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Inhibition of human immunodeficiency virus expression by sense transcripts encoding the retroviral leader RNA

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Abstract

Towards gene therapy for the treatment of human immunodeficiency virus type 1 (HIV-1) infections, we tested the potency of several antiviral constructs in transient HIV-1 production assays. Whereas little effect was obtained with antisense- and TAR decoy-constructs, we measured efficient inhibition of HIV-1 mRNA translation and virion production in the presence of HIV-1 leader-containing transcripts. The infectivity of these virions was also reduced by this sense inhibitor RNA. These results suggest that leader-encoded functions, like the dimer-linkage structure, can be used to specifically inhibit HIV expression in *trans*.

Keywords: Gene therapy; Sense transcript; HIV-1; mRNA

1. Introduction

Gene therapy has been proposed for treatment of AIDS, for which there are currently no effective chemotherapeutic or vaccine therapies (Baltimore, 1988). Several molecular strategies were designed and shown to inhibit replication of the HIV-1 retrovirus in tissue culture systems. These anti-HIV approaches include RNA molecules such as antisense transcripts, ribozymes and sense/decoy motifs that mimic important HIV-1 RNA structures, such as the TAR or RRE elements (Graham and Maio, 1990; Rhodes and James, 1990; Sarver et al., 1990; Sczakiel et al., 1990; Sullenger et al., 1990; Joshi

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et al., 1991; Rittner and Sczakiel, 1991; Sczakiel and Pawlita, 1991; Sullenger et al., 1991; Weerasinghe et al., 1991; Dropulic et al., 1992; Tung and Daniel, 1993; Lori et al., 1994; Yu et al., 1994). The efficiency of such antiviral RNA molecules can be increased by addition of the retroviral packaging signal Ψ, which results in colocalization of the inhibitor transcript and the target HIV-1 genomic RNA within viral particles (Sullenger and Cech, 1993).

The HIV-1 replication cycle can be divided in two phases: the infection and expression phase. The infection is followed by reverse transcription of the RNA genome and integration of the DNA provirus in the host chromosome. Expression of the HIV-1 genome is mediated by the cellular transcription machinery. Full-length viral RNA genomes and several spliced mRNAs are synthesized, followed by assembly and release of new viral particles. It has been proposed that for greater synergy of antiviral effects, approaches that block the establishment of infection should be combined with approaches that block the production of virus from infected cells (Mitsuya et al., 1990). In this study, we tested the antiviral activity of transcripts with combinations of sense RNA decoys, antisense RNA domains, and the retroviral packaging signal. One feature of the inhibitor constructs used in this study is that they potentially target multiple steps of the retroviral replication cycle, with most obvious targets in the viral expression phase. Using HeLa cell cotransfections, we demonstrate that inhibition of HIV-1 production from the pLAI proviral DNA clone was primarily due to a reduction in protein expression levels. Furthermore, the virions produced were less infectious compared to wild-type HIV-1 particles. We show that the leader region of the HIV-1 genomic RNA is both required and sufficient to substantially inhibit HIV-1 expression.

2. Materials and methods

2.1. Plasmid constructions

The full-length molecular clones HIV-1 pLAI and HIV-2 ROD were a generous gift from Keith Peden (Peden et al., 1991). All plasmids were constructed by standard techniques. Nucleotide numbers refer to the positions on the transcript of the HIV-1 LAI isolate, with +1 being the capped G residue. The CMV-Tat plasmid (Koken et al., 1994) and defective LTR promoters with mutations in the TAR element (mutants L5 and B123) were previously described (Berkhout and Jeang, 1989). Plasmid LTR-Ψ contains the HIV-1 *Xba*I–*Cla*I fragment inserted into Bluescript KS + and was previously described as Blue-5′-LTR (Klaver and Berkhout, 1994a). LTR-Ψ contains the complete 5′-LTR promoter and HIV-1 leader sequences up to position 380 in the *gag* gene. Several HIV-1 fragments were cloned in either sense or antisense orientation in the *Sma*I site of the Bluescript polylinker downstream of the HIV-1 LTR-leader sequences (see Fig. 1 for a schematic representation of the DNA constructs). A 2683 bp HIV-1 fragment obtained by *Asp*718 digestion (position 5924–8607) was inserted into the polylinker *Asp*718 site, thus creating LTR-Ψ-S1 and LTR-Ψ-AS1, respectively. Likewise, plasmids LTR-Ψ-S2 and LTR-Ψ-AS2 were generated with the 2189 bp *Asp*718 fragment (position 3735–5924). The putative packaging region Ψ was deleted in the

