Dioxin Activates HIV-1 Gene Expression by an Oxidative Stress Pathway Requiring a Functional Cytochrome P450 CYP1A1 Enzyme

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We have studied the effect of several environmental chemicals on the transient expression of a chloramphenicol acetyltransferase (*cat*) reporter gene linked to the promoter sequences in the long terminal repeat (LTR) of the human immunodeficiency virus type 1 (HIV-1). Aflatoxin B_1, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; dioxin) and benzo[*a*]pyrene cause a significant increases in CAT expression in mouse hepatoma Hepa-1 cells. The induction of CAT after TCDD treatment is abolished by administration of N-acetyl-L-cysteine or 2-mercaptoethanol and does not take place in a mutant cell line that lacks CYP1A1 enzymatic activity. Linker-scanning mutational analysis of transcription factor binding sites in the promoter revealed that both the NFκB and an adjacent aromatic hydrocarbon response element (AhRE) are required for TCDD-dependent CAT expression. In addition, mutation of the NFAT/AP-1 binding sites in the negative regulatory region of the promoter increases the magnitude of the TCDD effect. We conclude that induction of a functional CYP1A1 monooxygenase by TCDD stimulates a pathway that generates thiol-sensitive reactive oxygen intermediates which, in turn, are responsible for the TCDD-dependent activation of genes linked to the LTR. These data might provide an explanation for findings that TCDD increases infectious HIV-1 titers in experimental systems and for epidemiologic reports suggesting that exposure to aromatic hydrocarbons, such as found in cigarette smoke, is associated with an acceleration in AIDS progression. *Key words*: benzo[*a*]pyrene, chloramphenicol acetyltransferase, c37, CYP1A1, dioxin, Hepa-1 cells, HIV-1, TCDD. *Environ Health Perspect* 103:366–371 (1995)

Halogenated aromatic hydrocarbons such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; dioxin) cause a profusion of apparently unrelated toxic effects in which the single common denominator is the aromatic hydrocarbon receptor-mediated transcriptional activation of the cytochrome P450 *CYP1A1* gene (*1–6*). In humans, exposure to dioxin and various other chlorinated phenolic agents causes chloracne, a long-lasting skin disease characterized by the hyperkeratinization of follicular sebocytes (*7,8*). In addition, recent long-term epidemiological studies have established a link between exposure to high doses of TCDD and certain types of cancers (*9,10*). Dioxin is one of the strongest tumor promoters ever tested in animal model systems; it causes an elevated incidence of hepatic carcinoma and pulmonary and skin tumors (*11–13*) and promotes tumor formation at one-hundredth the dose of the classical tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in the skin of hairless mice (*14–16*). During rodent embryogenesis, TCDD administration also causes craniofacial abnormalities such as cleft palate and hydronephrosis (*17–20*). Characteristic events of secondary palate formation, such as osteoblast differentiation and synthesis and mineralization of extracellular matrix, are inhibited by TCDD (*21*). Unlike in whole animal studies, TCDD has no toxic effect in tissue culture cells, although it causes a large elevation of intracellular calcium, which induces decreased ß-adrenergic responsiveness in cardiac myocytes (*22,23*), and causes apoptosis of immature thymocytes (*24,25*). In this regard, the developing immune system is a particularly sensitive target for TCDD, with thymic atrophy being the most common pathological consequence of exposure (*26*).

Work from our laboratory has shown that treatment of mouse hepatoma cells with polycyclic or halogenated aromatic hydrocarbons such as TCDD and benzo[*a*]pyrene (BaP) causes an increase in the steady-state mRNA levels of the proto-oncogenes c-*fos*, c-*jun*, *jun-B*, and *jun-D* and the concomitant increase of the DNA-binding activity of the transcription factor AP-1 (*27*). These results suggested the possibility that other transcription factors might also be activated by TCDD treatment and that genes which contain binding sites for these transcription factors in their regulatory domains might respond to TCDD or BaP treatment. We tested this hypothesis in mouse hepatoma cells by analyzing the effect of TCDD treatment on the activation of a chloramphenicol acetyltransferase (*cat*) reporter gene fused to the long terminal repeat (LTR) sequences of the human immunodeficiency virus-1 (HIV-1).

