Identification of a Novel Cis-acting Negative Regulatory Element Affecting Expression of the CYP1A1 Gene in Rat Epidermal Cells*

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Polycyclic aromatic hydrocarbons such as 3-methylcholanthrene are toxic to rat epidermal cells in low passages (3 to 6), but cultures of high passage (15) are resistant. Since such compounds can be metabolically activated by cytochrome P4501A1, we have examined the regulation of this gene in low and high passage cells. Consistent with this difference, little or no 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-inducible P4501A1 mRNA or enzyme activity was observed in high passage as compared to low passage cultures. Similarly, transfection of a luciferase reporter construct containing −1317 to +256 base pairs of the 5′-flanking region of the murine CYP1A1 gene was TCDD-inducible in low but not high passage cells. Ligand binding and transfection experiments demonstrated the presence of functional Ah receptor complexes in both high and low passage cells. Deletion analysis identified a 26-base pair negative regulatory DNA (NeRD) element contained within the upstream regulatory region of the CYP1A1 gene responsible for this effect. Nuclear extracts from both low and high passage cells contain a protein which specifically binds to NeRD-containing DNA. Thus, the loss of polycyclic aromatic hydrocarbon sensitivity in high passage rat epidermal cells appears to be due to decreased expression of CYP1A1, and this effect may be mediated by an altered NeRD binding factor(s) present in these cells.

Polycyclic aromatic hydrocarbons (PAHs)† are ubiquitous environmental contaminants, some of which have been identified as rodent (1) and human (2) skin carcinogens and are thus a threat to human health. Since the epidermis is the primary extrapulmonary barrier to toxic agents, understanding the mechanism by which PAHs are metabolically transformed by keratinocytes is important for assessing their potential risk. Elucidating the response of normal and neoplastic cells to these compounds will assist in evaluating the applicability of the rodent model to human pathology.

Numerous biochemical studies have demonstrated that PAHs induce cytochrome P4501A1 and are metabolized by this enzyme to DNA-damaging electrophiles that lead to mutations and cellular transformation (3). Mechanistically, the induction of cytochrome P4501A1 by PAHs and related chemicals such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin) is mediated by the Ah receptor (AhR), a ligand dependent, DNA-regulatory protein to which these chemicals bind with high affinity (4–8). After ligand binding, two molecules of hsp90 dissociate from the ligand-AhR complex (9–11), the receptor translocates into the nucleus (12, 13), dimerizes with the Ah receptor nuclear translocator (ARNT) protein, and is converted into its high affinity DNA binding form (8, 13–16). The binding of the heteromeric ligand-AhR complex to its specific DNA sequence, the dioxin responsive element (DRE), leads to DNA bending, chromatin disruption, increased promoter accessibility, and increased CYP1A1 gene transcription (17–22). In addition to positive regulation, the CYP1A1 gene is also under negative control in which inducible and constitutive expression of the promoter can be affected (23–25).

Primary cultures of epidermal cells are known to respond to PAHs and TCDD with the induction of P4501A1 and increase in PAH metabolism. Previous work has demonstrated that rat epidermal cells lose PAH responsiveness as they are passaged and become immortalized (26, 27). In addition, the cells lose their T3 feeder layer dependence, become less stratified, dramatically increase their growth rate and colony forming efficiency and gradually lose their differentiated characteristics (27).‡ Previous studies of murine and human epidermal keratinocytes have demonstrated that PAH responsiveness (CYP1A1 inducibility) is dependent upon the differentiated state of the cells, with those committed to terminal differentiation being most responsive (28–31). However, the mechanistic basis for the differentiation-dependent change in PAH responsiveness is currently unknown. Although it is not clear whether the spontaneous loss of PAH responsiveness in high passage rat keratinocytes is related to their loss of differentiated phenotype, the availability of a variety of molecular probes to the CYP1A1 system allows us to examine the mechanism of the spontaneous loss of PAH sensitivity in rat epidermal cells in detail. Here we demonstrate that resistance of high passage (HP) rat epidermal keratinocytes to PAH-dependent toxicity is due to reduced levels of inducible P4501A1 activity. Transfection and deletion analysis have identified a novel cis-acting negative regulatory DNA (NeRD) element present upstream of the CYP1A1 promoter that acts to negatively regulate TCDD-inducible expression of the CYP1A1 gene in HP cells.

