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The specificity of the human immunodeficiency virus type 2 transactivator is different from that of human immunodeficiency virus type 1

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The recently described human immunodeficiency virus type 2 (HIV2) is significantly divergent in sequence from the more frequently isolated human immunodeficiency virus type 1 (HIV1). Both HIV1 and HIV2 encode a transactivator that is capable of strongly stimulating expression directed by the viral long terminal repeat (LTR). Here, we define the region of the HIV2 genome encoding the transactivator and show that the specificity of the transactivator differs from that of HIV1. By deletion analysis of the HIV2-LTR, we show that both HIV1 and HIV2 transactivators require sequences within 35 to 53 bp downstream of the start of transcription. However, in order to stimulate expression at full efficiency, the HIV2 transactivator further requires sequences unique to the HIV2-LTR between nucleotides +53 and +99. Hence, HIV2 poorly transactivates the LTR of HIV1, while two divergent isolates of HIV1 will efficiently transactivate the LTR of either HIV1 or HIV2. Nonetheless, *in vivo* competition between the transactivators of HIV1 and HIV2 suggests that they use a common mechanism.

Key words: human immunodeficiency virus type 2/*tat*/transactivation/retrovirus/transactivator responsive sequences

Introduction

Human immunodeficiency virus type 1 (HIV1) is the causative agent of most cases of acquired immune deficiency syndrome (AIDS) worldwide (Curran *et al.*, 1985). Recently, however, infection by a related retrovirus, called HIV2, has been found in patients originating from western Africa with AIDS and related symptoms (Clavel *et al.*, 1987). Serological evidence indicates that HIV2 and closely related variants are more widespread in some parts of western Africa than is HIV1 (Kanki *et al.*, 1987).

The genomes of HIV1 and HIV2 share only about 45% nucleic acid homology overall, and from 30% to 60% amino acid identity in different predicted gene products (Guyader *et al.*, 1987). Nonetheless, both are non-transforming retroviruses that appear to use the CD4 molecule as a receptor, cause cell fusion, and can elicit cytopathic damage in cell culture. In addition, both can transactivate expression directed from their long terminal repeat (LTR). In HIV1, a product of apparent mol. wt of 14 or 15 kd encoded by the *tat* gene is responsible for the transactivation (Goh *et al.*, 1986; Seigel *et al.*, 1986; Muesing *et al.*, 1987). This protein (called HIV1-TAT here) can increase LTR-directed expression by over two orders of magnitude and is essential for viral replication (Sodroski *et al.*, 1985a; Dayton *et al.*, 1986; Fisher *et al.*, 1986). As an activator of gene expression, HIV1-TAT is unusual in that its activity is dependent on a short sequence in the viral LTR required in *cis* that must be positioned immediately downstream of the start of transcription and is orientation-dependent (Rosen *et al.*, 1985; Wright *et al.*, 1986; Peterlin *et al.*, 1986; Muesing *et al.*, 1987). The mechanism of action of HIV1-TAT is at present unknown and may be comprised of transcriptional and/or post-transcriptional components (Rosen *et al.*, 1985, 1986; Cullen, 1986; Feinberg *et al.*, 1986; Peterlin *et al.*, 1986; Wright *et al.*, 1986; Muesing *et al.*, 1987).

Based on homology between HIV1 and HIV2, an HIV2-TAT is predicted to share 42% amino acid identity with HIV1-TAT, but would contain 130 amino acids versus 86 for HIV1-TAT (Guyader *et al.*, 1987). Because of the importance of the *tat* gene in HIV1 viral replication, and because the unusual nature of HIV1 transactivation offers an interesting model of gene regulation, we have undertaken a molecular analysis of HIV2 transactivation in order to determine the consequences of the sequence divergence between HIV1 and HIV2 on their systems for transactivation.

We have previously shown that HIV2 encodes a gene capable of transactivating its own LTR. Moreover we showed that while HIV1-TAT could efficiently transactivate the HIV2-LTR (called cross-transactivation), HIV2-TAT could not as efficiently cross-transactivate the HIV1-LTR (Guyader *et al.*, 1987). Here we show that the transactivator of HIV2 requires at least two segments of the R region of the HIV2-LTR in *cis* in order to function at full efficiency. Only one of these two regions is conserved with the HIV1-LTR and is needed by the transactivator of HIV1. This can explain the non-reciprocal cross-transactivation that we observed previously, and which we extend here using different conditions of transfection and divergent isolates. We also define more precisely the region of the HIV2 genome that encodes the transactivator and present results from *in vivo* competition between the HIV1 and HIV2 *tat* gene products that suggest that HIV1 and HIV2 use a common mechanism of transactivation.

