JOURNAL OF VIROLOGY, Oct. 1991, p. 5524–5530
0022-538X/91/105524-07$02.00/0

Vol. 65, No. 10

# Inhibition of Human Immunodeficiency Virus Type 1 Multiplication by Antisense and Sense RNA Expression

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Received 25 February 1991/Accepted 10 June 1991

**Human immunodeficiency virus type 1 (HIV-1) primarily infects CD4$^+$ lymphocytes and macrophages and causes AIDS in humans. Retroviral vectors allowing neomycin phosphotransferase (*npt*) gene expression were engineered to express 5′ sequences of HIV-1 RNA in the antisense or sense orientation and used to transform the human CD4$^+$ lymphocyte-derived MT$_4$ cell line. Cells expressing antisense or sense RNA to the HIV-1 *tat* mRNA leader sequence, as part of the 3′ untranslated region of the *npt* mRNA, remained sensitive to HIV-1 infection. In contrast, resistance to HIV-1 infection was observed in cells expressing antisense RNA to the HIV-1 primer-binding site or to the region 5′ to the primer-binding site as part of the 3′ region of the *npt* mRNA. Cells expressing the *tat* mRNA leader sequence in the sense orientation as a precise replacement of the 5′ untranslated region of *npt* mRNA were also resistant to HIV-1. These results indicate that sense and antisense approaches can be used to interfere with HIV-1 multiplication.**

AIDS is caused by a retrovirus, human immunodeficiency virus type 1 (HIV-1) (3). This virus primarily infects CD4$^+$ lymphocytes and macrophages. Although other cell types also become infected, HIV-1 seems to preferentially kill CD4$^+$ lymphocytes (17). These lymphocytes, as well as the other fully differentiated cells within the hematopoietic system, including mature myeloid and lymphoid cells, are maintained by the continuous proliferation and differentiation of relatively small numbers of stem cells. Thus, if a stem cell could be made resistant to HIV-1 by the transfer of anti-HIV-1 constructs with retroviral vectors, then its progeny, including cells within the immune system, might also be HIV-1 resistant. Toward this aim, retroviral vectors are being developed and tested in vitro and in vivo with hematopoietic stem cells, fibroblasts, hepatocytes, and endothelial cells (6, 10, 16, 29).

It may be possible to interfere with HIV-1 multiplication at the level of replication and/or gene expression by using antisense (35) or sense (26, 33) RNA strategies. Antisense RNA, complementary to a specific portion of an RNA molecule, could, upon hybridization with target RNA sequences, disrupt reverse transcription, processing, translation, and/or transport of this RNA. Antisense RNAs have been shown to alter the expression of selected genes in several cell systems, including bacteria (25), *Xenopus* oocytes (22), *Drosophila* embryos (30), and plant (8) and mammalian (12) cells. Avian retrovirus (34) and human T-cell lymphotropic virus type I (36) replication has also been shown to be inhibited in antisense RNA-expressing cell lines. The degree of inhibition obtained in these studies was variable and depended on many factors, including size, hybridization location, secondary structure, and level of expression of both the antisense RNA and the target mRNA whose expression was being modulated.

A sense RNA approach has been used to block replication of the genome of a plant RNA virus by using the origin of replication located at the 3′ end of the genome as a competitive inhibitor for viral replicase (26). RNA-RNA and RNA-protein interactions are crucial for HIV-1 replication, *trans* activation, transcription, transport, translation, and packaging, and the HIV-1 RNA sequences involved in these interactions are known (3). RNA containing these sequences in a sense orientation followed by non-HIV-1 sequences could compete with HIV-1 mRNAs for binding of RNA and/or protein and result in inhibition of HIV-1 multiplication.

HIV-1 replication takes place by using virally encoded reverse transcriptase and cellular tRNA$_3^{Lys}$, which binds to the HIV-1 primer-binding site (PBS) via an 18-nucleotide-long sequence complementarity (3). This region is highly conserved among all HIV-1 isolates. Upon cDNA synthesis to the 5′ region of the HIV-1 RNA and RNase H degradation of the template RNA, this cDNA hybridizes to the complementary sequences present at the 3′ end of the HIV-1 RNA. Interference at the level of initiation of HIV-1 replication could be achieved by expression of antisense RNA to the PBS that could compete with the primer tRNA$_3^{Lys}$ for binding to the HIV-1 PBS. Antisense RNA to the region 5′ to the HIV-1 PBS could further prevent elongation of HIV-1 replication. In fact, exogenously added short synthetic oligodeoxyribonucleotides have been shown to inhibit HIV-1 multiplication (21). Alternatively, interference with HIV-1 replication may be achieved by expressing sense RNA to the 5′ sequences of HIV-1 that could compete with HIV-1 RNA during the strand-switching process.

