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Optimal Tat-mediated activation of the HIV-1 LTR promoter requires a full-length TAR RNA hairpin

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ABSTRACT

HIV-1 transcription from the LTR promoter is activated by the viral Tat protein through interaction with the nascent TAR RNA hairpin structure. The mechanism of Tat-mediated transcriptional activation has been extensively investigated with LTR–CAT reporter genes in transient transfections and, more recently, in infection experiments with mutant HIV-1 variants. Several discrepancies between these two assay systems have been reported. For instance, whereas opening of the lower part of the TAR RNA stem does not affect the promoter activity of an LTR–CAT plasmid in transient assays, the corresponding virus mutant is fully replication-impaired. With the aim to resolve this controversy, we have examined the activity of a set of TAR RNA mutants in transient transfection experiments with a variety of cell types. We now demonstrate that truncated TAR motifs exhibit a severe, but cell-type dependent transcription defect. Whereas full LTR activity is measured in COS cells that have been used regularly in previous transfection assays, a severe defect is apparent in a variety of human cell lines, including T cell lines that are typically used in HIV-1 replication studies. These results suggest the presence of a human protein that participates in Tat-mediated transcriptional activation through binding to the lower part of the TAR stem. Several candidate co-factors have been reported in literature. This study resolves the discrepancy between transfection and infection studies on the requirements of the lower TAR stem structure. The evidence also implies that LTR transcription studies should be performed preferentially in human cell types.

INTRODUCTION

The pathogenic human immunodeficiency virus type 1 (HIV-1) encodes not only the three structural genes (gag, pol and env) common to all retroviruses but also six unique gene products, including the Tat protein (1). This protein potently activates transcription from the viral long terminal repeat (LTR) promoter. Tat gains access to the DNA promoter region by binding to the trans-acting-responsive DNA element (TAR) that folds into a stable stem–loop structure as part of the nascent transcript. The HIV-1 Tat–TAR axis has been one of the most intensively investigated viral regulatory mechanisms (see reviews, 2,3). Extensive mutational analyses have identified the active domains in both the Tat protein and the TAR RNA element. The initial mutagenesis studies on the TAR RNA motif were done primarily in transiently transfected cells containing a Tat expression plasmid and a second plasmid encoding a reporter gene under transcriptional control of the HIV-1 LTR promoter (4–9). Furthermore, appropriate cell-free assay systems have been developed to study the Tat–TAR interaction and the mechanism of transcriptional activation (10–17). More recently, replication studies with mutant HIV-1 variants demonstrated that both Tat protein (18–22) and its TAR RNA target sequence (23–27) are essential for viral replication, although some level of TAR-independent replication has been reported in activated T lymphocytes (28) and in astrocytic glial cells (29).

Several transient transfection studies suggested that the integrity of the TAR stem, in particular of the upper domain including the single-stranded bulge and loop elements, is important for efficient transcriptional activation by Tat (8,9,30). The identity of the base pairs surrounding the tri-nucleotide bulge was found to be critical both for efficient trans-activation (31) and high-affinity binding of the Tat protein (32). In comparison, no or relatively moderate defects were scored for mutations in the lower stem domain of TAR (5,31). For instance, the detailed mutational analysis by Jakobovits et al. (5) in human epithelial 293 cells reported 34–39% LTR activity for TAR variants with a triple base substitution in the +7/+18 region of the stem. This defect is relatively minor compared with triple nucleotide substitutions in either the single-stranded bulge or loop element [6 and 2% LTR activity, respectively (5)].

We reported previously a discrepancy between transient transfection assays and virus infectivity studies with TAR-mutated HIV-1 LTR promoter motifs (23). Specifically, we found that a mutant HIV-1 virus with base substitutions in the lower TAR stem is replication-incompetent, although the same TAR mutant is fully transcriptionally active in transient transfections with an LTR–CAT (chloramphenicol acetyltransferase) reporter construct (31). These combined results may suggest an additional role of the TAR hairpin structure in the virus replication cycle, but the observed difference in TAR requirement may also reflect a variation in transcription in these two experimental settings. For

