Expression of Chimeric tRNA-Driven Antisense Transcripts Renders NIH 3T3 Cells Highly Resistant to Moloney Murine Leukemia Virus Replication

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NIH 3T3 cells infected with Moloney murine leukemia virus (MoMLV) express high levels of virus-specific RNA. To inhibit replication of the virus, we stably introduced chimeric tRNA genes encoding antisense templates into NIH 3T3 cells via a retroviral vector. Efficient expression of hybrid tRNA-MoMLV antisense transcripts and inhibition of MoMLV replication were dependent on the use of a particular type of retroviral vector, the double-copy vector, in which the chimeric tRNA gene was inserted in the 3' long terminal repeat. MoMLV replication was inhibited up to 97% in cells expressing antisense RNA corresponding to the *gag* gene and less than twofold in cells expressing antisense RNA corresponding to the *pol* gene. RNA and protein analyses suggest that inhibition was exerted at the level of translation. These results suggest that RNA polymerase III-based antisense inhibition systems can be used to inhibit highly expressed viral genes and render cells resistant to viral replication via intracellular immunization strategies.

Antisense RNA-mediated gene inhibition is a widely used experimental strategy for the inhibition of the expression of specific genes in diploid cells and constitutes a powerful tool for the manipulation of gene expression in eucaryotic cells. Regulation of gene expression via antisense RNA was first recognized as a naturally occurring mechanism in procaryotes (reviewed in reference 17). The potential of using complementary sequences to inhibit specific gene expression grew from the observations of Paterson et al. (30), who used complementary nucleic acids to inhibit the translation of specific mRNA species in vitro. However, the pioneering work of Zamecnik and Stephenson (49) first demonstrated the experimental inhibition of the expression of specific genes in live cells with synthetic oligonucleotides. Inhibition of specific genes with stably integrated antisense DNA templates (also called antisense genes or referred to as stable antisense RNA-mediated gene inhibition) was first demonstrated in bacteria by Pestka et al. (31) and Coleman et al. (8) and in eucaryotic cells by Izant and Weintraub (20). In these cases, the cells are genetically altered to continuously express antisense RNA, which leads to the inhibition of the target gene in the transduced cells and their progeny (reviewed in reference 17, 41, and 45). Genetic alteration of cells mediated via gene transfer and leading to the inhibition of viral replication was termed intracellular immunization (3), and the use of antisense RNA represents one approach to achieving such inhibition. To et al. (39) have shown that the replication of a recombinant avian retrovirus is inhibited in quail cells expressing corresponding antisense RNA, and von Ruden and Gilboa (43) have shown that primary human T-lymphoid cells can be made partially resistant to the replication of human T-cell lymphotropic virus type I via retrovirus-mediated antisense RNA inhibition.

The efficacy of antisense RNA-mediated gene inhibition is partially dependent upon the presence of a considerable

excess of antisense RNA over the target RNA in the cell. Several studies have shown that an apparent 30- to 100-fold excess of antisense RNA is required to obtain a 10- to 20-fold reduction in gene expression (20, 21, 24), while other studies have shown that a moderate excess (3- to 10-fold) may also lead to an observable inhibition (5, 29, 40). For a comprehensive review and discussion of this issue, see reference 41. Central to the development of effective and stable antisense inhibition systems is the design of antisense templates that are capable of expressing high levels of antisense RNA in eucaryotic cells. The promoter used to drive the expression of antisense RNA is of primary importance in the design of efficient antisense templates; however, the role of sequence elements which control the processing, cellular localization, or stability of the antisense RNA transcripts must also be considered. To the best of our knowledge, RNA polymerase II-based transcription units have been used exclusively in studies in which the stable expression of antisense RNA was sought (reviewed in reference 41).

The use of RNA polymerase III (pol III) transcription units represents an alternative approach for expressing high levels of RNA transcripts in cells. In mammalian cells, pol III-transcribed tRNA molecules are about 100-fold more abundant than is polyadenylated RNA on a molar basis (12), and since tRNA^{Met} species which are expressed from up to 12 different genes represent 5% of the tRNA population (35), it can be estimated that the number of tRNA transcripts expressed from one tRNA^{Met} gene is equal in number to about 25% of the total polyadenylated RNA present in the cell. Hence, a tRNA gene is an extremely efficient transcription unit.

Several reports have described the use of pol III-based promoters to express RNA in eucaryotic cells. Lewis and Manley (27) and Sisodia et al. (36) fused the adenovirus VA-1 promoter to various DNA sequences (the herpesvirus thymidine kinase gene, beta-globin gene, and beta-tubulin gene) and used transient transfection protocols to introduce the chimeric DNA constructs into cultured cells, resulting in

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the transient synthesis of corresponding RNA in the transduced cells. de la Pena and Zasloff (13) obtained expression of a tRNA-herpesvirus thymidine kinase fusion DNA construct upon microinjection of this construct into frog oocytes. Jennings and Molloy (22) constructed an antisense template by fusing the VA-1 gene promoter to a DNA fragment derived from the Simian virus 40 large T antigen. Cotransfection of COS-1 cells with the antisense gene and a plasmid containing a simian virus 40 origin of replication resulted in transient expression of antisense RNA and partial inhibition of simian virus 40 replication. More recently, Cotten and Birnstiel (9) microinjected a ribozyme-tRNA DNA construct into *Xenopus* oocytes, resulting in the expression of a corresponding RNA transcript and the generation of an apparently functional ribozyme. Common to the above-cited studies is that the pol III-based DNA templates were introduced into cells by a gene transfer procedure which led to only transient synthesis of pol III-driven RNA transcripts in the cells. Furthermore, since the number of active DNA templates present in the transiently transduced cells could not be determined in most cases, the efficiency of pol III-based transcription was not determined.