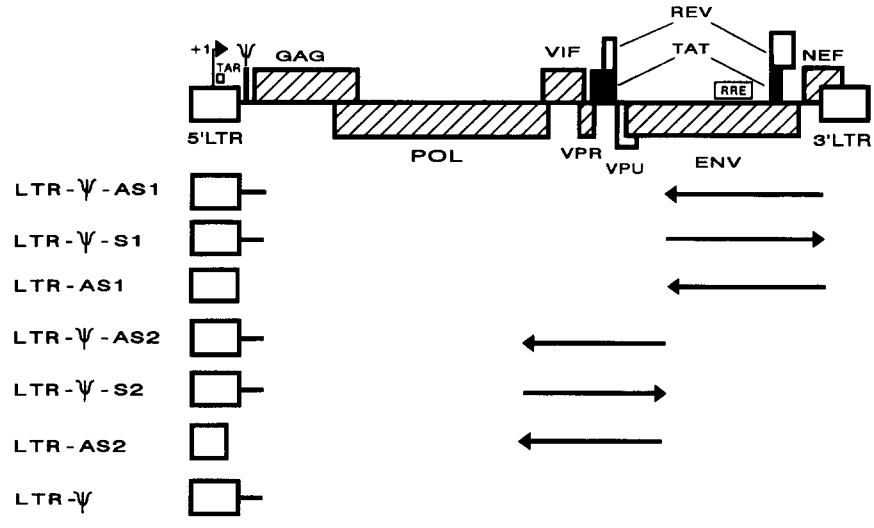

Fig. 1. Schematic representation of anti-HIV constructs. The genomic organization of HIV-1 proviral DNA is shown at the top. All constructs contain the 5'-LTR promoter and the start site of transcription is indicated by an arrow (position +1). The basic construct LTR-Ψ transcribes the complete untranslated leader region, including the TAR RNA motif at position +19/+42, the major splice donor at position 293 and the packaging signal Ψ. Sequences up to position 380 in the *gag* gene were included (*gag* start codon is at position 336). Additional HIV-1 sequences from the middle or 3'-region of the genome were cloned in either sense (S) or antisense (AS) orientation into LTR-Ψ. The LTR-Ψ-AS1/2 constructs were further modified by deletion of the packaging signal, thus creating the LTR-AS1/2 vectors.

two antisense constructs. For LTR-Ψ-AS1, the Ψ-region was removed as *Nar*I–*Sal*I segment (position 182-polylinker) by double-digest, Klenow treatment and subsequent ligation. For LTR-Ψ-AS2, the Ψ region was deleted as *Hind*III–*Xho*I fragment (position 76-polylinker). The resulting plasmids are termed LTR-AS1 and LTR-AS2. All constructs were verified by restriction site mapping.

2.2. Cell culture, plasmid transfection, virus and viral RNA / protein analyses

HeLa cells were grown in Dulbecco's modified Eagle's medium containing 5% fetal calf serum and transfected with plasmid DNA by means of DEAE-dextran (Berkhout and Jeang, 1989). The infectious molecular clones pLAI (HIV-1) and pROD (HIV-2) were cotransfected with inhibitor constructs into HeLa cells. We usually used a two-fold molar excess of the inhibitor construct, e.g. 600 pmol pLAI (5 μg) and 1200 pmol anti-HIV plasmid (2.5 μg Bluescript, 3.3 μg LTR-Ψ or 5.0 μg LTR-Ψ-AS1). All transfections were performed on at least three separate occasions. After 48–72 h, cells and supernatants were harvested and stored at −20°C. Cells were analyzed for HIV-1 proteins on a Western blot or assayed for CAT activity, supernatants were quantitated for reverse transcriptase (RT) and Gagp24 and kept as virus stock for subsequent titration on C8166 cells. The tissue culture infectious dose ($TCID_{50}$) was calculated according to Reed and Muench (1938).

For Western analysis, cells were washed once with phosphate-buffered saline and resuspended in reducing sodium dodecyl sulfate (SDS) sample buffer (50 mM Tris pH 7.0, 2% SDS, 10% β-mercaptoethanol, 5% glycerol). Proteins were resolved in a 10% SDS-polyacrylamide gel, transferred to Immobilon-P (16 h, 60 V), and subsequently blocked with phosphate-buffered solution (PBS) buffer containing 5% non-fat dry milk, 3% bovine serum albumin (BSA) and 0.05% Tween-20. Filters were incubated with serum of an HIV-1 infected individual (patient H) for 1 h at room temperature, washed and subsequently developed using the BCIP-NBT protocol.