Results

For full activity HIV2-TAT requires sequences in the R region of the HIV2-LTR which are not found in the HIV1-LTR

In HIV1, the region required in *cis* for transactivation by the *tat* gene product (the TAR region) lies between nucleotides −17 and +54 in the LTR (where +1 is the first transcribed base at the junction of the U3 and R regions: Sodroski *et al.*, 1985b; Wright *et al.*, 1986; Muesing *et al.*, 1987). We constructed a series of deletions in the HIV2-LTR in order to locate the TAR region of HIV2-TAT (Figure 1). Each HIV2-LTR plasmid contains the bacterial chloramphenicol acetyltransferase (CAT) gene (Gorman *et al.*, 1982) 3′ to the LTR sequences as a marker gene for LTR-directed expression. The basal level of expression of the CAT gene from each of the HIV2-LTR−CAT plasmids transfected was approximately equivalent (data not shown).

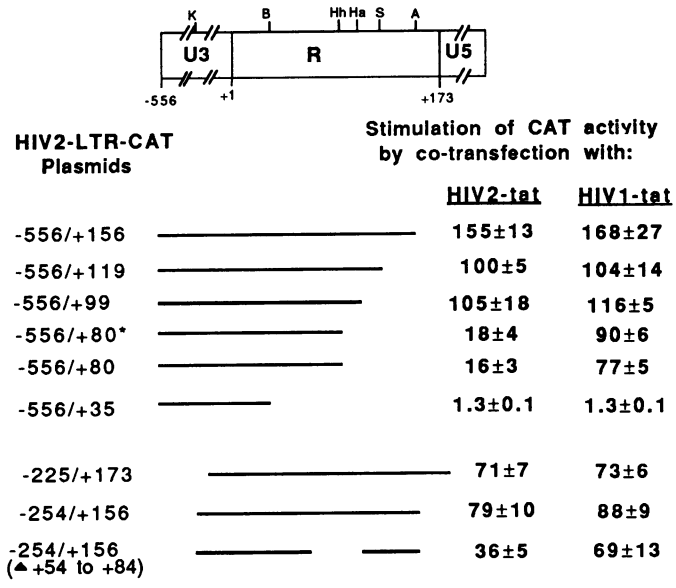


Fig. 1. Tat-responsive region of the HIV2-LTR. The stimulation of CAT activity is the fold-increase in CAT activity resulting from co-transfecting each HIV2-LTR−CAT plasmid with one of the HIV-*tat*-containing plasmids relative to the activity of each HIV2-LTR−CAT plasmid transfected with salmon spern DNA alone. Each HIV2-LTR−CAT plasmid is named by the nucleotides in U3 followed by the nucleotides in R it contains, and is represented by the dark lines. −556/+80* and −556/+80 are identical except that a *Hin*dIII linker was added to −556/+80* at the site of the deletion. Restriction sites correspond to the enzymes used to make each HIV2-LTR−CAT plasmid except the deletion between +54 and +84 (which was made by site-directed mutagenesis, see Materials and methods). A = *Alu*I, B = *Ban*II, Ha = *Hpa*II, Hh = *Hph*I, K = *Kpn*I, S = *Sau*96A. 1.0 μg of each LTR−CAT plasmid was transfected both with and without 1 μg of HIV2-*tat* expressed from the SV40 promoter (pROD221, Figure 3), or HIV1-*tat* expressed from the SV40 promoter (SV-HIV1-*tat*). The average value of three or four transfections is given followed by the standard deviations.

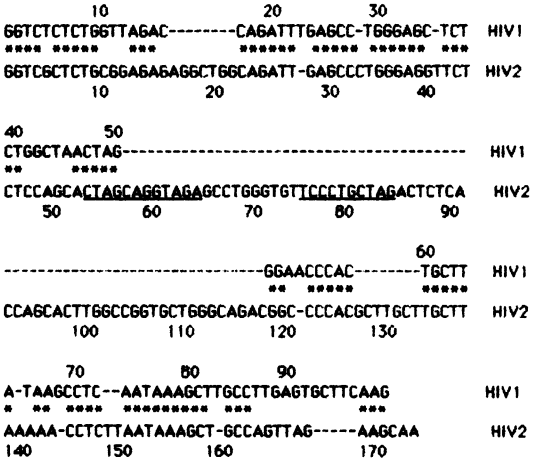


Fig. 2. Alignment of the R region of the LTR of $HIV1_{BRU}$ and $HIV2_{ROD}$. Seqeunce of $HIV1_{BRU}$ is from Wain-Hobson *et al.* (1985). Sequence of $HIV2_{ROD}$ is from Guyader *et al.* (1987). An asterisk indicates a base in an identical position between HIV1 and HIV2. Dashed lines indicate insertions introduced into the sequences to optimize the alignments. An imperfect inverted repeat is underlined.

