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Packaging of intron-containing genes into retrovirus vectors by alphavirus vectors

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ABSTRACT **Efficient and controllable expression of a transgene usually requires the presence of intron sequences and much efforts have been made to produce retrovirus vectors that can transduce and integrate genes with introns. However, this has proven difficult because the viral RNA is spliced when it is synthesized in the nucleus of a producer cell. We describe a novel approach to avoid this problem. In our system the retroviral RNA is synthesized in the cytoplasm of the cell, not in the nucleus, in a reaction driven by the Semliki Forest virus (SFV) expression system. The approach was tested with a recombinant Moloney murine leukemia virus genome containing the chloramphenicol acetyltransferase (CAT) gene in association with an intron. This was inserted into a SFV transcription plasmid and the corresponding SFV vector RNA was transcribed *in vitro*. BHK-21 cells were then transfected with this vector RNA together with two additional SFV vectors that encode the Moloney murine leukemia virus packaging proteins. Retrovirus vectors containing intron-CAT sequences were produced at titers up to 1.3×10^6 infectious particles per ml during a 5-hr incubation period. The vectors faithfully transduced the intron-containing CAT gene into NIH 3T3 cells, where the intron-CAT RNA was subjected to efficient splicing and used for high level enzyme expression. Thus, the results show that intron containing genes can be efficiently packaged into retrovirus vectors by the SFV expression system.**

Vectors based on retroviruses have established themselves as useful tools for stable gene transfer into animal cells both *in vitro* and *in vivo* (1, 2). These RNA viruses carry a reverse transcriptase, which converts their genome into double-stranded DNA and further an integrase, which catalyses the insertion of the genome into the DNA of host chromosomes (3). The integrated viral genome, or provirus, is copied by transcription into RNA molecules that are transported into the cytoplasm and then encapsidated into virus particles. When used as vectors, the proviral DNA is engineered and transfected into packaging cells (4). These cells provide all retroviral structural proteins and enzymes in trans. One major drawback with the retrovirus vector system is that it is in practice limited to transduce cDNA forms of processed mRNAs. These minigenes lack important control elements that sometimes leads to disappointingly low expression levels (5–7). In particular, it is difficult to use the retrovirus vector system for transduction of genes with intervening sequences (introns). The reason is that any intron present in an engineered provirus will be removed from the RNA form of the viral genome by the nuclear splicing machinery (8, 9). Here we describe a novel strategy to package intron containing genes into retroviruses. This is based on the transient expression of the RNA form of the viral genome directly in the cytoplasm of the cells using an alphavirus RNA vector.

MATERIALS AND METHODS

Cell Culture. Baby hamster kidney (BHK)-21 cells (American Type Culture Collection, Rockville, MD) were cultured in BHK-21 medium (GIBCO) containing 5% fetal calf serum, 10% tryptose phosphate broth, 20 mM Hepes, and 2 mM glutamine. NIH 3T3 cells were cultured in DMEM (GIBCO) supplemented with 10% fetal calf serum, 4,500 mg/l glucose, and 2 mM glutamine. HeLa cells were cultured in MEM alpha medium (GIBCO) supplemented with 10% fetal calf serum.

Plasmid Constructs. The maps of pSFV1-I-CAT and pSFV1-CAT constructs are shown in Fig. 1. A *Bgl*II-*Bam*HI fragment containing the simian virus 40 (SV40) early promoter, a chimeric intron, the CAT gene, and the SV40 late poly(A) signal was excised from pCAT-promoter vector (Promega) and inserted into the pSFV1/LN3i (10) downstream of the neo^R gene by subcloning to make pSFV1-I-CAT. To make the intron-free pSFV1-CAT we first removed the intron from the pCAT-promoter vector with *Hin*dIII and then proceeded as described for the pSFV1-I-CAT construction.

Production of Retroviral Vectors. The plasmid DNA was linearized with *Nru*I and used as template for RNA synthesis *in vitro* by SP6 polymerase. The RNA was introduced into BHK-21 cells together with SFV-C/gag-pol RNA and SFV1/Pr80env RNA or SFV1/AMenv RNA (10) by electroporation. The transfected BHK-21 cells were diluted into complete BHK medium and plated onto a 60-mm culture dish (Nunclon, Naperville, IL). The cells were incubated at 37°C for 5 hr, and then the media were replaced with 2 ml aliquots of fresh complete BHK-medium. After 5 hr incubation, the media were collected, passed through a 0.45 μm filter, and stored at −130°C. Neo^R-transduction-competent retrovirus particles were titrated on NIH 3T3 cells as described (10).

