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Identification of a Region within the Human Immunodeficiency Virus Type 1 Long Terminal Repeat That Is Essential for Transactivation by the Hepatitis B Virus Gene X

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Hepatitis B virus (HBV) X-gene product activates transcription of the chloramphenicol acetyltransferase (CAT) gene under control of the human immunodeficiency virus type 1 (HIV-1) long terminal repeat (LTR). To identify a *cis*-acting regulatory sequence within the HIV-1 LTR which is responsive to the HBV X-gene *trans*-activating function, we examined the effects of HBV X-gene expression in cells with a series of LTR/CAT deletion mutants. A region of the HIV-1 LTR containing the previously identified κB-like enhancer element was found to be responsive to HBV X-gene activation, and this effect was independent of, and additive with, the effect of the HIV-1 tat-III protein on CAT expression. Since κB-like enhancer sequences are known to regulate transcription of a variety of viruses and cellular genes, our results suggest that the X gene could activate such a gene during HBV infection and replication.

Hepatitis B virus (HBV) is a small DNA virus with only four recognized genes. A viral DNA replication mechanism (which includes a reverse transcription step and genome sequence homologies) suggests a phylogenetic relationship with retroviruses. Three HBV genes are known to encode virion structural proteins, but the role of the fourth and smallest gene (the X gene) during HBV infection is not clear. Recently, it was shown that the HBV X gene can activate transcription of genes under the control of β-interferon gene regulatory sequences (23), the complete human immunodeficiency virus type 1 (HIV-1) long terminal repeat (LTR) (22a), and a simian virus 40 virus DNA fragment containing a viral enhancer (22a). The HIV-1 LTR was also activated in HepG2 cells constitutively producing HBV particles and not in uninfected control HepG2 cells, suggesting that the X gene is expressed during this HBV infection (22a).

Several factors have been identified which appear to act on *cis*-acting sequences in the HIV-1 LTR in regulating HIV-1 gene expression. Certain HIV-1 genes appear to have regulatory functions. One of these is the transactivator gene (*tat*) the action of which requires a specific *trans*-activator responsive (TAR) sequence contained in the HIV-1 LTR (21). A far-upstream *cis*-acting negative regulatory element appears to respond to the viral *nef* gene product to inhibit viral transcription (1). In addition, several exogenous factors, including mitogens, phorbol esters (13, 25), and certain heterologous viruses (10, 19, 20, 22, 22a), have been shown to activate HIV-1 transcription. Phorbol esters appear to activate cellular transcription factors which act on a κB-like enhancer sequence in the HIV-1 LTR (15).

Here, we identify a 5′ upstream sequence within the HIV-1 LTR that appears to be necessary for activation by the HBV X gene. HepG2 cells were used for these experiments to facilitate the use of plasmid constructs with the HBV X gene under control of the HBV enhancer and X-gene promoter, which have liver cell requirements for optimal function, and because an ultimate goal of our research is to understand the role of the X gene during HBV infection of liver cells.

To investigate specific regions of the HIV-1 LTR for responsiveness to the HBV X gene, deletion mutants used previously to identify sequences in the HIV-1 LTR required for *trans*-activation by the *tat*-III product (21) were used. HepG2 cells were cotransfected with each deletion mutant separately with the plasmid pTWU17, which contains the HBV X gene under control of the HBV enhancer and X-gene promoter (23). HepG2 cells were used because the HBV enhancer and promoters exhibit specificity for liver cells of human origin and because activation of the HIV-1 LTR by the HBV X gene was previously shown in HepG2 cells (22a). The intact HIV-1 LTR/CAT hybrid gene (−423/+80) and its 5′ deletion mutants lacking 256 nucleotides (deletion −167/+80), 303 nucleotides (deletion −120/+80), and 319 nucleotides (deletion −104/+80) were all activated by the HBV X gene (Fig. 1). However, when the deletions from the 5′ end of the HIV-1 LTR extended to −57 and −45 base pairs upstream from the transcription initiation site, resulting in loss of HIV-1 LTR enhancer sequences (21), activation of chloramphenicol acetyltransferase (CAT) activity by the HBV X gene was completely abolished. The −167/−17 and −167/+21 mutants, deleted from the 3′ *tat*-III TAR element (21), were responsive to the HBV X gene. The −167/+45 mutant, which retains both the enhancer sequences and the TAR region of the HIV-1 LTR, was activated by the HBV X gene. We conclude that the HIV-1 enhancer sequences or closely adjacent sequences in the HIV-1 LTR are critical for *trans*-activation of HIV-1 by the HBV X gene.

