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NOTES

TAR Loop-Dependent Human Immunodeficiency Virus *trans* Activation Requires Factors Encoded on Human Chromosome 12

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The *trans*-activator response region (TAR) RNA in the human immunodeficiency virus type 1 (HIV-1) and HIV-2 long terminal repeat forms stem-loop secondary structures in which the loop sequence is essential for *trans* activation. We investigated how the HIV *trans*-activation mechanism encoded on human chromosome 12 relates to the TAR RNA loop-dependent pathway. DNA transfection experiments showed that *trans* activation in human-hamster hybrid cells with the single human chromosome 12 and human T-cell lines was highly dependent on the native sequences of the HIV-1 TAR loop and the HIV-2 5′ TAR loop. In nonhuman cell lines or hybrid cells without chromosome 12 that supported *trans* activation, the cellular mechanism was independent of the HIV-1 TAR loop and the response to mutations in the HIV-2 TAR loops differed from that found in human T-cell lines and human-hamster hybrid cells with chromosome 12. Our results suggest that the human chromosome 12 mechanism interacts directly with the TAR RNA loop or indirectly by regulating TAR RNA-binding proteins.

Replication of the human immunodeficiency virus (HIV) requires the viral *trans*-activator protein, Tat, and recruitment of host cell factors to *trans* activate the viral long terminal repeat (LTR) (for reviews, see references 9 and 35). A major cellular mechanism in human-hamster hybrid cells that supports Tat-directed *trans* activation of the HIV type 1 (HIV-1) and HIV-2 LTRs has been traced to human chromosome 12 (22, 23, 30). Tat-directed *trans* activation is dependent on the viral *trans*-activator response region (TAR) in the HIV-1 and HIV-2 LTRs (for reviews, see references 9 and 35). HIV-1 TAR and HIV-2 TAR are predicted to form RNA stem-loop secondary structures (3, 4, 14) with native loop sequences vital for high-level Tat-directed *trans* activation in vivo (7, 15, 16, 28) and for binding of cellular proteins that increase LTR-directed transcription in vitro (27, 33). Tat protein and cellular proteins bind to separate regions of HIV-1 TAR RNA (7, 12, 17–19, 27, 28, 31, 33, 37), and TAR RNA is thought to mediate the Tat-host protein *trans* activation of the LTR (8, 14). Because the chromosome 12-encoded factors and the predicted viral TAR RNA loop sequences are critical for Tat *trans* activation, we investigated the interdependence of these cellular and viral regulatory components.

Tat-directed *trans* activation in cells was assayed by transfection of DNA plasmids that express the HIV-1 *tat* (tat_1) or HIV-2 *tat* (tat_2) gene under control of the simian virus 40 early promoter together with plasmids that have the bacterial chloramphenicol acetyltransferase (CAT) gene positioned downstream of, and under transcriptional control of, the HIV-1 or HIV-2 LTR (TAR-1 and TAR2, respectively) (22, 23). The TAR-1 mutant (TAR-1Δ) has the +31 to +34 wild-type loop sequence UGGG replaced by CAAA (16) but retains the predicted secondary structure proposed for the RNA TAR stem-loop (Fig. 1A) and the ability to bind tat_1 protein in vitro (31). Thus, differences in TAR-1 and TAR-1Δ support of Tat-directed *trans* activation in vivo should reflect the activity of cellular factors that interact with the RNA loop sequences.

TAR-1 loop-dependent *trans* activation was examined in nine cell lines (three human, two human-hamster hybrid, two rodent, one mink, and one bovine). The human-hamster hybrid cell clones 271 and 867 (HHW271 and HHW867) are derived from fusion of human peripheral blood mononuclear cells with the Chinese hamster ovary (CHO) cell line UCW56 (10). These hybrid cell clones were grown under culture conditions to maintain their human chromosome content (10). Karyotype analysis (32) confirmed the chromosome content of the human-hamster hybrid cells as reported previously (22). DNA transfection experiments showed that the variation between TAR-1 and TAR-1Δ support of basal HIV-1 LTR activity (minus tat_1) in individual cell lines was 2.2-fold or less (Table 1). The activities of the HIV-1 LTR containing wild-type TAR-1 or TAR-1Δ suggested that species- or tissue-specific factors that control basal LTR activity do not act through the TAR loop sequences. Cotransfection of the nine cell lines with TAR-1 and tat_1 plasmid DNA showed a 52-fold spread in *trans* activation between the cell lines with the lowest and highest activities (HHW867 and RD, respectively; Fig. 2A). Although human RD cells and human-hamster HHW271 cells supported the highest levels of *trans* activation (212- and 53-fold, respectively), mink MU1 cells and human CEM and HUT78 T cells supported *trans* activation at nearly identical levels (21-, 22-, and 24-fold, respectively). These results agree with previous reports that show that comparing *trans*-activation activities of unrelated cell lines will not necessarily distinguish species-specific *trans*-activation activities (6). Cotransfection of

