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Helper-Independent Retrovirus Vectors with Rous-Associated Virus Type O Long Terminal Repeats

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We have constructed nonpermuted replication-competent avian retrovirus vectors that derive from Rous sarcoma virus (S. H. Hughes, J. J. Greenhouse, C. J. Petropoulos, and P. Sutrave, J. Virol. 61:3004–3012, 1987). We describe here the construction and properties of corresponding vectors in which the long terminal repeats (LTRs) of the parental virus have been replaced by the LTRs of the endogenous chicken virus Rous-associated virus type O. The Rous-associated virus type O LTR vectors replicated approximately 1/10 as well as the parental vectors and expressed a test gene, chloramphenicol acetyltransferase, approximately 1/30 to 1/50 as well.

We have constructed a series of helper-independent retrovirus vectors, the prototypes of which are called RCAN and RCAS, that have avian leukosis virus (ALV) long terminal repeats (LTRs) (11). These vectors derive from a cloned copy of the genome of the Schmidt-Ruppin strain of Rous sarcoma virus. The *src* gene was removed by manipulation of the cloned DNA in vitro, and a unique *Cla*I site that can be used for the insertion of foreign DNA has replaced the *src* gene (9, 20, 21). The vectors RCAS and RCAN differ in that RCAN lacks a splice acceptor required to express an inserted gene from the LTR promoter via spliced mRNA. RCAS has the splice acceptor. We have also reported the construction of a series of plasmids, called adaptors, that make it simple to insert a wide variety of DNA sequences into the RCAS and RCAN vectors (11).

In contrast to the exogenous ALVs, the related endogenous virus (Rous-associated virus type O [RAV-O]) has little, if any, oncogenic potential (2, 15). Natural and constructed recombinants have localized two determinants of oncogenicity, one in sequences outside the LTR region and the other in the LTR (16–18). The contribution of sequences outside the LTR to oncogenicity is not well understood; however, it is generally accepted that the differences in oncogenicity of the ALV and RAV-O LTRs derive from the presence of a powerful transcriptional enhancer in the ALV LTR that is absent from the RAV-O LTR (3–5, 13, 22). Oncogenic potential and toxicity are important criteria in the design of vectors for use in vivo, and we have already described a RAV-O LTR vector that is sufficiently benign to be inserted into the germ line of chickens (12, 18, 19).

We report here the construction and properties of a new vector called RCOS (replication competent, RAV-O LTR, splice acceptor) that has the ALV LTRs of the RCAS vector substituted by a RAV-O LTR. The adaptor plasmids can be used with the RCOS vector. Judging by the levels of reverse transcriptase activity, RCOS viruses replicate 5 to 10% as well as the corresponding ALV constructions. We have examined the expression of chloramphenicol acetyltransferase in RCOS and found levels of expression substantially lower than those seen with the corresponding RCAS constructions.

The LTR used in the construction of RCOS is derived from an unintegrated circular RAV-O DNA containing a single copy of the LTR. The sequence of this particular clone is known (10). BAL 31 deletions were used to introduce *Cla*I sites at positions upstream of the LTR (10). The various clones were named on the basis of the position of the *Cla*I site relative to the LTR. −170 RAV-O had a *Cla*I site 170 base pairs upstream of the LTR, just at the end of the *env*-coding region. The −170 RAV-O segment was digested with *Cla*I, which cleaves upstream of the LTR, and *Sst*I, which cleaves in the leader between the LTR and *gag*. The ALV vector 779/2795 (9) was digested with *Cla*I and *Sst*I, the ALV LTR was removed, and the −170 RAV-O LTR segment was inserted in place of the ALV LTR. The resulting vector is called 779/−170 RAV-O (12). The ALV vectors were named for the position of the *Cla*I site relative to a conserved *Eco*RI site in *env*; e.g., 779/2795 has the *src* gene deleted, with one side of the deletion 779 bases beyond the *Eco*RI site and the other 2,795 bases from *Eco*RI (6, 9).

