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# Basis for the Loss of Aryl Hydrocarbon Receptor Gene Expression in Clones of a Mouse Hepatoma Cell Line

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## SUMMARY

Rare benzo[*a*]pyrene-resistant clones were previously isolated from the mouse hepatoma cell line, Hepa-1 (Hepa1c1c7), and shown to be deficient in induction of CYP1A1 mRNA by ligands for the aryl hydrocarbon receptor (AHR). Clones belonging to complementation group B were shown to have reduced levels of ligand binding to AHR. It is shown here that all 15 independently derived B clones analyzed had much reduced levels of AHR mRNA, but in each case, the mRNA was normal in size. Infection of B clones with a retroviral expression vector for AHR restores CYP1A1 inducibility (although viral AHR expression is progressively silenced and CYP1A1 expression progressively diminishes as the cells are maintained in culture). Treatment of the B clones with the histone deacetylase inhibitors sodium butyrate or trichostatin A restores AHR expression and also restores CYP1A1 inducibility to nearly 100% of the cells in the treated cultures. Fusion of a representative B clone with a rat hepatoma cell line restores expression to the mouse *AHR* gene encoded by the B clone's genome. These results demonstrate that the loss of CYP1A1 inducibility in B clones is probably totally ascribable to their reduced levels of AHR and that the clones are most probably not mutated in the *AHR* gene but are deficient in its expression. The evidence suggests that the reduction in expression of mRNA encoded by the endogenous *AHR* gene in the B clones is not due to an epigenetic alteration in chromatin structure but that the clones are probably defective either in a transcription factor for the *AHR* gene or in a protein required for generating an open chromatin configuration over the gene.

The AHR binds a number of xenobiotic compounds, including PAHs and certain HAHs, such as TCDD and polychlorinated biphenyls, and mediates pathogenesis (including carcinogenesis) by these compounds. The 89-kDa nonliganded AHR forms part of a multimeric cytoplasmic complex of approximately 300 kDa, which also contains two molecules of HSP90, and perhaps another 43-kDa protein (1). After ligand binding, AHR dissociates from this complex and dimerizes with the 87-kDa ARNT protein (2). In the nucleus, this dimer acts as a transcription factor. Most, if not all, of the pathological effects mediated by AHR seem to depend on modulation of transcription by the receptor. The mechanism of transcriptional control is best understood for the *CYP1A1* gene. The AHR/ARNT dimer binds to several xenobiotic responsive elements in the 5′-flanking region of this gene and stimulates transcriptional initiation of the gene (for review, see Ref. 3).

The AHH activity of CYP1A1 metabolizes PAHs (such as BP) into cytotoxic as well as carcinogenic products. The mouse hepatoma cell line, Hepa-1 (Hepa1c1c7), is highly inducible for CYP1A1 by PAHs and HAHs. We isolated clones of Hepa-1 cells that are resistant to the toxicity of BP. These arose spontaneously at the relatively low rate of $2 \times 10^{-7}$ events per cell generation, and their frequency was increased markedly by mutagenesis. All of the clones had much reduced or undetectable CYP1A1-dependent AHH activities after treatment with concentrations of PAHs and HAHs that lead to maximal induction in WT Hepa-1 cells (4, 5). Analysis of somatic cell hybrids between individual clones and the Hepa-1 parental line demonstrated that most of the clones are recessive to the WT cells, whereas a few of the clones are dominant. Somatic cell hybridization experiments performed between the recessive clones permitted their assignment to four complementation groups (6, 7). One complementation group corresponds to the *CYP1A1* gene (8, 9). Clones as-

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**ABBREVIATIONS:** AHR, aryl hydrocarbon receptor; AHH, aryl hydrocarbon hydroxylase; ARNT, aryl hydrocarbon receptor nuclear translocator; BP, benzo[*a*]pyrene; HAH, halogenated aromatic hydrocarbon; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; HSP90, 90-kDa heat-shock protein; PAH, polycyclic aromatic hydrocarbon; PCR, polymerase chain reaction; WT, wild-type; kb, kilobase-pair(s)

signed to the other three complementation groups are all affected in functioning of AHR. Clones in complementation group C are defective in activity of the ARNT protein (10, 11). The one clone isolated in group D has moderately reduced levels of AHR, as assessed by ligand binding, but is more severely affected in translocation of AHR into the nucleus after binding ligand (7). The B clones all possess low but measurable AHH activities and similarly low but detectable levels of CYP1A1 mRNA [0.5–10% of the corresponding values in Hepa-1 cells, after treatment of cell cultures with PAH or HAH ligands for AHR (6, 12)]. Representative B clones were shown to have much reduced levels of ligand binding to AHR, but the small amount of AHR that binds ligand is capable of translocating into the nucleus. The dose-response curve and the $EC_{50}$ value for induction of AHH activity were identical for a representative B clone and Hepa-1 cells (13). The B clones and clones isolated by Whitlock and co-workers (7, 14) that are in the same complementation group have been used extensively to investigate the role and mechanism of action of AHR. In this study, we provide convincing evidence that the B clones are not mutated in the *AHR* gene; instead, they are defective in expression of the gene. We describe further insights into the regulation of *AHR* gene transcription provided by studies of the B clones.

## Materials and Methods

**Cell culture.** The growth of the Hepa-1 cell line and its derivatives, the cell fusion procedure, the benzo[*ghi*]perylene plus near-UV reverse selection (performed in cells without prior treatment with TCDD) (15), and the G418 selection (16) were carried out as described. *N*-butyric acid (Sigma Chemical, St. Louis, MO) was added directly to the medium, which was then adjusted to pH 7.2 with NaOH. Trichostatin A (Wako Pure Chemicals, Osaka, Japan) was added from a stock solution prepared in ethanol (to give a final concentration of 0.0005% ethanol). Fusion between H5-6 cells and either WT or B variant cells (the latter two strains carry markers for ouabain resistance and 6-thioguanine resistance) was induced by 50% (w/w) polyethylene glycol (PEG-1350) and somatic cell hybrids were selected with hypoxanthine, aminopterin, thymidine, and ouabain medium (17) supplemented with 0.2 μg/ml 12-*O*-tetradeconyl-phorbol-13-acetate (6). Staining of cellular DNA with propidium iodide and analysis of DNA content by flow cytometry was performed essentially as described previously (6) using a FacScan flow cytometer (Becton Dickinson, San Jose, CA.)

