The ability of retrovirus vectors to deliver genes to a variety of dividing cell types in vitro and in vivo has been applied in basic research and gene therapy largely over the last 20 years. As the interest in gene therapy is expanding from single gene disorders to multiple gene diseases and combined gene therapies, new reliable polycistronic vectors capable of transducing several genes together are required. Tetracistronic retrovirus vectors derived from Moloney murine leukemia virus (MoMLV) are currently available (de Felipe & Izquierdo, 2000). These vectors carry two different internal ribosome entry segments to construct and characterize three independent tetracistronic retroviroes of different sizes. Efficient co-expression of the five proteins was successful and titres obtained for these tetracistronic virus vectors (final genome size ~7-9 kb) were comparable to those of vector systems with shorter genomes. Other vectors constructed that exceeded the genome length of the wild-type virus suffered frequent deletions.

The picornavirus foot-and-mouth disease virus 2A sequence was combined with three different internal ribosome entry segments to construct and characterize three independent pentacistronic retroviroes. Efficient co-expression of the five proteins was successful and titres obtained for these pentacistronic virus vectors (final genome size ~7-9 kb) were comparable to those of vector systems with shorter genomes. Other vectors constructed that exceeded the genome length of the wild-type virus suffered frequent deletions.

The three pentacistronic retrovirus vectors of increasing lengths (Fig. 1) were made by standard genetic-engineering techniques and were maintained as retrovirus plasmids. The autocleavable FMDV IRES(previously (de Felipe & Izquierdo, 2000), introducing a BglII FMDV IRES-luc cassette at the unique BglII site between the neo gene and the EMCV IRES. The FMDV IRES-luc cassette came from plasmid pBIC (Martı́nez-Salas et al., 1993), filling the Bfml single-stranded ends with Klenow and ligating it to the also filled BgIII site of pSXL1-TK (Sugimoto et al., 1994). The construction of Penta-7.9 was achieved by deleting the luc gene from Penta-8.5 and replacing it with the hygro gene via the BglIII site downstream of the FMDV IRES. The hygro gene comes from pBabeHygro (Morgenstern & Land, 1990) and was amplified by PCR incorporating BamHI and BgIII sites at both ends of the gene. The hygro gene and the junction between FMDV IRES and hygro were verified by standard sequencing procedures.

All vectors share the same structure (Fig. 1) and the final length of each one (7-9, 8-5 and 9-3 kb) is given by the sizes of the genes carried: luc (1797 bp) substitutions for hygro (998 bp) in Penta-8.5 and plap (1574 bp) substitutes for egfp (719 bp) in Penta-9.3.

Plasmid retrovirus vectors were used to transiently transfected ectopic packaging ¥CRE mouse cells and their supernatants to infect amphotropic ¥CRIP cells, as described by Miller & Rosman (1989). Cells were selected with 2 µ puromycin ml⁻¹ for 6 weeks to obtain a population of resistant cells. Titres were estimated by infecting NIH 3T3 cells as described previously (Izquierdo et al., 1997). In all experiments, a plate of uninfected NIH 3T3 cells was placed under the same selection conditions as the infected plates to ensure that a lethal dose of antibiotic selection was used. Cells in the ¥CRIP-infected plates used to harvest the supernatant were counted after collection and the titres expressed as transducing units (TU) ml⁻¹ per cell. The luciferase activity assay was performed as described by...

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Martín et al. (2000) with minor modifications (1·3 × 10^5 cells were used and 10 μl of the total 100 μl of cell extract was taken to measure luciferase activity). To estimate plap expression, cells were fixed and stained with BCIP/NBT, as described previously (de Felipe & Izquierdo, 2000).

Supernatants from ψCRIP populations showed titres of about 2 × 10^5 TU ml^-1 for Penta-7.9 (equivalent to 0.02 TU ml^-1 per cell) and somewhat surprisingly, titres were higher in the larger vectors (0.36 and 0.15 TU ml^-1 per cell for Penta-8.5 and Penta-9.3, respectively). Expression for all of the reporter genes was positive in cell lawns from Penta-7.9 but low or negative for the last two genes in lawns from Penta-8.5 and Penta-9.3, respectively. Expression was also very weak or negative for all genes in Penta-9.3, except for the selection gene pac (data not shown). These results suggested the presence of rearrangements in the larger vectors. No replication-competent retroviruses were detected (de Felipe & Izquierdo, 2000) using the same supernatants from producer ψCRIP cells.

As lawns may represent a mixture of different integration events, it is always preferable to work with individual clones descendant from a single cell. Retrovirus-containing supernatants from producer ψCRIP cells were used to infect NIH 3T3 fibroblasts. These cells were selected in medium containing 2 μg puromycin ml^-1 and surviving colonies were picked and expanded for gene expression analysis. Four NIH 3T3 cell clones harbouring the vector Penta-7.9 were obtained and studied. Expression of egfp was monitored by flow cytometry (Fig. 2a) under the conditions described previously (de Felipe & Izquierdo, 2000).

