

The Human Immunodeficiency Virus Type 1 Tat Protein Transactivates Tumor Necrosis Factor Beta Gene Expression through a TAR-Like Structure

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Received 6 October 1993/Accepted 14 January 1994

We have previously shown that the Tat protein of human immunodeficiency virus type 1 (HIV-1) transactivates tumor necrosis factor alpha and beta (TNF α and TNF β) gene expression in HIV-1-infected and in *tat*-transfected T-lymphocytic and monocytic cell lines. The product encoded by the first exon of the *tat* gene (amino acids 1 to 72) is sufficient for this transactivation. Here we show that (i) the NF- κ B and Sp1 binding sites of the TNF β promoter are required for Tat-mediated transactivation and (ii) a predicted stem-loop structure in the TNF β mRNA leader region, which resembles the Tat-responsive element of the HIV-1 long terminal repeat (TAR) and which is therefore termed TAR-like, is essential for TNF β transactivation by Tat. These data suggest that similar promoter regulatory elements are necessary for Tat-mediated transactivation of both TNF β and HIV-1 gene expression. This represents the first demonstration of a cellular gene with a regulatory element downstream of the transcriptional initiation site that, like TAR, may function as an RNA element.

The human immunodeficiency virus type 1 (HIV-1) genome encodes several regulatory proteins that control the virus life cycle (11, 14, 16). One of these gene products, the Tat protein, *trans* activates HIV-1 long terminal repeat (LTR)-directed gene expression and is essential for virus replication (1, 11, 16, 40). Tat is an 86-amino-acid protein encoded by two exons (1, 14, 40). The product of the first exon of *tat* (amino acids 1 to 72) is sufficient for transactivation of the HIV-1 promoter (37). Tat-induced HIV-1 LTR transactivation requires regulatory elements located upstream of the transcriptional initiation site of the HIV promoter, such as the NF- κ B and Sp1 binding sites and the Tat-responsive element (TAR) located downstream (+19 to +42), which forms a stable stem-loop structure when transcribed into RNA (4, 5, 15, 17, 23, 27, 32) (Fig. 1B). Previous observations indicated that Tat binds to a bulge structure in the TAR RNA element (5, 33, 34). Other studies suggested that a cellular protein(s), binding to the TAR loop, acts in concert with Tat to activate the HIV-1 promoter (18, 19). The Tat-TAR interaction results in an increased rate of transcription and increased efficiency of transcriptional elongation and facilitates the initiation of complex formation (6, 22, 24, 25, 31, 38). Deletions introduced in the TAR region that perturb the loop sequence, the stem structure, or the binding of cellular proteins result in a marked decrease of Tat-mediated transactivation (15, 21, 22, 34, 38). Nevertheless, when the NF- κ B and Sp1 binding sequences of the HIV-1 LTR are deleted, Tat-mediated transactivation is greatly reduced, even in the presence of a functional TAR (4), indicating that for efficient viral transactivation both upstream and downstream (TAR) promoter regulatory elements are required (4).

We have previously reported that the HIV-1 Tat protein

transactivates the expression of the tumor necrosis factor beta (TNF β) gene in monocytic (U937), T-lymphocytic (H9 and Jurkat), and epithelial (COS-1) cell lines transiently or permanently expressing the *tat* gene (8). In T-cell lines acutely or chronically infected with HIV-1, TNF β promoter activation is associated with increased TNF α and - β mRNA steady-state levels and with enhanced production and secretion of these cytokines (8). Similar to data obtained in previous studies with the HIV-1 promoter (28, 39, 41), we also found that the product of the first exon of *tat* is sufficient for TNF activation (8). These results suggested that Tat-mediated activation of TNF gene expression requires promoter regulatory elements similar to those involved in HIV-1 transactivation. Thus, we searched for regions in the TNF β promoter analogous to those present in the HIV-1 LTR and known to be required for Tat-mediated transactivation. Putative binding sites for the NF- κ B and Sp1 cellular transcription factors are present in the TNF β promoter region at positions -98 to -88 and -64 to -57, respectively (29, 30, 42). Similarly, computer analysis performed to predict the secondary structure of the TNF β 5' untranslated region revealed the presence of stable secondary stem-loop structures between the nucleotides at positions +1 and +115 containing sequences closely related to those present in the TAR region of HIV-1 (Fig. 1A). On the basis of these similarities, this region was termed TAR-like. In the present study, we have analyzed the functional role played by these regulatory regions in Tat-mediated transactivation of TNF β gene expression. The results indicate that both the upstream and downstream (TAR-like) promoter elements of the TNF β gene are necessary for transactivation by the HIV-1 Tat protein.

