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Adaptor Plasmids Simplify the Insertion of Foreign DNA into Helper-Independent Retroviral Vectors

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We have previously described several helper independent vector constructions (S. Hughes and E. Kosik, Virology 136:89–99, 1984; J. Sorge and S. H. Hughes, J. Mol. Appl. Genet. 1:547–599, 1982; J. Sorge, B. Ricci, and S. Hughes, J. Virol. 48:667–675, 1983), all of which derive from Rous sarcoma virus. In this report we describe three improvements in the earlier constructions. First, the vectors have been restructured as proviruses, which considerably improves the efficiency of virus production following acute transfection. Second, a series of miniplasmids have been developed, which we call adaptors, and these miniplasmids can be used to convert virtually any DNA segment into a *Cla*I fragment suitable for insertion into the retroviral (or other) vectors. Adaptors have been developed that supply regions of functional significance, including a splice acceptor and an initiator ATG. Finally, the region of *env* defining subgroup specificity, A in the original vectors, has been substituted by the corresponding regions of subgroup B and D viruses, giving vectors with additional subgroup specificities and increased host ranges.

Helper-independent retroviral vectors have significant advantages: there is no need for a helper cell or helper virus, which considerably increases vector stability since it eliminates rearrangements due to recombination with helper sequences; in general the titer of helper-independent vectors is substantially higher than that of defective vectors; and finally, with a helper-independent system there is no need to introduce a selectable marker into the vector, since any sequence present in the vector will be carried along passively during replication.

We have previously described a series of helper-independent retroviral vectors that derive from Rous sarcoma virus (RSV). In all of these vectors the region encoding the oncogene *src*, which is dispensable for viral replication, has been excised by manipulation of a cloned DNA copy of the viral genome and replaced by a synthetic DNA linker containing the recognition site for the restriction enzyme *Cla*I. Foreign segments can be inserted into the *Cla*I site of these vectors in place of *src*. In RSV the *src* gene is expressed as a spliced mRNA. A splice donor just 3′ of the *gag* ATG joins a splice acceptor 5′ of the *src* ATG. The splice acceptor originally located upstream of *src* is necessary for the production of spliced mRNA and consequently for the expression of sequences inserted in place of *src*.

The *src* gene of wild-type RSV is flanked by direct repeats, which in the SR-A strain are 110 base pairs (bp) in length (3). Recombination between these direct repeats, presumably during reverse transcription, results in the loss of *src*. Vectors that retain the direct repeats undergo similar recombination events that cause the loss of the inserted sequences. Refinements of the original vector constructions included several derivatives that had one of the two copies of the direct repeat removed, and the resulting constructions have been successfully used as vectors (8).

We report here a new series of RSV-derived vectors which offer significant advantages over those described previously. The new vector constructions have been restructured in the form of proviruses, which considerably increases expression of the cloned vector DNA in the acute transfection used to initiate viral replication. Since the initial burst of virus is greater, considerable time is saved in preparing a high-titer stock. More importantly, the number of rounds that the virus replicates before the stock reaches high titer is substantially reduced. This gives the vectors less opportunity for rearrangement, and this series of vectors is exceptionally stable.

Vectors with subgroup B and D *env* genes have been produced by exchanging the relevant portion of the *env* gene of the original subgroup A vectors with the corresponding region of viruses that carry subgroup B and D *env* genes. With these new vectors, it is possible to infect C/A chicken cells and to introduce two vectors carrying different genes into the same cell, and with the subgroup D vectors, it is possible to infect mammalian cells in culture.

To simplify the insertion of foreign DNA into the *Cla*I cloning site in the vectors, a series of adaptor plasmids has been built that permits the conversion of almost any DNA segment into a *Cla*I fragment. Adaptor plasmids have been produced that supply a DNA insert with initiator ATGs and splice acceptors. The ATG-containing plasmids have their polylinker arrays derived from the *Escherichia coli* expression plasmid pUC12N, which has an *Nco*I site at the initiator ATG. This means that the same DNA segment can be introduced into the viral vectors via the eucaryotic adaptor plasmid and in parallel into the *E. coli* plasmid pUC12N. In this fashion, the corresponding constructions give rise in both eucaryotic and procaryotic cells to precisely the same polypeptide. In addition, since both plasmids have a cloning site at the initiator ATG, the proteins produced need not be fusions. Although the adaptor plasmids were originally designed for use with the helper-independent retroviral vectors, the adaptors can be used with a variety of other vector systems. The specific properties of the adaptor plasmids are covered in the Discussion.

MATERIALS AND METHODS

Chemicals and enzymes. *E. coli* DNA polymerase I, restriction endonucleases, and DNA linkers were purchased from New England BioLabs, T4 polynucleotide kinase was

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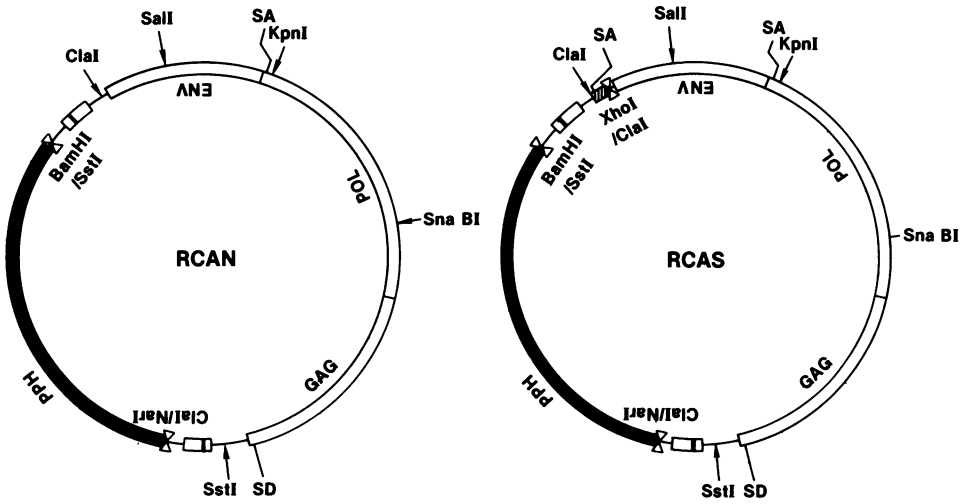

