The U3 Region Is Not Necessary for 3' End Formation of Spleen Necrosis Virus RNA

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Primary transcripts of retroviruses contain two poly(A) sites, one near the 5' and one near the 3' end of the transcript, but only the 3' poly(A) site is used for 3' end formation of viral RNA. It was hypothesized on the basis of experiments with U3-deleted vectors of spleen necrosis virus that the U3 region contains sequences required for this RNA 3' end formation: the titer of a U3-deleted vector was 150 times lower than that of the parental vector, and the addition of the simian virus 40 poly(A) signal sequence increased the titer of the U3-deleted vector (J. P. Dougherty and H. M. Temin, *Proc. Natl. Acad. Sci. USA* 84:1197–1201, 1987). However, we now show that the U3 region transcribed from the 3' long terminal repeat is not required for RNA 3' end formation and that the experiments of Dougherty and Temin led to an erroneous conclusion. We show here that the deletion of the U3 region did not decrease the steady-state level of viral RNA or shift the site of poly(A) addition. The added simian virus 40 poly(A) signal sequence was used preferentially over the poly(A) signal of spleen necrosis virus, and it increased the levels of RNA transcribed from vectors with and without deletion of the U3 region. Our results indicate that alteration of regulatory sequences in retroviral vectors can change the steady-state RNA levels and titers of the vectors in an unpredictable manner.

Retroviruses are RNA viruses that replicate through a DNA intermediate, the provirus. Retroviral replication consists of two phases of genome synthesis, involving two sets of enzymes. The first phase synthesizes the DNA genome from the RNA genome by using the virus-encoded reverse transcriptase; the second phase synthesizes the RNA genome from the DNA genome by using cellular enzymes.

The provirus has long terminal repeat (LTR) sequences at both ends. Although the LTR sequences are identical, they play different roles in the synthesis of viral RNA. Viral RNA synthesis is initiated at the 5' end of the R region in the 5' LTR, and viral RNA is polyadenylated at the 3' end of the R region transcribed from the 3' LTR.

With spleen necrosis virus (SNV) vectors containing an internal simian virus 40 (SV40) promoter, it was found that deletion of the U3 region in the 3' LTR resulted in a decrease in the virus titer by a factor of 150 relative to the titer in a wild-type SNV vector (5). Furthermore, addition of the SV40 late poly(A) signal sequence at the 3' end of the U3-deleted vector resulted in a titer almost equal to that of the wild-type vector (5). These two observations led to the hypothesis that the U3 region contains sequences required for 3' end formation of retroviral RNA (5).

Deletion of the U3 region did not affect expression of SNV vector RNA. To localize the sequences required for 3' end formation of SNV RNA, we constructed a series of SNV-derived retroviral vectors that contained deletions of various lengths in the U3 region in the 3' LTR. These vectors contained deletions of 53, 124, 188, and 404 bases in the U3 region in the 3' LTR. D17 cells (a dog osteosarcoma cell line which is permissive for SNV replication) were transfected with these plasmids as described previously (12). Approximately 48 h after transfection, cytoplasmic RNA was isolated from the cells (1) and was treated with DNase I to digest the contaminating plasmid DNA. The RNA was

analyzed by Northern (RNA) blot hybridization, as described previously (11). All deletion mutants expressed RNA of the size expected if there was normal 3' end formation and at a level similar to that of the parental vector (data not shown).

Since this result was inconsistent with the hypothesis of Dougherty and Temin (5), we tested the ability of the U3-deleted LTR to form the 3' end of the viral RNA. pJD220SVHy (Fig. 1A) is a derivative of SNV vectors deleted for the U3 region in the 3' LTR. The SV40 early promoter driving expression of the gene encoding hygromycin phosphotransferase B resistance (Hygro^r) is located in this vector. The SV40 late poly(A) signal sequence is positioned 3' to the U3-deleted LTR. Thus, even if the deletion of the U3 region destroyed the normal 3' end formation of viral RNA, the SV40 poly(A) sequence should replace that function. To generate stocks of JD220SVHy virus, DSN helper cells (6) were transfected with pJD220SVHy and were selected with hygromycin (Fig. 1A). After the selected helper cells became confluent, the virus was harvested. D17 cells were infected with this virus and were selected with hygromycin. During infection of D17 cells, the U3 sequence in the 5' LTR of the provirus was not formed because of the U3 deletion in the parental viral genome; consequently, RNA was transcribed only from the SV40 internal promoter in the infected D17 cells. RNA was isolated from the hygromycin-resistant D17 cells and was analyzed by Northern blot hybridization. If the SNV poly(A) signal sequence in the provirus with the U3 deletion was functional for RNA 3' end formation, RNA transcribed from the JD220SVHy provirus would be homogeneous in size in the infected cells (1.3 kb [Fig. 1A]). However, if the LTR with the U3 deletion was not functional for RNA 3' end formation, RNA from JD220SVHy would be heterogeneous in size because the RNA would be processed at poly(A) sites in the cellular genome and not at the normal poly(A) site of the vector (Fig. 1A). As a positive control, DSN helper cells were transfected with pJD214Hy, the parental vector containing an

