A Benzo[a]pyrene-induced Cell Cycle Checkpoint Resulting in p53-independent G1 Arrest in 3T3 Fibroblasts*

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The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor of the basic helix-loop-helix family. Although physiological ligands for the AhR have not been identified, carcinogenic polycyclic aromatic hydrocarbons such as Benzo[a]pyrene (B[a]P) are high affinity AhR ligands that induce nuclear translocation and sequence-specific DNA binding of the AhR. AhR-regulated genes include members of the cytochrome P-450 family that are known to oxidize B[a]P to form genotoxic (DNA-damaging) metabolites. Murine Swiss 3T3 cells express high levels of AhR. Treatment of Swiss 3T3 cells with B[a]P during the G1 phase of the cell cycle resulted in growth arrest, as shown by inhibition of CDK, cyclin-dependent kinase.

tetrachlorodibenzo-3T3 cells with B[a]P. The AhR antagonist α-naphthoflavone prevented B[a]P-induced growth arrest, further demonstrating that cessation of cell growth was mediated by the activated AhR. A nongenotoxic AhR ligand (2,3,7,8-tetrachlorodibenzo-p-dioxin) did not elicit growth arrest, showing that ligand activation of the AhR alone was insufficient to block cell cycle progression. However, genomic DNA from B[a]P-treated Swiss 3T3 cells contained covalent adducts, whereas that from 2,3,7,8-tetrachlorodibenzo-p-dioxin-treated cells did not, showing that G1 arrest correlated with DNA damage resulting from genotoxic B[a]P metabolites. B[a]P-induced DNA damage and growth arrest was coincident with elevated levels of nuclear p53 protein and induction of the p53-regulated mdm-2 proto-oncogene. However, Swiss 3T3 fibroblasts expressing “dominant negative” mutant p53, as well as primary fibroblasts from p53−/− “knockout” mice, also underwent growth arrest in response to B[a]P. Therefore, B[a]P-induced growth arrest occurs via p53-independent mechanisms.

Benzo[a]pyrene (B[a]P)† is a ubiquitous environmental pollutant that is generated during the combustion of fossil fuels. Carcinogenic and mutagenic effects of B[a]P have been well documented in animals and mammalian cell systems (1, 2). Although B[a]P is not itself genotoxic, it is oxidized to toxic and reactive species within target cells. Many of the biological effects of B[a]P and related polycyclic aromatic hydrocarbons (PAHs) are suspected to be mediated by the aryl hydrocarbon receptor (AhR).

The AhR is a ligand-activated transcription factor of the basic helix-loop-helix family, which displays high affinity binding to certain planar aromatic compounds (3, 4). Such compounds include PAHs (typified by B[a]P) and halogenated aromatic hydrocarbons (typified by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)). The unliganded AhR exists in the cytosol, in a complex with HSP 90. On binding to aryl hydrocarbons, the AhR dissociates from HSP 90 and translocates to the nucleus, where it is thought to form a complex with thearyl hydrocarbon nuclear transporter protein (4, 5). The ligand-activated AhR-aryl hydrocarbon nuclear transporter complex binds to specific enhancer sequences (termed xenobiotic response elements), present within the promoter region of aromatic hydrocarbon-inducible genes. The most widely studied AhR-responsive genes are the aryl hydrocarbon-inducible members of the P-450 cytochrome family. Ligand activation of the AhR results in transcriptional activation of genes of the P-450 subfamily, specifically P-450A1A1/1A2 and P-450B1 (6). PAHs such as B[a]P not only induce expression of P-450 genes but are themselves substrates for P-450-mediated oxidation and thereby have the potential to generate reactive electrophilic derivatives that can form covalent adducts in nucleic acids and proteins.

PAHs are known to be carcinogens and mutagens under experimental conditions, and their biological effects may contribute to initiation and progression of the transformed state in many human malignancies. It is becoming increasingly apparent that many carcinogens are likely to exert their effects on cellular growth and differentiation by perturbing the signal transduction pathways involved in the normal control of the cell cycle. To test our hypothesis that PAHs influence progression through the replicative cell cycle, we have investigated the effects of B[a]P activation of the AhR on growth factor-regulated mitogenic responses in murine 3T3 fibroblasts.

