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RNA transcripts of the human immunodeficiency virus transactivation response element can inhibit action of the viral transactivator

(decoy RNA/antisense RNA/tandem DNA/intracellular immunity)

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ABSTRACT **Tandem repeats of the transactivation response element (TAR) of the human immunodeficiency virus 1 (HIV-1) were generated using a specially constructed "tandemizing" plasmid, pGem-Tan. This plasmid exploits the rotational nonequivalence of *Ava* I restriction sites to generate multiple copies of an inserted sequence. Twelve tandem repeats of the TAR were then placed in sense and antisense orientations behind a strong human β-actin gene promoter. The TAR constructs were transfected with an appropriate HIV-1-driven reporter and *tat* gene expression plasmids into NT2/D1 cells, a pluripotential human embryonic teratocarcinoma cell line. Twelve tandem TAR repeats in the sense orientation suppressed 85–90% of the transactivating function of the virus-encoded tat protein, whereas the antisense construct or constructs containing single copies of TAR in either sense or antisense orientations were relatively ineffective. The suppression was specific for reporter gene constructs containing an intact HIV-1 long terminal repeat: Reporter genes driven by other promoters or by an HIV-1 long terminal repeat lacking the TAR were not suppressed. Suppression of activation by tat required transcription into RNA: Similar constructs containing the TAR repeats but lacking a eukaryotic promoter failed to suppress tat activation. In the absence of tat, the TAR DNA stimulated 2- to 5-fold the expression of gene constructs driven not only by the HIV-1 long terminal repeat but also by the human β-actin gene and the simian virus 40 promoters.**

Expression of the AIDS virus (human immunodeficiency virus 1; HIV-1) genome is autostimulatory. The product of the viral gene *tat* (transactivator) greatly stimulates viral gene expression by acting on the viral transactivation response element (TAR), which is part of the long terminal repeat (LTR) and occurs at the beginning of all HIV-1 transcripts (1). It is not known whether the tat protein acts directly on TAR or causes a cellular factor to do so.

The ability to prevent stimulation of TAR by tat might yield insights into the mechanism of this stimulation and also provide a concrete basis for the proposed anti-AIDS therapy of "intracellular immunity" (2). One possible method of inhibiting the tat–TAR interaction is to provide an excess of TAR "decoys": TAR sequences (DNA or RNA) that competitively bind factors mediating transactivation and prevent them from acting (3, 4).

A problem in the use of TAR decoys may be the inability to put enough copies into a target cell to be effective. A solution is to assemble many copies of the TAR in a head-to-tail tandem array and insert them as a single transcriptional unit—ideally behind a strong promoter. We have constructed an array of 12 TAR copies behind a strong promoter, the human β-actin gene promoter, and have shown that the transcripts so produced in human cells can indeed interfere with the tat–TAR interaction *in vivo*.

MATERIALS AND METHODS

Plasmids. pGem-Tan, the plasmid used for generating tandem arrays of the TAR, is a derivative of plasmid pGEM-2 (Promega). The multiple cloning site was altered with the use of a synthetic oligonucleotide and is shown in Fig. 1*A*. The rest of the plasmid is unchanged. To construct the TAR 12-mer, we modified a published technique (6) to generate tandem repeats that exploited the dyad asymmetry of *Ava* I cleavage sites. These were incorporated into the synthetic oligonucleotide with a *Hinc*II site for blunt-end insertions. The starting monomer unit was a 100-base-pair (bp) fragment from the plasmid pU3RIII, which contains the U3 and part of the R region of the HIV-1 LTR driving the bacterial chloramphenicol acetyltransferase (CAT) gene (7). The fragment containing TAR was excised by *Pvu* II and *Hin*dIII cleavage at positions −19 and +81, respectively, from the transcription start site. The fragment was then blunt-ended with the Klenow fragment of DNA polymerase I. The transfer of a single TAR-containing element into the *Hinc*II site of pGem-Tan also yielded a plasmid containing the fortuitous insertion of three copies in head-to-tail orientation. We then used a modification of the Hartley–Gregori technique (6) to create a head-to-tail tetramer of this trimer. The modification consisted of omitting the dideoxynucleotide-blocking step: instead, we digested pGem-Tan with both *Ava* I and *Xba* I and followed this with the tandem additions of the TAR trimers. Klenow polymerase was used to make all ends blunt and the ends were then sealed with T4 DNA ligase. The final product, pGT/TAR-12, is shown in Fig. 1*B*. The TAR 12-mer was then transferred in sense or antisense orientations to other plasmids (Fig. 1 *C* and *D*).