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1 The abbreviations used are: PAH, polycyclic aromatic hydrocarbon; AhR, aromatic hydrocarbon receptor; MeSO, dimethyl sulfoxide; DRE, dioxin responsive element; NeRD, negative regulatory DNA; TCDBF, 2,3,7,8-tetrachlorodibenzo-furan; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; HP, high passage; LP, low passage; MMTV, mouse mammary tumor virus; 3-MC, 3-methylcholanthrene; bp, base pair(s); PCR, polymerase chain reaction.

2 A. A. Walsh, K. Tullis, R. H. Rice, and M. S. Denison, unpublished observations.

MATERIALS AND METHODS

Chemicals—TCDD used for cell culture was purchased from Ultra Scientific (Kingston, RI) and 3-methylcholanthrene (3-MC) was obtained from Sigma. [3H]TCDD (36 Ci/mmol), unlabeled TCDD, and...
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The constructed plasmid (pGudLuc5.0) was digested with NcoI and SalI of the mouse CYP1A1 regulatory region containing the desired deletions. The internal deletion constructs pGudLuc5.1, 5.2, 5.3, and 5.4 were prepared by restriction digestion of pGudLuc5.0, blunting with Klenow and religating. The specific areas of the mouse 5′-flanking region deleted in each construct were as follows: pGudLuc5.1 (−643 to −648 bp deleted), pGudLuc5.2 (−815 to −848 bp deleted), pGudLuc5.3 (−816 to −848 bp deleted), pGudLuc5.4 (−816 to −863 bp deleted) of the mouse CYP1A1 5′-flanking region. Finner internal deletion analysis mutants of pGudLuc5.0 were prepared by PCR and the resultant constructs and the specific regions deleted are as follows: pGudLuc5.01 (−816 to −803 bp deleted), pGudLuc5.015 (−816 to −791 bp deleted), pGudLuc5.02 (−816 to −753 bp deleted), and pGudLuc5.03 (−816 to −704 bp deleted). The upstream primers were as follows: 5′-CCGGGATCTCTCTCTACACTTAGATAG3′ (5.01), 5′-GGTGGATCACCAGGTAGATGCTGCTGCAACCG-3′ (5.015), 5′-GGGGGATCCATGCTGCTGCAATGATGCGC-3′ (5.02), 5′-GGGGGATCACCAGGAGGCTACGCGGCTGAC-3′ (5.03), and the down-stream primer was 5′-AAAGAAGAGAATAAGAGGTTTGCGGG-3′. A BamHI site was added to each upstream primer, and the PCR products were digested with NcoI (each in internal PCR product) and BamHI as was the 3′ construct pGudLuc5.0. The PCR products were then ligated into the BamHI and NcoI sites of pGudLuc5.0 to recreate the upstream regulatory region containing the desired deletions.