These plasmids were then co-transfected with a plasmid which expressed the HIV2-*tat* gene. HIV2-TAT stimulated the expression of the CAT gene approximately equally from plasmids which contained the first 156, 119 and 99 bp of the R region (Figure 1). However, a deletion which left only the first 35 bp of R (−556/+36) completely eliminated the response to HIV2-TAT (Figure 1). This indicates that like the HIV1 transactivation system, there are sequences 3′ to the initiation site of viral RNA transcription which are absolutely required for a response of the HIV2-LTR to HIV2-TAT. In addition, a deletion which left only the first 80 bp of the R region (−556/+80*) reduced the ability of HIV2-TAT to transactivate this LTR to under 20% of the response of LTRs with an R region of 156, 119 or 99 bp (Figure 1). We also obtained the same result from a plasmid with the same deletion as the −556/+80* plasmid but which does not contain a *Hin*dIII linker at the site of the deletion (−556/+80, Figure 1). These results indicate that although only the first 80 bp are required to produce an LTR-directed response to HIV2-TAT, sequences further 3′ to +80 are required for full transactivation.

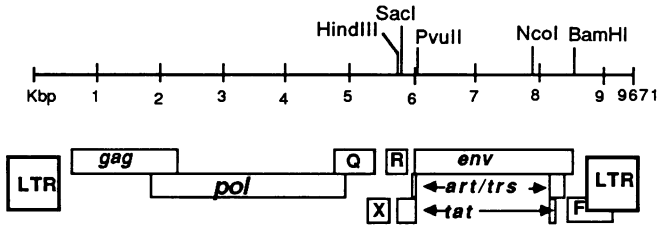
Inspection of the alignment of the R regions of both HIV1 and HIV2 (Figure 2) shows that the region between +58 and +119 of the HIV2 R region has no counterpart in the much shorter HIV1 R region (173 bp in HIV2 versus 97 bp in HIV1). Additionally, the region between +54 and +85 in the HIV2 R region contains an imperfect inverted repeat separated by 10 bp. Therefore, we employed site-directed mutagenesis to make a further deletion in the HIV2-LTR that removed the inverted repeat. This plasmid (−254/+156, Δ+54 to +84; Figure 1) shows an intermediate amount of transactivation by HIV2-TAT. Thus, disruption of the inverted repeat does not fully explain the lack of response of the −556/+80 plasmids. On the other hand, the intermediate response of the deletion between +54 and +84 indicates that there must be sequences between +35 and +53 in the HIV2-LTR which are necessary for stimulation of expression by HIV2-TAT.

We tested HIV1-TAT against these same HIV2-LTR deletions to determine if HIV1-TAT used the same or different sequences as HIV2-TAT when it transactivated the HIV2-LTR. Indeed, we found that HIV1-TAT, like HIV2-TAT, absolutely requires sequences in R downstream of +35 in order to transactivate the HIV2-LTR (Figure 1). We also noted that there was ~2-fold reduction in the response to both HIV1-TAT and HIV2-TAT of plasmids which did not contain the entire U3 region compared to those that did (Figure 1).

The response of HIV1-TAT of the HIV2-LTR plasmids with deletions to −156, +119 and +99 was about the same as their response to HIV2-TAT. In contrast, however, HIV1-TAT stimulated expression from the HIV2-LTR plasmids with only the first 80 bp of the R region about five times better than HIV2-TAT stimulated these plasmids. Thus, sequences between +99 and +80 are more important for HIV2-TAT than for HIV1-TAT. In addition, the HIV2-LTR with the deletion between +54 and +84 was stimulated almost twice as well by HIV1-TAT as by HIV2-TAT (Figure 1). These results indicate that sequences which are important for transactivation by HIV2-TAT, but not by HIV1-TAT, lie mostly, or entirely, within a region of the HIV2-LTR which is an insertion relative to the HIV1-LTR (Figure 2).

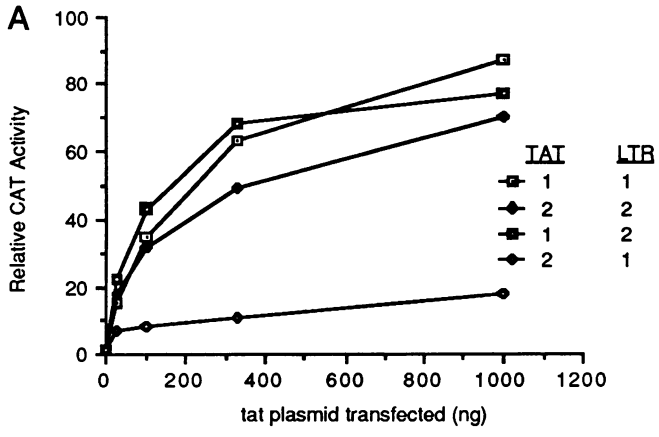
A short open reading frame encodes the HIV2 transactivator

To determine whether or not the *tat* gene in HIV2 was encoded solely by regions of the genome which share homology with HIV1-*tat*, or if other regions were necessary, we made deletions in a plasmid that contained the entire 3′ half of an HIV2 provirus and some flanking cellular DNA. The *tat* gene of HIV1 consists of three exons—a non-coding leader, a coding exon in the 'central region' between the *pol* gene and the *env* gene, and