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FIG. 1. (A) Protocol to test RNA 3' end formation in the U3-deleted LTR. Symbols: $\square$, LTRs; —, viral sequences; ▨ , gene encoding hygromycin phosphotransferase resistance (Hygro^r); ▩ , SV40 poly(A) signal sequence; ▧ , the SV40 early promoter; —, expected RNAs; ▬ , flanking sequences either in the plasmid or in the cellular genome; and AAAA, poly(A) tails. The size (in kilobases) of the expected RNA is shown above the line. (B) JD220SVHy RNA in D17 cells after infection. D17 cells were infected with helper-free viruses of JD214Hy or JD220SVHy (harvested from DSN helper cells) and were selected with 80 μg of hygromycin per ml (Calbiochem). RNA isolation and Northern blot hybridization were performed as described previously (1, 11). The names of the vectors and the time (in hours) of exposure are listed above the lanes. The positions of marker RNAs are listed on the left side. In the JD214Hy lane (63 h of exposure) of the blot, a band larger than 9.5 kb was detected. We do not know the nature of this band.

intact U3 region in the 3' LTR; the RNA from a JD214Hy provirus was expected to be 2.2 kb (Fig. 2, row 1).

The results of Northern analysis are shown in Fig. 1B. RNA from JD214Hy-infected cells was 2.3 kb, as expected. RNA from JD220SVHy-infected cells was 1.6 kb. This size is consistent with the poly(A) site being functional for RNA 3' end formation [1.3 kb plus the poly(A) tail]. Even on an autoradiogram that was exposed for 63 h, no other bands and no smearing pattern were observed (Fig. 1B).

Bands from this blot were excised, and the ^{32}P counts were measured and normalized to the counts of hybridization to the RNA of the endogenous β -actin gene (10). When the signal of JD214Hy was taken as 1.00, the signal of JD220SVHy was 0.16. This difference could be explained as the result of a difference of promoter strengths (3). The result of this experiment suggested that the U3 region is not necessary for 3' end formation of SNV RNA and was inconsistent with the hypothesis of Dougherty and Temin, that is, after infection of cells by JD220SVHy, signals for RNA 3' end formation should be provided by the cellular genome (5). To provide further evidence on the properties of the U3-deleted LTR, we analyzed RNA of a different U3-deleted SNV vector by Northern blot and RNA protection assays.

D17 cells were transfected with JD214Hy, KI3' Δ 0, and JD217Hy (Fig. 2, rows 1, 2, and 4). Forty-eight hours after transfection, RNA was isolated and was subjected to Northern blot hybridization (Fig. 3A, lanes 1, 2, and 4). Transfection efficiencies were normalized to levels of chloramphenicol acetyltransferase (CAT) activity resulting from cotransfection with pCMV-CAT (Fig. 3B).

To quantify data from Northern blot analyses, the same

experiment was repeated at least three times for each construct (Fig. 2). When the level of JD214Hy RNA was taken as 1.0 (Fig. 2, row 1), the level of KI3' Δ 0 RNA was 1.0 (Fig. 2, row 2), suggesting that the insertion of an *Xho*I linker itself did not affect the level of steady-state RNA. The level of JD217Hy RNA was 0.8 (Fig. 2, row 4). This result also supported the hypothesis that the deletion of the U3 region did not significantly decrease the steady-state RNA level.

