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trans Activation of Human Immunodeficiency Virus Type 1 Is Sequence Specific for Both the Single-Stranded Bulge and Loop of the *trans*-Acting-Responsive Hairpin: a Quantitative Analysis

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We have used site-directed mutagenesis to delineate sequence specific domains within the human immunodeficiency virus type 1 (HIV-1) *trans*-acting-responsive (TAR) RNA element that are required for *trans* activation by the viral Tat protein. Our data in part corroborate a recent report [S. Feng and E. C. Holland, Nature (London) 334:165–167, 1988] that five nucleotides within the loop (+29 to +33) of the TAR hairpin are important for *trans* activation. We, however, found no absolute requirement for the CUGGG loop sequence. Mutants with substitutions within the loop retained between 9 and 50% activity compared with the wild type. A second sequence, important for *trans* activation, was found in the 3-base bulge loop (+22 to +24) of the TAR hairpin. Cross-*trans*-activation studies of mutant HIV-1 TAR elements with the HIV-2 Tat protein suggest that a similar recognition event(s) forms the basis for *trans* activation of HIV-1 and HIV-2.

Human immunodeficiency virus type 1 (HIV-1) encodes a regulatory protein (Tat) that is essential for viral replication (3, 6). The target for Tat-mediated *trans* activation is the TAR (*trans*-acting-responsive) element. Mutagenesis studies have defined TAR as being positioned between nucleotides +19 and +42 with respect to the initiation site of viral transcription (7, 9, 10, 14). This 24-nucleotide long terminal repeat (LTR) segment is present in all HIV-1 mRNAs and forms the upper half of an extended stem-loop RNA secondary structure (11; Fig. 1A).

The functional TAR hairpin must maintain a base-paired stem, although the actual sequence of the stem is not important for Tat responsiveness (5, 7, 14). The identity of five of the six unpaired loop nucleotides was recently reported to be essential for *trans* activation (5). We considered a strict requirement for this $^{+29}$CUGGG^{+33} pentanucleotide surprising, since there is considerable variability in its sequence among different HIV-1 isolates (12). Therefore, we generated an identical collection of TAR loop mutants in order to quantify their relative activities.

The five loop mutants tested (L1, L2, L3, L4, and L5) are listed in Fig. 1B. The mutant LTRs were cloned into a plasmid carrying the *cat* reporter gene and were transfected into COS cells in the presence or absence of a plasmid encoding the HIV-1 Tat protein. Cell lysates were tested for CAT enzyme activity (Fig. 2A), and CAT mRNA was measured by S1 nuclease protection (Fig. 2B).

Uninduced promoter activity of all mutant constructs was comparable with that of the wild type (data not shown). The wild-type TAR construct responded dramatically to Tat (Fig. 2A, compare lanes 1 and 2; Fig. 2B, compare lanes 3 and 4). We quantitated *trans*-activation levels of approximately 200-fold (data are summarized in Fig. 1B). All loop mutants showed reduced responsiveness to Tat (Fig. 2A, lanes 3 to 7; Fig. 2B, lanes 5 to 7). Substitutions at position L1, L3, and L5 affected *trans* activation most severely. However, even these mutants were substantially activated by Tat. For example, the L3 mutant retained 9% of the wild-type activity, which is still a 17-fold increase over basal expression. Other loop mutations, such as those in L2 and L4, showed even less reduction in *trans*-activation efficiencies, retaining 94- and 60-fold increases over the uninduced level.

Our results seem to conflict with those from experiments performed with Jurkat cells (5), in which point mutations in the loop pentanucleotide resulted in complete loss of biological response to Tat. Therefore, we also tested the TAR constructs in Jurkat cells. As in COS cells, reduced activities were found for the five loop mutants compared with the wild type (data not shown). This result suggests that no tissue- or species-specific factor(s) is involved in *trans* activation of TAR. However, lymphoid cells are less efficiently transfected than COS cells and the absolute level of *trans* activation is correspondingly reduced. We saw only a 50-fold *trans* activation for wild-type TAR, and the reduction in overall activity made quantitative assessment of the most severe mutants (L1, L3, and L5) difficult. We therefore believe that numerically meaningful measurements of *trans* activation have to be performed by using an efficient transfection system.

