The U3 Region of Moloney Murine Leukemia Virus Contains Position-independent Cis-acting Sequences Involved in the Nuclear Export of Full-length Viral Transcripts

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Background: Self-inactivating retroviral vectors are characterized by the deletion of enhancer/promoter sequences in the U3 region.

Results: The U3 region of Mo-MLV-derived vectors contains sequences necessary for the nuclear export of full-length viral transcripts.

Conclusion: Sequences in the U3 and leader regions could be involved in the regulated nuclear export of full-length RNAs.

Significance: These findings provide new insights into the molecular mechanism underlying retroviral RNA nuclear export.

The distinguishing feature of self-inactivating (SIN) retroviral vectors is the deletion of the enhancer/promoter sequences in the U3 region of the 3’ long terminal repeat. This design is used to overcome transcriptional interference and prevent downstream transcription from the 3’ long terminal repeat. SIN vectors were derived from a number of different retroviruses. Studies of SIN vectors show that extensive U3 deletions in HIV-based vectors do not alter viral titers or the in vitro and in vivo properties of the vectors. However, deletion of the U3 sequences in γ- and α-retroviruses correlates with defects in 3’ RNA processing and reduces viral titers by >10-fold. Here, we studied the steps in the retroviral life cycle that are affected by the deletion of sequences in the 3’ U3 region of Moloney murine leukemia virus-derived retroviral vectors. The results show that the amounts of both full-length and internal RNA transcripts of U3-minus vectors are reduced in the nuclei of transfected cells, an effect that is probably due to a general defect in 3’ RNA processing. Furthermore, full-length RNA transcripts were also defective in terms of nuclear export. This defect was complemented by transferring the U3 region to another position within the retroviral vector, indicating that the U3 region contains position-independent cis-acting sequences that are required for the transport of full-length viral transcripts. The results also suggest that the leader region of Moloney murine leukemia virus contains inhibitory/regulatory sequences, which prevent export and mediate nuclear retention of full-length viral RNA.

Retroviral vectors are useful tools for delivering therapeutic genes to primary cells in vitro and have been used in a number of gene marking and gene therapy trials in humans (1). The principal advantages of retroviral vectors include highly efficient gene delivery, integration into the host cell genome, and high levels of gene expression. Another advantage of retroviruses is the fact that they can be pseudotyped by the incorporation of heterologous glycoproteins, allowing extension of the host range. Because the early steps in the viral life cycle do not depend on viral protein synthesis, retroviral vectors contain no viral coding regions and include only the viral sequences or cis-acting viral elements required for high efficiency transfer. All of the retroviral proteins are usually provided in trans by the retroviral packaging cells or by co-transfected packaging plasmids. As a result, retroviral vectors can be used to transfer only cloned sequences (2).

An improvement in retroviral vector design and safety was achieved by deleting the enhancer/promoter sequences in the U3 region of the 3’ long terminal repeat (LTR) (which renders the vectors self-inactivating (SIN)) (3–5). Subsequent to reverse transcription in a host target cell, the SIN deletion in the U3 region of the 3’ LTR is transposed to the U3 region of the 5’ LTR in the proviral DNA, thereby preventing the expression of full-length vector RNA. This approach has been used to overcome transcriptional interference between the enhancers and promoters in the viral LTRs and the internal promoters. It also increases the safety of retroviral vectors by preventing downstream transcription from the 3’ LTR into genomic DNA and the synthesis of full-length genomic transcripts in target cells. SIN vectors have been derived from several retroviruses, including Moloney murine leukemia virus (Mo-MLV), spleen necrosis virus (SNV), avian leukemia virus, and human immunodeficiency virus type 1 (HIV-1) (3–7). However, for γ- (SNV and Mo-MLV) and α-retroviruses (avian leukemia virus), the deletion of most of the sequences in the U3 region correlates

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4 The abbreviations used are: SIN, self-inactivating; Mo-MLV, Moloney murine leukemia virus; SNV, spleen necrosis virus; eGFP, green fluorescence protein; MFI, mean fluorescence intensity; RSV, Rous sarcoma virus; RSL, R region stem-loop.
with a defect(s) in 3′ RNA processing, and viral titers are reduced by >10-fold (5, 6, 8, 9). By contrast, extensive U3 deletions in HIV-based vectors do not alter vector titers or their in vitro and in vivo properties (7, 10).