Murine 3T3 fibroblasts have provided a valuable cell system for the study of growth factor-regulated signal transduction and cell cycle-regulated events (reviewed in Refs. 7 and 8). 3T3 fibroblasts are immortal, nontransformed cell lines, originally derived from mouse embryos. The growth properties and growth factor requirements of 3T3 cells have been well characterized. Moreover, much is known regarding the molecular mechanisms that regulate cell cycle progression in these cells. For exponential growth, 3T3 fibroblasts require exogenously added growth factors, usually supplied by supplementation of the growth medium with 10% serum. On serum withdrawal, 3T3 cells undergo growth arrest and remain in a quiescent state termed G0. However, when stimulated with fresh serum or certain defined mitogenic growth factors (such as platelet-
derived growth factor), the cells simultaneously re-enter the G_{1} phase of the cell cycle. G_{2} is defined as the time interval between G_{0} (or mitosis) and the initiation of DNA synthesis (S phase) and is characterized by a cascade of growth factor-stimulated signaling events, including growth factor-induced synthesis of second messenger molecules, activation of small GTP-binding proteins, protein kinase cascades, and the transcriptional activation of genes with protein products that are thought to be necessary for progression through the cell cycle (7–9). Thus, the cell cycle comprises a complex program of temporally ordered events that mediate cellular responses to external mitogenic stimuli.

Deregulation of the proliferative cell cycle may potentially be achieved by agents that interfere with components of the growth factor-stimulated signaling cascade, which mediates cell cycle progression. Here we show that B[a]P treatment of synchronized AhR-expressing murine 3T3 cells results in DNA damage and inhibition of growth factor-regulated cell cycle progression. As with other genotoxic agents, B[a]P-induced DNA damage is associated with elevated levels of nuclear p53 and increased transcription of the p53-regulated mdm-2 gene. However, in contrast with certain other “classic” genotoxic stimuli, which require p53 to mediate cell cycle arrest, we show here that p53 is not necessary for B[a]P-induced G_{1} arrest.

MATERIALS AND METHODS

Cells and Culture—Swiss 3T3 fibroblasts were obtained from the American Type Culture Collection and were grown in Dulbecco’s modified Eagle’s medium containing 10% heat-inactivated donor calf serum, supplemented with penicillin, streptomycin, and glucose. Dog amphotropic packaging cells were obtained from previously described sources (10). Primary fibroblast lines from p53+/− and p53−/− mice were kindly provided by Earlene Schmitt and Tyler Jacks (Massachusetts Institute of Technology, Cambridge, MA).

Plasmids and Probes—The murine mdm-2 cDNA was kindly provided by Dr. Donna L. George (University of Pennsylvania, Philadelphia, PA). The p21 DNA probe was amplified from mouse genomic DNA as described previously (11). The wild-type and mutant p53 constructs originally described by Elyiayhu et al. (12) were sent to us by Dr. A. Nigel Carter (University of Dundee, Dundee, United Kingdom).

AhR Ligands—B[a]P, α-naphthoflavone (ANF), and A89 were kindly provided by Dr. David H. Sherr (Boston University School of Public Health). The ligands were dissolved in ME2SO (B[a]P and ANF) or acetone (TCD) immediately prior to use and were added directly to the tissue culture medium as 1000× stocks to give the desired final concentration.

Preparation of Cytosolic Extracts and Nuclei for Immunoblotting—Monolayers of Swiss 3T3 cells in 10-cm culture dishes were rinsed with 10 ml of phosphate-buffered saline. The washed monolayers were detached by addition of 1.5 ml of 10 mM Tris (pH 7.0)/150 mM NaCl/1 mM EDTA for 10 min at room temperature. Detached cells were scraped off the dish, placed in a Microfuge tube, and centrifuged at 10,000 × g for 10 s. The washed cell pellets were lysed in 100 μl of lysis buffer (20 mM Hepes (pH 7.4)/150 mM NaCl/5 mM MgCl_{2}/1 mM EGTA/0.5% Nonidet P-40), vortexed vigorously, and incubated on ice for 5 min. The lysates were centrifuged at 10,000 × g for 5 min. The resulting supernatants (cytosolic extracts) were removed and frozen at –70 °C prior to immunoblotting. Nuclear pellets were resuspended in 100 μl of lysis buffer containing 2 units of RNaseA (Promega). Chromatin was digested on ice for 10 min. Digested nuclei were then frozen at –70 °C prior to SDS-polyacrylamide gel electrophoresis.