RT assays were performed as previously described (Willey et al., 1988). Each reaction contained 10 μl of virus-containing culture supernatant in 50 μl of RT buffer (60 mM Tris pH 8.0, 75 mM KCl, 5 mM $MgCl_2$, 0.1% NP-40, 1 mM EDTA, 4 mM DTT) supplemented with 0.25 μg polyA and 8 ng oligo(dT)18 primer and 0.1 μl α-^{32}P-dTTP (3000 Ci/mmol, 10 mCi/ml). After 4 h at 37°C, 10 μl was spotted onto DE-81 paper, which was washed 3 times in 5% Na_2HPO_4, once in ethanol and dried. RT activity was measured in the linear range of the assay and radioactive spots were quantitated on a Molecular Dynamics β-Imager. CAT assays were performed by the phase-extraction protocol (Seed and Sheen, 1988) and Gagp24 ELISA (Koken et al., 1992) were described previously. All values were determined within the linear range of the assays.

Total cellular RNA was isolated by the hot phenol method and analyzed on Northern blots as previously described (Klaver and Berkhout, 1994b). Blots were probed with ^{32}P-labeled HIV-1 DNA fragments generated by the random oligoprimer method. We used either an HIV-specific probe (*Xba*I–*Hind*III, position −455 to +77) or a vector-specific probe (*Asp*718–*Xba*I fragment of LTR-Ψ).

3. Results

3.1. Design of multivalent anti-HIV constructs

We constructed a set of HIV-based expression vectors with several combinations of putative inhibitory sequence elements (Fig. 1). These antiviral features included sense RNA decoys (TAR, RRE) and antisense RNA directed against different regions of the HIV-1 genome. All plasmids use the Tat-inducible LTR enhancer/promoter of the HIV-1 virus. The capability of such vectors to efficiently transcribe homologous or heterologous genes in the presence of the Tat *trans*-activator is well-known. Furthermore, expression of the LTR-encoded TAR RNA motif has been shown to inhibit HIV-1 replication by sequestration of critical cellular cofactors involved in Tat-mediated transcriptional activation (Graham and Maio, 1990; Sullenger et al., 1990, 1991; Lori et al., 1994). In order to facilitate packaging of the inhibitor RNAs into virions, we included the HIV-1 packaging signal in the basic LTR-Ψ transcription unit (Fig. 1). There is, however, still considerable uncertainty about the extent of the HIV-1 sequences required for optimal packaging. Part of the Ψ-signal is located between the major splice donor and the *gag* initiation codon at position 336 (Lever et al., 1989; Aldovini and Young, 1990; Clavel and Orenstein, 1990; Hayashi et al., 1992), but sequences in the

5′-region of the *gag* gene (Buchschacher and Panganiban, 1992; Parolin et al., 1994) and *env* sequences overlapping the RRE (Richardson et al., 1993) have recently been reported to contribute to the packaging function. Our basic construct LTR-Ψ included the complete leader region and part of the *gag* gene up to position 380, which should facilitate efficient packaging (Parolin et al., 1994).

Several fragments of the HIV-1 genome were individually inserted into the LTR-Ψ plasmid in either sense (S) or antisense (AS) orientation (Fig. 1). S1 encodes the complete RRE RNA motif, which may act as decoy by binding cellular factors involved in Rev-mediated regulation of HIV-1 mRNA processing. In addition, S1 will encode the secondary packaging function present in the *env* gene (Richardson et al., 1993). The antisense AS1 transcript is targeted not only to the full-length HIV-1 genomic RNA, but also to several spliced mRNAs encoding Tat, Rev, Env and Nef. The sense S2 insert is unique in that it has the potential to encode the *trans*-activator protein Tat, albeit a truncated one-exon form. This form of the Tat protein was previously shown to be at least partially active in LTR-induction assays (Siegel et al., 1986; Jeang et al., 1993). The AS2 transcript can hybridize both to the HIV-1 RNA genome and several subgenomic mRNA species encoding Vif, VpR, VpU, Tat and Rev. In order to analyze the specific contribution of the Ψ-signal, we modified the two antisense plasmids by deletion of the packaging function, thus creating constructs LTR-AS1 and LTR-AS2.

3.2. Northern blot analysis of LTR-Ψ transcripts

We would like to note that no heterologous splicing and polyadenylation signals were included in the HIV-1-based plasmids shown in Fig. 1. Since it is well-known that the absence of such RNA processing motifs may negatively influence the intracellular stability of recombinant transcripts (Okayama and Berg, 1983), we measured steady-state transcript levels in transfected HeLa cells by Northern blot analysis (Fig. 2A). This experiment clearly indicates that transcripts of distinct length are synthesized by all constructs.