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Figure 1. Nucleotide sequence and RNA secondary structures of the wild-type and mutant TAR constructs. The HIV-1 TAR hairpin was mutated in the 5′/3′ segment of the lower stem in variants 5′s (Xho+10), 5′d, 3′s, 3′d and model TAR. Substitutions are marked by a grey box, △ represents a deletion. The Xho+10/5′s mutation was tested previously in the context of the replicating virus. Nucleotide numbers refer to the position of the TAR element on the genomic RNA, with +1 being the capped G residue. The RNA secondary structures were predicted by free energy minimization with the Zuker algorithm (62), the free energy ΔG (in kcal/mol) was calculated including the terminal stacks. The wild-type TAR stem consists of 23 bp, which is reduced to 11 bp in mutants Xho+10/5′s, 5′d and model TAR and 17 bp in mutants 3′s and 3′d.

instance, the difference may reflect transcription from an integrated versus an unintegrated LTR promoter in the infected and transfected cells, respectively (33). Furthermore, whereas infections are routinely performed in CD4+ T cells, transfections are frequently performed in the non-T cell line HeLa or the non-human cell line COS. Here, we present evidence that a full-length TAR element is required for efficient LTR-transcription in human cell types, but this effect is less obvious in other cells.

MATERIALS AND METHODS

DNA constructs

Details on the LTR–CAT plasmids used in this study can be found in previous publications (31,34); the wild-type LTR–CAT contains the complete HIV-1 LTR U3 region upstream, and the cat reporter gene downstream of TAR (cloned in the HindIII site at position +78). Nucleotide numbers refer to the position on the HIV-1 RNA genome, with +1 being the transcriptional start site. The revertant TAR motifs that restore replication of the Xho+10 mutant virus were described previously (23). The proviral sequences were subcloned into LTR–CAT by exchange of the PvuII–HindIII fragment (position −22/+76). The Tat-expression plasmid pcDNA3-Tat contains the Tat-Rev genomic segment of the HIV-1 isolate pLAI (35) inserted downstream of the cytomegalovirus immediate early promoter in pcDNA3 (Invitrogen). Details of this vector will be described elsewhere [Verhoef and Berkhout, in preparation, see also (36)].

Cell culture, DNA transfection and CAT enzyme analysis

The lymphocytic T cell lines (SupT1, A3.01, MT2, C8166) were maintained at 37°C and 5% CO₂ in RPMI 1640 medium containing 10% fetal calf serum (FCS). Adherent cell lines (COS, HeLa) were grown in Dulbecco’s modified Eagle’s medium with 10% FCS. Transient transfections were performed with DEAE-dextran [COS and HeLa cells, see (7)] or by electroporation [all T cell lines, see (37)]. Co-transfection of COS cells (∼70% confluency on 60 mm dishes) was performed with 1 µg LTR–CAT and 0.1 µg pcDNA3-Tat. HeLa cells (∼70% confluency on 60 mm dishes) were transfected with 1 µg of each plasmid, and all T cell lines (5 × 10⁶) were electroporated with 2 µg LTR–CAT and 5 µg pcDNA3-Tat plasmid. We did verify that these Tat levels are within the linear range of LTR activation. To measure basal promoter activity, we used 30 µg LTR–CAT to transfect SupT1 cells. Cell extracts were prepared at day 3 post-transfection and assayed for CAT activity with butyryl-CoA in combination with the phase-extraction method as described (38). CAT activities were quantitated in the linear range of the reaction.

RESULTS

Truncated TAR hairpins are transcriptionally defective in human cells

The necessary and sufficient sequences for Tat-mediated transactivation have been reported to map between nucleotides +19 and +42 in the HIV-1 LTR [reviewed in (34)]. This region folds the upper half of the TAR hairpin with the typical 3 nucleotide (nt) bulge and 6 nt loop. This structured RNA motif is generally considered to be recognized as part of the nascent RNA transcript by Tat protein and cellular co-factors in the process of transcriptional activation. The Xho+10 mutant contains a sequence substitution upstream of the minimal TAR domain (Fig. 1, mutated segment at position +3/+16 is boxed), resulting in opening of the lower TAR stem. This mutant was previously...
Severely truncated TAR elements demonstrate a transcriptional defect in human cells. Three cell types (the COS African green monkey kidney cell line, the HeLa human epithelial, cervix carcinoma cell line and the human SupT1 T cell line) were transfected with the indicated LTR–CA T constructs. The CA T activity measured in transfections of the wild-type LTR–CA T in the presence of Tat was standardized at 100% for each individual cell line. The results shown represent average values of three (COS and SupT1) or four (HeLa) independent DNA transfections, with deviations from the average in the range of 10–20%.