The TAR RNA contains, besides the hairpin-loop, a second, single-stranded region (a 3-base bulge UCU from position +22 to +24; Fig. 1A). To assess the contribution of this bulge element in the *trans*-activation response, we constructed several bulge mutations. A 3-base substitution mutant, B123 (Fig. 1B; UCU to AAG), was made to change completely the sequence of the bulge while not affecting the base pairing of the adjacent nucleotides. Individual point mutations were also generated by using the same three base changes (B1, B2, and B3; Fig. 1B). A deletion mutant (BΔ, Fig. 1B) removed the entire 3-base bulge structure.

trans-Activation assays for the bulge mutants are shown in Fig. 3 and are quantitated in Fig. 1B. The most dramatic reduction in *trans*-activation level was observed for the mutants that either substituted completely or deleted completely the bulge element (B123 and BΔ in Fig. 3A, lanes 3 and 7). We measured a 12% (B123) and 15% (BΔ) *trans*-activation efficiency compared with that of the wild type. Analysis of the three point mutants showed that Tat responsiveness is most clearly reduced by the $^{+22}$U to A change in the mutant B1 (Fig. 3A, lane 4, and Fig. 3B, lane 2).

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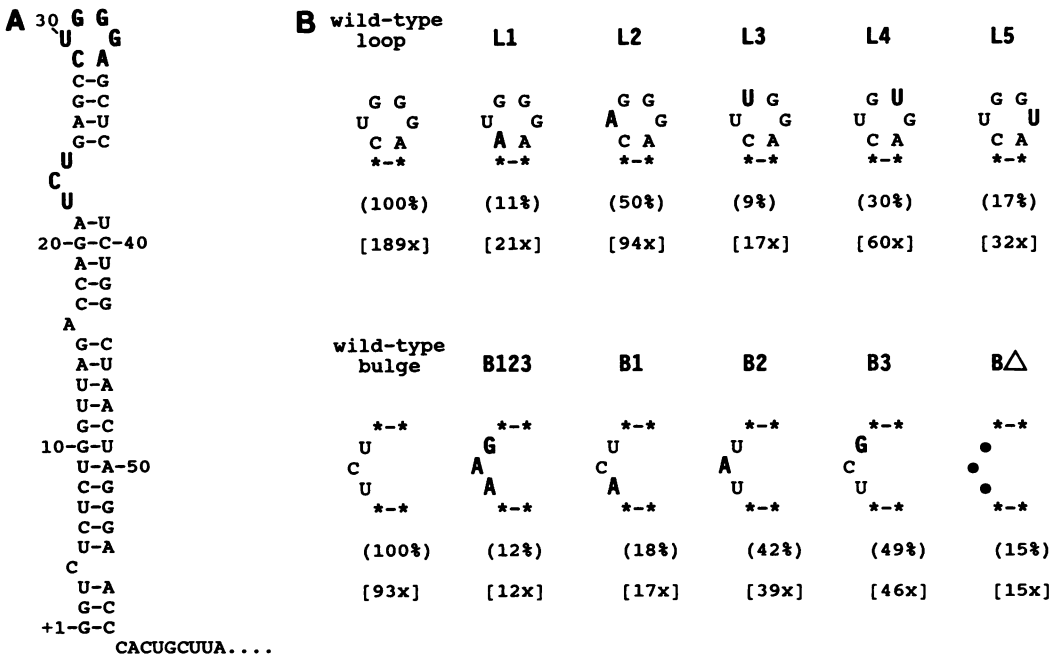

FIG. 1. (A) Secondary structure model for TAR RNA (11). Numbering is relative to the capped G residue at position +1. Single-stranded bulge and loop elements are indicated in boldface. (B) Summary of loop (L) and bulge (B) mutants. Base substitutions are indicated in boldface; ●, deletions in BΔ. * — *, Base pair that closes the hairpin loop or the base pairs that are adjacent to the bulge. The *trans*-activation efficiencies of the mutants are shown in parentheses (wild-type TAR = 100%). The fold increase in expression compared with the basal level is shown in brackets. The experimental data are shown in Fig. 2 and 3. Note that multi-base deletions in the loop region produce an absolutely inactive TAR element (data not shown).