The high-level expression vector pHβAPr-1 (5) contains the human β-actin gene promoter followed by a multiple cloning site and simian virus 40 3′ processing signals. pU3RIII carries the HIV-1 3′ LTR driving the CAT gene as described above. For convenience in identification, this plasmid is here named pHIVCat. pSVtat [originally termed pPL12 (8)] contains the simian virus 40 early promoter driving the HIV-1 *tat* gene. pHβAPr-1-CAT is similar to pHβAPr-1 but carries the human β-actin promoter driving the CAT gene (5). pHβ/AStat contains the HIV-1 *tat* gene from pCV-1 (8) inserted in antisense orientation in pHβAPr-1 (Fig. 1*C*). pHβ/α-10 contains 10 tandem repeats of a 171-bp cloned monomer unit of African green monkey component α-DNA (9). To make pΔTAR4CAT, the HIV-1 LTR in pHIVCat was digested with *Hin*dIII and *Sac* I, blunted with S1 nuclease,

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Abbreviations: CAT, chloramphenicol acetyltransferase; HIV-1, human immunodeficiency virus 1; LTR, long terminal repeat; TAR, transactivation response element.

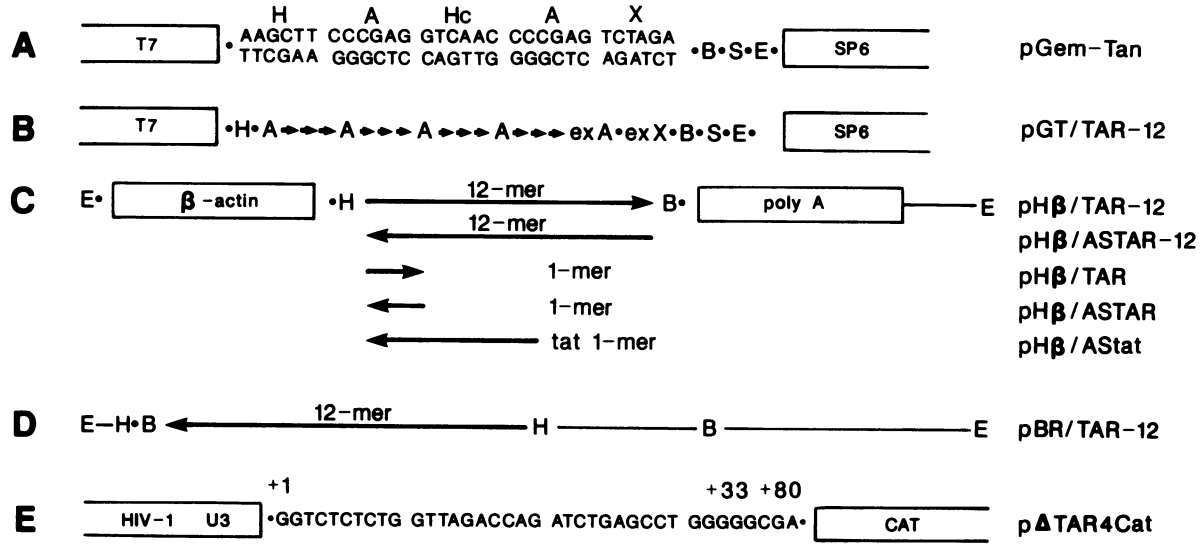

FIG. 1. Construction and maps of plasmids. (*A*) pGem-Tan was constructed by cleaving pGEM-2 (Promega) with *Hin*dIII and *Xba* I. A synthetic duplex oligonucleotide with compatible ends was inserted between these sites and the result was confirmed by sequencing. The multiple cloning sites and the locations and orientations of the SP6 and T7 promoters are shown. (*B*) The construction of pGT/TAR-12, a derivative of pGem-Tan containing 12 tandem repeats of the HIV-1 TAR region. (*C*) The TAR 12-mer from pGT/TAR-12 was inserted into pHβAPr-1 (5) to create pHβ/TAR-12 and pHβ/ASTAR-12 in which sense and antisense transcripts are driven by the human β-actin promoter. Monomer copies of TAR (nucleotides −19 to +79 relative to the transcription start site in the HIV-1 LTR) were inserted into pHβAPr-1 to create pHβ/TAR and pHβ/ASTAR. (*D*) The TAR 12-mer was inserted into the *Hin*dIII site of pBR322 to create pBR/TAR-12. This plasmid does not contain a eukaryotic promoter. (*E*) pΔTAR4Cat. The entire U3 region and the first 33 nucleotides of the R region of the HIV-1 LTR are included as shown, but sequences responsible for tat responsiveness were removed. The diagrams are not drawn to scale. A, *Ava* I; B, *Bam*HI; E, *Eco*RI; Hc, *Hin*cII; H, *Hin*dIII; S, *Sac* I; X, *Xba* I.

