

C

FMN

FIGURE 3 Binding pattern of [³H]BaP to cytoplasmic and nuclear proteins in ChaGo cells grown in the presence of 7,8-benzoflavone or cyclohexamide or FMN. Conditions for growth of cells, treatment with [³H]BaP, isolation of cytoplasm and nuclei, and electrophoresis in SDS PAGE system were all the same as described in legend to Fig. 2. 100 μg of cytoplasmic (CYT) or nuclear (NUC) proteins were loaded in each lane. A shows the fractionation profile of different samples of cytoplasmic or nuclear proteins as observed after staining of the gel with Coomassie Blue (SG) and after fluorography (F). Cells were exposed to BF (2 μg/ml) 18 h before the addition of [³H]BaP and were grown for an additional 24 h. Cytoplasmic and nuclear fractions were isolated from cells exposed to [³H]BaP in absence (−) or presence (+) of BF. B shows the pattern of [³H]BaP binding to cytoplasmic and nuclear proteins of ChaGo cells in the absence (−) or presence (+) of cycloheximide (0.25 μg/ml, 24 h). C shows the pattern of binding of [³H]BaP to cytoplasmic and nuclear protein in the absence (−) or presence (+) of FMN (2 μg/ml, 24 h). For fluorographic analysis, the gel was subjected to treatment with EN³HANCE (New England Nuclear, Boston, MA) and dried under vacuum. Exposure of the dried gel to X-ray film is for 4 wk at −80°C. Numbers indicated by the arrows show the electrophoretic mobility
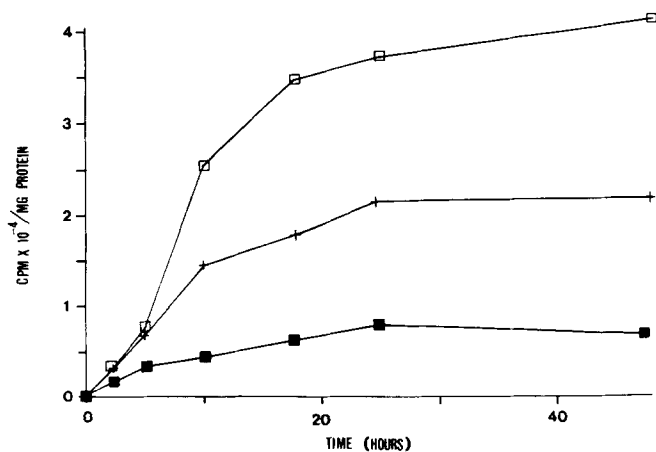

FIGURE 4 Levels of [³H]BaP-metabolites in ChaGo cells grown in presence of BF. Cells were grown in 60-mm dishes in the presence of [³H]BaP (0.25 μg/ml, 25 μCi/ml; specific activity, 30 Ci/mmol) alone (□) (control), or in presence of 1 μg/ml (+) or 2 μg/ml (♦) of BF for the specified period of time. The cells were then washed free of [³H]BaP by rinsing with Hanks' buffered saline five times. The cells were then harvested and lysed by osmotic shock followed by homogenization in a loose-fitting Dounce homogenizer. The lysed cell suspension was then centrifuged at 1,000 g for 5 min. An equal volume of 0.5 N NaOH in ethanol (80%) was then added to the supernatant, and extracted three times with 2 vol of hexane according to the method of DePierre et al. (24). The radioactivity in the aqueous phase is measured after neutralization of an aliquot of the supernatant in liquid scintillation counter. Protein content of the cell extract is determined according to the method of Lowry et al. (36).

to the method of DePierre et al. (24). The unmetabolized parent compound is effectively extracted with hexane. Results presented in Fig. 4 demonstrate that aqueous soluble [³H]-BaP-metabolite formation increased with time of incubation of ChaGo cells with [³H]BaP. Treatment of ChaGo cells simultaneously with [³H]BaP (0.2 μg/ml) and BF (1 or 2 μg/ml), significantly reduced the level of NaOH–ethanol extractable radioactivity. BF being an established potent inhibitor of AHH activity, these results suggest that the formation of the NaOH–ethanol extractable radioactive derivatives in the cell extract is a true measure of cellular AHH activity, and reflects the state of metabolism of [³H]BaP catalyzed by the enzyme AHH.