In analogy to the mechanism of polyadenylation in HIV-1, where 5′-LTR-initiated transcripts ignore the polyA signal at position +73 but efficiently polyadenylate in identical sequences of the 3′-LTR (Böhnlein et al., 1989; Dezazzo et al., 1991; Valsamakis et al., 1991; Weichs an der Glon et al., 1991; Cherrington and Ganem, 1992; Gilmartin et al., 1992; Valsamakis et al., 1992; Klaver and Berkhout, 1994b), it can be predicted that LTR-Ψ constructs will only utilize the polyA signal present in the unique viral LTR element upon second passage over this sequence. The observed transcript lengths are consistent with this idea. For instance, LTR-Ψ and LTR-Ψ-AS1 transcripts are approximately 3900 and 6600 nucleotides in length (Fig. 2A, lanes 2 and 3, respectively). Furthermore, the presence of vector-encoded sequences in these transcripts was verified by hybridization to a probe specific for plasmid sequences (Fig. 2C). Additional evidence for LTR-mediated polyadenylation comes from the analysis of LTR-AS2, which carries a large Ψ-deletion just downstream of the polyA signal. This construct lacks the G/U-rich motif that was previously shown to stimulate polyadenylation at the upstream polyA signal (Böhnlein et al., 1989). Indeed, despite the 200-nucleotide deletion in LTR-AS2, we detected a longer transcript compared to the

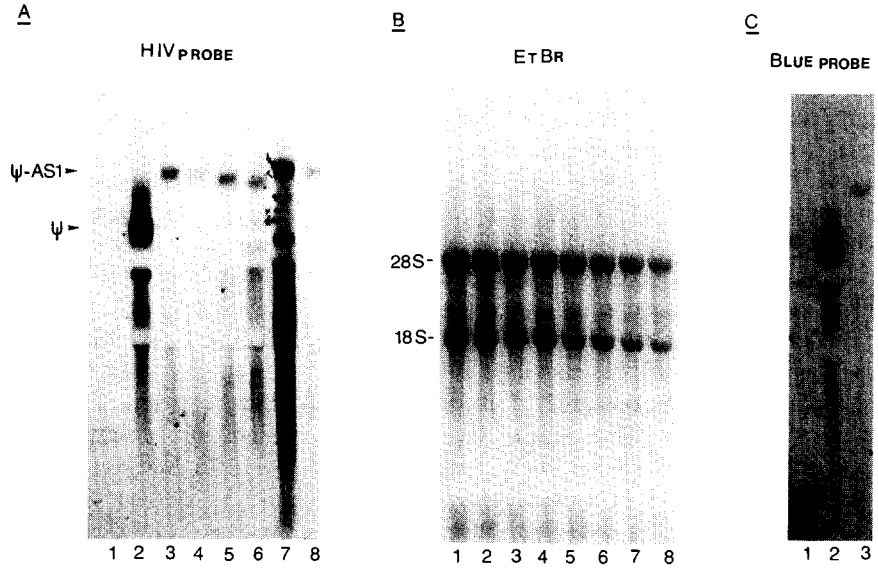

Fig. 2. Northern blot analysis of LTR-Ψ transcripts in transfected HeLa cells. HeLa cells were transfected with 30 μg LTR-Ψ derivative and 3 μg pTat plasmid. Total cellular RNA was isolated after 3 days by the hot-phenol method and analyzed by Northern blotting. Lanes: 1, an RNA sample of mock-transfected cells; 2, LTR-Ψ; 3, LTR-Ψ-AS1; 4, LTR-Ψ-S1; 5, LTR-AS1; 6, LTR-Ψ-AS2; 7, LTR-Ψ-S2; 8, LTR-AS2. Please note that an approximate 2-fold molar excess of LTR-Ψ was used compared to all other constructs, resulting in a more intense signal in lane 2. Northern blot analysis used a HIV-specific probe (panel A) or a Bluescript-derived probe designed to detect read-through transcripts (panel C). The ethidium bromide-stained gel is shown in panel B. The position of the Ψ and Ψ-AS1 transcripts is indicated on the left of panel A (lanes 2 and 3, respectively). The 18*S* and 28*S* rRNA markers are indicated on the left of panel B.

parental construct LTR-Ψ-AS2 (Fig. 2A, compare lanes 8 and 6, respectively). This finding is consistent with inactivation of the polyA signal in LTR-AS2, giving rise to extended transcript forms.

This Northern blot analysis also suggests that LTR-Ψ transcripts are not spliced. Thus, although all Ψ-RNAs contain the HIV-1 major splice donor, there is apparently no appropriate splice acceptor signals present in these transcripts. We believe this situation to be beneficial for HIV-inhibition because splicing at the HIV-1 splice donor would result in removal of at least part of the downstream sequences involved in genome dimerization and packaging.