The feasibility of using a tRNA-driven antisense RNA expression system to stably express high levels of antisense RNA in cells was tested by attempting to inhibit the replication of Moloney murine leukemia virus (MoMLV) in NIH 3T3 cells. The MoMLV genome represents a highly efficient transcription unit. The viral long terminal repeat (LTR)-initiated transcripts in the infected cells represented 1 to 5% of the polyadenylated RNA in the cells (42). Hence, effective inhibition of MoMLV replication would necessitate the use of a very efficient transcription system able to generate high levels of antisense RNA. Chimeric tRNA-MoMLV antisense genes were constructed and introduced into NIH 3T3 cells via retroviral vectors. Cell lines which constitutively expressed high levels of MoMLV-specific antisense RNA were established. Replication of MoMLV in cells expressing tRNA-driven antisense RNA was inhibited up to 97%. Inhibition of viral replication was exerted at the level of viral protein synthesis. Successful use of a tRNA-driven antisense RNA transcription system was dependent on the use of a particular type of retroviral vector, the double-copy (DC) vector, in which the chimeric tRNA gene was inserted in the viral LTR.

MATERIALS AND METHODS

Generation of MoMLV antisense retroviral vectors and derivation of cell lines. The MoMLV-based N2 retroviral vector (2), which contains the bacterial neomycin resistance gene (*neo*), was modified by the insertion of a 52-bp polylinker (containing the unique restriction sites 5'-*ApaI*-*BglII*-*SnaBI*-*SacII*-*MluI*-3') into the *NheI* restriction site present in the U3 region of the 3' LTR. The polylinker-modified N2 vector was designated N2A (19). A 500-bp DNA fragment containing the $\Delta 3$ -5 mutant human tRNA^{Met} gene (1) was excised from plasmid pH2D with the restriction enzymes *Bam*HI and *Stu*I and cloned into the *Sna*BI site present in the 3' LTR of the N2A vector to generate the construct LTR:tRNA. The DC:antisense vectors, DC:antigag and DC:antipol, were generated by cloning two antisense oligonucleotides (see Fig. 1B) between the *Sac*II and *Mlu*I sites immediately downstream of the $\Delta 3$ -5 tRNA gene. A 550-bp DNA fragment containing the chimeric tRNA-antigag sequences was excised from the DC:antigag vector and cloned

into the *Xho*I site of the N2A vector in either orientation to generate vectors SC:antigag and SC:antigagR. The LTR:antigag vector was generated by excising the $\Delta 3$ -5 tRNA gene from the DC:antigag vector by *Bgl*II and *Sac*II digestion and recircularization of the plasmid. A schematic diagram of the various vector constructs is shown in Fig. 1C. Vector DNA was converted to corresponding virus by established procedures. Plasmid DNA (1 μ g) was electroporated into the amphotropic packaging cell line AM12 (28) with a Bio-Rad gene pulser, and productively transduced cells were selected with G418 (0.7 mg/ml). G418-resistant colonies were pooled, and virus-containing supernatant was used to infect NIH 3T3 cells in the presence of 8 μ g of Polybrene per ml. G418-resistant colonies were isolated by ring cloning, expanded to cell lines, and used in subsequent experiments. The vector-transduced cell lines were free of replication-competent virus, even after extensive culturing in vitro. The presence or absence of replication-competent virus was determined by a highly sensitive method described previously (28).

DNA and RNA analyses. Chromosomal DNA was isolated by the guanidinium isothiocyanate method and centrifugation through a CsCl cushion (6). DNA (10 μ g) was digested with restriction endonucleases, fractionated on a 1% agarose gel, transferred to a nylon membrane (Biotrans; ICN) with a Bio-Rad electroblotter, and UV cross-linked to the membrane with a Stratagene UV Stratalinker. Membranes were prehybridized at 65°C in 25 ml of 1% bovine serum albumin-1 mM EDTA-0.5 M Na₂PO₄ (pH 7.2)-7% sodium dodecyl sulfate (SDS). A ³²P-labeled *neo* gene-specific probe (10⁷ cpm) generated with an oligolabeling kit (Pharmacia) was added directly to the prehybridization solution, and hybridization was carried out overnight at 65°C. Hybridized membranes were washed twice in 2 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.1% SDS for 20 min at room temperature and once in 0.2 $\times$ SSC-0.1% SDS for 20 min at 65°C. Washed membranes were exposed to X-ray film (XAR5) at -70°C for 1 to 3 days in the presence of an intensifying screen. Total cellular RNA was isolated by the guanidinium isothiocyanate-hot phenol method (14). Cytoplasmic RNA was isolated by first lysing cells with 50 mM Tris hydrochloride (pH 8.0)-100 mM NaCl-5 mM MgCl₂-0.5% Nonidet P-40 and then removing nuclei by centrifugation. Guanidinium isothiocyanate was added to the cytoplasmic fraction, and the mixture was processed as described above. To analyze low-molecular-weight RNA, we fractionated 20 μ g of total RNA on an 8% polyacrylamide-urea gel. Transfer and hybridization were performed as described above, with the following modifications. The prehybridization, hybridization, and last washes were all carried out at 55°C. The hybridization probe used was either a ³²P-labeled tRNA^{Met} gene-specific probe (a 550-bp DNA fragment excised with restriction enzyme *Nhe*I from plasmid LTR:tRNA) generated with the oligolabeling kit or a ³²P-end-labeled antigag-specific oligonucleotide generated with T4 polynucleotide kinase (Boehringer Mannheim). Note that the sequences of mouse tRNA^{Met} and human tRNA^{Met} are identical (18).