Materials and Methods

Aflatoxin B_1 was a gift of Howard G. Shertzer, and TCDD was a gift of the Dow Chemical Company; all polycyclic aromatic hydrocarbons used were purchased from the National Cancer Institute Chemical Carcinogen Repository. The mouse cell lines used in these studies were the wild-type Hepa-1 hepatoma line (*28*) and its CYP1A1 metabolism-deficient derivative, c37 (*29–31*), a variant that carries two missense mutations in the *Cyp1a1* gene, rendering the resulting enzyme nonfunctional (*31*). These cells were grown in α-minimal essential media supplemented with 5% fetal bovine serum.

The bacterial chloramphenicol acetyltransferase (*cat*) gene was used as a reporter in transient transfection experiments. The chimeric plasmid pBennCAT, carrying a fusion of the *cat* gene sequences to the HIV-1 U3 LTR was obtained from the National Institutes of Health AIDS Research and Reference Program. This plasmid contains approximately 500 base pairs of uncharacterized human DNA sequences (*32*), which were removed by standard recombinant DNA techniques, giving rise to plasmid pHIVLTRCAT. Several plasmid constructs carrying mutations in the transcription factor binding sites in the LTR were derived from the wild-type pHIVLTRCAT by linker-scanning mutagenesis (*33*). The sequence of the relevant portion of the U3 LTR is shown in Figure 1; the sequences that were replaced indicated by a single overline. In all cases, 10 or 30 nucleotide residues (1 or 3 helical turns) were replaced by 10 residues (one helical turn), thus preserving the relative position of the unaffected binding sites on the DNA helix. Mutagenesis was carried out by polymerase

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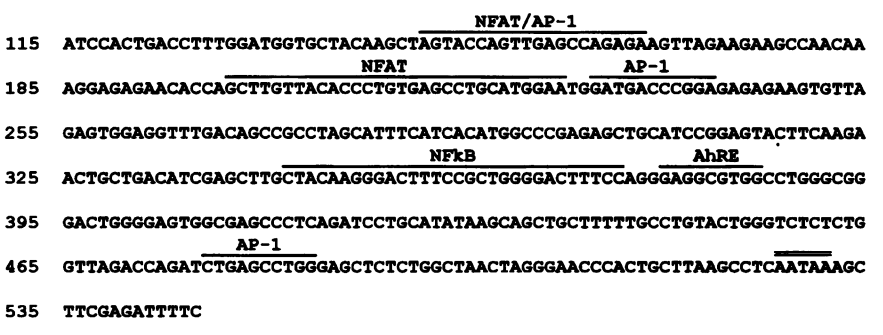

Figure 1. Transcription factor binding sites in the human immunodeficiency virus-1 long-terminal repeat. The sequence shown is from GenBank locus *hivhxb2cg*. Transcription factor binding sites, indicated by the overline, were determined using the transcription factor site database (TFSITES) in combination with the MAP algorithm of the DNA Analysis Package (Genetics Computer Group, Madison, Wisconsin). The double overline indicates the TATA box; transcription initiation is at residue 547. The three contiguous Sp1 binding sites located between residues 376 and 416 are not highlighted.

chain reaction (PCR) of the wild-type plasmid using primers complementary to the flanking sequences of the site to be deleted and containing a *Not*I restriction enzyme site sequence at their 5′ end. The PCR products were digested with *Not*I, ligated, and used to transfect electrocompetent *E. coli* DH5α. In mutant pLS1, the site replaced was the composite NFAT/AP-1 binding site located between residues 146 and 165. Mutants pLS2 (NFAT site at 200–230), pLS3 (AP-1 site at 233–243), and pLS4n (two NFκB sites at 344–374) were constructed in a similar manner. An *Xho*I site was used instead of *Not*I for mutant pLS4a (AhRE site at 378–388), and an *Xba*I site was used for mutant pLS5 (AP-1 site at 477–487). Double and triple mutants were constructed from the single mutants by *in vitro* recombinant techniques using recombination of fragments generated by appropriate restriction enzymes. All constructs were confirmed by restriction enzyme analysis and DNA sequencing.

