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# Structural Requirements for *trans* Activation of Human Immunodeficiency Virus Type 1 Long Terminal Repeat-Directed Gene Expression by *tat*: Importance of Base Pairing, Loop Sequence, and Bulges in the *tat*-Responsive Sequence

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**In order to elucidate the molecular mechanisms of action of the *tat*-responsive sequence, mutational analysis of the *tat*-responsive sequence was carried out. The most critical region comprised nucleotides +18 to +44 and included the 3-nucleotide bulge at positions +23 to +25, the loop sequence, and an intact stem. In addition, base pairing up to nucleotide +52 was required for the full magnitude of the *trans*-activation response. Single-nucleotide bulges at positions +5 to +17 were dispensable. Analysis of truncated and full-length transcripts demonstrated that a transcriptional antitermination model does not fully account for *trans* activation.**

Two viral genes encode regulatory proteins essential for human immunodeficiency virus type 1 (HIV-1) replication: *tat* (1, 4, 9, 11, 28, 33, 34) and *rev* (7, 28, 32, 35). Both genes *trans* activate HIV-1 gene expression. The *tat*-responsive sequence (TAR) was originally mapped to the viral long terminal repeat between nucleotides −17 and +80 and thus includes sequences present at the 5′ end of all HIV-1 mRNAs (27). Both nucleotide substitutions and 3′ deletions were used to delimit the TAR region to nucleotides −17 to +44 (15), +19 to +42 (16), +18 to +44 (10), or +14 to +45 (30). The mRNA transcribed from the TAR region can form a stable stem-and-loop structure in vitro (22), which has been proposed to have functional significance (2, 8, 22, 24).

To assess the requirements for intact base pairing in the stem structure of the TAR region, we made several sets of mutations in predicted duplex regions, including and extending beyond the region previously shown to be important for TAR function (see Fig. 1A). Three sets of 3- or 4-base mutations were introduced by site-directed mutagenesis (41) on either side of the stem and are predicted to decrease the stability of the stem when present alone. The mutations were designed so that when individual mutations are combined with those at the corresponding position on the opposite side of the stem, complete base pairing is restored. This approach allows a clear distinction between changes in primary nucleotide sequence and secondary structure. The mutations were subcloned in the HIV-1 long terminal repeat-driven reporter gene pU3RIII (34). The *trans*-activation activity, measured after transient transfection by the DEAE-dextran method (12), was defined as the chloramphenicol acetyltransferase (CAT) activity obtained in an established HeLa-*tat* cell line (26) compared with that in the parental cell line. Results, expressed as the average of two to five independent experiments, are summarized in Table 1. Mutations predicted to disrupt base pairing resulted in a moderate (TARI, TARII), severe (TARI′), or near-complete (TARIII, TARIII′, TARI,III, and TARI′,III′) loss of *trans*-activation activity, with the exception of the TARII′ mutant, which had a slight stimulatory effect. These results indicate that nucleotides in TAR outside of the boundaries previously described (10, 15, 16, 30) are important for full activity. Possible explanations for this discrepancy are discussed below. In all cases, compensatory mutations predicted to restore base pairing also restored TAR function to wild-type levels. These sets of mutations emphasize the requirement for secondary structure in the TAR region for *trans* activation, especially in mutant TARI,III,I′,III′, which has 16 nucleotide changes