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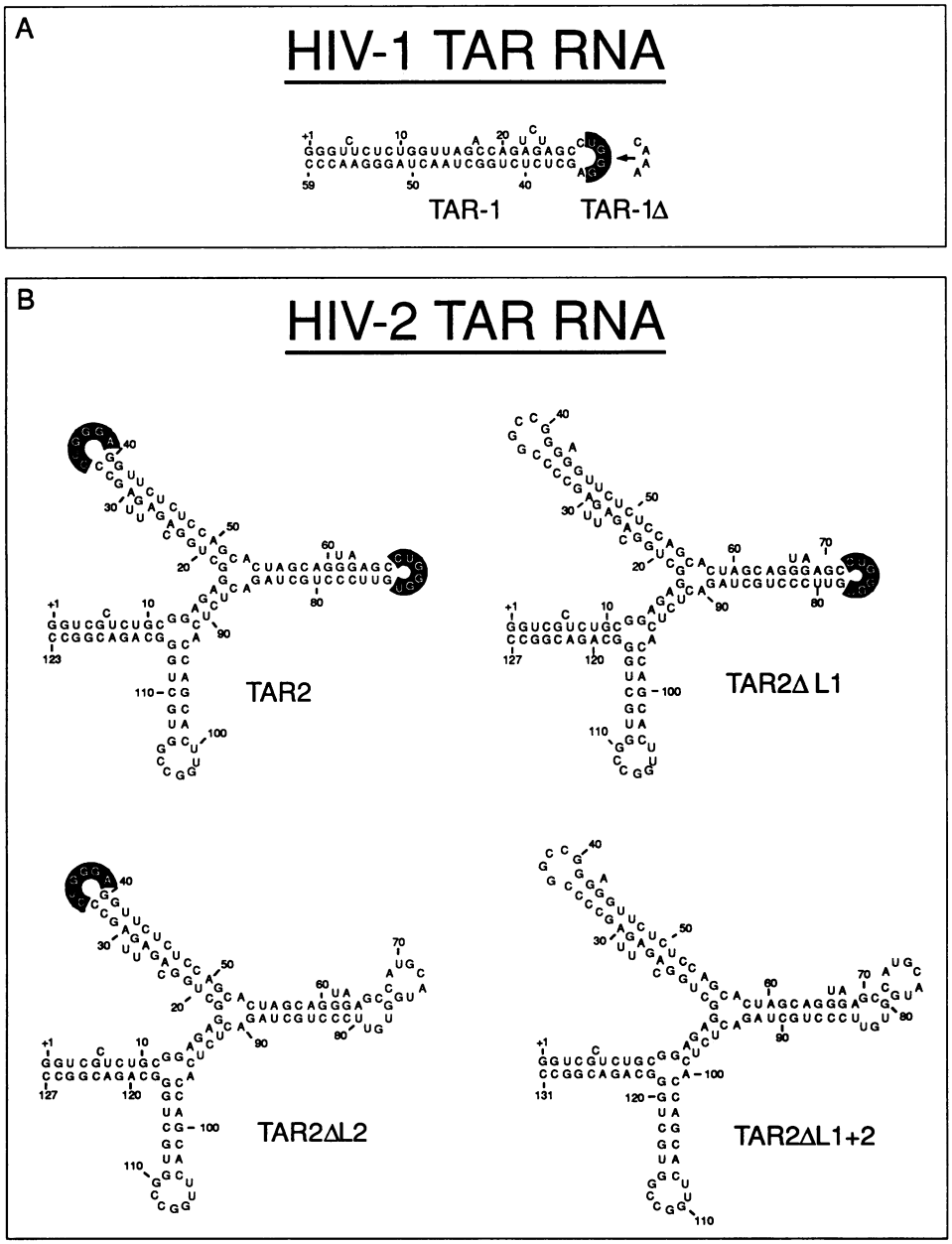

