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Overexpression of the Aryl Hydrocarbon Receptor (AhR) Accelerates the Cell Proliferation of A549 Cells[1]

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The arylhydrocarbon receptor (AhR) is a ligand-activated transcription factor that mediates a spectrum of toxic and biological effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds. Although the physiological ligand for the AhR has not yet been identified, several reports have suggested that the AhR may play important roles not only in the regulation of xenobiotic metabolism but also in the maintenance of homeostatic functions [Singh *et al.* (1996) Arch. Biochem. Biophys. 329, 47–55; Crawford *et al.* (1997) Mol. Pharmacol. 52, 921–927; Chang *et al.* (1998) Mol. Cell. Biol. 18, 525–535]. Several lines of evidence suggest that one of the possible physiological roles of the AhR is regulation of cell proliferation. In this study, we first showed that treatment of A549 cells with the AhR agonist stimulates cell proliferation. The effect was antagonized by co-treatment with α-naphthoflavone. To obtain direct evidence that the AhR regulates cell proliferation, we isolated the clones that overexpress the AhR. These clones grow faster than control cells, and the rate of growth is proportional to the amount of the AhR. Cell cycle analysis revealed that the acceleration of cell growth by overexpression of the AhR is most probably due to shortening of the late M to S phases. Studies on the expression profiles of cell cycle regulators showed that the AhR or AhR ligand induces the expression of DP2, PCNA, and RFC38. DP2 is the transcription factor that forms the functional dimer with E2F and regulates the expression of several genes involved in DNA synthesis. Interestingly, both PCNA and RFC38 are target genes of E2F and the DP complex. Also, both of these factors are involved in regulating DNA polymerase δ activity. E2F activity was substantially increased in both the AhR-overexpressing cells and the AhR-agonist treated cells, suggesting that AhR-activated E2F/DP2 may induce the expression of PCNA and RFC38 and subsequent DNA synthesis. Down-regulation of the expression of the Arnt by RNAi diminished the effects of the AhR on the cell proliferation of the A549 cells. Consequently, we conclude that the AhR, presumably in collaboration with the Arnt, activates the DNA synthesis and the subsequent cell proliferation in A549 cells.

Key words: A549 cells, aryl hydrocarbon receptor, cell cycle, cell proliferation, dioxin.

The arylhydrocarbon receptor (AhR) is a ligand-activated transcription factor that mediates a spectrum of toxic and biological effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds. Although a physiological ligand for the AhR likely exists, no such molecule has yet been identified. Several reports have shown constitutive activation of the AhR in the absence of an exogenous ligand, suggesting that the AhR may play important roles not only in the regulation of xenobiotic metabolism but also in the maintenance of homeostatic functions (*1–3*). AhR-knockout mice exhibit decreased liver size, hepatic portal fibrosis, decreased constitutive expression of certain xenobiotic-metabolizing enzymes such as CYP1A2, and decreased body size over the first 4 weeks of life relative to their littermate controls (*4–6*). The treatment of cultured embryos with AhR antisense oligonucleotides resulted in both a significantly lower incidence of blastocyst formation and significantly smaller mean numbers of embryo cells (*7*). In cell differentiation, the expression of the AhR is increased during differentiation toward keratinocytes and monocytes (*8, 9*). In contrast, the amount of AhR protein is found to decrease with ongoing adipose differentiation, resulting in the loss of functional response to xenobiotics (*10*). We recently showed that expression of high levels of the AhR significantly inhibits the accumulation of lipid as well as the induction of adipose-specific genes. In contrast, the lowering of AhR levels in 3T3-L1 cells, via antisense AhR mRNA, induced much greater differentiation (*11*). Consequently, it is likely that the AhR is involved in some aspects of the developmental and differentiation processes.

Several lines of evidence suggest that one of the possible

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physiological roles of the AhR is to regulate cell growth. Ma and Whitlock showed that AhR-defective Hepa 1c1c7 cells exhibited delayed cell growth and longer doubling time than wild-type cells (*12*). Stable transfection of AhR cDNA into AhR-defective mouse hepatoma cells has shown that the AhR plays important roles in controlling cell cycle progression and differentiation, and that no exogenous ligands are required for this function (*12*). By increasing TGF-β production, embryonic fibroblast cells from AhR-null mice showed a slower cell growth rate than the wild-type cells (*13*). In rat hepatoma cells, AhR activation with TCDD severely delays cell cycle progression through the G1 phase (*14*). This is due to direct transcriptional regulation of $p27^{kip1}$ mRNA by the TCDD-activated AhR (*15*). A direct interaction between the AhR and retinoblastoma protein has also been described (*16*). Dohr and Abel reported that TGF-β inhibits cell proliferation of A549 cells, but not of MDA-MB231 cells, and co-regulates mRNA expression of the AhR and cell cycle regulating genes in A549 cells (*17*).