We have previously demonstrated that the HIV-1 LTR is activated in a cell line, HepG2T14, constitutively producing HBV and not in HepG2 cells without HBV (22a). The HIV-1 LTR mutants were tested for the ability to express CAT in HepG2T14 cells. The results were consistent with the findings shown in Fig. 1 with pTWU17 in that all the HIV-1 LTR deletion mutants except −57/+80 and −45/+80 were *trans*-activated by the HBV X gene (Fig. 2). These results indicate that the HIV-1 LTR-directed gene expression can be acti-

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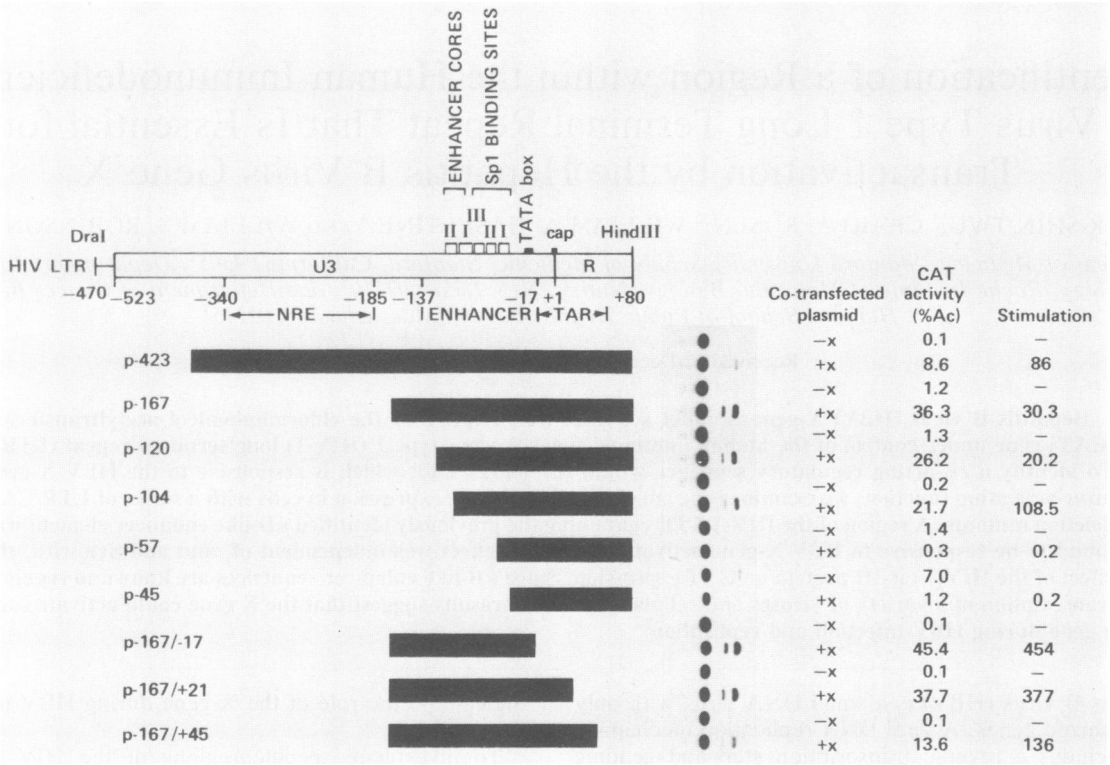

FIG. 1. Effect of the HBV X gene under control of the HBV enhancer and X-gene promoter on HIV-1 LTR deletion mutants in HepG2 cells. Cells (10^6) were transfected with 1 μg of each deletion mutant and 10 μg of pUC9 (without X gene [−x]) or pTWU17 (with X gene [+x]) (23) by the calcium phosphate method of Wigler et al. (24). Cells were harvested 44 to 48 h after transfection, and CAT assays were performed essentially as described by Gorman et al. (12). CAT assays were carried out by using the same amount of total protein for each sample within individual experiments (5). Spots corresponding to nonacetylated and acetylated forms of [^{14}C]chloramphenicol recovered from thin-layer chromatography plates were counted in a liquid scintillation counter to quantitate CAT activity. The *trans*-activation of each deletion mutant by the HBV X gene was expressed as the ratio of chloramphenicol conversion by extracts from cells transfected with the HIV-1 deletion mutant plus pTWU17 (with X gene [+x]) and with the mutant plus pUC9 (without X gene [−x]). In three repeats of each experiment, results differed by no more than 10%. The schematic representation of the HIV-1 LTR (above the deletion map) shows the positions of the RNA cap site (designated +1), a negative regulatory element, two enhancer core sequences, three binding sites for transcription factor Sp1, the TAR sequence which is responsive to the HIV-1 *trans*-activator protein (tat), and a TATA box.