CAT Assay. The CAT gene expression was measured by the CAT enzyme assay system with reporter lysis buffer (Promega) according to the instructions of the manufacturer. In brief, the cells were lysed by addition of 400 μl of 1× lysis buffer into each dish and incubated at room temperature for 15 min. The lysates were then heated at 60°C for 10 min. An aliquot of each lysate (100 μl) was mixed with 3 μl of [^{14}C]-labeled chloramphenicol (0.05 mCi/ml, DuPont/NEN; 1 Ci = 37 GBq), 5 μl of n-butyryl CoA (5 mg/ml), and 17 μl of H_2O and incubated at 37°C for 16 hr. The reaction was terminated by addition of 300 μl of xylenes (Aldrich) followed by mixing. The upper xylene phase was removed and back-extracted twice with 0.25 M Tris·HCl, pH 8.0. A portion of the xylene phase (200 μl) was

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Abbreviations: CAT, chloramphenicol acetyltransferase; I-CAT, intron-containing CAT; SFV, Semliki Forest virus; Mo-MuLV, Moloney murine leukemia virus; LTR, long terminal repeat; RT, reverse transcription; BHK, baby hamster kidney; SV40, simian virus 40.
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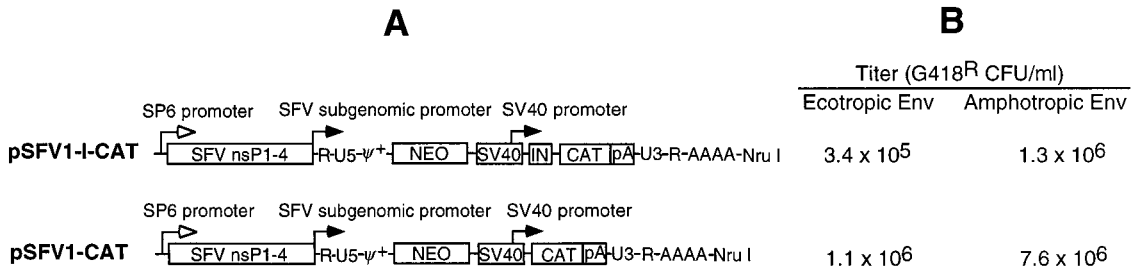

FIG. 1. (*A*) Schematic representation of pSFV1-I-CAT and pSFV1-CAT constructs. Only the SFV recombinant regions are shown. The SP6 promoter is indicated by an open arrow. The subgenomic SFV promoter and the SV40 promoter are indicated with solid arrows. SFVnsp1–4 represents the coding region of the SFV nonstructural proteins 1 to 4. The recombinant retroviral genome (R-U5-ψ^+-NEO-SV40-IN-CAT-pA-U3-R) is inserted downstream of the subgenomic SFV promoter. The SV40 early promoter is used to initiate the transcription of the CAT gene. A chimeric intron (IN) is inserted between the SV40 promoter and the CAT gene in pSFV1-I-CAT. In pSFV1-CAT, this intron has been removed. An internal, SV40-derived poly(A) signal (pA) is present downstream of the CAT gene in both constructs. (*B*) Titers of retrovirus vector preparations. RNA was prepared from plasmid constructs and used for vector production in BHK-21 cells. Media were harvested and titrated for neo^R-transduction competent retrovirus vectors on NIH 3T3 cells. The titers shown are the number of neomycin-resistant colonies obtained from 1 ml of medium. The maps are not drawn to scale.

mixed with 3 ml scintillation fluid and the radioactivity ([^{14}C] cpm) measured in a liquid scintillation counter.