TABLE 1. Effects of TAR mutations on *trans* activation

| Construct | Mutated nucleotides | $\Delta G_0$ of stem-loop structure (kcal/mol)[a] | % *trans* activation of wild-type[b] (no. of experiments) |
|---|---|---|---|
| TAR (wt) | None | −38.4 | 100 (6) |
| TARI | +9 to +12 | −26.6 | 31 (4) |
| TARI′ | +49 to +52 | −26.6 | 8 (5) |
| TARI,I′ | +9 to +12, +49 to +52 | −44.8 | 78 (5) |
| TARII | +14 to +16 | −28.6 | 30 (4) |
| TARII′ | +45 to +47 | −28.6 | 127 (3) |
| TARII,II′ | +14 to +16, +45 to +47 | −38.9 | 113 (3) |
| TARIII | +18 to +21 | −19.3 | 0.3 (2) |
| TARIII′ | +41 to +44 | −19.3 | 0.5 (2) |
| TARIII,III′ | +18 to +21, +41 to +44 | −38.9 | 100 (2) |
| TARI,III | +9 to +12, +18 to +21 | −7.5 | 0.5 (2) |
| TARI′,III′ | +41 to +44, +49 to +52 | −7.5 | 0.3 (2) |
| TARI,III,I′,III′ | +9 to +12, +18 to +21, +41 to +44, +49 to +52 | −45.4 | 169 (2) |
| TAR5C | +5 (deleted) | −41.2 | 100 (1) |
| TAR17A | +17 (deleted) | −41.2 | 100 (1) |
| TAR24UCU | +23 to +25 (deleted) | −42.8 | 0.3 (2) |
| TAR31/34 | +31 to +34 | −38.4 | 1.0 (2) |

[a] Calculated according to the energy rules of Salser (29).
[b] The average fold *trans* activation was 750.

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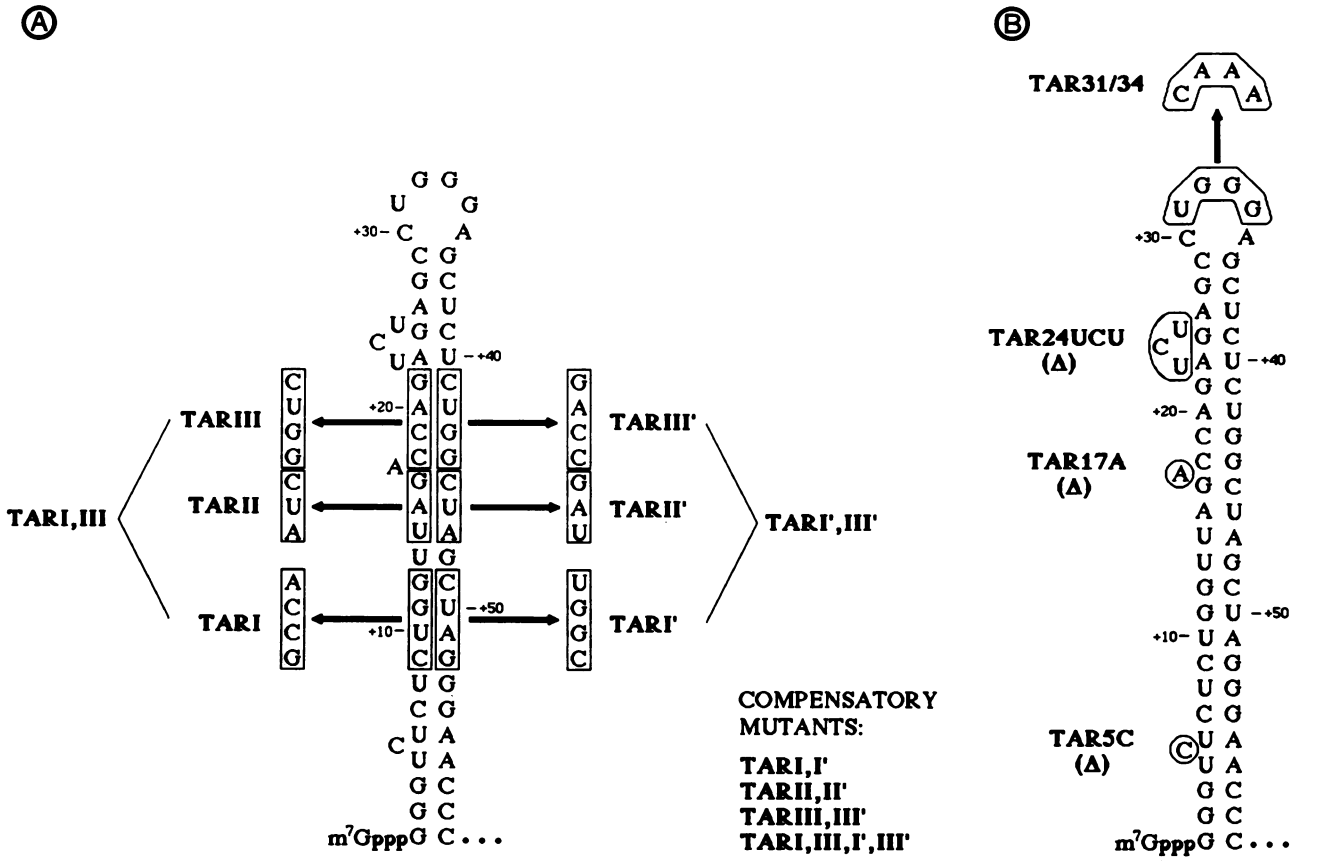


