JOURNAL OF VIROLOGY, Nov. 1990, p. 5475–5484
0022-538X/90/115475-10$02.00/0

Vol. 64, No. 11

Rearrangements in Unintegrated Retroviral DNA Are Complex and Are the Result of Multiple Genetic Determinants

JOHN C. OLSEN,[1] CAROL BOVA-HILL,[1]† DUANE P. GRANDGENETT,[2] THOMAS P. QUINN,[2]‡ JOHN P. MANFREDI,[1]§ AND RONALD SWANSTROM[1]*

Department of Biochemistry and Lineberger Cancer Research Center, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599,[1] and Institute for Molecular Virology, St. Louis University Medical Center, St. Louis, Missouri 63110[2]

Received 22 May 1990/Accepted 16 August 1990

We used a replication-competent retrovirus shuttle vector based on a DNA clone of the Schmidt-Ruppin A strain of Rous sarcoma virus to characterize rearrangements in circular viral DNA. In this system, circular molecules of viral DNA present after acute infection of cultured cells were cloned as plasmids directly into bacteria. The use of a replication-competent shuttle vector permitted convenient isolation of a large number of viral DNA clones; in this study, over 1,000 clones were analyzed. The circular DNA molecules could be placed into a limited number of categories. Approximately one-third of the rescued molecules had deletions in which one boundary was very near the edge of a long terminal repeat (LTR) unit. Subtle differences in the patterns of deletions in circular DNAs with one versus two copies of the LTR sequence were observed, and differences between deletions emanating from the right and left boundaries of the LTR were seen. A virus with a missense mutation in the region of the *pol* gene responsible for integration and exhibiting a temperature sensitivity phenotype for replication had a marked decrease in the number of rescued molecules with LTR-associated deletions when infection was performed at the nonpermissive temperature. This result suggests that determinants in the *pol* gene, possibly in the integration protein, play a role in the generation of LTR-associated deletions. Sequences in a second region of the genome, probably within the viral *gag* gene, were also found to affect the types of circular viral DNA molecules present after infection. Sequences in this region from different strains of avian sarcoma-leukosis viruses influenced the fraction of circular molecules with LTR-associated deletions, as well as the relative proportion of circular molecules with either one or two copies of the LTR. Thus, the profile of rearrangements in unintegrated viral DNA is complex and dependent upon the nature of sequences in the *gag* and *pol* regions.

A variety of viral DNA forms can be found in retrovirus-infected cells (56). Early after infection, replicative intermediates are seen which include an incomplete first-strand DNA species and a short defined second-strand DNA species (plus strong stop). These intermediates are precursors to linear duplex DNA which serves as the substrate for integration into the host genome (5). Linear viral DNA has a direct repeat at its ends called the long terminal repeat (LTR). Sequences at the outer boundaries of the LTR structures, which compose an inverted repeat, are required for proper integration.

In addition to linear viral DNA, circular DNA molecules which often contain a variety of rearrangements within the viral sequences have been found (17, 24, 27, 35, 45, 46, 53, 54). The major forms of circular DNA contain either two copies of the LTR in tandem, presumably formed by blunt-end ligation of linear DNA, or the equivalent molecule with a single copy of the LTR (56, 57). The LTR sequence is frequently associated with rearrangements in circular DNA. Four forms of rearranged circular molecules have been described previously: (i) molecules that have undergone an autointegration event in which a segment of the genome bounded by LTRs is inverted (45, 46, 53, 54); (ii) dimers and trimers of viral DNA (17, 27); (iii) circles with one copy of the LTR sequence that have a deletion starting near the LTR boundary (35, 46); and (iv) circles with two copies of the LTR in tandem with a deletion starting near the outer U5 boundary (35). The latter two classes of molecules include those that are similar to defective, deleted proviruses found within the c-*myc* locus in bursal tumors induced by avian leukosis virus (14, 40). In addition to the above-described forms, we have also detected molecules that are greater than unit length because of either duplication of a portion of the viral genome or acquisition of cellular sequences (J.C.O. and R.S., unpublished data).

We examined rearrangements in circular viral DNA as a model system for genetic instability of viral DNA. Circular DNA was isolated by using a shuttle vector system to obtain large numbers of viral DNA clones. By examining a large number of independent clones of viral DNA, we were able to define the population of circular molecules that is present after acute infection. Analysis of these molecules revealed unexpected complexity in the variety of their structures. A consideration of these structures suggests mechanisms that may be at work in rearranging viral DNA.

* Corresponding author.
† Present address: Glaxo Research Laboratories, Research Triangle Park, NC 27709.
‡ Present address: Duke University Medical Center, Durham, NC 27710.
§ Present address: Department of Physiology, School of Medicine, University of Zimbabwe, Harare, Zimbabwe.

MATERIALS AND METHODS

Cells and viruses. Fertilized chicken eggs were obtained from the Regional Poultry Research Laboratory, East Lansing, Mich. Chicken embryo fibroblasts (CEF), prepared from 11-day-old embryos, and QT6 cells, a continuous quail cell line (33), were maintained as previously described (35).

Virus stocks were obtained following either Polybrene-mediated transfection (26) or lipofection (11) of CEF with infectious plasmid clones. The titers of neomycin resistance gene (*neo*)-containing viruses, in G418-resistant CFU per milliliter, were determined as described previously (34). G418 was obtained from Life Technologies, Inc., Gaithersburg, Md.

Plasmids and bacteria. pANV-A was derived from plasmid p779NC327AC28F (obtained from S. Hughes), which contains a complete copy of the Schmidt-Ruppin A (SR-A) strain of the Rous sarcoma virus (RSV) genome (9) with a unique *Cla*I site in place of the v-*src* gene (23). To construct pANV-A, the plasmid pOneo was linearized with *Cla*I and inserted into the *Cla*I site of p779NC327AC28F. pOneo was constructed from plasmids pBR322 (2), pSV2neo (48), and pBRneo (48). Briefly, the 1.5-kilobase *neo*-containing *Hin*dIII-*Bam*HI fragment from pBRneo was used to replace the 2.3-kilobase *neo*-containing *Hin*dIII-*Bam*HI fragment of pSV2neo. The resulting plasmid was digested with *Acc*I and *Bam*HI, made blunt ended with the Klenow fragment of DNA polymerase I by a fill-in reaction, and ligated with the 581-base-pair (bp) origin-containing *Tha*I fragment of pBR322. This ligation resulted in regeneration of the *Bam*HI site. To generate pOneo, the resulting plasmid was linearized with *Bam*HI and made blunt ended by a fill-in reaction and a *Cla*I linker was inserted by using the linker-tailing method (28).

Molecular clones of the Prague A strain of RSV (pJD100) and mutant JD105 have been previously described (38, 39). Rous-associated virus type 2 (RAV-2) sequences were derived from λ–RAV-2 (37; obtained from G. Payne and H. E. Varmus) and subcloned into a plasmid designated pRAV-211 (unpublished data). All plasmids were maintained in *Escherichia coli* DH5α.

Analysis of unintegrated viral DNA. Unintegrated viral DNA was isolated from whole cells by the method of Hirt (22) as described previously (35). Agarose gel electrophoresis, transfer of DNA to nitrocellulose, and hybridization with a nick-translated [^{32}P]pANV-A probe were performed as described previously (35).

Cloning of unintegrated DNA into *E. coli*. Approximately 2 × 10^6 cells, seeded the day before onto 10-cm-diameter plates, were infected with 10^5 to 10^6 CFU of the indicated virus for 2 h at 37°C. The virus was then removed and replaced with fresh medium. At 24 h postinfection, extrachromosomal DNA was isolated by the method of Hirt (22) and covalently closed circular DNA was further purified by acid-phenol extraction (35, 60). The purified DNA was used to transform competent *E. coli* DH5α (Life Technologies) or by high-efficiency electroporation (10). Bacterial transformants were selected by growth on agar plates containing 50 μg of kanamycin (Sigma Chemical Co., St. Louis, Mo.) per ml.