In an effort to complement the defect in 3′ RNA processing observed in SIN SNV-based vectors, it was shown that the addition of an SV40 poly(A) site increased the viral titer, although it was still lower (by a factor of 5) than that of the parental vector (5). In addition, it was also shown that the LTRs in the some of the U3-minus SNV vectors were reconstituted with unusually high frequency (11). This indicated that there is a strong selectivity for reversion. Improved SIN vectors derived from SNV were developed by replacing the U3 region in the 5′ LTR with the cytomegalovirus immediate-early genes enhancer/promoter (CMV). These vectors produced viral titers close to those of the parental vectors, with the exception of differences in transcripational efficiency between the CMV and SNV promoters (12). SIN vectors were also derived from hybrid avian leukosis virus vectors in which the U3 region was extensively deleted or replaced with a heterologous region that led to a significant reduction in viral titer (6).

Mo-MLV-derived SIN vectors were the first vectors produced using this configuration. However, the employment of these vectors in the experimental works showed their low transduction efficiency (3, 4). Although introducing exogenous poly(A) sites into the SIN vectors led to an increase in viral titers, this improvement was not >2–3-fold (8, 13). On the other hand, replacing the U3 region in the 5′ LTR with Rous sarcoma virus (RSV) or a tetracycline-inducible promoter led to a marked increase in the viral titer. It has been suggested that promoter competition is the major limitation for the production of γ-retroviral SIN vectors (9). It is important to note that analysis of gene expression using different internal promoters produced identical results with γ-retroviral and lentiviral SIN vectors (14).

This work was addressed to study the stages in retroviral RNA production that are affected by deletions in the U3 region of Mo-MLV-derived retroviral vectors. We examined the replication of vectors harboring U3 deletions of various lengths, the expression of marker genes with the different internal promoters by SIN and parental vectors, and the properties of marker gene expression with respect to full-length and internal RNA transcripts. The results showed impaired production of both full-length and internal RNA transcripts in the nuclei of transfected cells, which is probably due to defective 3′ RNA processing. Furthermore, we identified an additional defect in nuclear–cytoplasmic transport that affected only the full-length RNA transcripts in the packaging cells.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—The Mo-MLV-derived retroviral vector pDSVneo was described previously and was used as the progenitor for the construction of vectors harboring U3 deletions of various lengths (15). For this purpose, the 709-bp ClaI-BamHI fragment containing the 3′ U3 region of pDSVneo was inserted into the ClaI and BamHI sites of the pBluescript KSII+ ΔXS plasmid. All deletions in the U3 region were made in the resulting plasmid, pU3 (114 bp between the XbaI and SacI restriction sites, 263 bp between Nhel and Xbal restriction sites, and 381 bp between Nhel and SacI restriction sites). The retroviral vectors pΔAXS, pΔNX, and pΔNS were generated by replacing the Clal-BamHI fragment of pDSVneo with the corresponding fragment of the pU3ΔXS, pU3ΔNX, or pU3ΔNS plasmids, respectively.

The retroviral vectors, pLNRSVgfp.wt/pLNRSVgfp.sin and pLNFGfp.wt/pLNFGfp.sin, were constructed via several steps. First, the eGFP gene was amplified with the primers HGD 5′-cgccgagctctggagcagggc-3′ (forward) and CGR 5′-cgccgagctctggagcagggc-3′ (reverse) using the pWPXL vector as a template (29). The vectors were then digested with restriction enzymes and cloned into the HindIII and ClaI sites of the pNCX vector (17). The 3′ LTR of the resulting plasmid, pLCNFgfp, was then replaced with the U3-deleted LTR from pDANS after two-step cloning. The EFlα promoter was amplified with the primers Ed 5′-cgccgagctctggagcagggc-3′ (forward) and Er 5′-cgccgagctctggagcagggc-3′ (reverse) using the pWPXL vector as a template, and the RSV promoter was amplified with the primers Rd 5′-cgccgagctctggagcagggc-3′ (forward) and Rr 5′-cgccgagctctggagcagggc-3′ (reverse) using the pLP2 plasmid (Invitrogen) as a template. The CMV sequences of the pLCNFgfp and pLCNFgfp.sin plasmids were then replaced with these sequences via the BamHI and HindIII restriction sites.

Retroviral vectors pLgfpSN, pLgfpSNsin, and pLgfpNSsin were derived from the pLXSN vector (17) by the same technique used to produce the previous plasmids. The eGFP gene was amplified with the primers EGD 5′-cgccgagctctggagcagggc-3′ (forward) and XGR 5′-cgccgagctctggagcagggc-3′ (reverse) and cloned into the EcoRI and Xhol sites of pLXSIN. The Mo-MLV U3 region was amplified with the primers MD2 5′-cgccgagctctggagcagggc-3′ (forward) and MR2 5′-cgccgagctctggagcagggc-3′ (reverse) and cloned into the BamHI and HindIII sites of the pLgfpSNsin in place of the SV40 early genes enhancer/promoter sequences. The amplified regions of the vectors were analyzed by sequencing. All DNA manipulations were performed according to standard procedures (30).