To analyze MoMLV-specific RNA, we fractionated 1 μ g of total or cytoplasmic RNA on 1% formaldehyde agarose gels and visualized it by ethidium bromide staining to ensure that equal amounts were loaded in each lane. Transfer to nylon membranes and hybridization were carried out as described for DNA blot analysis with an MoMLV U3-specific probe. Hybridized membranes were washed twice in 2 $\times$ SSC-0.1% SDS for 20 min at room temperature and once

FIG. 1. Structures of chimeric tRNA antisense templates and retroviral vectors. (A) The primary transcript of a tRNA gene is processed at both the 5' end and the 3' end to generate the mature tRNA species. $\Delta 3-5$ is a derivative of a human tRNA^{Met} gene (11 bp of the gene (11 bp of the mature tRNA product)), which abolishes processing of the primary tRNA transcript. Insertion of a foreign sequence between the $\Delta 3-5$ tRNA gene and a transcription termination signal generates a chimeric transcript in which the foreign sequence is fused to the 3' end of the tRNA, as shown by Adeniyi-Jones et al. (1). (B) Antisense and antipol oligonucleotides correspond to nucleotides 1645 to 1674 in the MoMLV gag gene and nucleotides 4513 to 4543 in the pol gene. Additional sequences were added to generate an RNA pol III transcription termination signal (shaded boxes) and *SacII* and *MluI* ends. The lower strand in each oligonucleotide corresponds to the viral RNA (sense strand). (C) The chimeric tRNA-MoMLV antisense DNA templates (tRNA-oligo) were inserted into the retroviral vector N2A. Vectors SC:antisense and DC:antisense were generated by cloning the tRNA-antisense oligonucleotide fusion product in either orientation into the unique *XhoI* site present downstream from the coding sequences of the *neo* gene. Transcripts initiated from the viral LTR or the tRNA gene are represented by solid lines with arrowheads. MoMLV antisense sequences are indicated by the striped arrows. The DC:antisense vectors, DC:antisense and DC:antipol, were generated by the insertion of the tRNA-MoMLV antisense DNA templates into the polylinker sequence present in the 3' LTR of the vector N2A. In the target cell, the chimeric tRNA gene is duplicated and is present in the 5' LTR as well. In this case, the tRNA-derived transcription unit is placed outside the viral transcription unit. Symbols are as described above. In vectors LTR:antisense and LTR:tRNA, the antisense oligonucleotide and the $\Delta 3-5$ tRNA^{Met} gene, respectively, were cloned separately into the U3 region of the 3' LTR of the N2A vector. *EcoRI*, *XbaI*, *BglII*, and *BstXI* are restriction sites used in the DNA analysis. For additional information, see Materials and Methods and references 2 and 19.

in $0.2\times$ SSC-0.1% SDS for 20 min at 65°C and exposed to X-ray film (XAR5).

Quantitation of virus secreted from cells infected with MoMLV. NIH 3T3 cells were incubated with virus-containing supernatant in the presence of 8 μ g of Polybrene per ml for 2 h. Cells were washed once, and fresh culture medium was added. The appearance of reverse transcriptase (RT) in the medium was determined as described by Goff et al. (16). The infectious titer was determined by indirect immunofluorescence (46). NIH 3T3 cells were incubated with serial dilutions of virus-containing supernatant and stained 2 days later in situ with an MoMLV envelope-specific monoclonal antibody, ID11 (7).

Fluorescence-activated cell sorting analysis of MoMLV-infected NIH 3T3 cells. Cells were trypsinized, resuspended in culture medium at 10^6 cells per ml, incubated for 3 h in a polystyrene tube, washed twice in phosphate-buffered saline (PBS), and resuspended in 100 μ l of PBS containing 5% fetal calf serum and the monoclonal antibody ID11, which is specific for the MoMLV envelope protein (7). Cells were incubated on ice for 30 min, washed twice with PBS, and resuspended in 100 μ l of PBS containing 5% fetal calf serum and a goat anti-mouse fluorescein-conjugated immunoglobulin antibody (Becton-Dickinson) at a 1/50 dilution. Cells were again incubated on ice for 30 min, washed twice with PBS, and resuspended in 1 ml of 4% paraformaldehyde. Five thousand cells were used for sorting in a FACSscan apparatus (Becton-Dickinson).

Immunoprecipitation analysis. Labeling of virions with [35 S]methionine was accomplished by infecting 2×10^5 cells with MoMLV at a multiplicity of infection (MOI) of 2.0 and adding at 2 days postinfection 250 μ Ci of [35 S] methionine to the culture medium for 17 h. Secreted virus was collected by centrifugation of the cell supernatant through a 25% sucrose gradient. Pelleted virus was resuspended in 0.5 ml of phosphate lysis buffer (1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 0.1 M sodium phosphate [pH 7.5]). The extract (100 μ l) was immunoprecipitated with a goat anti-MoMLV antiserum (lot 76S000124; National Cancer Institute, Rockville, Md.) and protein A from fixed *Staphylococcus aureus* (Sigma). Precipitates were collected by centrifugation, resuspended in 50 μ l of electrophoresis sample buffer, boiled for 10 min, and electrophoresed in a 10% SDS-polyacrylamide gel. Gels were analyzed by fluorography with 2,5-diphenyloxazole, dried, and exposed to X-ray film (XAR5) for 1 to 3 days at -70°C. Analysis of newly synthesized MoMLV *gag* proteins was accomplished by preincubating cells with methionine-free medium for 30 min at day 3 postinfection with an MOI of 2.0 and labeling cells with 150 μ Ci of [35 S]methionine for 45 min. Cells were washed once with PBS and lysed with 0.5 ml of phosphate lysis buffer. The labeling efficiency was determined by trichloroacetic acid precipitation, and 10^6 cpm were immunoprecipitated as described above with a goat anti-p30 *gag*-specific antibody (lot 77S000174; National Cancer Institute). Electrophoresis, fluorography, and exposure were performed as described above.