The 779/−170 RAV-O vector has sequence homology flanking the *Cla*I site (12). To remove this homology, the plasmid was linearized with *Cla*I and digested with small amounts of BAL 31 exonuclease. The exonuclease-digested DNA was treated with the Klenow fragment of *pol*I in the presence of all four deoxynucleotide triphosphates and religated in the presence of *Cla*I linkers (New England BioLabs, Inc.). Clones containing the *Cla*I linkers were identified by *Cla*I digestion, and the approximate position of the *Cla*I sites was estimated by restriction endonuclease digestions and precisely determined by Maxam-Gilbert sequencing (14) in both directions from the *Cla*I site.

A clone that had lost 20 nucleotides from the ALV side of the *Cla*I site and 43 nucleotides from the RAV-O side was found. This clone was called 759/−127 RAV-O (Fig. 1). The deletion completely eliminates the homology flanking the *Cla*I insertion site and leaves the *env* gene and the RAV-O copy of the direct repeat sequences intact (Fig. 1). Virus rescue experiments performed with this construction and the parent 779/−170 RAV-O showed that 759/−127 RAV-O replicates as well as the parent (data not shown).

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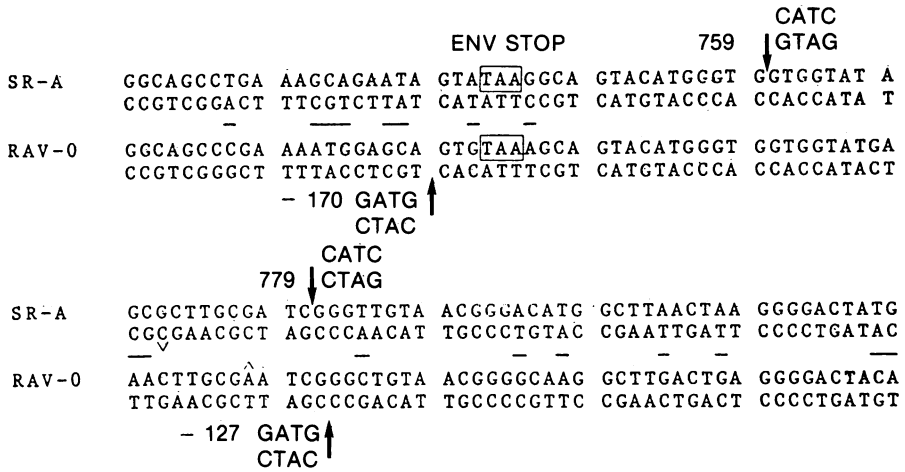

FIG. 1. Sequences at the joints between the ALV and RAV-O parents in 779/−170 RAV-O and 759/−127 RAV-O (RCON). Sequences are from the SRA-2 clone and the RAV-O clone used to construct the RAV-O LTR vectors. The positions where the linkers are inserted are indicated by arrows, and the sequence of the half linker that is present in the final constructions is shown next to the arrows. The precise sequence of the vectors can be deduced by reading from the SRA sequence up to the point of insertion of the linker through the half linker and then picking up the sequence at the half linker at the appropriate site and continuing into the RAV-O sequence.

By using a protocol similar to one used to convert the circular permuted ALV vector 779/2795 into the nonpermuted vector RCAN (11), the circular permuted ALV vector 759/−127 RAV-O was converted into the nonpermuted vector RCON. 759/−127 RAV-O was digested with a mixture of *Sal*I and *Bam*HI, and the segment containing *env*, the LTR, and a portion of *gag* was purified and ligated to *Sal*I-*Bam*HI-digested pPH, a derivative of pBR322 that lacks the *Cla*I site. An appropriate pPH clone was isolated and digested with a mixture of *Bam*HI and *Sst*I, treated with the Klenow fragment of *pol*I, and religated. In contrast to the corresponding RCAN construction (11), the *Bam*HI site was not reconstituted in this construction. The plasmid was cleaved with *Sal*I and *Nar*I, and the large fragment was purified on a gel. This segment was ligated to the *Cla*I to *Sal*I portion of 759/−127 RAV-O. The reconstructed nonpermuted vector, RCON (replication competent, RAV-O LTR, no splice acceptor), is shown schematically in Fig. 2.