FIG. 1. (A) Secondary structure of the TAR region and stem mutants. Nucleotides shown in boxes were changed to those indicated by site-directed mutagenesis. Mutations were combined by further mutagenesis (TARI,III and TARI',III') or by subcloning, using the *Sac*I site at +34 (all other double mutants). The names of double mutants which restore base pairing in the stem are indicated (bottom, right). (B) Bulge and loop mutants. Bulged nucleotides at positions +5, +17, and +23 to +25 were deleted, and the sequence of the loop at positions +31 to +34 was mutated to the indicated sequence.

but can still be as well *trans* activated by *tat* as the wild-type construct.

One possible explanation for the discrepancy between this report and others is the type of transfection protocol used. All other reports investigating the boundaries of the TAR region (10, 15, 16, 30) have used cotransfection of the reporter gene with a *tat*-expressing plasmid, often in systems which allow efficient replication of the transfected plasmids (15, 30), whereas we have used transfection of the reporter gene into cells stably expressing *tat*. To determine whether there were major differences between the two protocols, we cotransfected the wild-type pU3RIII or mutant TARI, TARI', or TARI,I' with a simian virus 40-based *tat* expression vector, in COS cells. In this system, the mutations had little or no effect (data not shown). However, in cotransfected HeLa cells, in which the plasmid remains in low copy number, there were no qualitative differences in the results obtained compared with those described above, as evidenced by the inhibitory effect of TARI' and the restoration of activity in TARI,I' (data not shown). Thus, it is likely that the copy number of the transfected plasmids has effects on the phenotypes of some TAR mutants. Although unlikely, we cannot exclude the possibility that the mutant phenotypes of TARI and TARI' are species dependent.

A second set of mutations in the TAR region was made to determine the importance of structural features other than base pairing in the stem (Fig. 1B). Several studies have shown that bulged nucleotides in other RNA stem-and-loop structures are required for protein binding (3, 14, 25, 39). To determine whether such nucleotides are important for TAR function, bulged nucleotides at positions +5, +17, and +23 to +25 were deleted. In addition, the sequence of the loop was mutated (positions +31 to +34). *trans*-Activation activity of these mutants, as assessed by transfection in the HeLa-*tat* cell line, is summarized in Table 1. TAR5C and TAR17A, in which single-nucleotide bulges at positions +5 and +17, respectively, were deleted, had no effect on *trans* activation. However, both TAR24UCU and TAR31/34 had very low activities (less than 5% of wild type) in HeLa-*tat* cells. These results indicate that the 3-nucleotide bulge near the top of the stem is also critical for TAR function. We are currently performing additional mutagenesis in order to better define the requirement for the 3-nucleotide bulge, by changing the sequence of these nucleotides and assaying for *trans* activation. In this context, it is important that conservation of the UCU bulge is not absolute among different isolates of HIV-1, since HIV-$1_{BRU}$ contains UUU instead (38). Also, the TAR region of HIV-2, which contains loop sequences identical to those in HIV-1 (CUGGG), has a bulge of 2 uridine residues in one of the proposed stem-loop structures (8, 13). Such differences may reflect a modulation of the ability of these viruses to respond to *trans* activation

