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NOTES

An Adenosine at Position 27 in the Human Immunodeficiency Virus Type 1 *trans*-Activation Response Element Is Not Critical for Transcriptional or Translational Activation by Tat

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Tat protein binds to the *trans*-activation response (TAR) element of human immunodeficiency virus type 1 RNAs and activates gene expression at the level of transcription in mammalian cell lines and translation in *Xenopus* oocytes. Certain residues within TAR are important for Tat binding in vitro, including residue A-27, which appears to be able to be modified in a Tat-dependent manner in *Xenopus* oocytes (L. Sharmeen, B. Bass, N. Sonenberg, H. Weintraub, and M. Groudine, Proc. Natl. Acad. Sci. USA 88:8096–8100, 1991). Activation by Tat in oocytes occurs via a covalent modification of TAR-containing RNA. We have found that in both mammalian cells and *Xenopus* oocytes, conversion of A-27 · U-38 to U-27 · A-38 or C-27 · G-38 reduces activation. However, conversion to G-27 · U-38 or G-27 · C-38 had little or no effect on activation, and in oocytes, these mutant RNAs were still covalently modified. These data exclude a specific role for the adenosine at residue 27 for Tat activation but suggest a requirement for a purine at this position.

The replication of human immunodeficiency virus type 1 (HIV-1) is critically dependent on the virally encoded Tat protein (10). The Tat target sequence (*trans*-activation response [TAR] element) is located between +14 and +44 at the 5′ end of nascent HIV RNAs (13, 14, 20, 24) and adopts a stable secondary structure in vitro (17) (Fig. 1A). Tat binds specifically to TAR RNA in vitro (11, 32), and important residues have been identified. In particular, U-23 in the bulge is essential (29) and the sequence and base pairing in the upper stem at G-26 · C-39 and A-27 · U-38 (referred to as G-25 · C-40 and A-26 · U-39 in some studies [4, 33]) appear to be important (33). Mutations in the bulge diminish Tat activation of transcription (3, 11, 21) and translation (19), and in one study, transversion of A-27 · U-38 to U-27 · A-38 reduced activation in HeLa cells (4). In contrast to the in vitro binding data, mutations in the loop abolish Tat activation, implying a requirement for cellular factors to facilitate Tat binding and/or activation (9, 11, 22). A number of TAR-binding proteins have been identified, although the precise function of these has yet to be determined (12, 16, 26).

We have shown that when TAR RNA is microinjected or expressed from an HIV long terminal repeat (LTR) template in *Xenopus* oocytes, there is no translation unless Tat is provided (6, 7). The mechanism of translational activation is unusual in that Tat will only activate an RNA target when it is injected into the nucleus. Also, as a consequence of activation, the TAR RNA becomes covalently modified, so that subsequent translation in fresh oocytes is Tat independent (8).

Most if not all cells contain an enzyme activity in the nucleus that unwinds duplex RNA and concomitantly converts up to 50% of the adenosine residues to inosine (2, 31). It has recently been proposed that TAR functions as a substrate for the A-to-I unwindase with a Tat-dependent specific modification of residue 27 (25), although the stem is much shorter than the usual target duplex and an activity on TAR has not been observed in all studies (18). The Tat specificity of this modification and the fact that the enzyme activity is located in the nucleus could implicate this modification in the Tat activation of translation that we have observed.

In an attempt to confirm the importance of adenosine 27 in Tat activation, we have constructed a number of mutations in the N-27 · N-38 base pair of the TAR upper stem. The mutations and their theoretical effects on the structure of this region of TAR in terms of duplex stability are shown in Fig. 1A.

Although mutant M1 (G · U) contains a non-Watson-Crick pairing, it is known to form a stable mismatched pair within RNA helices, providing a favorable free-energy increase (30); thus, an internal G · U pair is only slightly less stable than an A · U pair (28). Mutants M2 (G · C), M3 (U · A), and M4 (C · G) show only minor effects on the theoretical stability of TAR, and both the thermal stability and nuclease sensitivities of short RNAs containing these mutations reflect their predicted stabilities (33). Thus, it would appear likely that the mutations made in this study are energetically stable within the upper stem of TAR and are unlikely to adopt alternative secondary structures, such as the "migrating" bulge shown by other mutations in this region (33). The mutations in M3 and M4 alter the potential recognition sites for proteins in the major and minor grooves on either face of the stem by switching the purine and pyrimidine residues at this position (23).