Expression of the AhR gene has been examined in various tissues of human, rat and mouse (*18–20*). The human AhR mRNA was detected in all tissues examined, and the highest expression of the AhR was found in the lung (*18*). These results suggest that the AhR may play some functional roles in the lung. A549 cells were established from a human alveolar cell carcinoma and found to exhibit several features typical of type II alveolar epithelial cells of the lung (*21*). These include multilamellar cytoplasmic inclusion bodies and the unique pathway for phospholipid synthesis. Thus, A549 cells have been widely used as an in vitro model for lung. In this study, we isolated clones overexpressing the AhR from A549 cells and showed that the activation of the AhR accelerates the proliferation of A549 cells by shortening the late M/early S phases. The mechanism involves the induction of the factors responsible for DNA synthesis. These results strongly suggest that the AhR may regulate cell proliferation *via* activation of DNA synthesis in A549 cells.

MATERIALS AND METHODS

Cell Culture and Stable Transfection—A549 cells, obtained from the Human Science Research Resources Bank (Osaka), were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum. To construct the AhR mRNA expression vector, full-length human AhR cDNA was subcloned into the mammalian expression vector pRc/CMV2 (Invitrogen), which contains a selective marker, the neomycin resistance gene. The cells were transfected by using Lipofectin (Invitrogen) and were allowed to grow in nonselective medium for 48 h. The cells were then cultured in medium containing G-418 (650 μg/ml). After 2 to 3 weeks, clones were isolated and expanded individually. The expression of AhR protein in each clone was analyzed by Western blotting.

Immunoblot Analysis—The cells, grown in 60-mm dishes, were rinsed with ice-cold phosphate-buffered saline (PBS). The rinsed cells were scraped off the dish, placed in a microcentrifuge tube, and centrifuged at 5,000 ×*g* for 1 min. The resulting pellets were suspended in the lysis buffer [50 mM Hepes KOH (pH 7.8), 420 mM KCl, 0.1 mM EDTA, 5 mM $MgCl_2$, 1 mM DTT, 0.5 mM PMSF, 0.0002% leupeptin and 20% glycerol], vortex-mixed, and rocked at 4°C for 60 min. The suspensions were centrifuged for 15 min at 10,000 ×*g* and the resulting supernatants were then frozen until further analysis. The protein concentration of the extracts was determined according to the method of Bradford, using bovine serum albumin as standard (*22*). Protein samples were denatured by heating to 90°C in SDS-reducing buffer and resolved by electrophoresis on 10% SDS–polyacrylamide gels. After transfer to nitrocellulose membranes, the filters were probed with specific antibodies. For the detection of the AhR, color visualization was performed with secondary antibodies conjugated with alkaline phosphatase and nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate substrate solution (Promega). For the detection of the Arnt, Phototope HRP Western blot detection kit (Cell Signaling Tech., Beverly, MA) was used.

Immunofluorescence Staining—Cells were grown on glass slides coated with collagen, fixed with 2% paraformaldehyde in PBS, and treated with 0.2% Triton-X. Cells were then stained with anti AhR antibodies and the secondary antibodies labeled with FITC. Experiments were performed at least three times, and, on average, 20 fields were evaluated on each slip.

Analysis of mRNA Expression Using cDNA Arrays—Total RNA from the clones was isolated using the TRIzol reagent (Invitrogen). Poly-A RNA was purified using the Poly A Ttract mRNA isolation system (Promega) according to the manufacturer's instructions. RNA integrity was confirmed by gel electrophoresis and spectrophotometric measurements. The expression of genes related to the cell cycle was studied by hybrid selection of the radioactively labeled cDNA on high-density arrays of membrane-bound cDNA probes. The cDNA arrays used in this study were the Atlas human cell cycle cDNA arrays from BD Clontech Laboratories (Palo Alto, CA). Preparations of radioactively labeled cDNA and subsequent hybridizations to the cDNA arrays were conducted as recommended by the manufacturer of the arrays, using reagents provided by the manufacturer. In brief, a labeled cDNA probe was produced from 1 μg of poly-A RNA by incorporation of [α-^{32}P]dATP during a standard reverse transcription. The cDNA expression array filters were prehybridized in ExpressHyb (BD Clontech Laboratories) for 30 min at 68°C and hybridized with the ^{32}P-labeled first-strand cDNA probes (*ca.* 5×10^6 cpm) overnight at 68°C. After hybridization and washing, the array filters were sealed in plastic bags and exposed to X-ray film with an intensifying screen for 4 days at –80°C, and mRNA