Provirus Analysis. Proviruses were analyzed by PCR. DNA was isolated from cells with the TRIzol Reagent (GIBCO/BRL) and used in PCR together with the *pfu* DNA polymerase (Stratagene). The DNA was first denatured at 95°C for 5 min. The PCR reaction was then carried out at 94°C for 45 sec, 65°C for 45 sec, and 72°C for 3 min and repeated in 30 cycles. The two primers used to detect the provirus were primer 1 (5′-GAAGAGCTTGGCGGCGAATG-3′) and primer 2 (5′-TATCGAACCCCAGAGTCCGT-3′). The upstream primer 1 hybridizes to the 3′ region of the neo^R gene and the downstream primer 2 to a region just downstream of the SV40 fragment carrying the poly(A) signal (Fig. 3 *A* and *B*). The PCR products were analyzed on a 0.7% agarose gel.

Analysis of CAT Gene Transcription. Transcription of CAT genes was analyzed by reverse transcription (RT)–PCR. Total RNA were isolated from cells with the TRIzol Reagent and used in RT-PCR according to the Access RT-PCR System (Promega). The first strand DNA was synthesized by avian myoblastosis virus reverse transcriptase at 48°C for 45 min. Next, the RNA/DNA hybrids were denatured and the avian myoblastosis virus reverse transcriptase was inactivated by incubation at 94°C for 2 min. Second strand DNA synthesis and DNA amplification were done by *Tfl* DNA polymerase. The DNA amplification was carried out at 94°C for 45 sec, 65°C for 45 sec, and 68°C for 3 min and repeated in 45 cycles. The primers used for RT-PCR were primer 1, primer 2, primer 3 (5′-TGCAGAAGTTGGTCGTGAGG-3′) and primer 4 (5′-TAGTGAGGAGGCTTTTTTGG-3′). Primer 3 hybridizes to a region just in front of the 5′ splice site of the CAT-associated intron and primer 4 to a region just downstream of the SV40 promoter (Fig. 3 *C–F*). The RT-PCR products were analyzed on a 0.7% agarose gels.

RESULTS

Efficient Production of Retroviral Vectors with Intron-Containing CAT Genes. We have shown previously that SFV-derived RNA vectors can be used to synthesize recombinant RNA genomes of the Moloney murine leukemia virus (Mo-MuLV) in the cytoplasm of the cell and that these genomes can be packaged into transduction competent retrovirus particles (10). In these experiments the Mo-MuLV proteins were co-expressed from two additional SFV RNA vectors. We reasoned that the cytoplasmic SFV expression system should also facilitate an uncompromised production of Mo-MuLV vectors with intron-containing genes. To test this possibility we constructed two new SFV vector plasmids (Fig. 1*A*). These were designed to express a recombinant Mo-MuLV RNA genome containing a CAT gene with and without an intron (pSFV1-I-CAT and pSFV1-CAT). In both constructs the CAT gene is preceded by an internal promoter and followed by an internal poly(A) signal, both derived from SV40. In addition the constructs contain the neo^R gene, under the control of the Mo-MuLV promoter, in their 5′ long-terminal repeats (LTRs). The corresponding SFV RNA vectors were transcribed *in vitro* and used for transfection of BHK-21 cells together with the SFV RNA vectors that specified expression of the Mo-MuLV gagpol and env precursor proteins. We used both the ecotropic and the amphotropic env gene in the coexpression experiments. Media from cotransfected cells, collected between 5–10 hr after electroporation, were analyzed for neo^R transducing vectors on NIH 3T3 cells. The titers ranged from 3.4×10^5 to 7.6×10^6 colony-forming units/ml (Fig. 1*B*). This shows that neo^R-transduction competent retroviral vectors were formed efficiently in the transfected BHK-21 cells.