**Protein assays.** Western blot analysis of cytosolic extracts was performed as described previously (11) using affinity-purified antibodies to AHR or an IgG fraction prepared from polyclonal antiserum to mouse HSP90 (a gift from Dr. Steven Ullrich, National Institutes of Health, Bethesda, MD). Antigen-antibody complexes were detected with a goat antirabbit antibody coupled to horseradish peroxidase (Pierce Chemical, Rockford, IL), using the enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL). The AHH assay and *in vivo* AHR ligand-binding assay were performed as described previously (7). For the latter assay, the amounts of ligand receptor in both cytosol and nucleus were analyzed by sucrose gradient analysis after culturing the cells with [$^3$H]TCDD.

**Retroviral expression vector for AHR.** The plasmid pSRα(*Not*1) (see Fig. 1, *top*) was derived from pSRαMSVTKNEO (18) and was a gift from D. Rawlings and O.N. Witte (School of Medicine, University of California Los Angeles). The mouse AHR cDNA was generated from pcDNA1/Neo/AHR by PCR using a 5′ primer containing an *Xba*I restriction site and a 3′ primer containing a *Hin*dIII site. The PCR product was ligated into pSRα(*Not*1) using the *Xba*I and

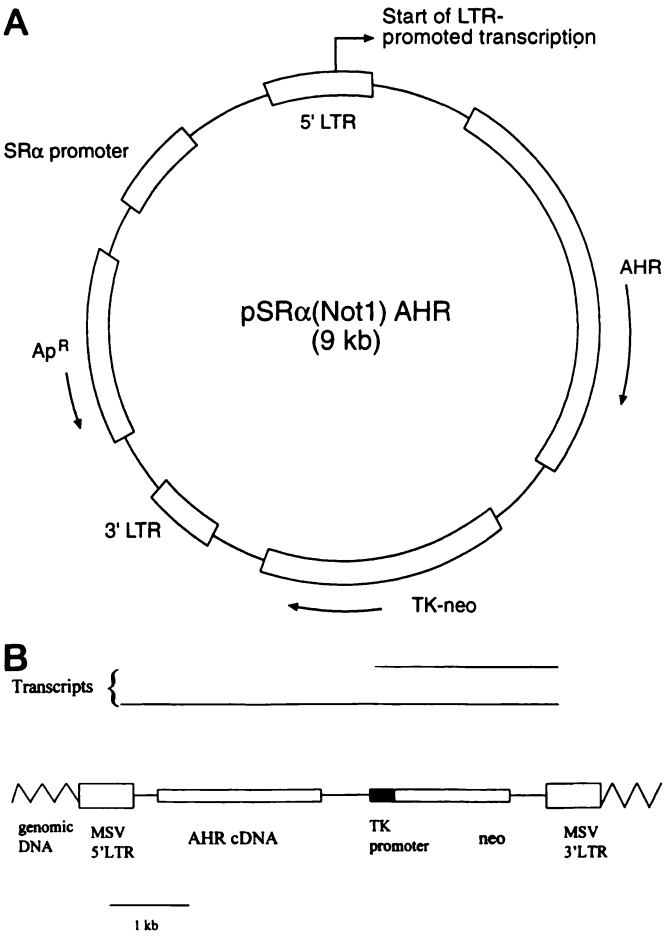


**Fig. 1.** Structure of the retroviral expression vector. *Top*, pSRα(*Not*1)AHR vector. *Bottom*, Proviral form of the vector.

*Hin*dIII sites located in its multiple cloning site, to generate pSRα(*Not*1)AHR. Infectious retroviruses derived from pSRα(*Not*1)AHR were prepared using a rapid procedure developed by Muller *et al.* (18), as follows. pSRα(*Not*1)AHR and the ecotropic packaging vector pSV-ψ$^-$-E-MLV were cotransfected into COS-7 cells using the calcium phosphate precipitation method of Chen and Okayama (19). A transcript corresponding to the two long terminal repeats and the intervening sequence (containing the *neo* gene and AHR cDNA) is thereby packaged into an infectious retrovirus in the COS-7 cells. [See Fig. 1, *bottom*, for the structure of the proviral product derived from pSRα(*Not*1) AHR.] The virus was harvested from the culture medium starting 3 days after cotransfection. Hepa-1 cells and the B clones in 100-mm tissue culture dishes received 3 ml of viral suspension in growth medium supplemented with 8 μg/ml Polybrene (Abbot Laboratories, Columbus, Ohio). Four hours later, an additional 7 ml of growth medium was added to each dish. Two or 3 days later, the infected cells were trypsinized and plated in normal growth medium or growth medium supplemented with G418.

**RNA analysis.** Cellular mRNA was isolated using the FastTrack mRNA isolation kit (InVitrogen, San Diego, CA), fractionated on 1% agarose-formaldehyde gels, and transferred to nylon membrane (Hybond-N; Amersham). The filters were probed with cDNA fragments labeled with [$^{32}$P]dCTP by random priming. The cDNAs were obtained either by restriction digestion or by PCR from appropriate vectors. Filters were washed in 0.2× standard saline citrate (1× = 150 mM NaCl, 15 mM sodium citrate, pH 7.0), 0.1% sodium dodecyl sulfate at 55° and exposed to Kodak XAR-5 or Kodak Biomax MR (Eastman Kodak, Rochester, NY) film at −70° with intensifying screens.