TABLE I. **List of gene-specific primers.**

Gene	Forward primer	Reverse primer
Cyclin A	AACTTCAGCTTGTGGGCACT	AGGCTGCTGATGCAGAAAGT
Cyclin B1	TTGGTGTCACTGCCATGTTT	GATGCTCTCCGAAGGAAGTG
Cyclin B2	TCAACCCACCAAAACAACAA	AGGGTTCTCCCAATCTTCGT
Cdk2	AAATTCATGGATGCCTCTGC	CAGGGACTCCAAAAGCTCTG
Cdk4	GGGCAAAATCTTTGACCTGA	GAAAGGCAGAGATTCGCTTG
Chk1	CTGAAGAAGAGTCGCAGTG	GCATGCCTATGTCTGGCTCT
PCNA	TGGCGCTAGTATTTGAAGCA	CCGTCTTTTGCACAGGAAAT
DP2	AACCATTTGGCTGCTGATTC	CGCTTCTGCTTTATCCGTTC
RFC38	ACTCCACAAAGGCTCCTTGA	GGCTTTGCTACCCAGCTGTA
Ubiquitin	GGAAACAGCTGGAAGATGGA	AGGGATGCCTTCCTTGTCTT

expression levels were analyzed by scanning the films with a densitometer (*23*).

Analysis of mRNA Expression by RT-PCR—Total RNA was extracted as described above, and the RNA (1 μg) was then reverse transcribed using oligo dT primer. A portion of the cDNA (corresponding to 0.1 μg of total RNA) was amplified by PCR in 50 μl of a mixture of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, and 2μCi of [α-^{32}P]dCTP (3,000Ci/mmol). PCR comprised 22 cycles for GAPDH and 25 cycles for others, with denaturing at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min. The PCR products were analyzed by running reactions on 10% acrylamide gels, and the radioactivity was evaluated by autoradiography as described above. The linearity of the quantitation of RT-PCR products was ascertained by the experiment using varied amounts of total RNA obtained from A549 cells. The PCR products were also subcloned in pGEM-T vector (Promega), and the sequence was confirmed. Table I lists the primer pairs used.

Thymidine Incorporation—Cells in log phase were synchronized in M phase by treatment with nocodazole (0.4 μg/ml) for 10 h. Mitotic cells were detached by gentle agitation and were collected by mild centrifugation (1,000 ×*g* for 5 min). The cells were washed twice with ice-cold PBS and reseeded in plates at 5 × 10^4 cells per 35-mm diameter plate. The cells were pulsed with [^{3}H]thymidine (1 mCi/ml) for 45 min at different times after seeding. Cells were washed twice with ice-cold PBS and lysed with 0.075% sodium dodecyl sulfate. Trichloroacetic acid (TCA) was added to a final concentration of 10%, and the resulting precipitate was collected by filtration on Whatman GF/C filters, washed with ice-cold 5% TCA, and analyzed for radioactivity by liquid scintillation counting.

Flow Cytometry—Cells were collected by mild trypsinization and gentle centrifugation, then fixed in 70% ethanol. The fixed cells were washed twice with PBS and resuspended in propidium iodine (PI) solution (100 ×*g* of DNase-free RNase A and 10 ×*g* of PI per ml of PBS). DNA content in the cells was analyzed with FACScan (Becton Dickinson).

Transient Transfection and Reporter Gene Assay—Cells were transfected with 0.5 μg of reporter construct and 0.5–1 μg of expression construct as described above. To correct for variation in transfection efficiency, we co-transfected with pRL-SV40 control vector (Promega Biotech). Luciferase activities were measured 36 h after transfection, using a kit from Promega Biotech according to the manufacturer's instructions. All experiments were repeated a minimum of three independent times.

Preparation of Double-Stranded RNA—To design target-specific siRNA duplexes, we selected sequences of the type AA (N19) (N, any nucleotide) from the open reading frame of the Arnt mRNA. A selected siRNA sequence was also submitted to a BLAST search to ensure that only one gene

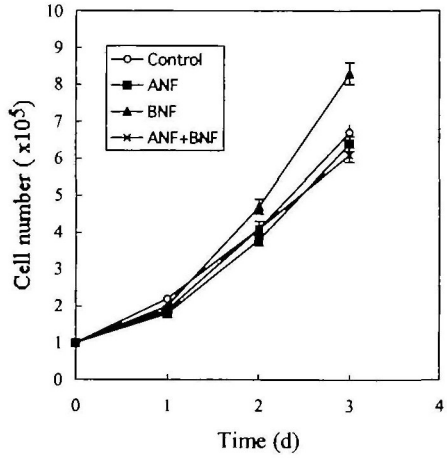


Fig. 1. **Effect of AhR ligands on cell proliferation in A549 cells.** A549 cells were treated with 10 μM β-naphthoflavone (BNF) or 10 μM α-naphthoflavone (ANF). The AhR ligands were dissolved in dimethylsulfoxide (DMSO), and control cells were given the same amounts (2 μl) of DMSO. Cell numbers were determined by hemocytometer cell counts. Each time point represents a triplicate determination, and each experiment was performed twice with similar results. Symbols are defined in the figure.