FIG. 1. Proviral vectors; maps of the proviral form of the retroviral vectors. The maps are approximately to scale. The entire plasmid is approximately 11.5 kilobases. The *E. coli* replicon (pPH) is shown in the drawing as a thick black bar. The approximate extent of the viral genes *gag*, *pol*, and *env* is indicated as open boxes. The LTRs are shown as open boxes with a heavy vertical bar denoting the R region. The recognition site for several enzymes that cut the construction once, *Sst*I, *Sna*BI, *Kpn*I, *Sal*I, and the unique site used for the insertion of foreign DNA, *Cla*I, are shown on the map. These two constructions, RCAN (left) and RCAS (right), differ by the presence, in RCAS, of a 100-bp insert just upstream of the *Cla*I insertion site that contains a functional splice acceptor (SA). The position of the splice acceptor used to produce *env* mRNA (SA) and the splice donor in *gag* (SD) are also indicated.

purchased from P-L Biochemicals, agarose was from Sigma Chemical Co., and acrylamide was purchased from Bio-Rad Laboratories.

Cells and transfections. C/E fibroblasts, which were derived from 12-day-old chickens of the EV-O strain, were grown in Dulbecco modified Eagle medium containing 5% calf serum, 5% fetal calf serum, 3% (wt/vol) tryptose phosphate broth, penicillin, and streptomycin.

In standard transfections, 5 μg of $CsCl_2$-banded DNA was introduced into chicken cells by $CaPO_4$ transfection (8, 21).

Plasmid constructions. (i) Proviruses. The new proviral vectors were all derived from the circularly permuted vectors described previously (8, 16, 17). The first step in conversion of the circularly permuted vectors into proviral form was to eliminate one of the tandem long terminal repeats (LTRs). This was accomplished by digesting the circularly permuted vector to completion with *Pvu*I. *Pvu*I cuts the construction in three places, once in the *amp* gene of the procaryotic replicon and once in each of the two tandem LTRs. The total digest was religated and used to transform *E. coli*. Clones were selected for resistance to ampicillin, and the resulting plasmids all contained a reconstructed *amp* gene. A clone was chosen that had lost the internal *Pvu*I fragment from the tandem LTRs and as such was identical to the parental vector 779/2795 except that it had a single LTR.

This single-LTR plasmid was digested with a mixture of *Sal*I and *Bam*HI, and the *Sal*I-*Bam*HI segment containing a portion of *env*, the LTR, and a portion of *gag* was purified on an agarose gel and ligated to *Sal*I-*Bam*HI-cut pPH, a derivative of pBR322 that lacks the *Cla*I site. An appropriate clone was isolated and recut with *Bam*HI and *Sst*I, treated with the Klenow fragment of *E. coli* DNA polymerase I, and religated. Klenow should remove the 3′ overhang on the *Sst*I side of the *gag* and fill in the 5′ overhang at *Bam*HI. Subsequent religation should restore the *Bam*HI site. The clone chosen had the expected *Bam*HI site. This plasmid was recut with *Sal*I and *Nar*I, and the large fragment was isolated on an agarose gel. This segment was ligated to the *Cla*I-*Sal*I portion of the single-LTR derivative of 779/2795. The final construction encoded the same viral vector as 779/2795 except that the retroviral portion was now in the form of a provirus, with the *E. coli* replicon between the LTRs. This vector was called RCAN (see Results).

To introduce a splice acceptor into the RCAN provirus, we inserted into RCAN a fragment (called 989-1089; see reference 8) that contains the *src* splice acceptor site. For convenience, the splice acceptor-containing segment was taken from a circularly permuted vector, 779/989-1089/2795 (8), as a *Kpn*I-*Cla*I fragment, and this fragment was used to replace the corresponding *Kpn*I-*Cla*I segment in RCAN. The resulting vector, RCAS, contained the same viral vector sequences as 779/989-1089/2795, except that the new vector (RCAS) was no longer circularly permuted (Fig. 1).

The subgroup B and subgroup D derivatives were made by excising the *Kpn*I-*Sal*I segment of both RCAN (the 779/2795 provirus) and RCAS (the 779/989-1089/2795 provirus) and replacing that segment with the homologous segment from a cloned Rous-associated virus type 2 (RAV-2) genome (subgroup B [12]) and from a Schmidt-Ruppin D clone (subgroup D [13]).

(ii) Adaptors. All the adaptor segments are carried by the miniplasmid 327ACRH. The plasmid 327 (15) was digested with *Hin*dIII and *Ava*I, and the digested DNA was treated with the Klenow fragment of *E. coli* DNA polymerase I and religated. As expected, this ligation recreated the *Hin*dIII site. The resulting plasmid was called 327AC. The *Eco*RI site was removed by digesting with *Eco*RI, treating with Klenow, and religating. The *Hin*dIII site was removed by a similar protocol. The plasmid with neither *Hin*dIII nor *Eco*RI sites was called 327ACRH. The polylinker from pUC12N was inserted into the *Cla*I site of 327ACRH by using small fragments derived from pBR322. To connect the *Hin*dIII end of the polylinker to *Cla*I, the segment of pBR322 from *Hin*dIII to *Cla*I was used. A special segment was created to connect the *Eco*RI end of the polylinker to *Cla*I. A 10-base oligonucleotide containing an *Eco*RI site was