FIG. 1. Predicted secondary structures of wild-type and mutant HIV-1 and HIV-2 TAR RNAs. The TAR RNA structures are shown in their most stable secondary form as computed by the method of Zuker and Stiegler (38) adapted for the DNASIS DNA sequence analysis system (Hitachi Software Engineering America, Ltd.). (A) Wild-type HIV-1 TAR (ΔG = −37.6 kcal/mol [ca. −157 kJ/mol]) and the loop mutant TAR-1Δ (ΔG = −37.6 kcal/mol [ca. −157 kJ/mol]) are predicted to have the same secondary RNA structure, as reported previously (19). TAR-1 loop sequences +31 to +34 (white letters on black background) are replaced with the TAR-1Δ sequence, 5′-CAAA-3′. (B) Predicted wild-type TAR2 (ΔG = −80.5 kcal/mol [ca. −337 kJ/mol]) contains the 5′ loop (+34 to +39) and 3′ loop (+68 to +73) (black background with white letters) as reported previously (15). The 4-base insertions at +36 to +39 (TAR2ΔL1; ΔG = −87.5 kcal/mol [ca. −366 kJ/mol]), at +68 to +71 (TAR2ΔL2; ΔG = −81.2 kcal/mol [ca. −340 kJ/mol]), or at both (TAR2ΔL1+2; ΔG = −88.2 kcal/mol [ca. −369 kJ/mol]) are predicted to modify only the distal regions of their respective stem-loop configurations.

the TAR-1Δ and tat_1 plasmids, however, showed definite species-specific responses (Fig. 2A). In the HHW271 hybrid clone, which contained the single human chromosome 12 and the normal CHO chromosome complement, and in the human cell lines (CEM, HUT78, and RD), TAR-1Δ maintained only 0 to 3% of the Tat_1-directed *trans* activation supported by wild-type TAR-1. In the hybrid clone without chromosome 12 (HHW867) and in nonhuman cells (MU1, CHO, L929, and MDBK), TAR-1Δ supported 37 to 114% of the Tat_1 *trans* activation found with TAR-1. An interesting result was the totally TAR loop-independent *trans* activation in mink cells (MU1). Previous reports show that MU1 cells transfected with HIV-1 proviral DNA support extracellular virus production comparable to that of human and primate

TABLE 1. Effects of TAR mutations on basal HIV-1 and HIV-2 LTR-CAT activities[a]

Cell type	HIV-1 activity		HIV-2 activity			
	TAR-1	TAR-1Δ	TAR2	TAR2ΔL1	TAR2ΔL2	TAR2ΔL1+2
CEM[b]	0.1	0.2	0.1	0.2	0.2	0.1
HUT78[b]	0.9	1.4	0.2	0.2	0.1	0.1
271[b]	3.0	3.8	0.9	1.3	0.5	1.7
RD[b]	63	85	10	24	9.3	28
MU1	8.2	3.8	0.2	0.2	0.3	0.2
867	4.8	3.7	0.5	0.5	0.6	1.0
CHO	8.2	10.1	5.3	6.8	3.5	5.8
L929	2.0	3.5	0.5	0.6	0.4	0.6
MDBK	1.4	1.2	1.1	0.9	0.5	0.6

[a] CAT activity was quantitated in the linear range of the assay. Values for CEM and HUT78 are from CAT assays (20) with an 18-h incubation; CAT values from the other cell lines were from a 10-min incubation or were normalized to a 10-min incubation. Values for individual cell lines are the average of two or more experiments, with a variation between experiments of <20%.

[b] Human-hamster hybrid clone HHW271 with human chromosome 12 present in >70% of the cells or human cell lines.

cells (1, 26). Our results show that MU1 cells and other nonprimate cells may support significant levels of Tat_1-directed *trans* activation, but the cellular mechanisms do not mimic the major HIV *trans*-activation pathway in human cells. The identical response of human and HHW271 cells to the TAR-1Δ loop mutation, in combination with our previous data (22), indicates that chromosome 12-encoded factors provide the species-specific component of HIV-1 TAR loop-dependent *trans* activation.