Approximately 10 μg of the appropriate plasmid was transfected by standard calcium phosphate techniques (*34,35*) into semiconfluent Hepa-1 or c37 cells. In some experiments, to determine the effect of the viral transactivator Tat protein (*36,37*), we co-transfected the cells with plasmid pCV-1 (obtained from the NIH AIDS Research and Reference Program) that expresses HIV-1 Tat under the control of the SV40 early promoter and enhancer. We found that Tat expression increased basal CAT levels and induced CAT levels by approximately the same magnitude; all the experiments reported here were done in the absence of Tat expression. As a negative control we used pSV0CAT, containing a promoterless *cat* gene, and as a positive control we used pSV2CAT, carrying the *cat* gene under the control of the SV40 early promoter and enhancer sequences. In initial experiments, we also used TPA treatment as a positive control for HIV LTR-dependent expression. To control for variations due to differences in transfection efficiency, all cultures were co-transfected with plasmid pCMVßgal (CloneTech, Palo Alto, California), which expresses the bacterial ß-galactosidase gene under the control of the cytomegalovirus immediate-early enhancer and promoter. Expression of ß-galactosidase under regulation by this enhancer is independent of treatment to the cells. Twelve to 16 hr after transfection, the cells were fed with low serum (0.1%) α-minimal essential media to deplete preexisting transcription factors that respond to stimulation by serum, and 24–48 hr later the cells were treated with TCDD or other compounds or with an equivalent amount of dimethylsulfoxide vehicle. We prepared cell extracts 18 hr later by three cycles of freeze–thawing, and expression of CAT and ß-galactosidase activities was determined. We measured CAT activity by the phase extraction method (*38*) using 0.2 μCi of [^{14}C]chloramphenicol (Amersham, Arlington Heights, Illinois) as the substrate. Chloramphenicol conversion to acetylated forms was 1–25%, well within the linear range [0–50% (*38*)] of the assay. We determined ß-galactosidase activity using a kit from Promega BioTech (Madison, Wisconsin). Data were normalized for differences in transfection efficiency by determination of the relative amount of chloramphenicol converted to acetylated forms per unit of ß-galactosidase. Experiments were repeated at least three times, and the values shown are the means ± SEM.

Results

We first tested the effect of aflatoxin B_1, TCDD, and several polycyclic aromatic hydrocarbons on transient CAT expression directed by the wild-type HIV-1 LTR. Mouse hepatoma Hepa-1 cells were transfected with pHIVLTRCAT; 24 hr after transfection, the cells were treated with the compounds indicated in Figure 2. Two of these compounds, aflatoxin B_1 and BaP, caused approximately a fivefold increase in CAT activity over the dimethylsulfoxide control. Four others, anthracene, benzo[*a*]anthracene, TCDD, and 3-methylcholanthrene, were next in potency, causing a two- to threefold increase in CAT activity. The effects of the last two, dimethylbenzanthracene (DMBA) and dibenzo[*a,h*]anthracene (DBA), were not significantly different from the control (Fig. 2). In initial control experiments, we estimated that the effect of BaP on CAT expression was approximately 50% of maximal activation obtained with TPA (data not shown).