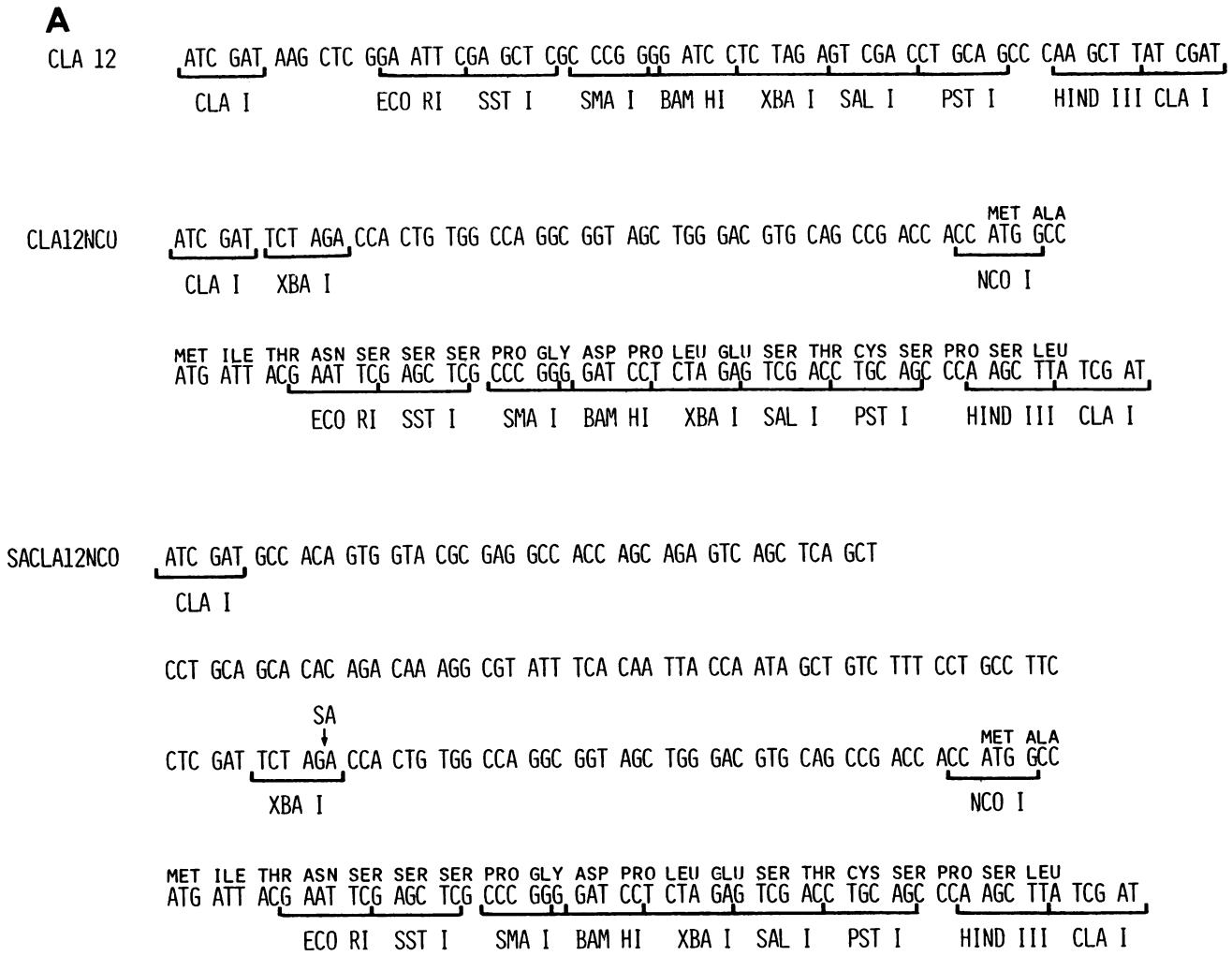

FIG. 2. (A) Sequence of the *Cla*I inserts in the adaptor plasmids. The sequence of *Cla*I-*Cla*I inserts in each of the three adaptors Cla12, Cla12NCO, and SA Cla12NCO is shown. The recognition sites for the 6-base specificity restriction enzymes are indicated beneath each sequence. For the Cla12NCO and SA Cla12NCO adaptors that contain a eucaryotic initiator ATG, the amino acids encoded by the region downstream of the initiator ATG are given. The position of the splice acceptor in SA Cla12NCO is also indicated (SA). While each of these adaptors was assembled from segments that have been sequenced and digestion with several restriction enzymes yielded only the expected fragments, these adaptors were not resequenced after being assembled. (B) Restriction maps of the adaptor plasmids. The restriction maps of the three adaptor plasmids Cla12 (a), SA Cla12NCO (b), and Cla12NCO (c) are given. The approximate positions of restriction endonuclease recognition sites in the polylinker and in the *E. coli* replicon are shown, as are the sites where before manipulation there were sites for *Eco*RI (RI^-) and *Hin*dIII ($HindIII^-$) in the plasmid. The *amp* gene (AMP) and the origin of replication (ORI) of the *E. coli* replicon are shown.

inserted at the *Hin*dIII site of pBR322. The small *Eco*RI-*Cla*I segment (from the linker to *Cla*, not from the original *Eco*RI site) was used to convert the *Eco*RI end of the polylinker to *Cla*I. The resulting *Cla*I-*Cla*I segment was inserted into the *Cla*I site of 327ACRH in the orientation so that the *Eco*RI site in the polylinker was near the *Ssp*I site in the plasmid, which was called Cla12. The sequence of the *Cla*I-*Cla*I segment of this plasmid is given in Fig. 2.

To facilitate the expression of certain segments, two derivatives of Cla12 were prepared, both of which contained a eucaryotic initiator ATG. The first such derivative was prepared by substituting the region between *Cla*I and *Eco*RI with a segment composed of the *Cla*I-*Nco*I portion of the *src* plasmid L-*src* and the *Nco*I-*Eco*RI portion from pUC12N. This plasmid was called Cla12Nco, and the sequence of its polylinker is given in Fig. 2. The third of these plasmids was prepared in the same manner by using the *Cla*I-*Nco*I segment from 779/1089-989/1079-2795/2795 virus and the *Nco*I-*Eco*RI segment from pUC12N. This plasmid, called SA Cla12Nco, contained in addition to a functional initiation ATG a functional splice acceptor (8). The sequence of the *Cla*I-*Cla*I segment is given in Fig. 2.

In all cases the plasmids were constructed by transformation of the DH-5 strain of *E. coli* by the high-frequency system developed by Hanahan (7). Plasmids used for chicken cell transfection were purified by banding twice in $CsCl_2$-ethidium bromide gradients.