These mutations have been analyzed for their effects on Tat activation of transcription in HeLa cells and on Tat activation of translation and TAR modification in *Xenopus* oocytes. To allow quantitation of any subtle effects of the mutations on transactivation in HeLa cells, we established

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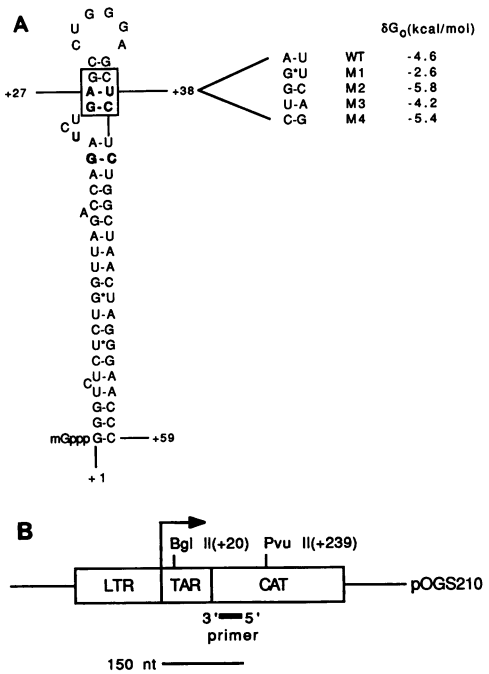

FIG. 1. (A) Secondary structure of TAR and upper-stem mutants. Nucleotide positions refer to the position relative to the transcriptional start site (+1). Mutations of the base pair at positions +27 and +38 are indicated (M1 through M4). WT, wild type. The free energy of a 3-bp section of the upper stem (boxed) is given for each of the TAR constructs used. These were calculated by the rules described by Turner et al. (30). Only the theoretical free energy of an internal 3-bp region of the helix has been presented in this study because the effect of the mutations at A-27 · U-38 on the calculated overall free energy of the stem-loop will be limited to this region. Estimation of the total free energy of the TAR stem-loop by the same parameters (30) gives a value of −27.4 kcal/mol, which is consistent with other estimates (29). Base pairs implicated as being essential in a specific interaction with a Tat peptide (33) are shown in boldface, and a single less-essential base pair is shown in open letters. Mismatched G · U pairs are separated by an asterisk. (B) Structure of expression plasmid for wild-type and mutant TAR sequences (pOGS210). Only the restriction sites used for subcloning mutant sequences into pOGS210 are shown. The primer used for primer extension analysis of RNAs is indicated below the plasmid and corresponds to nucleotides +33 to +13 in the CAT gene. The 150-nucleotide (nt) extension product is also shown.

limiting transfection conditions for both the target (TAR) and effector (Tat) templates. At 1 μg of target DNA and 10 ng of Tat-expressing plasmid per 10^6 cells, Tat activation activity was between 10 and 84% of maximum activity. All of the mutants were compared with the wild type under these limiting conditions.

Mutant TAR sequences were inserted into the plasmid pOGS210 (Fig. 1B) (1), which contains the HIV-1 LTR linked to the chloramphenicol acetyltransferase (CAT) reporter gene, and wild-type or mutant TAR plasmids were cotransfected into subconfluent HeLa cells with or without the Tat-expressing plasmid pOGS213 (1). Protein extracts were assayed for CAT activity after 48 h, and RNA was analyzed by quantitative primer extension with the primer shown in Fig. 1B. Typical results are shown in Fig. 2A.