## Results

**Expression of AHR, ARNT, and HSP90 in the B mutants.** Fifteen B clones were analyzed for expression of AHR and ARNT mRNA. These clones had been isolated in five different BP selection experiments, from three different subclones of Hepa-1 cells, or from different cultures of Hepa-1 cells that originated from a small number of cells (1000 or fewer) (5, 6)[3]. Most of the clones are therefore of independent origin. All 15 B clones had much reduced but detectable levels of AHR mRNA and normal levels of ARNT mRNA. In all of the mutants, the low level of AHR mRNA that was detected was normal in size (Fig. 2). The amount of the constitutively expressed tubulin mRNA in each lane of Fig. 2 provides a control for differences in loading. A representative B mutant also was shown to express normal levels of HSP90 protein as assessed by Western blot analysis (data not shown). The above characteristics of the B clones suggest that they are not mutated in the *AHR* gene but are defective in its expression, because if they were mutated in the *AHR* gene, they would probably be heterogeneous with regard to the amount of AHR mRNA they express [as we observed for CYP1A1 mRNA expressed by our group of clones mutated in the *CYP1A1* gene (14)] and also potentially with regard to its size.

**Rescue of the B clone phenotype by infection with a retroviral vector expressing AHR.** We constructed an ecotropic recombinant retrovirus containing both the *neo* gene and the AHR cDNA. A diagram of the proviral version of the vector, pSRα(*Not*1)AHR, is illustrated in Fig. 1, *bottom*. G418-resistant clones were obtained at a frequency of approximately $10^{-3}$ from cultures of Hepa-1, c12, and c15 cells (the latter two are B clones) infected with either pSRα(*Not*1)AHR or the parental vector lacking the AHR insert. G418-resistant clones from each infection were pooled and, 2 weeks after infection, subjected to the benzo[*ghi*]-perylene plus near-UV reverse selection, which selects for cells that possess inducible CYP1A1 activity (20). The pools derived from the pSRα(*Not*1)AHR-infected c12 and c15 cells survived the reverse selection at frequencies of 50% and 85%, respectively [viability was determined 4 hr after application of the reverse selection, when viable cells are clearly distinguishable, microscopically, from dead cells (20)], whereas the equivalent pools of pSRα(*Not*1)-infected c12 and c15 cells only survived the reverse selection at frequencies of 0.01% (Table 1; Fig. 3). Thus, the AHR cDNA was highly efficient at complementing the B defect. The G418-resistant cells from each infection were pooled, grown up in G418, and assayed for AHH activity 4 weeks after infection (Table 1). The G418-resistant pools of c12 and c15 cells possessed 3.8% and 22% of the inducible AHH activity of Hepa-1 cells, respectively. This lack of concordance with their survival frequencies in the reverse selection is most probably caused by instability of AHR expression in the infectants and the fact that they were assayed for AHH activity at a time longer after infection than when they were assayed for survival in the reverse selection. This interpretation is consistent with the observation (Table 1) that the G418-resistant pool of pSRα(*Not*1)AHR-infected c12 cells that survived the reverse selection had substantially higher activities than the equivalent cells not subjected

[3] O. Hankinson, unpublished observations.

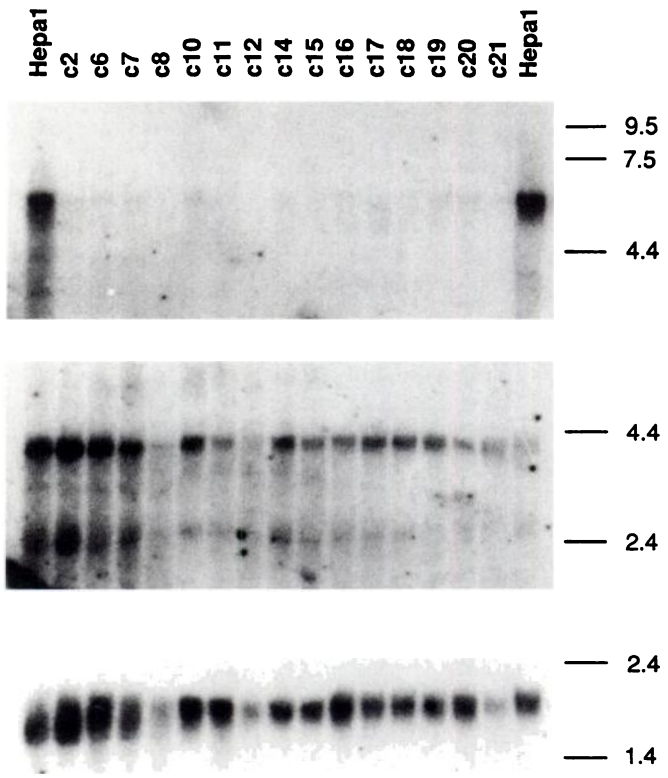


**Fig. 2.** AHR messenger RNA levels in the B clones. A Northern blot containing 5 μg of polyadenylated RNA from Hepa-1 and 15 B clones, as indicated, was probed sequentially with cDNAs for mouse AHR (*top*), mouse ARNT (*middle*), and mouse tubulin (*bottom*). *Right*, size markers (kb).

to the reverse selection (15.9% versus 3.8% of the induced Hepa-1 activity) and with the observation that the infectants continually declined in inducible AHH activity and ability to survive the reverse selection as they were cultured in G418-containing medium. (For example, the TCDD-inducible AHH activity of the G418-resistant pool derived from pSRα(*Not*1)AHR-infected c12 cells that had been subjected to the reverse selection was 15.9% of that of Hepa-1 cells 4 weeks after infection and only 7% 12 weeks after infection.) The G418-resistant pool derived from SRα(*Not*1)AHR-infected c12 cells that had been subjected to the reverse selection also expressed higher levels of the AHR protein than uninfected c12 cells or c12 cells infected with the parental vector but lower levels than Hepa-1 cells (Fig. 4). [The faster migrating bands in Fig. 4 most probably correspond to proteolytic degradation products of AHR (21).] The reduced level relative to Hepa-1 cells is again probably caused by instability of AHR expression in the infectants, because they were assayed 10 weeks after infection.

