Activation of human immunodeficiency virus by herpesvirus infection: Identification of a region within the long terminal repeat that responds to a trans-acting factor encoded by herpes simplex virus 1

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ABSTRACT Herpes simplex virus 1 (HSV-1) infection induces transcription of the chloramphenicol acetyltransferase (CAT) gene directed by the long terminal repeat (LTR) of human immunodeficiency virus (HIV) in both transiently and permanently transfected cells containing the HIV-LTR/CAT hybrid gene. To define the mechanism by which HSV-1 stimulates the HIV LTR, we examined the effects of isolated regulatory genes from HSV-1. The results of cotransfection assays with the immediate-early (IE) genes of HSV-1, IE110 (ICP0) and IE175 (ICP4), showed that the IE110 protein, either alone or in combination with the IE175 protein, can activate the HIV LTR. Cotransfection with the IE175 gene alone or with the Vmw65 gene (coding for a virion transcription factor) alone did not lead to HIV-LTR activation. The lack of requirement for the IE175 or Vmw65 gene products in transient-expression assays was confirmed in permanent cell lines containing the HIV-LTR/CAT hybrid gene by using temperature-sensitive mutants defective in the IE175 gene product or in uncoating functions. By deletion analysis, we localized a 73-bp-long region (positions –104 to –32) from the HIV LTR that responded to HSV-1 activation; when this region, which is distinct from the previously identified trans-activating responsive (TAR) region, was ligated to a heterologous, HSV-1-nonresponsive gene (α_4 -interferon/CAT), it conferred inducibility by both HSV-1 infection and IE110/175 cotransfection. Both simian and human cytomegalovirus also induced the HIV-LTR/CAT hybrid gene. However, we failed to detect specific upstream sequence requirements for induction by cytomegalovirus. Our results indicate that infection with unrelated viruses can alter the expression of HIV in an infected cell.

Two types of interaction between human immunodeficiency virus (HIV) and infected cells may be important in the pathogenesis of acquired immunodeficiency syndrome (AIDS) (reviewed in ref. 1). The first is a productive infection that can be demonstrated *in vitro* where the majority of infected cells undergo cytopathic changes followed by cell death within 10–14 days after infection. The second is a persistent, noncytopathic infection that has been detected both in a small population of T lymphocytes surviving the primary infection and also in monocytes/macrophages, B lymphocytes, and other cell types. *In vivo*, HIV replication can be demonstrated only in a small population of T cells, and virus persists for a prolonged time without inducing any pathological symptoms. The sequence of events needed to trigger the conversion of a persistent HIV infection to a productive cytolytic infection is not known.

We and others (2–4) have demonstrated that heterologous viruses can activate the HIV long terminal repeat (LTR). Using model systems that appear to mimic the latent integrated HIV provirus, we showed (2) that reactivation of the bacterial chloramphenicol acetyltransferase (CAT) gene initiated from the HIV LTR can be induced in stable DNA transfected cell lines by infection with herpes simplex virus 1 (HSV-1) (2). In the present report, we describe identification of a 5' upstream sequence within the HIV LTR that appears to contain a specific recognition sequence necessary for the activation by HSV-1 and examine the possible roles of three different HSV-1-encoded regulatory proteins in the HIV LTR activation. Further, we show that the HIV LTR can be reactivated by cytomegalovirus (CMV) infection as well.

MATERIALS AND METHODS

Cells and Viruses. Ltk[–] cells (thymidine kinase-deficient murine fibroblast line), Vero cells (African green monkey kidney cell line), and HeLa cells (epithelial line derived from a human cervical carcinoma) were grown as described (5). Virus stocks HSV-1(MPcl-20), HSV-1(17)tsK and HSV-1(HFEM)tsB7 were prepared and titered as described (5). Infections with human CMV [HCMV(Towne)] and simian CMV [SCMV(Colburn)] were carried out as described (6).