Analysis of cloned DNA. Plasmid DNAs from individual colonies were isolated from overnight cultures by the alkaline lysis method (30). Restriction endonucleases were purchased from Life Technologies or New England BioLabs, Inc. (Beverly, Mass.). LTR-containing fragments were identified by hybridization with an RNA probe specific for the 239-base *Sph*I-*Bst*EII fragment from RSV strain SR-A containing all of U5 and R and 136 bases of U3. This probe was synthesized by using as the template a pIBI31 (International Biotechnologies, Inc., New Haven, Conn.) plasmid containing a portion of the LTR, T7 RNA polymerase (Pharmacia LKB Biotechnology, Piscataway, N.J.), and [α-^{32}P]UTP (800 Ci/mmol; Dupont, NEN Research Products, Boston, Mass.). Nucleotide sequence analysis was performed by using the dideoxy-chain termination method (41, 59).

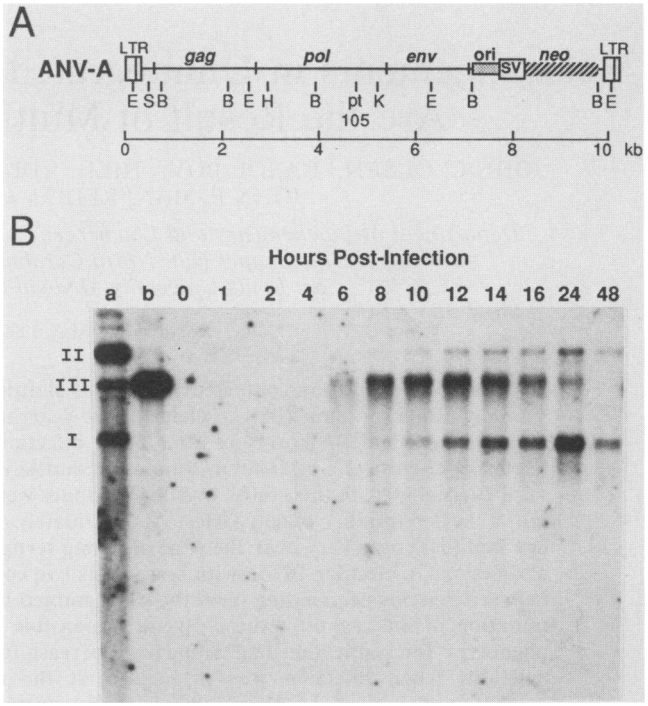


FIG. 1. (A) Structure of the ANV-A shuttle vector. Thin line, sequences derived from the SRA-2 clone of RSV strain SR-A (23) bounded by LTR units. Stippled box (ori), sequences from the pBR322 origin of DNA replication. Open box (SV), sequences from the simian virus 40 early enhancer-promoter region. Cross-hatched line, sequences from the Tn*5* *neo* gene, including the promoter region. Restriction endonuclease sites: E, *Eco*RI; S, *Sst*I; B, *Bam*HI; H, *Hpa*I; K, *Kpn*I. pt105 indicates the position of the Pro-to-Ser mutation in the IN domain of the *pol* gene that is responsible for the temperature-sensitive phenotype for replication of RSV mutant JD105 (39). kb, Kilobases. (B) ANV-A DNA synthesis in QT6 cells. Approximately 5.5 × 10^5 QT6 cells were seeded onto 6-cm-diameter tissue culture dishes. At 18 h after seeding, each dish was infected with approximately 5 × 10^5 CFU of ANV-A. At various times postinfection, unintegrated DNA was isolated. The unintegrated DNA was subjected to electrophoresis in 1% agarose, transferred to nitrocellulose, hybridized with a ^{32}P-labeled viral DNA probe, and detected by autoradiography. Lanes: a, marker DNA (pANV-A) showing the positions of form I, II, and III ANV-A unintegrated DNAs; b, marker DNA (*Sst*I-digested pANV-A) showing the position of form III ANV-A unintegrated DNA.

RESULTS

Construction of a replication-competent shuttle vector. We used as starting material a replication-competent viral vector derived from the SR-A strain of RSV (9, 23). The *neo* gene, including its promoter, from Tn*5* (1) and the pBR322 plasmid origin of DNA replication were inserted into a *Cla*I site downstream of the RSV *env* gene. In addition, sequences containing the simian virus 40 early promoter-enhancer region were placed upstream of the *neo* gene. The resulting plasmid, pANV-A (Fig. 1A), contains all of the *cis*- and *trans*-acting sequences necessary for replication as a retrovirus (avian *neo* virus subgroup A [ANV-A]), while the presence of the *neo* gene under the transcriptional control of

the simian virus 40 promoter allows for selection of virus-infected cells with the antibiotic G418. Furthermore, the plasmid origin of DNA replication and *neo* gene within the viral genome allow viral DNA to be propagated and selected for in bacteria.

Replication of ANV-A in avian cells. pANV-A was introduced into CEF by DNA transfection by using the Polybrene-dimethyl sulfoxide method (26). Virus production was measured by reverse transcriptase activity. Virus appeared in the culture medium 4 to 7 days posttransfection. To examine the integrity of the viral genome, virus obtained 7 days posttransfection was used to initiate a new infection of QT6 cells and the virus-specific DNAs synthesized shortly postinfection were analyzed (Fig. 1B). Both linear and circular DNAs of the sizes expected for the intact ANV-A genome were synthesized after infection in an ordered manner, as expected for avian retrovirus DNA synthesis in QT6 cells (19, 55). Linear DNA was observed within 6 h postinfection. The level of linear DNA peaked at about 12 h postinfection and decayed steadily during the following 36 h. Circular DNA forms were seen at 8 h postinfection, and they increased to peak levels at 24 h postinfection. Analysis of unintegrated DNA with restriction endonucleases yielded fragments of the expected sizes as detected by the ^{32}P-labeled viral DNA probe (data not shown). Thus, we conclude that ANV-A can replicate intact in CEF and without extensive rearrangement or deletion, at least for the time (7 days) required to achieve peak virus production following transfection.

Rescue of ANV-A sequences in bacteria. Previously, by using a replication-defective retrovirus shuttle vector to clone unintegrated DNA, we found that a large fraction of the circular viral DNA from infected cells had deletions in which one boundary was within or near the U5 domain of the LTR (35). We wanted to confirm and extend this observation by using replication-competent ANV-A as the shuttle vector. The use of a replication-competent shuttle vector also makes possible the isolation of a large number of independent clones compared with the relatively small number of clones we obtained with a replication-defective vector. ANV-A was used to infect QT6 cells at a multiplicity of infection of 0.5. Unintegrated DNA was fractionated from total cells by the procedure of Hirt (22) at 24 h postinfection, and covalently closed circular DNA was purified by acid-phenol extraction (35, 60). This DNA was used to transform *E. coli* DH5α to kanamycin resistance. In a typical experiment, we were able to obtain approximately 3×10^3 bacterial colonies containing viral DNA by using circular viral DNA isolated from 10^6 acutely infected QT6 cells.

Characterization of the rescued genomes. The plasmids from 193 independently isolated colonies were analyzed by constructing physical maps with known restriction endonuclease sites. Each plasmid was mapped by using *Sst*I, *Eco*RI, and *Bam*HI. These enzymes were sufficient to characterize most of the plasmids. Plasmids with complex structures were mapped further, as necessary, by using known sequence information as a guide (3, 9, 23, 42, 50, 52). By using this strategy, we were able to place all of the plasmids into six categories (Fig. 2), which can be summarized as follows: **(i) Intact molecules.** Approximately 50% of the clones were intact and unrearranged. Among these molecules, there was an equal distribution between clones with one copy of the LTR and clones with two copies of the LTR in tandem (48 versus 52%).