The HIV-1 LTR is a regulatory domain approximately 500 bases long that contains several sequence motifs recognized by cellular transcription factors. These recognition motifs include binding sites for AP-1, AP-2, AP-3, AP-4, NFAT-1, USF, URS, NFκB (*39,40*), as well as the TAR sequence, recognized by the viral Tat protein (*37*). The initial rate of proviral transcription is determined by interactions between these transcription factors and their cognate sequences in the LTR (*36,37,39–42*). Since TCDD induces AP-1 activity (*27*), one possible explanation for the stimulation of CAT activity by TCDD was that it resulted from TCDD-induced increases in AP-1. Alternatively, CAT stimulation could be due to activation of transcription factors other than AP-1. To address this question and to analyze which transcription factor binding sites were responsible for CAT induction by TCDD, we prepared a collection of single, double, and triple mutant derivatives of the reporter plasmid and measured CAT expression directed by these mutants in transient expression assays. Mutation of the proximal AP-1 in LS5 had no effect on basal expression levels but caused an increase in TCDD-dependent CAT expression, whereas mutation of the distal NFAT and AP-1 sites in LS1, LS2, and LS3 had a negligible effect on both basal and TCDD-induced CAT expression (Fig. 3). Mutation of the NFκB sites in LS4n or of the cryptic AhRE site in LS4a almost completely abolished basal and TCDD-stimulated CAT expression (Fig. 3). Double mutations caused diverse effects: LS12 abolished TCDD induction and increased basal expression slightly, whereas LS15 and LS35 showed both elevated basal expression levels and absence of TCDD stimulation; LS23 and LS25 had low basal levels and a TCDD stimulation factor of 6- to 7-fold (Fig. 3 and Table 1). The LS123 triple mutant exhibited extremely elevated basal as well as TCDD-stimulated

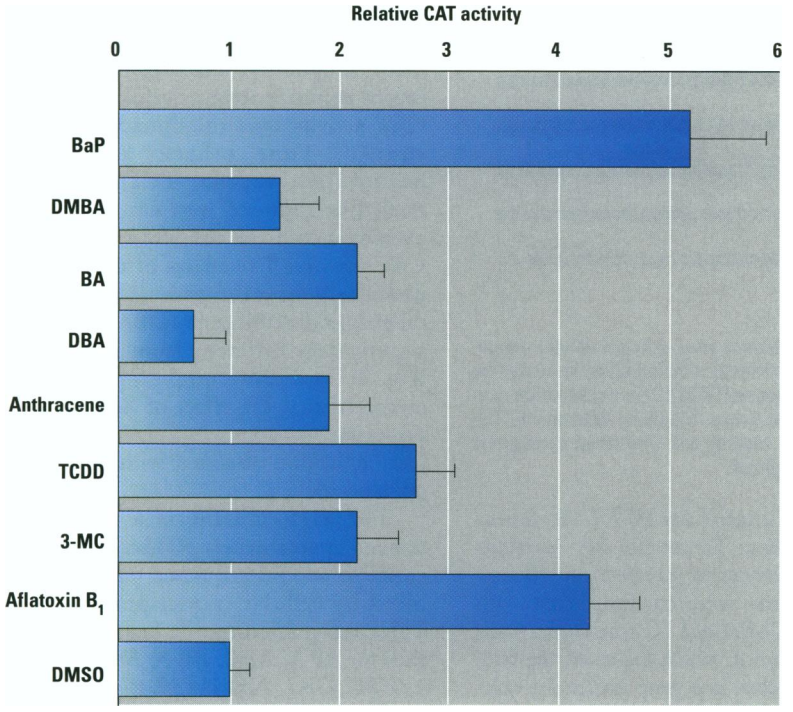

Figure 2. Activation of pHIVLTRCAT expression by various foreign chemicals. Forty-eight hours after transfection, cells were treated for 24 hr with the following compounds: BaP: 10 μM benzo[*a*]pyrene; DMBA: 20 μM 7,12-dimethylbenzo[*a*]anthracene; BA: 20 μM benzo[*a*]anthracene; DBA: 20 μM dibenzo[*a,h*]anthracene; anthracene: 20 μM anthracene; TCDD: 15 nM TCDD; 3-MC: 30 μM 3-methylcholanthrene; aflatoxin B_1: 100 μM aflatoxin B_1; DMSO: dimethylsulfoxide vehicle control at a final concentration of 0.1%. Stocks of all compounds were prepared as 1000-fold concentrated solutions in DMSO to ensure that in all cases the DMSO concentration in the cultures did not exceed 0.1%. The extent of chloramphenicol conversion ranged between 3 and 25% and was normalized to ß-galactosidase activity. The values shown are relative to those of the DMSO control.