and religated. Nucleotides 33–80 containing one-half of the potential stem–loop sequences of TAR were removed (Fig. 1*E*). This plasmid shows no response to transactivation by tat.

Cells and DNA Transfections. The pluripotential human embryonic teratocarcinoma cell line NT2/D1 (10) was used in these studies. DNA transfections by the calcium phosphate procedure and CAT assays in the linear ranges of time of incubation and protein concentration were carried out as described (11). CAT relative specific activities were calculated in nmol of chloramphenicol converted to acetylated forms per min per mg of protein. Labeling of DNA by nick-translation, Northern blot transfers, and preparation of single-stranded RNA probes with SP6 polymerase were according to standard procedures (12).

RESULTS

Ineffectiveness of Single Copies of Sense or Antisense TAR Elements in Blocking tat Transactivation. The expression of CAT activity driven by the HIV-1 LTR in transient transfection experiments with pHIVCat was greatly increased by cotransfection with pSVtat. This plasmid expresses tat activity under the control of the simian virus 40 early gene promoter (8). In NT2/D1 cells, the addition of 1 μg of pSVtat to 1 μg of pHIVCat increased the CAT activity of the cell extracts 100- to 200-fold when compared to cells similarly transfected with 1 μg of pHIVCat in the absence of tat (results not shown). To determine conditions for blocking this transactivation, we inserted single copies of the HIV-1 TAR in both sense and antisense orientations behind the human β-actin gene promoter in pHβAPr-1 to yield the plasmids pHβ/TAR and pHβ/ASTAR (Fig. 1*C*). We also inserted a single copy of the *tat* gene in the antisense orientation in pHβAPr-1 to yield pHβ/AStat. Fig. 2*A* shows that none of these plasmids inhibited transactivation of the HIV-1 LTR although they were present in 8-fold excess over pHIVCat and pSVtat.

Effect of Tandem TARs in Sense or Antisense Orientations in Blocking tat Transactivation. We then assembled a head-to-tail tandem array of 12 TARs inserted in the sense orientation behind the human β-actin promoter in pHβAPr-1 (Fig. 1*C*). The resultant plasmid, pHβ/TAR-12, was used to drive transcription of the 12 tandem TAR sequences. We then compared the effect of tat transactivation of pHIVCat in the presence of 8 μg of pHβ/TAR-12 or in the presence of an equal amount of the vector pHβAPr-1. The results, repeated in 13 experiments, showed consistent inhibition of 85–90% of the tat–TAR interaction as revealed by CAT assays (Fig. 2*B*).