vated in cells with replicating HBV presumably via the HBV X-gene product and that a region of the HIV-1 LTR containing the viral enhancer sequence is necessary for *trans*-activation by the HBV X gene in these cells.

It was recently shown that the HBV X-gene product and the HIV-1 *tat*-III protein have an additive or cooperative effect in activating the HIV-1 LTR (22a). The HIV-1 LTR deletion mutants were used to further investigate this cooperative effect. HepG2 cells were cotransfected with each of the HIV-1 LTR deletion mutants separately with pTWU17, pcDEBtat (an HIV-1 *tat*-III gene construct) (9), or pTWU17 and pcDEBtat. The cooperative effect of the HBV X gene and the HIV-1 *tat*-III gene was prominent on the HIV-1 LTR deletion mutants −423/+80, 167/+80, −120/+80, and −104/+80 (Table 1). However, this effect was abolished when an HIV-1 LTR region containing the HIV-1 enhancer was deleted (−57/+80 and −104/+80 mutants) but not when the TAR element (−167/−17 and 167/+21 mutants) was deleted. These results suggest that the corporation between the HBV X-gene product and the HIV-1 tat-III protein requires the HIV-1 transcriptional enhancer region and that the effect is unlikely to involve a posttranscriptional event.

These results demonstrate that an HIV-1 LTR region containing the viral enhancer is required for *trans*-activation by the HBV X gene and for a cooperative effect of the HBV X-gene product and the HIV-1 tat-III protein in augmenting HIV-1 LTR-directed gene expression. Our findings are not in complete agreement with those of Seto et al. (22), who suggest that multiple *cis*-acting sites, including far-upstream, enhancer, and TAR regions within the HIV-1 LTR, are required for full response to the HBV X-gene *trans*-activating function. We found that deletion of the negative regulatory element and the TAR element from the HIV-1 LTR did not affect its responsiveness to the HBV X-gene *trans*-activating function although as expected TAR deletion abolished responsiveness to the HIV-1 *tat*-III gene product. The magnitude of *trans*-activation of the HIV-1 LTR by the HBV X protein was not more than 10-fold in their experiments. The discrepancy between our findings and those of Seto et al. could be due to a number of differences in the two studies. First, the X-gene constructs used by the two laboratories contained different regulatory elements (native HBV promoter and enhancer versus simian virus 40 early promoter and enhancer) which could have resulted in different patterns or levels of X-gene expression in the recipient cells used. Second, different cells were used to test X-gene *trans*-activation on the HIV-1 LTR, and it is well known that different regulatory sequences function differently in different cells (11). Finally, other experimental conditions, such as the transfection methods used, were different.