HIV-1 mRNAs used for synthesis of various HIV-1 proteins contain 287 identical nucleotides at their 5′ end. This region contains a *trans* activation-responsive (TAR) element that allows for a 1,000-fold increase in HIV-1 gene expression upon interaction with the HIV-1 *tat* protein. Interference with HIV-1 gene expression at the level of translation may be achieved by expressing antisense RNA to the 5′ leader sequence of HIV-1. Similarly, sense RNA to HIV-1 leader sequence could inhibit gene expression (i.e., *trans* activation) by acting as a competitor for factors which normally bind to the HIV-1 mRNAs.

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In this study, various retroviral vectors were engineered to express chimeric RNA containing antisense or sense RNA to the 5′ sequence of HIV-1 RNA in human CD4$^+$ lymphocyte-derived cell lines, and cells were tested for susceptibility to HIV-1 infection.

## MATERIALS AND METHODS

**Enzymes and chemicals.** All restriction enzymes were purchased from Bethesda Research Laboratories, Inc. T4 DNA ligase, T4 polynucleotide kinase, and DNA polymerase I Klenow fragment were obtained from Pharmacia. Calf intestinal alkaline phosphatase and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside were obtained from Boehringer Mannheim. Isopropyl-thio-β-D-galactoside was obtained from P-L Biochemicals, Inc. Bovine calf serum, geneticin (G418), antibiotics (containing penicillin, streptomycin, and amphotericin B [Fungizone]), L-glutamine, α-minimal essential medium and RPMI 1640 medium were purchased from GIBCO. P24 antigen detection kit was obtained from Abbott Laboratories.

**Plasmid constructions.** Unless stated otherwise, all recombinant DNA techniques were performed as described in reference 19. Site-specific in vitro mutagenesis was done essentially as described in reference 15 with minor modifications (13). DNA sequences were confirmed by previously described procedures (23, 31). Nucleotide sequences from the HIV-$1_{HXB2}$ strain (28) were used to design all antisense and sense RNA-expressing vectors.

Moloney murine leukemia virus-derived retroviral vectors pUCMoTN and pBRMoTN (18) were used in this study. In these vectors, the 3′ untranslated region of neomycin phosphotransferase (*npt*) mRNA contains about 1,400 nonessential nucleotides between the *Asu*II and *Cla*I sites. These nucleotides were therefore deleted in the vector pUCMoTNΔAC by *Asu*II-to-*Cla*I deletion and religation at these compatible sites.

pUCMoTN-PBS(−) and pUCMoTN-5′PBS(−) vectors were constructed as outlined below. The following oligonucleotides, PBS(−) and 5′PBS(−), containing the reverse complement of the sequences desired to be present on the RNA were synthesized:

PBS(−): pCTCTATAGGCTTCAGCTGGGGGttcgaa*TT TTT*TTTTGGCGCCCGAACAGGGACTTGAA*TTTTT* atcgatCCCCCAACTAGAGCCTGGACCA

5′PBS(−): pCTCTATAGGCTTCAGCTGGGGGatcgat*G GGGGT*TGTGTGGAAAATCTCTAGCAG*GGGGG*ttcga aCCCCCAACTAGAGCCTGGACCA

Nucleotides shown with a double underline can base pair with each other, nucleotides shown in italics with an underline cannot base pair with each other, nucleotides shown in boldface correspond to the HIV-1 PBS or to the region 5′ to the PBS, and nucleotides shown in lowercase letters correspond to restriction sites (*Asu*II or *Cla*I) inserted for future cloning purposes.

These oligonucleotides were used separately for in vitro mutagenesis of the vector M13 containing the *Sph*I-to-*Sst*I fragment of the vector pUCMoTNΔAC. The correct clones were identified by restriction enzyme analysis and confirmed by DNA sequencing. The *Sph*I-to-*Sst*I fragment, containing the DNA sequences mutagenized with the oligonucleotide PBS(−) or 5′PBS(−), was then used to replace the unmutagenized portion of the pUCMoTN vector. The resulting vectors were called pUCMoTN-PBS(−) and pUCMoTN-5′PBS(−).

pUCMoTN-L(5′+) vector was constructed as follows. In this vector, the 5′ leader sequence of the *npt* mRNA was precisely replaced with the HIV-1 *tat* mRNA leader sequence (12a). Essentially, five double-stranded oligonucleotides were synthesized for this purpose. Starting from the 5′ end, these oligonucleotides contained *Pst*I and *Cla*I restriction sites, part of the herpes simplex virus thymidine kinase (*tk*) promoter region from the *Mlu*I site to nucleotide −1, nucleotides +1 to the AUG of the HIV-1 *tat* mRNA 5′ leader sequence, part of the *npt* coding region from AUG to *Eag*I, and *Asu*II and *Eco*RI restriction sites. These oligonucleotides were ligated together and cloned in M13 at the *Pst*I and *Eco*RI sites. Correct clones were identified by restriction enzyme analysis, and the nucleotide sequence of the resulting vector, M13-L, was further confirmed by DNA sequencing. The *Mlu*I-to-*Eag*I fragment of M13-L was then used to replace the unmutagenized portions in a plasmid containing the *Eco*RI-*Sph*I fragment of pUCMoTN. The *Eco*RI-*Sph*I fragment of the resulting vector, containing the HIV-1 *tat* mRNA leader sequence, was then cloned in the pUCMoTN vector at the *Eco*RI and *Sph*I sites. The resulting vector was called pUCMoTN-L(5′+).

pBRMoTN-L(3′+) vector was constructed by cloning the *Asu*II-to-*Cla*I fragment of the M13-L vector by blunt-end ligation at the *Bam*HI site in the vector pBRMoTN. The correct clones containing the HIV-1 *tat* mRNA leader sequence in the sense orientation were selected by restriction enzyme analysis.

