Retrotransposition of Nonviral RNAs in an Avian Packaging Cell Line

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Retroviruses produced from the quail packaging cell line SE21Q1b predominantly contain cellular RNAs instead of viral RNAs. These RNAs can be reverse transcribed and integrated into the genomes of newly infected cells and are thereafter referred to as newly formed retrogenes. We investigated whether retrogene formation can occur within SE21Q1b cells themselves and whether this occurs intracellularly or via extracellular reinfection. By using packaging cell line mutants derived from the SE21Q1b provirus and selectable reporter constructs, we found that the process requires envelope glycoproteins and a retroviral packaging signal. Our results suggest that extracellular reinfection is the primary route of retrotransposition of nonviral RNAs.

Mobile genetic elements play a significant role in shaping eukaryotic genomes (4). These elements may provide a genome with plasticity for adapting to a changing environment. Mobile elements that transpose via an RNA intermediate require reverse transcription and are called retrotransposons. Retrotransposons are divided into viral (long terminal repeat [LTR]) and nonviral (non-LTR) families (35). The former include short and long interspersed nuclear elements. Processed pseudogenes are deficiency in viral envelope glycoproteins and we developed reporter genes to interact in cis with the viral proteins in order to detect retrotransposition events. By using a genetic selection scheme, we could not find evidence for an intracellular pathway for autoretrofection. However, we found that cellular RNAs that contain a packaging sequence undergo autoretrofection via extracellular reinfection.

MATERIALS AND METHODS

Recombinant proviruses and packaging cell lines, pSEEmv+ and pSEEmv− are recombinant proviral molecular constructs derived from the SE21Q1b proviral molecular clone. The SE21Q1b proviral molecular clone, pSE21Q1b, has been previously described (1). pSEEmv+ was constructed by replacing the pSE21Q1b KpnI-MluI fragment with a 2.2-kb fragment from pRCAStB (23). A hygromycin phosphotransferase-encoding gene (hph) was added to enable selection. A 1.3-kbp hph gene was removed from pBSHgy by SalI and Spel digestion (12). Clal linkers were attached and the 1.3-kbp fragment was ligated to the Clal site in the 2.2-kb KpnI-MluI fragment. pSEEmv− was constructed by replacing the pSE21Q1b KpnI-MluI fragment with a fragment containing a deletion in the envelope. An 800-bp XbaI-NcoI fragment was removed from the KpnI-MluI fragment to create an out-of-frame env deletion. pSEEmv+pol−1 and -2 were constructed by digesting pSEEmv+, which has a restriction site in pol, with HpaI, followed by Bal31 exonuclease digestion and blunt-end ligation. Proviral molecular clones were sequenced to identify deletions. Clone 1 contains a 20-bp deletion (starting at position 2720 of avian leukosis virus [ALV]; GenBank accession no. M37980), and clone 2 has a 31-bp deletion (starting at position 2723 of ALV). Both pol mutants lead to frameshifts that are predicted to result in premature termination. Mass cultures of packaging cells were established by transfecting the viral constructs into quail QT35 cells and selecting colonies that were resistant to 100 µg of hygromycin/ml (3). Packaging cells were named QTSEEmv+, QTSEEmv−, and QTSEEmv+pol−1 and -2.

Construction of selectable reporter genes, transfection, autoretrofection assay, and coculture. To create the retrotransposition reporter construct p611, a 2.8-kb HindIII-NotI DNA cassette encoding a cytomegalovirus immediate-early (CMVie) promoter–reverse-oriented intron–phleomycin resistance gene was inserted into the unique SmaI site in pRSVneo (11) between neo and the simian virus 40 polyadenylation signal. This cassette is designed to allow phleomycin selection only after reverse transcription and integration of spliced RNAs transcribed from the LTR promoter, because a polyadenylation signal in the intron prevents the formation of readthrough transcripts from the CMV promoter.