BaP Metabolism and Induction of the Alpha-hCG Gene Expression in ChaGo Cells

The results presented above suggest that [³H]BaP is metabolized in ChaGo cells by adopting the pathway for PAH metabolism similar to the one established in other cell systems. The significance of metabolism of [³H]BaP and the covalent interaction of the metabolites with cellular proteins in relation to observed BaP-induced alpha-hCG gene expression was then verified. The stimulation of alpha-hCG mRNA synthesis in BaP and DMBA-treated cells was examined in cells simultaneously treated with the PAHs and the inhibitors of PAH metabolism. The interaction of [³H]BaP metabolites with cellular proteins in the presence of BF and cycloheximide was inhibited under these experimental conditions (Fig. 3, A

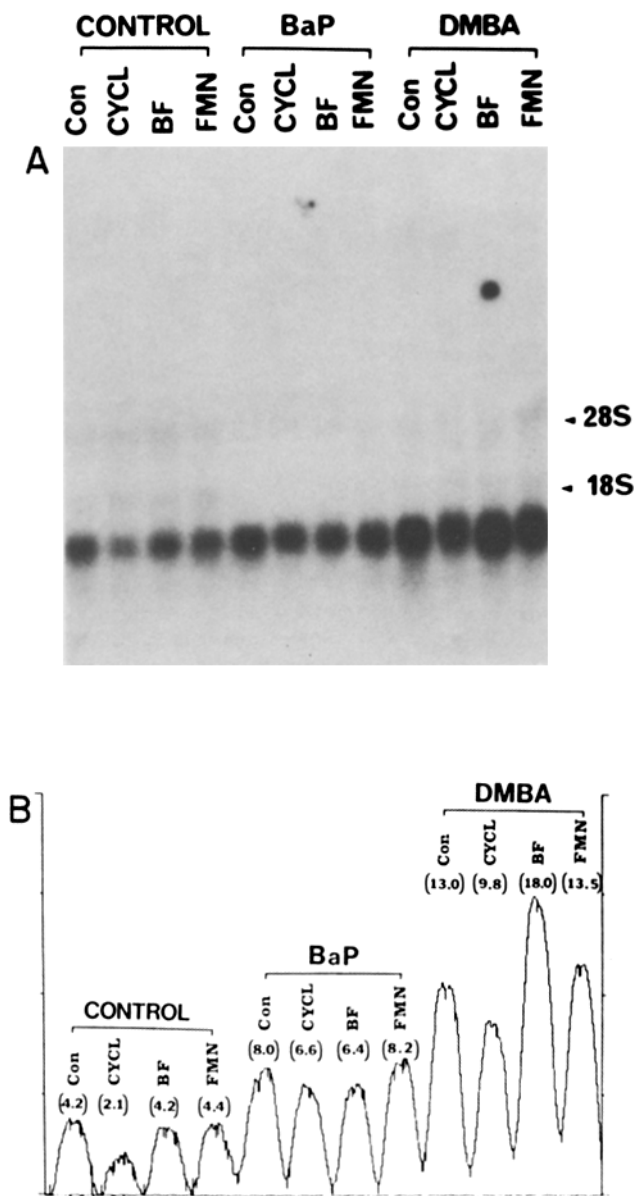of standard protein markers of known molecular size, electrophoresed under identical conditions.

FIGURE 5 Level of mRNA-alpha-hCG sequences in control, BaP-, or DMBA-treated cells grown in the presence of cycloheximide, BF, and flavin mononucleotide (FMN). (A) Experimental details of the isolation of cytoplasmic RNA and analysis by Northern blot technique are described in Materials and Methods. 50 μg of RNA from control or treated cells were denatured and applied to each lane. BaP and DMBA concentrations administered in the growth medium were both at 0.25 μg/ml. 7,8-Benzoflavone(BF), cycloheximide (CYCL), and FMN concentrations in the growth medium are 2 μg/ml, 0.1 μg/ml, and 2 μg/ml, respectively. The ^{32}P-labeled cDNA-alpha-hCG is used as probe to detect the alpha-hCG mRNA sequences. 1 × 10^7 cpm of ^{32}P-labeled cDNA-alpha-hCG (specific activity, 1 × 10^8 cpm/μg of DNA) are used in 5 ml of hybridization solution. Hybridization is for 48 h and autoradiography is for 4 d at −80°C in the presence of intensifier. (B) The autoradiographic signals generated by Northern blot analysis of cytoplasmic RNA isolated from control and PAH-treated cells are further analyzed by scanning densitometry (E-C Apparatus Corp., St. Petersburg, FL). The peak area of the densitometric scan of each signal is then integrated using the Zeineh Electrophoresis Reporting Integrator Program (Biomed Instruments, Inc., Fullerton, CA) connected to an Apple IIe computer. The numbers in parenthesis indicate the percent peak area. Treatment of cells with different drugs are described above.

and B). Results presented in Fig. 5 demonstrate that the BaP/DMBA-induced increased levels of alpha-hCG mRNA sequences were not significantly affected by simultaneous treatment either with BF (lanes 3, 7, and 11) or cycloheximide (lanes 2, 6, and 10) or with FMN (lanes 4, 8, and 12).