3.3. Inhibition of HIV-1 production by Ψ-containing transcripts

To determine the effectiveness of LTR-Ψ-mediated inhibition of HIV-1 expression, the infectious pLAI molecular HIV-1 clone and the anti-HIV constructs were cotransfected into HeLa cells. We used a two-fold molar excess of the inhibitor plasmid. The anti-HIV genes are induced in *trans* by the Tat protein encoded by pLAI, and these transcripts may subsequently interfere with HIV transcription, translation or virion

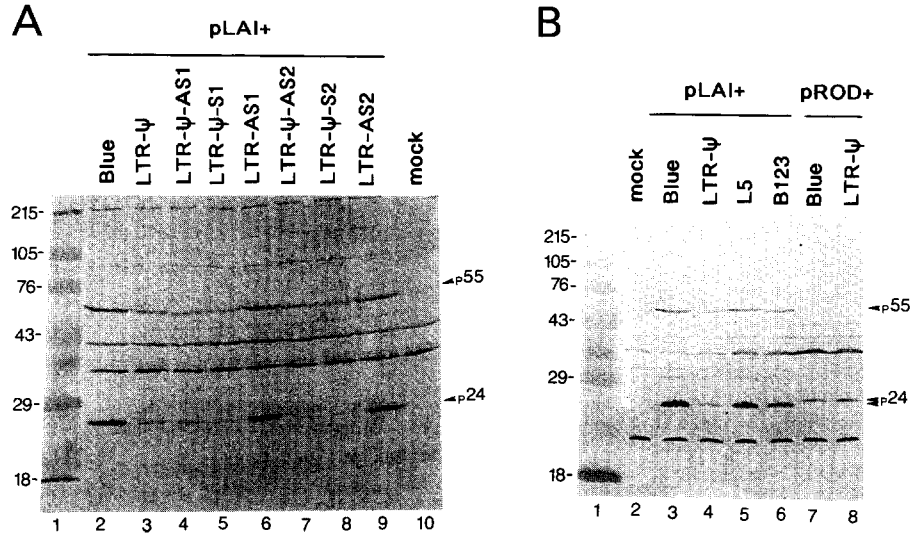

Fig. 3. Inhibition of HIV-1 protein expression in transiently transfected HeLa cells. (A) The pLAI molecular clone was cotransfected into HeLa cells with an anti-HIV construct (indicated on top of panel) in a constant molar ratio of 1:2 (pLAI:inhibitor plasmid). In control experiments, cells were either mock-transfected (lane 10) or transfected with pLAI and the plasmid Bluescript (Blue in lane 2). Forty-eight hours after transfection, cells were solubilized in SDS-sample buffer and HIV-specific proteins were assayed by immunoblotting using serum of AIDS patient H. The position of the Gag-p55 precursor and processed Gag-p24 protein is indicated on the right-hand side. Protein markers are presented in lane 1, with their molecular weight (in kDa) indicated on the left-hand side. The supernatants of these cultures were subjected to Gag-p24 antigen analysis, RT enzyme assays and the virus titer was scored on the C8166 T-cell line. All these results are summarized in Table 1. (B) HeLa cells were transfected with HIV-1 clone pLAI (lanes 3–6) or HIV-2 clone pROD (lanes 7–8) in the absence or presence of a 2-fold molar excess of inhibitor construct (LTR-Ψ or the control Bluescript plasmid). L5 and B123 are modified LTR-Ψ vectors containing an inactivating mutation in the TAR hairpin motif, which is essential for LTR-transcription. A protein sample of mock-transfected cells is shown in lane 2 and protein markers in lane 1. Supernatant Gag-p24 values of samples corresponding to lanes 3–6 were quantitated and are shown in Fig. 4. See panel A for further experimental details.

assembly. Furthermore, the infectivity of the virions produced may be affected by copackaged inhibitor RNAs. Several parameters of HIV-1 expression were measured 3 days after transfection. Cell extracts and culture supernatants were analyzed for Gag protein expression using Western blot and ELISA technique, respectively (Fig. 3 and Table 1). In addition, we measured virion-associated RT activity in the supernatant samples and the infectivity of the virions was quantitated in titration assays on the C8166 T-cell line. All results are summarized in Table 1 with the relative percentage HIV-production in parentheses (values obtained with pLAI in the absence of inhibitor were set at 100%).

HIV-1 protein expression was significantly inhibited by all of the LTR-Ψ constructs (Table 1). In particular, cotransfection with Ψ-encoding plasmids inhibited HIV-1 protein expression, whereas relatively little inhibition was measured with the Ψ-deleted LTR-AS1/2 vectors. A concomitant decrease was scored for RT and Gag values in the

Table 1
LTR-Ψ-mediated inhibition of HIV-1 expression

pLAI co-transfection with	Cells	Supernatant		
	Gag [a]	Gag p24 [b]	RT [c]	Virus titer [d]
Bluescript	+ + +	11 (100)	2870 (100)	7943 (100)
LTR-Ψ-AS1	+	1 (9)	191 (7)	79 (1)
LTR-Ψ-S1	+	0 (0)	191 (7)	126 (2)
LTR-AS1	+ + +	4 (36)	1092 (38)	2512 (32)
LTR-Ψ-AS2	+	1 (9)	83 (3)	21 (0)
LTR-Ψ-S2	+	0 (0)	121 (4)	79 (1)
LTR-AS2	+ + +	3 (27)	792 (28)	794 (10)
LTR-Ψ	+	3 (27)	264 (9)	251 (3)