An RNA protection assay was employed to locate the positions of poly(A) sites in KI3' Δ 0 and JD217Hy (Fig. 2, rows 2 and 4). The probes used are shown in Fig. 4A. With a KI220 probe with KI3' Δ 0 RNA, a 526-base protected fragment (fragment 2) was expected if the poly(A) site was positioned at the 3' end of the R region. With a JD220Hy probe with JD217Hy RNA, a 419-base protected fragment (fragment 4) was expected if the poly(A) site was positioned at the 3' end of the R region.

With KI3' Δ 0 RNA, the protected fragment was about 530 bases (Fig. 4B, lane 1), indicating that the poly(A) site was at the 3' end of the R region, as expected. With JD217Hy RNA, the protected fragment was about 420 bases, (Fig. 4B, lane 6), indicating that the deletion of the U3 region did not alter the location of the poly(A) site. No other band appeared or increased significantly in level in this lane (Fig. 4B, lane 6) compared with background bands, (Fig. 4B, lane 8). RNA transcribed through the SNV poly(A) site in JD217Hy (483-base fragment) could not be detected; at least, the pattern of the bands around 480 bases was not different from the pattern of the background bands (Fig. 4B, cf. lanes 6 and 8). These results demonstrate that RNAs were polyadenylated at the 3' end of the R region in the presence or absence of the U3 region.

FIG. 2. Structures and expected sizes of vector RNAs. pJD214Hy, pJD217Hy, pJD220Hy, pJD217SVHy, and pJD220SVHy were described previously (5). The U3 region in the 3' LTR was deleted by replacing the *SacI*-*AvaI* fragment in the LTR with an *XhoI* linker (5'-CCTCGAGG-3'). pKI3'Δ0 was made by inserting an *XhoI* linker into the *AvaI* site (Klenow treated) at the junction between the U3 and R regions in the 3' LTR of pJD214Hy. Other KI constructs were made on the same basis as the constructs described above. Recombinant DNA techniques were as described by Maniatis et al. (13). Details of the constructions will be given on request. Symbols are as described for Fig. 1. A, C, H, SI, SII, Xb, and Xh, *AvaI*, *Clal*, *HindIII*, *SacI*, *SacII*, *XbaI*, and *XhoI* sites, respectively. The asterisk (*) shows the position of the *XhoI* linker. The sizes (in kilobases) of the RNAs are shown below the solid lines. The relative RNA levels were determined as follows: D17 cells were cotransfected with each SNV vector construct and pCMV-CAT (pwt760 in reference 15); transfection was performed as described previously (12). RNA was isolated as described elsewhere (1) and was treated with DNase, separated by electrophoresis, and blotted to nitrocellulose (11). The nitrocellulose was hybridized to the radioactive *XbaI*-*Clal* fragment of the Hygro^r gene labeled by a random priming procedure (8). The relative RNA levels were obtained by normalizing the level of RNA of each construct to the level of JD214Hy RNA, which was taken as 1.00; transfection efficiencies were normalized by the relative levels of CAT activity. The relative RNA levels were mean values of at least three experiments. The standard deviations were less than 60% of the mean values.

Further analysis of readthrough RNA at the SNV 3' poly(A) site showed that deletions of the SNV genomic sequences including the U3 region in KI3'Δ0 (Fig. 2, row 2) increased the percentage of read-through RNA from 4% to 18% (K. Iwasaki and H. M. Temin, *Genes Dev.* in press). This result was consistent with the different levels of steady-state RNA of JD217Hy and KI3'Δ0 (Fig. 2, rows 2 and 4).

The SV40 poly(A) sequence altered expression of vector RNAs with and without the U3 deletion. Dougherty and Temin previously reported that the SV40 late poly(A) signal sequence increased levels of steady-state RNA synthesized from SNV vectors containing a U3 deletion in the 3' LTR (5). They hypothesized that the increase of the RNA levels of these vectors was the result of compensating for the lack of RNA 3' end formation by the SNV sequence with the SV40 poly(A) signal sequence (5). Therefore, to test the effects of addition of the SV40 poly(A) sequence downstream of the vectors, the levels of RNA of KI3'Δ0, KI220, JD217Hy, and JD220Hy (Fig. 2, rows 2 through 5), were investigated by transfection and Northern blot analysis as described above. (Transfection efficiencies were normalized to levels of CAT activity resulting from cotransfection with pCMV-CAT [Fig. 3B].) The addition of the SV40 poly(A) signal sequence increased the steady-state levels of RNA of vectors with and without a U3 deletion in the 3' LTR (KI3'Δ0 versus KI220 and JD217Hy versus JD220Hy [Fig. 2, cf. rows 2 and 3 and rows 4 and 5; Fig. 3A]).