Plasmid	Sequences of HIV2	Relative Activity of Cotransfected HIV2-LTR-CAT
pROD215	HindIII to 3' flanking	33
pROD219	SacI to 3' flanking	1.6
pROD214	HindIII to BamHI	165
pROD216	HindIII to NcoI	152
pROD221	HindIII to PvuII	134
carrier DNA	none	1.0

Fig. 3. Region of HIV2 necessary and sufficient to code for the HIV2 transactivator. **Top:** scale of the HIV2 genome that starts at the beginning of the R region in the 5′ LTR and ends at the end of the R region in the 3′ LTR. Only the restriction enzyme sites used to construct the plasmids tested as transactivators are shown. **Middle:** representation of the genome of HIV2 showing the position of the major open reading frames and the LTRs. Each open reading frame is named for its homology to an open reading frame in HIV1, except for X which has no counterpart. The Q gene is also called the *sor* gene and the F gene is also called the 3′ *orf* gene. Other small open reading frames also exist (Guyader *et al.*, 1987). **Bottom:** plasmids tested as transactivators are defined by the enzyme sites used to construct the plasmid. pROD215 and pROD219 contain 2 kbp of cellular DNA flanking the 3′ LTR. All plasmids contain the HIV2 U3-R region as the promoter except for pROD221 which contains the SV40 early promoter. 3 μg of each plasmid was transfected with 1.5 μg of HIV2-LTR−CAT (−556/+156 in Figure 2). CAT activity is expressed relative to a transfection of HIV2-LTR−CAT with salmon sperm DNA alone.



B

TRANSACTIVATOR	TRANSACTIVATION OF LTR-CAT		
	$HIV1_{BRU}$	$HIV1_{MAL}$	$HIV2_{ROD}$
HIV1-tat_{BRU}	82	87	115
HIV2-tat_{ROD}	11	15	119
$HIV1_{MAL}$ (provirus)	42	51	58
SIV_{MAC} (provirus)	10	9	49

Fig. 4. Non-reciprocal cross-transactivation of HIV1 and HIV2. **Panel A:** 1 μg of HIV1-LTR−CAT or HIV2-LTR−CAT was transfected with different concentrations of plasmids which express the HIV1-*tat* or the HIV2-*tat* from the SV40 promoter (SV-HIV1-tat and pROD222, see Materials and methods). Both LTRs contain all of U3 and either the first 77 bp (HIV1) or the first 156 bp (HIV2-LTR) of R. Results are expressed relative to the amount of CAT activity from HIV1-LTR−CAT and HIV2-LTR−CAT without transactivators. Open squares represent HIV1-LTR−CAT plus HIV1-*tat*; closed diamonds represent HIV2-LTR−CAT plus HIV2-*tat*; closed squares represent HIV2-LTR−CAT plus HIV1-*tat*; and open diamonds represent HIV1-LTR−CAT plus HIV2-*tat*. **Panel B:** 1 μg of LTR−CAT plasmid was transfected both with and without 2 μg of DNA containing a transactivator. Each LTR−CAT plasmid contains all of U3 and most of R (see Materials and methods). HIV1-tat_{BRU} and HIV2-tat_{ROD} are the same as in Panel A. $HIV1_{MAL}$ is a complete provirus of a Zairian isolate in pUC18; SIV is a complete provirus of macaque isolate 142 cloned in phage lambda. Results represent the increase in CAT activity of the LTR−CAT plasmids transfected alone compared to the CAT activity in co-transfections with a transactivator.

a coding exon in an alternative reading frame of the *env* gene (Arya *et al.*, 1985; Sodroski *et al.*, 1985b; Muesing *et al.*, 1987). The third exon has been shown not to be essential for the transactivation function of the protein (Sodroski *et al.*, 1985b; Seigel *et al.*, 1986; Wright *et al.*, 1986; Muesing *et al.* 1987). There are two regions of HIV2 which share homology with the coding sequences of the *tat* gene of HIV1, and are found in analogous regions of the genome (39% homology in the first coding exon, 59% in the second coding exon: Guyader *et al.*, 1987). The first translation initiation codon after the start of transcription in plasmid pROD215 begins the open reading frame which shares homology with the first coding exon of HIV1-*tat*. A small deletion was made at the 5′ end of pROD215 which removed the initiator codon and the following eight putative amino acids encoded by the open reading frame. This mutation (pROD219) nearly reduced to background levels the ability of this plasmid to transactivative the co-transfected CAT gene under control of the HIV2-LTR (Figure 3). This indicates that, as in HIV1-*tat*, this open reading frame encodes an essential part of the *tat* gene.