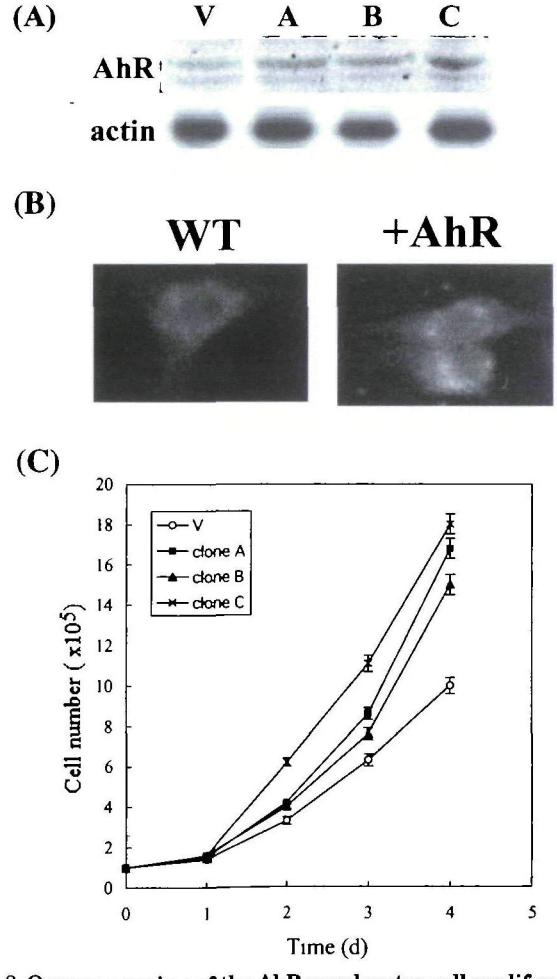


Fig. 2. **Overexpression of the AhR accelerates cell proliferation in A549 cells.** (A) Western blot analysis of the AhR and the actin was performed on whole cell extracts (5 μg of protein) resolved by electrophoresis on 10% SDS/polyacrylamide gel. V: cells expressing the vector mRNA. (B) Immunofluorescence microscopy of A549 cells stained for the AhR. WT; normal A549 cells, +AhR; cells expressing AhR sense mRNA. (C) Cell growth of the clones was measured as described in the legend for Fig. 1. Data represent means of determinations made on three independent dishes. The analysis was reproduced twice with similar results. Symbols are defined in the figure.

of the human genome was targeted. The RNAs were prepared by using silencer siRNA construction kit (Ambion, TX). The cells were transfected with 20 nM RNAs by using oligofectamine reagent (Invitrogen) according to the manufacturer's instruction.

RESULTS

Effects of the AhR Ligand on Cell Proliferation in A549 Cells—In the first set of experiments, we examined the effects of two AhR ligands, β-naphthoflavone (BNF) and α-naphthoflavone (ANF), on cell growth in A549 cells (Fig. 1). As shown in Fig. 1, BNF, which is an AhR agonist but has less affinity than TCDD, stimulated the growth of A549 cells. ANF, which can bind to the AhR but is unable to transform the AhR to its DNA-binding form, alone had no effect on the growth of A549 cells (Fig. 1). The effects of BNF on cell growth were reversed by the co-treatment of the cells with ANF (Fig. 1). These results strongly suggest that activation of the AhR is involved in the control of cell proliferation in A549 cells.

Overexpression of the AhR Accelerates Cell Proliferation—Although most of the actions of the AhR ligands, such as TCDD and BNF, depend upon the AhR, these chemicals also have AhR-independent actions (*24*). Also, dioxin can suppress the expression of the checkpoint protein MAD2 in an AhR-independent manner and consequently affect cell proliferation (*25*). Therefore, to assess the role of the AhR in cell proliferation directly, we established stable clones overexpressing the AhR and studied their effect on cell proliferation. A549 cells were stably transfected with either a vector expressing high levels of full-length sense AhR mRNA or a control vector. After selection and expansion of stable clones, the level of the AhR protein was examined by Western blotting (Fig. 2A). On the basis of AhR protein level, three independent clones overexpressing the AhR were chosen for the next set of experiments (Fig. 2A). To characterize these cells, we examined the subcellular localization of the AhR protein by immunofluorescence staining (Fig. 2B). The AhR in normal cells is mostly localized in the cytoplasm. As with the AhR-overexpressing cells, the stain-