The wild-type TAR construct was fully responsive to Tat, with an estimated 185-fold transactivation over basal levels of CAT activity. This activation was reflected by a comparable increase in the detectable levels of steady-state CAT mRNA in the cell. Subtle changes in the conformation of the upper stem generated by mutating A-27 to another purine (G) have little effect on transactivation by Tat regardless of base-pairing at this position, since both mutants with a purine at position 27 (M1 and M2) show less than a twofold reduction in transactivation compared with the wild type. In contrast however, conversion of A-27 to a pyrimidine (mutants M3 and M4) reduced transactivation by Tat more severely, to a level between three and five times lower than that of wild-type RNA.

Given that M3 and M4 displayed some activity, it appears that the nature of the base pair was not absolutely critical for Tat action. The most likely explanation for their phenotype was therefore that they were displaying a reduced affinity for Tat rather than an absolute inability to bind. This was confirmed by testing the effect of the mutations in excess Tat. At 10-fold-higher Tat concentration, these mutations behaved like the wild type (shown for M3 in Fig. 2C). This is in contrast to a mutant in which the bulge has been deleted, which could not be rescued at any concentration of Tat tested (Fig. 2C). The observation that the copy number of transfected plasmids can affect the phenotype of TAR mutants confirms the results of others (22) and underlines the importance of studying the effects of mutations within TAR under appropriate limiting conditions.

We also introduced the same TAR mutant plasmids into *Xenopus* oocytes in order to determine whether the mutation within the N-27 · N-38 base pair has a similar effect on translational activation (Fig. 2B). Under limiting conditions for Tat and TAR (8), the effect of the N-27 · N-38 mutations on Tat transactivation in oocytes was similar to that in HeLa cells. The TAR sequences containing a purine at position 27 (M1 and M2) maintained a wild-type phenotype, and those containing a pyrimidine at this position (M3 and M4) were unable to support significant levels of Tat-mediated transactivation. Furthermore, all of the mutant TAR RNAs were dependent on Tat for translation, indicating that subtle alteration of the upper stem cannot lead to Tat independence. It therefore seems unlikely that the modification of adenosine to inosine at position 27 observed by Sharmeen et al. (25) could be responsible for the Tat-independent translation of TAR RNA in *Xenopus* oocytes (8).

The effects of the mutations were less extreme in HeLa cells than in oocytes, as mutant TAR elements were neither transactivated to wild-type levels nor completely unresponsive to Tat (Fig. 2). These differences may reflect the more heterogeneous nature of a plate of a million HeLa cells compared with 30 identical oocytes. The overall effect, however, on Tat transactivation in both cell types was the same, i.e., a requirement for a purine at position 27 for efficient Tat activation. Thus, the process by which Tat contributes to the increased production of full-length mRNA in mammalian cells may have common features with the process of conferring translational competence to the RNA in *Xenopus* oocytes.

Given that TAR RNA with a G · U pair (N-27 · N-38) was fully responsive to Tat in oocytes, it was likely to act as a substrate for the Tat-dependent modification. To test this, RNA identical to that produced in vivo by mutant plasmid M1 was synthesized in vitro, and mutant (G · U) and wild-type (A · U) RNAs were injected into the nuclei of *Xenopus* oocytes in the presence and absence of Tat protein (Fig. 3). Both wild-type and mutant RNAs were expressed at similar levels and only in the presence of Tat (lanes 1 and 2 and lanes 7 and 8). Total RNA prepared from oocytes injected with the mutant RNA (both with and without Tat) was reinjected into fresh oocytes, again in the presence or absence of Tat