The vector pUCMoTN-L(−), containing HIV-1 *tat* mRNA 5′ leader sequence in the antisense orientation, was constructed by cloning the *Asu*II-to-*Cla*I fragment of M13-L into the pUCMoTN vector at the *Asu*II-to-*Cla*I sites. The correct clones containing these restriction sites were selected by restriction enzyme analysis.

**Cell lines.** Psi-2 (20) and PA317 (24) packaging cell lines were cultured in α-minimal essential medium supplemented with 2 mM L-glutamine, antibiotics (penicillin, 100 units/ml; streptomycin, 100 μg/ml; amphotericin B, 0.25 μg/ml), and 10% fetal calf serum at 37°C in a humidified atmosphere with 6% $CO_2$. The human CD4$^+$ lymphocyte-derived $MT_4$ (suspension) cell line was cultured in RPMI 1640 medium supplemented with L-glutamine (2 mM), antibiotics, and fetal calf serum (10%) at 37°C in an atmosphere of 6% $CO_2$.

**Transfection and infection of mammalian cell lines.** Six hours after $2 \times 10^5$ Psi-2 cells were seeded on 60-mm tissue culture dishes in 4 ml of medium, the cultures were transfected with 1 μg of plasmid DNA by previously described procedures (11, 37). Sixteen hours later, the cells were washed and cultured. Once confluent, the cells were trypsinized and transferred to 100-mm tissue culture dishes and cultured in medium containing antibiotics, 10% calf serum, and G418 (200 μg/ml). The selective medium was changed every 4 to 5 days, and the number of colonies was counted after 14 days.

Vector particles released from transformed Psi-2 cells at 50 to 100% confluency were obtained by filtering the culture medium through a 0.22-μm-pore-size filter. These particles were used to infect PA317 cells as described previously (14). Essentially, $2 \times 10^5$ cells in 4 ml of medium were seeded for 6 h in 60-mm tissue culture dishes, after which this medium was replaced by 1 ml of medium containing 8 μg of Polybrene per ml and 100 μl of vector particles. After 2 h of incubation at 37°C, 3 ml of medium was added and the incubation was continued for 16 more hours. Cells were then trypsinized and transferred to 100-mm tissue culture dishes in the presence of selective medium containing 200 μg of

G418 per ml. The selective medium was changed every 4 to 5 days, and the number of colonies was counted after 14 days.

The transformed PA317 cell lines releasing retroviral vector particles were used to infect $MT_4$ cells by cocultivation (1). $MT_4$ ($10^6$) cells in 10 ml of RPMI 1640 medium containing L-glutamine, antibiotics, and 10% fetal calf serum were placed on a 100-mm tissue culture dish containing transformed PA317 cells (50% confluent). After 24 h of cocultivation at 37°C, the infected $MT_4$ cells in suspension were transferred to a 100-mm petri dish and incubated in the presence of the above medium supplemented with 400 μg of G418 per ml. This selective medium was changed every 4 to 5 days for up to 3 to 4 weeks (time required for untransformed cells to die). The pool of stably transformed $MT_4$ cells (without further cloning) was then used for RNA analysis and subjected to HIV-1 challenge.

**Northern (RNA) blot and slot-blot analysis.** RNA was isolated as described in reference 4. Essentially, cells were lysed in a denaturing solution containing guanidinium isothiocyanate, and the total RNA was pelleted by centrifugation through a CsCl cushion. For Northern blot analysis, total cellular RNA (9 μg) was loaded onto a 1.5% formaldehyde gel and subjected to electrophoretic separation. Fractionated RNA was capillary blotted to a GeneScreen Plus membrane (Dupont), which was then probed with the $^{32}P$-labeled *Sst*I-*Sst*I fragment (188 nucleotides long) of pUC MoTN-L(5′+) vector.

For slot-blot analysis, RNA was isolated as described in reference 5. Essentially, total RNA was recovered from cell lysates by a single extraction with a mixture of phenol and chloroform. Total cellular RNA (9 μg) was immobilized onto a GeneScreen Plus membrane by gentle suction with a blotting manifold (Bethesda Research Laboratories). The membrane was then probed with the $^{32}P$-labeled 188-nucleotide-long *Sst*I-*Sst*I fragment.