SDS–Polyacrylamide Gel Electrophoresis and Immunoblotting—100-μg aliquots of each sample were denatured by heating to 90 °C in SDS reducing buffer and were separated by electrophoresis on 7.5% SDS-polyacrylamide gels. After transfer to nitrocellulose, the filters were probed with polyclonal antisera to recombinant murine AhR (Bio-mol), or a monoclonal antibody against murine p53 (Oncogene Sciences). Bound antibodies were detected using alkaline phosphatase-conjugated secondary antibodies (Bio-Rad).

Generation of p53-expressing Packaging Cell Lines and Swiss 3T3 Fibroblasts—Exponentially growing cultures of dog amphotropic packaging cells were transduced with 10 μg of the pLTRp53c5 plasmid (encoding a dominant negative murine p53 with mutations in amino acids 168 and 234) or pLTRp53Nc9 (encoding wild-type murine p53) plus 20 μg of salmon sperm carrier DNA, by calcium phosphate coprecipitation. After 12 h, the transfection medium was removed and replaced with fresh culture medium. 12 h later the cells were split into medium containing 0.5 mg/ml G418. After selection in G418-containing medium, resistant colonies of cells were pooled and placed in fresh G418-free medium. 24 h later, the virus-containing medium was removed and used to infect exponentially growing cultures of Swiss 3T3 fibroblasts. G418-resistant Swiss 3T3 cells were obtained as described above. Individual colonies of G418-resistant cells were isolated, expanded, and screened for expression of wild-type or mutant p53. Individual colonies expressing high levels of dominant negative (designated Sc6) or wild-type (designated Nc9) p53 were selected for use in the experiments described here.

Growth Factor–stimulated [3H]Thymidine Incorporation Assays, FACS Analysis, and RNA Blot Analysis—These were performed as described previously (13).

Postlabeling of DNA Adducts—Genomic DNA from 3T3 fibroblasts was analyzed for adducts using the Nuclease P1-enhanced postlabeling assay exactly as described by Reddy and Randerath (14).

RESULTS

B[a]P Inhibits Growth Factor-stimulated DNA Synthesis in AhR-expressing Fibroblasts—Swiss 3T3 fibroblasts expressed high levels of a 95-kDa protein that was recognized by antisera to the AhR (Fig. 1A). By contrast, other cell lines tested, including NIH 3T3 (Fig. 1A), Balb 3T3, 3T3-L1, and KBalb (data not shown), did not express detectable levels of AhR protein. Cultures of near-confluent Swiss 3T3 and NIH 3T3 fibroblasts were induced to exit the replicative cell cycle and undergo growth arrest by serum starvation for 48 h. Quiescent (G_{0}) cultures were then stimulated with PDGF in the presence of [3H]thymidine with or without varying doses of the AhR ligand B[a]P. 24 h later [3H]thymidine incorporation into genomic DNA was determined to provide an index of PDGF-stimulated DNA synthesis. As shown in Fig. 1B, PDGF elicited a strong mitogenic response in both Swiss and NIH 3T3 cells.

B[a]P caused a dose-dependent inhibition of PDGF-stimulated DNA synthesis (IC_{50} 0.1 μM, maximal effect at 0.5 μM) in Swiss 3T3 cells. We routinely observed between 50 and 75% inhibition of growth factor-stimulated mitogenic responses at maximally effective concentrations of B[a]P (Fig. 1B). As shown in Fig. 1B, the inhibitory effects of B[a]P on PDGF-induced DNA synthesis were prevented by co-administration of the AhR partial antagonist ANF. By contrast with Swiss 3T3 fibroblasts, PDGF-stimulated DNA synthesis was unaffected by B[a]P in NIH 3T3 cells (and all other fibroblast lines we have tested; data not shown), which lack detectable levels of AhR.

Even the highest B[a]P concentration tested (30 μM) had no effect on PDGF-stimulated DNA synthesis in NIH 3T3 fibroblasts (not shown). Cell viability assays using trypsin blue exclusion showed that no necrosis or apoptosis occurred as a result of B[a]P treatment in any of the cell lines we used (not shown).