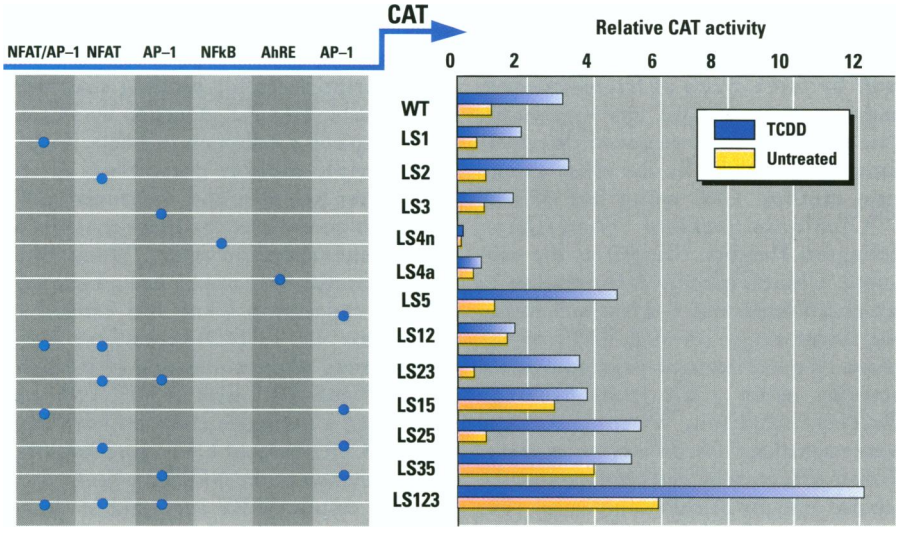

Figure 3. Effect of TCDD on CAT expression directed by linker-scanning mutants. The diagram on the left shows the approximate position of the mutated sites, with the individual mutations denoted by a blue circle (see Figure 1 for the actual coordinates and the text for a complete description of each mutated site). Relative CAT activity values were determined as indicated in Figure 2.

levels, but the extent of induction was not significantly different than in the wild-type (Fig. 3 and Table 1). These results indicate that the NFκB sites mutated in LS4n are required for basal and TCDD-stimulated CAT expression. Furthermore, a previously unrecognized Ah receptor binding site, the canonical GCGTG AhRE site at position 380 mutated in LS4a, was also found to be essential for expression in hepatoma cells. In addition, the region between residues 115 and 255 that contains the NFAT and AP-1 sites appears to dampen the stimulation by TCDD because mutation of these sites resulted in increased levels of CAT activity after TCDD treatment. This region is known to contain negative regulatory elements for HIV-1 expression (*39,40*). The proximal AP-1 site mutated in LS5 seems to behave in a similar fashion because its absence increases the effect of TCDD. The values for the fold induction by TCDD for the different mutants tested are shown in Table 1 and are discussed in more detail in the next section.

Table 1. Induction by TCDD of CAT activity directed by HIV long-terminal repeat linker-scanning mutants

Mutant	Fold induction[a]
WT	3.2
LS1	2.8
LS2	3.7
LS3	2.3
LS4n	2.0
LS4a	1.5
LS5	4.3
LS12	1.2
LS23	6.6
LS15	1.3
LS25	6.1
LS35	1.3
LS123	2.1

[a]The values shown are calculated from the data in Figure 3.