**HIV-1 infection and p24 antigen detection.** The titer of the HIV-$1_{IIIB}$ strain replicated in $MT_4$ cells (32) was determined by using the same cell line. Without further propagation in our laboratory, the titer of the HIV-$1_{NL4-3}$ strain, replicated in A3-01 cells transfected with the NL4-3 provirus DNA, was also determined by using $MT_4$ cells. For both HIV-1 strains, 10 μl of virus preparation was used to infect $10^6$ $MT_4$ transformants (G418 resistant) in 1 ml of RPMI 1640 medium containing L-glutamine, antibiotics, 10% fetal calf serum, and 400 μg of G418 per ml. The cells were incubated at 37°C in an atmosphere of 6% $CO_2$. Two hours later, the cells were washed to remove unadsorbed virus particles and the incubation was continued. Culture supernatant (100 μl) was collected at various intervals and tested for p24 antigen (24-kDa HIV-1 *gag* gene product) production by enzyme-linked immunosorbent assay (ELISA). An optical density value of 1 corresponds to 200 pgs of HIV-1 p24 antigen. The maximum optical density value reported is 2 since values greater than this could not be accurately measured owing to the sensitivity of the ELISA reader.

## RESULTS AND DISCUSSION

Moloney murine leukemia virus-derived retroviral vectors pBRMoTN, pUCMoTN (18), and pUCMoTNΔAC, containing the herpes simplex virus *tk* promoter driving *npt* gene expression (conferring G418 resistance), were used in this study. These vectors were engineered to express antisense or sense RNA molecules to HIV-1 as part of the 3′ or 5′ untranslated region of the *npt* mRNA. These vectors were used to generate transformed $CD4^+$ lymphocyte-derived $MT_4$ cell lines, which were then tested for antisense or sense RNA expression and for their susceptibility to HIV-1 infection.

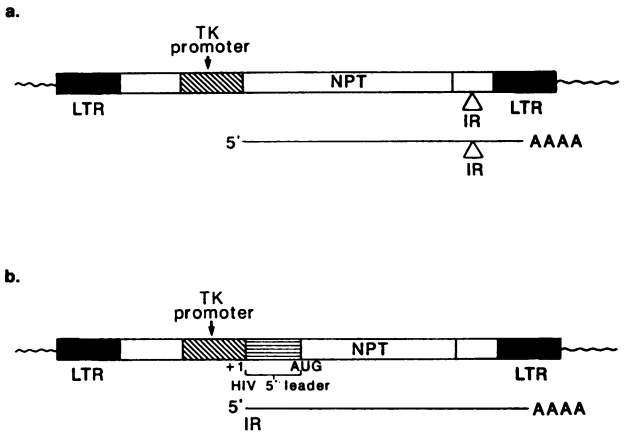


FIG. 1. Retroviral vectors expressing antisense or sense RNA molecules to HIV-1. (a) Interfering RNA (IR) corresponds to antisense RNA to the HIV-1 PBS [MoTN-PBS(−)], to antisense RNA to the region 5′ to the PBS [MoTN-5′PBS(−)], or to the HIV-1 *tat* mRNA 5′ leader sequence cloned in the antisense [MoTN-L(−)] or sense [MoTN-L(3′+)] orientation. These sequences were cloned as part of the 3′ untranslated region of the *npt* mRNA. (b) Interfering RNA corresponds to the 5′ leader sequence of HIV-1 *tat* mRNA that was used to precisely replace the 5′ leader sequence of *npt* mRNA [MoTN-L(5′+)]. TK, thymidine kinase; LTR, long terminal repeat.

**Antisense RNA-expressing vectors.** Antisense RNAs to HIV-1 were expressed as part of the 3′ untranslated region of *npt* mRNA between the *npt* stop codon and the polypurine tract (PPT). The following retroviral vectors were constructed (Fig. 1a): pUCMoTN-PBS(−), expressing antisense RNA to the HIV-1 PBS (18 nucleotides long); pUCMoTN-5′PBS(−), expressing antisense RNA to the 18-nucleotide-long region 5′ to the HIV-1 PBS; and pUCMoTN-L(−), expressing antisense RNA to the HIV-1 *tat* mRNA 5′ leader sequence. This leader sequence contains 343 nucleotides, of which the first 287 nucleotides are common to all HIV-1 mRNAs. Antisense RNAs to the HIV-1 PBS (18 nucleotides long) and to the 18-nucleotide-long region 5′ to the PBS are of relatively short length. Therefore, to render them available for hybridization to the HIV-1 target mRNA, it seemed important to express them as part of a loop structure. This was achieved by selecting within the 3′ untranslated region of *npt* mRNA a stable stem-and-loop structure as predicted by Zucker and Streigler's computer program (38) and replacing the sequences within this loop with the HIV-1 antisense RNA sequences. To further ensure that a stem structure will form and that the antisense RNA sequences to HIV-1 will be located at the center of the loop, base pairing in the stem region was increased by the insertion of 9 bp and the base of the loop was changed by the insertion at either end of 5 nucleotides that cannot base pair with each other.