Reported to be fully active in transient COS cell transfections (34), but the same mutation is detrimental to virus replication in human T cells (23). We therefore reexamined the activity of this TAR mutant in a variety of cell types, including monkey kidney COS cells, human epithelial HeLa cells and the human T cells SupT1. LTR–CAT reporter plasmids with the wild-type or Xho+10-mutated TAR element were transfected in the presence of a second plasmid encoding Tat. Cell lysates were prepared 3 days post-transfection and tested for CA T enzyme activity (Fig. 2). Construct Xho+10 retained <20% of the wild-type expression level in both human cell lines HeLa and SupT1. In contrast, >80% transcriptional activity was consistently measured in the monkey COS cells.

To verify this cell type-specific defect in TAR function, we tested a larger set of structurally altered TAR mutants (Fig. 1). Mutant 5′d contains a deletion in the same region that was substituted in mutant Xho+10 (in analogy to the names of the other mutants, we also refer to the Xho+10 mutant as 5′s). Both Xho+10/5′s and 5′d mutants encode an amputated TAR stem (11 bp, ΔG = −14.7 kcal/mol) compared with the wild-type TAR hairpin (23 bp, ΔG = −24.8 kcal/mol). The activity spectrum of 5′d in the three cell lines was indistinguishable from that of Xho+10/5′s; significant activity was scored exclusively in COS cells, and severely reduced expression was measured in HeLa and SupT1 cells (Fig. 2). The 3′s and 3′d mutations affect the 3′ side of the TAR stem, thereby truncating the TAR stem to 17 bp (Fig. 1, ΔG = −18.4 and −18.2 kcal/mol, respectively). These plasmids were transcriptionally active in human cells (Fig. 2), indicating that a duplex RNA structure of ∼17 bp is sufficient for optimal trans-activation in human cells. Another TAR mutant was used previously as model TAR motif in detailed mutational analysis of the upper TAR domain (31). This mutant, model TAR, combines the Xho+10/5′s substitution as in Xho+10 and a deletion on the 3′-side of TAR (Fig. 1; stem consists of 11 bp, ΔG = −14.7 kcal/mol), and a severe transcription defect was scored specifically in the human cell types (Fig. 2). These results suggest that the integrity of the TAR stem is critically important for function in human cells, consistent with the observed replication defect of the corresponding virus mutant in human T cell lines.

The lower TAR stem, but not the loop or bulge elements, function in a cell type-specific manner

Although many HIV-1 functions have been reported to contribute to viral replication in a cell type-specific manner, such an effect...
Figure 4. Nucleotide sequence and RNA secondary structure of the Xho+10 mutant and revertant TAR hairpins. An HIV-1 variant with the Xho+10 TAR mutation is severely defective in replication, but can give rise to spontaneous revertant viruses. We previously described in detail two reversion experiments [(23); routes I and II, upper and lower part]. The mutated segment in Xho+10 is marked by a grey box. The mutations that became fixated in the revertant genomes are listed next to the arrows of the two evolutionary pathways (e.g. A3U is an A to U transversion at position +3, Δ-8CTGTA-4 represents a 5 nt deletion upstream of the transcriptional start). These acquired mutations are highlighted in the RNA hairpin structures by a black box. These structures were predicted by the Zuker algorithm (62), and the calculated free energies (in kcal/mol, including terminal stacks) are indicated. The number of base pairs in the RNA helixes is as follows; Xho+10 mutant, 11; revertant Ib12, 16, Ib1, 17; Id15, 21; II, 18. The wild-type TAR hairpin (23 bp) is included in the lower right box for comparison.