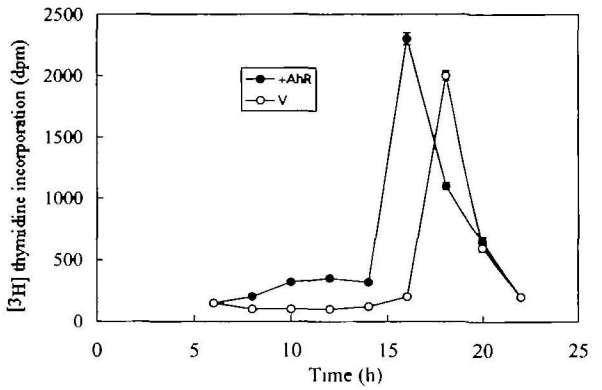


Fig. 3. **Overexpression of the AhR stimulates DNA synthesis in A549 cells.** Cell clones were synchronized in G2/M phases by treatment with nocodazole, released from the mitotic block, and pulse labeled with [^{3}H]thymidine as described in "MATERIALS AND METHODS." Data represent means and standard deviations from three samples for each time point. Each experiment was performed twice using independent clones, with similar results. Symbols are labeled in the figure. V: cells expressing vector mRNA; +AhR: cells expressing AhR sense mRNA.

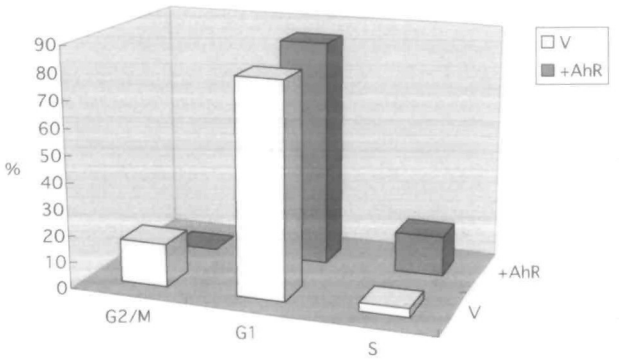


Fig. 4. **Cell cycle analysis of the cell clones.** Cell clones were synchronized in G2/M phases by treatment with nocodazole, released from the mitotic block, and fixed with ethanol. The cells fixed were stained with PI. The percentage of cells in each phase was determined by flow cytometry. Data represent means of determinations made on three independent clones. The analysis was reproduced twice using independent clones, with similar results. V: cells expressing vector mRNA; +AhR: cells expressing AhR sense mRNA.

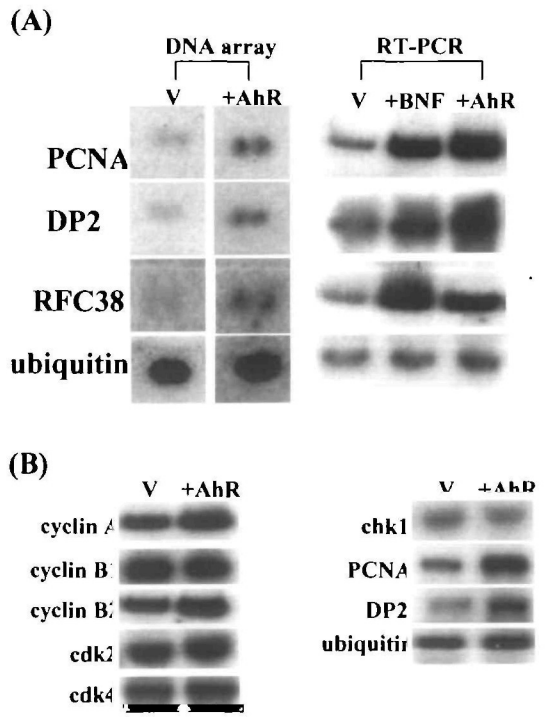


Fig. 5. **Effects of the AhR on gene expression profile of cell cycle–related genes.** (A) Expressions of the selected cell cycle–related genes were analyzed on DNA arrays (left panel). Cells were treated with 10 μM BNF for 16 h, and the RNA was isolated. The differences in expression were confirmed by semiquantitative RT-PCR as described in "MATERIALS AND METHODS." The linearity of the quantitation of RT-PCR products was ascertained by the experiment using varied amounts of total RNA obtained from A549 cells. The sequences of the PCR products were also confirmed. (B) Expression of the representative genes, which showed no significant differences on DNA array, was analyzed by RT-PCR. The analysis was performed three times using independent clones, with similar results. V: cells expressing vector mRNA; +AhR: cells expressing AhR sense mRNA.

