Amphotropic Retrovirus Vector System for Human Cell Gene Transfer†

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Retroviral vectors have been constructed for gene transfer in mammalian and avian cells, however most retroviral vector systems are complicated by the spread of a replication-competent helper virus. This problem has been circumvented by segregating the viral genome into *cis*- and *trans*-acting components. By establishing helper cell lines that produce the *trans*-acting viral gene products, one can propagate the *cis*-acting component in them and harvest defective viral particles that contain only the *cis*-acting component. The *cis*-acting component can provide a useful vehicle for the highly efficient transfer of genes into target cells. The defective vector systems described to date, however, are restricted in host range to murine, avian, rat, and dog cells. We describe a helper-free vector system based entirely on an amphotropic murine virus with a wide mammalian host range, including the ability to carry out efficient gene transfer into human cells. We also describe a double mutation constructed in the *trans*-acting genome which reduces the frequency of replication-competent recombinant viruses to undetectable levels.

Retrovirus vectors provide an efficient means for transferring genes into cells (7, 14, 15, 18, 21, 24, 29). Ideally a gene vector should convey genetic information from one site to another without distorting the host system in which it operates. Unfortunately, the presence of replicating viruses can cause many undesired effects (7, 14, 15, 18, 21, 24, 29). To eliminate this problem, retroviral vectors are being constructed such that viral replication is blocked subsequent to provirus integration, thus prohibiting viral spread (12, 27).

The retroviral genome in these vector systems has been segregated into two or more components. One component contains most of the *cis*-acting elements, such as the polypurine tract, a long terminal repeat (LTR), the primer binding site, and the sequences necessary for viral RNA packaging. This *cis*-acting component, which lacks the genes that encode viral proteins, may be used to carry nonviral genes of interest. The other component(s) (*trans*-acting) contains the genes that encode the viral proteins along with control elements necessary to express them. By introducing *cis*-acting mutations in the *trans*-acting genome, one can limit its ability to be replicated at a stage soon after RNA transcription. The *trans*-acting genome can thus direct the synthesis of viral proteins but cannot be replicated to produce progeny virus. Since neither component alone contains enough genetic information for indefinite viral replication, cell lines containing both components do not produce replication-competent virus. However, the *cis*-acting genome can utilize the viral proteins produced by the *trans*-acting genome and can be exported within virus particles produced by cells containing both genomes. Since retrovirus particles contain all of the viral proteins necessary for virus penetration, reverse transcription, and DNA integration, the *cis*-acting genome can reach the provirus stage without *de novo* protein synthesis. However, once integrated, the *cis*-acting genome cannot produce viral proteins, and therefore the replication cycle ends. The provirus simply becomes a transcription unit that expresses the exogenous gene that

was introduced into the *cis*-acting vector. Transcription is controlled by the elements present in the viral LTR.

Our efforts have been directed toward constructing such a vector which can be used in a wide range of target cells. Since there is substantial interest in the transfer of genes into human cells, we developed a vector that can infect human as well as many other mammalian cells efficiently. This vector system is free of helper viruses and, under controlled conditions, may be used without biochemical selection. We have called our *cis*-acting vector *cis* and our *trans*-acting genome *trans* (since it contains mutations in the packaging and polypurine regions).

MATERIALS AND METHODS

Preparation of cloned retrovirus DNA. All viral genomes were derived from a permuted DNA clone of murine amphotropic virus 4070, called Ampho 8 (a gift from A. Oliff [2]). Figures 1 and 2 show how Ampho 8 DNA was manipulated to yield the various constructions shown in Fig. 3. All DNA manipulations were performed in *Escherichia coli* by standard cloning protocols (11). Ampho 8 DNA was digested with enzymes *Cla*I and *Eco*RI, and the purified LTR-containing fragment was ligated to an ampicillin-sensitive, kanamycin-resistant derivative of pBRNeo (23) called Rheo. The cloned product, A8CR, was digested with *Cla*I, the ends were made flush with Klenow polymerase in the presence of all four deoxynucleotide triphosphates, and subsequent ligation yielded a plasmid, A8CRC, resistant to *Cla*I digestion. Cloned plasmid A8CRC was digested with *Pvu*II, treated with BAL 31 exonuclease, and ligated to *Cla*I linkers. Transformed *E. coli* cells were screened for plasmids that had lost the sequence between nucleotides 250 and 415 (Fig. 4). A plasmid, A8PBC, had lost the sequences downstream of nucleotide 269 in which a *Cla*I linker had been inserted (determined by nucleotide sequencing [13]). The extent of the deletion into the *gag* gene was not determined. The plasmid A8CRC was also digested partially with *Pst*I, treated with S1 nuclease and Klenow polymerase, and ligated to *Cla*I linkers. A cloned plasmid, A8PPC, that contained a *Cla*I linker at the *Pst*I site proximal to the LTR was isolated. The plasmid A8CR was also digested with *Pvu*II, treated with