We performed Northern blot analysis on infectants 8 weeks after infection. The Hepa-1 cells expressed the endogenous AHR transcript (of about 5.4 kb), whereas this mRNA was not detected in B cells infected with pSRα(*Not*1) (Fig. 5A). The pool of B cells infected with pSRα(*Not*1)AHR and subjected to the reverse selection expressed an AHR-hybridizing mRNA, also of about 5.4 kb, at approximately the same level as Hepa-1 cells. Hepa-1 cells infected with the AHR-containing retroviral vector expressed very high levels of the 5.4kb mRNA. The AHR-hybridizing mRNA expressed in the infectants corresponds to the longer transcript originating from

TABLE 1
**Survival in the reverse selection and AHH activities of cells infected with the AHR retroviral expression vector**

| Strain | Retrovirus | Selection | Time after infection (weeks) | Survival in reverse selection (%) | AHH specific activity[a] | |
|---|---|---|---|---|---|---|
| | | | | | −TCDD | +TCDD |
| Hepa-1 | None | None | 0 | 100 | 1.4[b] | 100 |
| | pSRα(*Not*1) | G418 | 2 | 100 | | |
| | | | 4 | | 2.5 | 59[b] |
| | pSRα(*Not*1)AHR | G418 | 2 | 100 | | |
| | | | 4 | | 2.9 | 156[b] |
| c12 | pSRα(*Not*1) | G418 | 2 | 0.01 | | |
| | | | 4 | | <0.1 | 0.1 |
| | pSRα(*Not*1)AHR | G418 | 2 | 50 | | |
| | | G418 | 4 | | <0.1 | 3.8 |
| | | G418 + RS[c] | 4 | | <0.1 | 15.9[b] |
| | | G418 + RS[c] | 12 | 8 | <0.1 | 7.0 |
| c15 | None | None | 0 | 0.01 | 1.0 | 1.4 |
| | pSRα(*Not*1)AHR | G418 | 2 | 85 | | |
| | | | 4 | | 0.8 | 22.1 |

[a] Percentage of the specific activity in TCDD-treated Hepa-1 cells analyzed on the same occasion.
[b] Mean of two independent determinations. All other values are means of quadruplicate assays on a single extract.
[c] Culture derived from cells surviving the reverse selection applied 2 weeks after transfection.

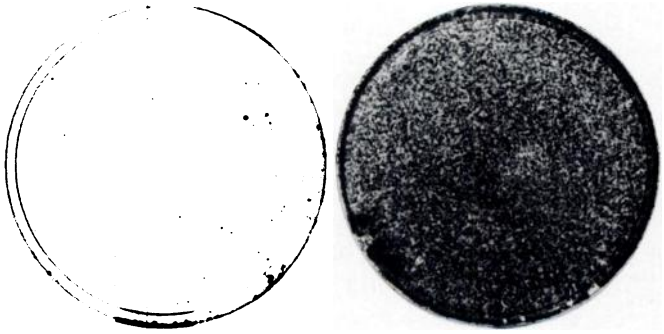

**Fig. 3.** Resistance of pSRα(*Not*1)AHR-infected c12 cells to the reverse selection. c12 cells infected with pSRα(*Not*1) (*left*) or pSRα(*Not*1)AHR (*right*) were selected with G418. Two weeks after infection, surviving cells were subjected to the reverse selection at $5 \times 10^5$ cells per dish. The dishes were stained after an additional 9 days of incubation.

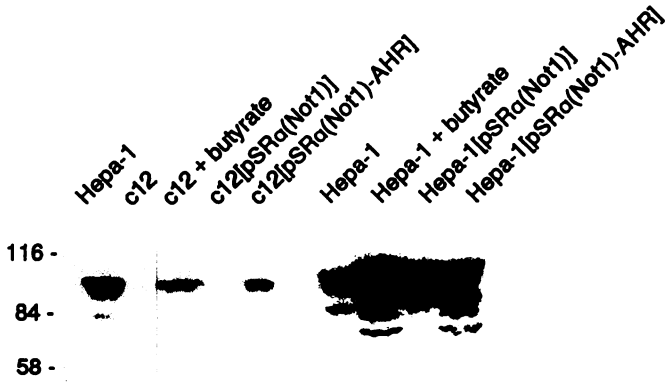


**Fig. 4.** Restoration of AHR protein expression to c12 cells infected with pSRα(*Not*1) AHR or treated with butyrate. c12 cells and Hepa-1 cells infected with the indicated retroviruses were assayed by Western blot analysis using a polyclonal antiserum to AHR 10 weeks after infection. The c12[pSRα (*Not*1)-AHR] culture had been subjected to the reverse selection 2 weeks after infection. Western blot analysis was also performed on extracts prepared from cells cultured at $5 \times 10^5$ cells per 100-mm dish in the presence or absence of 5 mM butyrate for 24 hr.

the retroviral provirus (Fig. 1, *bottom*) and, coincidentally, is the same size as the endogenous AHR mRNA. All extracts contained equal amounts of mRNA corresponding to the constitutively expressed gene, *CHOb* (22), confirming that equal amounts of mRNA from each culture were loaded on the gel. The same filter was washed and then reprobed with the *neo* gene. All infectants expressed approximately equal levels of the shorter transcript originating from the retroviral provirus but varying amounts of the longer transcript, which anneals with this probe as well as the *AHR* probe (Fig. 5B). The B-infectant culture expressed much less of the longer transcript than the infected culture of Hepa-1 cells. Thus, the longer transcript (which encodes AHR but not the neo protein, because translation cannot reinitiate in the middle of this transcript) was preferentially silenced relative to the shorter transcript in the B-infectant culture.

The results of the retroviral expression vector experiments demonstrate that the AHR-expressing retrovirus is capable of rescuing the CYP1A1-deficient phenotype of the B clones, but that expression of AHR encoded by the vector is unstable in the infected cells. The rate of loss of AHR expression described above in the c12 infectants was particularly rapid. Expression was lost less rapidly in most other pSRα(*Not*1)AHR infected cultures that we studied (e.g., the infected culture of Hepa-1 shown in Fig. 5A and data not shown).

**Restoration of AHR expression by treatment of B cells with butyrate.** As discussed above, the properties of the B clones suggest that they are deficient in transcription of the *AHR* gene. Butyrate treatment has been shown to increase the rate of transcription of a number of genes in cell culture systems. We therefore investigated whether butyrate treatment can affect expression of the *AHR* gene in the B clones. Butyrate inhibits histone deacetylase, which leads to an increase in the degree of acetylation of certain histone proteins and the unfolding of chromatin (for review, see Ref. 23).