(ii) U5-associated deletions. Approximately 30% of the clones had deletions in the 5′ portion of the viral genome. In each case, one end of the deletion was near the outside U5 boundary of an LTR while the other end of the deletion was found a variable distance away (32 bases to 6.6 kilobases). Of the molecules in this category, most (93%) had a single LTR unit.

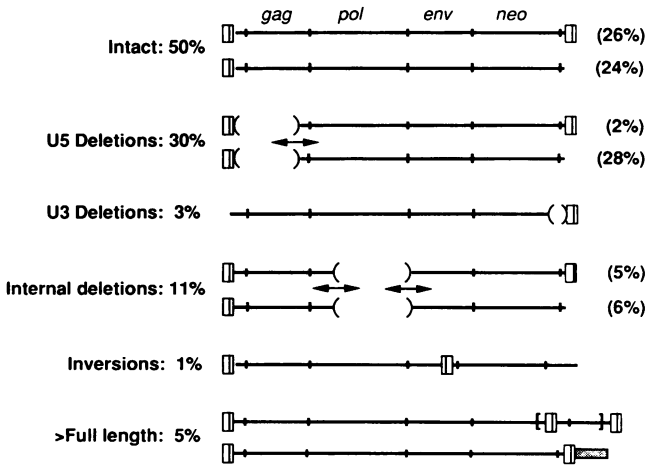


FIG. 2. Summary of the structures of 193 clones of ANV-A unintegrated circular DNA as determined by restriction endonuclease mapping. Thin line, sequences derived from ANV-A. Large open box, U3 domain of LTR. Small open box, U5 domain of LTR. The percentages on the left indicate the relative proportions of molecules in the designated categories. The percentages on the right indicate the breakdown of clones with one and two copies of the LTR. The double-headed arrows indicate that the deletion endpoints of individual clones in the category were not clustered. About 12% of molecules with deletions extending from the U5 boundary also had internal deletions. The data for molecules with both types of deletions are included in the category with U5 deletions. Two types of greater-than-full-length molecules were observed. One type had a duplication of viral sequences (square brackets). The second type included insertion of a cell-derived sequence (shaded box).

(iii) U3-associated deletions. Approximately 3% of the clones had deletions in which one endpoint was near the outside boundary of the U3 domain of the LTR. All of these molecules had a single LTR unit.

(iv) Internal deletions. Approximately 11% of the clones had deletions that were located internally within the genome, i.e., localized between the *Sst*I restriction endonuclease site and the *neo* gene. These deletions ranged in size from about 50 to 5,700 nucleotides. It was found that 12% of the clones with deletions extending from the U5 boundary also had internal deletions, suggesting that these two lesions arose independently.

(v) Inversions. One molecule that appeared to be of genome length but had a single large inversion was found. Each endpoint of the inverted sequence was mapped to a position near a solitary LTR unit. This clone is similar to previously described molecular clones which are thought to result from an intramolecular autointegration event (45, 46, 53, 54).

(vi) Greater-than-full-length molecules. Approximately 5% of the clones were larger than the 10.2-kilobase-pair (kbp) size expected for a full-length genome. The sizes of these molecules ranged from 10.4 kbp to greater than 20 kbp. The structures of many of these molecules are complex and will be described in detail elsewhere (J.C.O. and R.S., unpublished data).

During these experiments, several smaller libraries of unintegrated circular ANV-A DNA were prepared. We

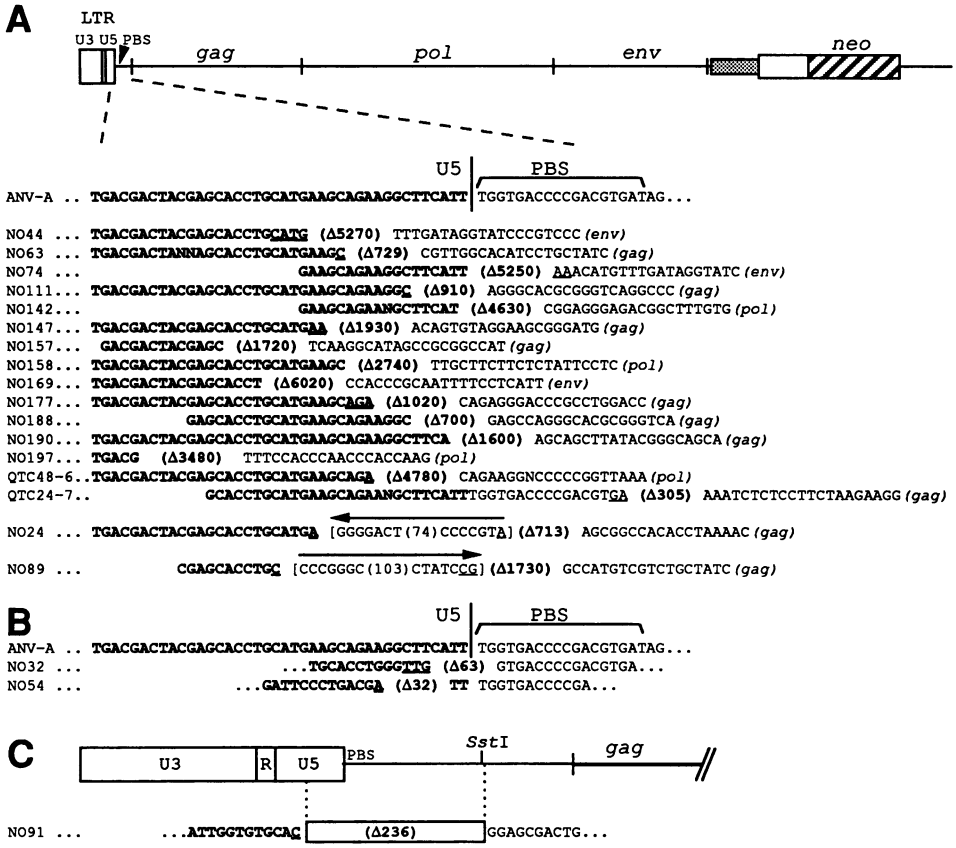


FIG. 3. Nucleotide sequence analysis at the sites of deletions extending from the U5 boundary in clones with a single LTR. Boldface sequences are from the LTR. A delta (Δ) followed by a number indicates the size of the deletion, which is rounded off when sequences from the Prague C strain of RSV were used for the alignment. Underlined sequences denote common nucleotides present at each boundary of the deletion. (A) Clones with one deletion endpoint clustered near the U5 boundary and the other deletion endpoint in a viral replicative gene. PBS denotes the binding site for the $tRNA^{trp}$ primer used to initiate the first (minus) strand of DNA synthesis. The arrows show the presence of duplicated sequences present at the deletion site in an orientation that is opposite to (NO24) or the same as (NO89) that of the original sequence. N indicates that the sequence was ambiguous at that position. (B) Clones with one deletion endpoint near a U5 boundary and the other extending further into the LTR. (C) One clone with the deletion endpoint further away from the U5 boundary. The 5′ end of the deletion is 68 to 69 bases upstream of the U5-PBS boundary. The 3′ end of the deletion includes sequences required for efficient packaging of RNA into virions (25).

found that the percentage of clones in each of the major categories was reproducible, particularly in the larger libraries (containing greater than about 60 clones). We also showed that the described rearrangements in circular viral DNAs were not due to a cloning artifact by performing a mixing experiment with the parental pANV-A plasmid and an uninfected cell lysate. The plasmid DNA was isolated from the lysate and used to transform *E. coli*. We examined 100 clones of DNA recovered from the lysate and found no alterations in their structure.