p611 was constructed by replacing the LTR promoter of p611 with the LTR, primer binding site (PBS), and 3′-containing sequence from LA611, an ALV vector containing the phleomycin cassette (17). Both p611 and LA611 were digested with ScaI and NheI, and a 4-kb fragment from p611 was ligated to a 5-kb fragment from LA611 to construct pΨ+611. QT35 cells, SE21Q1b cells, and the quail packaging cell lines QTSEEmv+, QTSEEmv−, and QTSEEmv+pol− were transfected with p611 or pΨ+611, and mass cultures were selected in 150 µg of G418/ml. These cell lines were named...
Mass cultures were expanded, and 33 amide plus 10% dextran sulfate at 42°C with 10^6 cpm of probe/ml. Filters were analyzed after 32P random-primed DNA labeling (Boehringer Mannheim, Indianapolis, IN). Enhanced chemiluminescence (ECL) was performed in Stark’s buffer, which consists of 50 mM NaCl, 5 mM EDTA, 10 mM Tris-Cl (pH 7.4), 0.5% sodium dodecyl sulfate, and 200 µg of salmon sperm DNA/ml, and 50% formamide.

Southern blot analysis and hybridizations. Genomic DNAs (15 µg) were digested with the restriction enzymes Apal and NsI and then transferred on to GeneScreen (NEN, Boston, Mass.) hybridization transfer membrane by alkaline transfer (21) and hybridized with 32P-labeled DNA probes. Hybridizations were performed in Stark’s buffer, which consists of 5× SSC (0.75 M NaCl, 75 mM Na3 citrate [pH 7.0]), 25 mM NaHPO4, 0.02% bovine serum albumin, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 250 µg of salmon sperm DNA/ml, and 50% formamide plus 10% dextran sulfate at 42°C with 10^6 cpm of probe/ml. Filters were washed in 0.2× SSC at 65°C and exposed to X-ray film at −80°C. Both a 1.4-kb DNA fragment spanning the CMVie promoter and phleomycin gene and an EcoNI-Xhol 500-bp DNA intron fragment from p611 were used as probes after 32P random-primed DNA labeling (Boehringer Mannheim, Indianapolis, IN). Prior to hybridization with the 32P-labeled 500-bp probe, the 32P-labeled 1.4-kb probe was removed from the Southern blot by incubating the blot three times in 10 mM Tris-Cl (pH 8.0), 1 mM EDTA, and 0.1% sodium dodecyl sulfate for 10 min at 90°C.

Nestred PCR. Nested PCR analysis (see Fig. 2B, bottom) for the detection of the retrotransposed reporter gene was performed as follows. Total cellular DNAs (200 ng) were used in nested PCRs. The first-round PCR mixture consisted of 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 2 mM MgCl2, 50 µg of gelatin/ml, 0.1 mM deoxynucleoside triphosphates, 1 U of AmpliqTaq (Perkin-Elmer), Branchburg, N.J.), 100 ng of PH2 primer (5’GGCGGTAGGCCTGTACGG TG3’), CMV2 (2 × 10^6 cpm) at >10^6 cpm/µg was included in a 75-µl reaction mixture overlaid with mineral oil. CMV2 was 32P end labeled by polynucleotide kinase and [γ-32P]ATP and then passed through a G-50 Sephadex column to rid it of unincorporated radionucleotides. Thermocycle incubation was carried out as described above except the first round except the 72°C step was 1.5 min and there were only 25 cycles.

Quantitative competitive PCR (QC-PCR) was performed on genomic DNAs that were digested with ClaI and Rnase A and then incubated in 50 mM NaOH at 65°C for 1 h, neutralized with an equal volume of 5 M NH4 acetate, and precipitated with ethanol. Precipitates were collected by centrifugation, and the concentration was determined spectrophotometrically. A 218-bp PCR fragment containing the sequences for primers PH1 and CMV2 at the ends was used as a competitive template. The 218-bp fragment was purified through low-melting-point agarose, quantified spectrophotometrically, and diluted with 50 µg of glycogen/ml to prevent DNA loss due to adsorption to tube walls. PCR was carried out with 200 ng of genomic DNA with various amounts of 218-bp competitor DNA. The PCR mixture consisted of 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 2 mM MgCl2, 50 µg of gelatin/ml, 0.1 mM deoxynucleoside triphosphates, 1 U of AmpliqTaq (Perkin-Elmer), and 100 ng each of primers PH1 (5’GAG ACCCGAAGCCGACCTGG3’) and CMV2 (5’GGCGGTAGGCCTGTACGG TG3’). CMV2 (2 × 10^6 cpm) at >10^6 cpm/µg was included in a 75-µl reaction mixture overlaid with mineral oil. CMV2 was 32P end labeled with [γ-32P]ATP and polynucleotide kinase and then passed through a G-50 Sephadex column to rid it of unincorporated radionucleotides. Thermocycle incubation was carried out by preincubating the reaction mixture at 95°C for 5 min and then thermocycling it at 94°C for 1 min, at 66°C for 1 min, and 72°C for 2 min for 30 cycles. The second-round PCR consisted of adding 2 µl of the initial PCR mixture to the above-described reaction mix after replacing the primers with the internal primers PH1 (5’GAG CACCGAAGCCGACCTGG3’) and CMV2 (5’GGCGGTAGGCCTGTACGG TG3’).