Cell Culture—Rat epidermal keratinocyte cultures of passages 2–30 were supported with an irradiated 3T3 feeder layer, while those of passages 30–60 were propagated in the absence of 3T3. Previous experiments have demonstrated no difference in cell growth or PAH responsiveness (e.g. 3-MC induced toxicity or AhR inducibility) between high passage cells grown in the presence or absence of the 3T3 feeder layer (data not shown). Cell growth experiments were performed with feeder layer support. In experiments using low passage cells, the feeder layer was removed either by rinsing with 0.5 mM EDTA in PBS or by the advancing keratinocyte cultures that they reached confluence and therefore did not complicate interpretation of the results. Cells were grown in a 3:1 mixture of Dulbecco-Vogel Eagle’s and Ham’s F-12 media supplemented with 5% fetal bovine serum, hydrocortisone (0.4 μg/ml), epidermal growth factor (10 ng/ml), chola toxin (10 ng/ml), transferrin (5 μg/ml), insulin (5 μg/ml), adrenalin (0.18 μM), triiodothyronine (20 μM), and antibiotics (36). Mouse hepatoma (Hepa1c1c7) cells were grown as described previously (18, 19). Aaryl Hydrocarbon Hydroxylase (AHH) Assay—AHH activity was determined as described previously (26, 37). Near confluent cultures were incubated for 12 h with 5 nM TCDD or solvent alone (0.05% MeSO), rinsed twice, and then incubated in 5 ml of serum-free medium containing 3.5 μM [14C]benzo(a)pyrene (30 Ci/mmol). After 4 h the cultures were subjected to 6 h of 1,000 g followed by the addition of 3.4 ml of MeSO. Unmetabolized [14C]benzo(a)pyrene was removed by two extractions with hexane, and the concentration of labeled metabolites in the aqueous layer determined by acidification counting after neutralization with 1 N HCl. The protein concentration was determined by Coomassie G-250 dye binding (38) using bovine serum albumin as the standard.

Ah Receptor Assays—The luciferase activity of the cells was measured as described previously (39, 40). Briefly, cells were incubated with 10 nM [3H]TCDD in the absence or presence of 1 μM unlabeled TCDD for 2 h, followed by removal of free and loosely bound [3H]TCDD with dextran-coated charcoal (0.1 mg of charcoal/ml of cytosolic protein). Sample aliquots (300 μl) were layered over 10–30% sucrose gradients with 10 Ci/ml [3H]formaldehyde-labeled serum albumin (Sigma) and catalase (11.3 S) included as internal sedimentation markers (41). After centrifugation, fractions were collected and the radioactivity in each fraction determined by liquid scintillation.

Northern Analysis—Confluent cultures were incubated with 5 nM TCDD or solvent alone for 24 h, washed with phosphate-buffered saline, and dissolved in 1 ml of 4 M guanidine thiocyanate, 0.1 M Tris-HCl (pH 7.5) and 0.5 M NaCl. The construct was sheared by passage through a 20-gauge needle several times and RNA isolated by centrifugation through CsCl (42). Total cellular RNA (20 μg/lane) was electrophoresed through a 1% agarose gel containing 0.6 M formaldehyde and subsequently transferred to a nylon membrane (Schleicher and Schuell). The membrane was prehybridized in 1× NaCl, 100 mM Na2HPO4 (pH 6.5), 10 mM EDTA, 1% SDS, 200 μg/ml yeast tRNA, and 200 μg/ml salmon sperm DNA at 65 °C for 4 h. Mouse P4501A1 (American Type Culture Collection, number 63006), P4501B1 (43), and rat glyceraldehyde-phosphate dehydrogenase (44) cDNA probes were labeled with [α-32P]dCTP by random priming and added to the hybridization mixture (105 cpm/ml). After 24 h at 65 °C the membrane was washed twice each at 65 °C for 20 min with 1× NaCl, 50 mM Na2HPO4, 1% SDS, 0.1× SSC, and 0.5× SSC and once with 75 mM NaCl, 7.5 mM sodium citrate. Blots were autoradiographed overnight at ~80 °C, radioactivity quantitated using PhosphorImaging and values normalized to glyceraldehyde-phosphate dehydrogenase.