Sequence analysis of clones with a deletion extending from the U5 region of a single LTR. The nucleotide sequence across the deletion site was determined for 20 randomly chosen clones with deletions extending from the U5 boundary of the LTR that appeared by restriction enzyme mapping to possess a single LTR unit. The results of the sequence analysis are summarized in Fig. 3. In 18 of 20 clones, the deletions were simple in that no additional nucleotides or inversions were present at the deletion site. The average size of the simple deletions in sequenced clones with a single LTR unit was 2,300 bp (range, 32 bp [NO54] to 6,020 bp [NO169]). In 16 of 20 clones, the 5′ endpoint of the deletion was in the U5 domain, within 35 nucleotides of the U5 boundary, and extended into a viral replicative gene (Fig. 3A). In one clone (QTC24-7), the 5′ endpoint was to the right of the U5 boundary in the tRNA primer-binding sequence (PBS) region (Fig. 3A). In three clones (NO32, NO54, and NO91), the deletion endpoints were smaller and confined to the LTR and adjacent noncoding sequences (Fig. 3B and C).

In two clones (NO24 and NO89), extra sequences representing duplications of viral sequences present near the 3′ end of the genome were present at the deletion site (Fig. 3A). In NO24, an 87- to 89-bp sequence from the 3′ untranslated region (39 to 126 bp upstream of the U3 boundary) was duplicated in an inverted orientation at the position of a 713-bp deletion site that extended from U5 into *gag*. In NO89, a 115- to 118-bp sequence beginning 172 bp downstream of the *neo* termination codon was duplicated in the same orientation at the site of a 1,730-bp deletion that extended from U5 into *gag*.

Sequence analysis of clones with a deletion extending from the U5 region of tandem LTRs. Only 7% of the molecules with 5′ deletions had tandem LTR units. The nucleotide sequence at the deletion site was determined for seven such clones (Fig. 4). The average size of the deletions in clones with tandem LTR units was 5,400 bp (range, 3,700 to 6,600

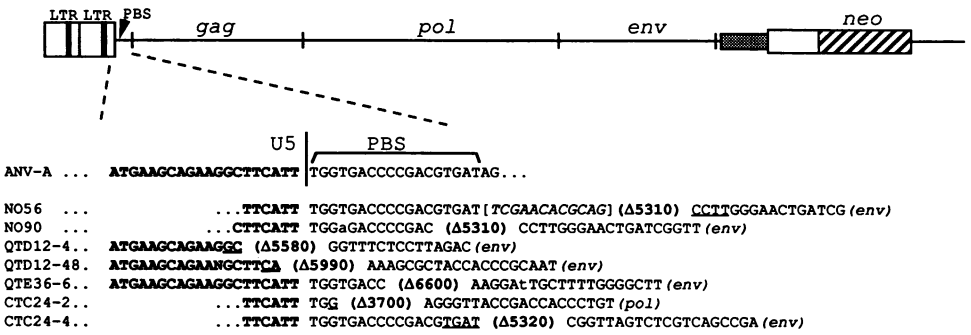


FIG. 4. Nucleotide sequence analysis at the sites of deletions extending from the U5 boundary in clones with tandem LTR units. See the legend to Fig. 3 for definitions. Lowercase letters indicate base changes relative to the parental ANV-A sequence. The dodecanucleotide sequence in brackets for NO56 was derived from the $tRNA^{trp}$ primer used to initiate DNA synthesis. It is likely that during formation of plus strong-stop DNA, termination of synthesis did not take place at the usual site but rather after copying of an additional 12 nucleotides of tRNA.

bp). In contrast to molecules with a single LTR, the 5′ deletion endpoint in molecules with two LTR units was usually (five of seven cases) found in the PBS region to the right of the U5 boundary. In one clone, NO56, an additional dodecanucleotide (TCGAACACGCAG) was present at the deletion site adjacent to a complete copy of the tRNA PBS. This dodecanucleotide sequence is also found in the same position in the tryptophan tRNA used to prime minus-strand DNA synthesis. Normally, during DNA synthesis, 18 nucleotides of the tRNA are copied; it is likely that the extra sequence in pNO56 arose because of DNA synthesis beyond the normal termination site. Colicelli and Goff (7) have previously reported the structure of a clone in which a tRNA molecule was aberrantly copied during reverse transcription.

Sequence analysis of clones with a deletion extending from the U3 region of a single LTR. The nucleotide sequence across the deletion site in molecules with deletions extending from the U3 boundary was determined for eight clones (Fig. 5). In each case, one of the deletion endpoints was near the left edge of the U3 boundary of a molecule with a single LTR unit. In six clones, the deletion extended towards the *neo* gene (Fig. 5A and C); in the two others (NO216 and QTD12-13; Fig. 5B), the deletion extended in the opposite direction, further into the U3 domain.

Effects of the host cell and time postinfection. To determine whether the high frequency of rescued molecules with deletions extending from the U5 boundary was due to the use of heterologous host cell line QT6, a similar analysis of unintegrated viral DNA isolated from CEF cells acutely infected with ANV-A was performed. At 24 h postinfection of CEF, 15 (39%) of 38 independent viral DNA clones were full-length, while 14 (37%) of 38 clones had deletions in the 5′

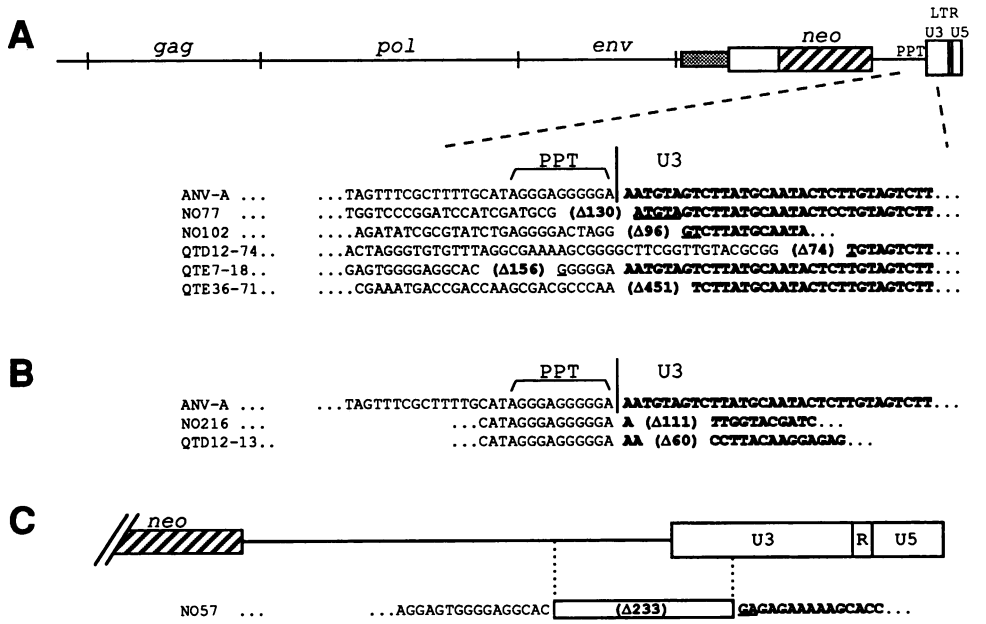


FIG. 5. Nucleotide sequence analysis at the sites of deletions extending from the U3 boundary in clones with single LTR units. See the legend to Fig. 3 for definitions. PPT denotes the site for the RNA primer used to initiate synthesis of plus strong-stop DNA. (A) Clones with one deletion endpoint clustered near the U3 boundary and the other endpoint located to the left. (B) Clones with one deletion endpoint near the U3 boundary and the other extending further into the LTR at the right. An extra T (italic) was present at the deletion site in NO216. (C) Clone with U3-associated deletion further away from the U3 boundary. The 3′ end of the deletion endpoint is 71 to 73 bases to the right of the PPT-U3 boundary.