[a] Westernblot analysis of transfected HeLa cells (see Fig. 3).
[b] Gag-p24 ELISA (pg/100 μl), less than 20% variation was seen in duplicate experiments.
[c] RT-activity (counts) in polyA-oligo(dT) assay, less than 15% variation was seen in duplicate experiments.
[d] Titration on C8166 cells ($TCID_{50}$/ml), performed in quadruplicate.

culture supernatant. An even more dramatic reduction in the amount of infectious virus was measured in some supernatant samples. For instance, even though significant levels of Gag-p24 were found in the supernatant of LTR-Ψ transfected cells, hardly any infectious virus was measurable (27 and 3%, respectively). This suggests that the inhibitor RNA not only inhibits virus production, but also decreases the infectivity of those virions, presumably through copackaging of the inhibitor transcript. Further evidence for this idea comes from a comparative analysis of transcripts with or without the Ψ-packaging signal. A somewhat reduced number of HIV-1 particles were expressed in the LTR-AS1 transfection (36%), but no further decrease in infectivity was measured (32%) compared to wild-type HIV-1 virions. In contrast, of the viral particles produced in the presence of LTR-Ψ-AS1 (9%), only a few were infectious (1%). These combined results suggest that LTR-Ψ-based constructs can reduce HIV-1 infectivity at multiple points in the HIV-1 replication cycle.

Of particular interest is the sense LTR-Ψ-S2 construct, which initially may have seemed inappropriate for inhibition because it encodes the exon-1 form of the Tat protein that can activate expression of the HIV-1 virus in *trans*. Apparently, the inhibitory functions of this construct (TAR decoy, Ψ-signal) are dominant over the potential helper function of Tat. It seems likely that a positive Tat effect is not observed because pLAI itself can synthesize saturating amounts of Tat protein. This was indeed observed in cotransfection assays with LTR-CAT reporter genes (data not shown). In fact, overexpression of Tat protein can lead to inhibition of LTR function, probably through squelching of cellular cofactors, and may therefore add to the inhibitory potential of this construct.

It is important for putative antiviral reagents to be active against different HIV-1 isolates and members of the less homologous HIV-2 and SIV virusgroups. We therefore wished to investigate whether the HIV-1 LTR-Ψ construct was able to interfere with HIV-2 expression in a cotransfection assay. HeLa cells were transfected with the pROD molecular clone and HIV-2 protein expression was analyzed on Western blot (Fig. 3B,

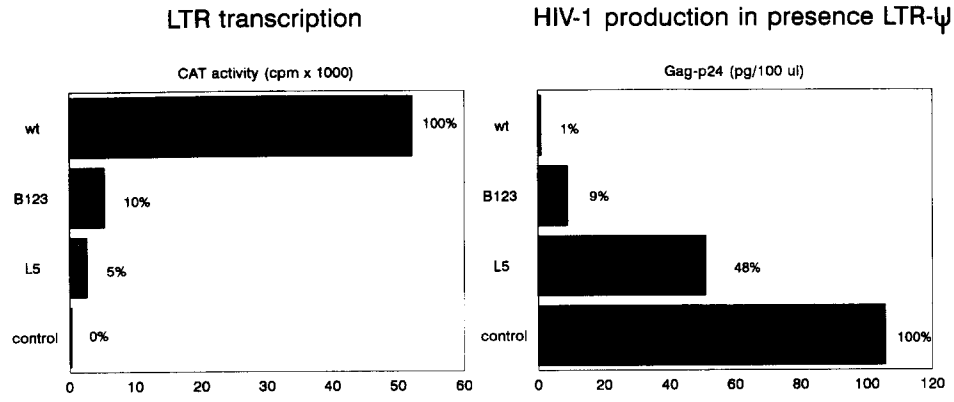

Fig. 4. Reduced HIV-inhibition with transcriptionally defective LTR-Ψ plasmids. Transcriptional activity of the wild-type (wt) and mutant LTRs was tested with LTR-CAT constructs (left panel). HeLa cells were transfected with 0.5 μg of the individual LTR-CAT plasmids indicated in the presence of 0.2 μg pTat. The control sample was from mock-transfected cells. Total cell lysates were prepared at day 3 and assayed for CAT activity. Relative CAT activities were compared with that obtained with the wt LTR, which was set at 100%. Inhibition of HIV-1 production was assayed in cotransfections of pLAI with either wild-type or mutated LTR-Ψ constructs (right panel). The control transfection was with pLAI in the presence of the Bluescript vector. HIV-1 production was measured by Gag-p24 ELISA in the culture supernatant. Gag-p24 values obtained in the cotransfection of pLAI with the Bluescript control plasmid were standardized at 100%. The cell samples of these transfections were solubilized in SDS-sample buffer and analyzed on Western blot (Fig. 2B, lanes 3–7).

lanes 7 and 8). We measured no significant reduction in the level of HIV-2 protein expression, suggesting that the inhibitory sequences in the HIV-1 leader/Ψ-element are not cross-reactive with HIV-2 genomes.