Plasmid DNA. The construction of plasmids pU3RIII (HIV-LTR/CAT), pU3RI [human T-lymphotropic virus type I (HTLV-I)-LTR/CAT], and 5' or 3' deletion mutants of pU3RIII has been described (7). The plasmids pGR212, pXhoI-C, and pIGA-15 contain intact genes for the M_r 65,000 virion transcription factor Vmw65, the M_r 175,000 immediate-early (IE) protein (IE175, also called infected-cell protein 4, ICP4), and the M_r 110,000 IE protein (IE110, or ICP0) of HSV-1, respectively (8). The plasmids pSV2cat (9) and pA₁₀cat₂ (10) contain the CAT gene under the control of the simian virus 40 (SV40) early regulatory region with and without the 72-base-pair (bp) repeats, respectively. Plasmid pPOH3 contains a HSV-1 thymidine kinase (TK)/CAT hybrid gene (8). Plasmid p α CAT is a 5', –39 deletion mutant containing the mouse α_4 -interferon promoter directing CAT activity (11).

CAT Assays and RNA Analysis. CAT activity was assayed 24 hr after either virus induction in the permanently transfected cells or glycerol shock in transient-expression

Abbreviations: HIV, human immunodeficiency virus; HSV-1, herpes simplex virus 1; CMV, cytomegalovirus; HCMV, human CMV; SCMV, simian CMV; SV40, simian virus 40; AIDS, acquired immunodeficiency syndrome; LTR, long terminal repeat; CAT, chloramphenicol acetyltransferase; IE, immediate-early; TK, thymidine kinase.

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assays (5). RNA was isolated and analyzed as described (2, 5).

DNA Transfection. Permanently transfected Vero (VNHIV-CAT Pool) and Ltk⁻ (LNHIV-CAT Pool) cells containing the HIV-LTR/CAT hybrid gene were established by pSV2neo cotransfection and selection for G418 resistance as described (2, 5). For transient-expression assays, DNA transfection was carried out by a calcium phosphate precipitation procedure (5) and expression was analyzed within 36 hr after transfection. Transfection mixtures for 100-mm dishes (5×10^6 cells) contained 1 μ g of supercoiled plasmid DNA (for CAT assay), and for T80 flasks (7.5×10^6 cells), 1.5 μ g (for RNA isolation). After overnight incubation, the transfected cells were shocked briefly with glycerol, then infected with virus when indicated and subjected to enhancement with 5 mM butyrate until harvested.

RESULTS

Determination of the HSV-1 Gene Product(s) Involved in HIV-LTR Induction, Permanent cell lines. We have shown (2), using a HSV-1 temperature-sensitive (ts) uncoating mutant, tsB7, that (i) Vmw65, a late virion structural component that specifically stimulates the expression of the HSV-1 IE genes (12, 13), was not involved in HIV-LTR induction and (ii) HIV-LTR induction required HSV gene expression. Of the five IE genes expressed after HSV-1 infection, the IE175 gene clearly performs an essential role in HSV-1 delayed-early gene expression and viral replication (14). To test whether the IE175 protein was involved in the reactivation of the HIV LTR in our pooled, permanently transfected Ltk⁻ (LNHIV-CAT Pool) and Vero (VNHIV-CAT Pool) cells (2), we took advantage of the availability of the HSV-1 temperature-sensitive mutant tsK, which produces a defective IE175 protein (15). No constitutive expression was observed at either the permissive (33°C) or the nonpermissive (39°C) temperature (Fig. 1A, Mock). However, infection with HSV-1 wild type or tsK mutant at either temperature reactivated HIV-LTR/CAT transcription in the VNHIV-CAT Pool cells (Fig. 1A). Similar results were obtained with the LNHIV-CAT Pool cells (data not shown), and parallel infections with HSV-1 tsB7 at the nonpermissive temperature, as reported previously (2), failed to induce CAT expression in either cell type. Control immunofluorescence assays of these cultures revealed that at the nonpermissive temperature (39°C), the tsK infection gave efficient synthesis of the IE175 protein but produced no delayed-early major DNA-binding protein, whereas tsB7 infection at 39°C failed to yield either class of proteins. These results suggest that the IE175 protein, or at least the particular lesion of the tsK mutant, is not involved in the reactivation of the HIV LTR by HSV-1 infection.