TABLE 1. Summary of clones of unintegrated circular viral DNA isolated from QT6 cells infected with a viral mutant deficient in integration

Virus	Infection temp (°C)	No. of DNA clones analyzed	% Full-length with:		% with U5 deletions
			1 LTR	2 LTR	
AJD100	37	104	39	15	19
AJD100	42	111	40	18	20
AJD105	37	255	31	36	18
AJD105	42	266	45	33	7

region of the genome similar to those described above (Fig. 3). Therefore, the high frequency of deletions extending from the U5 boundary was not due to the QT6 host cells.

In the above-described experiments, circular ANV-A DNA isolated 24 h postinfection was analyzed. In one experiment, we analyzed circular viral DNA molecules that were cloned 12 and 60 h postinfection of QT6 cells. For the 12-h infection, 28 (70%) of 40 clones appeared to be full-length while 10 (25%) of 40 clones had deletions in the 5′ region of the genome. For the 60-h infection, 16 (41%) of 39 clones were full-length, while 20 (51%) of 39 clones had deletions in the 5′ region. Although the sample size was smaller in these analyses, it is clear that circular molecules with deletions extending from the LTR boundary into the *gag* gene are common both early and late after acute infection.

Effect of a viral mutant deficient in integration. One possible model for generation of molecules with deletions near the boundaries of the LTR involves binding and aberrant nicking by integration protein (IN)-associated endonuclease activity (35). To test this possibility, we made use of a temperature-sensitive RSV isolate defective for replication because of a single amino acid substitution (Pro to Ser) at amino acid residue 115 of IN (39). This missense mutation results in a replication-competent virus that synthesizes normal levels of viral DNA but integrates its DNA at only 10 to 20% of wild-type levels under permissive conditions (39).

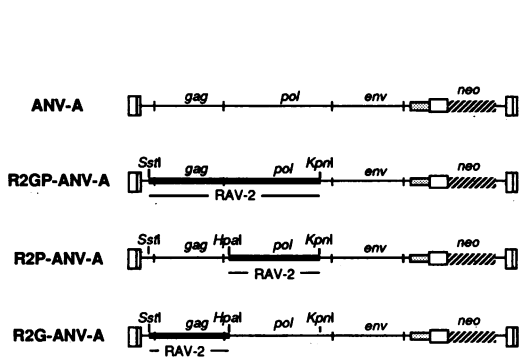
To place the mutated sequence into pANV-A, the 2.3-kbp *Hpa*I-*Kpn*I fragment of pANV-A (Fig. 1A) was replaced with the equivalent fragment from a molecular clone (pJD105) of the Prague A strain of RSV carrying this mutation, resulting in recombinant pAJD105. We also made the equivalent recombinant (pAJD100) carrying the wild-type Prague A sequences (from pJD100) in this region. Virus from pAJD100 and pAJD105 was generated in parallel in CEF cultures at 37°C following DNA-mediated transfection (11). These viruses were used to infect QT6 cells in parallel at 37 and 42°C. At 24 h postinfection, unintegrated circular viral DNA was isolated from the cells. This viral DNA was used to transform bacteria, and a collection of rescued plasmid clones was characterized by restriction endonuclease mapping. The results are summarized in Table 1.

Two of the effects observed differed between wild-type and mutant viruses. (i) The fraction of intact circular molecules with two LTRs was greater (approximately twofold) for the mutant AJD105 virus than for the AJD100 virus at each temperature. (ii) For the AJD100 virus, similar percentages (19 to 20%) of molecules with deletions extending from the U5 boundary were rescued at each temperature. However, for the mutant AJD105 virus, the percentage of rescued molecules with deletions extending from the U5 boundary decreased approximately 2.5-fold when infection was done at 42°C rather than 37°C. These results suggest that IN or the IN domain located on the β subunit of the reverse transcriptase plays a role in the generation of at least some of the deletions extending from the U5 boundary, as well as affecting the formation of intact circles containing two copies of the LTR.

Differences between the *gag* and *pol* genes of SR-RSV and RAV-2. Sudol et al. (49) reported that a recombinant of RSV strain SR-A in which the 2.5-kbp *Eco*RI-*Kpn*I *pol*-containing fragment was replaced with the equivalent sequences from a molecular clone of the Bryan high-titer strain of RSV replicated to 100-fold higher infectious virus titers than the parental SR-A strain of RSV. A similar recombinant (R2GP-ANV-A) was constructed in which a 4.7-kbp *Sst*I-*Kpn*I fragment containing the *gag* and *pol* sequences of pANV-A (Fig. 1A) was replaced with the equivalent sequences from a RAV-2 DNA clone (originally derived from RSV Bryan high-titer strain stocks). We found that R2GP-ANV-A grew to 25-fold higher virus titers, as measured by G418 CFU and reverse transcriptase activity. R2GP-ANV-A also replicated with faster kinetics than ANV-A when equivalent amounts of the viral genomes were introduced into CEF by DNA transfection (data not shown). Differences between the two viruses were also found by analyzing the profile of unintegrated circular DNA rescued by cloning in bacteria (Fig. 6). Recombinant R2GP-ANV-A, when compared with ANV-A analyzed in parallel, yielded more intact clones (68 versus 43%), fewer clones with deletions extending from the U5 boundary (14 versus 29%), and a different ratio of intact clones with a single LTR unit to those with tandem LTRs (5:1 versus 1:1). Southern analysis of *Bam*HI-digested unintegrated DNA isolated 24 h postinfection also showed a bias for circles with a single LTR over circles with tandem LTR units in R2GP-ANV-A infections, whereas the ratio was about 1:1 in ANV-A infections (data not shown).

An attempt was made to localize further the sequences responsible for the above-described differences in DNA metabolism between the two viruses. DNA fragments were exchanged, resulting in recombinant viruses with the *gag* gene (R2G-ANV-A) or the *pol* gene (R2P-ANV-A) from RAV-2 in the ANV-A background (Fig. 6). R2P-ANV-A grew with kinetics and peak titers in CEF similar to those of ANV-A. R2G-ANV-A grew to peak titers similar to those of ANV-A but had slightly delayed kinetics (data not shown). When the profile of rescued unintegrated circular DNAs was analyzed, it was found that R2P-ANV-A was similar to ANV-A (Fig. 2 and 6). In contrast, R2G-ANV-A yielded significantly more molecules with deletions extending from the U5 boundary upon comparison with ANV-A (62 versus 29%) and had a higher ratio of intact clones with a single LTR unit to those with tandem LTR units (9:1). The ratio of clones with single LTR units to clones with tandem LTR units in the population of molecules with deletions extending from the U5 boundary was not significantly different for any of the viruses (Fig. 2 and 6).

Since neither R2G-ANV-A nor R2P-ANV-A behaved like R2GP-ANV-A in the profile of unintegrated circular DNA molecules or in replication rate and high-titer production of virus, we assume that multiple determinants in the *gag-pol* region are responsible for these features. The fact that R2G-ANV-A was similar to R2GP-ANV-A in the altered ratio of circles with one LTR to tandem LTRs suggests that determinants adjacent to or within the *gag* gene can influence this aspect of viral DNA metabolism.



	Number of DNA Clones Analyzed	% Full Length 1 LTR	% Full Length 2 LTR	% U5 Deletion	Number of U5 Deletions with: 1LTR	Number of U5 Deletions with: 2LTR
ANV-A	72	19	24	29	20	1
R2GP-ANV-A	72	57	11	14	10	0
R2P-ANV-A	60	22	27	37	20	2
R2G-ANV-A	60	27	3	62	34	3

FIG. 6. Summary of clones of unintegrated circular DNA isolated from QT6 cells infected with RAV-2–ANV-A chimeric viruses. The thin line represents sequences derived from ANV-A. The thick line represents sequences derived from RAV-2. The *Hpa*I recognition site is 228 bp downstream from the position of the amino terminus of the mature reverse transcriptase.