3.4. Inhibition is proportional to transcriptional activity of the LTR-Ψ construct

The introduction of large amounts of promoter/enhancer DNA sequences into cells may switch off transcription of cellular or other exogenous genes (Scholer and Gruss, 1984). This squelching phenomenon is thought to be due to competition for limiting cellular factors involved in transcription, and similar effects have been observed for the HIV-1 LTR DNA (Hammes et al., 1989). In order to verify that HIV-inhibition observed in our transient system is dependent on the anti-HIV transcripts rather than DNA motifs of the transfected plasmid, we performed inhibition studies with transcriptionally inactive variants of the LTR-Ψ plasmid. In order to do so, we introduced mutations in the TAR RNA binding site for the Tat *trans*-activator protein. In this RNA hairpin motif, we either changed the sequence of the bulge (mutant B123) or the loop (mutant L5), mutations that were previously shown to inhibit LTR-transcription to varying degrees (Berkhout and Jeang, 1989). We measured 10 and 5% transcriptional activity of these mutant promoters in LTR-CAT assays (Fig. 4, left panel). In parallel cotransfections of the corresponding LTR-Ψ plasmids with pLAI, we measured reduced inhibitory potential of the mutant LTR constructs (Figs. 3B and 4, right panel). HIV-1 production

was reduced to 1% by cotransfection with wild-type LTR-Ψ, whereas 9% HIV-1 production levels were measured with the LTR-mutant B123 and up to 48% with the more severely inactive L5 mutant. Although we cannot formally exclude the possibility that inhibition is exerted by TAR DNA sequences encompassing the bulge-loop region, these observed differences between transcriptionally active and inactive forms of the LTR-Ψ constructs strongly suggest that inhibition of HIV-1 expression is fully dependent on the inhibitor sequences as RNA transcript.

4. Discussion

In this study we report that an LTR-driven sense transcript encompassing the leader region of the HIV-1 RNA genome does significantly reduce virus expression in transient cotransfection assays in HeLa cells. Inhibition was prominent in transfection mixtures with the HIV-1 plasmid and the inhibitor construct in a 1:2 molar ratio. We demonstrated that most potent inhibition is obtained with constructs that synthesize both TAR RNA and the downstream regions of the HIV-1 leader transcript. TAR RNA by itself was previously shown to act as an efficient transcriptional decoy element (Graham and Maio, 1990; Sullenger et al., 1990, 1991; Lori et al., 1994). These studies, however, used either polymeric TAR constructs (Graham and Maio, 1990; Lori et al., 1994) or powerful RNA polymerase III transcription units (Sullenger et al., 1990, 1991) to obtain high intracellular concentrations of TAR RNA. We observed efficient inhibition of HIV expression at relatively low concentrations of the leader RNA sequences, suggesting that this anti-HIV mechanism may be more effective than TAR RNA decoys. Furthermore, we observed little additional inhibition when antisense sequences were incorporated into the transcription unit. This comparative analysis of anti-HIV sequences suggests that leader RNA motifs may be especially potent inhibitors of the HIV production phase. We should point out that HIV-inhibition by sense constructs was previously reported in microinjection experiments (Sczakiel et al., 1990) and stably transfected cell lines (Joshi et al., 1991; Tung and Daniel, 1993).

An unexpected observation in this study was that Ψ-containing transcripts interfere with HIV mRNA-translation; and, although antisense RNA is generally known to inhibit mRNA translation (Eguchi et al., 1991), we found the sense HIV-1 leader-RNA to be primarily responsible for this effect. Furthermore, we measured no correlation between inhibitory potential and stability of the different LTR-Ψ transcripts, suggesting that the inhibitory potential of some of the constructs is dependent on their specific sequence. These combined results suggest that a function of the HIV-1 leader RNA overlapping the Ψ-signal can inhibit HIV-1 mRNA translation in *trans*.