To further confirm that inhibition of DNA synthesis by B[a]P resulted from arrest in G_{1}, we obtained nuclear DNA profiles of unstimulated, PDGF-stimulated, and PDGF- and B[a]P-treated cells. Near-confluent cultures of 3T3 cells were placed in 0.5% serum-containing medium for 24 h to induce quiescence. The quiescent fibroblasts were left untreated or were stimulated with PDGF (30 ng/ml) in the presence and absence of B[a]P (1 μM) for a further 20 h. At this time the cells were fixed, stained with propidium iodide, and subjected to FACScan analysis as described previously (13). Representative nuclear DNA profiles for unstimulated, PDGF-stimulated, and PDGF- and B[a]P-stimulated cells are shown in Fig. 2. Cell cycle analysis showed that only 4.2% of the serum-starved cells were in S phase (Fig. 2, upper panel). 20 h of PDGF stimulation induced 50.5% of the cells to enter S phase (Fig. 2, middle panel). However, only 18.1% of the PDGF-stimulated cells entered S phase in the presence of 1 μM B[a]P (Fig. 2, lower panel). These
results demonstrated that B[a]P inhibited PDGF-stimulated entry into S phase by 64%. This value is similar to the 50–75% inhibition of PDGF-stimulated mitogenesis that resulted from B[a]P treatment in previous experiments (Fig. 1). Therefore, the FACScan analysis corroborated our \[^3H\]thymidine incorporation data, showing that B[a]P inhibited mitogen-stimulated entry into S phase and caused growth arrest in G1.

B[a]P-induced growth arrest was not overcome by increasing concentrations of PDGF (Fig. 3). Indeed, inhibition of \[^3H\]thymidine incorporation was apparent at supraphysiological doses of PDGF (30 ng/ml). Inhibition of growth factor-dependent DNA synthesis was not specific for PDGF, since B[a]P inhibited DNA synthesis in response to all fibroblast mitogens we tested (including basic fibroblast growth factor, epidermal growth factor, insulin-like growth factor 1, and phorbol esters; data not shown). Therefore, B[a]P affected a common distal step in the growth factor-stimulated signaling pathways leading to DNA synthesis.

B[a]P Treatment during G1 Inhibits Subsequent Entry into S Phase—We performed kinetic experiments to identify the temporal window of B[a]P sensitivity following growth factor-stimulated entry of Swiss 3T3 cells into the cell cycle. Quiescent (G0) murine 3T3 fibroblasts require continuous PDGF treatment for approximately 12 h prior to entry into S phase (7, 8). 12 h of growth factor treatment results in progression through G1 to a restriction point (designated “R,” equivalent to “START” in yeast), beyond which point cells become growth factor-independent and commit to enter S phase and undergo DNA replication. Serum-starved quiescent cells were treated with PDGF and \[^3H\]thymidine. B[a]P was added to the cultures simultaneously with, or at frequent intervals following, PDGF treatment. 24 h after addition of PDGF, the cultures were fixed and assayed for entry into S phase by measurements of \[^3H\]thymidine incorporation. As shown in Fig. 4, cells were most susceptible to the inhibitory effects of B[a]P during the first 8–12 h following PDGF-induced entry into G1. Therefore, the entire duration of G1 preceding R was sensitive to the growth inhibitory effects of B[a]P. However, once PDGF-stimulated cells initiated DNA synthesis, they became refractory to the effects of B[a]P.

B[a]P-induced DNA Damage and Nuclear p53 Accumulation in Swiss 3T3 Cells—Treatment of PDGF-stimulated cultures of Swiss 3T3 cells with the nongenotoxic (and nonmetabolizable) AhR ligand TCDD (at concentrations of up to 100 nM) did not prevent PDGF-stimulated entry into S phase (not shown). This demonstrated that AhR activation alone was insufficient to account for the inhibition of growth factor-stimulated DNA synthesis in response to B[a]P. Cytochrome P-450-catalyzed oxidation of B[a]P is known to generate genotoxic metabolites that can form adducts with DNA and other cellular macromolecules (15). To test whether B[a]P was causing DNA damage under conditions of cell cycle arrest, we performed postlabeling analysis (14) of genomic DNA from control and B[a]P-treated cultures of Swiss 3T3 cells. As shown in Fig. 5, 12 h of B[a]P treatment indeed resulted in the formation of several species of DNA adducts. Although we have not determined the identities of the adducts formed under our experimental conditions, the patterns of migration of these species on four-dimensional TLC chromatograms are similar to the chromatographic properties of adducted nucleotides formed following in vivo B[a]P administration (14, 16). B[a]P-induced adduct formation in Swiss 3T3 cells was fully prevented by ANF (data not shown). By contrast, with Swiss 3T3 cells, DNA adducts were barely detectable in NIH 3T3 fibroblasts during the first 12 h of B[a]P treatment, although more prolonged exposure to B[a]P (24–36 h) did result in similar adduct levels to those present in B[a]P-treated...
Swiss 3T3 cells (data not shown). By contrast with B[a]P, TCDD (10–100 nM) did not induce adduct formation at any time point tested (data not shown). Thus, the ability of AhR ligands to induce DNA damage during G1 correlated with the ability to cause cell cycle arrest.