RESULTS

Construction of a selectable reporter gene to study autoretrofection. Figure 1 diagrams two possible autoretrofection pathways (B and C). One pathway is by an intracellular route (B), and the other is via extracellular reinfection (C). Packaging cell lines were derived from SE21Q1b proviral mutants that lack Psi and that either contain the envelope gene (env+) or lack a functional envelope gene (env−). In the absence of envelope glycoproteins, the intracellular route is predicted to be the predominant one.

The selectable reporter constructs p611 and pΨ+611 were used to identify cells which are capable of autoretrofection. Figure 2 shows the selectable reporter constructs p611 (Fig. 2A) and pΨ+611 (Fig. 2B). pΨ+611 has a completeLTR,
PB3, and the packaging sequence, Ψ. In both constructs Phl' is conferred if the intron is removed. The expected pathway leading to Phl' is diagrammed for pΨ+611 (Fig. 2B); a phleomycin resistance retrogene is formed after splicing of the RNAs, reverse transcription, and integration of cDNAs. The polyadenylation site in the intron prevents readthrough transcripts from the CMVie promoter, which encodes phleomycin resistance.

Env+ packaging cells are not capable of autoretrofection with RNAs lacking Ψ but form unusual episomal retrogenes. In order to test whether cellular RNAs can undergo autoretro-
rfection, p611 was transfected into SE21Q1b cells and QT35 cells. After selection in G418, SE21Q1b-derived SE611 and QT35-derived QT611 cells were obtained and expanded into mass cultures which were treated with phleomycin. As presented in Table 1, approximately three SE611 Phlr clones were obtained per $6 \times 10^6$ cells. This frequency is 10 times greater than that obtained with nonvirus-producing QT611 cells.

PCR was performed on some of these clones and mass cultures with primers that flank the splice junction (Fig. 2B, bottom), and this occasionally resulted in a 233-bp product indicative of a spliced retrogene (Fig. 3, lanes 2, 3, and 6). Sequence analysis of this product showed that the splice junction was correctly formed, thus providing evidence that an RNA intermediate was involved in the creation of a new retrogene. However, only 2 of 15 SE611 Phlr clones produced the 233-bp PCR product (Table 1) while the majority of clones did not produce a PCR product or generated a product of another size (Fig. 3, lanes 4 and 5).

Southern blot analysis was performed on the genomic DNAs extracted from the SE611 Phlr clones to see whether integrated copies of the predicted retrogene were formed. Unexpectedly, we found that it was not possible to detect a restriction fragment corresponding to the expected retrogene (data not shown). To analyze the source of the 233-bp PCR products further, we used QC-PCR to determine the number of retrogene copies per genome (24). Quantitation is based on the ability of the competitor template to be amplified to the same extent as the target template when both are present in equal amounts. Figure 4, lanes 10 to 16, shows the results of QC-PCR when genomic DNA containing a single copy of the retrogene is used with the competitor. The equivalence point corresponds to approximately $6.25 \times 10^4$ copies per $200 \mu$g of genomic DNA. This value agrees well with the calculated number of $8.7 \times 10^4$ copies of a single-copy sequence present in $200 \mu$g of chicken fibroblast DNA with one genomic equivalent of 2.3 pg (26). Lanes 3 to 9 show the results from a SE611 Phlr clone which was positive in the PCR assay. This DNA reaches equivalence between $1.25 \times 10^3$ and $6.25 \times 10^2$ copies of the competitor. Hence, the retrogene in the SE611 Phlr clone is present in only about 1 to 2% of the cells. We have further found that the retrogene in the SE611 Phlr clone is enriched in the low-molecular-weight fraction of Hirt supernatants and in the cytoplasmic fraction of the cellular extract (data not shown). These results suggest that the retrogene is present as unintegrated episomal DNAs that may be localized in a cytoplasmic compartment. Since these retrogenes are not integrated, autoretrofection does not occur in SE21Q1b cells when they are assayed with p611.