DISCUSSION

The main conclusions of this study are that (i) rearrangements are found at an extraordinarily high frequency in the population of unintegrated circular DNA, and (ii) both quantitative and qualitative aspects of rearrangements in circular DNA are dependent upon multiple determinants in the viral genome. Our analysis more fully defines the range and proportions of rearranged DNA molecules present after acute infection and points to significant complexity in the structures of these molecules.

Complexity of circular viral DNA. In the case of ANV-A, approximately one-half of all of the molecules isolated had a single LTR sequence and over half of these molecules had deletions with one endpoint near the U5 boundary of the LTR (Fig. 2). In most (16 [80%] of 20), of the deleted molecules, the endpoint within the LTR was within 35 nucleotides of the U5-PBS boundary, with the rest of the molecules having deletion endpoints either further upstream in the U5 domain or in the adjacent PBS sequence (Fig. 3). The fraction of intact molecules with two copies of the LTR sequence was similar to the fraction with one copy of the LTR sequence. However, only a small percentage of molecules with two copies of the LTR had deletions with an endpoint near the LTR boundary and most of those with deletions had endpoints within the PBS sequence adjacent to the outer U5 sequence. Several molecules, including NO24, NO56, NO89, and NO216 (Fig. 3 to 5), had additional sequences present at the site of the LTR-associated deletion.

The U3 boundary of the LTR also was a site of clustering of deletion endpoints, although at a 10-fold lower frequency. In a small sample, the deletion endpoints clustered just within the U3 boundary in molecules that had a single copy of the LTR. For a small number of molecules (NO32, NO54, NO216, and QTD12-13) with a single copy of the LTR, the deletion endpoint was near either the U3 or the U5 boundary but the deletion extended into the LTR (Fig. 3 and 5).

It is likely that all of the above-described rearranged viral DNA forms arose during the acute infection just before cloning, since each lacks *cis*-acting sequences necessary for virus replication. We also detected molecules with internal deletions which would also be replication defective but could have arisen during previous rounds of replication (Fig. 2).

Role of viral proteins in DNA rearrangements. We examined the role of viral proteins in the generation of circular molecules in two experimental settings. In the first, we used a mutant (AJD105) with an amino acid substitution in IN which results in a temperature-sensitive phenotype for replication (39). With this mutant, we observed a 2.5-fold decrease at the nonpermissive temperature in the fraction of molecules with deletions with an endpoint at the LTR boundary (Table 1). This observation is consistent with the hypothesis that IN could mediate these deletions. However, since the viral DNA polymerase (β subunit) retains the IN domain as part of its polypeptide chain, this interpretation is not unequivocal. An alternate possibility, which we cannot exclude, is that the mutation adversely affects some function of the reverse transcriptase that is important for deletion formation.

The second approach to examining the role of viral proteins in the generation of different forms of circular DNA involved exchanging sequences from the *gag-pol* region between different virus strains. The results of these experiments suggest that other regions in the viral *pol* gene and, possibly, within the *gag* gene have effects on the composition of circular DNA. A recombinant carrying the Prague A strain of the *pol* gene in the SR-A background (AJD100; Table 1) had an increased percentage of circles with one LTR sequence and half as many circles containing two LTR sequences, compared with equal numbers of each when the virus with *gag* and *pol* genes derived from the SR-A RSV strain was used (Fig. 2). Similarly, a recombinant with *gag* and *pol* derived from RAV-2 gave a disproportionate number of circles with one copy of the LTR compared with circles with two copies of the LTR, i.e., a fivefold difference (R2GP-ANV; Fig. 6). However, in this case, a sequence containing the entire RAV-2 *gag* gene and the amino-terminal portion (about 76 amino acids) of *pol* appeared to have the determinants that altered this ratio (R2G-ANV-A; Fig. 6). Also, the R2G-ANV-A recombinant had determinants which gave rise to higher numbers of circles with deletions with an endpoint near the LTR boundary (Fig. 6). These results point to a complex interaction between multiple determinants in *pol* and, possibly, *gag* that affect both the frequency of circles with one or two copies of the LTR sequence and the frequency of deletions emanating from the boundary of the LTR. We do not know what components are responsible for these phenomena, although it is known that for murine leukemia viruses the viral capsid protein (CA) is present in an intracellular protein complex that is competent for viral DNA integration (4). It has been suggested that viral protein NC has a role in viral DNA synthesis (8, 43), as well as virion RNA packaging (12, 16, 31, 32). Thus, each of these *gag* proteins, in addition to the viral DNA polymerase and IN protein, could affect the metabolism of viral DNA.

Consideration of mechanisms of deletion formation associated with the LTR. The LTR sequence plays a central role in virus replication (56, 57). The LTR is bounded by primer sites for the first and second strands of DNA synthesis, synthesis past a template strand discontinuity is required to create the sequence organization of the LTR, strand displacement synthesis is required to duplicate the LTR sequence, the LTR bounds linear DNA which is the precursor to both integrated DNA and circular DNA, and the inverted repeats at the ends of the LTR play a key role in the integration process (18). Deletions starting near the LTR boundary are likely to occur at one of these steps either during or after DNA synthesis. Mechanisms proposed for the generation of these deletions must account for several observations, including the lack of extended homology at the deletion site, the clustering of deletion endpoints, and the recurring insertion of viral genomic sequences at the deletion site.

One possibility is that the deletions arise as a result of aberrations during reverse transcription. This could occur during the synthesis of either the first or second strand and may be due to aberrant jumping of reverse transcriptase from one position on the template to another position on the same template or even to another template. Multiple jumps would account for the apparent insertion of viral sequences normally present elsewhere in the genome (NO24 and NO89; Fig. 3). Aberrant jumping by reverse transcriptase has previously been suggested to play a role in rearrangements, including transduction of cellular sequences (13, 20, 51), high-frequency recombination (6, 47), and deletion formation (36, 44, 58). More recently, this mechanism has been suggested to account for deletions and small insertions that have been detected after replication of a spleen necrosis virus vector (36a).

Generation of LTR-bounded deletions in circles with two copies of the LTR is fairly rare (less than 10% of circles with two LTR sequences; Fig. 2) and must not interfere with the completion of viral DNA synthesis as judged by the presence of both LTRs. An inappropriate jump of an incomplete minus strand to the PBS region of the plus strong-stop DNA intermediate (or a jump by an incomplete plus strand to an inappropriate position on the minus strand) would result in an LTR-bounded deletion which would retain part of the PBS sequence yet have the potential to complete DNA synthesis.

Circles with one copy of the LTR and an LTR-bounded deletion probably do not arise from a pool of linear molecules with these deletions that recombine within the LTR sequences to circularize. If this were the case, then the deletions in circles with one LTR should appear more similar to the deletions in circles with two LTRs, which are thought to circularize by blunt-end ligation of linear DNA. While similar mechanisms for deletion formation may be at work in the generation of circles with either one or two copies of the LTR, application of these mechanisms to the generation of circles with one LTR must take into account their greater abundance (relative to deletions in circles with two LTRs), the general absence of the PBS sequence, and the inability to complete synthesis of the second LTR sequence.