To convert RCON, which lacks a splice acceptor, into a vector that can express genes from the LTR via a spliced mRNA, segment 989-1089 (9), which contains the *src* splice acceptor, was introduced into RCON. The resulting virus,

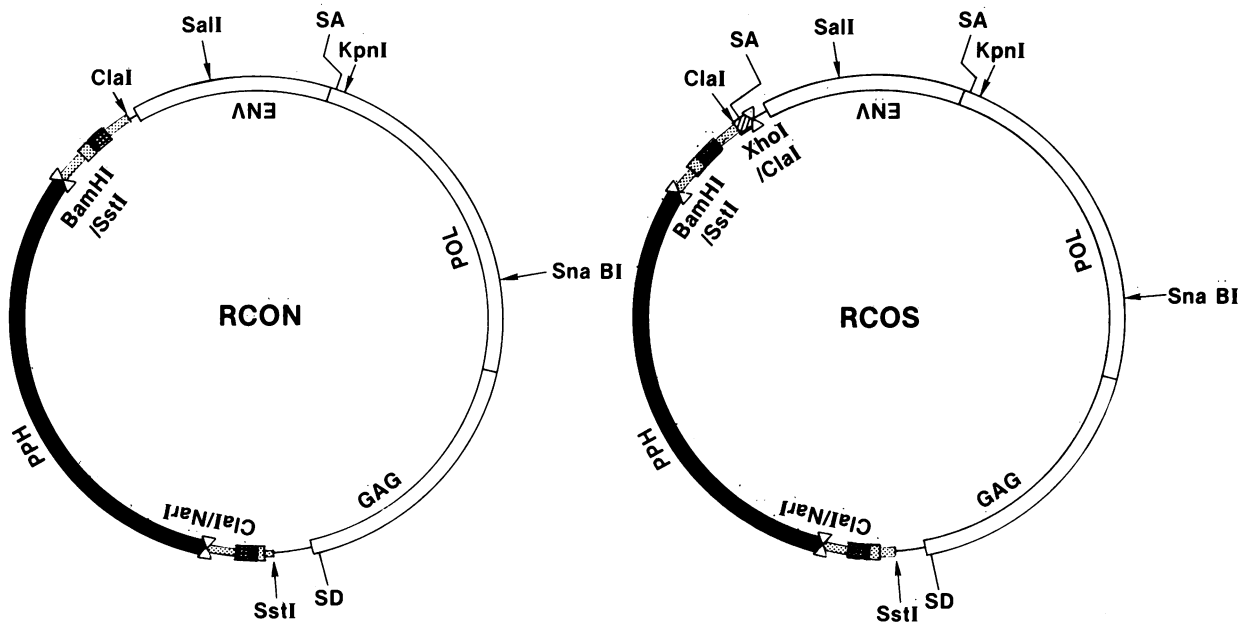

FIG. 2. Maps of the RCOS and RCON plasmids. Maps of the plasmids are drawn approximately to scale. The entire plasmid is 11.3 kilobases. Symbols: ■, *E. coli* replicon (pPH); □, retroviral genes *gag*, *pol*, and *env*; ▩, ▮, and ▯, U_3, R, and U_5 region of the LTRs; ▭, portion of RCON and RCOS deriving from the RAV-O parent. The constructions RCON and RCOS differ from each other only in that RCOS contains a 100-base-pair insert (labeled SA) that contains a functional splice acceptor. Also shown are the positions of the recognition sites for some of the enzymes that cleave the plasmids once, including the *Cla*I site, where foreign DNAs are inserted into the retroviral vectors.