**Cells Lines and Transfection**—NIH3T3 (murine cell line) and GP+envAM12 (amphotropic 3T3-based packaging cell line with murine leukemia virus gag, pol, and env genes) (31) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum.

Cell clones stably producing retroviral vectors were established by transfecting GP+envAM12 cells with vector plasmids using the dimethyl sulfoxide-Polybrene method (32). A total of 4 × 10⁵ cells were seeded into 25-cm² flasks at 24 h before transfection. For transfection, 10 μg of vector DNA was used per flask. Cells were selected by incubation with G418 (800 μg/ml (Invitrogen)) starting 24 h after transfection and lasting for at least 12–14 days. To compare viral titers and eGFP expression levels among geneticin-resistant cell clones expressing different retroviral constructs, cells were further cultured in a monolayer.

**Virus Propagation and Flow Cytometry**—Viral infection was performed immediately after harvesting the virus. The supernatants were harvested from 90% confluent stable producer cell
clones after 16-h intervals and filtered through the 0.45-μm filters. Infections were performed in the presence of 8 μg/ml Polybrene (Sigma) for 2 h at 37 °C. G418-resistant colony forming unit titers were determined using the infection of NIH3T3 cells by end-point dilution.

The number of cells expressing eGFP as well as the mean fluorescence intensity (MFI) of positive cells was determined with a FACS analyzer (BD Biosciences FACSScanto I). Transfected cells were analyzed by FACS immediately after using the medium supernatant for infection experiments. Infected cells were analyzed 72 h post-infection.

**RNA Isolation**—RNA was isolated from virus particles using a NucleoSpin RNA Virus column (Macherey-Nagel) according to the manufacturer’s instructions. To prepare nuclear and cytoplasmic RNA, cells (5 × 10⁶) were collected and treated according to the protocol described by Blissenbach, M. et al. (33) with several modifications. Briefly, cells were washed in 1 ml of phosphate-buffered saline (PBS) and resuspended in 175 μl of RLN buffer (50 mM Tris-HCl (pH 8.0), 140 mM NaCl, 1.5 mM MgCl₂, 0.5% Nonidet P40, 1000 units/ml RNAse inhibitor (Thermo Scientific), and 1 mM dithiothreitol) and incubated for 2.5 min on ice. Debris and nuclei were pelleted by centrifugation (300 × g, 2 min, 4 °C), and the cytoplasmic fraction was transferred to a tube containing 750 μl of TRI Reagent (Sigma). To isolate RNA from the cell nuclei, cells were incubated in 175 μl of RLN buffer for 15 min on ice. The nuclear pellet was washed in 500 μl of PBS, pelleted by centrifugation for 3 min at 300 × g, resuspended in 175 μl of RLN buffer, and added to a tube containing 750 μl of TRI Reagent (Sigma). RNA from the nuclear and cytoplasmic fractions was extracted according to the manufacturer’s protocol (Sigma).

**Western Blotting**—Total protein lysates were prepared by lysing GP + envAM12 packaging cells in radioimmune precipitation assay buffer, which contained 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and a mixture of protease inhibitors (Santa Cruz Biotechnology). Nuclear and cytoplasmic fractions were obtained as described for the RNA analyses and were used to prepare protein lysates. Equal amounts of total protein were resolved on 12% SDS-PAGE gels and transferred to nitrocellulose membranes (Bio-Rad). Membranes were stained with antibodies against histone H4 and α-tubulin (sc-25260 and sc-5286, respectively; Santa Cruz Biotechnology, Heidelberg, Germany).

**Quantitative RT-PCR Analysis**—The full-length and internal RNA transcripts were measured by real-time RT-PCR using primers specific for the eGFP sequence and the neo gene. The primers and probes were homologous to a region of eGFP: 214f (gcagtgcttcagccgctac), 309r (aagaagatggtgcgctcctg), and 234F (6-carboxyfluorescein-cgacacatggaagcacacct-BHQ1) (34). The primer sequences used for the neo gene real-time RT-PCR assay have been published previously (8): 364f (cgcgctgctgtgctgctgtg), 529r (atgcgacagcgcaggc), and probe 423p (6-carboxyfluorescein-aactagcctgagcagct-BHQ1), which is homologous to pre-GAPDH RNA. Serial dilutions of nuclear RNA were used as standards, and the ratio of cytoplasmic to nuclear pre-GAPDH RNA levels was determined.