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FIG. 1. Schematic diagram of plasmid constructions. The thick lines represent procaryotic DNA elements; the thin lines represent retroviral DNA elements. The box represents an LTR composed of U_3 , R, and U_5 regions. The viral genes *gag*, *pol*, and *env* are indicated where present. PP, polypurine tract; C, *Clal*; H, *HindIII*; P1, *PvuI*; Ps, *PstI*; RI, *EcoRI*; RV, *EcoRV*; S, *SalI*; Sc, *SacI*. All long arrows represent DNA manipulations followed by a cloning step in *E. coli*.

FIG. 2. Representation of the isolated fragments used to construct the genomes shown in Fig. 3. Abbreviations are as given in the legend to Fig. 1.

FIG. 3. DNA genomes used in this study. Large boxes represent the LTRs. The 5' and 3' splice sites represent the major sites of RNA splicing. Pack represents a *cis*-acting packaging locus. The ATG represents the translation start site for the *gag* gene. The TAG represents the translation stop site for the *env* gene (although nucleotide sequence analysis has shown this to be a TGA in the amphotropic virus). The *gag*, *pol*, and *env* genes have not been drawn to scale. The polypurine tract is represented by PP. Neo represents the Tn5 gene for neomycin resistance restricted with enzymes *Hind*III and *Sma*I and modified with *Cla*I linkers. The clone prA8 is derived from the permuted clone amphi 8 from 4070 virus (2). To create prA8, the amphi 8 DNA was rearranged to resemble a provirus and cloned into plasmid pBRNeo. The *trans*-acting plasmid, transpack, was derived from prA8 by deleting nucleotides 269 through 555 (nucleotide 1 being the left-most nucleotide of R). The transpapp plasmid is similar to transpack, except nucleotides -395 through -35 have been deleted as well (nucleotide -1 is the right-most nucleotide of the downstream U₃ region). The pappy plasmid is similar to transpapp, except the packaging locus has not been removed. The *cis*-acting vector, cistor, was derived from prA8 by deleting the DNA between nucleotides 550 (a natural *Pst*I site) and -517 (a natural *Cla*I site). The *Pst*I site has been converted to a *Cla*I site. Cistorneo results from the insertion of the modified neomycin resistance gene into the *Cla*I site of cistor. NIH/3T3 cells were cotransfected with a neomycin-resistant plasmid pKONeo (a gift from D. Hanahan) and one of the *trans*-acting genomes. G418-resistant colonies were cloned, and reverse transcriptase levels in the culture media were measured. The clones expressing the highest levels of reverse transcriptase were expanded and named as shown in the figure.

BAL 31 exonuclease, and ligated to *Eco*RI linkers. A cloned plasmid, A8PBR, that had lost the sequences downstream to nucleotide 185 was isolated. An *Eco*RI linker was attached to nucleotide 185; the extent of the deletion into the *gag* gene was not determined. Plasmid A8PBR was digested with *Eco*RI and then religated to delete the *gag*, *pol*, and *env* sequences. The resulting plasmid was called DTR. DTR was digested with *Eco*RV, treated with BAL 31 exonuclease, digested with *Sac*I, treated with S1 nuclease and Klenow polymerase in the presence of all four deoxynucleotide triphosphates, and then ligated to *Sal*I linkers. A cloned plasmid, PAPP, that had lost the sequence between nucleotides -395 and -35 (Fig. 4) was isolated. DNA sequencing revealed that a single *Sal*I linker had replaced the deleted region.

Figure 2 illustrates how the viral DNA variants shown in Fig. 3 were constructed with isolated fragments from the

plasmids shown in Fig. 1. All of the viral DNA constructs except prA8 were cloned in plasmid pUC9 (25) with *Hind*III and *Eco*RI restriction sites. The prA8 DNA was cloned into pBRNeo (23) with *Hind*III and *Pvu*I sites.