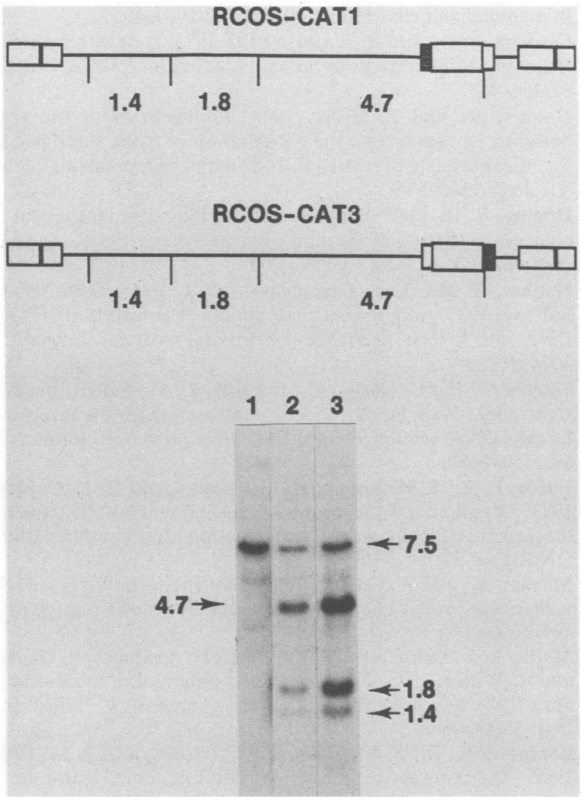

FIG. 3. Analyses of the provirus DNA in RCOS-CAT-1- and RCOS-CAT-3-infected line 0 cells. The top portion shows the *Bam*HI sites in RCOS-CAT-1 and RCOS-CAT-3 constructions. CAT is shown as an open box in the 3′ end of the viral genome. The only difference in the constructions is in the orientation of the polylinker used to insert CAT (smaller boxes surrounding the CAT insert). CAT was introduced into the *Bam*HI site of the polylinker of the adaptor plasmid *Cla* 12 as a *Sau*III A fragment (11). Both orientations of the CAT segment with respect to the polylinker have created a *Bam*HI site downstream of the CAT region. In the lower portion are shown *Bam*HI-digested DNAs from uninfected EV-O cells (1) (lane 1), RCOS-CAT-1-infected cells (lane 2), and RCOS-CAT-3-infected cells (lane 3). The DNAs were probed with SRA-2 DNA, which also detects c-*src* (top band in lanes 1 through 3); the c-*src* bands serve as an internal control for differences in DNA concentration. Numbers in both panels are fragment sizes, in kilobases.

using the old nomenclature, would be 759/989-1089/−127 RAV-O. For convenience it is now called RCOS. We have made separate tests of the properties of RCOS and RCON. The RCOS viruses were tested for their ability to express genes from the LTRs, and those data are reported below; RCON has been tested with genes under the direction of internal promoters, and those data will be reported elsewhere (C. Petropoulos and S. Hughes, unpublished observations).

The ability of the RCOS vector to carry and express a foreign gene was tested by inserting two versions of the bacterial chloramphenicol acetyltransferase (CAT) gene into RCOS and comparing the behavior of the two RCOS-CAT constructions with that of the corresponding ALV LTR (RCAS) CAT vector constructions. Like the RCAS vector, RCOS can be used with *Cla*I adaptor plasmids (11). The insertion of CAT into the adaptor plasmid *Cla* 12 in both orientations relative to the *Cla* 12 polylinker has been

TABLE 1. Reverse transcriptase activity in transfected cells[a]

Virus	Relative RT
RCAS-CAT-1	1.0
RCOS-CAT-1	0.05–0.1
RCAS-CAT-3	1.1
RCOS-CAT-3	0.05–0.1

[a] Cultures fully infected with the virus were measured for reverse transcriptase activity (RT) by using the standard assay (9). The data are normalized relative to the level of reverse transcriptase found in cultures infected with the wild-type strain of Rous sarcoma virus (3×10^5 cpm incorporated per hour). In such assays, twofold differences are not significant. All samples are from viruses grown in line 0 fibroblasts (1).

described (11), as have the ALV LTR vectors RCAS-CAT-1 and RCAS-CAT-3, which differ from each other only in the orientation of the adaptor sequences surrounding the CAT gene (Fig. 3). The corresponding RCOS constructions, RCOS-CAT-1 and RCOS-CAT-3, were constructed by inserting the CAT gene from *Cla* 12-CAT-1 and *Cla* 12-CAT-3 (as a *Cla*I fragment) into RCOS in the proper orientation (Fig. 3) (11).