RESULTS

Antisense templates and retroviral vector design. $\Delta 3$ -5 is a derivative of a human tRNA^{Met} gene from which the 3' processing signal has been deleted (1). Consequently, insertion of foreign sequences between the transcribed region of the tRNA gene and a tRNA transcription termination signal should result in the formation of chimeric RNA transcripts

(Fig. 1A). Figure 1B shows the sequence of two oligonucleotides that were fused to the 3' end of the $\Delta 3$ -5 tRNA^{Met} gene. Antigag oligo contains 30 nucleotides which correspond to a sequence present in the MoMLV *gag* gene, nucleotides 1645 to 1674, and antipol oligo contains a sequence of 31 nucleotides corresponding to nucleotides 4513 to 4543 in the *pol* gene. Additional nucleotides were included to generate a pol III transcription termination signal, as well as *Sac*II and *Mlu*I ends for subsequent cloning. Note that the MoMLV coding strand in both oligonucleotides is the bottom strand, so that fusion of the *Sac*II end to the 3' end of the $\Delta 3$ -5 tRNA gene will generate the corresponding antisense transcript and also provide a transcription termination signal. The retroviral vectors used to introduce the $\Delta 3$ -5 tRNA^{Met}-oligo fusion constructs (hereafter referred to as antisense templates) into recipient cells are based on the high-titer N2 retroviral vector (2) and are shown in Fig. 1C. The SC:antigag vector construct represents the conventional strategy for retroviral vector design, in which the foreign gene, the antisense template in this case, was inserted between the two LTRs downstream of the *neo* gene, parallel as well as antiparallel to the viral transcriptional unit (SC: antigag and SC:antigagR, respectively; SC [single-copy vector] refers to the fact that the antisense template is present as a single copy in the proviral DNA). In this case, the structure of the proviral DNA in the infected cell is identical to the structure of the vector DNA, and the chimeric tRNA template is encoded within the vector LTR transcription unit. Because such positioning may adversely affect the activity of the tRNA promoter (11, 19, 23, 34), we created a second vector design in which both the *gag* and *pol* antisense templates were introduced into the U3 region of the 3' LTR of the N2A vector, only in the parallel orientation (DC: antisense vectors, DC:antigag and DC:antipol). It is a consequence of the mechanism of replication of retroviruses that the antisense templates will be duplicated and will thus be present in both LTRs of the proviral DNA (hence, the name of this vector, DC [double-copy vector]). In this case, the copy of the tRNA antisense template present in the 5' LTR is placed outside the viral transcription unit (Fig. 1C), possibly leading to the increased transcriptional activity of the tRNA promoter. (Retroviral vectors carrying antisense templates will be referred to hereafter as antisense vectors.) Two additional vector constructs were used in this study; they contain the *gag* antisense oligonucleotide or the $\Delta 3$ -5 tRNA gene cloned separately in a DC vector (Fig. 1C, LTR:antigag and LTR:tRNA constructs, respectively).

Vector DNA was converted to corresponding virus and used to infect NIH 3T3 cells (see Materials and Methods). Individual G418-resistant clones were isolated and expanded to cell lines for further analysis. The structure of the proviral DNA was analyzed by DNA blotting (Fig. 2). The predicted duplication of the antisense templates in the proviral DNA derived from DC vectors was confirmed with the restriction enzymes *Xba*I and *Bgl*II. DC vector DNA contains two *Xba*I sites in the viral LTRs and a unique *Bgl*II site in the antisense template (Fig. 1C). Thus, only faithful duplication of the antisense templates will generate a 3.8-kb DNA fragment upon digestion with *Bgl*II, equal in length to the restriction fragment generated by digestion with *Xba*I (Fig. 2A). *Eco*RI digestion was used to show that the antisense templates are present in the SC vector-derived proviruses (Fig. 2B), and *Bst*XI digestion was used to show that the *gag* antisense oligonucleotide was present and duplicated in the proviral DNA derived from the LTR:antigag vector (Fig. 2B).

FIG. 2. DNA blot analysis of the proviral structure. (A) DNA was isolated from three cell lines harboring a DC:antigag vector (cell lines DC:antigag1 to DC:antigag3) and from two cell lines harboring a DC:antipol vector (cell lines DC:antipol1 and DC:antipol2), digested with restriction enzymes as indicated, subjected to electrophoresis in agarose gels, blotted to nylon paper, and hybridized with a 32 P-labeled *neo*-specific probe. The DC:plasmid was DC:antigag vector DNA. (B) DNA was isolated from cell lines harboring SC:antigag, SC:antigagR, and LTR:antigag vectors and analyzed as described for panel A. The SC:plasmid and the LTR:plasmid were SC:antigag and LTR:antigag vector DNAs, respectively.