Since the intensity of the autoradiographic signal generated in Northern blot analysis is proportional to the concentration of the specific mRNA sequences complementary to the specific ^{32}P-labeled DNA probe used in the hybridization mixture, a quantitative evaluation of the autoradiographic signals generated in Fig. 5A was made by densitometric scanning of these signals. These results are shown in Fig. 5B. As stated above, either BaP or DMBA treatment of ChaGo cells increased the levels of cytoplasmic alpha-hCG mRNA sequences. We have previously reported that DMBA stimulation of alpha-hCG synthesis was somewhat higher than that observed after BaP treatment of ChaGo cells (14). This was further substantiated by the results presented in Fig. 5 of this report in which the level of alpha-hCG mRNA sequences in DMBA-treated cells was greater than that observed in BaP-treated cells. The stimulation of alpha-hCG mRNA synthesis by either BaP or DMBA was not inhibited by treatment of the cells simultaneously with the three inhibitors of PAH metabolism. The reasons for the further stimulation of alpha-hCG mRNA synthesis in cells treated simultaneously with DMBA and BF are not clear as yet. The small reduction in the levels of alpha-hCG mRNA sequences observed in all cycloheximide-treated (48 h) samples (Fig. 5) may be due to the effect of the drug on general protein synthesis in these cells. Similar effect of cycloheximide was observed by Harris et al. (25) on AHH activity induced by benzanthracene. These results demonstrate that inhibition of metabolite formation or their interaction with cellular macromolecules did not affect the increased synthesis of alpha-hCG mRNA sequences in BaP or DMBA-treated cells, suggesting that metabolism of BaP/DMBA may not be an obligatory step for the induction of alpha-hCG gene expression.

Cytotoxic Effect of BaP on ChaGo Cells in the Presence of BF

Results presented in Figs. 2 and 3 established that BaP metabolism is necessary for forming covalent complexes with cytoplasmic and nuclear proteins. At the same time results presented in Fig. 5 demonstrate that BaP metabolism is not obligatory for the induction of alpha-hCG gene expression.

It is believed that the cytotoxic effects of chemical carcinogens are due to their genotoxic/DNA damaging effects caused by reactive metabolites of BaP. We have previously reported that BaP is cytotoxic to ChaGo cells at concentrations of these chemicals of 0.3 μg/ml and higher (14). The possible role of BaP metabolism in the cytotoxic effect of the chemical was verified by growing the cells in the presence of BaP with and without the metabolic inhibitor BF. These results are presented in Fig. 6. The growth of cells in the presence of the indicated concentrations of the chemicals were determined by measurement of total cell DNA (25). The results demonstrate that 0.5 μg/ml of BaP was cytotoxic to ChaGo cells (Fig. 6). BF (1 or 2 μg/ml) by itself did not have any effect on the growth of ChaGo cells. However, BF at concentrations 1 or 2 μg/ml prevented the cytotoxic effect of BaP (0.5 μg/ml) to the extent of 25 and 60%, respectively.
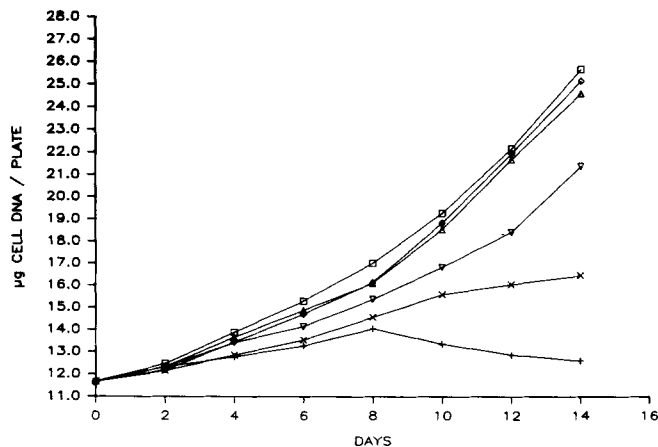
Figure 6 Cytotoxicity of ChaGo cells in the presence of BaP and BF. ChaGo cells were grown in 60-mm tissue culture dishes in 4 ml of Ham's 7-10 medium supplemented with 10% fetal calf serum. Growth of cell is determined by measurement of cell DNA (26). Each point represents an average of DNA determinations in duplicate plates. □, control; +, BaP (0.5 µg/ml); ◇, BF (1.0 µg/ml); △, BF (2.0 µg/ml); ×, BaP (0.5 µg/ml) + BF (1.0 µg/ml); ▽, BaP (0.5 µg/ml) + BF (2.0 µg/ml).