**Sense RNA-expressing vectors.** The following retroviral vectors were constructed: pUCMoTN-L(5′+), expressing HIV-1 *tat* mRNA leader sequence (+1 to AUG; 343 nucleotides) precisely replacing the *npt* mRNA 5′ leader sequence (Fig. 1b); and pBRMoTN-L(3′+), expressing HIV-1 *tat* mRNA leader sequence as part of the 3′ untranslated region of *npt* mRNA (Fig. 1a).

**Transformation of $MT_4$ cell lines with retroviral vector**

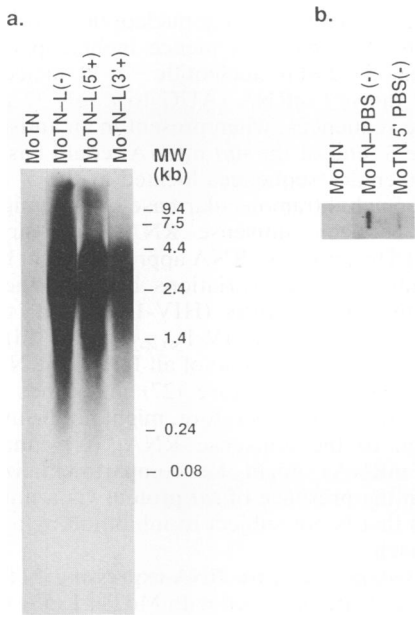


FIG. 2. Antisense and sense RNA levels in $MT_4$ transformants. (a) Northern blot analysis of antisense and sense RNA to the HIV-1 *tat* mRNA 5′ leader sequence expressed in $MT_4$ cells containing MoTN-L(−), MoTN-L(5′+), and MoTN-L(3′+). $MT_4$ cells containing MoTN served as a control. MW, molecular weight. (b) Slot-blot analysis of antisense RNA to the HIV-1 PBS or to the region 5′ to the PBS expressed in $MT_4$ cells containing MoTN-PBS(−) or MoTN-5′PBS(−), respectively.

**particles.** The retroviral vectors engineered to express antisense [pUCMoTN-PBS(−), pUCMoTN-5′PBS(−), and pUCMoTN-L(−)] and sense [pUCMoTN-L(5′+) and pBRMoTN-L(3′+)] RNAs to HIV-1, as well as the vector (pUCMoTN) lacking the test sequences, were used to transfect the ecotropic Psi-2 packaging cell line. The retroviral vector particles [MoTN-PBS(−), MoTN-5′PBS(−), MoTN-L(−), MoTN-L(5′+), MoTN-L(3′+), and MoTN] released from these transformants were used to infect the amphotropic PA317 packaging cell line. These transformants, releasing amphotropic retroviral vector particles, were then used in cocultivation experiments to infect the $MT_4$ cell line, and stable $MT_4$ transformants were selected by growth for up to 1 month in medium containing G418.

**Antisense and sense RNA levels in $MT_4$ transformants.** Northern blot analysis of RNA isolated from the $MT_4$ transformants was performed to assess the level of expression of various antisense and sense RNA molecules to HIV-1 (Fig. 2a). The probe used in these experiments was the $^{32}P$-labeled 188-nucleotide-long *Sst*I-*Sst*I fragment of pUC MoTN-L(5′+) containing part of the HIV-1 *tat* mRNA 5′ leader sequence, including the HIV-1 PBS and the 18-nucleotide-long region 5′ to the PBS. This probe should hybridize to the vector and *npt* mRNAs containing the HIV-1 antisense or sense RNA sequences and not to RNAs lacking these sequences. As shown in Fig. 2a, the presence of RNAs containing antisense or sense RNA molecules to HIV-1 was clearly detectable in the $MT_4$ transformants containing MoTN-L(−), MoTN-L(5′+), and MoTN-L(3′+). It is noteworthy that similar levels of Moloney murine leukemia virus long terminal repeat and *tk* promoter-driven RNAs containing antisense and sense RNA sequences to HIV-1 were expressed from all three vectors (Fig. 2a; data not shown). Antisense RNA to the HIV-1 PBS or to the region 5′ to the PBS could not be detected in this experiment (data not shown), most likely because base pairing between these antisense RNAs and the probe was only over 18 nucleotides; for other sense and antisense RNAs, whose expression could be detected, base pairing occurred over a stretch of 188 nucleotides. Slot-blot analysis, at a lower stringency, was therefore performed with the same probe to detect the presence of RNAs containing the 18-nucleotide-long antisense RNA sequences to the HIV-1 PBS and to the region 5′ to the PBS. As shown in Fig. 2b, the presence of HIV-1 antisense RNAs expressed in the $MT_4$ transformants containing MoTN-PBS(−) and MoTN-5′PBS(−) was clearly detectable under these conditions.

It is noteworthy that cells transformed with the MoTN-L(5′+) vector allowing *npt* gene expression under control of the *tk*-TAR fusion promoter were G418 resistant and allowed high-level expression of *npt* mRNA (Fig. 2a). These results indicate that this fusion promoter can allow constitutive gene expression in the absence of *tat* protein.