The involvement of an AhRE site on CAT expression suggested the possibility that the Ah receptor and a TCDD-inducible cytochrome P450 CYP1A1 enzyme might participate in the stimulation of CAT expression observed after TCDD treatment. We tested this hypothesis by comparing the transient expression of CAT activity directed by the pHIVLTRCAT plasmid in wild-type Hepa-1 cells and in the c37 derivative that lacks CYP1A1 enzymatic activity. If CYP1A1 activity were involved in stimulation of CAT expression by TCDD, this stimulation would not take place in cells lacking the CYP1A1 enzyme. As shown in Figure 4, this expectation was correct; stimulation of CAT activity was found at normal 2- to 2.5-fold levels in Hepa-1 cells, but was absent in the c37 derivative.

These results hinted at the possibility that oxidative stress mediated by TCDD-inducible CYP1A1 activity could be responsible for the effect of TCDD on LTR-directed CAT expression. To determine whether thiol-sensitive reactive oxygen species were involved in this effect, pHIVLTRCAT-transfected cells were grown in the presence of various concentrations of *N*-acetyl-L-cysteine (NAC) or 2-mercaptoethanol prior to treatment with TCDD for 16 hr and determination of CAT activity. We observed a clear decrease of TCDD stimulation of CAT activity

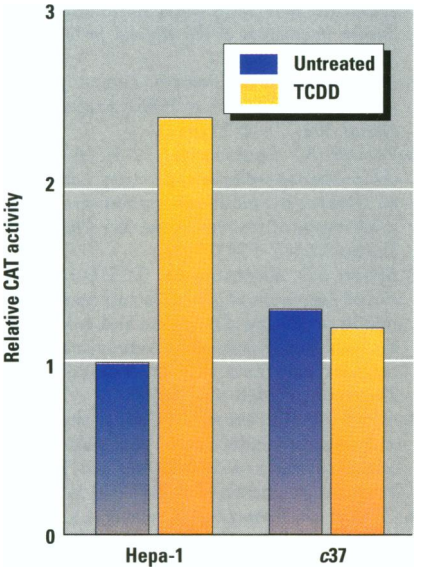

Figure 4. TCDD-dependent CAT expression in wild-type and CYP1A1-deficient cells. Forty-eight hours after transfection, wild-type Hepa-1 cells and their CYP1A1-deficient variant, c37, were treated with TCDD or left untreated. The activities of CAT and ß-galactosidase were measured 24 hr later. The values shown are relative to the ratio of CAT/ß-galactosidase activities in untreated Hepa-1 cells.

with increasing doses of either compound. At a dose of 1 mM NAC or 20 µM 2-mercaptoethanol, the effect of TCDD was completely abolished (Fig. 5), suggesting that, indeed, CYP1A1-dependent oxidative stress might be responsible for the effect of TCDD on LTR-directed CAT expression.