High CAT Expression in Cells Infected with Vectors Carrying a CAT Gene with an Intron. NIH 3T3 cells that have been infected with ecotropic vectors were used to measure CAT activity. This was done with cells that had been selected in G418 medium for neo^R expression as well as in unselected cells that had been incubated for only two days after infection. Fig. 2*A* shows the CAT activity in unselected cells. Lysates from cells infected with retroviral vectors from the SFV1-CAT RNA transfected BHK-21 cells contained very low CAT activity. In contrast, lysates from cells infected with retroviral vectors from SFV1-I-CAT RNA transfected BHK-21 cells had high CAT activity. Fig. 2*B* shows the corresponding analyses of cells that had been selected in G418 containing media for 6 days. Cells infected with the I-CAT vectors retained their high CAT activity whereas cells infected with the CAT vectors had lost most of their activity. Fig. 2*C* shows the CAT activity in nine G418-selected I-CAT and CAT cell clones, respectively. The activity of most I-CAT cell clones was high and that of all CAT clones barely above background level. We conclude that both vectors can transduce the internal CAT expression unit into the recipient cells in a functional form. However, only the intron-containing construct can promote a stable CAT expression at high level. Very similar CAT expression was found in HeLa cells infected with the amphotropic I-CAT and CAT vectors (data not shown). Altogether these data confirm the general notion that intron-containing genes are more efficiently expressed than genes lacking introns. Indeed, inefficient expression of CAT from a retrovirus vector containing a SV40 promoter-driven, CAT expression unit without introns has been observed before (11).

Intact Intron-Containing CAT Genes Are Packaged into Retroviral Vectors and Transferred into Recipient Cells. The structures of the I-CAT and CAT genes in the transduced cell

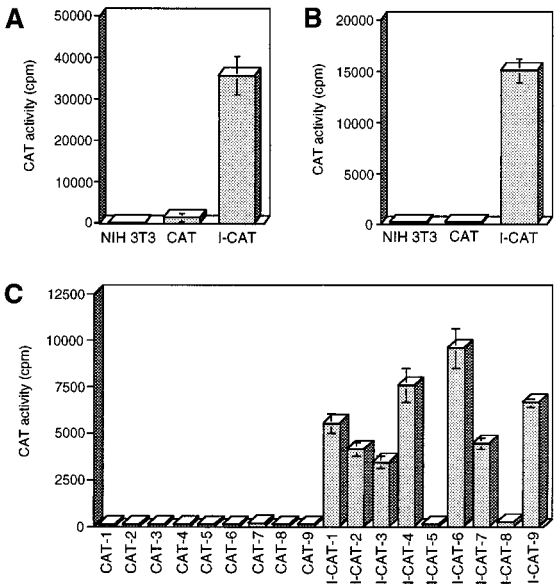

FIG. 2. CAT expressions in cells infected with ecotropic retroviral vectors carrying an I-CAT gene or an intron-free CAT gene (CAT). (*A*) Transient expression of the CAT gene. NIH 3T3 cells were plated into 60-mm culture dishes at 5×10^5 cells per dish 24 hr before infection. The cells in one dish were incubated with 1×10^5 infectious retroviral vectors carrying the I-CAT or the intron-free CAT gene at 37°C for 24 hr in the presence of 4 μg/ml polybrene. Then the medium was replaced with fresh medium and the cells were further incubated at 37°C for 28 hr. The CAT expression was measured as described in *Materials and Methods*. Noninfected NIH 3T3 cells were used as a control. (*B*) CAT gene expression in G418 selected cells. NIH 3T3 cells were infected with the I-CAT or CAT retroviral vectors and then incubated for 6 days in the presence of 600 μg/ml G418. The cells were trypsinized, replated (1×10^6 cells per 60-mM dish), cultured at 37°C for 24 hr, and tested for CAT activity. (*C*) CAT gene expression in cell clones. The infected cells were incubated for 11 days in the presence of 600 μg/ml G418. Nine resistant colonies were picked from each infection (CAT 1–9 and I-CAT 1–9) and expanded by incubation for 11–18 days. The CAT activity of the cells was measured as described in *B*. Bars = SD.