Southern transfers. DNA was isolated from confluent plates by extraction either with sodium dodecyl sulfate and pronase or with guanidine thiocyanate. DNA samples were

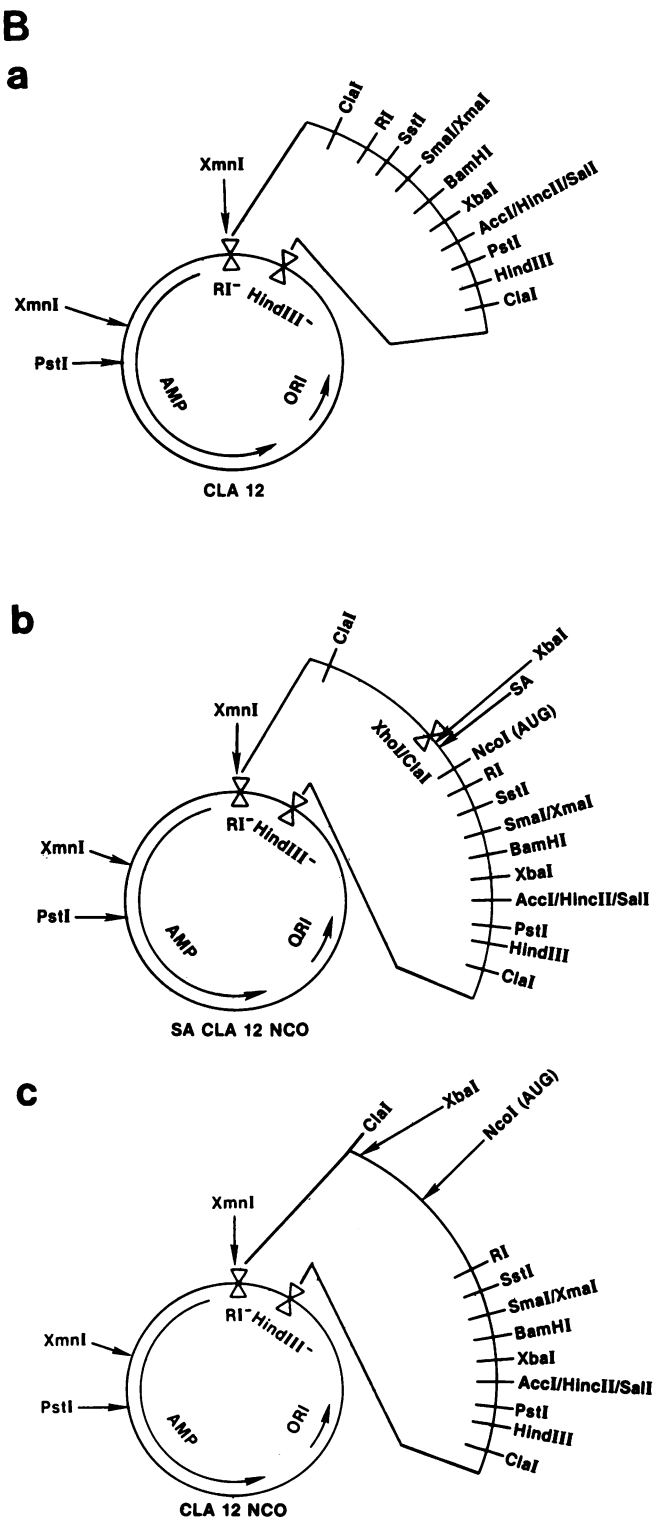

FIG. 2—*Continued.*

digested to completion; the extent of digestion was monitored by testing a portion of the reaction mixture with an internal plasmid control (9). From 5 to 7 μg of DNA was fractionated by electrophoresis in each lane. In situ denaturation of the DNA, transfer to nitrocellulose sheets, hybridization with nick-translated probes, and autoradiographic detection of the hybridizing bands were done according to standard protocols (8–10).

CAT assays. Extracts were prepared by freeze-thaw lysis. Cells from a 100-mm-diameter plate were lysed in 100 μl of 0.25 M Tris chloride, pH 7.8 (6). Protein concentrations were determined by the Bio-Rad reagent system, and the concentration of the extracts was adjusted accordingly. Chloramphenicol acetyltransferase (CAT) activity was determined by standard protocols (6).

RESULTS

Although the helper-independent retroviral vectors we developed previously are entirely functional, two obvious deficiencies existed. To facilitate construction, the original vectors were circularly permuted. The restructuring of the vectors as proviruses simplifies their use, since a vector in the proviral form does not have to be digested and religated before transfection. The proviral form of the vector is more efficient in transfections since every molecule has the proper structure rather than a small fraction produced by digesting and religating a circularly permuted vector.

Because of the distribution of restriction sites present in the viral vector, we chose to use a synthetic *Cla*I site for the insertion of foreign sequences (8, 16, 17). This meant that each fragment to be inserted had to have *Cla*I-compatible ends. We have built a series of miniplasmids called adaptors that can be used to convert virtually any segment into a fragment with *Cla*I ends. The design and use of these adaptor plasmids is described in detail below.

Proviral vectors. The vectors we originally called 779/2795 and 779/989-1089/2795 have been restructured as proviruses. The details of this construction are given in Materials and Methods. The final form of the plasmids carrying the proviral vectors is illustrated schematically in Fig. 1. The original vectors were named when we were comparing a series of closely related constructions that differed slightly in the region surrounding the position at which new information was inserted. It was necessary to differentiate these closely related vectors, and a nomenclature was used that precisely defined the viral segments. In each case the virus was named based on the positions at which the *Cla*I linkers were inserted, relative to the *Eco*RI site in *env*. The endpoints of the *src* deletion then defined the name of the virus, e.g., 779/2795 was a virus with an upstream *Cla*I site at a position 779 bases beyond the *Eco*RI site and a downstream *Cla*I 2,795 bases beyond *Eco*RI. A splice acceptor was carried on fragment 989-1089, so that the viral vector, with its splice acceptor, was 779/989-1089/2795. Since the endpoints of the construction have now been established, we have adapted a simpler nomenclature. All the vectors have been given four-letter designations; 779/2795 is, in the proviral form, now called RCAN. RC stands for replication competent, A for avian leukemia virus (ALV) LTR, and N for no splice acceptor. 779/989-1089/2795 is now called RCAS (RC, replication competent; A, ALV LTR; S, with a splice acceptor).