Transfections and Luciferase Assay—Promega Profection and Luciferase Assay kits were used. Briefly, cells were grown to 50% confluence, residual 3T3 cells (if any) were removed with EDTA (45) and fresh medium was added 1 h before addition of precipitated DNA. Plasmid DNA, precipitated with 2 μl calcium phosphate and 2× Hepes-buffered saline for 30 min, was added to cultures at 15 μl/cm2 dish. Cultures were incubated for 24 h, washed once with serum-free medium and fresh medium with 5 nM TCDD or MeSO was added for an additional 24 h. Cultures were harvested by scraping cells in lysis buffer (Promega), lysed by sonication for 10 s, centrifuged, and luciferase activity of the cleared supernatants measured using a Turner luminometer. Luciferase activity was normalized to protein and the activity expressed as relative light units per mg of protein. Triplicate dishes were used for each experiment; the values were averaged and expressed as a percentage of the control in each experiment. The values in the first set of transfections were lysates from MeSO-treated cells transfected with pGudLuc4.1. Since pGudLuc1.1 contained a different promoter (MMTV), TCDD-induced luciferase activity was compared to that of MeSO-treated cells. For the remaining transfections the control was pGudLuc5.0, not treated with MeSO. Each set of transfection experiments was performed a minimum of three times. The values graphed were expressed as the means (normalized to control values) and standard deviation among experiments.

Gel Retardation Analysis—Complementary pairs of synthetic oligonucleotides containing the sequences 5′-GATCGGCGAGTTGGATGAAAGACC-3′ (corresponding to the AhR binding site of DRE2 (19) and designated as the DRE oligonucleotide) and 5′-GATCGCCAGTATCCCTGTGGGATTG-3′ (19) were used as a control for the retardation binding assay (46). The direct repeats within the NeR element and referred to as the NeR oligonucleotide), were synthesized, purified, and annealed, the NeR oligonucleotide was radiolabeled with [γ-32P]ATP using T4 polynucleotide kinase as described (46). Gel retardation analysis of nuclear extracts prepared from low and high passage rat keratinocytes and mouse hepatoma (Hepa1c1c7) cells were determined as described (18, 19, 46).

RESULTS

Rat epidermal cells lose their sensitivity to 3-MC inhibition of colony expansion between passages 3 and 15 (Fig. 1). Cultures were plated at low cell density and treated with 2 μg/ml 3-MC for 14 days and then fixed and stained. A loss of sensitivity was noted beginning at passage 6 and was complete by passage 15. Colony forming efficiencies of the rat epidermal
cells increased from 3% at passage 3 to 12% at passage 15 (data not shown) indicative of spontaneous immortalization (27). Later passages displayed the same results as passage 15 and were used subsequently for convenience.

The loss of 3-MC sensitivity in high passage cells was correlated with a decrease in constitutive and inducible AHH activity (Fig. 2). AHH activity was induced by TCDD treatment by 3.5-fold in low passage cultures and was not induced in high passage cultures. To determine whether reduction in inducible AHH was due to a reduction in mRNA levels of P4501A1 and/or another TCDD-inducible P450, CYP1B1, Northern blot analysis of message levels in the high and low passage cultures were examined. As is evident in Fig. 3, TCDD induced P4501A1 and B1 mRNAs in low passage cultures by a factor of 6- and 10-fold, respectively, while in high passage cultures the induction was only 1.2- and 0.9-fold, respectively. Thus, inducible expression of both CYP1A1 and B1 is suppressed in high passage cells.

To determine whether the reduction in inducible P4501A1/B1 expression in high passage cells was due to a loss of the AhR, we measured AhR ligand binding using the sucrose density centrifugation assay (Fig. 4). These experiments demonstrated the presence of an 8.5–9.0 S [3H]TCDD:AhR binding peak in both low and high passage cells. Moreover, quantification of [3H]TCDD specific binding revealed that low and high passage cells contained comparable levels of [3H]TCDD specific binding (~37 fmol/mg of protein).