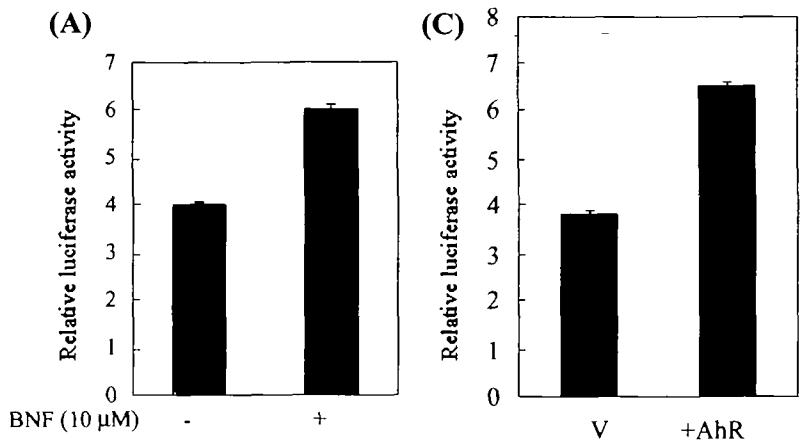
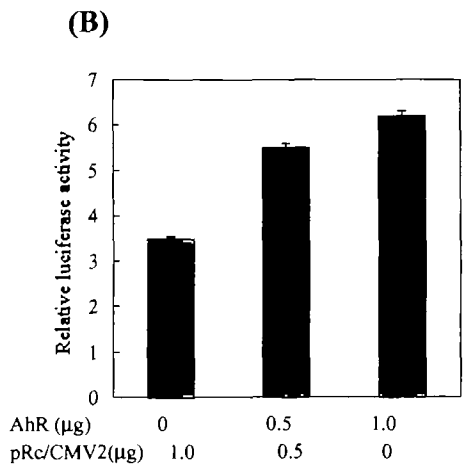


Fig. 6. **The AhR enhances E2F binding activity in A549 cells.** (A) A549 cells transfected with E2F/luciferase gene were treated with 10 μM BNF for 16 h. Control vector, pRL-SV40, was cotransfected to correct for differences in transfection efficiency. Data represent means of determinations made on three independent dishes. (B) Increasing amounts of the AhR expression vector were transfected into A549 cells with the reporter plasmid E2F/luciferase. For all transfections, pRL-SV40 control vector was cotransfected to correct for differences in transfection efficiency. Data represent means of determinations made on three independent dishes. (C) Control cells and the AhR-overexpressing cells were transfected with E2F/luciferase gene and the control vector, pRL-SV40. Data represent means of determinations made on three independent dishes. V: cells expressing the vector mRNA. +AhR: cells expressing AhR sense mRNA.



ing pattern is similar to those in the control cells, *i.e.*, the enhanced AhR had perinuclear and cytoplasmic distribution (Fig. 2B). Also, diffused nuclear distribution of the AhR was observed in the AhR-overexpressing cells (Fig. 2B). Comparison of cell proliferation rates between these clones showed that the rate accelerated as the amount of AhR increased (Fig. 2, A and C). These results strongly suggest that activation of the AhR is involved in the proliferation of A549 cells.

Overexpression of the AhR Accelerates Entry in S Phase—To obtain further insight into the AhR-dependent cell growth acceleration, the cells were synchronized at M phase and the following cell cycle progression was determined. In the AhR-overexpressing cells, the onset of DNA synthesis began 14 h following the release from the mitotic block and peaked 16 h after release (Fig. 3). DNA synthesis in the control cells began 16 h and peaked 18 h after release (Fig. 3). Flow-cytometric analysis of the distribution of the cell population 12 h after release of the mitotic block revealed that, in control cells, approximately 90% of the cells were in the G1 phase, whereas 10% of the cells were still in the G2/M phases and no cells were detected in the S phase (Fig. 4). In contrast, 10% of the AhR overexpressing cells were already in the S phase, whereas there were no detectable cells in the G2/M-phases (Fig. 4). These results clearly demonstrated that overexpression of the AhR shortened the interval from the late M to S phase.