Expression of tRNA-driven antisense RNA transcripts in NIH 3T3 cells. Total RNA isolated from NIH 3T3 cell lines transduced with antisense vectors was subjected to electrophoresis in 8% polyacrylamide-urea gels, transferred to nylon paper, and hybridized with a $\text{tRNA}_{\text{Met}}^{\text{Met}}$ -specific probe (Fig. 3A and C) or a *gag*-specific oligonucleotide probe (Fig. 3B). The two major low-molecular-weight RNA species detected in NIH 3T3 cells by hybridization with a $\text{tRNA}_{\text{Met}}^{\text{Met}}$ probe represent the primary and mature $\text{tRNA}_{\text{Met}}^{\text{Met}}$ species expressed from (some of) the 12 copies of $\text{tRNA}_{\text{Met}}^{\text{Met}}$ genes present in the genome (35). Minor species detected with the tRNA probe may represent partially processed tRNAs or aberrant tRNA-like transcripts expressed from $\text{tRNA}_{\text{Met}}^{\text{Met}}$ pseudogenes (50). Cells transduced with DC:antisense vectors expressed a novel RNA species (Fig. 3A, arrows), the size of which corresponded to the predicted size of the tRNA-driven antisense transcripts. The two closely migrating bands (clearly distinguishable in cells infected with the *gag* antisense vectors) may represent the primary and 5'

FIG. 3. RNA blot analysis of chimeric tRNA transcripts present in NIH 3T3-derived cell lines. Total RNA was isolated from three cell lines harboring the DC:antigag vector (DC:antigag 1 to DC:antigag3), two cell lines harboring the DC:antipol vector (DC:antipol1 and DC:antipol2), three cell lines harboring the SC:antigag vector (SC:antigag1 to SC:antigag3), and four cell lines harboring

processed transcripts (Fig. 1A). The difference in migration between the putative *gag* and *pol* antisense transcripts cannot be explained by the 1-nucleotide difference between the two RNA species (Fig. 1B) and may be due to conformational differences. RNA samples (Fig. 3B) run in parallel with the samples shown in Fig. 2A were hybridized with a *gag* sense oligonucleotide probe (the 5'-end 32 P-labeled oligonucleotide shown in Fig. 1B, antigag oligo, bottom strand). As expected, this probe detected only the putative antigag RNA species, which was also detected with a $\text{tRNA}_i^{\text{Met}}$ -specific probe, confirming the identity of this RNA species. From this analysis, the tRNA-driven chimeric RNA species appear to be expressed very efficiently in the DC: antisense vector-transduced cells. By differential exposure of the RNA blot shown in Fig. 3A and densitometric analysis, it was estimated that the chimeric tRNA species represent about 3 to 5% of the endogenous mature $\text{tRNA}_i^{\text{Met}}$ species. Since tRNA species are 100-fold more abundant than is poly(A)-containing RNA and since $\text{tRNA}_i^{\text{Met}}$ species represent about 5% of the total tRNA, it is estimated that the tRNA-driven antisense transcripts expressed in the DC vector-infected cells correspond to about 15 to 25% of poly(A)-containing RNA in the cells on a molar basis. Figure 3C shows the RNA blot analysis of low-molecular-weight RNA expressed in cells harboring SC:antigag vectors. In contrast to the results with DC vector-containing cells, chimeric tRNA transcripts were not detected, even after longer exposures of the autoradiogram. Therefore, expression of the chimeric tRNA antisense template is dependent upon the use of the DC vector design, testifying to the importance of retrovirus vector design in efficient expression of transduced genes.

Inhibition of MoMLV replication in cells expressing tRNA-driven antisense RNA. To determine the effect of antisense RNA expression on a single cycle of viral replication, we infected cells with MoMLV at an MOI of 2.0 and determined the formation of progeny virus by monitoring the appearance of RT activity in the cell supernatant 3 days postinfection. The results of such an experiment (Fig. 4) showed that the formation of progeny virus in two independently derived cell lines expressing antisense RNA corresponding to the *gag* region (DC:antigag) was reduced up to 97%, compared with that in cell lines harboring similar vectors lacking either the antisense oligonucleotide (LTR:tRNA) or the tRNA moiety (LTR:antigag). Note that cells harboring the LTR:antigag vector expressed the antigag sequence from the viral LTR (Fig. 1C) which, by itself, is not capable of rendering the cells resistant to MoMLV replication. Vectors containing an unrelated oligonucleotide fused to the $\Delta 3$ -5 tRNA and expressing the chimeric tRNA at levels comparable to those in DC:antigag-infected cells did not inhibit MoMLV replication (data not shown). In contrast to cells harboring the DC: antisense vectors, cells harboring the SC:antisense vectors (SC:antigag and SC:antigagR), which did not express the chimeric tRNA transcripts, showed no inhibition of MoMLV replication. Therefore, inhibition of viral replication is dependent on the expression of detectable levels of tRNA-

the SC:antigagR vector (SC:antigagR1 to SC:antigagR4), subjected to electrophoresis in polyacrylamide-urea gels, blotted to nylon paper, and hybridized with a 32 P-labeled human $\text{tRNA}_i^{\text{Met}}$ -specific probe (A and C) or an MoMLV *gag* oligonucleotide-specific probe (B). The primary $\text{tRNA}_i^{\text{Met}}$ transcript is 89 nucleotides long, and the mature $\text{tRNA}_i^{\text{Met}}$ transcript is 72 nucleotides long. Bands corresponding to the chimeric tRNA transcripts are indicated by arrows.