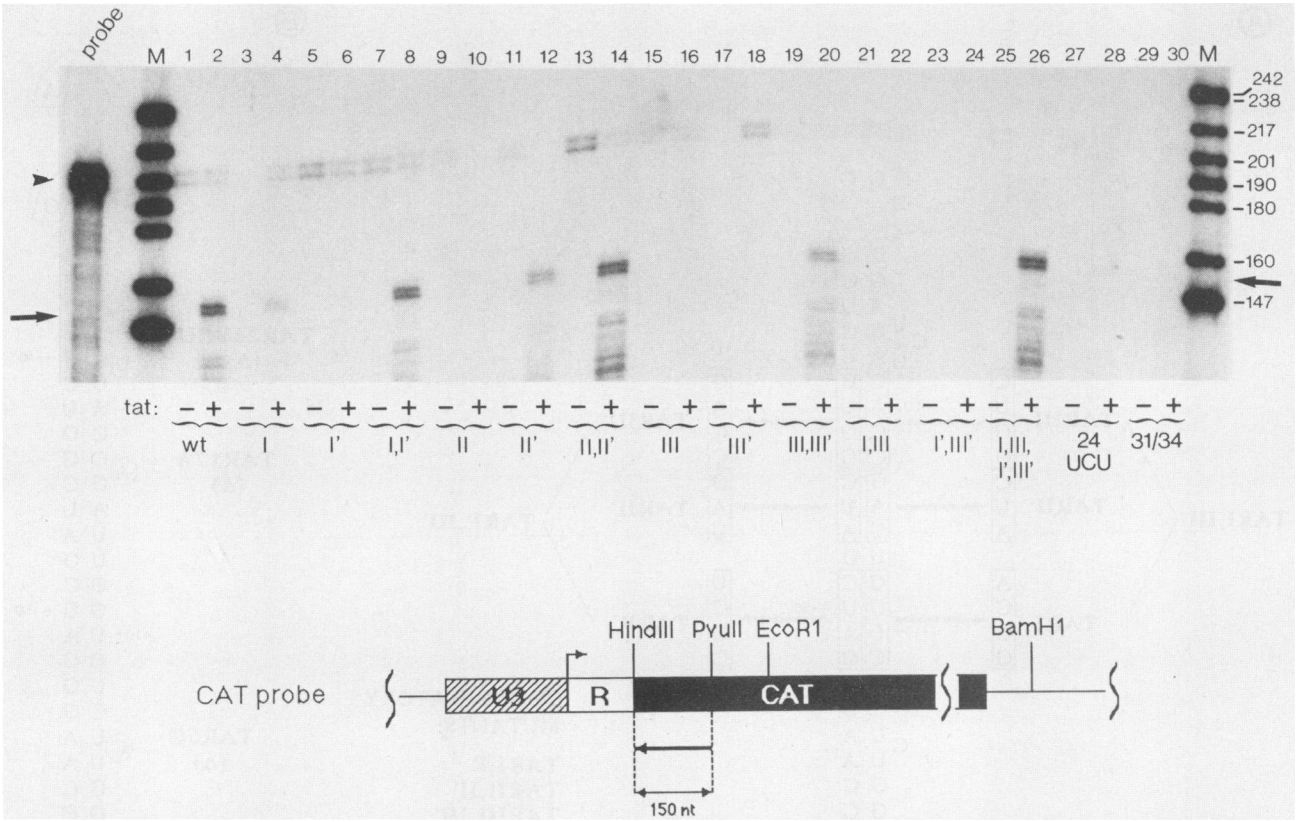


FIG. 2. Quantitative RNase protection analysis of TAR-CAT mRNA in transfected cells. Cells were transfected and 20 μg of cytoplasmic RNA was analyzed as described in the text. The unprotected probe migrated as a doublet at approximately 200 nucleotides ( ► ). Lane M, DNA size markers (end-labeled pBR322 cut with *Msp*I); odd-numbered lanes, transfected HeLa cells (−); even-numbered lanes, transfected HeLa-*tat* cells (+). Arrows indicate the position of the protected probe fragment (150 nucleotides). An autoradiogram from a 3-day exposure is shown. The plasmid used for transfection is indicated below the autoradiogram. The position of the probe relative to the structure of the TAR-CAT mRNA is also diagrammed. wt, Wild type.

or may reflect reduced structural or primary sequence requirements within the bulges.