Overexpression of the AhR Induces the Expression of Factors Involved in DNA Replication—The cell cycle analysis revealed that the AhR accelerates entry into the S phase (Figs. 3 and 4). Thus, in the next set of experiments, expression of the factors involved in cell cycle regulation was analyzed on DNA arrays. An Atlas Human Cell Cycle Array (Clontech) was hybridized with radioactive cDNA prepared from either control cells or AhR-overexpressing cells. The array contains 111 distinct cDNAs spotted in duplicate. To ensure reproducibility, the experiments were performed three times. In each experiment, RNA was freshly isolated from the independent clones expressing the same amounts of AhR as contained in clone C, as described in Fig. 2A. Of the 111 genes tested, 108 genes varied by less than 20% on average between the two blots, whereas the expression of DP2 (transcription factor), RFC38 (replication factor), and PCNA (replication factor) showed 4- to 5-fold increases in the AhR-overexpressing cells (Fig. 5A). No genes were observed to have been down-regulated by the AhR in this experiments. The changes in gene expression were also confirmed by semi-quantitative RT-PCR (Fig. 5A). We also analyzed the expression of the representative cell cycle regulators, which showed no significant differences with the expression on the DNA array. Again, no differences in the expression of these factors, including cyclin and the related kinase, were observed by RT-PCR (Fig. 5B). The gene expression profile in the AhR-overexpressing cells is almost identical to those in BNF treated cells (Fig. 5B). Interestingly, DP2 is an E2F dimerization partner, and PCNA and RFC38 are known as target genes of E2F (*26*). Furthermore, the AhR interacts with pRB, another dimerization partner of E2F (*16*). Therefore, one can argue that the activity of E2F is increased in the AhR-overexpressing cells. In the next set of experiments, a reporter construct containing the luciferase gene driven by E2F was used to examine the effect of the AhR on the E2F-dependent transactivation activity. As shown in Fig. 6, E2F-dependent luciferase activity was increased in A549 cells by the treatment with BNF (Fig. 6A). Transfection of increasing amounts of the AhR expression vector led to dose-dependent increase in luciferase activity (Fig. 6B). The results are similar to those in the previous study (*27*). Finally, the AhR-overexpressing cells exhibited higher luciferase activity than the control cells (Fig. 6C).

The Arnt is Required for Cell Proliferation of A549 Cells—Most of the actions of the AhR are performed as the

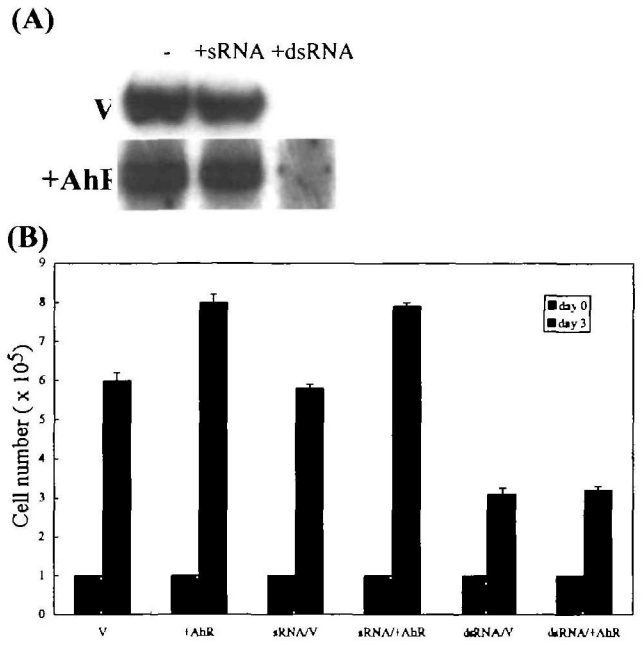


Fig. 7. **Knock-down of the Arnt expression inhibits cell proliferation in A549 cells.** (A) The cells were transfected with 20 nM RNAs. Western blot analysis of the Arnt was performed on whole cell extracts (10 μg of protein) resolved by electrophoresis on 10% SDS/polyacrylamide gel. V: cells expressing the vector mRNA. +AhR: cells expressing AhR sense mRNA. +sRNA: sense RNA. +dsRNA: double-stranded RNA. (B) Cell numbers were measured for 3 days as described in the legend for Fig. 1. Data represent means of determinations made on three independent dishes. V: cells expressing the vector mRNA. +AhR: cells expressing AhR sense mRNA. +sRNA: sense RNA. +dsRNA: double-stranded RNA.

dimer with Arnt. Therefore, in next set of the experiments, we knocked down the expression of the Arnt by use of RNAi. The transfection of the double-stranded RNA corresponding to nucleotides 19 to 39 of the Arnt resulted in the slower cell growth in both the control cells and the AhR-overexpressing cells (Fig. 7B). The acceleration of cell proliferation by the AhR was completely diminished (Fig. 7B). Western blotting showed that the Arnt protein in the cells was decreased by the transfection of the dsRNA (Fig. 7A). The transfection of the sense RNA did not alter the cell growth rate (Fig. 7B). These results indicate that the Arnt is required for cell proliferation of A549 cells.