The TAR 12-mer sequence was also inserted in the antisense orientation in pHβAPr-1 to yield pHβ/ASTAR-12 (Fig. 1*C*). In cotransfections with pSVtat, pHβ/ASTAR-12 showed only weak (40%) inhibition of tat transactivation of pHIVCat (Fig. 2*B*). These findings indicate that the orientation of the TARs relative to the promoter is important for inhibition and that transcription might be required.

To test further the need for transcription, we inserted the TAR 12-mer into the *Hin*dIII site of pBR322 and tested the resultant plasmid pBR/TAR-12 (Fig. 1*D*) in excess amounts in cotransfection assays with pHIVCat and pSVtat. The 12-mer in this case did not inhibit tat transactivation at all (Fig. 2*C*). pBR/TAT-12 is not expected to be transcribed in mammalian cells in the absence of a eukaryotic promoter. A Northern blot transfer of RNA from NT2/D1 cells transfected with pBR/TAR-12 detected no TAR-containing RNA (Fig. 3*A*). Thus, the untranscribed tandem DNA copies of TAR were ineffective in blocking tat transactivation.

Specificity of tat–TAR 12-mer Interaction. A transcript containing 12 copies of the TAR would probably have much secondary structure. Under some circumstances, such RNA secondary structure can be a strong general inhibitor of translation (13). Therefore, it was necessary to determine whether inhibition by pHβ/TAR-12 was specific for the tat–TAR interaction. Accordingly, we compared the effects of pHβ/TAR-12 when cotransfected in excess with pSV2Cat, pHβAPr-1-Cat, or pHIVCat in the absence of pSVtat. We also tested pΔTAR4Cat, a derivative of pHIVCat that does