clones were studied by PCR. DNA was extracted from each of the cell clones of the two series and used as template for primers to the regions that were flanking the I-CAT and CAT expression units (primers 1 and 2 in Fig. 3 *A* and *B*). Polymerization reactions with DNA from the I-CAT and CAT cells should yield a single DNA fragment of 1,540 bp and 1,317 bp, respectively. Bands corresponding to the two fragments were seen when pSFV1-I-CAT and pSFV1-CAT plasmid DNAs were used as control templates (Fig. 3*A* and *B*, "positive 1" and "positive 2," respectively). The DNA analyses of the cell clones showed that the larger PCR fragment was generated in all reactions with DNA from I-CAT cells (Fig. 3*A*) and the smaller one in all reactions with DNA from CAT cells (Fig. 3*B*). In Fig. 3*G*, lanes 2 and 3, samples of reactions with DNA from I-CAT-1 and CAT-1 cells were run next to each other to clearly demonstrate the size difference between the two fragments. No distinct bands were found in gel analyses of PCR reactions done in the absence of DNA (Fig. 3*B*, "negative") or with DNA extracted from untransduced NIH 3T3 cells (Fig. 3*A*, "NIH 3T3"). Thus, these results suggest that all I-CAT clones carry an intron-containing CAT expression unit whereas all CAT clones contain a CAT unit without intron. Additionally the data suggest that both units contain the SV40-derived poly(A) signal (pA in Fig. 1*A*). We conclude that we have been able to package an intron-containing gene efficiently into retrovirus vectors using the cytoplasmic expression system of SFV.

The CAT-Associated Intron Is Efficiently Removed by the Nuclear Splicing Machinery in Transduced Cells. To find out how efficiently the nuclear splicing machinery can remove the CAT-associated intron from proviral transcripts, we analyzed the RNA of the I-CAT cell clones by RT-PCR. The CAT cell clones were analyzed in parallel as controls. The recombinant provirus is expected to drive RNA transcription both from the viral promoter in the 5′ LTR and from the internal SV40 promoter (Fig. 3, *C–F, Upper*). We first used primers 1 and 2 to follow the splicing of the intron in the LTR-derived transcript (Fig. 3*C*). These primers should generate a 1,540-bp fragment from the unspliced form of the transcript and a 1,407-bp fragment from the spliced one. A gel analyses of the reaction mixtures with RNA from the I-CAT clones showed, in each case, two closely migrating bands that corresponded to the expected fragments. This band doublet migrated between the 1,587-bp and 1,375-bp DNA markers and the position is indicated with an arrow in Fig. 3*C*. The two bands of the I-CAT-1 sample were more clearly shown in Fig. 3*G*, lane 4. The faster migrating band was more intense than the slower one in all samples. This suggested that most of the I-CAT RNA was present in a spliced form. The corresponding analyses of reactions with RNA extracted from CAT cell clones showed only one band, which corresponded to the expected 1,317-bp fragment (Fig. 3 *D* and *G*, lane 5).

Additional RNA analyses were done with two different upstream primers (primers 3 and 4). With primer 3, DNA polymerization is initiated just upstream of the CAT-associated intron. When this is used together with the downstream primer 2 and I-CAT RNA as the template, a 1,201-bp fragment should be generated from the unspliced, LTR- or SV40-driven transcripts and a 1,069-bp band from their spliced forms. The results in Fig. 3*E* and in Fig. 3*G*, lane 6, show the corresponding band doublet. The faster migrating band predominates, again suggesting that most of the transcripts have been subjected to RNA splicing in the cells. When RNA from CAT cells was subjected to RT-PCR with primer 3 and 2 no distinct products were generated (data not shown). This was expected as the template region for primer 3 is lacking in the CAT construct.

Primer 4 binds to a region that corresponds to the 5′ end of the SV40 transcript (Fig. 3*F*). This region is present in both the I-CAT and the CAT constructs. When the primers 4 and 2 were used with the I-CAT RNA in a RT-PCR reaction a band-doublet was seen as in the reaction with primer 3 and 2 (data not shown). This doublet corresponded to the fragments expected from the unspliced and spliced form of LTR- or SV40-driven transcripts. The reaction with RNA from the CAT clones yielded only a single band, which corresponded to the expected 1036-bp fragment (Fig. 3 *F* and *G*, lane 7). Thus, the RNA analyses with primers 3 and 4 confirmed the efficient splicing of the CAT-associated intron from proviral transcripts. This fact would, most likely, make it very difficult to produce I-CAT containing retrovirus vectors by the conventional procedure, where a provirus is used to transfect a packaging cell line.

It should be noted that the LTR transcripts also contain a splice donor and acceptor site in the ψ^+ region (1). These, however, are known to mediate only inefficient splicing. Therefore we did not analyze their possible effects on the proviral RNA processing in the CAT and I-CAT cell clones.