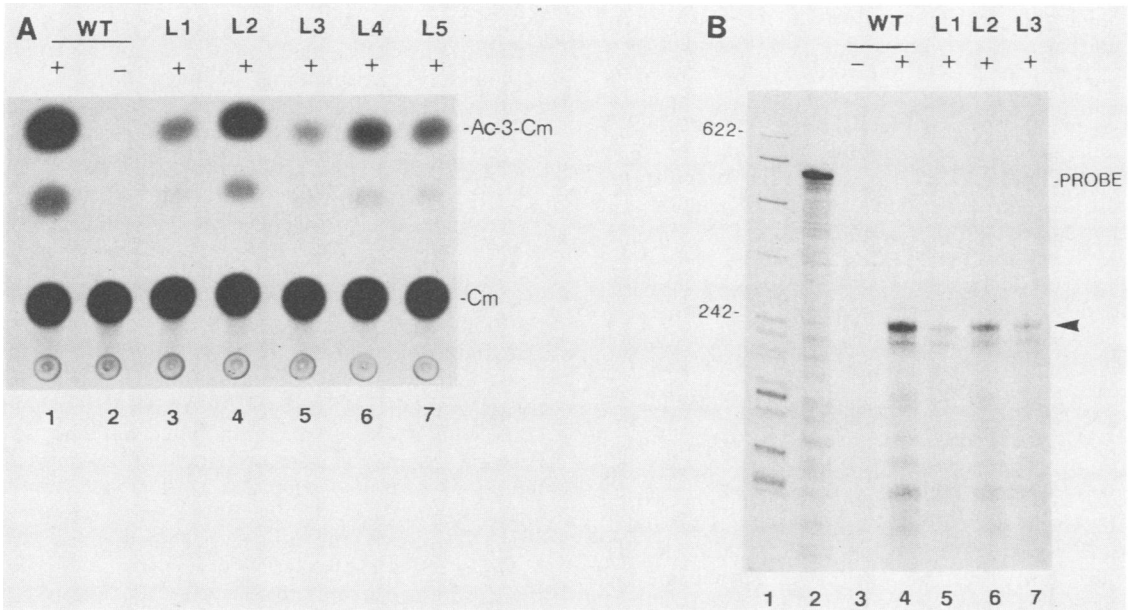

FIG. 2. Tat-mediated *trans* activation of loop (L) mutants. COS cells were transfected with 0.5 μg of LTR-CAT DNA (indicated on top of the autoradiogram) in the absence (–) or presence (+) of a Tat expression vector. At 48 h after transfection, cell lysates were tested for CAT enzyme activity (A) or total cellular RNAs were isolated and analyzed by S1 nuclease protection (B). (A) CAT enzyme activity was monitored by using a standard CAT assay analyzed by thin-layer chromatography. The positions of nonacetylated [^{14}C]chloramphenicol (Cm) and acetylated chloramphenicol (Ac-3-Cm) are indicated. (B) S1 nuclease protection was done with a uniformly labeled 522-nucleotide single-stranded CAT-DNA probe (lane 2). A 256-nucleotide fragment (◄) is expected from S1 nuclease digestion in the presence of CAT mRNA. End-labeled pBR322-*Hpa*II digest was used as a molecular weight marker (lane 1). Primer extension experiments indicated that all constructs used the same transcription initiation site (not shown).