**Challenge of antisense or sense RNA-expressing $MT_4$ cells with HIV-1.** Retroviral vectors engineered in this study were designed to express HIV-1 antisense or sense RNA sequences corresponding to HIV-$1_{HXB2}$, which is one of the clones obtained from HIV-$1_{IIIB}$, a pooled virus. Nucleotide sequences of other viruses present in HIV-$1_{IIIB}$ are unknown. These isolates, depending on their nucleotide sequence, might escape inhibition by the antisense or sense RNAs expressed from retroviral vectors engineered in this study and multiply. This, in turn, could obscure the protective effect of antisense or sense RNA molecules to the HIV-$1_{HXB2}$ isolate. Therefore, the ideal solution would be to use homogeneous virus released from a cell line containing a cloned provirus whose sequence is identical or at least very similar to that of HIV-$1_{HXB2}$. Subsequently, one such cloned virus, HIV-$1_{NL4-3}$, was used in our studies. Its sequence within nucleotides +1 to +287 is quite similar to that of HIV-$1_{HXB2}$. This sequence identity is 100% for the PBS and for the 18-nucleotide-long region 5′ to the PBS. Both HIV-$1_{IIIB}$ and HIV-$1_{NL4-3}$ were used in HIV-1 challenge experiments described below.

**HIV-1 resistance of antisense RNA-expressing $MT_4$ transformants.** $MT_4$ cells transformed with MoTN-PBS(−), MoTN-5′PBS(−), and MoTN-L(−) vectors expressing antisense RNAs to HIV-1 were challenged with HIV-$1_{IIIB}$ (Fig. 3a and b) and HIV-$1_{NL4-3}$ (Fig. 3c). Infection of $MT_4$ cells transformed with MoTN vector lacking the test sequences served as a control. After infection, HIV-1 production was monitored at various time intervals by measuring the level of p24 antigen (HIV-1 *gag* gene product) in cell culture supernatant.

The $MT_4$ transformants expressing antisense RNA to the PBS or to the region 5′ to the PBS were partially resistant to both strains of HIV-1 tested, HIV-$1_{IIIB}$ (Fig. 3a) and HIV-$1_{NL4-3}$ (Fig. 3c), as virus production was decreased significantly. The expression of these antisense RNAs at the center of a stable loop structure might have contributed to the observed partial resistance. Using retroviral vectors allowing strong constitutive and/or *tat*-inducible expression, it is tempting to speculate that the vector MoTN-PBS(−) will be able to confer resistance to all HIV-1 isolates as this region is highly conserved.

The cells expressing antisense RNA to the HIV-1 *tat* mRNA leader sequence (343 nucleotides long) failed to prevent multiplication of both strains of HIV-1 tested (Fig. 3b and c). This negative result could be explained in several