Env-deficient packaging cells are not capable of autoretrofection. Experiments with the envelope-deficient packaging cells were performed as described above in order to test whether autoretrofection occurs intracellularly. Before testing the envelope-deficient packaging cells for autoretrofection with p611, it was necessary to see whether the envelope-producing packaging cells established by transfecting QT35 cells with pSE\_env\_1 could recapitulate the results with SE21Q1b cells. As shown in Table 1, QT611SE\_env\_1 cells are able to generate Phlr cells at about the same frequency as SE611 cells. Furthermore, when pooled colonies of Phlr cells were analyzed...
by PCR, it was possible to detect the correctly spliced 233-bp PCR fragment (Fig. 5, lanes 9 to 11).

The envelope-deficient packaging cells, QT611SEv−, produced a two- to threefold-lower frequency of Phl cells than its env+ counterpart (Table 1). However, this difference is unlikely to be significant since the total number of Phl cells from both packaging lines was very low. PCR analysis of these Phl cells did not reveal the correct 233-bp fragment. Instead, aberrant fragments of different sizes or no products other than the unspliced fragment were detected (Fig. 5, lanes 2 to 8). It has not been possible to find any QT611SEv− Phl cells that create a correctly spliced retrogene; hence, there is no evidence that autoretrofection occurs in these cells with the p611 reporter construct. There is a faint PCR fragment of approximately the correct size in lane 2. Sequence analysis of this band and other PCR products with aberrant sizes showed that they contained deletions of the intron and were not properly spliced (data not shown).

**Autoretrofection of Ψ-containing RNAs occurs in envelope-expressing packaging cells.** Since autoretrofection did not occur with p611, increasing the likelihood of the event was possible by increasing the packaging efficiency of the reporter RNA. Hence, a derivative of p611, pC1611, which contains a Ψ sequence as well as the PBS used for minus-strand DNA synthesis was constructed. When pΨ+611 was assayed for autoretrofection in an envelope-producing packaging cell line (QTΨ+611SEenv+), there was an increase in the number of Phl cells during culture. As seen graphically in Fig. 6, the number of Phl cells increased from approximately 50 to 500 per $6 \times 10^6$ cells during weeks 3 to 5. This is a 100-fold increase in the number of Phl cells compared to that when p611 was used (compare to data in Table 1). The low frequencies of generating Phl cells in QTΨ+611 cells and QT611 cells as well as in packaging cells harboring p611 did not change during continuous culture.