Other genotoxic agents, such as UV light, ionizing radiation, and certain chemicals, are also known to induce growth arrest by a molecular mechanism requiring the p53 tumor suppressor gene product (17–20). When activated and translocated to the nucleus, p53 induces the expression of genes that mediate withdrawal from the cell cycle (i.e. growth arrest or apoptosis; reviewed in Ref. 21). Since B[a]P elicited growth arrest in Swiss 3T3 cells, we tested whether B[a]P treatment also induced accumulation of p53 in the nucleus. Quiescent cells were stimulated with PDGF in the absence or presence of B[a]P for 12 h. Nuclei were then isolated and analyzed for p53 content by immunoblotting with anti-p53 antibodies. PDGF treatment alone resulted in some accumulation of p53 (Fig. 6A). This finding has been reported by other investigators, who have shown that expression of p53 protein (as well as its subcellular distribution) is regulated in a cell cycle-dependent manner (22). However, the addition of B[a]P resulted in markedly increased levels of nuclear p53 protein (Fig. 6A). Moreover, B[a]P-dependent induction of p53 was prevented by the AhR antagonist ANF. Therefore, p53 accumulated in the nucleus following B[a]P treatment, most probably because of DNA damage due to adducted B[a]P metabolites. The cytosolic levels of p53 protein were also occasionally slightly elevated following B[a]P treatment (not shown). p53 mRNA levels were not altered in response to B[a]P (Fig. 6B), indicating that the changes in nuclear p53 protein levels that we observed occurred...
spond to 32P-labeled adducted nucleotides derived from genomic DNA.

**FIG. 5.** Formation of DNA adducts under conditions of B[a]P-induced cell cycle arrest. Quiescent cultures of Swiss 3T3 cells were stimulated with PDGF (10 ng/ml) alone or in combination with 0.3 μM B[a]P. 12 h later, genomic DNA was harvested from the cultures and analyzed for the presence of adducts using the nuclease P1-enhanced postlabeling assay (14). The figure shows autoradiographs of the four-dimensional chromatograms used to analyze DNA from cells treated with PDGF alone (upper panel) or in combination with B[a]P (lower panel). The spots in the lower left each chromatogram corresponds to the origin, where radiolabeled samples were applied to the TLC plates. That B[a]P-induced spots present only on the lower chromatogram correspond to 32P-labeled adducted nucleotides derived from genomic DNA after nuclease digestion.

**FIG. 6.** B[a]P induces nuclear accumulation of p53 in PDGF-stimulated Swiss 3T3 cells. A, immunoblot showing accumulation of p53 in nuclei from B[a]P-treated cells. Quiescent cultures of Swiss 3T3 cells (→) were stimulated with 3 ng/ml PDGF (P), 0.3 μM B[a]P (B), 0.5 μM ANF (A), or combinations thereof (PB and PBA). 12 h later, nuclei were isolated from the cells as described under "Materials and Methods." 100-μg samples of nuclear proteins were separated by electrophoresis on a 7.5% SDS-polyacrylamide gel. Proteins were transferred to a nitrocellulose filter, which was hybridized with a 32P-labeled p53 cDNA probe as described under "Materials and Methods." Labeled probe bound to the filter was detected by autoradiography. Equivalent loading of samples was confirmed by comparison of ethidium bromide-stained 18 and 28 S RNAs in adjacent lanes (not shown). Similar results were obtained in two other experiments. B, RNA blot showing p53 mRNA levels in B[a]P-treated cells. Quiescent cultures of Swiss 3T3 cells (→) were stimulated with 3 ng/ml PDGF (P), 0.3 μM B[a]P (B), or PDGF and B[a]P (P+B). 12 h later, total cellular RNA was extracted from the cells as described under "Materials and Methods." 20 μg samples of RNA were separated by electrophoresis on a 1% denaturing agarose gel. The size-separated RNAs were transferred to a nitrocellulose filter, which was hybridized with a 32P-labeled p53 cDNA probe as described under "Materials and Methods." Labeled probe bound to the filter was detected by autoradiography. Equivalent loading of samples was confirmed by comparison of ethidium bromide-stained 18 and 28 S RNAs in adjacent lanes (not shown). Similar results were obtained in two other experiments.