The relative amounts of RNA extracted from the cytoplasmic and nuclear fractions were normalized in real-time RT-PCR using primers specific for 18 S ribosomal RNA (34). The assay used the following primers and probe: 343f (ccttcgacgctgcgctg), 409r (tcacctgctgtgac), and 370p (6-carboxyfluorescein-cggtgctgtgctgtgctg-BHQ1). Quantitative real-time PCR was performed with an iQ5 PCR system (Bio-Rad). Student’s t test was used for statistical analysis, and p values < 0.05 were considered significant.

**RESULTS**

**The Replication of Retroviral Vectors Harboring U3 Deletions of Different Lengths**—To determine the role of the U3 region in viral replication, deletions of various lengths were introduced into the U3 region of the 3’ LTR of a pDSVneo vector, which contained the marker gene, neo, under the control of an internal SV40 early gene enhancer/promoter (Fig. 1A) (15). The following regions were deleted: a 114-bp region between the XbaI and SacI restriction sites (positions −147 to −34) (16), which is adjacent to the R region (plasmid pΔAXS); a 263-bp region between the Nhel and XbaI (positions −414 to −152), which is adjacent to the 5’ end of the U3 region and includes both 72 bp enhancer repeats (plasmid pΔANX); a 381-bp region between the Nhel and SacI (positions −414 to −34), which is a deletion that combines both previous deletions (plasmid pΔANS). The latter plasmid (pΔANS) contained only 35 bp from the 5’ end and 33 bp from the 3’ end of the U3 region in the 3’ LTR (Fig. 1A).

Recombinant and parental plasmids were transfected into a GP + envAM12 packaging cell line, and several populations of virus-producing cells were selected for each construct. Virus titers were determined by the number of G418-resistant colonies formed after the infection of NIH3T3 cells. As shown in Fig. 1B, the titer of the pΔAXS vector decreased by only 2-fold compared with that of the parental vector, whereas the titer of the pΔANX vector decreased by a factor of 5.5. It is noteworthy that the titer of pΔANS, which harbored combined deletions in the U3 regions of the pΔAXS and pΔANX vectors, decreased by 11-fold. These results suggest that sequences in the U3 region could be functionally involved in different processes during retroviral RNA production.

**Expression Patterns Induced by Different Internal Promoters**—We also examined expression patterns using different internal promoters to compare the SIN and parental vectors. For this purpose the extensively deleted U3 region of pΔANS was transferred to the pLNCX plasmid (17). The resulting plasmid was designated as the SIN vector. The gene encoding eGFP was cloned into the polylinker of the plasmid, and the CMV promoter was replaced with the RSV or EF1α (human elongation factor 1α gene enhancer/promoter) promoter (Fig. 2A). Packaging cells containing these plasmids were selected with G418, and the expression patterns of eGFP were analyzed in both
transfected and target cells. Furthermore, to enhance the effect(s) of the U3 region, the amount of plasmid DNA input was reduced by 20 times (from 10 \( \mu \)g to 500 ng), and all transfections were performed in the presence of carrier DNA (1:10).

The replication and expression of these two pairs of retroviral vectors (pLNRSVgfp.wt/pLNRSVgfp.sin and pLNEFgfp.wt/pLNEFgfp.sin) were analyzed according to several parameters. As shown in Fig. 2B, the SIN vectors showed reduced transfection efficiency: 2-fold for pLNRSVgfp.sin compared with pLNRSVgfp.wt and 5-fold for pLNEFgfp.sin compared with pLNEFgfp.wt. However, there were small differences in the levels of eGFP expression in target cells induced by the internal promoter in the pLNRSVgfp.sin and pLNRSVgfp.wt vectors (1.3-fold), and there were no differences between the pLNEFgfp.sin and pLNEFgfp.wt vectors (Fig. 2C). Compared with the parental vectors, pLNRSVgfp.sin showed a 5.3-fold reduction in eGFP titers, and pLNEFgfp.sin showed a 13-fold reduction. These results correlated with the observed reduction in the amount of virion RNAs measured by real-time RT-PCR using primers specific for the neo gene (Fig. 2, D and E). These data indicate that there were no defects in virus replication after the packaging of RNA into viral particles.

The levels of eGFP expression were also estimated in transfected packaging cells. First, the number of SIN vector-transfected cells producing detectable levels of eGFP was 1.5-fold lower than that of cells transfected with the parental vectors. Second, similar differences were observed in eGFP expression levels (as measured by MFI; Fig. 3, A–C). Thus, despite the significant drop in virus titers and levels of virion RNA, we observed only minor differences in the levels of expression induced by the internal promoters in target and transfected cells (1.3- and 1.5-fold, respectively).