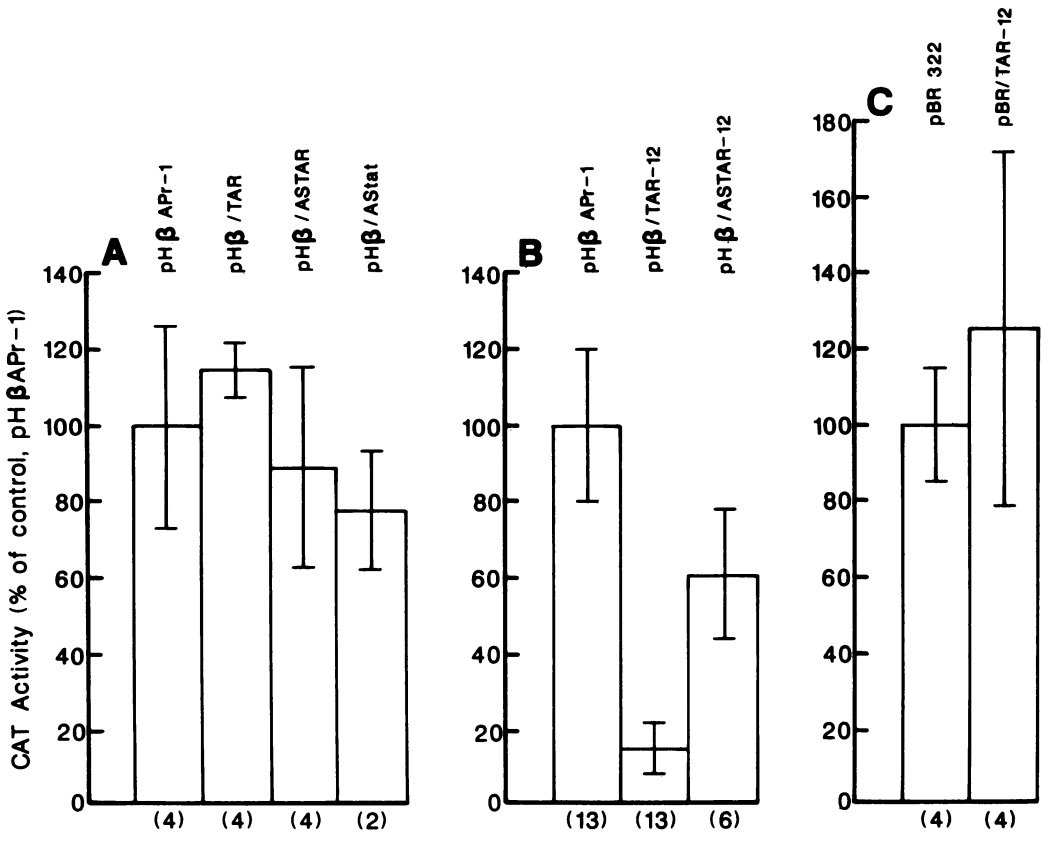

FIG. 2. Inhibition of the tat–TAR interaction by sense or antisense TAR RNA. NT2/D1 cells were transfected with 1 μg of pHIVCat plus 1 μg of pSVtat plus 8 μg of the indicated plasmid per dish. Cells were harvested after 44 hr and CAT activities in nmol of chloramphenicol acetylated per min per mg of protein were measured. Results of separate experiments are expressed relative to standard controls [8 μg of pHβAPr-1 (*A* and *B*) and 8 μg of pBR322 (*C*)]. The 100% activity varied among experiments but averaged about 20 nmol of chloramphenicol acetylated per min per mg of protein for both standard controls (pHβAPr-1 and pBR322). Standard deviations are indicated by error bars and the number of separate determinations is indicated by numbers in parentheses below the histograms. (*A*) Transfection with 8 μg of pHβAPr-1 (control), pHβ/TAR, pHβ/ASTAR, or pHβ/AStat. (*B*) Transfection with 8 μg of pHβAPr-1 (control), pHβ/TAR-12, or pHβ/ASTAR-12. (*C*) Transfection with 8 μg of pBR322 (control) or pBR/TAR-12.

not respond to tat because of deletion of one-half of the stem–loop sequence of TAR. This plasmid was tested in the presence and absence of pSVtat. Table 1 shows that pHβ/TAR-12 did not inhibit expression of any of these plasmids when compared with the parent vector pHβAPr-1 and that there was, on the contrary, a small but reproducible stimulation (1.5- to 4-fold) of all promoters. Thus, the inhibitory effect of pHβ/TAR-12 seen in Fig. 2*B* is specific for the tat–TAR interaction. pHβ/ASTAR-12 also stimulated heterologous promoters (pSV2Cat; data not shown).

Since both pHβ/TAR-12 and pHβ/ASTAR-12 inhibited transactivation, it seemed possible that any transcribed multimeric sequence might do this. To rule this out, we constructed a plasmid, pHβ/α-10, that carries 10 tandem copies of a 171-bp primate satellite DNA repeat (9) driven by the β-actin promoter and tested its effect on transactivation. This plasmid had no effect on tat transactivation (data not shown). Hence, inhibition by pHβ/TAR-12 and pHβ/ASTAR-12 is probably sequence specific.

The general stimulatory effect of pHβ/TAR-12 on cotransfected genes was unexpected and judged worthy of further investigation. To learn whether transcription was necessary for this effect, we cotransfected 1 μg of pSV2Cat, pHβAPr-1-Cat, pHIVCat, or pΔTAR4Cat with 9 μg of either pBR/TAR-12 or the parent vector pBR322. Under these conditions, the CAT activity of all four of these plasmids was stimulated 2- to 5-fold by cotransfection with pBR/TAR-12 (Table 2). The results indicated that the untranscribed DNA copies of the TAR 12-mer were sufficient for stimulation.