What is the role of the IN domain in deletion formation? One possibility is that the endonuclease activity of the IN protein is involved in nicking viral DNA either after DNA synthesis is complete or during DNA synthesis. We have previously suggested that IN could play a role in generating these deletions by inappropriate cleavage at the LTR boundary in circular DNA (35). Alternatively, aberrant nicking of plus strong-stop DNA could occur near the U5 boundary, preventing normal completion of DNA synthesis. An aberrant jump during DNA synthesis could then occur as described above, or recombination could take place to a point within the viral genome. Either of these processes could be aided by limited sequence homology. This would account for the observation that several of the deletions have short direct repeats at the deletion junction (Fig. 3). In this case, the IN mutation in AJD105 would result in less nicking and a decrease in LTR-associated rearrangements (Table 1). Alternately, the lesion in AJD105 could affect some other aspect of deletion formation, for example, the ability of reverse transcriptase to jump from template to template during DNA synthesis. This could explain the apparent paradox that this mutant is conditional for virus replication (39) and deletion formation (Table 1) but not for integration (39).

One limitation in establishing the origin of deletions in circular viral DNA is a lack of knowledge concerning the mechanism of circularization of linear viral DNA. Our ability to alter the ratio of circular DNA molecules with either one or two copies of the LTR unit by use of recombinant viruses (e.g., R2G-ANV-A; Fig. 6) should allow us to identify viral proteins that influence circularization.

Although we have studied molecules that are themselves likely to be dead-end products in the virus life cycle, their structures point to the potential for significant genetic instability in viral DNA. One process in which similar deletions may play a role in virus-induced pathologic changes is generation of bursal lymphomas by avian leukosis virus. A large fraction of the integrated viral DNA molecules found next to the activated c-*myc* gene in these tumors contain a deletion with an endpoint near the U5 boundary (14, 40), and proviruses with similar deletions are seen in human T-cell lymphotropic virus type I-associated leukemias (21, 29). A smaller fraction of these proviruses contain a deletion with an endpoint near the U3 boundary (14), similar to those described here. It would be of interest to determine the oncogenic potential of the RAV-2 recombinants described in this report which vary in the frequency at which these deletions occur. Furthermore, it has been suggested previously that rearrangements between viral DNA and cellular sequences could initiate transduction of cellular sequences (13, 15, 20, 51). Understanding the basis of genetic instability in retroviral DNA may provide insight into the unusual process of oncogene transduction.

ACKNOWLEDGMENTS

We thank Phillip McCauley and Pascale Nantermet for assistance with portions of this study. We thank Howard M. Temin for a helpful discussion.

This work was supported by Public Health Service grants RO1-CA33147 (R.S.) and RO1-CA16312 (D.P.G.) from the National Institutes of Health. This work was initiated while J.O. was a fellow of the Leukemia Society of America and R.S. was supported by an American Cancer Society Junior Faculty Award.

LITERATURE CITED

1. **Beck, E., G. Ludwig, E. A. Auerswald, B. Reiss, and H. Schaller.** 1982. Nucleotide sequence and exact localization of the neomycin phosphotransferase gene from transposon Tn5. Gene **19:** 327–336.
2. **Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heynecker, H. W. Boyer, J. H. Crosa, and S. Falkow.** 1977. Construction and characterization of new cloning vehicles. II. a multipurpose cloning system. Gene **2:**95–113.

3. **Bova, C. A., J. P. Manfredi, and R. Swanstrom.** 1986. *env* genes of avian retroviruses: nucleotide sequence and molecular recombinants define host range determinants. Virology **152**:343–354.
4. **Bowerman, B., P. O. Brown, H. E. Varmus, and J. M. Bishop.** 1989. A nucleoprotein complex mediates the integration of retroviral DNA. Genes Dev. **3**:469–478.
5. **Brown, P. O.** 1990. Integration of retroviral DNA. Curr. Top. Microbiol. Immunol. **157**:19–47.
6. **Coffin, J. M.** 1979. Structure, replication, and recombination of retrovirus genomes: some unifying hypotheses. J. Gen. Virol. **42**:1–26.
7. **Colicelli, J., and S. Goff.** 1986. Structure of a cloned circular DNA containing a tRNA sequence between the terminal repeats. J. Virol. **57**:674–677.
8. **Crawford, S., and S. Goff.** 1984. Mutations in *gag* proteins p12 and p15 of Moloney murine leukemia virus block early stages of infection. J. Virol. **49**:909–917.
9. **DeLorbe, W. J., P. A. Luciw, H. M. Goodman, H. E. Varmus, and J. M. Bishop.** 1980. Molecular cloning and characterization of avian sarcoma virus circular DNA molecules. J. Virol. **36**:50–61.
10. **Dower, W. J., J. F. Miller, and C. W. Ragsdale.** 1988. High efficiency transformation of E. coli by high voltage electroporation. Nucleic Acids Res. **16**:6127–6145.
11. **Felgner, P. L., T. R. Gadek, M. Holm, R. Roman, H. W. Chan, M. Wenz, J. P. Northrop, G. M. Ringold, and M. Danielsen.** 1987. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. Proc. Natl. Acad. Sci. USA **84**:7413–7417.
12. **Fu, X., R. A. Katz, A. M. Skalka, and J. Leis.** 1988. Site-directed mutagenesis of the avian retrovirus nucleocapsid protein pp12: mutation which affects RNA binding in vitro blocks viral replication. J. Biol. Chem. **263**:2134–2139.
13. **Goldfarb, M. P., and R. A. Weinberg.** 1981. Generation of novel, biologically active Harvey sarcoma viruses via apparent illegitimate recombination. J. Virol. **38**:136–150.
14. **Goodenow, M. M., and W. S. Hayward.** 1987. 5′ long terminal repeats of *myc*-associated proviruses appear structurally intact but are functionally impaired in tumors induced by avian leukosis viruses. J. Virol. **61**:2489–2498.
15. **Goodrich, D. W., and P. H. Duesberg.** 1988. Retroviral transduction of oncogenic sequences involves viral DNA instead of RNA. Proc. Natl. Acad. Sci. USA **85**:3733–3737.
16. **Gorelick, R. J., L. E. Henderson, J. P. Hanser, and A. Rein.** 1988. Point mutations of Moloney murine leukemia virus that fail to package viral RNA: evidence for specific RNA recognition by a zinc finger-like protein sequence. Proc. Natl. Acad. Sci. USA **85**:8420–8424.
17. **Goubin, G., and M. Hill.** 1979. Monomer and multimer covalently closed circular forms of Rous sarcoma virus. J. Virol. **29**:799–804.
18. **Grandgenett, D. P., and S. R. Mumm.** 1990. Unraveling retrovirus integration. Cell **60**:3–4.
19. **Guntaka, R. V., O. C. Richards, P. R. Shank, H. J. Kung, N. Davidson, E. Fritsch, J. M. Bishop, and H. E. Varmus.** 1976. Covalently closed circular DNA of avian sarcoma virus: purification from nuclei of infected quail tumor cells and measurement by electron microscopy and gel electrophoresis. J. Mol. Biol. **106**:337–357.
20. **Herman, S. A., and J. M. Coffin.** 1987. Efficient packaging of readthrough RNA in ALV: implications for oncogene transduction. Science **236**:845–848.
21. **Hiramatsu, K., and H. Yoshikura.** 1986. Frequent partial deletion of human adult T-cell leukemia virus type I proviruses in experimental transmission: pattern and possible implication. J. Virol. **58**:508–512.
22. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. **26**:365–369.
23. **Hughes, S. and E. Kosik.** 1984. Mutagenesis of the region between *env* and *src* of the SR-A strain of Rous sarcoma virus for the purpose of constructing helper independent vectors. Virology **136**:89–99.
24. **Ju, G., and A. M. Skalka.** 1980. Nucleotide sequence analysis of the long terminal repeat (LTR) of avian retroviruses: structural similarities with transposable elements. Cell **22**:379–386.
25. **Katz, R. A., R. W. Terry, and A. M. Skalka.** 1986. A conserved *cis*-acting sequence in the 5′ leader of avian sarcoma virus RNA is required for packaging. J. Virol. **59**:163–167.
26. **Kawai, S., and M. Nishizawa.** 1984. New procedure for DNA transfection with polycation and dimethyl sulfoxide. Mol. Cell. Biol. **4**:1172–1174.
27. **Kung, H. J., P. R. Shank, J. M. Bishop, and H. E. Varmus.** 1980. Identification and characterization of dimeric and trimeric circular forms of avian sarcoma virus-specific DNA. Virology **103**:425–433.
28. **Lathe, R., S. Skory, and M. P. Kieny.** 1984. Inserting new restriction sites by linker tailing. Focus **6**(4):1–7.
29. **Malik, K. T. A., J. Even, and A. Karpas.** 1988. Molecular cloning and complete nucleotide sequence of an adult T cell leukemia virus/human T cell leukemia virus type I (ATLV/HTLV-I) isolate of Caribbean origin: relationship to other members of the ATLV/HTLV-I subgroup. J. Gen. Virol. **69**:1695–1710.
30. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1989. Molecular cloning: a laboratory manual, 2nd ed., p. 1.25–1.28. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
31. **Meric, C., and S. Goff.** 1989. Characterization of Moloney murine leukemia virus mutants with single-amino-acid substitutions in the Cys-His box of the nucleocapsid protein. J. Virol. **63**:1558–1568.
32. **Meric, C., and P. F. Spahr.** 1986. Rous sarcoma virus nucleic acid-binding protein p12 is necessary for viral 70S RNA dimer formation and packaging. J. Virol. **60**:450–459.
33. **Moscovici, C., M. G. Moscovici, H. Jimenez, M. M. C. Lai, M. J. Hayman, and P. K. Vogt.** 1977. Continuous tissue culture cell lines derived from chemically induced tumors of Japanese quail. Cell **11**:95–103.
34. **Olsen, J. C., P. Furman, J. A. Fyfe, and R. Swanstrom.** 1987. 3′-Azido-3′-deoxythymidine inhibits the replication of avian leukosis virus. J. Virol. **61**:2800–2806.
35. **Olsen, J. C., and R. Swanstrom.** 1985. A new pathway in the generation of defective retrovirus DNA. J. Virol. **56**:779–789.
36. **Omer, C. A., K. Pogue-Geile, R. Guntaka, K. A. Staskus, and A. J. Faras.** 1983. Involvement of directly repeated sequences in the generation of deletions of the avian sarcoma virus *src* gene. J. Virol. **47**:380–382.