Deletions were also made in the 3′ end of pROD215. Plasmids pROD214, pROD216, and pROD221 all efficiently stimulated expression of the HIV2-LTR (Figure 3). The HIV2-derived insert in pROD221 is only 363 bp long and ends 12 bp after an in-frame stop codon which coincides with a consensus splice donor. Thus, these results indicate that no open reading frame other than the one partially homologous to the first coding exon of HIV1-*tat* is necessary for transactivation. Further experiments showed that the fact that plasmids pROD214, 216, and 221 all have higher activity than does pROD215 (Figure 3) is probably not due to expression of another viral gene with an inhibitory property (data not shown), but is more likely to be due to reduced expression of the *tat* gene in pROD215 by linked non-viral sequences (plasmid or 3′ flanking cellular DNA). We also tested pROD214, pROD216, and pROD221 for their ability to transactivate the HIV1-LTR and found that the presence or absence of the last exon made no difference to the amount of stimulation (data not shown).

Cross-transactivation under different conditions of transfection and with different isolates

Previously, experiments in which the *tat* gene (and other viral genes) was expressed from its own LTR showed that HIV1-TAT transactivates the HIV2-LTR as efficiently as it transactivates its own LTR, while HIV2-TAT transactivates the HIV1-LTR at only 10−20% of the efficiency at which it transactivates its own LTR (Guyader *et al.*, 1987; Arya *et al.*, 1987). To determine whether or not this phenomenon was general under different conditions

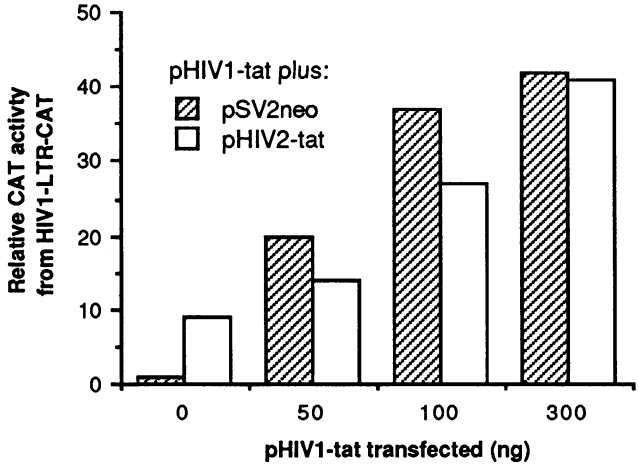


Fig. 5. Transactivation of HIV1-LTR−CAT in the presence of both HIV1 and HIV2-TAT. 250 ng of HIV1-LTR−CAT was transfected with 0, 50, 100 or 300 ng of pHIV1-*tat*, and 3000 ng of either pSV2neo (striped boxes) or pHIV2-tat (open boxes). pSV2neo and the plasmids used to express the HIV1 and HIV2 *tat* genes contain the SV40 early promoter. CAT activity is expressed relative to the amount of activity from a transfection of HIV1-LTR−CAT with no transactivator (0 ng pHIV1-tat plus pSV2neo in the figure). A representative experiment is shown.

of transfection, we co-transfected HIV1- or HIV2-LTR−CAT plasmids with a range of concentrations of plasmids that express the HIV1-*tat* or HIV2-*tat* genes expressed from the SV40 early promoter.

We found that HIV1-TAT stimulates HIV1-LTR- and HIV2-LTR-directed expression to essentially the same extent over the entire range of concentrations (30−1000 ng) of HIV1-*tat*-expressing plasmid transfected. In contrast, HIV2-TAT consistently transactivated the HIV1-LTR less efficiently than it transactivated its own LTR at all concentrations of DNA tested (Figure 4A). Depending on the concentration of HIV2-TAT transfected, the level of stimulation of HIV1-LTR-directed CAT activity by HIV2-TAT was from 10−30% of the amount of stimulation of HIV2-LTR-directed CAT activity of HIV2-TAT (Figure 4A). In addition, the amount of transactivation approached saturation at low transfected DNA levels (330 ng of *tat* plasmid) in all cases (Figure 4A). Since this occurred even in the case of the HIV2-*tat* co-transfected with HIV1-LTR−CAT, the failure of HIV2-TAT to efficiently transactivate the HIV1-LTR is not due to limiting amount of the HIV2-*tat* gene product.

In order to determine whether the amount of cross-transactivation was specific to each isolate or whether different isolates could be grouped according to their specificity, we repeated our co-transfections using as a target the $HIV1_{BRU}$-LTR (called HIV1-LTR above), the HIV2-LTR (ROD isolate), or the $HIV1_{MAL}$-LTR. As sources of the *tat* gene we used plasmids which express the $HIV1_{BRU}$ or HIV2 *tat* gene (the same as those used above) in addition to a complete provirus of the $HIV1_{MAL}$ isolate and a complete provirus of the simian immunodeficiency virus from macaque monkeys ($SIV_{MAC-142}$ isolate cloned in phage lambda: Daniel *et al.*, 1985; Charkrabarti *et al.*, 1987).