We compared the time required to produce a high-titer viral stock for two pairs of circularly permuted and nonpermuted constructions. The permuted vector 779/2795 was compared with its nonpermuted counterpart RCAN and the permuted vector 779/989-1089/2795 with the corresponding nonpermuted vector RCAS. None of these vectors contained an inserted gene, so they do not produce any phenotypic change in culture cells. The production of virus was monitored by measuring reverse transcriptase. The

TABLE 1. Replication and transformation[a]

Virus	Relative RT activity	Phenotype of cells
RCAS-CAT-1	1.0	NML
RCAS-CAT-3	1.1	NML
RCAN-SABRAS	0.75	TF
RCAS-BRAS(D)	0.9	TF
RCAS-BRAS(D) introduced into cells infected with RCAS-CAT(A)	ND[b]	TF

[a] Cultures fully infected with virus were measured for reverse transcriptase activity by a standard assay (8). The data are normalized relative to the level of reverse transcriptase (RT) found in cultures infected with the wild-type Schmidt-Ruppin A strain of RSV (3×10^5 cpm incorporated in 1 h). In such an assay, twofold differences are not significant. Cells were scored for phenotype: NML, normal; TF, transformed (see also Fig. 6).

[b] ND, Not done.

circularly permuted constructions were digested with *Sal*I to release the viral portion of the plasmid from the *E. coli* replicon and religated before transfection. The proviral form of the vector was transfected as supercoiled DNA. In each case approximately 5 μg of DNA was introduced into chicken embryo fibroblasts by the $CaPO_4$ method. Although there was some variation in absolute efficiency from one transfection to another, the appearance of high-titer virus was much more rapid with the proviral form of the vector, averaging 6 to 8 days after transfection, as opposed to 12 to 14 days with the circularly permuted constructions. Since the virus produced by transfecting 779/2795 was identical to the virus produced by transfecting RCAN, the virus produced by transfecting 779/989-1089/2795 was identical to the virus produced by transfecting RCAS, and the same transfection protocols were used in all experiments, we attribute differences in the time required to obtain a high-titer stock to the amount of virus produced following the initial transfection. This is to be expected since each of the circular RCAN and RCAS DNAs should contain a complete viral transcriptional unit, whereas religation of the corresponding circularly permuted vector should result in a number of incorrect structures. Since fewer rounds of replication are required to derive a high-titer stock, there is less opportunity for rearrangement.

Adaptor plasmids. Since the genome of the avian sarcoma virus that was used as the basis for the vector construction contains sites for most of the common restriction endonucleases, inserting a polylinker into the vector cloning site was of little practical value. Instead a series of small plasmids were created that had polylinker arrays flanked by *Cla*I sites. By cloning a segment of DNA into the polylinker, the segment could be readily converted to a fragment with *Cla*I ends.

In their simplest form the adaptor plasmids consist of an array of restriction sites. Although the segment carrying the polylinker did not have, by inspection of its sequence, any obvious initiator ATGs, polyadenylation sites, or other sequences likely to interfere with transcription or translation, a direct demonstration that the adaptor would not interfere with viral replication or gene expression was necessary. One such adaptor plasmid, Cla12, had the polylinker derived from the *E. coli* plasmid pUC12 (19). The details of the construction of the plasmid are given in Materials and Methods. The sequence of the *Cla*I-*Cla*I segment is given in Fig. 2A; a map of the plasmid is given in Fig. 2B.

A *Sau*IIIA fragment containing the bacterial *cat* gene was cloned into the *Bam*HI site of the Cla12 adaptor. The *cat* gene was chosen for two reasons. First, it is not a selectable marker in higher eucaryotic cells, and second, it is easily assayed in a quantitative fashion. It was important to know that the polylinker could be used in both orientations; so Cla12 clones were chosen that had the *Sau*IIIA fragment containing *cat* inserted in the adaptor in both orientations.

The *Cla*I fragment was excised from each of the clones and inserted into RCAS in the appropriate orientation for *cat* expression. The only difference between the viruses was in the polylinker segment surrounding *cat*; the *cat* coding region is in the same orientation. Each of the viruses was introduced into chicken embryo fibroblasts by $CaPO_4$ transfection. Reverse transcriptase activity was monitored and reached levels as high as wild-type RSV, indicating comparable virus titers (Table 1). Following transfection the cells were passaged every 2 or 3 days. Although we could routinely see high-titer virus a few passages after transfection, a minimum of 10 passages was made before cell extracts were prepared for enzymatic assays or DNA was prepared for restriction enzyme analyses. There were two reasons for this procedure. First, it is important to wait until all the cells in the culture have an opportunity to become fully infected. The extent of infection was always confirmed by at least two successive reverse transcriptase assays. Second, we wished to take advantage of the observation that although chicken cells can be acutely transfected with a variety of DNA sequences, chicken cells do not stably retain transfected DNA (2). These previous observations were confirmed in these experiments. DNA was isolated from the infected cells and digested with the restriction enzyme *Eco*RI or *Bam*HI, and the resulting fragments were analyzed by the Southern transfer procedure (18) (Fig. 3). All of the fragments seen could be accounted for by digestion of proviral DNA. In the *Bam*HI digestion, the segments from the ends of the provirus did not register because they were attached to different segments of cellular DNA in each infected cell. If DNA from the original transfection was present, additional bands representing the ends of the viral genome linked to plasmid sequences would also be seen. The absence of any additional fragments that cannot be accounted for in the proviral form of original vectors not only implies that the plasmid DNA used in the initial transfection has been lost, but also that the vast majority of the proviruses have no major rearrangement(s). Extracts were made from the infected cells and tested for CAT enzymatic activity (Fig. 4). Both constructions induced the synthesis of large amounts of CAT activity; there were no obvious differences in the efficiency of CAT expression between the two constructions. Since the *cat* gene does not provide a positive selection, the Southern transfer results, taken together with the CAT assay, strongly suggest that the viruses present in the cells at the end of the experiment have precisely the structure of the viral segment in the starting plasmid.