HIV-2 TAR $RNA_{(ROD)}$ has at least two predicted stem-loops, whereas HIV-1 TAR has only one (4, 14) (Fig. 1B). Two of the HIV-2 stem-loops (positions +18 to +52 and +54 to +85) contain the loop sequence CUGGGX, which is identical to the HIV-1 TAR-RNA loop sequence that binds cellular proteins and regulates HIV-1 LTR-directed transcription in vitro (27, 28, 33, 37). The HIV-2 LTR-CAT gene plasmids, which were constructed previously (15), have the wild-type loop sequences (TAR2) or mutations in the 5′ loop (CUGGGA to CCGGCCGGGA; TAR2ΔL1) or the 3′ loop (CUGGGU to CAUGCAUGGU; TAR2ΔL2) or both (TAR2ΔL1+2). These mutations retain the predicted overall secondary structure and the stem nucleotide base-pairings but have altered loop configurations (Fig. 1B). TAR2 mutations affected basal HIV-2 LTR activities (minus tat_2) less than twofold in individual cell lines except for human RD cells, which had a 2.4- to 2.8-fold increase in LTR activity with the 5′ loop mutation (TAR2ΔL1 or TAR2ΔL1+2) (Table 1). The increase in basal activity with the 5′ loop mutation appears to be cell type specific; previous reports show that other human and monkey cells do not support increased basal LTR activity with TAR2ΔL1 or TAR2ΔL1+2 (15) and that other 5′ loop mutations do not increase basal LTR activity in HUT78 cells (4).

In the eight cell lines tested, cotransfection of tat_2 and wild-type TAR2 produced a myriad of *trans*-activation levels (Fig. 2B). Bovine cells (MDBK) were refractory to *trans* activation (1.5-fold); increasing levels of *trans* activation were supported by mouse (L929, 6.4-fold), hamster (CHO, 7.9-fold), human-hamster hybrid (HHW867, 8.5-fold; HHW271, 49-fold), mink (MU1, 182-fold), and human (HUT78, 195-fold; RD,