The MAL isolate of HIV-1 is one of the most divergent from the BRU (also called LAV-1) prototype isolate (Alizon *et al.*, 1986). The R region of $HIV1_{MAL}$ is 90% homologous to that of $HIV1_{BRU}$, while their predicted *tat* gene products share only 76% amino acid identiy (Alizon *et al.*, 1986). SIV_{MAC}, on the other hand, is more similar to HIV2 than it is to any of the HIV1 isolates. The SIV R region is 95% homologous to that of HIV2 (Hirsh *et al.*, 1986, 1987; Chakrabarti *et al.*, 1987). While the predicted SIV_{MAC}-*tat* gene product shares only 59% identity with that of HIV2, it is still more like HIV2 in both sequence (48% amino acid identity to HIV1) and size (also 130 amino acids) than it is to HIV1 (Charkrabarti *et al.*, 1987).

We found that the HIV_{MAL}-LTR and the HIV_{BRU}-LTR responded nearly identically. None of the transactivators showed a significant preference for one HIV1-LTR over another (Figure 4B). Interestingly, despite the sequence divergence of their two *tat* genes, the HIV_{MAL} provirus-derived *tat* gene product behaved similarly to that of the HIV_{BRU}-TAT in stimulating expression from each LTR to about the same extent. On the other hand, the transactivator encoded by SIV_{MAC}, like HIV2-TAT, increased expression from the HIV2-LTR more than expression from either of the HIV1-LTRs (Figure 4B). Thus, the pattern of cross-transactivation falls into two groups; the HIV1 isolates which behave similarly, and the HIV2/SIV isolates.

Competition between HIV2-TAT and HIV1-TAT in vivo

Because the HIV2-TAT transactivates the HIV1-LTR at reduced efficiency, we wished to determine the effect of saturating amounts of HIV2-TAT on the ability of HIV1-TAT to transactivate the HIV1-LTR. Thus, we transfected cells with a fixed amount of both HIV1-LTR−CAT and HIV2-*tat* plasmids and with increasing concentrations of an HIV1-*tat* plasmid. We considered two possibilities. First, HIV2-*tat* plus HIV1-*tat* would lead to greater expression of the HIV1-LTR than in the case of HIV1-*tat* alone. This would indicate that HIV2-TAT could transactivate the LTR by a mechanism independent of that of HIV1 (see Discussion). Second, HIV2-*tat* plus HIV1-*tat* would lead to equal or somewhat reduced expression of the HIV1-LTR than in the case of HIV1-*tat* alone. This would suggest that the HIV1- and HIV2-TATs use a common mechanism to transactivate the HIV1-LTR.

The transfections were done such that the total amount of HIV2-*tat* gene transfected was in saturating amounts compared with the amount of HIV1-LTR−CAT (10:1 molar ratio, Figure 4A and data not shown). This ensured that the HIV1- and HIV2-TATs would be competing for limiting amounts of the LTR. In addition, transfections which did not include the HIV2-*tat* plasmid included the plasmid pSV2neo (the *neo* gene driven by the SV40 early promoter) to control for non-specific effects of additional plasmid and promoter sequences on the expression of the other genes. We found that the amount of HIV1-LTR-directed CAT activity in the presence of HIV2-*tat* and HIV1-*tat* was equal to, or less than, the amount of HIV1-LTR-directed CAT activity in the presence of HIV1-*tat* alone (Figure 5). In general, co-transfection of HIV2-*tat* could partially inhibit the activity of HIV1-*tat* only at low concentrations of HIV1-*tat* (Figure 5 and data not shown). However, in no case was the amount of transactivation by HIV1-TAT augmented by addition of saturating amounts of HIV2-TAT (Figure 5, and data not shown).

Discussion

We have characterized the transactivation of HIV2 by the virally encoded *tat* gene. We find that it is similar to the transactivator of HIV1 in that the essential sequences of *tat* are present in the HIV2 genome in a region analogous to where the *tat* is found in the HIV1 genome. In addition, both HIV1 and HIV2 transactivators require similar, but not identical, sequences in *cis* in the R region of the LTR. However, the sequence divergence between HIV1-TAT and HIV2-TAT is enough to introduce function differences. Specifically, in order to transactivate at all, both HIV1 and HIV2 *tat* gene products require (at least) sequences

in *cis* between +35 and +53 in the HIV2-LTR. HIV2-TAT, however, has an additional requirement for sequences between +53 and +99 in order to transactivate at full efficiency.