A culture of the B strain, c12, was treated with butyrate and then subjected to the reverse selection. Butyrate treatment increased the frequency of cells surviving the reverse selection. The effect was maximal with 5 mM butyrate (data not shown). When $5 \times 10^5$ c12 cells in a 100-mm dish were treated with 5 mM butyrate for 24 or 48 hr and then subjected to the reverse selection, approximately 30% survived, compared with a survival of approximately $10^{-4}$ for c12 cells not treated with butyrate. Survival was assessed microscopically

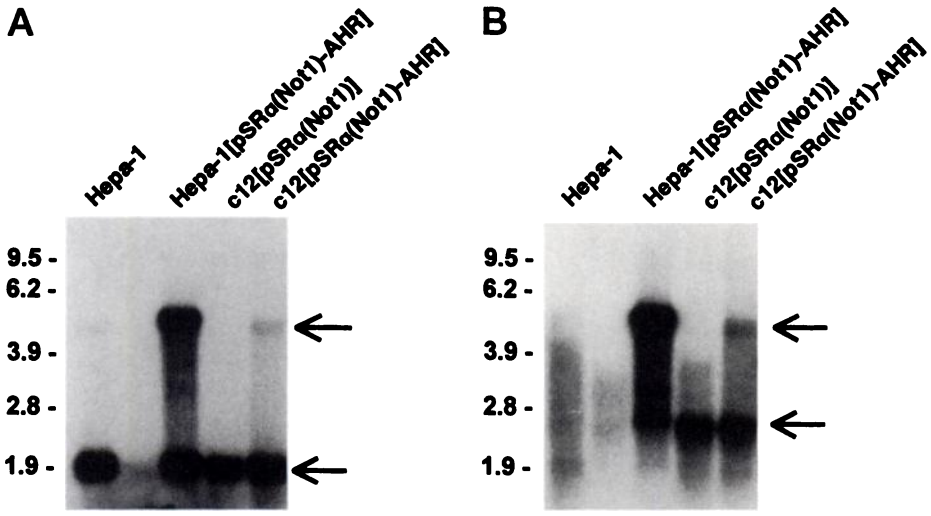


**Fig. 5.** Restoration of AHR mRNA expression to c12 cells infected with pSRα(*Not*1) AHR. A, polyadenylated RNA (5 μg) from each of the indicated strains was probed with a mixture of mouse AHR cDNA and CHOb cDNA. The c12[pSRα(*Not*1)-AHR] culture was subjected to the reverse selection 2 weeks after infection. All infectants were assayed 8 weeks after infection. *Upper arrow*, AHR mRNA; *lower arrow*, CHOb mRNA. *Left*, size markers (kb). B, the membrane was washed and reprobed with the neo cDNA. *Upper arrow*, larger proviral transcript; *lower arrow*, smaller proviral transcript. *Left*, size markers (kb).

4 hr after application of the reverse selection (data not shown). When 2000 c12 cells per 100-mm dish were treated with 5 mM butyrate, maximal survival in the reverse selection occurred when butyrate was applied for 48 hr, when nearly 100% of the cells that survived the toxic effect of butyrate also survived the reverse selection (Fig. 6). (Upon application of 5 mM butyrate, cell growth ceased immediately, and at later time points, a decline in cell number occurred.) We do not know the reason for the diminished effectiveness of butyrate at the higher cell density. Twice-daily refeeding of the culture containing $5 \times 10^5$ c12 cells per 100-mm dish with 5 mM butyrate over a 48-hr period did not increase subsequent survival in the reverse selection (data not shown), which argues against an explanation postulating a more rapid metabolic elimination of butyrate at the higher cell density. Because the effect of butyrate on $5 \times 10^5$ c12 cells per 100-mm dish seemed to be nearly as great at 24 hr as at 48 hr and because the longer treatment was more toxic to the cells, we used a 24-hr treatment for most subsequent studies.

The other fourteen B clones all responded to butyrate in a fashion similar to c12. Butyrate had no effect on survival in the reverse selection of the other classes of CYP1A1 noninducible mutants of Hepa-1. The cloning efficiencies in the reverse selection both with and without butyrate treatment for these mutants were as follows: c1 (complementation group A, mutated in the *CYP1A1* gene) $< 10^{-6}$; c4 (complementation group C, deficient in ARNT) $< 10^{-6}$; c35 (complementation group D, deficient in AHR function) $10^{-5}$; c31 (dominant, expresses a repressor that prevents binding of the AHR/ARNT dimer to the xenobiotic responsive element) $4 \times 10^{-4}$.

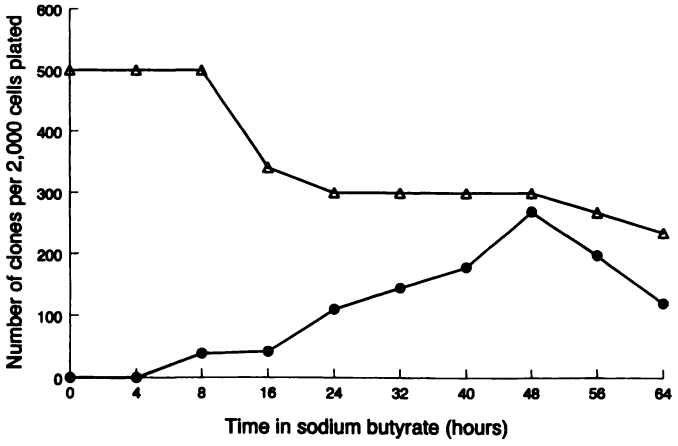


**Fig. 6.** Effect of duration of treatment with butyrate on the survival of c12 cells in the reverse selection: 2000 c12 cells per 100-mm dish were treated with 5 mM butyrate for the indicated periods and then either plated in normal growth medium (△) or subjected to the reverse selection (●).

The effect of butyrate on the B clones is reversible. When c12 cells were treated with butyrate for 24 hr, incubated in the absence of butyrate for 3 days, and then subjected to the reverse selection, they survived the selection at the same low frequency as c12 cells not treated with butyrate. Furthermore, when clones resistant to the reverse selection were isolated from a c12 culture treated with butyrate, grown up over a period of about 4 weeks and then retested for resistance to the reverse selection, they proved to be no more resistant than c12 cells not treated with butyrate (data not shown).