Posttranscriptionally. This finding is similar to the observations of other workers, who have suggested that p53 induction results from stabilization of protein levels, as opposed to increased transcription of the p53 gene (21). No nuclear accumulation of p53 was detected following B[a]P-treatment of NIH 3T3 cells, which lack detectable levels of AhR (not shown).

**B[a]P-induced Growth Arrest Occurs Independently of Changes in p21 Expression**—p53-induced cell cycle arrest is considered to result from the transcriptional induction of a cyclin-dependent kinase (CDK) inhibitor (23, 24), termed p21/CIP/WAF (hereafter referred to as p21). When expressed, p21 is thought to bind to and inactivate cyclin-CDK complexes, thereby preventing the phosphorylation of critical substrates that are necessary for cell cycle progression. We performed RNA blot analysis to test whether B[a]P-treatment induced p21 expression. RNA samples from PDGF-stimulated cells treated with or without B[a]P were separated on agarose gels and transferred to nitrocellulose. Filters were probed with 32P-labeled probes for p21 as well as for the mdm-2 proto-oncogene (Fig. 7), which is also known to be transcriptionally regulated by p53 (25). Although mdm-2 levels were induced by B[a]P at time points corresponding to both growth arrest and to accumulation of nuclear p53, we observed no B[a]P-dependent change in p21 transcript levels at these times (Fig. 7). Since p21 is a member of a family of CDK inhibitors that are assumed to have similar functions (26), we performed RNA blot analysis to test for B[a]P-dependent changes in the expression of other known CDK inhibitors. B[a]P treatment did not elicit changes in expression of RNAs corresponding to p27 or any other members of the INK family (p15, p16, p18, and p19) that we examined (data not shown). Therefore, B[a]P-dependent growth arrest did not correlate with induction of known CDK inhibitor transcripts.

**B[a]P-induced Cell Cycle Arrest is p53-independent**—Since B[a]P-induced growth arrest occurred in the absence of p21 expression, and p21 is considered to be the effector of p53-dependent growth arrest was also p53-independent. We tested the role of p53 in B[a]P-dependent cell cycle arrest using two experimental strategies: 1) ectopic expression of dominant negative p53 mutants in Swiss 3T3 cells, and 2) the use of primary cultures of fibroblasts from p53−/− "knockout" mice.

Retroviral vectors were used to generate Swiss 3T3 fibroblasts expressing dominant negative p53 (designated Sc5) or wild-type p53 (designated ScN9) as a control. Biologically active p53 is believed to be a tetramer, the DNA binding activity of which is regulated by conformational changes (21). Certain dominant negative mutant forms of p53 (such as the c5 mutant) are able to interact with the wild-type p53 protein yet form complexes that are unable to undergo the appropriate conformational changes necessary for nuclear translocation and DNA binding. Thus, dominant negative p53 can sequester endogenous cellular p53 in the form of inactive complexes. RNA blot analysis of total RNA samples from the cell lines we generated indicated that they expressed high levels of the wild-type and mutant p53 transcripts that were in excess of endogenous p53 RNA levels expressed by parental Swiss 3T3 cells. (Fig. 8A). The parental Swiss 3T3 fibroblasts and the Sc5 and ScN9 cells we generated were rendered quiescent by serum starvation and
then stimulated with PDGF in the presence of \[^{3}H\]thymidine and varying doses of B[a]P. As shown in Fig. 8B, Swiss 3T3, Sc5, and SNc9 cell lines were all susceptible to dose-dependent inhibition of PDGF-stimulated DNA synthesis by B[a]P. The SNc9 cells (overexpressing wild-type p53) were consistently contact-inhibited at lower cell densities than Swiss or Sc5 cells. This was reflected by the lower values we obtained for PDGF-stimulated \[^{3}H\]thymidine incorporation in these cells relative to parental Swiss 3T3 fibroblasts and Sc5 cells (Fig. 8B, upper panel). When the data were expressed as a percentage of growth factor-stimulated DNA synthesis in the absence of drug, the three cell lines exhibited identical dose dependencies for growth inhibition in response to B[a]P (Fig. 8B, lower panel). These data suggested that sequestration of cellular p53 by ectopically expressed dominant negative p53 did not preclude B[a]P-induced growth arrest.