Expression Patterns of Full-length RNA Transcripts—To study the expression patterns of full-length RNA transcripts from the SIN vectors, we used an experimental scheme in which eGFP could be expressed from the enhancer/promoter of viral LTR. The eGFP gene was cloned into the polylinker within the pLXSN plasmid (17). The 3' LTR in the resulting
plasmid (called pLgfpSNwt) was then replaced with the U3-deleted LTR from the pD/H9004NS plasmid (pLgfpSNsin, Fig. 4A). In addition, we generated a retroviral vector carrying the internal U3 region to determine its ability to complement the defects in the U3-minus vectors. In these vectors, the SV40 immediate early genes enhancer/promoter in the pLgfpSNsin plasmid was replaced with the following sequences: a 48-bp fragment of the 3′ untranslated region, the complete U3 region, and the first 3 bp from the R region (pLgfpMNsin, Fig. 4A). Plasmids were transfected into packaging cells according to methods used in the previous experiments.

The transfection efficiency of pLgfpSNsin was reduced by 2-fold compared with that of the parental vector, whereas the pLgfpMNsin and pLgfpSNwt vectors showed similar results (Fig. 4B). The amount of virion RNAs (RNA titer) for pLgfpSNsin decreased by 33-fold compared with that for the parental pLgfpSNwt vector, whereas that for pLgfpMNsin decreased by 16-fold (Fig. 4C). The expression patterns of eGFP
in transfected cells showed that, compared with the parental vector, the number of cells producing detectable levels of eGFP was reduced by 5.7-fold for pLgfpSNsin and by 4.8-fold for pLgfpMNsin and that the MFI decreased by 5.5- and 3.3-fold, respectively (Fig. 5, A–C). Next, to compare the expression efficiency of full-length and internal RNA transcripts, we determined the overall decrease in eGFP expression (decrease in MFI/ decrease in the number of eGFP-producing cells). The data (summarized in Table 1) correlated well with the decrease in RNA titer observed for both SIN vectors (31-fold for pLgfpSNsin and 15.8-fold for pLgfpMNsin). Overall, the expression of the internal RNA transcripts fell by 2.2-fold (Fig. 3, B and C; Table 1), whereas that of the full-length transcripts fell by 12.4-fold (31/2.5, corrected for transfection efficiency) (Fig. 5, B and C; Table 1). Thus, the difference in the efficiency of expression for internal and full-length RNA transcripts was 5.6-fold (12.4/2.2; Table 1). These results suggest an additional defect in the production of full-length RNA transcripts by the packaging cells.
We also generated the retroviral vectors, pLNSgfp.wt and pLNSgfp.sin (analogous to pLgfpSNwt and pLgfpSNsin), in which the neo gene was expressed in the full-length RNA transcript (Fig. 4A). We then used these vectors to estimate transfection efficiency and the RNA titer. The results were identical to those obtained in the previous experiments using the pLNEFgfp.sin and pLNEFgfp.wt vectors (a 5-fold decrease in transfection efficiency and a 13.5-fold decrease in RNA titer; Fig. 4, B and C; Table 1). Also, pLNMsfp.sin (a version of pLgfpMNSin) yielded the same results as pLNRSVgfp.sin (a 2-fold decrease in transfection efficiency and a 5.5-fold decrease in RNA titer; Fig. 4, B and C; Table 1). The overall decrease (decrease in transfection efficiency $\times$ decrease in RNA titer) in retroviral RNA production was nearly 65-fold for both versions of the vectors (2 $\times$ 33 for pLgfpSNsin/pLgfpSNwt and 5 $\times$ 13 for pLNSgfp.sin/pLNSgfp.wt and pLNEFgfp.sin/pLNEFgfp.wt; Table 1). Using the Mo-MLV U3 region as an internal promoter increased the production of pLgfpMNSin and pLNMsfp.sin full-length RNA by 4.2-fold (67/16) and 6.1-fold (67/11), respectively (Table 1). This indicates that the U3 region contains position-independent cis-acting sequences that play a role in retroviral RNA production. It should be noted that the U3 region of RSV (pLNRSVgfp.sin) increased RNA production by the same factor (5.3-fold, 63/12; Table 1).

**RNA Analysis of Full-length and Internal Transcripts in Transfected Cells**—Next, we examined the copy number of the full-length and internal RNA transcripts for the SIN (pLgfpSNsin) and parental (pLgfpSNwt) vectors in both the nucleus and cytoplasm of transfected cells. Full-length RNAs were quantified by real-time RT-PCR using primers specific for the eGFP sequences, and internal transcripts were analyzed using primers specific for the neo gene. However, because the neo gene sequences were also present in the full-length RNAs, the copy numbers of the internal tran-
scripts were quantified by calculating the ratio of the full-length and internal transcripts in transfected cells. Therefore, we used the same template to prepare the standards used in both RT-PCRs (gfp- and neo-specific) and calculated the ratio in the nucleus and cytoplasm of cells transfected with the parental pLgfpSNwt vector. The results showed that the ratio was nearly 2:1; therefore, this index was used for all subsequent calculations.