A 24-hr treatment with butyrate increased the TCDD-inducible AHH activity of c12 cells approximately 20-fold. A similar effect was seen in other B clones but not in Hepa-1 cells or in other classes of CYP1A1 noninducible mutants (Table 2 and data not shown). This treatment also increased the amount of AHR in both the cytosol and nucleus of c12

TABLE 2

**Effect of butyrate treatment on AHH activity and AHR ligand binding activity**

| Assay | Treatment | Strain | |
|---|---|---|---|
| | | Hepa-1 | c12 |
| AHH[a] | None | 1.3 | 0.16 |
| | 5 mM butyrate | 1.6 | 0.16 |
| | 10 nM TCDD | 100 | 0.98 |
| | 5 mM butyrate + 10 nM TCDD | 102 | 10.3 |
| Ligand binding in cytosol[b] | None | 334 | 6.6 |
| | 5 mM butyrate | ND[c] | 45 |
| Ligand binding in nucleus[b] | None | 691 | 5.1 |
| | 5 mM butyrate | ND | 75 |

[a] Expressed as a percentage of that in TCDD-treated Hepa-1 cells.
[b] fmol [$^3$H]TCDD/mg of protein.
[c] ND, not done.

cells, as measured by ligand binding analysis of cells treated in culture with [$^3$H]TCDD (Table 2). Treatment of cytosolic extracts of c12 cells with butyrate *in vitro*, however, did not increase the levels of ligand binding to AHR (data not shown). A 24-hr treatment with butyrate increased the amount of AHR protein in the cytosol of c12 cells (Fig. 4). This treatment increased the amount of AHR mRNA in the c12 and c15 B strains to the level present in untreated Hepa-1 cells. Butyrate treatment also increased the amount of AHR mRNA in Hepa-1 cells (Fig. 7). The observation that a 24-hr treatment of c12 cells with butyrate increased AHR mRNA levels to a greater degree than AHR protein, AHR ligand-binding activity, or AHH activity (the last two of which increased to only about 10% of the levels present in Hepa-1 cells) can be explained by the fact that the increases in the last three parameters are later responses than the increase in AHR mRNA.

**Restoration of AHR expression by treatment of B cells with trichostatin A.** As has been observed with other effects of sodium butyrate on cell behavior, relatively high concentrations of this compound were required to induce expression of AHR in the B clones. This raises the possibility that the effect of butyrate is not mediated by its effect on histone deacetylase but by an effect on another cellular parameter. Therefore, we tested the recently described, much more potent inhibitor of histone deacetylase, trichostatin A (for review, see Ref. 24), on the B clones. Trichostatin A treatment increased the frequency of B cells that could survive the reverse selection. A 24-hr treatment with 25 nM trichostatin A, which killed 49% of the cells (as assessed by the ability of cells plated at low density to form clones) permitted approximately 90% of the cells to survive the reverse selection (as assessed by microscopic examination of the cells 4 hr after completion of the reverse selection) (Fig. 8, and data not shown). Treatment with 25 nM trichostatin A also markedly increased expression of AHR mRNA in the c12 strain (Fig. 9). Testing of higher concentrations of trichostatin A was not practical because of toxicity.

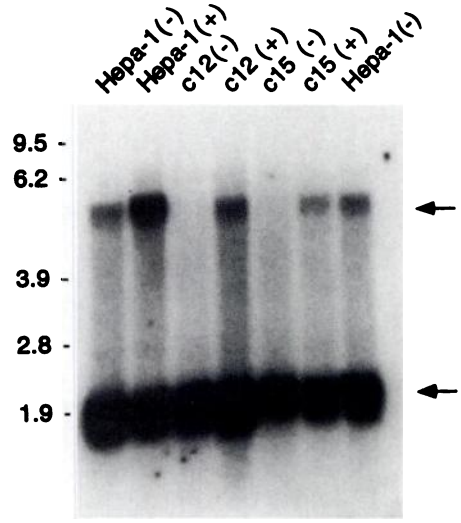


**Fig. 7.** Effect of butyrate treatment on AHR mRNA. Polyadenylated RNA was isolated from the indicated cell lines cultured at $5 \times 10^5$ cells per 100-mm dish in the presence (+) or absence (−) of 5 mM butyrate for 24 hr. Polyadenylated RNA (5 μg) from each culture was then subjected to Northern blot analysis using a mixture of AHR and CHOb cDNAs as probe. *Upper arrow*, AHR mRNA; *lower band*, CHOb mRNA; *left*, size markers (kb).

**Restoration of AHR expression in B cells after fusion with rat hepatoma cells.** Rat AHR (96 kDa) is different in size from AHR encoded by the mouse $Ah^{b-1}$ allele present in Hepa-1 cells (89 kDa), although both proteins migrate abnormally upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis, with apparent mobilities of approximately 106 kDa and 95 kDa, respectively (20, 25, 26). The rat hepatoma line, H5-6, is highly inducible for AHH activity by TCDD. We fused the B clone, c12, with H5-6 cells and selected somatic cell hybrids. The $G_1$ DNA content of each hybrid was equal to the sum of the $G_1$ DNA contents of its parental cells (data not shown), which is consistent with our previous observation that hybrids between rat and mouse hepatoma cells contain the full complement of chromosomes from both parents (17). Whereas mouse AHR could not be detected in c12 cells in this experiment and these cells expressed only very low levels of AHH activity, the B/H5-6 hybrids were highly inducible for AHH activity, and expressed the mouse AHR protein as well as the rat protein (Fig. 10). Thus, expression of the mouse *AHR* gene contained in the B clone genome was activated after fusion of B cells with the rat hepatoma cells.