Northern Blot Analysis of TAR 12-mer Transcripts. To verify that inhibition by the TAR 12-mer expression construct required transcription, NT2/D1 cells were transfected with pHβ/TAR-12, pHβ/ASTAR-12, or pBR/TAR-12. RNA was prepared from the cells 65 hr later and analyzed by Northern blot transfer using the TAR 12-mer insert as probe. Fig. 3*A* shows that pHβ/TAR-12 and pHβ/ASTAR-12 were efficiently transcribed, whereas no detectable RNA was transcribed from pBR/TAR-12. For unknown reasons, steady-state levels of RNA transcribed from pHβ/ASTAR-12 were lower than those derived from pHβ/TAR-12. We tested the strand specificity of the transcripts using radioactively labeled single-stranded RNA probes derived from the TAR 12-mer inserted in both orientations behind the SP6 promoter of pGEM-2. The Northern blots shown in Fig. 3*B* demonstrated that pHβ/TAR-12 produced mainly sense transcripts, whereas pHβ/ASTAR-12 produced mainly antisense transcripts.

DISCUSSION

We have demonstrated a method of increasing the concentration of specific DNA or RNA sequences within transfected cells by "tandemization" of a specific sequence, the HIV-1 TAR, behind a strong promoter. We have further shown that, with this method, concentrations of HIV-1 TAR RNA can be achieved that strongly inhibit transactivation of HIV-1 directed gene expression by the viral *tat* gene product. Although one copy of the TAR driven by the human β-actin

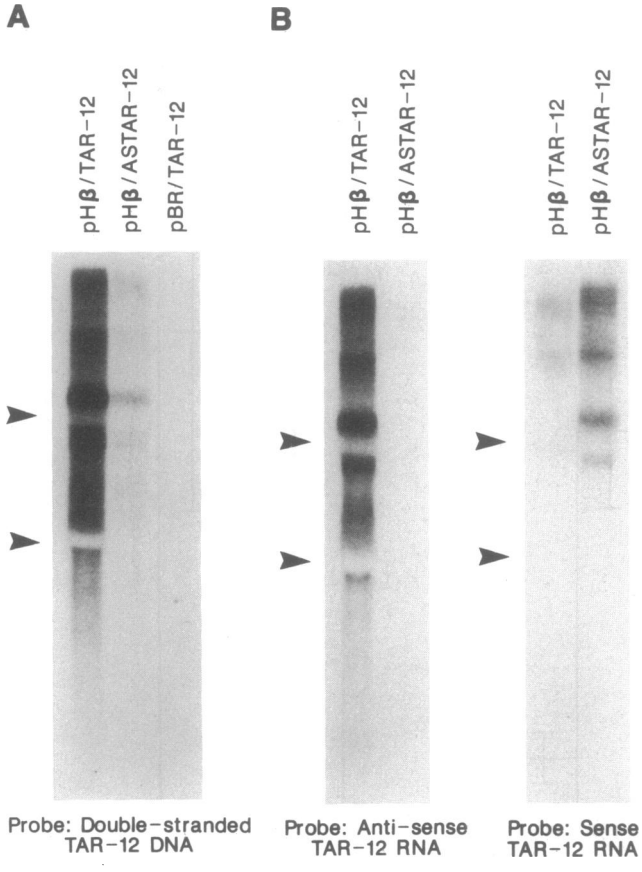

FIG. 3. Northern blot analysis of NT2/D1 cells transfected with plasmids containing TAR 12-mer sequences. (*A*) NT2/D1 cells were transfected with each plasmid at 10 μg per dish, as indicated above the corresponding lane. The cells were harvested 65 hr later, total cellular RNA was extracted, and 25 μg of RNA from each sample was separated in denaturing agarose gels. Northern blots were prepared and probed with nick-translated double-stranded DNA prepared from TAR 12-mer DNA. (*B*) NT2/D1 cells were transfected with pHβ/TAR-12 or pHβ/ASTAR-12 at 10 μg per dish, as indicated. Northern blots were prepared as in *A* and probed with radioactive single-stranded RNA corresponding to antisense TAR 12-mer or to sense TAR 12-mer. The RNA probes were prepared using SP6 polymerase and pGEM-2 derivatives in which the TAR 12-mer was inserted in sense or antisense orientations with respect to the SP6 promoter. Arrowheads mark the positions of 18S and 28S rRNAs.

promoter was ineffective, 12 copies inhibited transactivation 85–90%. The inhibition appeared to require the accumulation of TAR RNA, since the promoterless construct pBR/TAR-12 did not cause such an accumulation and showed no inhibitory activity.