Cells and transfections. NIH/3T3 and WI-38/VA-13 cells were grown in Dulbecco modified Eagle medium containing 10% fetal bovine serum in the presence of penicillin and streptomycin. The cells were plated onto 10-cm dishes at 30% confluency. Standard transfection techniques were used (30); however, cotransfections were performed without carrier DNA. The amounts of selectable marker plasmid and viral plasmid were 1 and 15 μ g, respectively. The cells were exposed for 2 min to 2 ml of 15% (vol/vol) glycerol in medium 4 h posttransfection. Reverse transcriptase activity was determined as described previously (4). Selection in the antibiotic G418 was as described (23), with concentrations of 350 μ g/ml for NIH/3T3 cells and 175 μ g/ml for VA-13 cells.

Isolation of RNA and S1 nuclease analysis. Total cellular RNA was isolated with guanidine isothiocyanate (3) and analyzed by S1 mapping as described previously (1, 28).

RESULTS

Figure 3 illustrates the genomes used. In transpack, a 280-base-pair region was deleted downstream of the 5' splice site (see Fig. 5 for splicing data), a region that generally encodes a *cis*-acting packaging locus (12, 26, 27). Transpack was cotransfected into NIH/3T3 cells with the dominant selectable marker pKONeo (a gift from D. Hanahan), and G418-resistant colonies were selected (23), cloned, and expanded. Reverse transcriptase levels in the culture medium of the expanded clones were variable, the highest being 15% of prA8 (T19-13Z; Table 1). Figure 5 demonstrates the presence of transpack RNA, but not wild-type RNA, in these cells. We could not detect transpack RNA in the culture medium with a sensitive S1 nuclease technique (data not shown), which is consistent with there being a deletion of the *cis*-acting packaging locus. Two weeks after culture medium from a plate of T19-13Z cells was placed on a plate of NIH/3T3 cells, the NIH/3T3 cells remained reverse transcriptase negative, and transpack RNA could not be detected by the S1 technique.

Because retroviruses are known to package low levels of cellular RNA (9), we suspected that a simple packaging mutant might be "leaky" at a low frequency. The transpack genome, shown in Fig. 3, was deleted further to remove the *cis*-acting polypurine locus (22) and most of the downstream U₃ region. Deletion of these regions should not affect viral RNA or protein production in the primary cell line. However, if a transpack genome were to be packaged into a virus particle, it would be blocked at later stages in replication: plus-strand DNA synthesis (polypurine mutation), DNA integration (deletion of inverted repeat in U₃ [17]), and second-generation gene expression (since the transcriptional promoter in the U₃ region of the upstream LTR is derived from the U₃ region of viral RNA). Cell lines containing

TABLE 1. Characteristics of cell lines^a

Cell line	RTase activity	Cistronero titers		Replication-competent virus production
		NIH/3T3	VA-13	
NIH/3T3	1.5×10^4	0	0	No
VA-13	1.5×10^4	0	0	No
T19-13Z	1.5×10^5	5×10^3	5×10^3	* ^b
T19-14X	8×10^4	1×10^3	1×10^3	No
T19-15W	1×10^5	2×10^4	2×10^4	Spontaneously
VT19-17-H2	7×10^4	1×10^3	1×10^3	No
VT20-43-D12	7×10^4	1×10^3	1×10^3	No
prA8	1×10^6	5×10^4	5×10^4	Yes

^a The reverse transcriptase (RTase) activity is shown as incorporated counts per minute. The cistronero titers were measured by transfecting the cell line listed with cistronero DNA, harvesting culture media after 48 h, passing the media through a 0.45- μ m filter, adding polybrene to the media to a concentration of 8 μ g/ml, and infecting either NIH/3T3 or VA-13 cells with the media. The infected cells were placed in G418 selection media 48 h later, and colonies were counted after 7 to 10 days. For the VT20-43-D12 cells, culture media was harvested without prior cistronero transfection (since these cells contain cistronero endogenously). Cell lines were tested for replication-competent virus production by harvesting and treating culture media as above, infecting NIH/3T3 cells and growing them under non-selective conditions, and testing the NIH/3T3 cells for reverse transcriptase activity 2 weeks later. Reverse transcriptase activity was found to be either below 2×10^4 cpm or above 1×10^6 cpm in these assays, with no intermediate values.

^b *, Occasionally after transfection with cistronero but not after transfection with nonviral DNA.