All four viruses were transfected into chicken embryo fibroblasts (8, 23), and the cells were passaged. Reverse transcriptase activity plateaued approximately 7 to 10 days after transfection for the RCAS-CAT viruses and 15 to 20 days after transfection for the RCOS-CAT viruses (Table 1). The cells were harvested for CAT assays and DNA approximately 25 days after transfection. DNA samples were

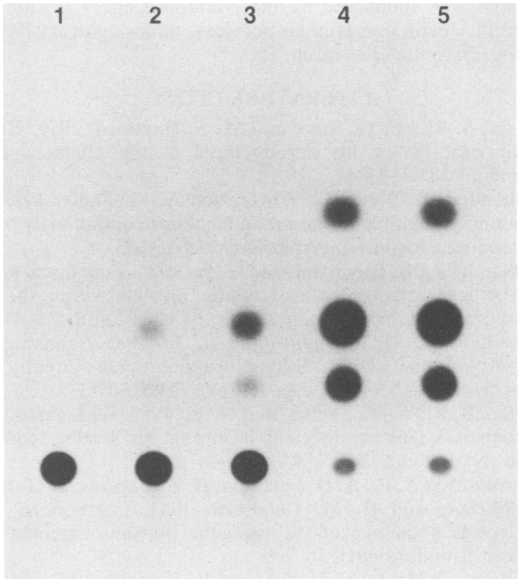

FIG. 4. CAT activity in RCOS-CAT- and RCAS-CAT-infected line 0 cells. To compare the levels of CAT made in RCOS-CAT- and RCAS-CAT-infected cells, extracts were prepared and the amounts of CAT enzymatic activity were compared in standard assays. Cells from a confluent 100-mm plate were lysed in 100 μl of buffer (7, 11), and the concentration of protein was measured by using the Bio-Rad reagent. The volumes of the lysates were then adjusted, on the basis of protein concentration, to give matching protein concentrations in all the extracts. The figure shows a photograph of an autoradiogram developed after 3 days of exposure. Lanes: 1, 50 μl of extract from uninfected cells; 2, 50 μl of extract from RCOS-CAT-1-infected cells; 3, 50 μl of extract from RCOS-CAT-3-infected cells; 4, 5 μl of extract from RCAS-CAT-1-infected cells; 5, 5 μl of extract from RCAS-CAT-3-infected cells.

digested with *Bam*HI, and the resulting fragments were separated on a 0.8% agarose gel, transferred to nitrocellulose, and probed with Rous sarcoma virus DNA, which would detect both deleted and undeleted forms of these proviruses. Both the RCAS-CAT viruses (11) and the RCOS-CAT viruses (Fig. 2) are stable, judging by this criterion. However, it is our experience that the stability of a particular vector construction often depends on the nature of the inserted sequences.

Samples of RCOS-CAT-infected cells were harvested and analyzed for CAT expression. Cells infected in parallel with RCAS-CAT viruses were also harvested and assayed. Standard CAT assays were done on the infected cells and uninfected controls (7, 11). Because CAT is efficiently expressed from the ALV LTRs, only 1/10 the amount of total cellular protein was assayed from the RCAS-CAT cells (Fig. 4). The RCAS-CAT cells contain approximately 30 to 50 times as much CAT enzymatic activity as do the RCOS-CAT cells; there is a small but reproducible difference in expression between RCOS-CAT-1 and RCOS-CAT-3 (Fig. 4).

We believe that the RAV-O LTR vector described in this report will have two principal uses. First, it may be used as a vector in vivo, especially in germ line infection experiments (12, 19). Second, the RCOS vector expresses a foreign gene at substantially lower levels than a corresponding RCAS vector, and as such, they can be used in conjunction with the RCAS to compare the effects of expressing the same protein at low and high levels. Experiments of this type will be considerably simplified, since both the RCOS and the RCAS vectors can be used with the adaptor plasmids (11).

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