These results suggested that metabolism of BaP was necessary to exert the cytotoxic effect of the carcinogen in ChaGo cells.

DISCUSSION

Production of alpha-hCG by ChaGo cells is an inappropriate expression of the gene. Treatment of the ChaGo cells with PAHs such as BaP or DMBA can further stimulate the synthesis of the hormone, more specifically the alpha subunit of the glycoprotein (14). Understanding of the molecular mechanism of BaP/DMBA–induced alpha-hCG gene expression has been the primary objective of this investigation.

It is generally believed that PAHs like BaP or DMBA follow one of the two or both the pathways to exert their effects on the target cell systems (4). BaP, upon entering the cell, interacts with specific receptor molecules. This complex is then translocated into nucleus of the cell and interacts with the target genes such as AHH gene, leading to the increased production of AHH enzyme. This enzyme is responsible for conversion of the parent compound into the reactive derivatives, and thus accelerates the metabolism of the parent compounds. Results presented in Fig. 2 and 3 show that inhibition of the AHH activity by BF or reduction in the level of the enzyme by treatment with cycloheximide drastically affected the covalent interaction of the [³H]BaP with cell proteins. These results thus clearly demonstrated that only the metabolites of [³H]BaP interacted in such fashion with ChaGo cell proteins.

The question posed at this stage is whether or not such metabolism of BaP is necessary for the induction of alpha-hCG gene expression. Results presented in Fig. 5 demonstrate that BaP-induced alpha-hCG gene expression was not affected by treatment of the cells with BF concentrations at which significant inhibition of AHH activity could be noticed (Fig. 4). Under the similar experimental conditions BF also reduced the cytotoxic effect of BaP (Fig. 6). These results suggest that metabolism of BaP/DMBA is not an obligatory step for the induction of alpha-hCG gene expression by these chemicals. Though significant levels of inhibition of metabolite formation and interaction with cellular proteins after incubation of

the cells with BF were observed, it may be argued that complete inhibition of the metabolite formation is not achieved under these conditions. Thus involvement of the metabolites in the BaP- or DMBA-induced alpha-hCG induction can not be completely ruled out. These results are suggestive of a possible role of the parent compound on the induction of alpha-hCG gene expression. Our previous results demonstrate that BaP-induced expression of alpha-hCG gene is mediated via hypomethylation of "C" residues in alpha-hCG gene (14). It will be of significant interest to know whether a similar mechanism is involved in the BaP induction of AHH gene expression.

Though the metabolic pathway of action of BaP via metabolism leading to the formation of reactive compounds does not seem to be necessary for alpha-hCG gene expression, it is apparent that this pathway is used in ChaGo cells to manifest the cytotoxic effect of BaP (Fig. 6).

The role of AHH in the metabolism of PAH is well established (4, 25–31). Thus its role in the ultimate action of the reactive derivative in the process of carcinogenesis may be defined. At this stage, the role of ectopic alpha-hCG synthesis in the development of tumor is not established. It is not clear whether it is just a circumstantial event associated with the process of neoplasm, such as the commonly observed inappropriate expression and suppression of unrelated genes; or whether such ectopic hormone production has any direct role in the process of tumor formation.

Stanbridge et al. (32) have shown that expression of alpha-hCG gene can be specifically correlated with the transformed phenotype in human cell hybrids. These authors suggest that alpha-hCG expression might allow the tumorigenic segregants to evade the putative growth regulatory signal or that the alpha-hCG gene expression is controlled by regulator(s) whose gene(s) is(are) located on chromosome(s) that contain the genetic information necessary for the suppression of tumorigenicity. Loss of the "suppressor" chromosome would involve concomitant loss of the alpha-hCG regulatory gene, thereby resulting in a "switch-on" situation for alpha-hCG synthesis. Bergtsson and Rydstrom (33) have demonstrated the positive regulation of PAH hydroxylases by gonadotropins. They suggest that a higher level of circulating gonadotropin can be correlated with an increased risk of developing tumors. Therefore, these genes whose expression can be modulated by PAH might be important in the tumor formation process. Their expression might be analogous to the "damage inducible genes" in the SOS response in Escherichia coli (34), i.e., DNA-damaging agents induce the expression of genes for repair/survival purposes at a cost of increased mutations, cytotoxicity, and maybe even carcinogenicity. In mammalian cell systems, it is possible that DNA-damaging agents, besides inducing repair functions, also induce undesirable cellular functions (e.g., transforming genes). Their induction might be similar to that of alpha-hCG gene in ChaGo cells, mediated probably through a reversible epigenetic phenomenon, i.e., via the parent unmetabolized compounds, and not via the metabolites. Such knowledge on the sequence of events is of significant importance for the elucidation of molecular mechanism of action of PAHs in biological systems.

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