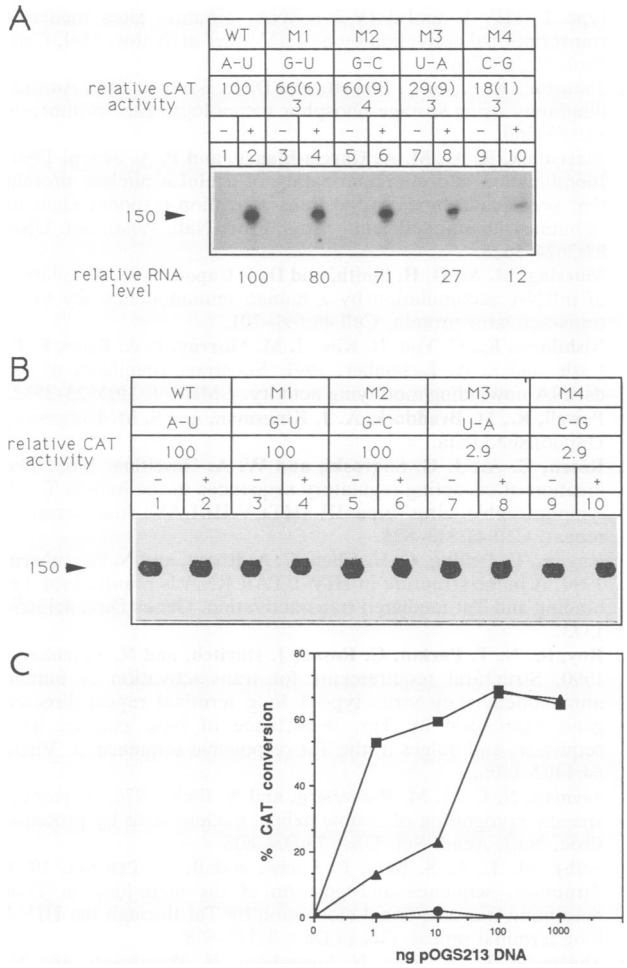

FIG. 2. Effect of mutations in the N-27 · N-38 base pair of TAR on Tat activation in vivo. (A) HeLa cells. Transfections were carried out with (+) or without (−) the Tat expression plasmid pOGS213. The efficiency of transfection into HeLa cells for plasmids carrying wild-type (WT) and mutant (M1 through M4) sequences was measured by cotransfecting with a cytomegalovirus-β-galactosidase plasmid and assaying for β-galactosidase activity (27). Protein extracts containing equivalent units of β-galactosidase activity were subsequently used in CAT assays. The relative CAT activity represents the proportion of chloramphenicol converted to acetylated forms in the presence of Tat and is set at 100% for wild-type TAR plus Tat. The standard deviation for each mutant is shown in parentheses, and the number of experiments carried out is shown underneath. In all cases, in the absence of Tat, the relative activity was less than 3% and is not shown. The relative RNA level represents direct quantitation of radiolabel in the 150-nucleotide primer extension product (arrowhead) from a typical experiment. (B) *Xenopus* oocytes. Oocytes were injected with 3 ng of plasmids carrying the wild-type (WT) or mutant (M1 through M4) sequences in the presence (+) or absence (−) of Tat protein, and protein extracts were assayed after 16 h. The relative CAT activity was calculated as for panel A, and a typical result is shown. The 150-nucleotide primer extension product obtained by using equal amounts of oocyte RNA from each sample is indicated with an arrowhead. (C) Effect of titrating Tat protein on activation of wild-type and mutant TAR sequences. Plasmids encoding wild-type (■), mutant M3 (▲), or bulge-deleted (●) TAR RNA (0.5 μg each) were cotransfected into HeLa cells with 0 to 1 μg of pOGS213 and a β-galactosidase expression plasmid. Protein extracts containing an equal number of β-galactosidase activity units were assayed for CAT activity.