Transient-expression assays. To identify more definitively which HSV-1 gene product(s) might be involved in HIV-LTR reactivation, we studied the induction of the HIV LTR in transient expression assays. We tested Vmw65, IE175, and IE110 genes (8, 13) and the combination of the IE110 and IE175 genes (16) that have been previously shown to have trans-activating ability. Cotransfection experiments were carried out in three cell types: Ltk⁻, Vero, and HeLa. Only the combination of the IE110 and IE175 genes induced HIV-LTR/CAT expression in all cell lines tested (Table 1). Cotransfection of the IE110 gene alone with the HIV-LTR/CAT gene induced CAT activity in Ltk⁻ and Vero cells but not in HeLa cells (Table 1). However, correctly initiated CAT RNA was detected both in HeLa cells at 24 hr (Fig. 1B) and in Ltk⁻ cells at 8 hr and 24 hr (data not shown) after cotransfections of the HIV-LTR/CAT gene with the IE110 gene. We assume that at early times, the nuclease S1 analysis is a more sensitive indicator of activation than the CAT assay. Similarly, correctly initiated HIV-LTR/CAT RNA

FIG. 1. Identification of the HSV-1 gene product required for HIV-LTR induction. (A) Nuclease S1 analysis of RNA isolated from permanently transfected Vero cells (VNHIV-CAT Pool, containing the HIV-LTR/CAT gene) 10 hr after infection with wild-type (wt) HSV-1(MPcl-20), HSV-1(17)tsK, or HSV-1(HFEM)tsB7 at permissive (33°C) or nonpermissive (39°C) temperature. "Mock" represents no infection. Immunofluorescence assays with monoclonal antibodies to the IE175 protein and the delayed-early major DNA-binding protein were used to confirm the infection phenotypes: the percentage of positive nuclei obtained for the wt-, tsK-, and tsB7-infected cultures, respectively, was 50, 30, and 12 for IE175 at 33°C; 60, 40, and 0.1 for IE175 at 39°C; 40, 25, and 8 for delayed-early major DNA-binding protein at 33°C; and 60, 0.3, and 0.01 for delayed-early major DNA-binding protein at 39°C. (B) Nuclease S1 analysis of RNA isolated from HeLa cells cotransfected with the HIV-LTR/CAT plasmid and an equal concentration of pBR322 (Mock), or IE110 or IE175 plasmid and from cells infected with HSV in transient-expression assays. RNA was isolated either 8 or 24 hr after glycerol shock. Hybridizations were done using a 495-nucleotide complementary RNA probe as previously described (2). Arrowheads show the positions of the full-length probe (495 bp long) and the correctly initiated HIV-LTR/CAT transcript (335 bp). Markers (bp) represent *Hpa* II digestion of pBR322 plasmid.

was present in cells either cotransfected with the combination of the IE110 and IE175 genes or infected with HSV (Fig. 1B). Essentially no CAT mRNA was observed in the mock transfections, and cotransfection with the IE175 gene alone gave only low levels of CAT mRNA. Thus the data from transient-cotransfection assays agree well with the results obtained for the permanently transfected cells infected with the tsK mutant and indicate that the IE110 gene product may play an important role in the activation of HIV LTR, although cotransfection of the IE175 gene with the IE110 gene resulted in more rapid accumulation of CAT mRNA.

Sequence Within the HIV LTR Responding to HSV-1 Trans-Activation. To identify functional regions within the HIV LTR responding to HSV infection, we tested the set of deletion mutants that were previously used to identify the location of cis-acting regulatory sequences in the HIV LTR required for trans-activation by the *tat-III* product (7). The activities of the deletion mutants were tested in HeLa cells by cotransfection with a mixture of IE110 and IE175 plasmids. The intact HIV-LTR/CAT hybrid gene (-453/+80) and its 5' deletion mutants lacking 286 nucleotides (deletion -167/+80), 333 nucleotides (deletion -120/+80), and 349 nucleotides (deletion -104/+80) were all inducible by the IE110/175 gene products (Fig. 2). A decrease in constitutive expression with a concomitant increase in IE110/175 induction (except for deletion -120/+80, this discrepancy was also observed in permanently transfected cells containing the -120/+80 HIV-LTR deletion) of CAT gene activity was observed with progressive deletions from the 5' end of the HIV LTR. However, when the deletions extended to -57 and -45 bp upstream from the RNA initiation site, the inducibility of the CAT activity was almost abolished (Fig. 2). Whereas the RSV-57/+80 mutant, which was previously shown to respond to tat activation (7), was not activated

Table 1. Trans-activation of HIV LTR and LTR deletion mutants by HSV-1

Cotransfection/ infection	CAT activity, % conversion*				
	Ltk ⁻			HeLa	Vero
	-453/+80	-104/+80	-57/+80	-453/+80	-453/+80
pBR322	2	<1	<1	7	2
Vmw65	7	ND	ND	5	ND
IE110	41	37	<1	11	65
IE175	2	ND	ND	13	5
IE110/175	40	38	<1	57	75
HSV	ND	34	<1	ND	ND

ND, not determined.