An RNA protection assay (7) was used to test whether the addition of the SV40 poly(A) sequence shifted the location of the poly(A) site. The probes used are shown in Fig. 4A. With a vector containing an intact U3 region in the 3' LTR, the poly(A) site was at the 3' end of the R region, as described above (Fig. 4B, lane 1). When the SV40 sequence was added to the 3' end of the vector, most of the RNA was polyadenylated at the poly(A) site of the SV40 sequence (Fig. 4B, lane 2). The amount of RNA of KI220 was increased compared with that of KI3'Δ0 as observed in the Northern blot analysis (Fig. 3A). For JD217Hy, the poly(A) site was also at the 3' end of the R region, as described above (Fig. 4B, lane 6). However, for JD220Hy (Fig. 4B, lane 7), most of the RNA was polyadenylated at the poly(A) site in the SV40 sequence and the RNA level was increased compared with that of JD217Hy (Fig. 4B, lane 6). The increases in amount of RNA were larger with the vectors containing a deleted U3 region than with the vectors containing an intact U3 region (Fig. 4B, cf. lanes 1 and 2 and lanes 6 and 7; Fig. 2, cf. rows 2 and 3 and rows 4 and 5).

These results demonstrated (i) that the SV40 poly(A) sequence was used preferentially over the SNV sequence for RNA 3' end formation when they were next to each other in the same constructs and the SV40 sequence was 3' to the SNV sequence and (ii) that the presence of the SV40 poly(A) sequence increased the level of RNA in the presence or absence of the U3 region.

Denome and Cole reported that when more than one poly(A) signal sequence are tandemly ligated and the distance between the poly(A) sites is short (about 250 bp), the downstream site is utilized predominantly over the upstream site (2). Our observation that the SV40 poly(A) site rather than the SNV poly(A) site was used is consistent with their observation.

However, in our case the SV40 poly(A) sequence also increased the steady-state level of RNA transcribed from the vectors containing it. Our preliminary results indicated that the increase of the RNA level by the addition of the SV40

FIG. 3. RNA of SNV vectors in D17 cells. (A) Northern hybridization analysis of SNV vectors. Transfection, RNA isolation, and Northern blot hybridization were performed as described in the legend to Fig. 2. The names of the vectors are listed above the lanes. The positions of marker RNAs are shown on the left side. (B) CAT assay to normalize transfection efficiency. The CAT assay was performed as described previously (9). The names of the SNV vectors cotransfected with pCMV-CAT are listed above the lanes.

poly(A) sequence was partly the result of a difference in RNA stability (data not shown).

The SV40 promoter sequence decreased levels of full-length

RNAs from vectors with and without the U3 deletion. One observation by Dougherty and Temin that supports the hypothesis that the U3 region transcribed from the 3' LTR is involved in 3' end formation of SNV RNA (5) is that the titer of an SNV vector containing the U3 deletion in the 3' LTR (JD217SVHy; Fig. 2, row 8) was 150 times lower than the titer of vectors containing an intact U3 region in the 3' LTR (JD214Hy; Fig. 2, row 1) and was 30 times lower than the titer of vectors containing the U3 deletion and the SV40 late poly(A) signal sequence (JD220SVHy; Fig. 2, row 9). Therefore, we tested whether the titer of JD217SVHy (Fig. 2, row 8) was lower than those of JD214Hy (Fig. 2, row 1) and JD220SVHy (Fig. 2, row 9).

C3 and DSN helper cell lines were transfected with the vector plasmids (6, 16). Viruses were harvested at 65 and 115 h after transfection, and D17 cells were infected with these viruses. After hygromycin selection of the infected D17 cells, the number of colonies was counted. Although the absolute titers varied from experiment to experiment (there were seven experiments), the relative titers of the vectors were similar in all the experiments. The average relative titers for JD214Hy, JD217SVHy, and JD220SVHy were 1.00, 0.05 ± 0.04 , and 0.65 ± 0.28 (mean $\pm$ standard deviation), respectively. A Wilcoxon rank-sum test indicated that the differences were significant ($P = 0.016$ against