For some applications it is important to have vectors that can supply an authentic eucaryotic initiator ATG and an acceptable untranslated leader sequence. Two such adaptors have been generated and tested; one contains only the leader and the ATG, and the other contains in addition a functional splice acceptor. In both of these adaptor constructions the leader segment and the initiator ATG came from the v-*src* gene of the Schmidt-Ruppin strain of RSV. The splice acceptor is a synthetic segment described previously (8). The v-*src* initiator ATG is contained within an *Nco*I site. The polylinker inserted into the adaptor plasmids derives from pUC12N, a version of the plasmid pUC12 in which the *lacZ* initiator ATG has been converted into an *Nco*I site by site-directed mutagenesis (11, 19). The structures of the two

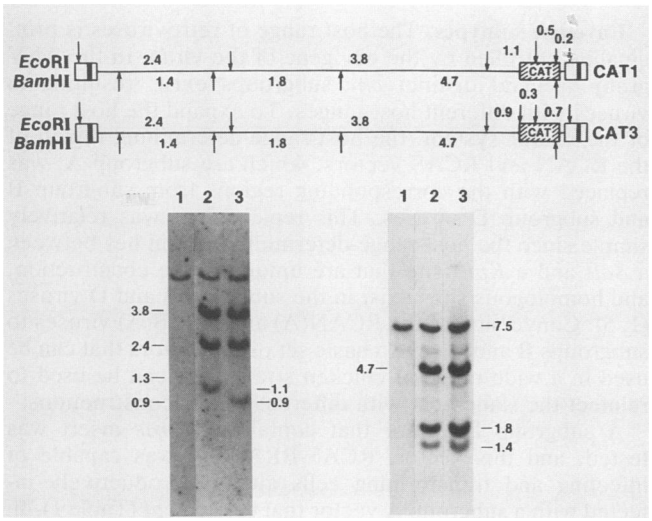

FIG. 3. Restriction maps of the CAT viruses. DNA was prepared from the cells used in the CAT assays shown in Fig. 3. From 5 to 7 μg of DNA was digested to completion, separated by electrophoresis on a 0.8% agarose gel, denatured, and transferred to nitrocellulose paper. Virus-specific fragments were detected with nick-translated DNA from the SRA-2 clone of RSV. At the top of the figure are scale drawings of the expected restriction sites in both the RCAS-CAT-1 and the RCAS-CAT-3 viruses. The expected size of each fragment (in kilobases) is given on the maps. At the outside of each set of three lanes are the sizes (in kilobases) of marker fragments and inside are the interpolated sizes (in kilobases) of the fragment(s) containing part or all of *env* and *cat*. Digests shown on the left were all done with *Eco*RI. Lanes: 1, uninfected cells; 2, RCAS-CAT-1; 3, RCAS CAT-3. The right-hand lanes are of the same DNAs cut with *Bam*HI. The interpolated sizes are in accord with the maps shown at the top of the figure. The probe used in this experiment was made from SRA-2 DNA, which does not detect *cat*-specific DNA, so there is no possibility of detecting the internal *cat* fragments resulting from digestion of the proviruses. In addition, these fragments are small and would not be efficiently transferred to nitrocellulose. The 0.7-kilobase fragment from CAT-1 and the 0.7-kilobase fragment from CAT-3 each contain about 200 bp of virus-specific information; previous experience suggests that this is insufficient to be detected with a probe to the entire viral genome (9, 14).

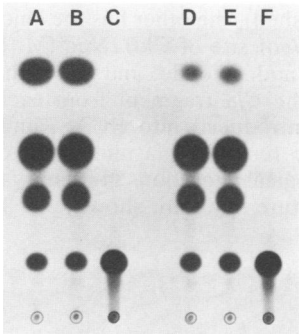

FIG. 4. Assay for CAT. The presence of enzymatically active CAT was assayed as described in Materials and Methods. For these assays, cells from a 100-mm plate were lysed in 100 μl of buffer. The protein concentration of each extract was determined (see Materials and Methods), and the extracts were adjusted to give matching protein concentrations. The figure shows an autoradiograph developed after an exposure of 4 days. In lane A, 50 μl of extract derived from chicken embryo fibroblasts infected with the RCAS-CAT-1 virus was reacted for 90 min. In lane B, 50 μl of extract from fibroblasts infected with RCAS-CAT-3 virus was incubated for 90 min. Lane C was reacted under the same conditions as lanes A and B, except that the extract was from uninfected chicken cells. All the chicken cells used in this experiment were derived from a single embryo. Lanes D, E, and F correspond to lanes A, B, and C, respectively, except that only 5 μl of each extract was used in each of the assays.

adaptors called Cla12Nco and SA Cla12Nco are given in Fig. 2B, and the sequence of the region between the *Cla*I sites is given in Fig. 2A. Having the same polylinker in the procaryotic and eucaryotic expression systems greatly facilitates making constructions that express the same proteins in both systems.

One of the significant advantages of the *Nco*I/ATG adaptors is that they can be used to express authentic polypeptides even when appropriate leaders and ATGs are not present in the original gene. We wished to test whether the adaptors would function with genes inserted at the *Nco*I site and introduced the v-*ras* gene from Harvey sarcoma virus into the adaptors. A pair of complementary synthetic oligonucleotides were used to replace the upstream end of the *ras* coding region, creating a segment that could be ligated to an *Nco*I site. The synthetic DNA replaced the segment of *ras* between the ATG and the *Hin*dIII site. The remainder of the *ras* gene (a *Hin*dIII-*Eco*RI segment) was isolated from a viral clone. This construction is depicted in Fig. 5. The *ras* gene was introduced into SA Cla12Nco and Cla12Nco between the *Nco*I and *Eco*RI sites. Appropriate clones were chosen for each construction and introduced into RCAN and RCAS, respectively. The final constructions differed only in the splice acceptor just upstream of the *ras* gene. Cells infected by the virus were monitored for reverse transcriptase activity; the titer of the *ras*-containing viruses measured by reverse transcriptase activity was the same as the titer of the parental RSV viruses (Table 1). Both constructions transformed primary chicken cells (Fig. 6), and analyses of cell extracts showed that both constructs made *ras* protein of the expected size (data not shown). DNA isolated from the cells was analyzed with restriction endonucleases and gave the expected bands. Two similar constructions have been prepared, one of which has the human *trk* gene inserted into Cla12Nco at the *Nco*I site (10; D. Martin-Zanca and S.