MATERIALS AND METHODS

Construction of the TNF β promoter deletion mutants. A series of 5' and 3' deletion mutants of the TNF β promoter region (relative to the initiation site of transcription) were engineered by the PCR methodology (43). To remove the

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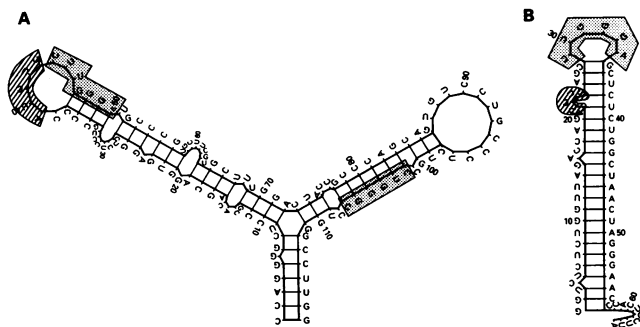


FIG. 1. Predicted secondary structure of the TNF β sequence 3' of the CAP site (+1 to +115) (A) and of the HIV-1 TAR (B). The similarities which the TNF β sequences share with the HIV-1 LTR bulge (▨; AUCUG) and loop (▩; CUGGGA) sequences are indicated. The numbers indicate nucleotide positions with respect to the start site of transcription (+1).

NF- κ B and Sp1 binding sites ($-$ NF κ B and $-$ Sp1 plasmids), two sets of primers (35-mers) (a to d) which consisted of 20 bases (3' end) complementary to the target sequence of the TNF β promoter and of 15 bases (5' end) containing the cleavage site for *Eco*RI (add-on sequence) were synthesized. These primers are listed in Table 1. The primers complementary to the TNF β sequences $-$ 305 to $-$ 286 and +96 to +115 (primers e and f, respectively) were utilized to generate the constructs. Both primers contained an add-on sequence at the 5' end for cloning in the unique *Hind*III site of pSV0-CAT (8). The PCR products were digested with *Eco*RI, and the appropriate fragments were ligated to each other (eb-cf and ed-af), digested with *Hind*III, and cloned in the pSV0-CAT plasmid, upstream of the chloramphenicol acetyltransferase (CAT) reporter gene (20). To delete the TNF β sequence 3' of the CAP site ($-$ TAR-like plasmid), primer e ($-$ 305 to $-$ 286) and a primer complementary to the +13-to-+32 coding region of TNF β , which contained an add-on sequence at the 5' end recognized by *Hind*III, were utilized for the amplification reaction. The latter primer is as follows: 5' ATTAGAAGCT TAGGAGAGCCTCACCTGCTGT 3'. The PCR product was digested with *Hind*III and cloned in the pSV0-CAT plasmid. The amplification reactions were carried out as previously described, but the number of cycles was reduced in order to prevent accumulation of unwanted mutations (43). Nucleotide sequence analysis of the plasmid DNAs, performed with the Sequenase kit (U.S. Biochemical Corporation), confirmed the deletions introduced in the TNF β promoter region and that the orientations of the subcloned fragments were correct.

Plasmids, cell cultures, transfections, and CAT assays. In addition to the plasmids containing the wild type (8) and the deletion mutants of the TNF β promoter, the CD7-CAT plasmid, which contains the wild-type HIV-1 LTR (13); the Δ BS-CAT plasmid containing the HIV-1 LTR lacking 4 bp at the top of the TAR stem-loop structure (13); and a human

T-cell lymphotropic/leukemia virus type I (HTLV-I) Tax-expressing vector (10) were utilized for the transfection experiments. The plasmids (10 μ g of total DNA) were transfected into a Jurkat cell line constitutively expressing Tat (Jurkat-Tat) and into the parental Jurkat cells by using a previously described electroporation procedure adapted to suspension cells (8). Alternatively, the *tat*-expressing plasmid pCV-TAT or its control plasmid pCV-0 (5 μ g each) (8) and the TNF β -CAT constructs (5 μ g each) were transiently cotransfected into Jurkat cells by the same procedure. Experiments to study phorbol 12-myristate 13-acetate (PMA) induction were performed by adding PMA (25 ng/ml) to the cells at 12 to 24 h after transfection and harvesting the cells 24 to 48 h later. Cellular extracts were prepared as previously described (8, 20), and protein concentrations were determined by the Bradford method (7). CAT assay reactions were performed for 15 min to 2 h at 37°C with equal amounts of total cellular proteins, by using butyryl coenzyme A as acyl donor (36). The butyrylated forms of chloramphenicol were either extracted with xylene and counted in a scintillation counter (36) or extracted with ethyl acetate and separated by thin-layer chromatography (20).