The above experiments measure only the ability of the AhR to bind ligand and do not address its ability to activate transcription in these cells. To address this, we carried out transient transfection experiments using a heterologous TCDD-inducible reporter gene construct. This vector, pGudLuc1.1, which contains only the CYP1A1 promoter, which confer TCDD/AhR responsiveness (32, 33). Transient transfections of this vector into low and high passage cells revealed that TCDD induced luciferase activity was observed in the low passage cells and was not in those of high passage (Fig. 5B), mimicking the results obtained with the endogenous CYP1A1 gene. These results argue against alteration in chromatin structure as the cause of the loss of responsiveness in the high passage cells and suggest that the CYP1A1 5′-flanking region contains a cis-acting element responsible for the decreased responsiveness. Alternatively, the reduced level of expression could result from the lack of a factor necessary for normal promoter activity in high passage cells. To explore this possibility, low and high passage cells were transfected with a construct (pGudLuc4.2, Fig. 5A) containing only the CYP1A1 promoter (inclusive of the first 248 bp upstream of the transcriptional start site). Transient transfections with this construct resulted in high levels of constitutive promoter activity in both the low and high passage cells (Fig. 5B), confirming that loss of promoter function was not responsible for the loss of responsiveness in late passage cells.

The above results support the existence of a NeRD element present in the upstream region at a position which lies between
the promoter and the DREs (within −248 to −818 upstream of the start site of transcription). To identify the specific region responsible for this effect, we prepared a series of constructs containing internal deletions within the remaining upstream region (Fig. 6A). TCDD-inducible luciferase activity was observed in low and high passage cells transfected with constructs pGudLuc5.2 and 5.4, while those transfected with 5.1 and 5.3 failed to induce (Fig. 6B). It is not clear why pGudLuc5.3 exhibits such high constitutive activity in the low passage cells. A likely possibility is that since the promoter already appears to be very highly active, little or no additional increase in luciferase can be observed following TCDD treatment. Overall, the deletion results indicate the high passage-specific negative regulatory region is located in the region between −2816 and −2643.

To define the negative region more precisely, small scale deletion analyses in the −2818 to −2643 bp region were generated (Fig. 7A). Transfection of high passage cultures with pGudLuc5.01 (containing a deletion of the region between −2818 and −2803 bp (Fig. 7B), revealed low levels of inducibility relative to that of pGudLuc5.0. Full TCDD inducible activity was restored when the region between −2816 and −791 bp was deleted (pGudLuc5.015). No significant increase in inducibility was observed with additional deletions. These results indicate that the negative regulatory effect in the high passage cells is modulated by a cis-acting NeRD element(s) contained within this 26-bp region. Moreover, inspection of this region suggests the presence of two NeRD elements, as shown in the alignment using upstream sequences from the mouse (32), rat (47), and human (48) CYP1A1 genes (Fig. 8). Sequence comparisons reveal a highly conserved direct repeat of CCTCCAC(C/T)(A/C)T in the rat and mouse upstream regions; limited homology was observed with the human sequence. The observation that some TCDD inducible reporter gene activity was observed with pGudLuc5.01 (where only one of the direct repeat sequences was deleted) and full inducible activity was observed with pGudLuc5.015 (where both sequences were deleted) suggest that each of these NeRD elements confers some negative regulatory activity.

The above results indicate that the cis-acting NeRD element is critical for the observed negative regulation of the CYP1A1 gene, but the mechanism and factors involved in this effect remain to be resolved. The simplest explanation of the loss of PAH responsiveness in rat epidermal keratinocytes is that the presence of a NeRD-binding protein(s) in high but not low passage cells is responsible for the negative regulatory activity. To test this hypothesis, gel retardation analysis was carried out to examine the binding of nuclear proteins from low and high passage cells to a 45-bp oligonucleotide containing the direct NeRD repeat (Fig. 9). Although several protein-DNA complexes were revealed using extracts from both passages, no unique high passage protein-DNA complex was observed. In fact, only one protein-DNA complex was demonstrated to be specific for the NeRD-containing DNA oligonucleotide (i.e. it could be com-
peted away by an excess of unlabeled NeRD-containing oligomer but not by an excess of DRE-containing oligomer) and this specific complex was present in both low and high passage nuclear extracts (Fig. 9). Additional experiments revealed no difference in protein-DNA complex formation using cytosol or nuclear extracts from Me2SO- or TCDD-treated low or high passage cells (data not shown). To determine whether the specific protein-NeRD DNA complex was more widely distributed or present only in rat epidermal keratinocytes, competitive gel retardation analysis was carried out using nuclear extracts from other cell lines. A protein-DNA complex which migrated to the same position and exhibited the same binding specificity as that observed using extracts from rat keratinocytes, competitive gel retardation analysis was carried out using nuclear extracts from other cell lines. A protein-DNA complex which migrated to the same position and exhibited the same binding specificity as that observed using extracts from rat keratinocytes, competitive gel retardation analysis was carried out using nuclear extracts from other cell lines. A protein-DNA complex which migrated to the same position and exhibited the same binding specificity as that observed using extracts from rat keratinocytes, competitive gel retardation analysis was carried out using nuclear extracts from other cell lines. A protein-DNA complex which migrated to the same position and exhibited the same binding specificity as that observed using extracts from rat keratinocytes, competitive gel retardation analysis was carried out using nuclear extracts from other cell lines. A protein-DNA complex which migrated to the same position and exhibited the same binding specificity as that observed using extracts from rat keratinocytes, competitive gel retardation analysis was carried out using nuclear extracts from other cell lines.