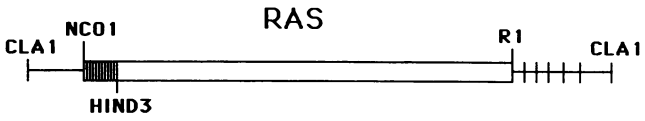

FIG. 5. Conversion of the *ras* gene into a *Cla*I segment by using the adaptor plasmids. Two of the adaptor plasmids, Cla12Nco and SA Cla12Nco, were digested to completion with *Nco*I and *Eco*RI. The *ras* coding region was introduced into the adaptors in two segments. The portion encoding the amino terminus of *ras* (*Nco*I-*Hin*dIII) derives from two complementary synthetic oligonucleotides. This segment is shown as a box marked with vertical lines. To recapitulate the exact *ras* coding sequence, the oligonucleotides do not restore the *Nco*I site following ligation. The rest of the *ras* gene (open box) derives from a *Hin*dIII-*Eco*RI segment isolated from a viral clone that contains an activated Harvey *ras* gene. The *ras* coding region was reassembled in a three-way ligation that included the digested plasmid, the synthetic DNA segment, and the viral DNA. Clones that contained all three segments were found by hybridization, and the structure of the clones was verified by restriction endonuclease mapping. The *Cla*I-*Cla*I segment shown in the drawing was isolated from adaptor plasmids containing the reconstructed *ras* gene by *Cla*I digestion and subsequently introduced into RCAN and RCAS (see text).

Hughes, unpublished), the other has the chicken c-*mos* gene inserted at the *Nco*I site of Cla12Nco (M. Schmidt, M. A. Oskarsson, G. Vande Woude, and S. Hughes, unpublished observations). The *Cla* fragment from each of these constructions was introduced into RCAS, and the virus was derived from the resulting plasmid. Both of these viruses caused morphological alterations in primary chicken embryo fibroblasts in culture (data not shown).

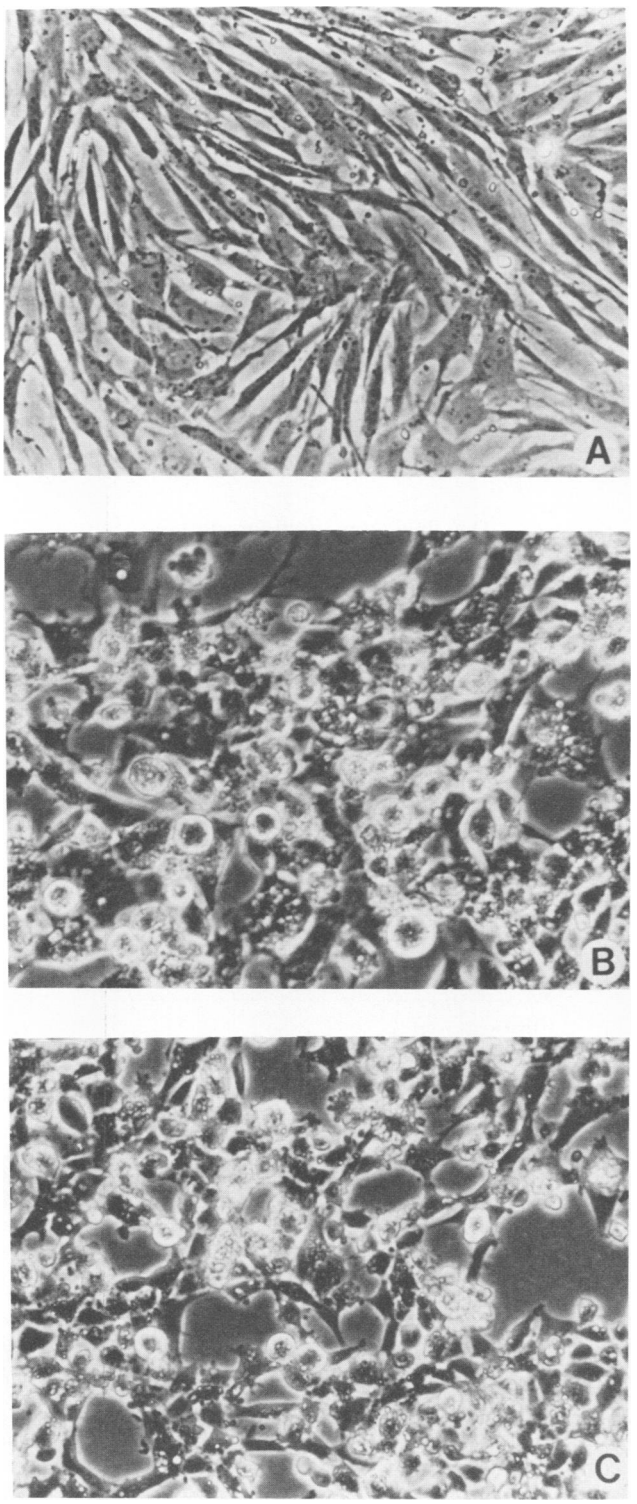

FIG. 6. Chicken cells infected with *ras* viruses. As described in the text, chicken cells derived from one embryo were transfected with 5 μg of DNA encoding either the RCAS-BRAS virus or the RCAN-SABRAS virus. Untransfected cells from the same embryo were passaged in parallel. Foci appeared 3 to 4 days after transfection on plates transfected with the *ras* viruses, and the entire plates took on a transformed appearance 6 to 7 days after the initial transfections. At that time, photographs were made of uninfected cells (A), cells infected with the RCAS-BRAS virus (B), and cells infected with the RCAN-SABRAS (C). Cells infected with CAT viruses have an appearance identical to that of uninfected cells (not shown).

Envelope subtypes. The host range of retroviruses is principally controlled by the *env* gene of the virus. In the ALV group, several distinct *env* subgroups exist, resulting in viruses with different host ranges. To expand the host range of the vector system, the host-range-determining region of the RCAN and RCAS vectors, which are subgroup A, was replaced with the corresponding regions from subgroup B and subgroup D viruses. This replacement was relatively simple since the host-range-determining region lies between a *Sal*I and a *Kpn*I site that are unique in the construction, and homologous sites exist in the subgroup B and D viruses (1, 5). Conversion of the RCAN(A) and RCAS(A) viruses to subgroups B and D gave a basic set of six vectors that can be used in a wide range of chicken strains and can be used to reinfect the same host with different vector constructions.