RESULTS

5' TNF β promoter regions involved in Tat-mediated transactivation. To determine the role played by the upstream regulatory regions of the TNF β promoter in Tat-mediated transactivation, 5' deletion mutants of the TNF β promoter region (relative to the transcription initiation site) were constructed by removing the putative binding sites for the cellular transcription factors NF- κ B ($-$ 98 to $-$ 88) and Sp1 ($-$ 64 to $-$ 57) (29, 30, 42) and by subcloning the resulting fragments in the promoterless pSV0-CAT plasmid (Fig. 2A). These constructs were transfected into Jurkat-Tat cells or into the parental Jurkat cells, and TNF β -CAT activity was measured and compared with the activity obtained with the wild-type TNF β -CAT plasmid. The removal of the NF- κ B and Sp1 binding sequences of the TNF β promoter resulted in a significant reduction of Tat-mediated transactivation (Fig. 2B and 3A). In particular, transactivation in Jurkat-Tat cells was 1.8- and 2.4-fold with the $-$ NF- κ B and the $-$ Sp1 plasmids, respectively, and 8.25-fold with the wild-type TNF β -CAT plasmid compared with transactivation in transfected Jurkat cells lacking Tat (Fig. 2B and 3A, lanes 1 to 3 and 7 to 9). Similar results were obtained with Jurkat cells transiently cotransfected with the *tat* gene (pCV-TAT) or its control plasmid (pCV-0) and the TNF β -CAT constructs (Fig. 3B). In particular, Tat-induced transactivation was 1.5- and 2-fold for the $-$ NF- κ B and $-$ Sp1 constructs, respectively (Fig. 3B, lanes 1 to 3 and 7 to 9). These results indicated that these *cis*-regulatory elements of the TNF promoter are required for Tat-mediated transactivation of TNF β gene expression.

A TAR-like structure present in the TNF β 5' untranslated region is required for Tat-mediated transactivation of the TNF promoter. Computer analysis revealed the presence of stable secondary stem-loop structures ($-$ 41.4 kcal of free energy

TABLE 1. Primers used in construction of TNF β promoter deletion mutants

Primer	Add-on sequence	Complementary sequence (positions)
a	5' AAATTGAATTC AAAA	TTCTCTATAAAGGGACCTG 3' ($-$ 29 to $-$ 12)
b	5' TTTTGAATTC AATTT	GGGCTTAGAAGATACTGCTG 3' ($-$ 118 to $-$ 100)
c	5' AAATTGAATTC AAAA	TAGAACC CGCCCGCTGCCTG 3' ($-$ 69 to $-$ 50)
d	5' TTTTGAATTC AATTT	TTCTAGGTCGGGCTGGGC 3' ($-$ 84 to $-$ 65)

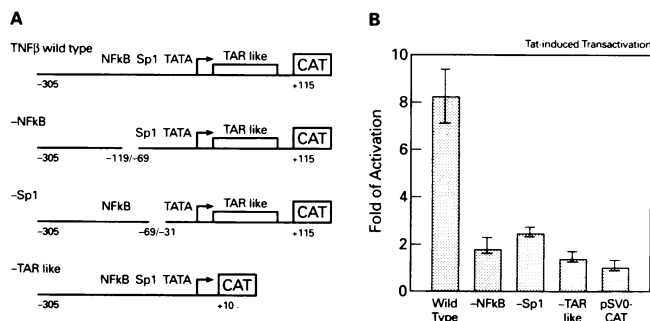


FIG. 2. (A) Schematic representation of the wild-type and mutant TNF β plasmids. The positions of the upstream and downstream regulatory sequences are indicated at the top of the figure. The initiation sites of transcription are marked by arrows. Specific nucleotide deletions are indicated in the corresponding constructs. (B) Fold transactivation of Tat-induced TNF β -CAT gene expression (CAT activity) relative to each TNF β promoter deletion mutant and wild-type construct. Fold increase of transactivation in the presence of Tat (Jurkat-Tat cells) was evaluated over the basal level of TNF β -CAT activity obtained by transfecting the constructs into the parental Jurkat cell line. The results represent the means for four independent experiments.