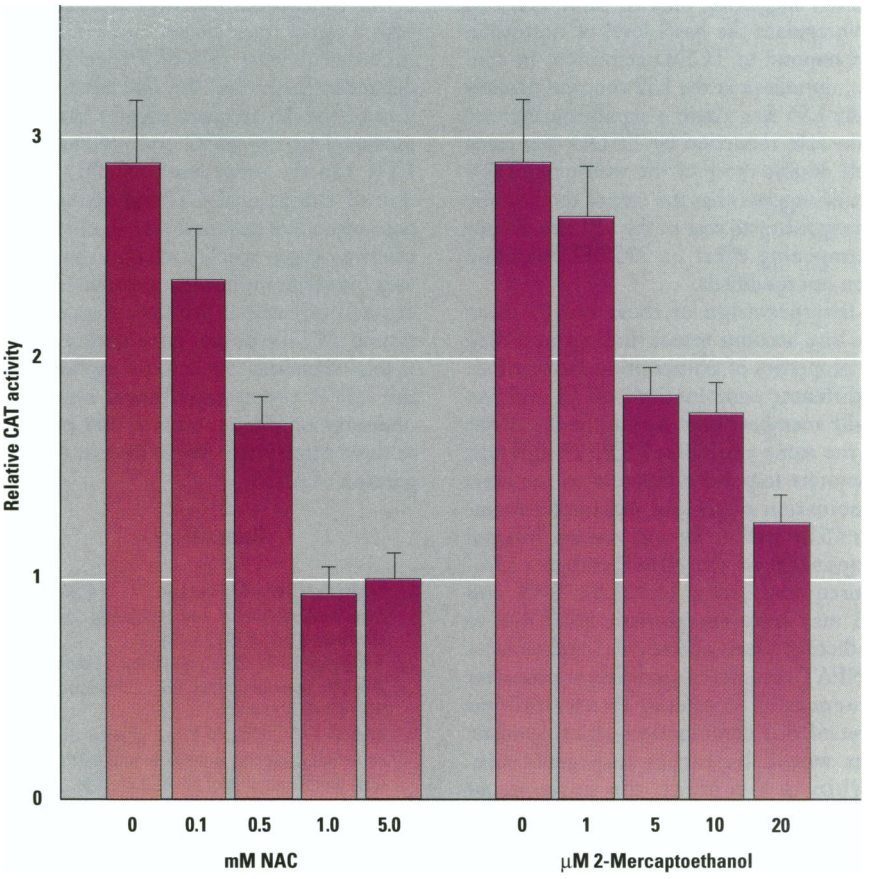

Figure 5. Inhibition of TCDD-dependent CAT activation by *N*-acetyl-L-cysteine (NAC) and 2-mercaptoethanol. Thirty to 60 min before TCDD treatment, NAC or 2-mercaptoethanol was added to the medium at the concentrations indicated and maintained throughout the duration of the TCDD treatment. The values shown are relative to the CAT activity in transfected cells not treated with TCDD.

Discussion

The results that we present in this article indicate that TCDD, aflatoxin B_1, and several polycyclic aromatic hydrocarbons (PAHs) can significantly activate the expression of genes linked to the LTR sequences of HIV-1. The magnitude of the activation appears to be different for the various compounds tested. In the case of TCDD, stimulated values are significantly higher than control values, although they do not usually exceed them by more than 2.5- to 3-fold. This stimulation is in agreement with observations by others that TCDD can cause an increase of infectious HIV-1 titers in experimental systems (*43,44*). As for PAHs, the highest levels of CAT activation that we observed were a result of BaP treatment, a finding that may provide a possible molecular explanation for the observation that cigarette smoking accelerates the progression of AIDS (*45–48*). It is, of course, likely that the effect of cigarette smoke on AIDS progression results from a combination of many different causes, of which gene activation by BaP is only one. Surprisingly, DBA had no effect on CAT expression, a finding that we cannot explain at present.

Using linker-scanning mutational analysis, we have identified several domains of the HIV-1 LTR responsible for basal as well as TCDD-stimulated CAT expression. We find that expression directed by the HIV-1 LTR is high in mouse hepatoma cells, in agreement with previous observations in human hepatoma cell lines (*49–51*), this suggests that the liver may be a primary virus reservoir. Mutation of the NFκB binding sites eliminates CAT expression, confirming the absolute requirement for NFκB. NFκB, however, is not the only transcription factor necessary for expression; we have uncovered an Ah receptor response element containing the canonical AhRE sequence GCGTG, which is also essential for basal expression. In addition, this site participates in the effect of TCDD on CAT activation because its mutation in pLS4a reduces drastically the fold induction by TCDD (Table 1). This AhRE site is embedded within the first of three adjacent Sp1 sites, which have been shown to interact cooperatively with NFκB in HIV enhancer activation (*52*). It could be argued that Sp1, and not the Ah receptor, was the transcription factor responsible for the loss of activity of pLS4a because both binding sites would be equally affected by the mutation. This possibility is unlikely because mutations in just one of the three Sp1 binding sites have little or no effect on HIV enhancer expression (*40*); this suggests that the Ah receptor, and not Sp1, is the relevant transcription factor whose binding and subsequent activity are affected by the LS4a mutation. As shown by the mutagenesis analysis, both NFκB and Ah receptor binding sites are responsible for the basal expression levels, and it is possible that both transcription factors function in synergy.