FIGURE 5. Expression patterns of full-length RNA transcripts in transfected cells. A, flow cytometry analysis of eGFP expression. Control untransfected cells and LgfpSNwt, LgfpSNsin, and LgfpMNsin packaging cells transfected with pLgfpSNwt, pLgfpSNsin, and pLgfpMNsin retroviral vectors, respectively. B, histograms showing the number of cells producing detectable levels of eGFP. ***, \( p < 0.001 \), compared with the control cells. C, representative histograms show the levels (MFI) of eGFP expression in the packaging cells. Relative values with respect to the pLgfpSNwt vector are shown. **, \( p < 0.01 \), ***, \( p < 0.001 \).

TABLE 1
Summary of full-length and internal RNA production by self-inactivating retroviral vectors

| Vector          | Efficiency of transfection\(^a\) | Level of eGFP expression (MFI)\(^a\) | Number of eGFP-producing cells\(^a\) | RNA titer\(^b\) | Overall decrease in eGFP expression\(^b\) | Overall decrease in full-length RNA production\(^c\) |
|-----------------|----------------------------------|--------------------------------------|-------------------------------------|----------------|-------------------------------------------|--------------------------------------------------|
| plNEFgfp.wt     | 1                                | 1                                    | 1                                   | 1              | 1                                         | 1                                                |
| plNEFgfp.sin    | 0.2                              | 0.68                                 | 1                                   | 0.082          | 0.46                                      | 0.016 (63)                                       |
| pLNRSVgfp.wt    | 1                                | 1                                    | 1                                   | 1              | 1                                         | 1                                                |
| pLNRSVgfp.sin   | 0.5                              | 0.62                                 | 1                                   | 0.169          | 0.41                                      | 0.085 (12)                                       |
| pLgfpSNwt       | 1                                | 1                                    | 1                                   | 1              | 1                                         | 1                                                |
| pLgfpSNsin      | 0.5                              | 0.18                                 | 0.18                                | 0.03           | 0.032                                     | 0.015 (67)                                       |
| pLgfpMNsin      | 0.5                              | 0.3                                 | 0.21                                | 0.062          | 0.063                                     | 0.062 (16)                                       |
| pLNsgfp.wt      | 1                                | 1                                    | 1                                   | 1              | 1                                         | 1                                                |
| pLNsgfp.sin     | 0.5                              | 0.18                                 | 0.21                                | 0.074          | 0.015 (67)                                | 0.091 (11)                                       |

\(^a\)Values obtained with the wild-type vectors were set to 1. These parameters were determined as described in the legends to Figs. 2, 3, 4, and 5.

\(^b\)The overall decrease in eGFP expression was expressed as the relative MFI \( \times \) relative number of eGFP-producing cells.

\(^c\)The overall decrease in full-length RNA production was expressed as the relative efficiency of transfection \( \times \) relative RNA titer.
Preliminary experiments were performed to determine the purity of cytoplasmic and nuclear RNA fractions. The pre-mRNA levels of endogenous mouse GAPDH in the nuclear and cytoplasmic fractions of extracted RNAs were determined by real-time RT-PCR using primers spanning the intronic sequences of pre-GAPDH. Because pre-GAPDH RNA is exclusively nuclear, it can be used to identify contamination of cytoplasmic fractions by nuclear RNAs. To prepare cytoplasmic fractions, the cell lysis protocol was optimized to ensure that the level of pre-mRNA in the supernatant was less than 1% that in the nuclear pellet. To prepare the nuclear fractions, lysis conditions were chosen such that the ratio of pre-mRNAs in the supernatant and nuclear pellet was relatively constant over a specific time period. Normalization of all samples was carried out with primers specific for 18 S rRNA. In addition, total cell lysates and nuclear and cytoplasmic fractions were analyzed by Western blotting with antibodies against α-tubulin and histone H4 (Fig. 6A). As expected, histone H4 was detected in the nuclear fraction but not in the cytoplasmic fraction, whereas α-tubulin was detected in the cytoplasmic fraction but not in the nuclear fraction.