[−173.2 kJ]) at positions +1 to +115 of the TNF β 5' untranslated region (Fig. 1A). This region contains nucleotide sequences similar to those present in the HIV-1 TAR region and essential for Tat-mediated transactivation of HIV-1 gene expression. Specifically, the first loop of the TNF β RNA secondary structure contains a sequence (+39-AUCUC−+43) which is 80% homologous (4 of 5 matching nucleotides) with the AUCUG sequence recognized by Tat in the bulge of the TAR (Fig. 1) (5, 33, 34). In addition, the loop sequence of the TAR (CUGGGA), believed to be the target for the binding of a cellular factor(s) (Fig. 1B) (18, 19), is also present in this TNF β region at positions +44 to +50 with a nucleotide insertion and a mismatch (CUUGGGC) and at positions +102 to +107 with a single mismatch (CUGGGC) (Fig. 1A). In neither case, however, does this sequence appear to be presented as a loop; therefore, it may not be accessible or necessary for the binding of cellular proteins. To assess the role played by the 5' untranslated region of TNF β in Tat-mediated transactivation, the sequences 3' of the CAP site of the TNF β gene were deleted (−TAR-like plasmid) (Fig. 2A). When this construct was transfected into Jurkat-Tat cells or cotransfected with pCV-TAT into Jurkat cells, no Tat-mediated transactivation of TNF β gene expression was observed, in contrast to what was observed with the wild-type TNF β -CAT construct (Fig. 2B and 3, lanes 1 and 4 and 7 and 10). This demonstrated that the sequences 3' of the CAP site (TAR like) are essential for Tat-mediated transactivation of the TNF β promoter.

Effects of Tax and PMA on the TNF β promoter lacking the TAR-like sequences. When the −TAR-like TNF β construct was transfected into the parental Jurkat cells, we found that the basal levels of TNF β -CAT activity were 5- to 10-fold lower than with the wild-type TNF β plasmid (Fig. 3, lanes 1 and 4). This suggested the following two different possibilities: (i) the untranslated (leader) mRNA region plays a critical role in the basal and Tat-induced expression of the TNF β promoter, or (ii) the −TAR-like TNF β construct contains an inactive promoter. To exclude the latter possibility, experiments were performed with PMA and the HTLV-I transactivation protein Tax, since both activate gene expression through the NF- κ B-responsive element, which is still present in the −TAR-like

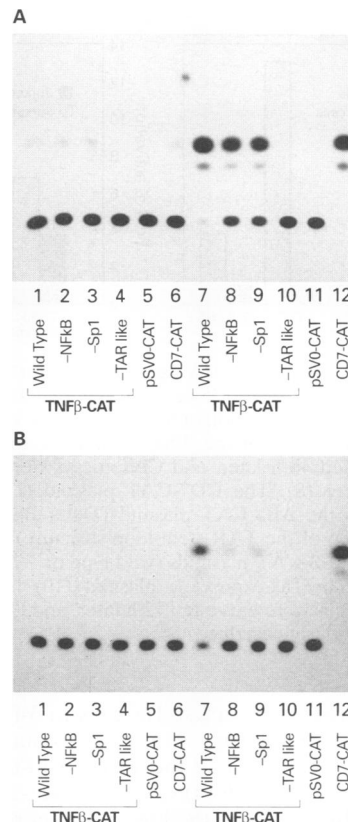


FIG. 3. (A) CAT assays after transfection of the TNF β -CAT constructs into Jurkat (lanes 1 to 6) and Jurkat-Tat (lanes 7 to 12) cell lines. (B) CAT assays after cotransfection of the TNF β -CAT constructs with pCV-0 (lanes 1 to 6) or pCV-TAT (lanes 7 to 12) plasmids into Jurkat cells. The transfected CAT-reporter plasmids are shown at the bottom of each lane. In both panels, the CD7-CAT plasmid (HIV-1 LTR) was used as a control of Tat expression (lanes 6 and 12) while the pSV0-CAT plasmid was used as the negative control (lanes 5 and 11). In order to obtain maximal transactivation levels with the TNF β -mutated promoters, CAT reactions were carried out for 2 h. Under these experimental conditions, the reaction is at the plateau for the CD7-CAT plasmid, explaining the slight difference in CAT activity between the wild-type TNF β -CAT construct and the CD7-CAT plasmid, in contrast with what was observed in previous comparative studies (8).