FIG. 4. Inhibition of RT production in cell lines harboring DC:antigag vectors. Two cell lines containing an LTR:tRNA, DC:antigag, SC:antigag, SC:antigagR, or LTR:antigag vector were infected with MoMLV at an MOI of 2.0. After one round of viral replication, i.e., 72 h postinfection, the appearance of RT activity in the cell culture medium was measured. A sample from the RT reaction was spotted on a DE81 filter and counted (A) or exposed to X-ray-sensitive film (B). Each column or spot represents RT activity secreted from one cell line.

driven antisense RNA (Fig. 3). The results of another experiment (Table 1) showed that while antisense RNA corresponding to a region in the *gag* gene was effective, leading to 94 to 95% inhibition, as measured by an infectious virus assay, or 80 to 94% inhibition, as measured by RT activity secreted into the supernatant, the expression of antisense RNA corresponding to a region in the *pol* gene had a minimal effect on viral replication. It is possible that a secondary structure in the viral RNA prevents efficient hybridization of the *pol* antisense RNA to its target.

TABLE 1. Inhibition of MoMLV replication in NIH 3T3-derived cell lines expressing antisense RNA

Cell line	Virus released		RT activity	
	Titer (no. of viruses/ml)	% Inhibition ^a	32 p cpm	% Inhibition ^a
LTR:tRNA1	2.2×10^7		1,659	
LTR:tRNA2	1.0×10^7		1,565	
DC:antigag1	ND ^b		299	82
DC:antigag2	7.0×10^5	95	103	94
DC:antigag3	9.0×10^5	94	326	80
DC:antipol1	ND		826	49
DC:antipol2	ND		1,097	32

^a Calculated against the average titer or RT activity measured in the two LTR:tRNA cell lines.

^b ND, Not done.

To test the effect of antisense RNA on the spread of virus in cultures, we infected cell lines harboring DC:antigag vectors and LTR:tRNA vectors with MoMLV at an MOI of 0.0002. The spread of virus through the culture was monitored by staining infected cells with a monoclonal antibody directed against the viral envelope and fluorescence-activated cell sorting. Such an analysis (Fig. 5) demonstrated that the spread of MoMLV in a cell culture harboring the DC:antigag vector was greatly inhibited. For example, on day 8 postinfection, 79% of cells harboring a control vector (LTR:tRNA) were infected with MoMLV, while only about 2% of the DC:antigag vector-containing cells were infected.

Antisense RNA-mediated inhibition of MoMLV replication, as measured by an infectious virus assay and/or an RT assay of the cell supernatant (Fig. 4 and Table 1), might be due to the generation of defective virus or might be due to an actual decrease in virions released into the medium. To distinguish between these two possibilities, we determined the amount of virus released into the supernatant biochemically by immunoprecipitation. DC:antigag and LTR:tRNA vector-harboring cell lines were infected with MoMLV at an MOI of 2.0, and cells were labeled with [³⁵S]methionine for 17 h on day 2 postinfection. Newly secreted virus was collected from the medium, and viral proteins were immunoprecipitated with MoMLV-specific goat antisera. The amount of virus secreted from cells expressing *gag* antisense RNA was reduced 80 to 94%, commensurate with the observed reduction in RT activity (Fig. 6).

Antisense RNA-mediated inhibition of MoMLV is mediated at the level of translation. Accumulation of MoMLV RNA in cell lines harboring *gag* antisense vectors and control vectors was analyzed by RNA blotting procedures (Fig. 7). MoMLV-infected cells express two viral RNA species, a 35S unspliced RNA form and a 21S spliced RNA form. Since comparable amounts of MoMLV-specific RNAs were present in both total and cytoplasmic RNAs isolated from cell lines harboring LTR:tRNA or DC:antigag vectors, the observed decrease in progeny virus formation in cells expressing *gag* antisense RNA must have occurred subsequent to the synthesis and accumulation of virus-specific RNA species in the cytoplasm. MoMLV RNA accumulates in infected cells to high levels, representing 1% to several percent of cytoplasmic poly(A)-containing RNA in the cells (42). (This fact is also illustrated in the autoradiogram in Fig. 7A, which contained 1 µg of unfractionated RNA per lane and which was exposed for only 2 min.) A portion of the RNA blot in Fig. 7A was exposed for 1 h (i.e., 30-fold longer than the other portions). The band which could be clearly seen in the uninfected lane represented the LTR-initiated transcript expressed from the antisense vector. By differential exposures of the autoradiogram, it was estimated that the MoMLV transcripts were about 100-fold more abundant than the vector LTR-initiated transcripts. Therefore, it is unlikely that the vector LTR-initiated transcripts were responsible for the measured inhibition. This observation provides indirect confirmation that the chimeric tRNA transcripts must be expressed very efficiently to engender such inhibition of the highly expressed MoMLV genes.

To determine if antisense RNA inhibition is mediated at the level of translation, we measured the rate of synthesis of viral proteins (Fig. 8). Cell lines containing DC:antigag and LTR:tRNA vectors were infected with MoMLV at an MOI of 2.0. Three days postinfection, cells were pulsed with [³⁵S]methionine for 45 min, and virus-specific *gag* proteins were immunoprecipitated with a *gag*-specific antiserum and displayed on SDS-polyacrylamide gels. An 8- to 10-fold

reduction in the amount of newly synthesized *gag* gene product was observed in cells harboring the DC:antigag vector, suggesting that inhibition of MoMLV replication in cells expressing corresponding antisense RNA is mediated at the level of translation.