The functional difference between HIV1 and HIV2 transactivators is also observed by the non-reciprocal amount of cross-transactivation between HIV1 and HIV2. HIV2, like SIV, transactivates expression from the HIV2 LTR more efficiently than from HIV1 LTRs. In contrast, a divergent isolate of HIV1, like the prototype HIV1, transactivates each LTR tested with the same efficiency. These observations can be explained by the fact that HIV2-TAT has a requirement for sequences in *cis* that are not found in the HIV1-LTR (Figures 2 and 3). The SIV R region is very similar to that of HIV2 (Hirsh *et al.*, 1986, 1987; Chakrabarti *et al.*, 1987), and presumably the SIV transactivator also requires sequences in *cis* that are not found in HIV1. On the other hand, the HIV2-LTR contains all of the sequences necessary for the full activity of the TATs of divergent HIV1 isolates.

The mechanism of HIV1-TAT action is not clear. It has been ascribed to both transcriptional and/or post-transcriptional events, probably depending on the experimental conditions (Chen, 1986). Although the specific binding of $p14^{tat}$ to the TAR sequence either in DNA or in RNA has not been reported, the differential ability of HIV1- and HIV2-TAT to transactivate some of the same LTRs rules out the possibility that transactivation is due to the induction by both HIV1- and HIV2-TAT of the same cellular protein. It has also been suggested that a stem and loop structure of the HIV1 RNA that has been found to exist in solution (Muesing *et al.*, 1987) may play a role in the mechanism of transactivation. In this regard, the R region of HIV2 has the potential to form a complicated secondary structure consisting of a long stem and loop using the bases conserved between HIV1 and HIV2, and two smaller stem and loops structures using sequences mostly unique to HIV2 (data not shown). However, deletion of one entire imperfect repeat unique to the HIV2-LTR could not entirely explain the difference in specificity between the HIV1 and HIV2 transactivators (Figure 1).

Although we have not directly tested here any particular model for the mechanism of transactivation, the fact that the HIV2-TAT competes with the HIV1-TAT for the transactivation of the HIV1-LTR strongly suggests that it uses the same mechanism as HIV1-TAT for transactivation. In contrast, activation of the HIV1-LTR by co-transfection with some DNA viruses (Gendelman *et al.*, 1986) or by stimulation of cells with T-cell activators (Nable and Baltimore 1987) occurs by mechanisms distinct from that of TAT because these agents augment the amount of LTR-directed expression in the presence of the *tat* gene and/or act through sequences 5′ to the transcriptional start site distinct from those needed by TAT.

The inability of HIV2-TAT to efficiently transactivate the HIV1-LTR could be the result of its poor affinity for the proper target either because of size or sequence divergence from HIV1-TAT. On the other hand, the ability of HIV2-TAT to partially inhibit low concentrations of HIV1-TAT from transactivating the HIV1-LTR (Figure 5) suggests the possibility that HIV2-TAT may be able to bind to the HIV1 target, while not being able to efficiently transactivate it. An activator protein with separate regions of binding and activation has previously been found in other systems (for example, Brent and Ptashne, 1985). Mutational analysis of the HIV2-*tat* should help identify function domains of the protein which it shares with HIV1-TAT and those which may be strain-specific. This identification may eventually be useful in the development of specific viral inhibitors.

Materials and methods

Cells, transfections and CAT assays

SW480 cells, a colon carcinoma line obtained from the American Type Culture Collection were maintained in Dulbecco-modified Eagle's medium with 7% fetal calf serum. The day before transfection they were replated at 8×10^5 cells per 60 mm culture plate and maintained in media with 10% fetal calf serum. Transfections were done with cesium chloride purified plasmids in the presence of 20 μg/ml salmon sperm DNA by the calcium phosphate precipitation method (Graham and Van der Eb, 1973) with a 15% glycerol shock after 6 h. Where appropriate, the amount of carrier DNA was reduced to keep the total DNA concentrations equal.

Two days after transfection, cells were washed and resuspended in 100 μl of 0.25 M Tris−HCl pH 7.8. Extracts were made by freeze/thawing the cells three times in a dry ice/ethanol bath. After discarding the insoluble cellular debris by centrifugation, extracts were used for CAT assays as described (Gorman *et al.*, 1982). After radiography, the percentage of acetylation was determined in a scintillation counter by cutting the acetylated and non-acteylated products from the chromatograph. Typical reactions used 10% of the extract from cells co-transfected with an LTR−CAT and *tat*-encoding plasmid, and 40% of the extract from cells transfected with an LTR−CAT plasmid alone. Trial experiments were performed to determine the linear range of response of the CAT assay with respect to incubation time and volume of cell extract in our hands. Thereafter, reactions were typically incubated for 20 min, but this was adjusted for each experiment such that the amount of acetylated chloramphenical fell into the linear response range of the assay. Thus, all values within a single transfection are normalized with respect to the amount of extract and/or incubation time of each reaction. Except as indicated, all transfections were performed on at least three occasions.

Plasmids

All plasmids were constructed by standard techniques. Nucleotide numbers for HIV1 are those of Wain-Hobson *et al.* (1985), while those of HIV2 are from Guyader *et al.* (1987).