construct. Tax, in particular, has been previously shown to activate TNF β gene expression (2, 26, 30, 41, 42). The addition of PMA did not induce any significant increase of TNF β -CAT expression (both wild-type and −TAR-like constructs) in Jurkat or Jurkat-Tat cells (Fig. 4A), as previously described (8, 30, 42). On the contrary, PMA induced HIV-1 LTR-CAT expression in Jurkat-Tat cells but not in Jurkat cells lacking Tat, in agreement with previous data (41). In addition, with Jurkat-Tat cells, transactivation was observed both with the HIV-1 LTR wild-type plasmid (CD7-CAT) (13-fold) and with an LTR construct lacking the top 4 bp in the TAR stem-loop structure (Δ BS-CAT) (3.8-fold) (13) (Fig. 4A). The lesser activation effect of PMA with the Δ BS-CAT plasmid may be partially explained by the loss of a cooperative action between Tat and PMA in the absence of a functional TAR region. When these experiments were repeated by cotransfecting the cells with a Tax-expressing plasmid, a 1.8-fold transactivation of the −TAR-like TNF β plasmid was obtained in Jurkat cells,

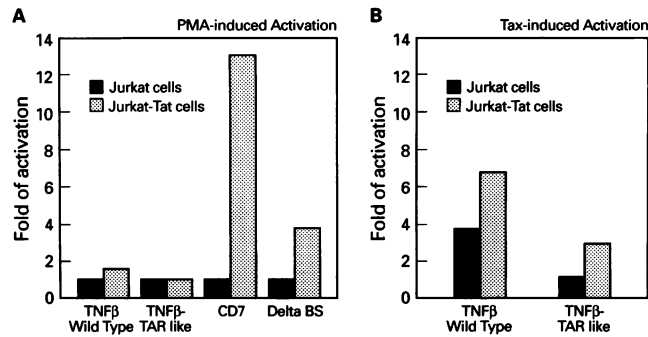


FIG. 4. Activation of TNF β -CAT gene expression by PMA (A) or by HTLV-I Tax (B). (A) PMA (25 ng/ml) was added to Jurkat-Tat and Jurkat cells 24 h after transfection of the cells with the wild type or with the TAR deletion mutant of the TNF β promoter and HIV-1 LTR. Cells were harvested 48 h later, and CAT assays were performed as described elsewhere (8). The CD7-CAT plasmid (CD7) (wild-type HIV-1 LTR) and the Δ BS-CAT plasmid (Delta BS) (HIV-1 LTR lacking the top 4 bp of the TAR stem-loop structure) (13) were used as controls. (B) TNF β -CAT plasmids (wild type or -TAR-like) were cotransfected with a Tax-expressing plasmid (10) into Jurkat and Jurkat-Tat cells. Cells were harvested 72 h later, and CAT assays were performed as previously described (8).

and this was increased in Jurkat-Tat cells to 3-fold (Fig. 4B). Similarly, and in agreement with recent data obtained by others (42), Tax transactivated the TNF β wild-type promoter both in Jurkat cells (3.75-fold) and in Jurkat-Tat cells (6.7-fold) (Fig. 4B). Interestingly, the lower level of responsiveness to Tax of the -TAR-like TNF β construct, in comparison with the wild-type TNF β promoter, resembles the results obtained with the HIV-1 LTR and its TAR deletion mutant (Δ BS) after stimulation with PMA.

These results demonstrated that the -TAR-like TNF β promoter is still transcriptionally competent and that the absence of transactivation in the presence of Tat (Fig. 3) is due specifically to the removal of the TAR-like sequence of the TNF β gene.

DISCUSSION

In this study, we have defined the promoter regions required for Tat-mediated transactivation of TNF β gene expression, a phenomenon previously reported by us (8) and others (35). The TNF β upstream promoter region contains the putative binding sites for the cellular transcription factors NF- κ B and Sp1, which are located at positions -98 to -88 and -64 to -57, respectively (29). The TNF β region 3' of the CAP site, between +1 and +115, contains nucleotide sequences similar to those present in the bulge and loop of the HIV-1 TAR RNA and which are responsible for the binding of Tat and cellular protein(s) (Fig. 1) (29). In addition, computer analysis predicted that this TNF β region forms stable secondary stem-loop structures resembling the HIV-1 TAR (Fig. 1A). On the basis of these similarities, we termed this region TAR-like. Transfection of the 5' and 3' TNF β promoter deletion mutants (Fig. 2A) in Jurkat cells transiently or permanently expressing the *tat* gene indicated that both the upstream and the downstream regulatory elements of the TNF β promoter are required for Tat-mediated transactivation (Fig. 2B and 3). In particular, the results suggest that a critical role in this biological effect is played by the 5' untranslated region (containing the TAR-like structure) (Fig. 2B and 3). The same region appears to be critical also for the basal transcriptional activity of the TNF β