## Discussion

Our data indicate very strongly that the B clones are defective in expression and particularly in transcription of the *AHR* gene but are not mutated either in the coding region or in a regulatory sequence for the gene. This evidence is as follows: (i) all of the B clones tested have considerably reduced (but nevertheless detectable) levels of AHR mRNA, despite the fact that most clones, if not all, were of independent origin; (ii) AHR expression and activity were reactivated in all B clones by treatment with agents that inhibit histone deacetylase; (iii) expression from the *AHR* gene of a B clone was reactivated after fusion of the B cells with rat hepatoma cells. Previous characterization of revertants of the c12 clone also failed to provide evidence that this strain carries a mutation in the coding region of *AHR* (27). We demonstrated previously that butyrate treatment leads to the *de novo* expression of AHR activity and CYP1A1 inducibility in a "dedifferentiated" rat hepatoma cell line. However, the efficiency with which butyrate produced this effect in the rat hepatoma cells was several orders of magnitude less than the efficiency with which it induced reactivation of AHR expression in the B clones of the Hepa-1 cell line. Furthermore, results from previous complementation analysis indicate that lack of AHR expression in the dedifferentiated rat hepatoma cells is ascribable to the inactivity of a gene different from the defective gene in the B clones (17).

We have attempted repeatedly to rescue the B clones via transfection with plasmid AHR expression vectors but have not obtained transfectants that survive the reverse selection in a stable fashion.[4] As demonstrated here, however, the B phenotype could be rescued efficiently if the AHR cDNA were introduced via a retroviral expression vector. Success with the retroviral expression vector may be ascribable, at least partly, to the fact that retroviral vectors, unlike plasmid vectors, possess a specific mechanism for integrating into

[4] J. Zhang and O. Hankinson, unpublished observations.

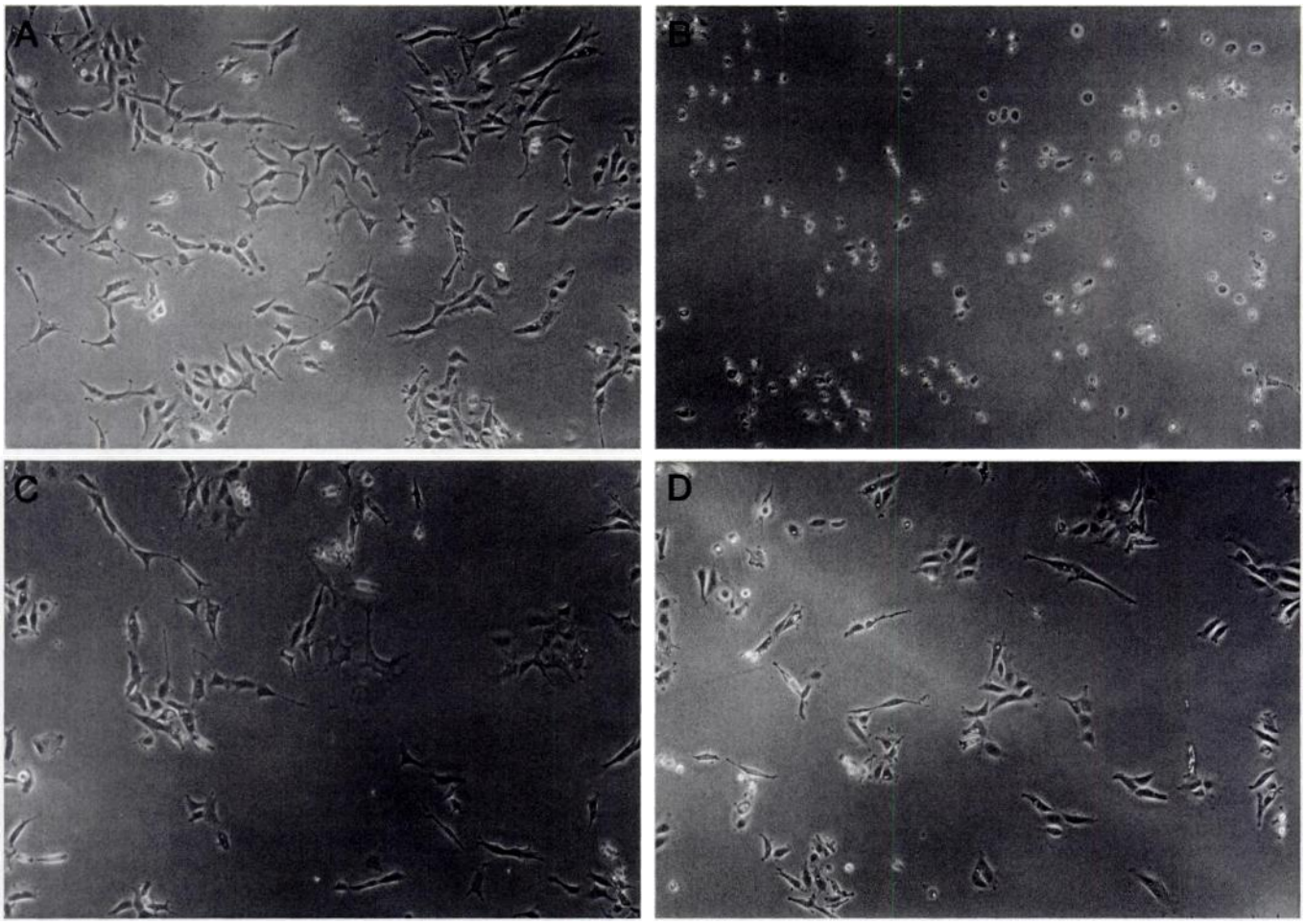


**Fig. 8.** Effect of trichostatin A on survival of c12 cells in the reverse selection. $5 \times 10^5$ c12 cells were inoculated per 100-mm dish. The following day, the cells were treated with 25 nm trichostatin A or vehicle (ethanol). After an additional day, they were subjected to the reverse selection. They were photographed under phase contrast optics 8 hr later. A, Vehicle control, no reverse selection. B, Vehicle control, reverse selection. C, Trichostatin A, no reverse selection. D, Trichostatin A, reverse selection.

genomic DNA. Our observation that the B clones could be rescued by the AHR retroviral expression vector demonstrates that the CYP1A1 noninducibility phenotype of the B clones is principally, if not completely, caused by the loss in AHR expression. We provide convincing evidence, however, that expression of the AHR cDNA is slowly and progressively silenced in the infectants as they are maintained in culture. We do not know the mechanism of this silencing. A much more rapid silencing process is probably at least partially responsible for the inability of the transfected plasmid AHR expression vectors to stably rescue the B clones. We previously observed that expression of the *CYP1A1* gene and of a plasmid ARNT cDNA expression vector introduced into Hepa-1 cells progressively declined over several weeks after transfection (9, 10), and we have observed that a β-galactosidase gene is rapidly silenced (but not lost) after transfection into Hepa-1 cells.[5] Instability of expression of transfected genes or cDNAs in Hepa-1 cells (even those that are presumably integrated) therefore seems to be a general phenomenon.