It was still formally possible that levels of expression of dominant negative p53 were insufficient to sequester all of the endogenous p53, and that sufficient endogenous (wild-type) cellular p53 remained in Sc5 cells to form transcriptionally active complexes and mediate growth arrest in response to B[a]P. To independently rule out this possibility, we obtained primary cultures of fibroblasts from transgenic p53/− cells (and from wild-type p53+/− mice as controls). These were tested for AhR expression and susceptibility to B[a]P-induced growth arrest. As shown in Fig. 9A, the p53+/− and p53−/− cell lines expressed equivalent levels of AhR protein. Cultures of p53+/− and p53−/− cells were rendered quiescent by serum starvation and stimulated with PDGF in the presence of \[^{3}H\]thymidine, with or without B[a]P. As shown in Fig. 9B, both cell lines synthesized DNA in response to PDGF treatment (although the -fold stimulation of DNA synthesis in response to PDGF was consistently lower in p53−/− fibroblasts, due to higher PDGF-independent proliferation). PDGF-stimulated DNA synthesis was inhibited by approximately 50% in p53+/− cells and by approximately 45% in p53−/− cells as a result of B[a]P treatment (Fig. 9B). B[a]P inhibition of entry into S phase was prevented by the AhR antagonist ANF in both cell lines. The percentage of inhibition of DNA synthesis in the primary cultures of p53+/− and p53−/− cells was always less than that elicited by B[a]P in immortal Swiss 3T3 fibroblasts (e.g. compare extent of B[a]P-inhibited mitogenesis in Fig. 9 with Fig. 1). Cell-specific differences arising from immortalization or strain-specific differences between the mice from which these cells were derived are likely to account for the differential B[a]P sensitivity we have observed. Nevertheless, the data obtained using the different cell lines were qualitatively similar and showed that AhR-mediated growth arrest in response to B[a]P occurred independently of p53.

**DISCUSSION**

We have shown here that B[a]P induces G1 arrest in 3T3 fibroblasts. The susceptibility of several different cell lines to B[a]P-induced growth arrest correlated with expression of the AhR, and B[a]P-induced G1 arrest was prevented by the AhR antagonist ANF. These results strongly suggest that the AhR mediates G1 arrest to B[a]P. Nevertheless, it remains formally possible that differences other than the levels of AhR expression in the cell lines we used resulted in differential sensitivity to B[a]P. Experiments are under way to determine whether ectopic expression of AhR in AhR-deficient NIH 3T3 cells confers G1 arrest in response to B[a]P.

The values plotted are means of duplicate determinations that differed by less than 5%. Error bars are omitted for clarity. Similar results were obtained in two other experiments.
and Methods." Similar results were obtained in two experiments. The values shown are means of duplicate determinations. A, cytosolic extracts were obtained from confluent monolayers of p53+/+ and p53−/− fibroblasts as described under "Materials and Methods." 100 μg of each extract was separated by electrophoresis on a 10% SDS-polyacrylamide gel. After transfer to nitrocellulose, separated proteins were probed with anti-AhR antisera as described under "Materials and Methods." B, quiescent cultures of fibroblasts were left untreated (−) or were stimulated with 3 ng/ml PDGF (P) in the presence of 0.3 μM B[a]P (P+B) without or with 0.5 μM ANF (P+B+A). 24 later, thymidine incorporation into genomic DNA was determined as described under "Materials and Methods." The values shown are means of duplicate determinations. Similar results were obtained in two experiments.