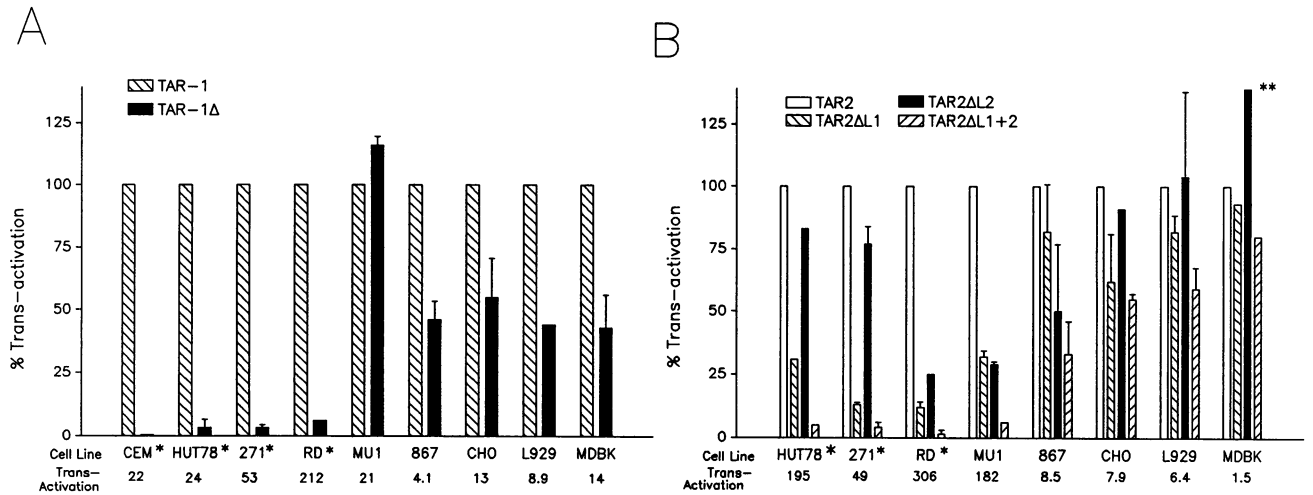

FIG. 2. Tat-directed *trans* activation of the HIV-1 and HIV-2 LTRs. Monolayer cultures of HHW271, HHW867, RD, MU1, CHO, L929, and MDBK cells were cotransfected with plasmid DNAs by the $CaPO_4$ technique described previously (23); suspension cultures of CEM and HUT78 cells were transfected by electroporation as described by Barry et al. (6). *trans*-activation values are the fold increase in CAT activity of cotransfections with LTR-CAT plus *tat* compared with LTR-CAT plus sonicated salmon sperm DNA. The percent *trans* activation, calculated for each cell type, is the fold *trans* activation supported by LTR-CAT constructs with wild-type TAR (100% *trans* activation) or TAR mutations. Quantitation of CAT activity was done in the linear range of the assay. The values presented here are the means of at least two independent transfection experiments per cell type. Similar results (<20% variation between experiments) were obtained in the independent experiments. Error bars represent the SDs of percent *trans*-activation values obtained in experiments with the TAR mutations compared with those with wild-type TAR. *, human chromosome 12 present in >70% of the human-hamster hybrid HHW271 cells or human cells; **, value out of range on graph (187% of wild-type TAR2 activity). (A) HIV-1 LTR *trans* activation by Tat_1. TAR-1 is an LTR-CAT construct with a wild-type TAR sequence; TAR-1Δ is the same construct except for a 4-nucleotide mutation at +31 to +34 in the predicted TAR RNA loop shown in Fig. 1 and previously (19). (B) HIV-2 LTR *trans* activation by Tat_2. TAR2 (the LTR-CAT construct with wild-type TAR loop sequences), TAR2ΔL1 (the 5′ loop mutant), TAR2ΔL2 (the 3′ loop mutant), and TAR2ΔL1+2 (containing both 5′ and 3′ loop mutations) are shown in Fig. 1.

306-fold) cells (Fig. 2B). As we observed for HIV-1, comparing the HIV-2 *trans*-activation levels of unrelated cell lines did not distinguish species-specific mechanisms.

Cells cotransfected with plasmids carrying the HIV-2 TAR loop mutations and tat_2 did show species specificity. In human and HHW271 cells, mutation of both native TAR loops (TAR2ΔL1+2) decreased *trans* activation to 3% ± 1.3% (average ± standard deviation [SD]) of TAR2 activity (Fig. 2B). This result paralleled the nearly total dependence of *trans* activation on the HIV-1 TAR loop sequences in all chromosome 12-containing cells (Fig. 2A). In contrast, when only one native 5′ or 3′ loop was present with the other loop mutation in HIV-2 TAR (TAR2ΔL1 or TAR2ΔL2; Fig. 1B), two patterns of TAR loop-dependent *trans* activation occurred (Fig. 2B). One pattern, observed for HUT78 and HHW271 cells, showed that HIV-2 *trans* activation was greatly dependent on the 5′ TAR loop; TAR2ΔL2 supported 80% ± 3% (average ± SD) of TAR2 activity. The native 3′ TAR loop (TAR2ΔL1) supported only 19% ± 8.7% (average ± SD) of TAR2 activity in these cells. The second pattern, observed in the nonlymphoid human RD cell, showed that the presence of both native TAR loops was necessary for efficient activity (Fig. 2B). The presence of only one native TAR loop, TAR2ΔL1 or TAR2ΔL2, supported 12 or 25%, respectively, of the TAR2 activity. This dependence on the 5′ plus 3′ TAR loops in RD cells also occurred in the nonlymphoid mink cell line (MU1), which supported high-level HIV-2 *trans* activation (Fig. 2B). For MU1, the HIV-2 5′-plus-3′-loop-dependent *trans* activation was strikingly the reverse of the totally TAR loop-independent HIV-1 *trans* activation in these cells (Fig. 2A). The two variations of TAR loop-dependent *trans* activation, those that depend on the HIV-1 TAR loop and the HIV-2 5′ TAR loop compared with the HIV-2 5′ plus 3′ TAR loops, suggest that more than one cellular mechanism is involved or that a single mechanism is modified, depending on cell type.

The HHW867 hybrid clone and nonhuman cells (CHO, L929, and MDBK) had low-level HIV-2 *trans* activation that was relatively TAR loop independent (Fig. 2B). The low-level *trans* activation in cells without chromosome 12 (CHO, HHW867, L929, and MDBK) was not due to the absence of cellular mechanisms that support Tat-induced LTR gene expression. The level of heterologous Tat_1 *trans* activation of the HIV-2 LTR in these cells was 5- to 10-fold higher than homologous HIV-2 *trans* activation and was also largely independent of the native TAR2 loops (data not shown). A number of reported tat_1 activities that are independent of TAR, including *trans* activation in human and rodent glial cells (34) and activation of human papillomavirus gene expression (36), support our previous observation of chromosome 12-independent pathways for tat_1 activity that are not cell or species specific (23).