*Percent conversion was calculated by dividing the radioactivity (cpm) present in the 3-acetylchloramphenicol spot by the sum of the radioactivity present in the chloramphenicol and 3-acetylchloramphenicol spots.

by IE110/175, the -167/+21 HIV-LTR deletion mutant, which does not contain the 3' tat-III trans-activating responsive (TAR) element, was inducible when cotransfected with the IE110/175 genes. These results suggest that the functional domain for tat-III trans-activation is not identical to that required for HSV-1 induction. As controls for the specificity of induction by IE110/175 genes, we tested four other promoter/CAT constructions. Only the HSV-1 TK promoter, as shown previously (8), and none of the heterologous promoters, was activated by the combination of the IE110 and IE175 genes (Fig. 2).

From these results, we conclude that the DNA sequence between positions -104 and -57 of the HIV LTR is critical for IE110/175 induction of the HIV-LTR/CAT hybrid gene.

HSV-1 Induction of a Heterologous Promoter Fused to a Portion of the HIV LTR. To assess further the ability of this

region within the HIV LTR to confer HSV-1-inducibility, we placed bp -104 to -32 of the HIV LTR upstream of a mouse α_4 -interferon promoter/CAT hybrid gene that contained 39 bp upstream from the cap site of the mouse α_4 -interferon gene (including the "TATA" sequence) and the proximal 22 bp of the untranslated leader region. The resulting plasmid, pHIV/ α CAT, contained 73 bp from the HIV LTR fused to the α_4 -interferon promoter, the CAT gene, and the SV40 polyadenylation site (Fig. 3A). Neither the intact p α CAT plasmid (-464/+22), assayed in stable transfected L929 cells (N.B.K.R., unpublished data), nor the minimal truncated p α CAT plasmid (-39/+22), assayed for transient expression in Vero cells, was induced by HSV-1 infection (Fig. 3B). However, when the 73-bp sequence derived from the HIV LTR was inserted 5' to the α_4 -interferon deletion mutant pHIV/ α CAT, HSV-1 infection induced CAT expression (Fig. 3B). Similarly, cotransfection with the IE110 gene or with the IE110 and IE175 genes into Vero cells induced CAT activity in pHIV/ α CAT, but not in the parental p α CAT plasmid. The inducibility of the CAT gene in the pHIV/ α CAT plasmid was confirmed at the RNA level. Properly initiated CAT mRNA was detected after HSV-1 infection of cells transfected with pHIV/ α CAT or after cotransfection of pHIV/ α CAT in the presence of the IE110 and IE175 plasmids or the IE110 plasmid but not after cotransfection of pHIV/ α CAT and the IE175 plasmid (Fig. 3C).

Activation of HIV-LTR/CAT by CMV Infection. Using the VNHIV-CAT Pool cells (2), we tested the ability of SCMV strain Colburn to induce the transcription of the HIV LTR. Infection of the VNHIV-CAT Pool cells with either HSV or SCMV resulted in activation of the integrated HIV-LTR sequences as shown by assays of CAT RNA (Fig. 4A) and enzyme activity (Fig. 4B). Although HSV and SCMV infections induced similar levels of CAT activity after 8 and 24 hr, SCMV infection induced (in three independent experiments) substantially less CAT mRNA; the reason for this difference is unknown, although these results suggest that CMV infection may posttranscriptionally regulate the HIV LTR. Nevertheless, these results indicate that the ability to reactivate the latent HIV-LTR sequences is common to at least two viruses of the herpesvirus group.