To define the level at which TAR mutations affect gene expression, we analyzed CAT RNA levels in transfected HeLa and HeLa-*tat* cells, using an RNase protection assay (40). Cytoplasmic RNA was isolated by using the Nonidet P-40 lysis procedure (20), including a DNase I digestion step. The probe was derived from the coding portion of the CAT gene (*Hin*dIII to *Pvu*II fragment, 150 base pairs) and subcloned in antisense orientation in pSP65 (21) and is expected to protect 150 nucleotides of the CAT mRNA from RNase digestion (Fig. 2). The $^{32}$P-labeled probe (approximate specific activity, $6 \times 10^8$ cpm/μg) was gel purified on a 5% polyacrylamide-urea gel, eluted overnight at 4°C in 0.5 M ammonium acetate–1 mM EDTA–0.1% sodium dodecyl sulfate, extracted with phenol-chloroform, and ethanol precipitated. The optimal hybridization temperature for the CAT probe was determined to be 55°C. Representative results are shown in Fig. 2 (note that the undigested probe and the protected CAT band are doublets, due to some heterogeneity in the probe). All protection assays were standardized by including a probe for the endogenous γ-actin mRNA (6) at the same time as the CAT probe; the signal for γ-actin was observed to be relatively constant between samples (with one exception; see below). We were not able to detect the CAT mRNA in the absence of *tat* (Fig. 2, odd-numbered lanes), presumably because of the very low level of expression. The signal obtained from HeLa-*tat* cells transfected with most plasmids (Fig. 2, even-numbered lanes) roughly (within a factor of two) paralleled the level of *trans* activation, with the exception of TARII (Table 1). For this construct, the CAT mRNA was barely detectable (Fig. 2, lane 10) although it had only a moderate inhibitory effect on *trans* activation. This discrepancy may be accounted for by a reduction in the amount of RNA used in the assay, since the signal for γ-actin was weaker in this sample than in others (data not shown).

We also examined the effect of TAR mutations on the reported antitermination activity of *tat* (17, 30, 36). This activity can be assayed by comparing the cytoplasmic levels of short RNA species of 59 to 65 nucleotides, suggested to be prematurely terminated transcripts, with those of full-length or read-through transcripts. Figure 3 shows the results of an RNase protection assay, using cytoplasmic RNA from HeLa or HeLa-*tat* cells transfected with wild-type TAR (lanes 1 and 2) or mutants TARI (lanes 3 and 4), TARI′ (lanes 5 and 6), and TARI,I′ (lanes 7 and 8) and corresponding antisense probes spanning the first 85 nucleotides of the HIV-1 untranslated region (*Pvu*II-*Hin*dIII fragment of pU3RIII), including the TAR element. The optimal hybridization temperature, for both short and full-length transcripts, was found to be 50 to 55°C. A low level of attenuated transcripts was detectable in HeLa cells transfected with pU3RIII (wild type; Fig. 3, lane 1) or with the double mutant TARI,I′ (Fig.