The inhibition was specific for the tat–TAR interaction. This was shown by the lack of inhibition that pHβ/TAR-12 had (*i*) on pHIVCat in the absence of tat, (*ii*) on pΔTAR4Cat in the presence or absence of tat, and (*iii*) on pSV2Cat and pHβAPr-1-Cat. This specificity indicated that the mechanism of inhibition did not involve the induction of a double-stranded RNA-dependent kinase (dsI), a known translation inhibitor (13). The induction of dsI might be expected with the TAR 12-mer expression construct, given the extensive secondary structure that should be present in the RNA. However, the separation between the mRNA cap site and the TAR secondary structures provided by the transcribed regions of the β-actin promoter may suppress the dsI activity. Perhaps this accounts for the apparent lack of dsI activity seen.

The specificity is puzzling since the tat protein is thought more likely to act through cellular factors rather than directly upon the TAR sequence itself (14, 15). What is the usual function of these factors? Are some endogenous mRNAs preceded by TAR-like sequences? If so, are there endogenous *tat*-like genes that are activated under special circumstances? These questions await further investigation.

Table 1. Effect of pHβ/TAR-12 on HIV-1 and non-HIV-1 promoters in the absence of tat or of a functional TAR

Plasmids	CAT activity, nmol per min per mg of protein
pHIVCat (2 μg) + pHβAPr-1 (8 μg)	0.16
pHIVCat (2 μg) + pHβ/TAR-12 (8 μg)	0.69
pΔTAR4Cat (2 μg) + pHβAPr-1 (8 μg)	0.10
pΔTAR4Cat (2 μg) + pHβ/TAR-12 (8 μg)	0.19
pΔTAR4Cat (1 μg) + pSVtat (1 μg) + pHβAPr-1 (8 μg)	0.08
pΔTAR4Cat (1 μg) + pSVtat (1 μg) + pHβ/TAR-12 (8 μg)	0.24
pSV2Cat (2 μg) + pHβAPr-1 (8 μg)	0.36
pSV2Cat (2 μg) + pHβ/TAR-12 (8 μg)	0.53
pHβAPr-1-Cat (2 μg) + pHβAPr-1 (8 μg)	0.93
pHβAPr-1-Cat (2 μg) + pHβ/TAR-12 (8 μg)	2.55
pHIVCat (1 μg) + pSVtat (1 μg) + pHβAPr-1 (8 μg)	25.2
pHIVCat (1 μg) + pSVtat (1 μg) + pHβ/TAR-12 (8 μg)	3.6

NT2/D1 cells were transfected with combinations of plasmids as indicated. Cells were harvested 44 hr later and CAT activities were determined. The values shown are the averages of two transfections. The last pair of entries showing stimulation by tat is included for comparison.

The data also showed that the TAR 12-mer had a slight but reproducible stimulatory effect on CAT gene constructs other than those regulated by the tat–TAR interaction. This included constructs driven by the human β-actin promoter (pHβAPr-1-Cat) or by the simian virus 40 early promoter (pSV2Cat) on the HIV-1 promoter after it was mutated to tat unresponsiveness in pΔTAR4Cat and on pHIVCat when the tat protein was absent. This effect, unlike the tat–TAR-specific inhibition, did not appear to require RNA synthesis because it was also induced by pBR/TAR-12, measured relative to the control transfections with the vector pBR322. The simplest interpretation is that the multiple TAR DNA copies bound some general negative regulatory factor. At least two proteins are known to bind to DNA sequences included within the monomer unit (16) but because neither is thought to be a general inhibitor, the observation remains unexplained.