If a limited supply of the *gag* gene product is responsible for the observed decrease in MoMLV replication in cells expressing antisense RNA, then the replication of defective retroviruses which lack a functional *gag* gene should be also inhibited. To test this hypothesis, we infected cells harboring the DC:antigag and LTR:tRNA vectors with MoMLV at an MOI of 2.0 and determined the resulting titers of MoMLV and *neo*-containing virus. (The *neo*-containing virus is generated by packaging of the N2-based antisense vector transcripts through the helper functions provided by MoMLV.) Production of *neo*-containing virus was inhibited to approximately the same extent (92%) as was production of MoMLV (91%) (Table 2). In addition, replication of a defective MoMLV-derived virus harboring the Mtx-resistant *DHFR* gene in the presence of MoMLV was also inhibited to a similar extent (90%) in cells expressing antisense RNA. On the other hand, replication of vesicular stomatitis virus in the presence or absence of MoMLV was reduced only slightly in cells harboring the DC:antigag vector (20 to 26%). The lack of inhibition of vesicular stomatitis virus replication in the presence of MoMLV in cells expressing MoMLV-specific antisense RNA argues against the possibility that inhibition of MoMLV replication is mediated via interferon.

DISCUSSION

The effectiveness of an antisense RNA inhibition strategy is dictated in part by the ratio of antisense RNA to its target sequence in the cell. The inability to stably express sufficient levels of antisense RNA in cells from an antisense RNA-synthesizing DNA template is a major impediment in the development of effective gene inhibition protocols in eucaryotic cells. To the best of our knowledge, RNA polymerase II-based transcription units have been exclusively used in studies in which stable expression of antisense RNA in cells was sought (reviewed in reference 41). The use of RNA pol III-based transcription units represents an alternative approach to expressing high levels of antisense RNA in cells. In this study, we showed that a chimeric tRNA gene stably integrated for the cell chromosome directs the synthesis of very high levels of antisense RNA in cells. On the basis of RNA analysis (Fig. 3) and tRNA and tRNA_{Met} distributions in the cell, it was estimated that, on a molar basis, the chimeric tRNA transcripts corresponded to about 15 to 25% of the polyadenylated RNA present in the cell.

Replication of MoMLV is significantly inhibited (over 90%) in cells expressing virus-specific antisense RNA from a chimeric tRNA gene (Fig. 3 and 4 and Tables 1 and 2). Since the MoMLV genome encodes a highly efficient transcription unit (the viral RNA transcripts representing 1 to 5% of polyadenylated RNA in the infected cells), the ability to inhibit the replication of MoMLV represents a stringent test for this experimental strategy. Antisense RNA directed to a region in the *gag* gene was found to be more effective than was antisense RNA directed to a region in the *pol* gene (Table 1), despite the fact that comparable amounts of antisense RNA were expressed in the cells (Fig. 3). The reason for this difference is not clear. One possibility may be that a secondary structure in the *pol* sequence limits access to the antisense RNA. This observation underscores the necessity to screen the target gene for regions which are

FIG. 5. Inhibition of MoMLV spread in a culture of NIH 3T3 cells expressing tRNA-antigag fusion transcripts. NIH 3T3 cells (10^5) harboring either the LTR:tRNA or the DC:antigag vector were plated in 6-cm plates and infected with MoMLV at an MOI of 0.0002. Cells were trypsinized at day 4 postinfection, a sample of the cells was used for fluorescence-activated cell sorting, and 2×10^5 cells were seeded on fresh plates. This procedure was repeated at days 6, 8, and 11 postinfection. NIH 3T3/MLV cells are NIH 3T3 cells chronically infected with MoMLV. The percentages of positively sorted cells is represented by the number shown in the upper-right corner. Positive cell gating was selected (vertical lines) to yield a 1.8% background (NIH 3T3).

FIG. 6. Immunoprecipitation analysis of MoMLV virions. Cells were infected with MoMLV at an MOI of 2.0, and [35 S]methionine-labeled virus was immunoprecipitated with an MoMLV-specific antiserum and displayed on an SDS-polyacrylamide gel. Cells were infected (+) or mock infected (-) with MoMLV. The virion components detected in this analysis, clearly seen in the supernatant derived from cells harboring the LTR:tRNA vector, are identified on the right. p30 levels were decreased 80 and 94% in the DC:antigag2 and DC:antigag3 cell lines, respectively, as determined by densitometric analysis. Molecular weight (MW) is shown (in thousands) on the left.

FIG. 7. MoMLV-specific RNA synthesis in various cell lines. Total or cytoplasmic RNAs isolated from cells infected (+) or mock infected (-) with MoMLV were subjected to electrophoresis in 1% agarose gels (1 μ g of RNA per lane), blotted to nylon paper, and hybridized to a 32 P-labeled MoMLV-specific probe. Two major MoMLV-specific bands corresponding to the viral unspliced and spliced RNA forms were detected. The RNA blot in panel A (representing total RNA) was exposed for 2 min, and the RNA blot in panel B (representing cytoplasmic RNA) was exposed for 60 min. A 60-min exposure of total RNA derived from the DC:antigag3 cell line is also shown. The arrow points to a single band in the lane containing mock-infected cells which corresponds to the LTR-initiated transcript expressed from the DC:antigag vector. Ethidium bromide staining was used to ascertain that comparable amounts of RNA were analyzed. Positions of ribosomal 28S and 18S RNAs and size markers (in kilobases) are shown on the left.

FIG. 8. Immunoprecipitation analysis of newly synthesized MoMLV proteins. Cells were infected (+) or mock infected (–) with MoMLV and pulsed with [35 S]methionine for 45 min. Proteins were immunoprecipitated with an MoMLV p30-specific antiserum (IP) and displayed on SDS-polyacrylamide gels. A sample of nonprecipitated material was also displayed (Total) to ascertain that comparable amounts of material were analyzed. The bands corresponding to the *gag* precursor Pr65 and the faintly seen p30 product are indicated by arrows on the right. Pr65 (*gag*) levels were reduced 88% in DC:antigag2 cells, as determined by densitometric analysis. The exact nature of the slowly migrating heterodisperse band in the molecular weight (M.W.) range from 85,000 to 90,000 is unclear. M.W.s are shown in thousands.

susceptible to antisense RNA-mediated inhibition. Additional studies will be required to determine whether general rules can be established to simplify the process.