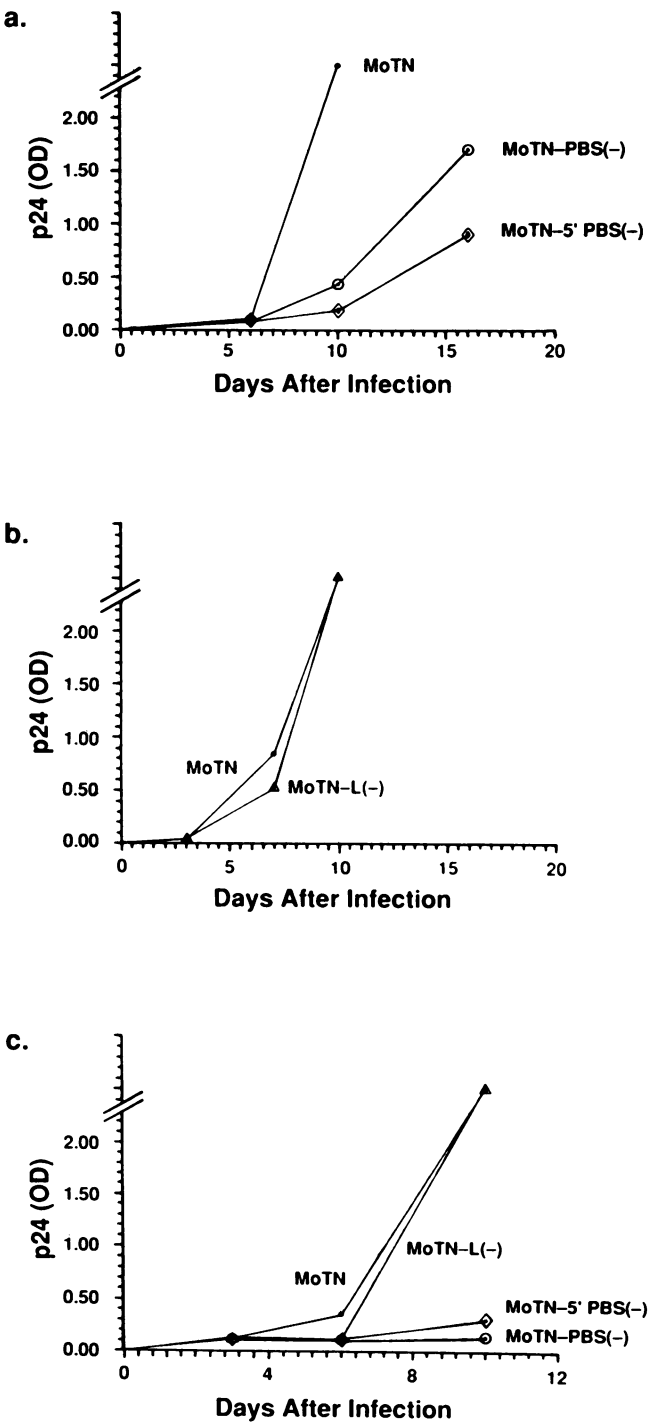


FIG. 3. Susceptibility of $MT_4$ cells expressing antisense RNAs to infection by both HIV-$1_{IIIB}$ and HIV-$1_{NL4-3}$. $MT_4$ cells transformed with MoTN (•), MoTN-PBS(−) (⊙), MoTN-5'PBS(−) (◇), or MoTN-L(−) (△) were infected with HIV-$1_{IIIB}$ (a and b) or with HIV-$1_{NL4-3}$ (c), and the level of p24 antigen released in the culture supernatant was determined by ELISA at various times after infection. Values greater than 2 are shown at an arbitrarily chosen point. OD, optical density.

ways. (i) The synthetic oligonucleotides containing the HIV-1 *tat* mRNA leader sequence include part of the *tk* promoter region (*Mlu*I to nucleotide −1; 13 nucleotides long) and part of the *npt* mRNA (AUG to *Eag*I; 37 nucleotides long). These sequences, when present in an antisense orientation at the 3′ end of the *npt* mRNA, could base pair with the complementary sequences located at the 5′ end of this *npt* mRNA. Such intramolecular base pairing might prevent interaction between antisense RNAs and target HIV-1 mRNAs. (ii) The antisense RNA approach might be impeded by nucleotide sequence variations between the antisense RNA and the pooled virus (HIV-$1_{IIIB}$) and, to a lesser extent, the cloned virus (HIV-$1_{NL4-3}$) used. (iii) The TAR sequence present at the 5′ end of all HIV-1 mRNAs forms a stable stem-and-loop structure (27) and binds to the *tat* protein (7). This region, therefore, might not be available for hybridization to the antisense RNA. (iv) Finally, TAR-containing mRNAs might be compartmentalized and/or translated in the presence of *tat* protein via a unique mechanism (2, 9) that is not subject to inhibition by an antisense RNA approach.

**HIV-1 resistance of sense RNA-expressing $MT_4$ transformants.** $MT_4$ cells transformed with MoTN-L(5′+) or MoTN-L(3′+) expressing sense RNA to the HIV-1 *tat* mRNA 5′ leader sequence as part of the 5′ or 3′ region of *npt* mRNA, respectively, were subjected to challenge by HIV-$1_{IIIB}$ (Fig. 4a) and HIV-$1_{NL4-3}$ (Fig. 4b). Cells containing the MoTN vector lacking test sequences served as controls. $MT_4$ cells transformed with MoTN-L(5′+), expressing the HIV-1 *tat* mRNA leader sequence as a precise replacement of the *npt* mRNA leader sequence, were quite resistant to HIV-$1_{IIIB}$ and HIV-$1_{NL4-3}$ infection. However, expression of sense RNA to HIV-1 within the 3′ region of *npt* mRNA had no protective effect as HIV-1 production in these cells transformed with MoTN-L(3′+) was similar to that obtained from cells containing MoTN (Fig. 4).

Differences in resistance to HIV-1 infection conferred by retroviral vectors in which the HIV-1 sequences are located within the 5′ leader sequence or the 3′ untranslated region of the *npt* gene might be due to differences in the ability of these transcription units to be up-regulated by the HIV-1 *tat* protein. For *trans* activation by the *tat* protein at the level of transcription, a particular spacing is required between the TAR region and the transcription factor-binding sites present within the promoter region (32). This spacing is conserved in the vector MoTN-L(5′+) but is too large in the vector MoTN-L(3′+). Thus, *tat*-inducible *trans* activation is only expected to occur from the *tk*-TAR promoter present in the vector MoTN-L(5′+). Levels of sense RNA to HIV-1 expressed in the absence of *tat* protein in cells containing MoTN-L(5′+) or MoTN-L(3′+) were very similar (Fig. 2a). However, because of *trans* activation in the presence of *tat* protein, the level of HIV-1 sense RNA expressed from the vector MoTN-L(5′+) could be higher than that expressed by the vector MoTN-L(3′+). The stability of these two sense RNA molecules could be different as well.