has not been described for Tat/TAR-mediated LTR transcription. Three explanations can be proposed for this cell-type dependent phenotype. First, it is possible that a cellular factor, involved in Tat-mediated trans-activation through binding to the lower TAR stem, is lacking or inactive in COS cells. Second, LTR transcription in COS cells may not require co-factors that bind the lower TAR stem. Third, the mechanism of LTR-transcription is a multistep process and it is possible that the rate of this process is controlled in COS cells at a slow, rate-limiting step that is not apparent in human cells. In all three scenarios, opening of the TAR stem will not influence the transcription rate in COS cells. If a particular rate-limiting step determines the level of LTR transcription in COS cells, other HIV-1 mutants defective in LTR-transcription should also be less overt in this cell type. We therefore tested the cell-type specificity for other TAR mutants and the partially defective Tat protein mutant Y26H. Two additional TAR mutants were tested, either with substitutions in the single-stranded bulge or loop domain (mutants B123 and L135, respectively, see legend to Figure 3 for further details on the substitutions in these mutants). Their transcriptional activity was scored upon transfection of COS and SupT1 cells (Fig. 3). Unlike the Xho+10 stem-mutant that was defective exclusively in SupT1 cells, these TAR mutants were equally defective in both cell types. Furthermore, the Y26H Tat mutant demonstrated a similarly reduced activity in both cell types (Fig. 3). These results suggest that the cell-type dependent phenotype is rather specific for lower TAR stem mutants, consistent with the first two models.

A previous study reported that the replication defect of TAR-mutated viruses can be complemented by activation of the host T cells by phorbol 12-myristate 13-acetate (PMA) and phytohemagglutinin (PHA) (28). Thus, it is possible that the cellular milieu of COS cells is similar to that of PMA/PHA-activated T cells with respect to the function of mutant TAR motifs. To directly test this possibility, we measured the activity of the Xho+10 mutant in the SupT1 T cell line upon activation by PMA, PHA or a combination of both reagents. We measured no increased transcriptional activity in these activated T cells (results not shown).
Repair of the truncated TAR stem restores transcriptional activity

The analysis so far indicates that an extended RNA hairpin structure is important for optimal TAR function in human cells. However, we cannot exclude alternative mechanistic explanations. For instance, the inactivity of the Xho+10/5′ and 5′d mutants in human cells may reflect a sequence-specific binding of a human co-factor to the +3/+16 TAR sequences, either in the RNA or DNA form. When the Xho+10 mutant virus was used to select for faster replicating revertants, we observed restoration of base pairing of the lower TAR stem by acquisition of additional mutations (23). Several of these TAR revertant structures, obtained in two independent reversion experiments, were now tested in the transient LTR–CAT assay. Three consecutive TAR variants of reversion route I (Fig. 4, upper pathway) and the final structure that evolved in route II (lower pathway) were subcloned into the LTR–CAT plasmid to measure their transcription properties.

In route I, base pairing is gradually restored in the consecutive TAR samples by acquisition of mutations (Fig. 4; Xho+10 mutant, 11 bp; revertants Ib12, 16 bp; Ib1, 17 bp; Id15, 21 bp). Whereas the Xho+10 mutation dramatically reduced the level of Tat-activated LTR transcription in SupT1 cells, the TAR revertants improved the expression level step-by-step and the final ID15 variant produced wild-type levels of CAT enzyme (Fig. 5A). The latter TAR mutant corresponds with a fast replicating revertant virus (23). Thus, the increased LTR expression levels correlated perfectly with the stability and length of the TAR RNA stem. Combined with the results obtained with the initial set of TAR mutants (Fig. 1), these results suggest a correlation between the length of the TAR duplex and its transcriptional activity in human cells.

To further corroborate these results we tested this set of TAR mutant-revertants in two other T cell lines, A3.01 and MT2, and we used COS cells to provide a cellular milieu in which the Xho+10 defect is not manifest. The results of several transfections are summarized in Figure 6 and clearly demonstrate the cell-type specific defect of mutant Xho+10 and the subsequent recovery of transcriptional activity in route I revertants. To test whether the transcriptional defect/repair was specific for Tat-mediated transcription from the LTR promoter, we tested this same set of mutant–revertant TAR motifs in the absence of the Tat trans-activator protein. We found that the basal LTR promoter activity was not sensitive either to opening of the TAR stem in Xho+10 or to the subsequent repair of this motif in the three TAR revertants of pathway I (Fig. 5B).