DISCUSSION

There is today a large number of gene expression studies, both in tissue culture cells and in transgenic animals, which show that intron-containing genes are expressed more efficiently than intronless ones. The introns have been shown to influence gene expression in several ways. In general the presence of an intron in the pre-mRNA appears to stimulate accumulation of

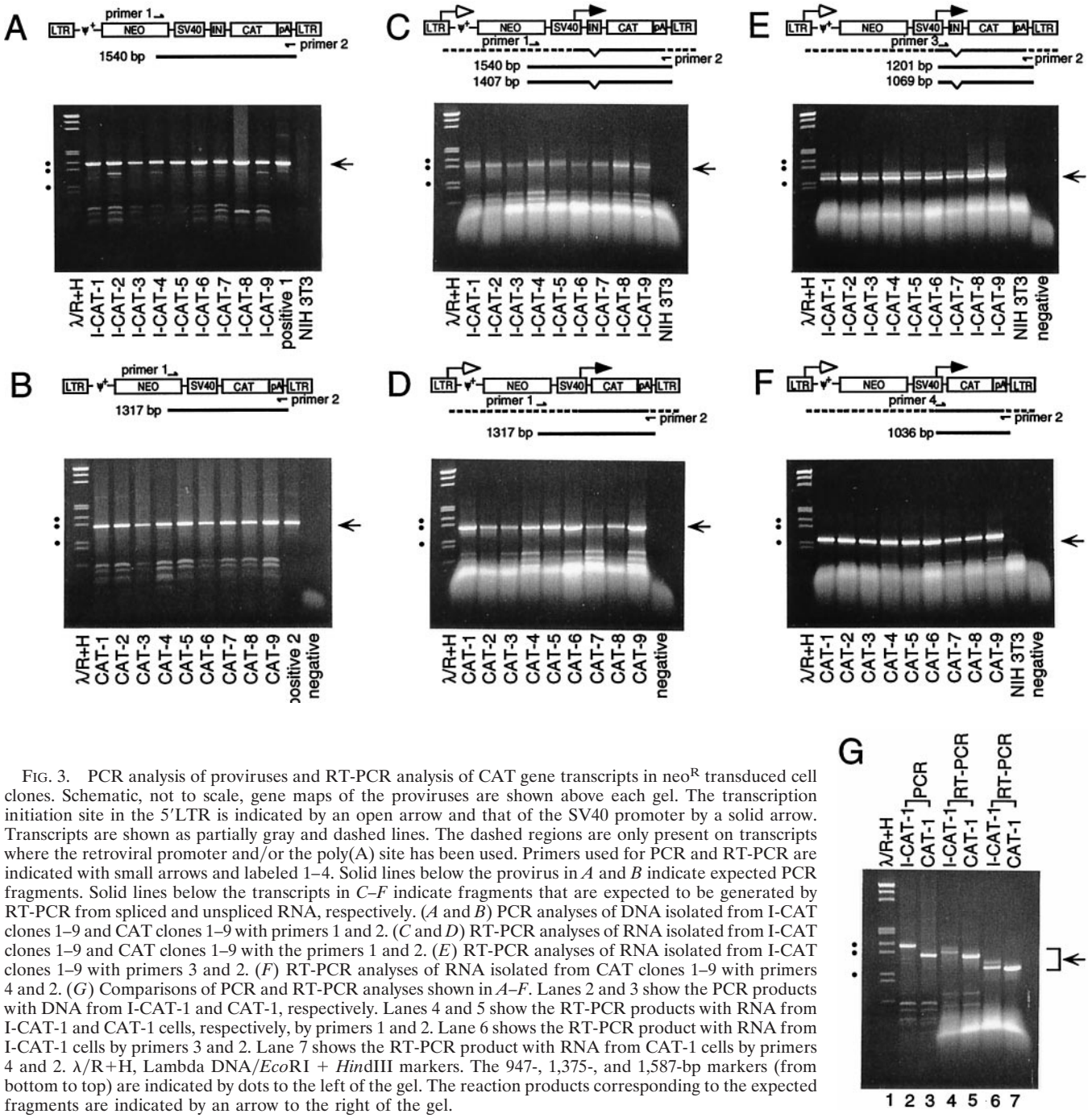

FIG. 3. PCR analysis of proviruses and RT-PCR analysis of CAT gene transcripts in neo^R transduced cell clones. Schematic, not to scale, gene maps of the proviruses are shown above each gel. The transcription initiation site in the 5′LTR is indicated by an open arrow and that of the SV40 promoter by a solid arrow. Transcripts are shown as partially gray and dashed lines. The dashed regions are only present on transcripts where the retroviral promoter and/or the poly(A) site has been used. Primers used for PCR and RT-PCR are indicated with small arrows and labeled 1–4. Solid lines below the provirus in *A* and *B* indicate expected PCR fragments. Solid lines below the transcripts in *C–F* indicate fragments that are expected to be generated by RT-PCR from spliced and unspliced RNA, respectively. (*A* and *B*) PCR analyses of DNA isolated from I-CAT clones 1–9 and CAT clones 1–9 with primers 1 and 2. (*C* and *D*) RT-PCR analyses of RNA isolated from I-CAT clones 1–9 and CAT clones 1–9 with the primers 1 and 2. (*E*) RT-PCR analyses of RNA isolated from I-CAT clones 1–9 with primers 3 and 2. (*F*) RT-PCR analyses of RNA isolated from CAT clones 1–9 with primers 4 and 2. (*G*) Comparisons of PCR and RT-PCR analyses shown in *A–F*. Lanes 2 and 3 show the PCR products with DNA from I-CAT-1 and CAT-1, respectively. Lanes 4 and 5 show the RT-PCR products with RNA from I-CAT-1 and CAT-1 cells, respectively, by primers 1 and 2. Lane 6 shows the RT-PCR product with RNA from I-CAT-1 cells by primers 3 and 2. Lane 7 shows the RT-PCR product with RNA from CAT-1 cells by primers 4 and 2. λ/R+H, Lambda DNA/*Eco*RI + *Hin*dIII markers. The 947-, 1,375-, and 1,587-bp markers (from bottom to top) are indicated by dots to the left of the gel. The reaction products corresponding to the expected fragments are indicated by an arrow to the right of the gel.