DISCUSSION

Several lines of evidence suggest that the AhR is involved in regulating cell proliferation. AhR-defective Hepa 1c1c7 cells exhibited delayed cell growth and longer doubling time than wild-type cells (*12*). Stable transfection of AhR cDNA into AhR-defective mouse hepatoma cells has shown that the AhR plays important roles in the control of cell cycle progression and differentiation and that no exogenous ligands are required for the function (*12*). Mouse embryonic fibroblast cells derived from AhR-null mice exhibited a lower proliferation rate than wild-type cells (*13*). The AhR may be involved in the control of DNA synthesis *via* interaction with p300 (*28*). In this study, we first showed that treatment of A549 cells with the AhR agonist stimulates cell proliferation (Fig. 1). The effect was antagonized by the treatment with α-naphthoflavone (Fig. 1). Although these results suggest that the AhR signaling pathway is involved in the regulation of cell proliferation in A549 cells, dioxin can suppress the expression of the checkpoint protein MAD2 in an AhR-independent manner and thereby affect cell proliferation (*25*). Therefore, to obtain direct evidence that the AhR regulates cell proliferation, we isolated clones overexpressing the AhR (Fig. 2A). These clones grew faster than the control cells, and their growth rate accelerated as the amount of the AhR increased (Fig. 2C). Accordingly, these data indicate that the AhR plays a role in the regulation of cell proliferation in A549 cells.

Cell cycle analysis revealed that the acceleration of cell growth by overexpression of the AhR is most probably due to a shortening of the late M to S phases (Figs. 3 and 4). A recent study showed that the induction of CYP 1A1 mRNA by TCDD is cell cycle–dependent and is markedly increased in late G1/early S cells (*29*). Vaziri *et al.* reported that the AhR expression is induced at the G1/S phase boundary and peaks in the S phase (*30*). Thus, it is likely that AhR activity peaks at the late G1/early S phases. One of the possible roles of the AhR in the late G1/early S phases is to induce factors that regulate DNA replication. Studies on the expression profiles of cell cycle regulators showed that the AhR or AhR ligand induces the expression of DP2, PCNA, and RFC38 (Fig. 5). Computer analysis revealed that there is no XRE sequence within the promoter region of DP2, PCNA and RFC 38 (data not shown), while these genes contain the functional E2F binding site (*26*). In the AhR-overexpressing cells and the AhR-agonist–treated cells, E2F activity was substantially increased (Fig. 6). Therefore, the induction of these genes may be due to the enhanced activity of E2F. DP2 is a transcription factor that forms a functional dimer with E2F and regulates the expression of several genes involved in DNA synthesis. Interestingly, both PCNA and RFC38 are target genes of E2F and DP complex. Also, both factors are involved in regulating of DNA polymerase δ activity, suggesting that E2F/DP2 activated by the AhR may induce the expression of PCNA and RFC38 and subsequent DNA synthesis. Consistent with this suggestion, AhR-dependent activation of DNA polymerase κ activity was recently reported (*31*). Also, the AhR is required for p300-mediated induction of DNA synthesis by adenovirus E1A (*28*). As shown in Fig. 7, the lowering of the Arnt protein resulted in the loss of the AhR's effects on cell proliferation. Thus, one can speculate that the Arnt may, at least partly, be responsible for the AhR-dependent acceleration of cell growth. Consequently, we conclude that the AhR, presumably in collaboration with the Arnt, regulates cell proliferation through the activation of DNA synthesis.

We do not exclude the possibility that other factors are involved in AhR- dependent cell cycle regulation. For instance, it is possible that the AhR induces growth factors, secretion of which stimulates cell proliferation. TGF-β, a proliferation inhibitor, was present at high levels in conditioned medium from AhR-null MEFs, suggesting that the AhR influences TGF-β production, thus leading to an alteration in cell cycle control (*13*). We examined whether or not the conditioned medium from the AhR-overexpressing cells contains such factors and alters the growth rate. We found that the medium from cultures of the AhR-overexpressing

cells had no effect on the growth of the control cells (data not shown). Therefore, it is unlikely that the AhR dependent acceleration of cell proliferation observed in this study resulted from activation of an autocrine loop of growth factors.

From a toxicological perspective, one of the questions remaining is whether the AhR-dependent effects of dioxins reflect the enhanced physiological function of the AhR, or the specific actions of the dioxin-AhR complex, or a combination of both. In this study, AhR-overexpressing cells exhibit several similarities to the AhR-agonist treated cells, *i.e.*, acceleration of cell growth and alteration of expression level of cell cycle–related genes. Thus, the toxicological effects of dioxins appear to be induced at least partly through the physiological AhR signaling pathway. If they are, the AhR may play several unidentified roles in maintaining homeostasis, because dioxins cause a variety of toxic reactions in living material (*32–35*). Understanding the detailed physiological roles of the AhR may reveal further mechanisms of the toxicological action of dioxins.

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