FIG. 5. S1 nuclease protection studies. A DNA probe was constructed by subcloning the *Kpn*I to *Bam*HI fragment shown into a bacterial plasmid. The plasmid was digested with *Kpn*I and 3' end labeled with terminal transferase. For each lane, 30 μ g of total cellular RNA was hybridized to a 10-fold molar excess of the DNA probe. S1 nuclease digestion and denaturing acrylamide gel electrophoresis were as described previously (1, 28). (A) RNA was purified from NIH/3T3 cells infected with a mixture of prA8- and cistronero-derived viruses and grown under G418 selection. RNA processed at the 5' splice site generates a 172-nucleotide band. RNA from cistronero generates a 523-nucleotide band. Unspliced RNA from prA8 generates a 980-nucleotide band. (B) RNA from uninfected cells. (C) RNA from T19-13A-C10 cells, a line carrying the transpack genome. Bands representing spliced RNA and unspliced RNA lacking the packaging region are seen. No wild-type RNA is seen. (D) RNA from the T19-13Z line carrying transpack. (E) RNA from the T19-14X cell line carrying transpack. (F) RNA from the T19-15W cell line carrying pappy.

transpapp were established as for T19-13Z. The highest level of reverse transcriptase detected in the culture medium was 8% of prA8 (T19-14X; Table 1). This clone was expanded. Again, culture medium from these cells failed to produce a viral infection in NIH/3T3 cells as assayed by reverse transcriptase and S1 mapping.

The pappy genome, shown in Fig. 3, was deleted only in the polypurine and U₃ regions. Several clonal cell lines were established. We noted that some clones expressed wild-type reverse transcriptase levels, and these were found to be producing replication-competent particles (assayed by infecting NIH/3T3 cells with culture fluid and measuring their reverse transcriptase activity 2 weeks later). Other clones with lower reverse transcriptase levels became replication-competent producers after serial passage. We see two possible explanations for the production of competent virus. The first involves DNA recombination or rearrangement during transfection of cells with pappy. As shown in Fig. 1 and 2, the A8CRC fragment used to construct pappy contains a normal polypurine tract and U₃ region near the *Hind*III site. Although this region should not be transcribed into RNA during transfection, a recombination event with the PAPP-derived region of pappy on a second plasmid could produce a template for transcription resembling a replication-competent genome. Such recombination events, involving two exogenous molecules, are common during transfection (10, 20) and are likely to have produced the replication-competent viruses we observed. An alternative explanation is that an endogenous provirus supplied the information necessary to repair the defect in the 3' end of the pappy genome. Precedent has been set for such a recombination event in cultured cells in which a functional 3' LTR was contributed by an endogenous virus (5). Regardless of the mode of replication-competent virus production, the pappy genome was unsuitable and was not used further.

The *cis*-acting vector, called cistor, is shown in Fig. 3 and was derived entirely from the amphotropic genome. The DNA sequence of this portion of the genome is shown in Fig. 4. The coding region of the Tn5 gene for neomycin resistance was inserted into this vector, and the recombinant was called cistorneo. Cistorneo virus can replicate in the presence of a helper virus in titers roughly equivalent to that of the helper. When T19-13Z cells were transfected with the cistorneo plasmid and culture medium was harvested 42 h later, ca. 5×10^3 virus particles per ml could be detected by infection of NIH/3T3 or VA-13 cells (transformed WI-38 human fibroblasts) and subsequent exposure of target cells to G418 selection (Table 1). The target cells usually remained reverse transcriptase negative and did not produce virus as assayed by infectivity in NIH/3T3 cells. However, in ca. one-third of the transfections involving cistorneo and T19-13Z cells, a replication-competent virus was generated and could be detected by reverse transcriptase and infectivity assays of the target cell culture medium 2 weeks after infection. In contrast, T19-14X cells transfected with cistorneo produced ca. 10^3 Neo viruses per ml, titered on either murine or human cells, without any occurrence of replication-competent virus production in multiple independent transfections (Table 1).

Since the T19-14X cell line did not produce replication-competent virus after transfection with cistorneo, we constructed a cell line that contained both transpapp and cistorneo integrated chromosomally. VA-13 cells were cotransfected first with transpapp and pSV2gpt, and selection was applied as described previously (16). Cells were cloned and assayed for reverse transcriptase production.