Lack of HIV-LTR Sequence Specificity in CMV Induction. To identify functional regions within the HIV LTR responding to CMV infection, we performed transient-expression assays with the same LTR deletion mutants that were used to test induction by HSV-1. For infection with HCMV strain Towne, we transfected the mutants into MG-63 human osteosarcoma cells, while for infection with the SCMV, we transfected the mutants into Vero cells. Unlike HSV-1, however, both HCMV and SCMV induced all of the deletion mutants tested (Table 2). From these data we conclude that the CMV-mediated trans-activation of HIV LTR does not show the same specificity as that observed for HSV, indi-

FIG. 2. Identification of the region within the HIV LTR responsive to HSV-1 trans-activation. HeLa cell extracts were assayed for CAT activity after transfection with various CAT plasmids in the absence (-) or presence (+) of an equimolar mixture of the HSV-1 IE110 and IE175 plasmid DNAs; autoradiographs show the 3-acetyl[¹⁴C]chloramphenicol spots visualized after TLC separation of the reaction products; % conversion represents the amount of [¹⁴C]chloramphenicol converted to its 3-acetylated derivative, as in Table 1. For the HIV-LTR/CAT plasmid (-453/+80) and its deletion derivatives, solid bars represent the portion of the HIV LTR remaining, with the open bar representing the Rous sarcoma virus (RSV) enhancer (-138 to -229); the name of each plasmid indicates the number of nucleotides present upstream to the RNA start (cap) site (+1). Other CAT plasmids tested were pSV2cat, pA₁₀cat₂, and plasmids containing HTLV-I-LTR/CAT and TK/CAT hybrid genes.

FIG. 3. Conferring HSV responsiveness to a heterologous promoter. (A) Schematic diagram of the plasmid DNAs used. Plasmid p α CAT contains bp -39 to +22 (cap site = +1) of the α_4 -interferon promoter directing expression of the CAT gene. Plasmid pHIV/ α CAT contains a region (-104 to -32) of the HIV LTR ligated 5' to the p α CAT construct. (B) CAT activity expressed from p α CAT or pHIV/ α CAT in transfected Vero cells. Cells were transfected with CAT plasmid alone (Mock) or in the presence of IE plasmids as indicated or were transfected with CAT plasmid and then infected with HSV. Cell extracts were assayed for CAT activity; autoradiographs show 3-acetyl[14 C]chloramphenicol product; numbers represent percent conversion of [14 C]chloramphenicol to its 3-acetylated derivative. (C) Nuclease S1 analysis of cellular RNA (8 hr) from Vero cells transfected with pHIV/ α CAT, either alone (Mock) or in the presence of IE plasmids, transfected with pHIV/ α CAT and then infected with HSV. Arrowheads show positions of the full-length probes [424 nucleotides (nt)] and the correctly initiated α -interferon (α IFN)/CAT transcript (272 bp). The construction of the RNA probe and the fragment generated after RNA hybridization and S1 treatment are shown in the diagram below the autoradiograph. E and H represent cleavage sites for the restriction enzymes *Eco*RI and *Hind*III, respectively, used in constructing the DNA from which the probe was transcribed; the black box represents the SP6 promoter used for transcription of the probe. Hatched area represents the mouse α_4 -interferon promoter region, and the cross-hatched area represents 250 bp of the CAT coding region.

cating that the molecular mechanisms of HIV-LTR activation by HSV-1 and CMV may not be identical.

DISCUSSION

This report describes experiments designed to investigate the mechanism of HIV-LTR responsiveness to HSV-1 infection. We previously showed that HSV-1 infection induced the transcription of HIV-LTR/CAT gene (2), and we have now identified an HSV-1-responsive element located within a 73-bp sequence between -104 and -32 bp from the HIV-

LTR cap site. The responsive region is within the region previously shown (7) to contain the HIV-LTR enhancer (-137 to -17). Transfer of this 73-bp region to a heterologous promoter conferred HSV-1 inducibility on a gene previously unresponsive to HSV-1 infection. Further, we established that the presence of the HSV-1 IE110 gene product, either alone or in combination with the IE175 gene product, is essential for the specific induction of the HIV LTR. The activation with CMV shows two interesting features. First, unlike the specific induction of the HIV LTR observed with HSV-1, both human and simian CMV infection showed no sequence specificity in transient-expression assays; all the transfected HIV-LTR deletion mutants showed enhanced CAT expression. Second, the significant difference between CAT activity and mRNA level at 8 hr after viral infection indicates that the CMV activation of the HIV LTR may be predominantly posttranscriptional, whereas HSV has been shown to activate transcription of the HIV LTR (2).