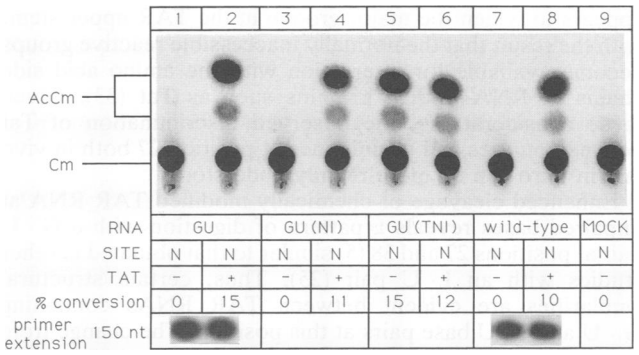

FIG. 3. Acquisition of Tat-independent phenotype in *Xenopus* oocytes by a G-27 · U-38 TAR mutant. Mutant RNA transcribed in vitro (G · U RNA), which is identical to RNA transcribed in vivo by mutant M1, was tested for the Tat-independent (modified) phenotype by our standard procedure, namely, injection of RNA into the nucleus, incubation for 16 h, rescue of RNA, and reinjection into the nucleus (N) of *Xenopus* oocytes in the presence (+) or absence (−) of Tat protein (8). GU (TNI) and GU (NI) refer to RNA rescued from oocytes after the initial injection in the presence and absence of Tat, respectively. Percent conversion refers to the proportion of radioactivity in the acetylated forms (AcCm) versus the unacetylated forms (Cm) of chloramphenicol. Primer extension products (150 nucleotides [nt]) are shown for RNA extracted after the initial injections, indicating that similar amounts of RNA were present in oocytes injected with wild-type and mutant RNAs. The CAT primer used is shown in Fig. 1B. CAT assays and primer extensions were analyzed by phosphor imaging (Molecular Dynamics, Sunnyvale, Calif.). Phosphor screens respond linearly to beta emissions over a 10^7-fold range and are considerably more sensitive than X-ray film (15). All samples were quantitated with the Molecular Dynamics phosphor imager.

protein (Fig. 3, lanes 3 to 6). This mutant RNA, which had previously been transactivated by Tat, was now expressed in a Tat-independent manner (lane 5) and had thus acquired the same modified phenotype as wild-type TAR RNA (8). We conclude therefore that the adenosine at position 27 in TAR RNA is not implicated in the mechanism of Tat-dependent modification that results in Tat-independent translation.

Weeks and Crothers have previously analyzed the effect of three of the mutations used in this work (G · C, C · G, and U · A) on the binding of a Tat peptide in vitro (33). Interestingly, the overall trend of progressively reduced in vitro binding affinities (G · C > U · A > C · G) mirrors the order of the reduction in transactivation seen here. However, although conversion of A · U to G · C at positions 27 and 38 reduced the affinity of Tat binding up to eightfold (33), we did not observe a comparable reduction in transactivation in vivo. Such a difference in the magnitude of the effect of mutation on binding and transactivation may simply reflect the shortcomings of in vitro binding studies, which will not include the complete array of interacting factors that are likely to influence Tat binding in vivo. We have studied one additional TAR configuration, G-27 · U-38, which shows nearly wild-type levels of transactivation by Tat. In contrast to one of the conclusions of Weeks and Crothers (33), this result suggests that there is not an absolute requirement for Watson-Crick base-pairing at this residue nor a requirement for the specific A · U pair. Rather, our data indicate a requirement for a purine at this position.

The ability of proteins to discriminate between purines and pyrimidines is largely dependent on the accessibility of the hydrogen bond donors and acceptors present in the major groove of the A-form RNA helix (23). The proximity of the trinucleotide TAR bulge to the N-27 · N-38 pair

appears to widen the major groove in the TAR upper stem, with the result that the normally inaccessible reactive groups become available for interaction with the amino acid side chains of RNA-binding proteins such as Tat (33). Given these considerations, the observed discrimination of Tat between purines and pyrimidines at position 27 both in vivo and in vitro can be more readily understood.

Enhanced cleavage of chemically modified TAR RNA at unpaired bases reveals a pattern of digestion with a G · U pair at positions 27 and 38 (5) similar to that obtained in other studies with an I · U pair (25). Thus, certain structural similarities are evident between TAR RNAs containing G · U and I · U base pairs at this position. The change from an A · U pair to a G · U pair at positions 27 and 38 in TAR does not confer a Tat-independent phenotype on TAR RNA in *Xenopus* oocytes, and TAR RNA containing a G at position 27 can still be modified. It therefore seems unlikely that a modification of adenosine to inosine at position 27 is involved in the Tat-dependent activation of translation in oocytes.

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