The clone VT19-17-H2 was found to produce 7% of wild-type levels. A plate of these cells was then transfected with cistorneo, and selection for both pSV2gpt and neo was applied. Resistant colonies were expanded and tested. In no case did the resistant cells produce more than 7% of the level of reverse transcriptase found in VA-13 cells infected with the wild-type virus. Culture fluid from the doubly resistant cells did not contain a replication-competent virus as assayed on NIH/3T3 and VA-13 cells. However, NIH/3T3 or VA-13 cells infected with culture medium from some of the doubly resistant cells were found to be resistant to G418 selection. Figure 6 shows an S1 nuclease analysis of RNA from the doubly resistant cell line VT20-43-D12 and from G418-resistant NIH/3T3 cells after infection with VT20-43-D12 culture medium. The VT20-43-D12 RNA contains both transpapp and cistorneo transcripts, whereas the G418-resistant infected NIH/3T3 cells contain only the cistorneo transcript. The high-molecular-weight bands seen in all lanes are probably due to reannealed probe and its degradation products. Thus, VT20-43-D12 cells produce cistorneo-containing virus particles in the absence of a replication-competent helper virus. Unfortunately, the titer of cistorneo produced by these cells does not exceed 10^3 particles per ml. The band representing the 5' RNA splice (172 nucleotides) is rather faint (Fig. 6, lane A). This suggests that these cells

FIG. 6. S1 nuclease analysis with the same DNA probe as described in the legend to Fig. 5. The diagram at the bottom of Fig. 5 applies to this figure as well. Each lane represents hybridization of 30 μ g of total cellular RNA to a 10-fold molar excess of DNA probe, followed by S1 nuclease digestion and electrophoresis on an 8% acrylamide denaturing gel. RNA samples were obtained from (A) VT20-43-D12 cells, (B) G418-resistant NIH/3T3 cells infected with media from VT20-43-D12 cells, and (C) uninfected NIH/3T3 cells; (D) ϕ X DNA digested with *Hinf*I.

may be splicing the transpapp RNA rather poorly, which may account for the low titer. In addition, there are two bands in the 150- to 200-nucleotide region, suggesting an alternate splicing pattern in this mutant. If the variant-sliced RNA produced a non translatable mRNA, the titer may also be lower.

DISCUSSION

Presuming that recombination during the replication of retroviruses occurs during reverse transcription (8), we interpret the data in Table 1 to suggest that the packaging mutation alone probably does not block RNA packaging completely. We suspect that after T19-13Z cells are transfected with cistorneo, a rare virus particle is produced that contains not only the cistorneo but the transpack transcript as well. Since these genomes together contain all of the information necessary to produce a replication-competent genome, it is conceivable that a recombination event leading to the production of a replication-competent genome might occur during reverse transcription. Since only a single recombination event between the cistorneo and transpack genomes is necessary to produce a genome resembling prA8, the event might occur at an observable frequency. In contrast, the transpapp genome, having two widely separated defects, would require a double recombination to produce a replication-competent genome. We suspect that although such an event may occur, its frequency is probably too low to be observed under routine conditions. An alternative explanation is that recombination occurs between exogenous cistorneo DNA and integrated transpack DNA during transfection. However, there is little precedent for such a process. The role of endogenous proviruses in the production of replication-competent genomes from transpack and transpapp is uncertain. However, we suspect their role to be minor, if anything, since the cell line T19-13Z does not produce replication-competent virus unless transfected with virus-related DNA.

Two other groups who have constructed packaging mutants do not report observing recombination (12, 27). In one report (27), the *env* gene is segregated from the *gag* and *pol* genes on a separate plasmid. This type of construction requires the extraction of genetic information from three separate DNA molecules to produce a replication-competent genome (an unlikely event). However, the other report (12) utilizes a Moloney murine leukemia virus construction similar to transpack. Perhaps slight differences in the extent of the packaging deletion or differences in virus type account for the difference in recombination frequency observed.

The current retroviral vector systems produce relatively low titers of recombinant virus. The titer we observed agrees with that reported by Watanabe and Temin (27) but is lower than that reported by Mann et al. (12). It has been shown that transfected retroviral DNA is not expressed as well as DNA integrated after viral infection (6). If the site of transpapp integration is influencing gene expression, a large number of colonies may need to be screened before high producer lines are found. Alternatively, the transcriptional control elements of transpapp may need to be altered to improve virus output.

In summary, we have developed a replication-defective retrovirus vector with broad host range. Because of its defective nature it does not produce a helper virus and should not cause a viremia if used in vivo. Its advantages arise from the efficient and predictable integration of the retroviral genome. Currently, gene expression is governed by the viral LTR. However, promoter mutagenesis or substitution should be possible if warranted. The major shortcom-

ing of the system, at present, is a low viral titer. However, this should be overcome by further modification of the transcriptional or splicing control elements, by inducing gene amplification in the helper cell line, or by screening a large number of cell lines looking for a rare high producer.

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