A subgroup D vector that contains the *ras* insert was tested, and this vector, RCAS-BRAS(D), was capable of infecting and transforming cells already productively infected with a subgroup A vector that carried *cat* (Table 1). In addition, the RCAN-BRAS(D) virus could infect and transform cultured rat and mouse cells (S. Sukumar and S. Hughes, unpublished observations), a characteristic of subgroup D viruses (20).

In addition, an RCAS-NEO(B) virus was built and was capable of converting C/A chicken cells to neomycin resistance (J. Brumbaugh, G. Smith, and S. Hughes, unpublished observations). The subgroup B *env* segment we used in these constructions has already been carefully tested for subgroup specificity (1).

DISCUSSION

The helper-independent ALV retroviral vectors have several distinct advantages. These vectors routinely achieve titers as high as the replication-competent parent virus, and as was shown previously with other constructions, there is no need to include a selectable marker of any kind in the construction. So long as the vector does not rearrange, whatever segment is introduced is carried along passively by the vector, and if the vector is appropriately designed, it can express unselected markers. We have tested the system with the bacterial *cat* gene, a marker that cannot be selected in the chicken fibroblast system. The proviruses present in cells infected by the CAT viruses have the correct restriction map, which together with the CAT expression data demonstrates that there has not been substantial rearrangement during viral propagation.

Additional experiments were done with vectors that express the oncogenes *ras*, *trk*, and c-*mos*; the expression of these oncogenes is at least potentially selectable in cultured cells. Several experiments suggest that unrearranged vectors express the oncogenes. Like the CAT constructions, the *ras* constructions were mapped with restriction endonucleases

and Southern transfer. No obvious rearrangements could be seen. More important, we observed transformation 1 week after the initial transfection, and the entire culture took on a transformed phenotype. It is exceedingly unlikely that a rare rearranged virus could take over the culture so quickly and completely before interference was set up. In previous experiments with vectors that carry but cannot express the *src* oncogene, one or two foci per plate were occasionally seen; however, these foci appeared weeks after the initial transfection event, not concomitantly with the initial appearance of virus. Furthermore, these rare foci did not spread and take over the culture (8).

The simplest interpretation of these data is that the unrearranged vectors express their inserts at high level and that there are no discernible rearrangements in the virus. There are two obvious reasons for this. The previously described circularly permuted helper-independent vectors, which have only one copy of the direct repeat sequence which originally flanked v-*src* (3, 8), are reasonably stable in culture. The restructuring of helper-independent retroviral vectors as proviruses has considerably improved their efficiency. The time to derive a high-titer vector stock has been reduced by approximately 50%, to 6 to 8 days. More important, however, than the saving of time is the reduction in the number of replication cycles required before all the cells are infected. Since the vectors only rearrange during replication, reduction in replication cycles makes it much easier to derive vector stocks free of rearranged viruses.

We plan to do additional experiments with the subgroup B and D envelope vectors; however, it is clear from the experiments that have been done that it is possible to introduce a second vector into an already infected culture, provided that the second vector is of a different subgroup from the first. In addition, it is possible to infect C/A chicken cells with the new vectors, and if the subgroup D vector is used, it is possible to infect mammalian cells in culture.

Before introduction into the vector, DNA fragments must be modified to produce segments with ends that can be ligated to *Cla*I sites. A series of adaptor plasmids have been created that have multiple cloning sites, and in each case the outermost sites in the array are *Cla*I sites. Any segment cloned into the polylinker can be mobilized as a *Cla*I fragment. In their simplest form, adaptor plasmids provide nothing more than an array of cloning sites chosen so as not to interfere with transcription or translation. The simple adaptor described here has the polylinker derived from the *E. coli* plasmid pUC12. The *Cla*I-*Cla*I segment from the adaptor can be used in the helper-independent retroviral vector system in either orientation without interfering with the stability of the vector or with the expression of the insert.

In some cases a simple array of restriction sites is not sufficient to render certain DNA segments suitable for expression in the retroviral vector. It is often necessary to supply an initiator ATG and an appropriate eucaryotic leader. We have created two adaptors which supply an initiator ATG and a eucaryotic leader. One of these supplies, in addition to these functional elements, a splice acceptor. Both of these plasmids have an entry site (*Nco*I) at the initiator ATG, which permits, with the use of synthetic oligonucleotides, the expression of the correct protein from virtually any gene or DNA segment. The polylinker portion of the adaptor plasmid downstream of the *Nco*I site derives from the *E. coli* expression plasmid pUC12N (11, 19). This means that the same DNA segment can be introduced into the *E. coli* expression plasmid and into the retroviral vector via the adaptor plasmid and that both systems will produce precisely the same protein, one in *E. coli*, the other in eucaryotes.

Although the adaptors were designed to be used with the helper-independent avian retroviral vectors, it should be clear that the adaptors can be used with a variety of eucaryotic promoters. The only real requirement is that the promoter have an appropriate *Cla*I cloning site available.

Several of the constructions described in this manuscript are potentially useful in other contexts. All the oncogenic vectors, those that express v-*ras*, *trk*, and c-*mos*, morphologically transform primary chicken cells in culture, although none is capable of immortalizing chicken embryo fibroblasts. The *ras* viruses also transform cultured quail cells (J. Bradac and S. Hughes, unpublished observations). Because these viruses are identical except for the inserted gene, direct comparisons can be made of their biological properties. In this context, we have already seen that the three new viruses each cause different morphological alterations in cultured chicken embryo fibroblasts. The next step will be to test the viruses in vivo. We are particularly interested in the in vivo properties of the *trk* virus, since there is no viral counterpart in nature, and there is no obvious way to test the biological activity of the *trk* gene in the natural host, humans (10).

In addition to viruses such as those described here that express their inserts from the LTR promoter, the helper-independent vector/adaptor system can be used to create viruses that express foreign genes from an internal promoter (C. Petropoulos and S. Hughes, unpublished observations).

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