promoter. The -TAR-like mutant, in fact, showed a 5- to 10-fold reduction of the constitutive promoter activity when compared with the TNF β wild-type plasmid after transfection in Jurkat cells (Fig. 3, lanes 1 and 4). This observation is consistent with previous data concerning the role of a TAR-like structure in the transcriptional activity of the JC virus late promoter (9). Experiments performed with activators of cellular genes known to induce gene expression by acting only on upstream regulatory sequences, such as PMA and the HTLV-I transactivator Tax (2, 26, 30, 41, 42), indicated that the -TAR-like plasmid is still transcriptionally active (Fig. 4). In particular, both wild-type and -TAR-like TNF β plasmids showed a significant responsiveness to the HTLV-I Tax protein (Fig. 4B). This effect was more evident in Jurkat-Tat cells, suggesting that Tat may have an additive effect on TNF β gene expression by binding to the TAR-like region or, when deleted, by interacting with cellular DNA binding proteins (17). On the contrary, PMA did not induce TNF β -CAT activity, wild type or -TAR-like, in either Jurkat or Jurkat-Tat cells, in agreement with previous results obtained by others (Fig. 4A) (30). However, in the presence of Tat, PMA induced transactivation of the TAR deletion mutant of the HIV-1 LTR (Δ BS-CAT) but at levels lower than those of the wild type. This resembles the results obtained with the -TAR-like TNF β deletion in the presence of Tax and Tat. Thus, the -TAR-like TNF β plasmid is still inducible by transcriptional activators requiring only upstream regulatory elements (NF- κ B), suggesting that the loss of Tat-mediated transactivation with this construct is due specifically to the deletion of the TAR-like region. The low-level promoter activity of the -TAR-like TNF β construct may be due to differences in TNF β mRNA stability after removal of the 5' untranslated region, as previously shown for the TAR-deleted HIV-1 LTR (17).

The data presented here show that TNF β transactivation by Tat requires both the upstream promoter regulatory elements and sequences in the RNA leader region. This is similar to results previously reported for the HIV-1 LTR (4, 5, 15, 17, 23, 27, 32) and the JC virus late promoter (9). Although additional experiments are required to define the molecular mechanism(s) involved in Tat-mediated transactivation of TNF β gene expression, these results represent the first demonstration of nucleotide sequences similar to those present in the HIV-1 TAR RNA and required for the activation of a cellular gene by Tat and demonstrate a novel role played by the region 3' of the CAP site in the basal transcriptional activity of the TNF β promoter. The biological relevance of this observation is linked to the fact that TNF β has been involved in the pathogenesis of AIDS and AIDS-associated disorders (3, 8, 12); therefore, its increased expression may play a role in AIDS.

ACKNOWLEDGMENTS

This work has been partially supported by the Italian Ministry of Health (Ricerca Corrente 1993) and by Istituto Superiore Sanità (VI Italian AIDS program).

Furthermore, we thank G. Barbanti-Brodano (Institute of Microbiology, University of Ferrara, Ferrara, Italy) for the Jurkat and Jurkat-Tat cell lines; P. Fitzgerald (Office of the Director, Division of Computer Research and Technology, National Institutes of Health [NIH], Bethesda, Md.) for RNA tertiary-structure computer analysis; E. Tschachler (Department of Dermatology, University of Vienna, Vienna, Austria), S. Daeffler (Department of Internal Medicine, New York University, New York, N.Y.), and M. L. Tornesello (Division of Viral Oncology, "Fond. G. Pascale," Naples, Italy) for helpful discussion; R. C. Gallo (Laboratory of Tumor Cell Biology [LTCB], National Cancer Institute [NCI], NIH, Bethesda, Md.) for continued support; and S. Arya and M. Reitz (LTCB, NCI, NIH, Bethesda, Md.) and E.

Beth-Giraldo (Division of Viral Oncology, "Fond. G. Pascale," Naples, Italy) for critical review of the manuscript.

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