FIG. 4. RNA protection assay to analyze poly(A) sites of constructs. (A) Probes and protected fragments in RNA protection experiment. Symbols are as described in the legends to Fig. 1. When RNA is polyadenylated at the poly(A) site in the SV40 sequence, the protected fragment of the KI220 probe is 732 bases. When RNA is polyadenylated at the poly(A) site in the LTR sequence, the protected fragment of the KI220 probe is 526 bases. If RNA is transcribed through the SNV poly(A) site in KI3'Δ0 (Fig. 2, row 2), the protected fragment should be 590 bases. When RNA is polyadenylated at the poly(A) site in the SV40 sequence, the protected fragment of the JD220Hy probe is 625 bases. When RNA is polyadenylated at the poly(A) site in the LTR sequence, the protected fragment of the JD220Hy probe is 419 bases. If RNA is transcribed through the SNV poly(A) site in JD217Hy (Fig. 2, row 4), the protected fragment should be 483 bases. (B) RNA (0.5 μ g or as indicated at the top of the lanes) was hybridized to the probes shown in panel A. The names of the vectors and probes are listed above the lanes. Positions of marker RNA are shown on the left side. Positions of the specific bands are indicated by arrows. The other bands are background, because they appear in the lanes labeled "none" (RNA protection assay with RNA taken from pCMV-CAT-transfected cells). Numbers next to the arrows are the numbers of the protected fragments in panel A. The RNA protection assay was performed as described previously (7). RNA probes were synthesized from the T3 promoter of pBluescript by T3 RNA polymerase (Stratagene) in the presence of [α - 32 P]UTP. After hybridization to probes (6×10^5 cpm), the RNA was digested with 10 μ g of RNase A and 3.2 μ g of RNaseT₁ per ml, separated in a 3% polyacrylamide-7 M urea gel, and analyzed by autoradiography.

FIG. 5. RNA from D17 cells that contain SNV vectors having the SV40 promoter. (A) Northern hybridization analysis of the SNV vectors. Transfection, RNA isolation, and Northern blot hybridization were performed as described in the legend to Fig. 2. The names of the vectors are listed above the lanes. The positions of marker RNAs are shown on the left side. (B) CAT assay to normalize transfection efficiency. The CAT assay was performed as described previously (9). The names of SNV vectors cotransfected with pCMV-CAT are shown above the lanes.

the hypotheses of no difference between JD214Hy and JD217SVHy and of no difference between JD217SVHy and JD220SVHy). These results are consistent with the previous results (5).

Cytoplasmic RNA from the transfected helper cells was isolated at the same time as the viruses were harvested and was analyzed by Northern blot analysis. The steady-state levels of full-length RNAs of JD217SVHy and JD220SVHy correlated with the titers of the vectors (data not shown).

Since the steady-state level of full-length RNA of JD217SVHy was eight times lower than that of full-length RNA of JD217Hy (Fig. 2, rows 8 and 4), it appeared that insertion of the SV40 promoter reduced the levels of full-length RNA of the vectors. Therefore, we decided to investigate the effects of insertion of the SV40 early promoter into other vectors. Two vectors containing the SV40 promoter were constructed, pKI3'Δ0SV and pKI221SV (Fig. 2, rows 6 and 7). The RNA levels of KI3'Δ0, KI3'Δ0SV, KI221SV, JD217Hy, JD217SVHy, and JD220SVHy (Fig. 2, rows 2, 6, 7, 4, 8, and 9) were investigated by transfection and Northern blot analysis as described above. (Transfection efficiencies were normalized to the levels of CAT activity resulting from cotransfection with pCMV-CAT [Fig. 5B].) The presence of the SV40 promoter decreased the level of full-length RNA from KI3'Δ0SV 2.5 times compared with the level in KI3'Δ0 (Fig. 5A, cf. lanes 2 and 1; Fig. 2, cf. rows 6 and 2). An internal transcript was also present in the transfected cells (Fig. 5A, lane 2). The addition of the SV40 poly(A) sequence to KI3'Δ0SV, giving KI221SV, increased the steady-state levels of full-length and internal RNAs 3.3- and 5.5-fold, respectively, compared with those of KI3'Δ0SV (Fig. 5A, cf. lanes 3 and 2; Fig. 2, cf. rows 7 and 6).