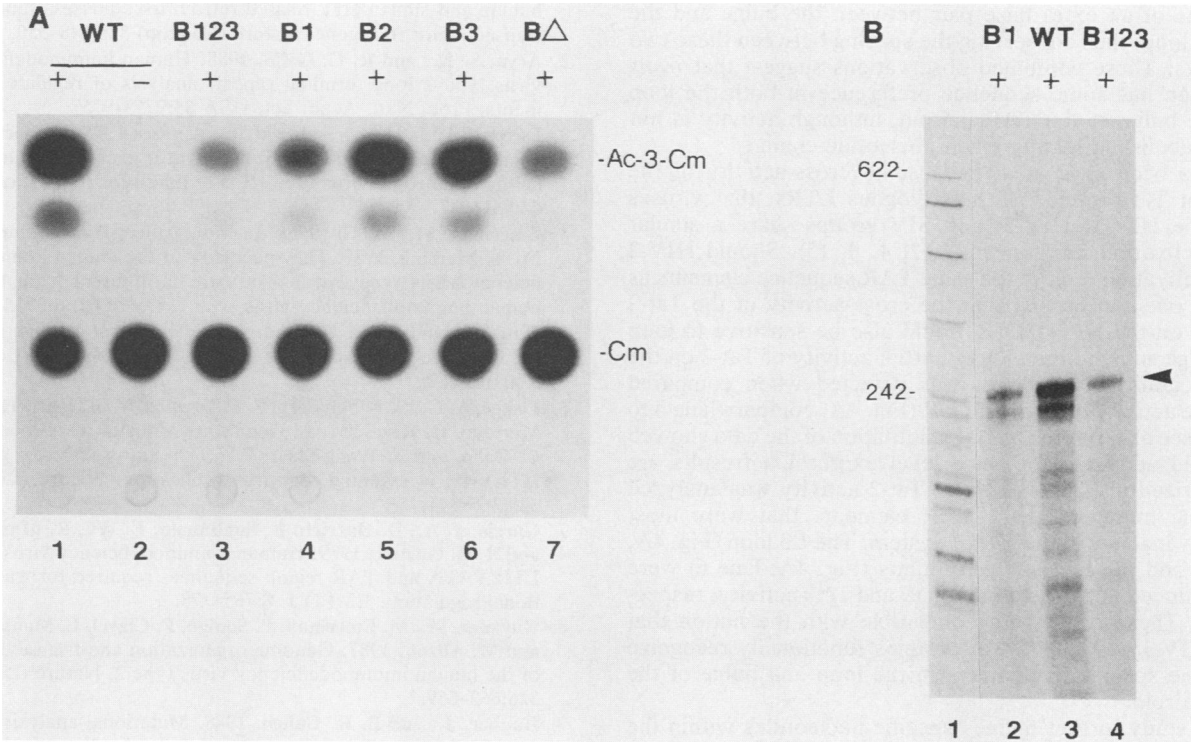

FIG. 3. Tat-mediated *trans* activation of bulge (B) mutants by a CAT enzyme assay (A) and by an S1 nuclease protection assay (B). See the legend to Fig. 2 for further details.

Phylogenetic comparisons also support the finding that bulge sequences are important for *trans* activation. While the $^{+22}$U residue is conserved in all HIV-1, HIV-2, and simian immunodeficiency virus (SIV) isolates, several changes are present in the $^{+23}$CU^{+24} bulge nucleotides. We note that the position of the bulge structure appears to be conserved. For example, a 2-base bulge, as seen for all the HIV-2 and SIV isolates, is always accompanied by the