DISCUSSION

The loss of AHH activity in high passage rat epidermal cells appears to be due to a dramatic reduction in inducible cytochrome P-450\(^3\) and implies that the reduction in PAH metabolism is responsible for the reduction in cytotoxicity. The loss of PAH-inducible CYP1A1 expression in high passage cells is due to an alteration of one or more novel trans-acting factors that negatively regulate CYP1A1 gene expression in high passage cells and involves two novel cis-acting NeRD elements located between \(-816\) and \(-791\) bp upstream of the CYP1A1 gene. In normal Ah-responsive cells the binding of the transformed AhR complex to the DRE results in derepression of the CYP1A1 gene promoter and activation of transcription. Previous studies have demonstrated several mechanisms by which the CYP1A1 gene is negatively regulated (23–25, 35, 49–51). Putative negative regulatory elements which repress constitutive promoter activity have been identified by deletion studies (23, 25, 50, 51). In the resting cell, little or no expression of CYP1A1 is observed. However, following TCDD treatment the transformed TCDD-AhR complex accumulates within the nucleus and the binding to DREs contained within the upstream regulatory region overcomes the inhibitory effect of the negative regulatory elements, resulting in high levels of CYP1A1 promoter activity (6, 35). This inhibitory domain resides between \(-780\) and \(-680\) bp upstream of the transcriptional start site of the murine CYP1A1 gene and within the upstream regulatory region of the rat (\(-843\) to \(-746\)) and human (\(-833\) to \(-558\)) CYP1A1 genes and appears to consist of multiple negative regulatory elements (23, 25). A second level of negative regu-

\(^3\) Refer to Nelson et al. (63) for a discussion of cytochrome P-450 enzyme and gene nomenclature.
lation of CYP1A1 has been described in which inducible expression of the gene fails to occur (24, 52) because access to the DRE is blocked by a constitutively bound protein factor(s). The inability of transformed AhR complex to bind to the DRE prevents inducible expression of the CYP1A1 gene.

The negative regulatory activity identified in our studies is distinct from those described above in at least four ways: 1) our NeRD element is located further upstream than the previously identified cis-acting negative regulatory elements 2) the negative regulatory elements identified by other investigators function only to repress constitutive promoter activity and are overcome by TCDD treatment, while the region defined in our studies represses TCDD-inducible expression as well; 3) unlike the DRE-dependent mechanism of negative regulation, the region defined in our studies does not involve the DREs nor any putative DRE repressor proteins; and 4) the inhibitory effect described in our studies has been detected only in high passage rat keratinocytes, unlike previously described negative regulatory elements which function in a variety of cell types.