The results of these experiments showed that the levels of full-length SIN vector RNA (pLgfpSNsin) in the nucleus and cytoplasm of transfected cells decreased by 6- and 36-fold, respectively, compared with those of the parental vector (pLgfpSNwt) (Fig. 6, B and C). However, the measurement of internal transcript levels revealed a similar decline in the nucleus and cytoplasm of transfected cells (3-fold; Fig. 6, B and C). This indicates that SIN vectors have an additional defect in the nuclear-cytoplasmic transport of genomic RNA molecules. The difference in the reduction of full-length and internal RNA transcripts in the nuclei of transfected cells was 2-fold (a 6- and 3-fold decrease, respectively). This may be due to differences in selection, as only the internal RNA transcript mediates the expression of the neo gene. In addition, we determined which defect was complemented, 3' RNA processing or nuclear
Mo-MLV U3 Sequences Are Involved in Viral RNA Nuclear Export

export, for the pLgfPMNsin vector at the RNA level. As shown in Fig. 6D, the levels of full-length RNA transcripts in the nucleus and cytoplasm of transfected cells decreased by the same magnitude (13-fold) compared with those of the parental vector (pLgfSNwt), indicating that U3 sequences restore nuclear export of full-length viral transcripts.

Thus, the decrease in full-length SIN vector RNA (pLgfSNsin) in the cytoplasm of transfected cells (36-fold, Fig. 6C) corresponded with reductions in expression efficiency (31-fold; Fig. 5, B and C; Table 1) and RNA titer (33-fold, Fig. 4C). Also, the decline in full-length RNA nuclear export (6-fold, 36/6) was consistent with the difference in the efficiency of expression between full-length and internal RNA transcripts (5.6-fold) as well as the levels of complementation observed for vectors bearing the internal U3 region (4.2-, 6.1-, and 5.3-fold for pLgfPMNsin, pLNMSgfp.sin, and pLNRSVgfp.sin, respectively). It should be noted that a 5-fold difference in the efficiency of nuclear export between full-length and internal RNA transcripts aptly explains the difference (2.5-fold) in transfection efficiency between the pLgfSNsin (2-fold) and the pLNSgfp.sin (5-fold) and pLNEFgfp.sin (5-fold) vectors when the ratio of their transcription rate is 2:1.

DISCUSSION

Here, we studied the steps in the retroviral life cycle that are affected by the deletion of sequences in the U3 region of the 3’ LTR. The results indicate that sequences within the U3 region, which are usually deleted in the SIN configuration of retroviral vectors, are involved in at least two different stages of retroviral RNA production. First, the levels of full-length and internal RNA transcripts of the U3-minus Mo-MLV-derived retroviral vectors decreased in the nuclei of transfected cells, an effect that is probably due to a general defect in 3’ RNA processing, as has been suggested before (5). Second, nuclear-cytoplasmic transport of the full-length RNA transcript was also impaired, indicating that Mo-MLV has a specialized mechanism for transporting genomic RNA molecules from the nucleus to the cytoplasm of infected cells.

Deleting the U3 sequences adjacent to the R region resulted in only a 2-fold reduction in viral titer (114 bp, pDSVneoΔXS; Fig. 1), whereas deleting most of the U3 sequences reduced the viral titer by 11-fold (381 bp, pDSVneoΔNS; Fig. 1). In the analogous vector, which harbored an extended U3 deletion (pLgfPNsin, Fig. 4), a 20-fold reduction in transfected DNA led to an even greater reduction in the virus titer (33-fold). Taken together, these results are in good agreement with the measurements of the level of full-length RNA in the nuclei of cells transfected with the pLgfPNsin vector (6-fold decrease, Fig. 6B) as well as with the results of other studies, demonstrating that the introduction of an additional polyadenylation signal into SIN vectors leads to no more than a 2-fold increase in the viral titer (5, 8, 13). These results also suggest that using high concentrations of plasmid DNA for transfection substantially compensates for the defect in 3’ RNA processing. This situation may be somewhat similar to those that occur when proviral DNAs integrate into transcriptionally active regions of the cellular genome, as this defect was weakly manifested in the target cells (Fig. 2C). One explanation for this could be the increased concentration of specific cellular factors within compartments that support high transcriptional activity. The 2-fold difference in the reduction of full-length and internal RNA transcripts for the pLgfSNsin vector (Fig. 6B) in the nuclei of transfected cells is likely to reflect differences in selective pressure rather than to result from other factors as it contradicts the above results.