Since TCDD (the most potent AhR-activating ligand known) did not inhibit growth factor-stimulated DNA synthesis, B[a]P-induced growth arrest was not solely due to AhR activation. Unlike TCDD, which is not metabolized, B[a]P is susceptible to P-450-dependent oxidation, resulting in production of several genotoxic metabolites (15, 27). It would appear most likely, therefore, that growth arrest in response to B[a]P represents a fail safe mechanism that prevents replication of damaged, B[a]P-adducted DNA. Similarly, other genotoxic stimuli (e.g., UV light, ionizing radiation, and chemicals) induce cell-specific growth arrest or apoptosis. This is thought to be a protective mechanism that allows repair of damaged DNA or serves to eliminate irreparably damaged cells.

The 53-kDa protein product of the p53 tumor suppressor gene is a transcriptional activator that is thought to play an important role in determining cellular responses to a variety of genotoxic agents (17–20). Induction of nuclear p53 activity following DNA damage is believed to result in expression of certain genes, including several that mediate DNA repair and/or withdrawal from the cell cycle (apoptosis or growth arrest). The gene encoding the p21 CDK inhibitor can be transcriptionally induced by p53 (as well as by other stimuli) and is considered to mediate growth arrest in response to p53 in many instances (23, 24). As has been observed with other genotoxic stimuli in different cell lines, B[a]P induced nuclear p53 accumulation in Swiss 3T3 fibroblasts. Interestingly, although expression of the p53-regulated mdm-2 proto-oncogene was elevated concomitantly with B[a]P-induced nuclear p53 and growth arrest, p21 mRNA was not induced following B[a]P treatment. Other workers have also noted differential expression of mdm-2 and p21 following p53 activation (28). The precise mechanism underlying the differential expression of these p53-inducible genes has not been identified. However, cell-specific differences in expression (or activities) of other trans-acting stimuli that regulate expression of p21 and mdm-2 genes may underlie this dichotomy.

Since we observed no change in p21 levels at times corresponding to B[a]P-induced growth arrest, and because p21 is thought to mediate growth arrest in response to p53, we considered the possibility that B[a]P-induced growth arrest might occur independently of p53. We used two strategies to test the involvement of p53 in B[a]P-dependent growth arrest: 1) dominant negative p53 mutants in the context of Swiss 3T3 cells, and 2) the use of p53-deficient fibroblasts from transgenic B[a]P−/− mice. The results from these experiments demonstrate that p53 is not necessary for B[a]P-induced growth arrest. Yet, as would appear likely from our results, B[a]P treatment of Swiss 3T3 cells results in genotoxic stress, and p53 induction may serve as a signal to begin repair of damaged DNA. Indeed, B[a]P metabolites are known to form DNA adducts (14–16), and the p53-regulated GADD 45 gene product was recently suggested to be involved in excision repair (29). Experiments are under way to investigate the role of p53 in DNA repair processes during B[a]P-induced G1 arrest.

The lack of involvement of p53 in B[a]P-induced growth arrest raises questions regarding the mechanism(s) mediating growth arrest. Other known tumor suppressors, for example, the retinoblastoma gene product, are possible candidate mediators of B[a]P-induced growth arrest. Retinoblastoma is known to regulate a restriction point in late G1. Phosphorylation of retinoblastoma by appropriate cyclin-CDK complexes during late G1 ordinarily releases the negative regulatory constraint on entry into S phase that is imposed by hypophosphorylated retinoblastoma (30). These events occur within the time frame
of B[a]P sensitivity during G1 in Swiss 3T3 cells. Experiments are under way to identify the mechanisms that mediate B[a]P-induced growth arrest. Defects in such putative mechanisms may cause failure of damaged cells to undergo growth arrest and might consequently result in replication of mutated genes. Transformation is thought to require multiple co-operating lesions (31). Progressive accumulation of mutations in critical growth regulatory genes, including those encoding cellular proto-oncogenes, may ultimately result in or contribute to malignant transformation. Any mutation or lesion that released cells from the B[a]P-activated checkpoint would likely dramatically increase the likelihood of PAH-induced mutagenesis with each cell cycle.

We have shown that B[a]P influences growth factor-stimulated mitogenesis, as well as at least one known tumor suppressor (p53) and one proto-oncogene (mdm-2). Our data indicate that the AhR is necessary for these biological effects. This work suggests a strong link between the AhR and carcinogenesis resulting from B[a]P. Further experiments are under way to understand the molecular mechanisms whereby B[a]P influences cell cycle control, since such putative mechanisms are likely to underlie the carcinogenic effects of PAHs and possibly other genotoxic agents.

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