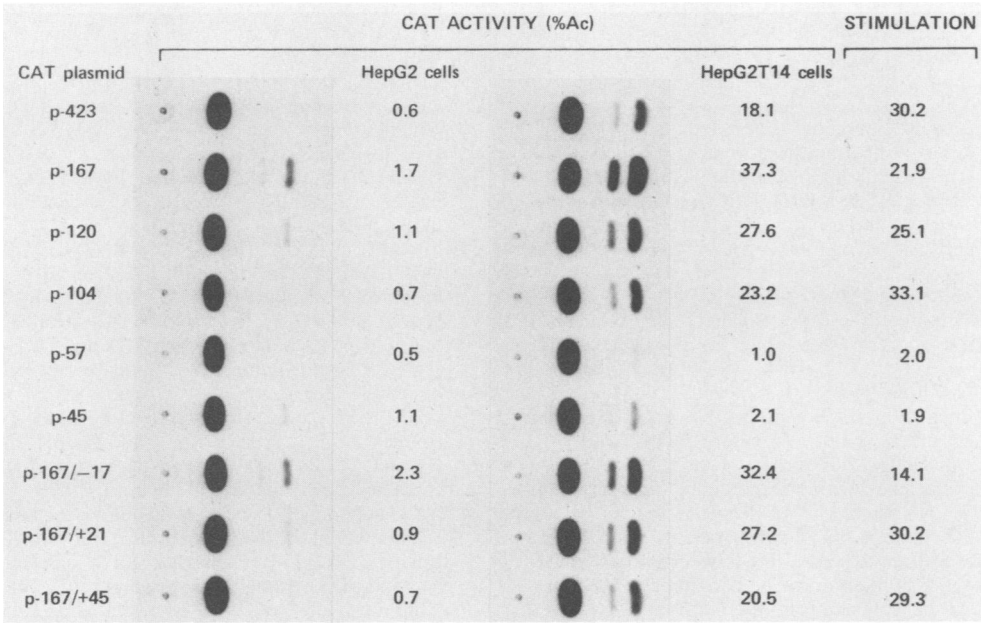

FIG. 2. Effect of the HBV X gene under control of the HBV enhancer and X-gene promoter on HIV-1 LTR deletion mutants in HepG2 cells and in HBV DNA-transfected HepG2 cells (HepG2T14). HepG2 or HepG2T14 cells (10^6) were transfected with 1 μg of each deletion mutant and 10 μg of pUC9. After 44 to 48 h, cells were harvested and assayed for CAT activity. Ac, Acetyltransferase.

The function of the HBV X gene during HBV infection and replication is not understood. The importance of our result is the indication that this HBV gene might activate transcription controlled by κB-like enhancer sequences. Our deletion mutant experiments localize the responsive region of the HIV-1 LTR to a small region containing the κB-like transcriptional enhancer of HIV-1. Previous work has shown that the HBV X gene can activate regulatory sequences of the human beta interferon gene (23) and simian virus 40 (18), both of which contain NF-κB responsive enhancer elements (6, 18; M. Lenardo, personal communication) and not the human T-cell lymphotropic virus type I or visna virus LTRs (18), which do not contain κB sequences. κB-like enhancer sequences have been found to regulate transcription of a variety of other viruses (4, 6, 7, 18, 21) and cellular genes (2, 3, 8, 14, 17). This suggests that during HBV infection the HBV X gene could activate a cellular gene regulated by a κB-like sequence and important for HBV replication. The HBV genome does not contain a κB-like sequence, and in unpublished experiments we have not been able to detect an effect of the HBV X gene on expression of any HBV genes.

There are several possible mechanisms by which the HBV X gene could activate HIV-1 LTR-directed transcription (22a). The HBV X gene might act directly on a sequence in the HIV-1 LTR (e.g., the κB-like sequences) as a transcription factor, it could activate a cellular transcription factor, or it could act by some other mechanism. Purification of the X protein will be important to characterize it and determine its ability to directly bind and activate the function of a specific sequence in the HIV-1 LTR.

Our finding that the HIV-1 LTR is activated in a cell line in which HBV is replicating raises the question of whether HBV could activate HIV-1 in vivo in a host infected with both viruses. It is well known that HBV can be found in T lymphocytes (a cell type known to be infected by HIV) of HBV-infected humans and chimpanzees (16) suggesting that both viruses might simultaneously infect some cells during natural infection of humans. This, however, seems unlikely to be a major mechanism regulating HIV expression in vivo.

TABLE 1. Responsiveness of HIV-1 LTR deletion mutants to the HBV X gene (pTWU17) and to the HIV-1 *tat*-III gene (pcDEBtat) in HepG2 cells

Plasmid	*trans*-Activation[a]		
	X	tat	X + tat
p-423	71	78	158
p-167	111	48	271.7
p-120	69	96.7	264.3
p-104	81.5	146.5	369
p-57	1	30	32
p-45	1	23	72
p-167/−17	412	3	428
p-167/+21	350	2	118
p-167/+45	170	151	409

[a] HepG2 cells (10^6) were cotransfected with 1 μg of the indicated HIV-1 LTR deletion mutant and 10 μg of pUC9, 10 μg of pTWU17, and/or 1 μg of pcDEBtat. CAT assays were performed with the same amount of cell lysate of each sample extracted 44 h after transfection. The values shown represent ratios of chloramphenicol conversion efficiencies of extracts prepared from cells cotransfected with a particular HIV-1 LTR deletion mutant and pTWU17 (X), pcDEBtat (tat), or pTWU17 and pcDEBtat (X + tat) to those of extracts prepared from cells cotransfected with a particular HIV-1 LTR deletion mutant and pUC9.

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