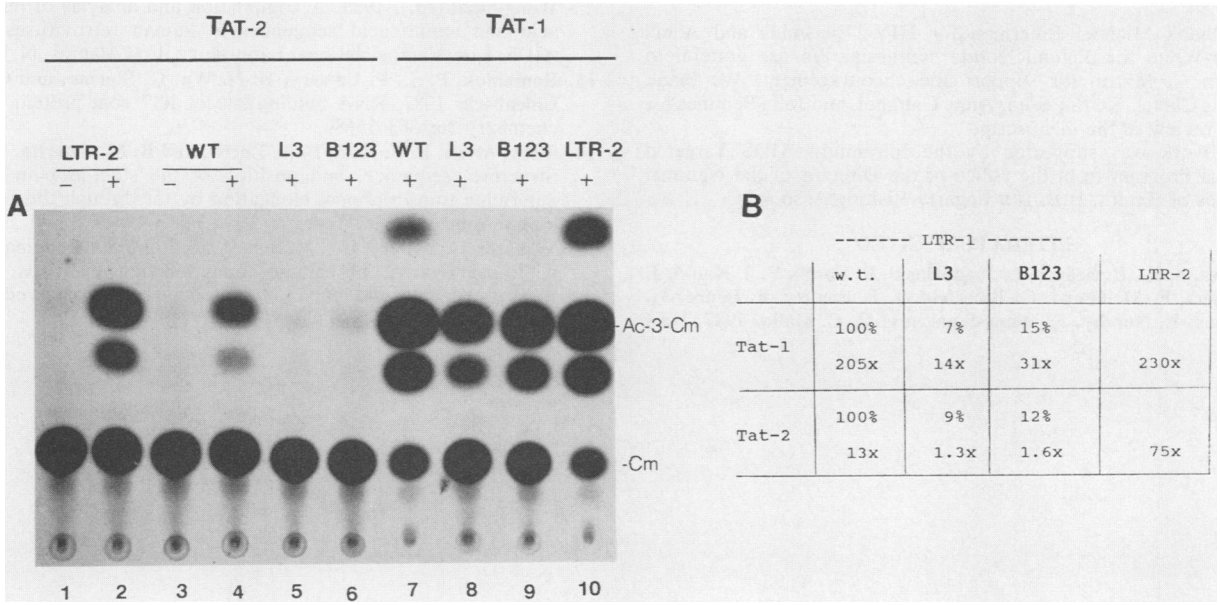

FIG. 4. Cross-*trans* activation of mutant HIV-1 TAR elements by the HIV-2 Tat (Tat-2) protein. (A) CAT assays were performed as described in the legend to Fig. 2, except that 20-fold more lysate was used per reaction. The plasmids used encode Tat-1 (pSV-tat) or Tat-2 (pROD214 in reference 4) and were transfected in combination with wild-type (WT) and mutant HIV-1 LTR-CAT constructs or the HIV-2 homolog (HIV2-LTR-CAT in reference 4). (B) Quantitative analysis of panel A. *Trans*-activation efficiencies (wild-type TAR = 100%) and the fold stimulation of basal level expression are shown.

presence of an extra base pair between the bulge and the hairpin loop, thus conserving the spacing between these two elements. These combined observations suggest that *trans* activation has some sequence preference in both the loop and the bulge of the TAR hairpin, although activity is not totally abolished by any given nucleotide change.

It has been suggested, because of cross-activity of the different Tat proteins on heterologous LTRs, that viruses from the HIV-1, HIV-2, and SIV groups share a similar *trans*-activation mechanism (1, 2, 4, 8, 15). Should HIV-2 *trans* activation require the same TAR sequence elements as HIV-1, one can predict that the cross-activity of the Tat-2 protein on the HIV-1 LTR would also be sensitive to loop and bulge substitutions. Only partial activity of Tat-2 on the heterologous HIV-1 target was detected when compared with the activity on its own LTR (Fig. 4A, compare lane 4 to lane 2; see also reference 4). Quantitation of the data showed a 13-fold activation of basal level expression (results are summarized in Fig. 4B). Next, Tat-2 activity was analyzed by using mutated HIV-1 TAR elements that were most severely inactive in the HIV-1 system. The L3 loop (Fig. 4A, lane 5) and the B123 bulge mutants (Fig. 4A, lane 6) were both reduced in *trans* activation (9 and 12% activity, respectively). These results are compatible with the notion that both HIV-1 and HIV-2 Tat proteins functionally recognize the same specific nucleotides in the loop and bulge of the TAR hairpin.

This study further defines specific nucleotides within the bulge and loop of the TAR hairpin that are most critically involved in *trans* activation. We believe it likely that the loss of *trans*-activation activity, as measured for loop and bulge mutants, is the result of interference with protein-RNA interaction. In other systems, similar nucleotide-specific contacts were reported between the single-stranded loop and bulge regions of a hairpin and viral protein (13). The identification of TAR-binding proteins awaits further study, and the description of phenotypic TAR mutants should facilitate this search.

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