TAR elements inserted as either a monomer or 12 tandem repeats in antisense orientations were relatively ineffective as inhibitors of tat transactivation. Tandemization of antisense

Table 2. Effect of TAR 12-mer sequences on heterologous promoters

Plasmids	CAT activity, nmol per min per mg of protein
pHIVCat (1 μg) + pBR322 (9 μg)	0.08
pHIVCat (1 μg) + pBR/TAR-12 (9 μg)	0.44
pΔTAR4Cat (1 μg) + pBR322 (9 μg)	0.05
pΔTAR4Cat (1 μg) + pBR/TAR-12 (9 μg)	0.20
pSV2Cat (1 μg) + pBR322 (9 μg)	0.35
pSV2Cat (1 μg) + pBR/TAR-12 (9 μg)	0.67
pHβAPr-1-Cat (1 μg) + pBR322 (9 μg)	0.26
pHβAPr-1-Cat (1 μg) + pBR/TAR-12 (9 μg)	1.16

NT2/D1 cells were transfected and CAT activities were determined 44 hr later as described in Table 1.

TAR increased its inhibition (Fig. 2 *A* and *B*) but only to 40%, whereas no inhibition was obtained with the antisense monomer sequence. Northern blot analysis of RNA from cells transfected with the TAR antisense 12-mer construct consistently showed lower steady-state levels of cognate RNA than were obtained with RNA from cells transfected with the same amount of the TAR 12-mer sense construct. The relative ineffectiveness of the TAR 12-mer antisense RNA as an inhibitor of the tat–TAR interaction may be related to this observation. On the other hand, tat specificity depends upon TAR sequence and structure; these would differ in the antisense RNA transcripts from those normally encountered and thus competition for essential transactivators such as tat or for cellular factors induced by tat might not occur. It was also expected that inhibition of expression through the formation of RNA·RNA hybrids might take place. The relative ineffectiveness of the antisense constructs as inhibitors suggested that either the formation of intrastrand secondary structure precluded RNA·RNA hybrid formation or sufficiently high levels of antisense RNA were not achieved in these experiments.

Exogenous DNA or RNA can selectively silence or downregulate gene expression by two general methods: competition (as described here) and antisense inhibition. Since both methods require a large excess of inhibitory sequences over target sequences, means of increasing the number of inhibitory sequences are valuable. The use of strong promoters and the use of multicopy plasmids (17) are two solutions; here we have described a third, that of assembling inhibitory sequences in a tandem array. Since in both competition (15, 16) and antisense (18) inhibition short sequences of 20–40 bp can be effective, arrays of up to 100 copies might eventually prove feasible (6).

This work was undertaken to determine whether high levels of sense or antisense RNAs might interfere with essential HIV-1 functions and prove useful in conferring cellular immunity as an approach to gene therapy in AIDS. Sense RNA transcripts of multiple TAR elements were capable of blocking tat-mediated transactivation of gene expression driven by the HIV-1 LTR, whereas the DNA copies of the same elements were ineffective. The results further indicated that these methods can be used to generate tandem arrays of duplexed oligomers if these have appropriate *Ava* I overhangs. This could be useful in Southwestern transfers (protein blots probed with oligonucleotides) and might facilitate, for example, the study of immunoglobin class switch sequences. Finally, it is worth noting that the *Hin*cII and *Ava* I sequences that separate the polymerized inserts do not contain stop codons and will not shift the reading frame. Thus, it might be possible to produce tandemly polymerized protein segments with multiple copies of a functional site or multiple slightly varying copies of a given epitope that can result in increased immunogenicity (19).

Note. While this work was in progress, Takeshita *et al.* (20) described a similar procedure for creation of tandem arrays of entire genes.

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