Only clones 1 and 3 were positive for expression. To detect the expression of other drug-resistance markers, clones were plated and selected for 7 days with 2 μg puromycin ml^-1, 1 mg G418 ml^-1 and 150 μg hygromycin ml^-1, either alone or in combination. Colonies 1, 2 and 3 showed resistance to the four situations. The combination of the three drugs reduced cell viability to 50%; this could be attributed to difficulties in expressing three resistant proteins at levels high enough to resist the combined triple selection. Despite this, we were able to obtain cell lines that were cultured for 4 weeks in each case (Fig. 2b). Clone 4 was resistant only to puromycin. Finally, puromycin-resistant cell clones were
plated and maintained in media containing either no drug or 1 μg GCV (ganciclovir) ml⁻¹ for 4 days. Again, the first three clones showed a good sensitivity to GCV (Fig. 2c).

Individual cell clones containing vectors Penta-8.5 and Penta-9.3 were also isolated by resistance to 2 μg puromycin ml⁻¹ and the expression of the first, third and fifth genes was assayed. These results are summarized in Fig. 1 by positive or negative symbols under each gene and corresponding clone number. The three NIH 3T3 clones isolated after infection with Penta-8.5 displayed the same pattern of gene expression as that with the YCRI population (lawn): resistance to puromycin and G418 (and the combination of both drugs), but absolute lack of GCV sensitivity. In clone number 2, full analysis was carried out and expression of egfp was detected, while no luciferase activity was monitored. A final group of three clones infected with Penta-9.3 showed only resistance to puromycin. The expression of plap was not determined in these clones as the lawn estimation for the gene was negative.

To verify the structural integrity of the proviruses, a long PCR was carried out on genomic DNA extracted from the 10 clones isolated (Fig. 3). The extraction of genomic DNA and the long PCR procedures were carried out as described previously (de Felipe & Izquierdo, 2000), except that

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<th>Vector</th>
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<td>Penta-7.9</td>
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### Fig. 2. Gene expression from NIH 3T3 cell clones infected with retrovirus Penta-7.9. (a) EGFP expression measured by flow cytometry. Vertical lines show the setting of the electronic gate used to distinguish between EGFP-negative and -positive cells. Percentage of total cells and mean values of fluorescence are also shown. (b) Resistance to 2 μg puromycin ml⁻¹, 1 mg G418 ml⁻¹, 150 μg hygromycin ml⁻¹ and the combination of the three, expressed as per cent cell survival. Survival in the presence of puromycin was considered arbitrarily as 100 %. Cells grown without drug selection were considered as 100 % survival. Under these conditions, it is not unusual to obtain survival values in the drug selection samples higher than in controls. (c) Sensitivity to 1 μg GCV ml⁻¹, expressed as per cent cell survival. Values for cells preselected in puromycin and without GCV were considered as 100 %.
elaboration of the amplified region begins at the centre of the pac gene and finishes at the 3’ LTR. The experiment enables us to correlate deficiencies in gene expression with major rearrangement in the vectors (involving changes in the size of the bands amplified by PCR). Clones 1, 2 and 3 from Penta-7.9 show a band that matches with the expected size fragment of 6-6 kb, but clone 4 seemed to have suffered a large deletion and produced a small band of about 1-6 kb. The only functional gene coded in this deleted provirus is pac, as the expression of all other genes is not detected. More difficult to explain is clone 2, in which a deletion is not apparent (within the detection limits of the electrophoretic analysis), but the second gene, egfp, is not expressed. Sequencing revealed a run of nine thymidine residues between the FMDV 2A gene and the ATG codon of egfp, a situation known to create a hot spot for frameshift mutations in retroviruses (Pathak & Temin, 1990; Burns & Temin, 1994). Two different PCR bands amplified from the genome of clone 2 showed 10 thymidine residues instead of the original nine, thereby creating a frameshift mutation.

Large deletions were detected in all Penta-8.5 and Penta-9.3 virus clones (Fig. 3), explaining the lack of expression of reporter genes downstream of the selection marker and the high titres observed. These results suggest that it may be risky to go close to or over the natural 8-3 kb size of the MoMLV genome. Nevertheless, previous reports show acceptable stabilities when short fragments of less than 1-5 kb are introduced into the full 8-3 kb retrovirus genome (Logg et al., 2001a, b). Highly manipulated viruses such as the ones we present here may be more susceptible to size limitations.

Despite the aforementioned restrictions, we have demonstrated that is possible to introduce five foreign cistrons into a retrovirus. Naturally, the number of cistrons increases, the genes cloned have to become smaller. In our vectors, all the genes carried are smaller than 2 kb; therefore, it may be difficult to acquire substantial additional space. To our knowledge, these are the first functional pentacistronic retrovirus vectors constructed to date that allow the expression of five independent proteins from a single transcription unit. There are reports of pentacistronic vectors using herpes simplex viruses (Krisky et al., 1998) or vaccinia virus (Carroll et al., 1998). However, these vectors, unlike the ones reported here, use several promoters along their large viral genomes. In the present study, we have constructed complex pentacistronic vectors able to co-express five independent genes provided that the size of the wild-type genome is maintained. These results increase our knowledge about the capability and limitations of retroviruses as polycistronic gene vehicles.