The conclusions obtained from our transient-cotransfection assays agree well with those obtained from experiments with permanently transfected cells in which temperature-sensitive mutants were used. For example, infection of both Vero and Ltk⁻ cell lines containing the HIV-LTR/CAT hybrid gene with HSV-1 tsK and tsB7 revealed that HSV-1 gene expression is required but that neither the Vmw65 nor the IE175 gene products alone were sufficient for HIV-LTR induction. Similarly, in transient-expression assays, cotransfection of the HIV-LTR/CAT hybrid gene together with either the Vmw65 or the IE175 gene failed to induce HIV-LTR/CAT expression.

FIG. 4. HSV and SCMV activation (8 and 24 hr) of the integrated HIV-LTR/CAT hybrid gene in the permanently transfected VNHIV-CAT Pool cells. (A) Nuclease S1 analysis of RNA. Arrowheads and probe used are described in the legend to Fig. 1. (B) CAT activity. Number below each lane represents percent conversion of chloramphenicol (c) to 3-acetylchloramphenicol (a). Position of 1-acetylchloramphenicol (b) is indicated.

Table 2. Trans-activation of HIV LTR and LTR deletion mutants by HCMV and SCMV

Cell line	Infection	CAT activity, % conversion*							
		-453/+80	-167/+80	-120/+80	-104/+80	-57/+80	-45/+80	RSV-57/+80	-167/+21
Vero	Mock	20	15	3	5	3	4	1	10
	SCMV	45	51	29	36	37	13	34	65
MG-63	Mock	11	1	0.2	0.4	0.7	0.3	0.6	5
	HCMV	78	41	35	37	15	8	13	32

*Calculated as described in the footnote to Table 1.

Previous reports (24, 25) have alluded to possible nonspecific effects of HSV-1 infection and HSV-1 IE trans-activating gene involvement in promoter activation in both transiently and permanently transfected cell lines. However, we have addressed the question of nonspecificity in our assays by including in each experiment hybrid plasmids that were not activated either by HSV-1 infection or by cotransfection with HSV-1 IE gene products, whereas the expected activation of a HSV-1 delayed-early gene promoter in the TK/CAT target was observed. In experiments involving the 73-bp sequence, we used the "parent" mouse α_4 -interferon promoter/CAT plasmid as a noninduced control. These controls, together with the magnitude of the HIV-LTR/CAT responsiveness, argue for specificity in HIV-LTR induction by HSV-1.

Although no previous studies have identified specific target sequences for the IE175 and IE110 trans-activators, even in HSV-1 delayed-early promoters, our results imply that an IE110 response element may exist in the HIV LTR. The 73-bp segment of the HIV LTR that responds to HSV-1 infection encompasses three tandem, closely spaced binding sites for transcription factor Sp1 that have been shown to increase the transcriptional efficiency of the HIV LTR *in vitro* (17). Interestingly, the HSV-1 IE175 (18) and TK (19) genes also contain multiple Sp1 binding sites within their promoters; however, Sp1 binding sites have been identified in several heterologous viral and cellular promoters, including the intact SV40 early promoter (20), which showed no enhancement after HSV-1 infection in our assays. Therefore, it is possible that the specificity resides in the particular arrangement of Sp1 sites (-76 to -45) and core enhancer elements (-104 to -80) within the LTR response region. Nabel and Baltimore (21) showed that a cellular protein (NF κ -B) binds to the core enhancer elements of the HIV LTR, with a concomitant induction of HIV-LTR expression. Because our 73-bp sequence contains the core enhancer elements, it may be that HSV-1 activation is mediated through a NF κ -B-like cellular transcription factor.

Some epidemiological studies (22) indicate five predictors that correlate with the development of AIDS in HIV-seropositive asymptomatic individuals. In these cases, opportunistic infections do not appear to be a major risk factor. In those studies, only the immune responses to HIV infection were analyzed and not the titer of HIV present in body fluids. However, other epidemiological studies (23) suggest that herpesvirus infection can increase an individual's susceptibility either to HIV infection or to disease progression. If opportunistic infections should result in an increase in HIV-LTR expression and higher virus titers in an asymptomatic HIV carrier, then infections with herpesviruses could be one

of the factors contributing to the onset and severity of the AIDS disease.

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