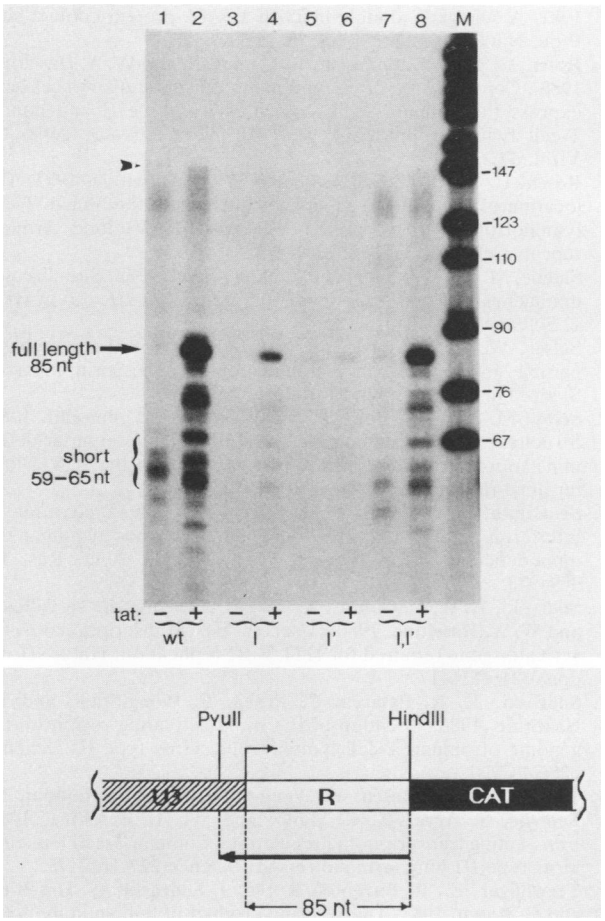


FIG. 3. RNase protection analysis of 5′ ends of TAR-CAT mRNAs. The relative abundance of full-length (85-nucleotide) and short (59- to 65-nucleotide) transcripts in cytoplasmic RNA preparations from HeLa (−) or HeLa-*tat* (+) cells transfected with pU3RIII (wild type [wt]) or mutant TARI, TARI′, or TARI,I′ was determined by using homologous RNA probes spanning the transcriptional start site (see diagram at bottom). A 10-μg amount of cytoplasmic RNA was used in the assay. Protected fragments were resolved on an 8% polyacrylamide-urea gel; an autoradiogram from a 7-day exposure is shown. Lane M, *Msp*I-digested, end-labeled pBR322. The migration position of the undigested probe is indicated ( ➤ ).

3, lane 7). However, these short RNA species were not detected in cells transfected with TARI (Fig. 3, lane 3) or TARI′ (Fig. 3, lane 5), indicating that secondary structure in this region is important for the generation and/or stability of the short transcripts. In HeLa-*tat* cells, full-length transcripts, whose relative amounts parallel those obtained by using the CAT probe, are readily seen (Fig. 3, even-numbered lanes). It is important, however, that there was an overall increase in the total amount of RNA detected in HeLa-*tat* cells compared with that in HeLa cells, with a preferential up-regulation in the expression of full-length transcripts. Therefore, unless there were major differences in the stability of the short transcripts in HeLa cells, relative to that of the full-length transcripts in HeLa-*tat* cells, the antitermination mechanism does not account for the full effect of *tat* in this system.

Since short transcripts were not detected in cells transfected with mutant TARI or TARI′ in the absence of *tat* and were barely detectable in the presence of *tat*, it is possible that these mutations affect the termination site, the transcription initiation efficiency, or the stability of terminated RNAs. One interesting possibility, for which evidence is being sought, is based on the recent observation that induction of beta interferon transcription by double-stranded RNA is mediated in part by NF-κB (19, 37). If the double-stranded nature of the TAR region could also induce NF-κB activity, perhaps via the double-stranded RNA-dependent p68 kinase (5, 31, 37), then this mechanism may contribute to HIV long terminal repeat-directed transcription, since the long terminal repeat contains two NF-κB-binding sites (23). The mutant RNAs TARI or TARI′, however, would be expected to be less potent activators of NF-κB and therefore to be transcribed less efficiently (Fig. 2 and 3).

This study, designed to analyze the structural requirements for TAR function, suggests that responsiveness to *tat* requires the bulge at positions +23 to +25, the loop sequence, and proper base pairing. The effects of mutations in nucleotides +9 to +12 and +49 to +52, although not as dramatic as those within the upper portion of the stem, are nevertheless significant. The importance of the lower region may not have been previously realized because it appears to be dependent on the transfection protocol and/or the cell type used to examine the *trans*-activation response.

Since the original submission of the manuscript, Laspia et al. reported similar results with respect to the contribution of antitermination to *trans* activation, using a recombinant adenovirus vector (18).

The first two authors contributed equally to this work.

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