A different TAR repair mechanism is seen in route II (Fig. 4). A 5 nt deletion upstream of the transcription start site (+1) shifts transcription initiation towards the +6 position within TAR (23). This modification apparently removes the 5′ dangling end and subsequent mutation of the new start site results in an abbreviated TAR hairpin with a closed stem. Consistent with the results obtained for the route I revertant, the route II revertant did increase its Tat-mediated transcriptional activity compared with the parental Xho+10 mutant (Fig. 5A). However, the route II revertant is somewhat exceptional in that its transcriptional activity is significantly higher than that of the wild-type LTR promoter. This increased transcription was measured for this TAR II revertant in all cell types tested (Fig. 6). Furthermore, basal
promoter activity of the TAR II revertant measured without Tat was also elevated compared with the wild-type LTR (Fig. 5B). Apparently, the route II revertant has improved the LTR promoter activity of the TAR II revertant measured without Tat previously measured sub-optimal fitness of the corresponding (39–41). Notwithstanding the fact that the TAR revertant II is previously to bind several proteins that repress basal LTR activity the 5 nt deletion (-8CTGTA-4) in a region that was reported over the upstream leader sequences, including the TAR element. Apparently, other steps in the viral replication cycle are negatively affected by the mutations in revertant II.

**DISCUSSION**

We suggested earlier that TAR RNA may have a role in the HIV-1 replication cycle separate from its contribution to transcription (23). This idea originated from an apparent discrepancy in the activity of a particular TAR mutant in two experimental systems. Specifically, a truncated TAR RNA hairpin was transcriptionally competent in transient transcription assays, but the corresponding virus mutant was replication-incompetent. The evidence presented in this study resolves this issue. Opening of the bottom part of the TAR stem inhibited the transcription function of the HIV-1 LTR promoter in human T cells, the cell type ordinarily used in HIV-replication studies. Previous transfection studies overlooked this transcription defect because it is cell-type dependent and not observed in COS cells. Thus, the integrity of the TAR RNA stem is critical for efficient Tat-mediated LTR transcription and the first definition of the borders of the minimal TAR domain [position +19 to +42, (34)] should be modified accordingly.

The cell-type specific behaviour of the lower TAR stem mutant is rather unique in that other transcriptionally defective TAR mutants display no such cell-type differences. This argues against a general phenomenon, for instance caused by a particular rate-limiting step during LTR-transcription in COS cells. We therefore favour a more direct explanation in which the lower TAR stem contributes in a cell-type specific manner to the transcription process by binding of a co-factor. LTR transcription in COS cells does either not require such a lower TAR stem binding factor, or this co-factor is absent from COS cells. Several TAR RNA-binding factors have been reported (42–55), but only those proteins that require an extended TAR stem for binding can explain the defect of TAR mutants such as Xho+10. The revertant data suggest that binding of this cofactor occurs without strict specificity for the sequence of the lower stem region. We cannot exclude an alternative mechanism, with a COS cell-specific factor that binds to the upper TAR region and that can overcome the requirement for an extended TAR stem.

The present study demonstrates that full-length TAR is required for optimal LTR transcription, thereby explaining the severe replication defect of HIV-1 variants with a truncated TAR stem. However, this does not rule out any additional role for this sequence/structure motif in the viral replication cycle, and several putative functions have been proposed [reviewed in (56)]. In this respect, we note that TAR is part of the repeat (R) region of the LTR that encodes a double hairpin motif [TAR and the “polyA-hairpin”, (57)] that is present at both the extreme 5’ and 3’ ends of all HIV-1 transcripts. These structures may actively participate in one of the many functions of the HIV-1 leader RNA that involve multiple RNA–RNA and RNA–protein contacts, e.g. RNA packaging, dimerization or reverse transcription. Interestingly, a recent study proposed a role for TAR RNA in the process of initiation of reverse transcription (58). In this process, a cellular tRNA primer that is annealed to the downstream primer-binding site (PBS) is extended by the viral reverse transcriptase enzyme over the upstream leader sequences, including the TAR element. Alternatively, relatively simple functions can be considered for the 5’- and 3’-terminal tandem hairpin motif. For instance, the structures may protect the viral RNA from degradation in the infected cell or virion particle.

Finally, this study underscores the concept that viral mechanism should be studied in experimental systems that model as closely as possible natural HIV infections. There is a growing list of controversies in HIV-1 research that are caused primarily by a difference in cell type used in the experiments. For instance, several HIV-1 accessory gene products are required for efficient virus replication in some cell types, but not in others (59) and the efficiency of reverse transcription was shown to differ significantly in T cell lines versus primary cells (60,61). This study reveals that the mechanism of Tat/TAR-mediated transcription should be performed preferentially in human T cell lines.

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