Three alternative hypotheses may account for the reduction in the amount of AHR mRNA in the B clones: (i) The endogenous *AHR* gene is silenced by an epigenetic alteration

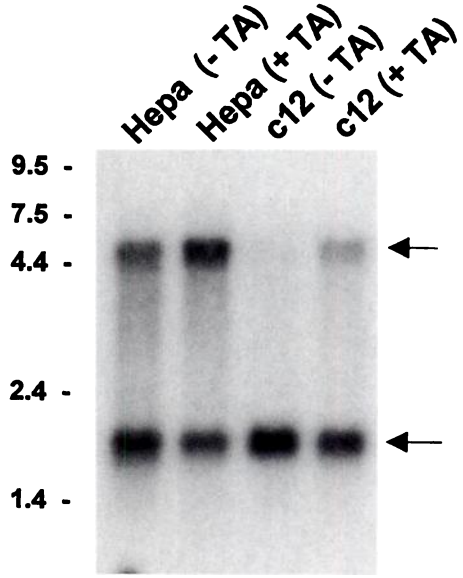


**Fig. 9.** Effect of trichostatin A treatment on AHR mRNA. RNA was isolated from the indicated cell lines cultured at $5 \times 10^5$ cells per 100-mm dish in the presence or absence of 25 nM trichostatin A for 24 hr. Polyadenylated RNA (2 μg) from each culture was analyzed as described in the legend to Fig. 7. *Upper arrow*, AHR mRNA; *lower arrow*, CHOb mRNA.

[5] J. Zhang and O. Hankinson, unpublished observations.

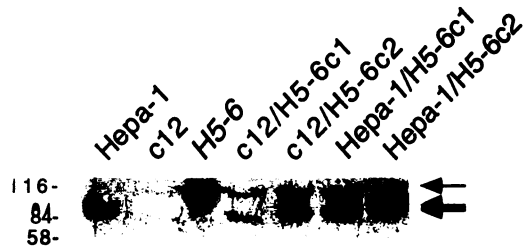


**Fig. 10.** Expression of AHR in B/H5-6 hybrids. Western blot analysis was performed using affinity-purified polyclonal AHR antibodies. *Lane 1*, Hepa-1 (100%); *lane 2*, c12 (2.1%); *lane 3*, H5-6 (40%). *lanes 4 and 5*, two independent c12/H5-6 hybrid clones (19% and 19%); *lanes 6 and 7*, two independent Hepa-1/H5-6 hybrid clones (20% and 34%). *Numbers in parentheses*, TCDD-induced AHH activities in the strains relative to Hepa-1 (averages of two independent determinations). *Upper arrow*, rat AHR; *lower arrow*, mouse AHR; *left*, molecular mass markers (in kDa).

in chromatin structure, such as heterochromatinization. [This mechanism of silencing may not necessarily be the same as that responsible for the silencing of the AHR cDNA (and other genes) that occurs after their infection or transfection into Hepa-1 cells.] Histone acetylation reverses this alteration. (ii) The B clones are mutated in a transcription factor required for expression of the endogenous *AHR* gene. An increase in histone acetylation leads to a more open chromatin configuration over the 5′ flanking region of the *AHR* gene, which allows access to another transcription factor that has no access to the *AHR* gene in its normal chromatin configuration, thereby increasing the rate of transcription of the gene. (iii) The B clones are mutated in a protein involved in the generation of an open chromatin configuration over the *AHR* gene that is required for its expression. An increase in histone acetylation circumvents the requirement for this factor. Several proteins in yeast (e.g., the SWI/SNF proteins and GRF2) and *Drosophila* (e.g., GAGA and the trithorax group of proteins) have been shown to be required for gene expression, most probably by affecting chromatin structure (for review, see Refs. 28–31), and homologs of some of these proteins have been identified in mammals (32). It should be noted that the putative factors invoked in hypotheses (ii) and (iii) would have to be active on only a restricted set of genes; otherwise, their loss would lead to cellular inviability.

The AHR deficiency of the B clones is very stable, because the clones revert to the AHH-positive phenotype at a frequency of less than $10^{-6}$ (4, 5, 27). Reactivation of mouse AHR expression in the B/H5-6 hybrids is less compatible with model (i) than models (ii) and (iii), because the AHR expression defect in the first model, but not in the other two, should act in a *cis*-dominant fashion. (However, it is conceivable that epigenetic silencing of the *AHR* gene could be reversed upon hybridization with the rat hepatoma cells.) Models (ii) and (iii) are also more compatible with the observation that the reversion frequency of a B clone was increased 20-fold by treatment with the chemical mutagen *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (27). Finally, the low spontaneous rate of origin of the B clones [$< 2 \times 10^{-7}$ events per cell generation (4)] is compatible with a mutational mode of origin, as required for hypotheses (ii) and (iii). On balance, therefore, the evidence favors hypotheses (ii) and (iii) over hypothesis (i), although it does not discriminate between hypotheses (ii) and (iii). Further studies should help determine which hypothesis is correct.

AHR is expressed to markedly different degrees in different tissues and at different times in development (25, 33). An important issue is whether the process (hypothesis i) or genes (hypotheses ii and iii) inferred in the current studies are involved in the tissue-specific or developmental regulation of AHR levels in the whole organism. Additional studies on the B clones also should provide further insights into epigenetic regulation of gene regulation [if hypothesis (i) is true] or the regulation of chromatin structure (hypothesis iii) or should lead to the identification of a transcription factor for the *AHR* gene (hypothesis ii).

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