Plasmids tested as transactivators—pSV-HIV1-*tat* was made by deleting pSV2neo between the *Hin*dIII site and *Eco*RI sites (the *neo* gene and its 3′ flanking SV40 sequences) and substituting HIV1 sequences between the *Sal*I site at nucleotide 5368 and the *Bam*HI site at nucleotide 8067 and a 500-bp *Bam*HI−*Eco*RI fragment containing the hepatitis B polyadenylation signal. pROD215 was made by inserting the *Hin*dIII to *Sal*I fragment of the cloned HIV2 provirus pROD35 (Clavel *et al.*, 1986) into the *Hin*dIII and *Sal*I sites of a pBR322-derived plasmid that contained a 400-bp cDNA of the ROD isolate of HIV2 with all of R and 225 bp of U3. Two of the four kbp of flanking cellular DNA were deleted between a *Cla*I site in the flanking DNA and the *Sal*I site. pROD219 was made from pROD215 by cutting at the *Hin*dIII site (nucleotide 5782) and the *Sac*I site (nucleotide 5871), repairing the ends with T4 polymerase for 5 min at room temperature, and religating. The deletion was verified by polyacrylamide gel analysis of restriction endonuclease digestions of sites close to either side of the deletion. pROD214 was described earlier, where it was called pME214 (Guyader *et al.*, 1987). It contains HIV2 sequences between the *Hin*dIII site and the *Bam*HI site (nucleotide 8569) and contains the HIV2 cDNA described above as a promoter. pROD216 was constructed by digesting pROD214 with *Bam*HI and *Nco*I (nucleotide 8761) repairing the ends with the Klenow fragment of DNA polymerase I and religating. pROD221 was constructed by inserting the *Hin*dIII−*Pvu*II (nucleotide 6156) fragment of pROD35 into pSV2gpt in place of the *gpt* gene (deleted by *Hin*dIII to *Eco*RV digestion). pROD222 was constructed by inserting the *Hin*dIII−*Bam*HI fragment of pROD215 into pSV-*gpt* in place of some of the *gpt* gene (deleted by *Hin*dIII to *Bgl*II digenstion). pMAL is a clone of unintegrated viral DNA of the MAL isolate containing a portion of the LTR at each end. It was made complete by permuting it in pUC18 around a unique *Eco*RI site in the middle of the genome. Before transfection, pMAL was digested with *Eco*RI, the viral fragment was isolated away from the pUC18, and ligated at high DNA concentration to form concatomers. $SIV_{MAC\text{-}142}$ (Chakrabarti *et al.*, 1987) was a gift of P.Sonigo.

All plasmids tested for their ability to be transactivated contain the CAT gene and SV40 polyadenylation signals from the plasmid pSVECAT (Gorman *et al.*, 1982). pHIV1-LTR−CAT was constructed by ligating the *Xho*I−*Hin*dIII fragment from the 3′ end of HIV1-BRU isolate into the *Sal*I to *Hin*dIII site of pSVECAT. −222/+171 was described previously (Guyader *et al.*, 1987) where it was called pHIV2-LTR−CAT. It contains the HIV2 cDNA described above and 15 bp of a poly(A) tail between the end of the R region and the beginning of the CAT gene. −556/+156 was made by ligating the *Ban*II−*Bam*II fragment of a longer HIV2 cDNA (E2, Clavel *et al.*, 1986), the *Ban*II−*Alu*I fragment of an HIV2 cDNA (*Hin*dIII linkers were added to the *Alu*I site) and the *Hin*dIII−*Bgl*II fragment of pHIV1-LTR−CAT. The only remaining HIV1 sequences in this plasmid are 200 bp of the F open reading frame 5′ to the HIV2 sequences. −556/+119, −556/+99, −556/+80 and −556/+35 were constructed by ligating the *Hin*dIII−*Bgl*II fragment of pHIV1-LTR−CAT to the *Bam*HI−*Sau*96a/*Hpa*II/*Hph*I or *Ban*II fragments of the E2 cDNA by blunt-end

ligation. −254/+156 was made by deleting an internal *Kpn*I fragment from the U3 to the 5′ flanking region of −556/+156. The *Kpn*I to *Hin*dIII fragment of −254/+156 was then subcloned into a phagmid vector (Bluescript, Vector Cloning System). Site-directed mutagenesis of this plasmid to delete the region between +54 and +84 was performed with standard techniques (Zoller and Smith, 1984) on the single-stranded template using a 20-mer (CTCTCCAGCCTCTCAC-CAGC). pHIV1$_{MAL}$-LTR−CAT was constructed by replacing the *Bgl*II to *Hin*dIII fragment containing the LTR of HIV1$_{BRU}$ in pHIV1-LTR−CAT with a *Bgl*II to *Hin*dIII fragment containing part of the *env* gene, all of U3, and 77 bp of the R region of the MAL genome (Alizon *et al.*, 1986).

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