PCR analysis of 10 QTΨ+611SEenv+ Phl clones showed
the correctly spliced 233-bp fragment (Fig. 7, lanes 2 to 11), indicating that retrogenes were being formed in these cells. Southern blot analysis of genomic DNAs isolated from these QTV\textsuperscript{P}+611SE\textsuperscript{env}+ Phl\textsuperscript{I} clones was performed to determine if integrated retrogenes were formed. Restriction enzyme digestion of pTV\textsuperscript{P}+611 with ApaI and NsiI results in a 2.4-kb fragment (Fig. 2B). This same fragment was expected on the Southern blot when pTV\textsuperscript{P}+611 was hybridized with a 1.4-kb probe containing CMV\textit{ie} and phleomycin gene sequences. If a retrogene is present, a 1.4-kb fragment was predicted to be formed after removal of the 1-kb intron by splicing. Surprisingly, the Southern blot showed that only 5 of the 10 clones contained the 2.4-kb ApaI-NsiI restriction fragment (Fig. 8, lanes 5, 8, 9, 11, and 12). The 1.4-kb spliced fragment which is indicative of an integrated newly formed retrogene is present in 9 of the 10 clones. In Fig. 8, lane 5, the DNA from this clone shows a strong hybridizing signal for the 2.4-kb fragment but no signal for the 1.4-kb fragment. Since this clone was PCR positive, we cannot preclude that this Phl\textsuperscript{I} clone arose by a mechanism similar to that observed during our p611 autoretrofection assays. The absence of the 2.4-kb pTV\textsuperscript{P}+611 restriction fragment and the presence of the spliced retrogene in some of the clones (Fig. 8, lanes 3, 4, 6, 7, and 10) are likely to be the result of retrofection during the initial selection in G418 cells after transfection of the packaging cells with pTV\textsuperscript{P}+611. That is, virus from QTV\textsuperscript{P}+611SE\textsuperscript{env}+ cells (which arose after transfection of QTSE\textsuperscript{env}+ cells with pTV\textsuperscript{P}+611) infected QTSE\textsuperscript{env}+ cells lacking the pTV\textsuperscript{P}+611 construct. Only 4 of the 10 clones show both the 2.4-kb and 1.4-kb fragments. The first five clones (Fig. 8, lanes 5 to 7) were isolated at 3 weeks, and the next five clones (Fig. 8, lanes 8 to 12) were isolated at 5 weeks. As indicated in Fig. 8, four of the five week 5 clones have the 2.4-kb fragment whereas only one of the five week 3 clones has this fragment. This result is likely to be an indication that retrofection of QTSE\textsuperscript{env}+ cells occurred early during G418 selection and that autoretrofection of QTSE\textsuperscript{env}+ cells predominated during continuous culture. In regard to the 10 clones analyzed, autoretrofection occurs in approximately 40% of the Phl\textsuperscript{I} cells. In order to confirm that the 1.4-kb ApaI-NsiI fragment does not contain the intron, the Southern blot was stripped of the first probe and rehybridized with a 500-bp intron probe. Two of these fragments are smaller than the 1.4-kb fragment and, hence, most likely have lost additional sequences along with the intron between the restriction enzyme sites. The other four are larger and may therefore be partial restriction enzyme digestion products or retrogenes that were formed with errors such that they lost additional sequences along with the intron between the restriction enzyme sites. The Southern blot shown in Fig. 8 was stripped from the 32P-labeled 1.4-kb DNA fragment containing both the CMV\textit{ie} promoter and phleomycin-resistant gene sequences, and the blot was rehybridized to a 32P-labeled 500-bp intron fragment from pTV\textsuperscript{P}+611. The filled arrow indicates the 2.4-kb-unspliced-intron-containing fragment. The 9-kb fragment seen in lane 2 (quail QT35 DNA) is present in lanes 3 to 13 and is the c-myc intron in quail cells which cross-hybridizes to the chicken c-myc intron probe from pTV\textsuperscript{P}+611. Molecular weights (in thousands) are noted at the left.

FIG. 8. Southern blot of QT\textsuperscript{P}+611SE\textsuperscript{env}+ Phl\textsuperscript{I} clones. Genomic DNAs were digested with the restriction enzymes ApaI and NsiI and hybridized to a 32P-labeled 1.4-kb DNA fragment containing both the CMV\textit{ie} promoter and phleomycin-resistant gene sequences. Lanes 1 and 15, 32P-labeled lambda-HindIII marker DNA; lane 2, QT35 DNA; lanes 3 to 12, DNAs from 10 QT\textsuperscript{P}+611SE\textsuperscript{env}+ Phl\textsuperscript{I} clones which were used in the PCR experiments shown in Fig. 7; lane 13, DNA from a Phl\textsuperscript{I} Pur\textsuperscript{I} clone obtained by coculturing QT\textsuperscript{P}+611SE\textsuperscript{env}+ cells with QT\textsuperscript{Babe} cells; lane 14, 40 pg of pTV\textsuperscript{P}+611. The filled arrow indicates the 2.4-kb-unspliced-intron-containing fragment, and the open arrow indicates the 1.4-kb-intron-minus-retrogene fragment. The six fragments that are marked with asterisks and the 1.4-kb fragments do not hybridize to the 500-bp intron probe. Molecular weights (in thousands) are noted at the left.