Alternatively, binding of the HIV-1 *tat* protein to the TAR sequence (7) is expected to decrease the amount of *tat* protein available for *trans* activation of HIV-1 gene expression (33). If one assumes that the binding of *tat* protein to the TAR sequence located at an internal position in RNAs expressed from the vector MoTN-L(3′+) is not possible, inhibition of *trans* activation of HIV-1 gene expression would only occur with RNAs containing the TAR sequence at the 5′ end expressed from the vector MoTN-L(5′+). Moreover, only RNAs containing the *tat* mRNA leader

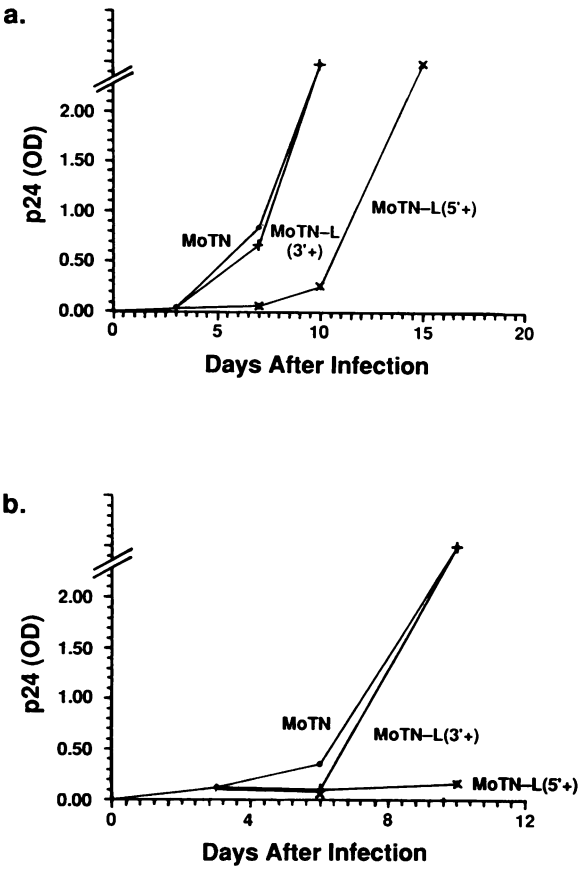


FIG. 4. Susceptibility of $MT_4$ cells expressing sense RNAs to infection by HIV-$1_{IIIB}$ and HIV-$1_{NL4-3}$. $MT_4$ cells transformed with MoTN (•), MoTN-L(5'+) (×), or MoTN-L(3'+) (+) were infected with HIV-$1_{IIIB}$ (a) or HIV-$1_{NL4-3}$ (b), and the level of p24 antigen released in the culture supernatant was determined by ELISA at various times after infection. For details, see the legend to Fig. 3.

sequence as a precise replacement of the *npt* mRNA leader sequence, expressed from MoTN-L(5'+), are expected to compete with translation of HIV-1 mRNAs.

Finally, during replication, possible strand switching onto the HIV-1 5' sequences contained in the sense orientation at either the 5' or the 3' end of the *npt* mRNA should have resulted in abortive replication. However, since no inhibition of HIV-1 multiplication was observed in cells containing MoTN-L(3'+), the sense RNA present at the 5' end of *npt* mRNA might interfere with HIV-1 multiplication at another level.

Sense RNA to HIV-1 *tat* mRNA leader in the retroviral vector MoTN-L(5'+) probably acts by binding to *tat* protein or to some other cellular factors. If so, this vector will inhibit multiplication of all HIV-1 isolates in which binding to these viral or cellular factors is maintained.

In conclusion, inhibition of HIV-1 multiplication was observed in $CD4^+$ lymphocyte-derived $MT_4$ cell lines transformed with retroviral vectors expressing antisense RNA to the PBS or to the region 5' to the PBS as part of a loop structure within the 3' untranslated region of *npt* mRNA. Inhibition was also evident with a vector expressing HIV-1 *tat* mRNA 5' leader sequence in the sense orientation as a precise replacement of the *npt* mRNA 5' leader sequence. The next step would be to determine at which step these sense and antisense RNAs interfere with the HIV-1 life cycle (i.e., replication, *trans* activation, or translation). Moreover, antisense RNAs expressed under the control of the Moloney murine leukemia virus long terminal repeat and the herpes simplex virus *tk* promoter might not be able to keep pace with HIV-1 mRNAs produced as a result of *trans* activation by the *tat* protein. These results might therefore be improved by using retroviral vectors allowing strong constitutive and/or *tat*-inducible gene expression.

## ACKNOWLEDGMENTS

We thank A. Yip, H. Smith, and J. Radul for oligonucleotide synthesis and DNA sequencing and R. L. Joshi for critical reading of the manuscript. The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: H9 cells infected with the HIV-$1_{IIIB}$ strain and HIV-$1_{NL4-3}$ clone produced from transformed A3.01 cells from R. Gallo, and the $CD4^+$ lymphocyte-derived $MT_4$ cell line from D. Richman.

This work was supported by a grant from MRC.

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