It is not yet clear how the NeRD elements contained within the −816 to −791 bp region exert their negative regulatory effect on the inducibility of the CYP1A1 gene, but deletion and transfection analysis has demonstrated that each of the NeRD elements exhibits some inhibitory activity on its own; full inhibitory activity required both elements. Studies in other biological systems suggest many scenarios that could explain the negative regulatory effect observed in our cells (55), the most likely of which is the silencing of expression through a direct interaction of a negative regulatory factor(s) with the NeRD element and subsequent interference with the formation or activity of the transcription machinery. Although it is possible that the NeRD-binding proteins could block or quench the functional activity of DREs themselves, they would have to negatively regulate at least 4 separate DREs which are distributed within 300 bp upstream of the NeRD element, a less likely scenario. Our gel retardation analyses, however, do not support the simplest possibility, the existence of a novel NeRD binding factor present only in high passage cells. Although it remains to be determined whether the NeRD-specific DNA binding factor identified here actually plays a role in the negative regulation of CYP1A1 gene expression, one can envision that an alteration in this factor (e.g. by phosphorylation) and/or a protein(s) to which it is associated occurs in high passage cells and is responsible for the inhibitory action. These changes would not likely be detected by the gel retardation assay used here, but would require more sophisticated analysis.

In addition to the specific factor which interacts with the NeRD element, it is not yet clear what specific nucleotides are involved in its regulatory activity. Sequence alignment of the NeRD elements reveals that they are highly conserved C-T-rich sequences (Fig. 8) which show homology to C-T-rich negative regulatory elements previously identified in the upstream regions of a number of other genes, including: insulin (54), CYP2C11 (55), glutathione S-transferase P (56) and collagen II, β-interferon, c-myc, and lysozyme (57). The specific relationship, if any, between the NeRD element identified in this report and the above elements remains to be determined. The presence of two similar C-T-rich silencer elements in the upstream region of the CYP2C11 gene (55) could provide a possible explanation for the observed decrease in constitutive AHH activ-
ity we observe in high passage cells, since this enzyme has been shown to be a major contributor to this activity (58). One can envision that increased activity/potency of negative regulatory factors in late passage cells could result in greater repression of this gene and a decrease in constitutive AHH activity, although this remains to be confirmed.

In addition to CYP1A1, we have observed that TCDD-inducible expression of the CYP1B1 gene is also negatively regulated in high passage cells (Fig. 3B). These results combined with the observed decrease in constitutive AHH activity suggest that this negative regulatory mechanism is not specific for CYP1A1 but may be a more general response in these cells. Although it remains to be determined whether other members of the Ah gene battery are also negatively regulated, analysis of the upstream regulatory sequences of the rat glutathione S-transferase Ya (59), quinone reductase (60), and aldehyde dehydrogenase-3 (61) have revealed several C-T-rich regions with homology to the NeRD element. Comparison of the inducible expression of these genes in low and high passage cultures will provide insight into the specificity/generality of this negative regulatory response.

Coincident with the loss of PAH sensitivity in high passage rat epidermal keratinocytes are a variety of changes in gene expression associated with cellular transformation, such as increased colony forming efficiency and decreased feeder layer dependence (27). A linkage of the loss of PAH sensitivity to immortalization and gradual loss of differentiation characteristics seems possible. In fact, studies in mouse and human epidermal keratinocytes (29, 31) demonstrated that inducible expression of CYP1A1 is dependent upon the cellular differentiation state of the cells, with those committed to terminal differentiation as being responsive to the inducing effects of TCDD. Thus, the negative regulatory effect observed in high passage rat keratinocytes could reflect a change in the differentiated characteristics of these cells and raise the possibility that the NeRD element and/or its associated binding protein(s) may have a role in regulating expression of other genes affected by changes in the cellular differentiation state. Determining the involvement of NeRD elements in the differentiation induced changes in CYP1A1 in mouse and human keratinocytes...
from the indicated cells were incubated with a32P-labeled NeRD-con
epidermal cultures, Dr. Colin R. Jefcoate for the mouse CYP1B1 probe,
function of the NeRD element and its binding factor(s) may
changes in expression of other genes. Thus, characterizing the
and approaches described here. Such experiments may even
could readily be accomplished using the reporter constructs
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position of the specifically bound protein-DNA complex.

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