FIG. 9. Rehybridized Southern blot of QT\textsuperscript{P}+611SE\textsuperscript{env}+ Phl\textsuperscript{I} clones. The Southern blot shown in Fig. 8 was stripped from the 32P-labeled 1.4-kb DNA fragment containing both the CMV\textit{ie} promoter and phleomycin-resistant gene sequences, and the blot was rehybridized to a 32P-labeled 500-bp intron fragment from pTV\textsuperscript{P}+611. The filled arrow indicates the 2.4-kb-unspliced-intron-containing fragment. The 9-kb fragment seen in lane 2 (quail QT35 DNA) is present in lanes 3 to 13 and is the c-myc intron in quail cells which cross-hybridizes to the chicken c-myc intron probe from pTV\textsuperscript{P}+611. Molecular weights (in thousands) are noted at the left.
There is incomplete resistance to superinfection. Table 2 shows

<table>
<thead>
<tr>
<th>Coculture</th>
<th>No. of Phl' Pur* cells on day:</th>
<th>Total no. of cells</th>
</tr>
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<tbody>
<tr>
<td>QTΨ + 611SEvm + with QTBabe</td>
<td>176 320 440 10^6</td>
<td></td>
</tr>
<tr>
<td>QTΨ + 611SEvm + with QTSEvm+Babe</td>
<td>18 80 144 10^6</td>
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*Cells (10^6) of QTΨ + 611SEvm + and cells (10^6) of QTBabe or QTSEvm+Babe were plated together on 10-cm-diameter tissue culture plates. Cells (10^6) from the coculture were treated with phleomycin and puromycin.

Cocultures were passaged over time and treated with drugs.

Average number of phleomycin and puromycin colonies from duplicate plates.

Phleomycin, there was no increase in the number of Phl' cells above that seen with QTΨ + 611(Fig. 6). Thus, autoretrofection is dependent on active reverse transcription.

**Autoretrofection is not blocked by viral interference.** Since autoretrofection occurred only in the presence of envelope, it likely occurs by extracellular reinfection. However, viral interference usually prevents superinfection of virus-producing cells. Therefore, it was important to know whether autoretrofection occurred because there was incomplete blockage to viral superinfection. In order to assess whether blockage to superinfection was incomplete, QTΨ + 611SEvm + cells were cocultured with either QTBabe or QTSEvm+Babe cells. QTBabe cells are QTTS cells which have been transfected with the plasmid pBabe to confer puromycin resistance. Likewise, QTSEvm + packaging cells were transfected with the same plasmid to establish puromycin-resistant QTSEvm+Babe cells. Both puromycin-resistant cell lines were used as marked cells in coculture experiments to test whether viral-envelope-expressing packaging cells were resistant to virus superinfection. Because these cells are puromycin resistant, viral interference can be determined by comparing the number of phleomycin- and puromycin-resistant (Phl' Pur*) colonies that arises in each of the cocultures with QTΨ + 611SEvm + cells. Virus produced from QTΨ + 611SEvm + cells can infect QT-Babe cells and transduce phleomycin resistance (data not shown). However, QTSEvm+Babe cells produce the same envelope glycoprotein and are infected only if there is incomplete resistance to superinfection. Table 2 shows that there is only a 10-fold reduction in the number of Phl' Pur* colonies when both cells in coculture produce virus. The frequency of phleomycin resistance in the presence of envelope glycoproteins is approximately 10^-3 contrasted to that of 10^-4 in envelope-minus cells. There is an increase in the total number of Phl' Pur* cells as the coculture is passaged over time. However, there is only a threefold reduction of Phl' Pur* cells between the QTBabe and QTSEvm+Babe cocultured cells after 2 weeks, indicating that viral interference is not complete and that the env+ cells are infected fairly efficiently. Filtered virus also gave only about a 10-fold reduction in titer on the env+ cells (data not shown).

**DISCUSSION**

We initiated these studies to test whether autoretrofection occurs via an intracellular pathway or via extracellular reinfection. Our results show that autoretrofection occurs via extracellular reinfection despite the presence of envelope glycoproteins that are expected to prevent superinfection. This extracellular reinfection is unusual, because highly efficient viral interference is most commonly seen with ALVs (32, 33, 36). However, lack of complete viral interference has also been noted (37). It is possible that the lack of interference is more pronounced in the continuous cell lines we used rather than in primary cultures. In our experiments to determine the level of resistance to superinfection, we used cell cocultures rather than cells infected with viral supernatants because the former are the most closely resemble our autoretrofection system. We therefore cannot exclude the possibility that cell-to-cell interactions in our cocultures also played a role in generating doubly resistant cells. However, we also saw low levels of interference using filtered virus.