The presence of the SV40 promoter also decreased the level of full-length JD217SVHy RNA eight times compared with that in JD217Hy (Fig. 5A, cf. lanes 5 and 4; Fig. 2, cf. rows 8 and 4), and the magnitude of the decrease was larger than that for the vector containing the U3 region, KI3'Δ0SV (Fig. 2, cf. rows 8 and 6). The addition of the SV40 poly(A) sequence to JD217SVHy, giving JD220SVHy, increased the level of RNAs in JD220SVHy, four- and sixfold for full-length and internal RNAs, respectively, compared with levels in JD217SVHy (Fig. 5A, cf. lanes 5 and 6, and Fig. 2, cf. rows 8 and 9).

The internal transcripts initiated from the SV40 promoter tended to be larger than expected (JD220SVHy RNA in Fig. 1; Fig. 2, rows 6 through 9; Fig. 5A, lanes 2, 3, 5, and 6). We analyzed the transcripts by an RNA protection assay. The transcripts were initiated at the proper site in the SV40 promoter and were polyadenylated at the proper poly(A) site of the SNV or SV40 poly(A) sequence (data not shown). Therefore, this size discrepancy may reflect the length of the poly(A) tails or poor resolution of the Northern gels.

Other hypotheses to explain the difference between the 5' and 3' poly(A) sites in retroviral RNA. In a previous study (5), an S1 nuclease assay did not reveal any discrete JD217Hy RNA. However, we found in Northern blot and RNA protection assays RNA of a size consistent with normal 3' end formation. Possible reasons that the previous S1 nuclease assay did not detect any signals from JD217Hy and JD217SVHy are that different probes were used to detect RNA transcribed from different constructs and that the protected fragments of JD217Hy and JD217SVHy in the S1 assay are 150 bases, not 140 bases as described in reference 5. (During our work, we found discrepancies in the RNA sizes from the expected sizes and in the presence and absence of several restriction enzyme sites. We sequenced the vectors used in this report and in the previous study [5]. We found that these vector sequences were different from the previously published sequences [14] and the sequences expected from the description of construction of the vectors [4]. [The sequencing data are not shown.] A faint band exists at around 150 bases in the lanes of both JD217Hy and JD217SVHy in the S1 gel shown in Fig. 3 of reference 5.) Therefore, we now conclude that the U3 region is not required for 3' end formation of the SNV RNA and that the experiments described in reference 5 led to an erroneous conclusion.

Thus, other hypotheses are necessary to explain the functional difference between the 5' and 3' poly(A) sites of the retroviral genome. Here we present three hypotheses. First, the RNA 3' end formation function of retroviruses is inefficient and allows a high level of readthrough RNA at both poly(A) sites. Second, non-LTR regions in the retroviral genome cause the functional difference between the two poly(A) sites. Third, the short distance between the cap and 5' poly(A) sites prevents 3' end formation of viral RNA at

the 5' poly(A) site. Our later experiments support hypothesis 3 (Iwasaki and Temin, in press).

Consequences for construction of retroviral vectors. The deletion of the U3 region by itself did not change the level of RNA of the vector (Fig. 2, cf. rows 2 and 4), and it did not alter the location of the poly(A) site of the vector RNA (Fig. 4B). These observations suggest that the U3 region is not necessary for 3' end formation of SNV RNA. The addition of the SV40 poly(A) signal sequence increased the levels of RNA transcribed from SNV vectors containing a deleted U3 or an intact U3 region. This phenomenon is not explained simply by compensation of the RNA 3' end formation function of the SNV poly(A) signal sequence by the SV40 poly(A) signal.

In addition, the magnitude of the decrease of the level of vector RNA by the presence of the SV40 promoter was larger for the vectors containing a deleted U3 region than for the vectors containing the intact U3 region (Fig. 2, cf. rows 6 and 8). The addition of the SV40 poly(A) signal sequence to these vectors also increased the levels of RNA transcribed from vectors containing a deleted or an intact U3 region. The magnitude of the increase in amount of vector RNA by the SV40 poly(A) sequence was slightly larger for the vectors containing a deleted U3 region than for the vectors containing the intact U3 region (Fig. 2, cf. rows 6 and 7 and rows 8 and 9). Compensation of the RNA 3' end formation function of the SNV poly(A) signal sequence by the SV40 poly(A) signal sequence was not sufficient to explain this phenomenon. Therefore, our results indicate that deletion and addition of regulatory sequences in retroviral vectors can change both the levels of steady-state RNA synthesized from the vectors and the vector titers in an unpredictable manner.

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