Examining the efficiency of expression and viral RNA molecules in cells transfected by SIN vectors showed that Mo-MLV full-length RNAs harboring extensive deletions within U3 are defective in nuclear-cytoplasmic transport. As was shown for two different retroviral constructs, this defect can be complemented by transferring the U3 region to another position within a retroviral vector. This indicates that the U3 region contains position-independent cis-acting sequences that are required for the transport of viral genomic RNAs. The results also suggest that RSV U3 sequences can complement this function with the same efficiency. These data are consistent with earlier work showing that after the introduction of an additional polyadenylation signal in the U3-minus vector of a related retrovirus (SNV), the titer was still 5-fold lower than that of the parental vector (5). For SNV, an unusually high frequency of reconstitution of the 3’ LTR in U3-minus vectors was observed (11). The necessity of a complete U3 region for maintaining the life cycle of retroviruses may explain the strict selectivity for reversion. Other studies also demonstrate that, in addition to extra polyadenylation signals, replacing the 5’ U3 region of SIN γ-retroviral vectors with a strong promoter-enhancer can markedly increase viral titers (8, 9, 12).

Impaired nuclear-cytoplasmic transport was observed only for full-length RNA transcripts. Full-length and internal RNA transcripts differ in terms of the 5’ end sequence, which includes the R, U5, and Ψ regions and part of the gag gene. This suggests that the leader region of Mo-MLV-derived vectors contains inhibitory sequences that prevent export and mediate the nuclear retention of full-length RNA. On the other hand, the internal transcript, which contains no inhibitory sequences, was efficiently transported to the cytoplasm and translated. This is consistent with other reports demonstrating that the efficiency of gene expression driven by identical internal promoters is similar for both γ-retroviral and lentiviral SIN vectors (14).

Viral sequences involved in the cytoplasmic accumulation of full-length RNA were found in the 5’ R region of murine leukemia viruses (SL3), known as the R region stem-loop (RSL) (18–20). The first 32 nucleotides of the R region form a secondary structure that is crucial for its activity. Deleting the RSL from the wild-type SL3 genome led to a specific reduction in the amount of cytoplasmic full-length viral transcripts (by ~5-fold) (20). It is important to note that including the 258-nucleotide downstream of the 5’ LTR in the intronless constructs increased the effect of the RSL by 2-fold in NIH3T3 cells and by 3–5-fold in CHO and K562 cells (18). Studies of a related retrovirus show that the SNV RU5 sequences facilitate the Rev/RRE-independent expression of intron-containing HIV-1 gag-sequences (21–24). Furthermore, similar properties were observed for the MPMV RU5 and HFV R regions (25, 26). The R and U3 regions are located at the ends of the retroviral genome. It is possible that the sequences within the regions that
are involved in the nuclear export of genomic RNAs are elements of the same system and that they perform similar or (and) different functions in this process. It is interesting to note that the use of the SFFV U3 region as an internal promoter seems to increase the titers of both Mo-MLV-derived and HIV-derived SIN vectors (9, 14).

The involvement of the ψ region in the nuclear export of Mo-MLV genomic RNA has been studied in two different systems (27, 28). One study showed that the deletion of stem-loop B or C (from four cis-acting stem-loops in the ψ region) leads to the accumulation of genomic RNAs in the nucleus (27). The results of another study indicate that most full-length RNAs are localized in the nucleus and that the deletion of different stem-loop motifs (from A to D), either alone or in combination, has different effects on their nuclear export. Deleting the C and D motifs produced the greatest increase in transport and the greatest decrease in splicing, deleting motifs A and B had an average effect, and deleting all four motifs did not affect the cytoplasmic level of full-length RNAs but led to a substantial increase in splicing (28). These data agree with the assumption that the leader region, including ψ, contains inhibitory sequences that are likely involved in regulating the splicing and nuclear export of full-length viral RNAs, and these sequences do not overlap with the splice donor site.

The majority of RNAs (pre-mRNA) contain introns, and their transport from the nucleus to the cytoplasm usually occurs only after splicing. Similar to retroviral genomes, specialized nuclear export mechanisms would allow alternatively spliced molecules to overcome the retention of unspliced forms of RNA in the nucleus. According to our experiments, RNA molecules (internal transcripts) that do not contain the inhibitory sequences and splice signals can be effectively expressed. Conversely, RNA molecules (full-length transcripts) that contain inhibitory sequences require a specific mechanism for nuclear export. Studies of the RSL properties were carried out using the pCAT3Basic construct, which is likely to contain inhibitory sequences in the long terminal repeat of a murine retrovirus specifically for efficient retrovirus-mediated gene transfer in avian cells revealed by self-inactivating vectors. J. Gen. Virol. 74, 39–46

To date, the mechanism underlying the nuclear export of γ-retroviral genomic RNA molecules remains unidentified. The results presented herein provide the first opportunity to study its properties. Research into the various retroviral elements responsible for nuclear export has raised questions about the general principles of this process and how its specific features affect the other functions of viruses. These data should also be useful for improving retroviral vectors, expression systems, and retroviral packaging constructs.

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