Our genetic selection for autoretrofection was designed to allow for detection of newly formed retrogenes. However, the results show that there is a background level of Phl' cells which arise independently of autoretrofection. The genetic selection allows detection only of bona fide retrogenes when there are greater than 10 Phl' colonies per million cells plated. When colonies form below this frequency, our biochemical analysis of these cells shows that they do not contain correctly spliced integrated retrogenes or contain correctly spliced episomal retrogenes. While episomes can be transcriptionally active (29), the absence of the retrogene in every cell calls into question its significance in conferring drug resistance. These Phl' cells survive in continuous culture in the presence of phleomycin, and hence, the majority of the cells are resistant to the drug even in the absence of a spliced retrogene. We suspect that readthrough transcripts from the CMVie promoter lead to this background level of Phl' cells, because RNase protection assays from some of these cells show readthrough transcripts in greater abundance than transcripts from a spliced retrogene (data not shown). Thus, the intronic polyadenylation signal might not be used in all cells, permitting ribosomes to successfully scan the 1-kb intron even in the presence of multiple termination codons.

We were able to detect autoretrofection only in packaging cells that express viral envelope glycoproteins in the presence of Ψ- and PBS-containing reporter RNAs. This is not unexpected, because the packaging sequence increases the number of viral particles containing the reporter RNA (8) and the PBS aids in reverse transcription. A curious aspect is that in our initial experiments, autoretrofection in SE21Q1b cells, as mentioned in the introduction, occurred with a marked RNA which did not contain Ψ or the PBS. Since these cells were passaged extensively for some time (over years), we surmise that autoretrofection can occur without viral cis sequences, albeit at a much lower frequency than with cis sequences.

Heidmann et al. have shown that in the absence of viral glycoproteins, mammalian retroviruses can retrotranspose through an intracellular pathway (14, 30). These investigators also reported that viral RNAs with Ψ sequences spilled out can be used more efficiently for retrotransposition than viral RNAs containing Ψ in the absence of viral proteins and that processed pseudogene formation can be detected in HeLa cells (20, 31). They propose that these events are not virus mediated but are due to long interspersed nuclear elements or to some endogenous cellular sources of reverse transcriptase acting upon their marked RNAs. Our results with a reporter construct containing the 5' LTR, PBS, and Ψ show that these cis sequences are not adequate for supporting the intracellular pathway. This pathway may require other cis sequences such as the polypurine tract and the 5' LTR to ensure completion of cDNA synthesis and the generation of cDNA ends competent for integration. Our results show that cellular RNAs are rarely able to undergo intracellular retrotransposition even in the presence of retroviruses in avian cells. However, our observation of the lack of retrotransposition of cellular RNAs in quail cells may be significant in that processed pseudogenes are rare.
in avian cells compared to their frequency in mammalian cells (10, 25, 35). Avian cells may lack retroelements which are actively involved in processed pseudogene formation.

The intracellular pathway that we studied is probably inefficient, because Gag and Pol proteins are not yet in their mature forms. It is generally thought that viral proteins require protease processing, and this processing is accomplished only by activated protease soon after budding (6, 16). For example, there is only minimal reverse transcriptase activity without protease processing and viruses defective in protease are not infectious (5, 27, 28). It may be necessary for the Gag polypeptide to be cleaved to form an active integration complex. Hence, mature integration-competent intracellular particles would be rare even if there were some intracellular protease activation. Even though the viral trans-acting proteins may not be fully active in the intracellular pathway due to inactive protease, the findings of Heidmann et al. which show that viral RNAs are able to use this pathway suggest that the cis sequences play a larger role in determining intracellular retrotransposition than the trans-acting proteins.

Curiously, we detected correctly spliced unintegrated retrogenes when p611 was used in the envelope-producing packaging cells. Rather than resulting from autoretrofection events, we think that these retrogenes are epimemes that may have resulted from viral budding into intracellular compartments (9, 19) or from retrogenes formed after extracellular reinfection that are blocked at integration. Since these retrogenes were unintegrated, we cannot conclude that autoretrofection occurred when p611 was used as the reporter. Even though retrofication of cellular RNAs lacking P611 allows detection of extracellular autoretrofection by allowing for a greater number of infectious virions to be cleaved to form an active integration complex.

REFERENCES