mRNA in the cytoplasm through its interaction with the nuclear splicing machinery (12–15). It has also been reported that the intervening sequences can increase 3′ processing of the mRNA (16). In other cases introns have been shown to contain enhancer elements that are required for differentiation-specific gene expression (17–20). Furthermore introns might facilitate packaging of genes into chromatin (21). Therefore much effort has been made to adapt the integrating retroviral gene-transduction systems to intron-containing genes. As earlier studies have already shown that intervening sequences of foreign genes are removed during synthesis and processing of viral RNA from engineered provirus, the general approach has been to introduce a complete foreign gene expression unit in reverse orientation into the provirus. Although this approach has resulted in successful transduction of some intron-containing genes, the experience today is that these vectors are often difficult to produce in packaging cells. The major reason seem to be that fortuitous RNA processing signals for splicing and polyadenylation are frequently found in the reversed sequence of the foreign gene (22–24). This is not surprising as the reversed stretch represent an unnatural sequence that has never been transcribed in a cell. Consequently the viral RNA may be prematurely terminated or rearranged with dysfunction of both the viral gene-transduction process and foreign gene expression as a result. Other potential problems with the reversed sequence approach are that the viral promoter and the reversed internal promoter in the engineered provirus compete for transcription and/or that the two different transcripts form a duplex through their complementary features (25, 26). Our present approach avoids all of these problems because our retroviral RNA transcription is driven by a cytoplasmic RNA expression system. This facilitates both the accumulation of unprocessed RNA directly in cytoplasm and its encapsidation into retroviral

particles. In this work we have clearly demonstrated the feasibility of this approach by using the SFV expression system. With this, we have produced retrovirus vectors, containing an internal CAT expression unit, including a promoter, an intron and a poly(A) signal, at high titers (up to 1.3×10^6 colony-forming units/ml). It is obvious that the benefits of a cytoplasmic expression strategy to produce retroviral RNA constructions is not limited to intron-containing genes but also includes genes in complex with promoter or enhancer elements as well as locus control regions. Such regulatory regions mostly consist of untranscribed sequences and therefore suffer from the same problems of fortuitous splicing and polyadenylation as the reversed genes do in the conventional retrovirus packaging system (23, 27, 28).

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