We would like to offer two hypothetical explanations for these observations. First, overexpression of HIV-1 leader transcripts may result in premature formation of RNA dimers, thereby interfering with the mRNA-function of HIV-1 transcripts. A few in vivo and in vitro observations are in support of this idea. Recent studies with HIV-1 and other retroviruses suggest that their genomes are already joined into some dimeric structure at the time of virus assembly (see discussion in Fu et al., 1994). Furthermore, spontaneous in vitro dimerization has been reported for retroviral leader RNAs in the absence of

protein (Bieth et al., 1990; Baudin et al., 1993; Berkhout et al., 1993; Sundquist and Heaphy, 1993; Skripkin et al., 1994) and such dimers were shown to be poor mRNAs in in vitro translation assays (Bieth et al., 1990). The inability to reduce HIV-2 expression with the HIV-1 leader RNA transcript (Fig. 3B) is also consistent with this hypothesis, because no HIV-1/2 RNA heterodimers can be formed in vitro (unpublished observations). By contrast, considerable interspecies activity was reported for some of the other leader-encoded functions. For instance, the HIV-1 Tat protein activates the HIV-1 and HIV-2 LTRs equally well (Emerman et al., 1987; Berkhout et al., 1990) and HIV-1 efficiently packages SIVmac genomic RNAs (Rizvi and Panganiban, 1993). Finally, the dimerization signal may also be responsible for the observed inhibition of HIV-1 virion infectivity through copackaging of the inhibitor RNA with HIV-1 genomic RNA. Indeed, constructs that were most effective in reducing virus production did also markedly reduce the infectivity of virus particles.

A second speculative interpretation of our inhibition studies is that the HIV-1 leader RNA contains critical regulatory sequences involved in mRNA translation. Overexpression of these motifs may result in titration of important RNA-binding proteins and *trans*-inhibition of HIV-1 mRNA translation. Translational enhancer motifs have been characterized in the leader RNA of other viruses, e.g. poliovirus, picornavirus and several plant viruses (Pelletier and Sonenberg, 1988; Carrington and Freed, 1990; Pilipenko et al., 1992). Furthermore, *trans*-inhibition of mRNA translation was recently demonstrated for the leader of the carlavirus potato virus S leader (Turner et al., 1994). However, the presence of such translational enhancer motifs in the HIV-1 leader remains to be unequivocally determined.

Our anti-HIV strategy employs several of the viral regulatory sequences necessary for viral replication, e.g. TAR RNA, the dimer-linkage structure (DLS) and the Ψ-domain. As discussed above, it has proven difficult to accurately map the position of some of these molecular signals on the HIV-1 genomic RNA. For instance, there is still some controversy on the exact location of the dimerization signal, which may play a key role in the initial steps of genome packaging (Fu et al., 1994). Further knowledge on these important replication signals should therefore aid the design of more efficient anti-HIV reagents. On the other hand, it seems possible that studies like this may be instrumental for a more detailed mapping of these critical viral functions. For instance, our preliminary deletion analysis indicates that leader sequences up to position 182 as in LTR-AS1 cannot efficiently inhibit HIV expression, which is consistent with the mapping of the HIV-1 dimerization signal at position 279 based on in vitro dimerization assays (Skripkin et al., 1994). It would also be interesting to further expand the inhibitory activity of these transcripts by the addition of other types of anti-HIV activities. In particular, one can think of RNA enzymes such as hammerheads, axehead or hairpin ribozymes, although it is currently unclear whether RNA enzyme-directed methods of gene inactivation are advantageous over "conventional" antisense inhibition (reviewed by Altman, 1993). An additional benefit of a combined approach is that it further minimizes the possibility of acquiring viral resistance, as is the case with current chemotherapeutic therapies.

All inhibitor constructs make use of the Tat-inducible LTR promoter/enhancer to drive expression of the inhibitor transcripts. Other groups have also designed Tat-induci-

ble vector systems (Venkatesh et al., 1990; Harrison et al., 1991; Buchschacher and Panganiban, 1992; Caruso and Klatzmann, 1992; Parolin et al., 1994). A major advantage of this homologous system is that high-level expression of the anti-HIV transcript is restricted to HIV-infected cells because the LTR promoter is largely dependent on the Tat *trans*-activator protein. If needed, the low levels of basal expression from the LTR promoter could be further reduced by removal of the NF-kB enhancer motifs. This manipulation was previously shown not to affect Tat-induced high level transcription (Berkhout and Jeang, 1992). A disadvantage of LTR-based vectors is that expression is triggered relatively late upon infection with the HIV-1 virus because virions do not contain Tat protein. Thus, induction of the anti-HIV gene will parallel expression of HIV-1 transcripts from the integrated provirus and this delayed expression of the inhibitor molecule may limit its efficacy in HIV-1 infection studies. We are currently investigating whether T-cell lines can be stably transfected with LTR-Ψ, or related constructs with constitutive promoter elements (e.g. CMV-Ψ), in order to further elucidate the mechanism of inhibition and to explore the potential in vivo applications of this system.

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