DNA constructs containing chimeric tRNA-driven antisense templates were introduced into NIH 3T3 cells via retroviral vectors. Expression of the chimeric tRNA genes, leading to the inhibition of MoMLV replication, was dependent on the use of a novel retroviral vector design in which the foreign gene is inserted into the 3' LTR of the retroviral vector (DC vector). Hantzopoulos et al. (19) showed that the expression of an adenosine deaminase minigene was significantly enhanced when a DC vector was used, and it was argued that the transfer of the adenosine deaminase minigene outside the viral transcription unit was responsible for the observed enhancement of gene expression. Similarly, it

TABLE 2. Replication of viruses in cells expressing MoMLV-specific antisense RNA

MoMLV	Other virus	Titer (no. of viruses/ml) in:		% Inhibition
		LTR:tRNA2	DC:antigag3	
+	None	1.0×10^7	9.0×10^5	91.0
+	<i>neo</i> containing	6.0×10^3	5.0×10^2	91.7
+	Mtx-resistant <i>DHFR</i> gene containing	1.0×10^3	1.0×10^2	90.0
+	Vesicular stomatitis virus	2.9×10^8	2.3×10^8	20.7
–	Vesicular stomatitis virus	4.2×10^8	3.1×10^8	26.2

appears that placement of the chimeric tRNA gene outside the viral transcription unit relieves it from the inhibitory effect of an upstream promoter. The chimeric tRNA genes were inactive when inserted into a retroviral vector in the traditional manner downstream of the *neo* gene within the LTR-initiated transcription unit of the N2 vector (Fig. 3 and 4) (15, 38). These observations underscore the importance of retroviral vector design for efficient expression of foreign genes.

Since the accumulation of MoMLV RNA in the cytoplasm of cells expressing antisense RNA was not affected (Fig. 7) but the appearance of newly synthesized viral *gag* polypeptides was reduced commensurate with the observed inhibition of MoMLV replication (Fig. 8), we tentatively conclude that the observed antisense RNA-mediated inhibition of MoMLV replication was mediated at the level of viral protein synthesis. In previous studies, it was shown that the inhibition of endogenous gene expression in cells stably expressing antisense RNA takes place in the nucleus, preventing the appearance of the target RNA in the cytoplasm (10, 24, 26). Differences in the experimental systems are most likely responsible for the different mechanisms of antisense RNA inhibition. An alternative interpretation is suggested by the observations of Bass and Weintraub (4), who have shown that the formation of duplex RNA between antisense RNA and target RNA in the nucleus of *Xenopus* oocytes results in the covalent modification of adenosines throughout the double-stranded RNA region. Wagner and Nishikura (44) have shown that a similar activity is also present in several mammalian cell types, including NIH 3T3 cells, and Kimelman and Kirschner (25) have described an activity in *Xenopus* oocytes which may be responsible for the destabilization of the developmentally regulated maternal fibroblast growth factor mRNA. It is thus possible that RNA duplexes formed between antisense RNA and MoMLV *gag* RNA may lead to RNA modifications which alter the coding capacity of the *gag* mRNA and lead to the observed decrease in *gag* polypeptide synthesis. To test this possibility, we generated DNAs from individual RNA transcripts isolated by the polymerase chain reaction and sequenced these DNAs. However, no evidence for adenosine to inosine conversion was found in 10 of 10 transcripts sequenced (unpublished results).

The potential formation of double-stranded RNA in MoMLV-infected (antisense RNA-expressing) cells also raises the possibility that the observed inhibition of MoMLV replication is the result of double-stranded RNA-mediated induction of interferon or interferon-induced antiviral activities (32). Two observations argue against this possibility. First, interferon-induced inhibition of retroviral replication in mouse fibroblast cells is exhibited at a posttranslational stage in the viral replication cycle (33). In our system, inhibition apparently occurs at the level of translation, not

posttranslation. Second, vesicular stomatitis virus replication, which is exquisitely sensitive to interferon, is not inhibited in cells expressing antisense RNA, whether Mo MLV is present or not (Table 2).

The use of an RNA pol III-based transcription system to stably express high levels of foreign RNA sequences in cells may have other important applications. Foremost, it may significantly improve the ability to inhibit endogenous genes in eucaryotic cells for the study of gene expression and function, whether antisense RNA, ribozymes, or competitors of sequence-specific binding factors are used. tRNA-driven transcription systems may be particularly useful for introducing "mutations" into the germ line, i.e., for generating transgenic animals or transgenic plants. Since tRNA genes are ubiquitously expressed in all cell types, the chimeric tRNA genes may be properly expressed in all tissues of the animal, in contrast to the more idiosyncratic behavior of RNA pol II-based transcription units (see, for example, references 37, 47, and 48). However, homologous recombination represents a more elegant although, at present, very cumbersome approach for introducing mutations into the germ line (see, for example, reference 51). In either case, the ability to generate transgenic animals or plants carrying defined mutations will be an extremely valuable experimental tool for studying gene function in a developmental context and for generating animal models for human genetic disorders. In addition, tRNA-driven gene inhibition strategies may also be useful in creating pathogen-resistant livestock and plants.

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