The NFAT and AP-1 binding sites in mutants LS1, LS2, and LS3, clustered in the negative regulatory region of the LTR, do not show a major effect on expression when altered individually. In double mutants that include LS1, as well as in the triple mutant LS123, basal level of expression is elevated, suggesting that the NFAT/AP-1 site at 146–165 is responsible for downregulating the basal level of expression. This is in agreement with earlier findings that this region of HIV-1 contains negative regulatory elements (*39,40*). As shown in Table 1, these double mutants and the triple mutant LS123 also show a low level of induction by

TCDD, suggesting that the same sites that downregulate the basal level of expression may respond to TCDD activation. In contrast, mutations at the LS2 site, and possibly at the LS5 site, cause a significant increase in the fold induction by TCDD that reach levels double those of the wild-type (Table 1); this suggests that the role of the LS2 site is antagonistic to that of the LS1 site, having a dampening effect on TCDD induction when not modified.

Interpretation of these results must take into account recent findings regarding the properties of transcription factor AP-1: 1) different combinations of *fos* and *jun* family members have very different effects on the same promoter (*53*); 2) different promoters respond differently to the same combination of *fos* and *jun* family members (*53*); and 3) *fos* and *jun* are integral components of the NFAT complex (*54*). Consequently, sites LS1, LS2, LS3, and LS5 may have antagonistic roles due to conflicting effects of free AP-1 and of AP-1 in NFAT complexes on different promoter sequences. The overall transcriptional effect of this combination of antagonistic sites would be rather unpredictable. Within this context, the outcome of TCDD exposure is likely to result from a combination of two opposing effects; on one hand, activation of expression may take place by means of the Ah receptor, the AhRE site, and a particular set of *fos/jun* members with positive effects on LTR expression. On the other hand, dampening of this induction of expression may occur by activation of other *fos/jun* members with negative regulatory functions.

TCDD toxicity has been proposed to result from epoxides and other derivatives of arachidonic acid metabolism catalyzed by TCDD-induced cytochrome P450 enzymes (*23,55,56*). In agreement with this hypothesis, we find that stimulation of CAT expression by TCDD is absent in the variant cell line c37 that lacks cytochrome P450 CYP1A1 activity, strongly suggesting that the effect of TCDD is mediated by the monooxygenase activity of CYP1A1. This activity might generate arachidonate metabolites responsible for the elevation of the pro-oxidant status of the cell, and indeed several cytochromes P450, including the TCDD-inducible CYP1A1 and CYP1A2 enzymes (*57–59*) and others (*60,61*), possess arachidonic acid epoxygenase activity. Our experiments, although not directly aimed at the identification of possible mediators, show that NAC and 2-mercaptoethanol eliminate the effect of TCDD, indicating that, as shown for NFκB activation (*62–64*), oxidative stress caused by thiol-sensitive reactive oxygen species is likely to be involved in the TCDD-dependent activation events.

In conclusion, our data are consistent with a signal transduction mechanism that includes at least two different TCDD-dependent pathways. On one hand, activation of the Ah receptor triggers expression mediated by the AhRE site present in the LTR. On the other hand, TCDD induction of a functional CYP1A1 monooxygenase stimulates generation of thiol-sensitive reactive oxygen species, which in turn activate transcription factors operative in LTR-directed expression. We find that, in addition to TCDD, several other toxic environmental chemicals can activate expression of the HIV-1 promoter-enhancer sequences, underscoring the importance that exposure to these compounds might have in the progression of AIDS.

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