The interdependence of human chromosome 12 and the predicted TAR RNA loop sequences has identified an important element in the human cellular mechanism for Tat-directed *trans* activation. Certain rodent cell lines are reported to support high-level HIV-1 *trans* activation (6) or virus production (29). Our results here and a previous report (5) suggest that a significant portion of the *trans*-activation mechanism in rodent cells is TAR independent. Furthermore, the high-level extracellular virus production reported for some rodent cells is accomplished through cellular mechanisms that support extremely high-level basal LTR activity and low-level *trans* activation (29). These mechanisms effectively circumvent the human cell phenotype of low basal LTR activity relative to the level of *trans* activation (for a review, see reference 9). We also found that the MU1 mink cell line, which supports HIV-1 virus production (1), *trans* activated independently of the HIV-1 TAR loop-dependent pathway.

The molecular mechanism that underlies the *trans* activation involving chromosome 12 plus the TAR RNA loop is not yet known. It is known, however, that the chromosome 12 mechanism works independently of HIV-1 LTR regions, upstream of TAR, that interact with the DNA-binding proteins NF-κB, SP-1, and LBP-1 (2). The TAR-1Δ loop mutation binds tat_1 protein in vitro (11, 31, 33) but loses the capacity to bind human cellular proteins and support Tat-directed *trans* activation in human cells (19, 27, 28, 33, 37) and in human chromosome 12-containing hybrid cells (Fig. 2A). Further experiments are needed to determine whether the TAR loop-binding proteins are encoded on human chromosome 12 or whether chromosome 12 encodes a pathway for the posttranslational regulation of TAR-binding proteins.

HIV-2 TAR RNA presents a more complex target for interaction with host factors than HIV-1 TAR RNA. In human Jurkat T cells and monkey COS kidney cells, the single native 3′ TAR loop supports low levels of *trans* activation while the single native 5′ TAR loop supports *trans* activation equal to that of wild-type TAR2 (15). Other reports show that nonlymphoid cells depend on both HIV-2 TAR RNA loops equally for *trans* activation (13, 25). Our results indicate possible cell-type-specific interactions with HIV-2 TAR; a predominant 5′ TAR loop dependence in human T cells and an equal dependence on the 5′ and 3′ TAR loops in nonlymphoid human cells. The T-cell-like response to TAR2 mutations in HHW271 cells is especially significant since these cells are derived from a fusion of primary human lymphocytes and CHO cells and contain the single human chromosome 12 (22, 23). It is possible that human chromosome 12 in the human-hamster hybrid cell HHW271 encodes the 5′ TAR loop-dependent activity of human T cells, a major target of HIV infection. The fibroblast human RD and mink MU1 cells had high-level HIV-2 *trans* activation, but the 5′ TAR loop activity, predominant in HHW271 and HUT78, shifted to an equal dependence on the 5′ and 3′ TAR loops, as reported for other nonlymphoid human cells (13, 25). Whether the TAR loop-binding proteins recognized for HIV-1 are the same for the HIV-2 5′ and 3′ TAR loops is not yet known. The same cell factors could be active in nonlymphoid cells but in a modified form that requires both the 5′ and the 3′ TAR loops for complete HIV-2 *trans* activation.

Protein kinase activity and the phosphorylation state of cellular proteins are reported to control HIV-1 *trans* activation (21, 24) through the regulation of TAR RNA stem-binding proteins (21). The TAR RNA stem-binding proteins are suggested to be part of a large ribonucleoprotein complex that stabilizes Tat interaction with TAR (21). Assignment of the TAR RNA loop-dependent activity of chromosome 12-associated factors to this ribonucleoprotein complex will require further study. The identification of chromosome 12-associated TAR-binding factors and their state of post-translational modification in lymphoid versus nonlymphoid cells may be important in the cell-type-specific TAR-dependent activities we are observing.

The results presented here have defined the chromosome 12-encoded pathway as an important human cellular mechanism for TAR RNA loop-dependent *trans* activation. Although all human cell types tested and the HHW271 hybrid clone containing chromosome 12 were dependent on the HIV-1 TAR loop for *trans* activation, only T cells and HHW271 were dependent predominantly on the 5′ loop of HIV-2 TAR. The two patterns of TAR2 activity in lymphoid and nonlymphoid cells suggest that either different factors

are involved or the same factors are modified, depending on cell type. Experiments to identify and compare TAR RNA-binding proteins from HHW271, human T cells, and nonlymphoid cells will be necessary to answer these questions.

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