36a. **Pathak, V. K., and H. M. Temin.** 1990. Broad spectrum of *in vivo* forward mutations, hypermutations, and mutational hotspots in a retroviral shuttle vector after a single replication cycle: deletions and deletions with insertions. Proc. Natl. Acad. Sci. USA **87**:6024–6028.

37. **Payne, G. S., S. A. Courtneidge, L. B. Crittenden, A. M. Fadly, J. M. Bishop, and H. E. Varmus.** 1981. Analysis of avian leukosis virus DNA and RNA in bursal tumors: viral gene expression is not required for maintenance of the tumor state. Cell **23**:311–322.
38. **Quinn, T. P., and D. P. Grandgenett.** 1988. Genetic evidence that the avian retrovirus DNA endonuclease domain of *pol* is necessary for viral integration. J. Virol. **62**:2307–2312.
39. **Quinn, T. P., and D. P. Grandgenett.** 1989. Avian retrovirus integration protein: structure-function analysis of viable mutants. Virology **173**:478–488.
40. **Robinson, H. L., and G. C. Gagnon.** 1986. Patterns of proviral insertion and deletion in avian leukosis virus-induced lymphomas. J. Virol. **57**:28–36.
41. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1974. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5468.
42. **Schwartz, D. E., R. Tizard, and W. Gilbert.** 1983. Nucleotide sequence of Rous sarcoma virus. Cell **32**:853–869.
43. **Schwartzberg, P., J. Colicelli, and S. Goff.** 1984. Mutations in the *gag* gene of Moloney murine leukemia virus: effects on production of virions and reverse transcriptase. J. Virol. **49**:918–924.
44. **Shimotohno, K., and H. M. Temin.** 1982. Spontaneous variation and synthesis in the U3 region of the long terminal repeat of an

avian retrovirus. J. Virol. **41**:163–171.
45. **Shoemaker, C., S. Goff, E. Gilboa, M. Paskind, S. W. Mitra, and D. Baltimore.** 1980. Structure of a cloned circular Moloney murine leukemia virus DNA molecule containing an inverted segment: implications for retrovirus integration. Proc. Natl. Acad. Sci. USA **77**:3932–3936.
46. **Shoemaker, C., J. Hoffman, S. P. Goff, and D. Baltimore.** 1981. Intramolecular integration within Moloney murine leukemia virus DNA. J. Virol. **40**:164–172.
47. **Skalka, A. M., L. Boone, R. Junghans, and D. Luk.** 1982. Genetic recombination in avian retroviruses. J. Cell. Biochem. **19**:293–304.
48. **Southern, P. J., and P. Berg.** 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. J. Mol. Appl. Genet. **1**:327–341.
49. **Sudol, M., T. L. Lerner, and H. Hanafusa.** 1986. Polymerase-defective mutant of the Bryan high-titer strain of Rous sarcoma virus. Nucleic Acids Res. **14**:2391–2405.
50. **Swanstrom, R., W. J. DeLorbe, J. M. Bishop, and H. E. Varmus.** 1981. Nucleotide sequence of cloned unintegrated avian sarcoma virus DNA: viral DNA contains direct and inverted repeats similar to those in transposable elements. Proc. Natl. Acad. Sci. USA **78**:124–128.
51. **Swanstrom, R., R. C. Parker, H. E. Varmus, and J. M. Bishop.** 1983. Transduction of a cellular oncogene: the genesis of Rous sarcoma virus. Proc. Natl. Acad. Sci. USA **80**:2519–2523.
52. **Swanstrom, R., H. E. Varmus, and J. M. Bishop.** 1982. Nucleotide sequence of the 5′ noncoding region and part of the *gag* gene of Rous sarcoma virus. J. Virol. **41**:535–541.
53. **Tamura, T., and T. Takano.** 1982. Long terminal repeat (LTR)-derived recombination of retroviral DNA: sequence analysis of an aberrant clone of baboon endogenous virus DNA which carries an inversion from the LTR to the *gag* region. Nucleic Acids Res. **10**:5333–5343.
54. **Van Beveren, C., E. Rands, S. K. Chattopadhyay, D. R. Lowy, and I. M. Verma.** 1982. Long terminal repeat of murine retroviral DNAs: sequence analysis, host-proviral junctions, and preintegration site. J. Virol. **41**:542–556.
55. **Varmus, H. E., S. Heasley, H. J. Kung, H. Oppermann, V. C. Smith, J. M. Bishop, and P. R. Shank.** 1978. Kinetics of synthesis, structure and purification of avian sarcoma virus-specific DNA made in the cytoplasm of actively infected cells. J. Mol. Biol. **120**:55–82.
56. **Varmus, H. E., and R. Swanstrom.** 1984. Replication of retroviruses, vol. 1, p. 369–512. *In* R. Weiss, N. Teich, H. E. Varmus, and J. Coffin (ed.), Molecular biology of tumor viruses: RNA tumor viruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
57. **Varmus, H. E., and R. Swanstrom.** 1985. Replication of retroviruses, vol. 2 (Suppl.), p. 75–134. *In* R. Weiss, N. Teich, H. E. Varmus, and J. Coffin (ed.), RNA tumor viruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
58. **Voynow, S. L., and J. M. Coffin.** 1985. Evolutionary variants of Rous sarcoma virus: large deletion mutants do not result from homologous recombination. J. Virol. **55**:67–78.
59. **Zagursky, R. J., K. Baumeister, N. Lomax, and M. L. Berman.** 1985. Rapid and easy sequencing of large linear double-stranded DNA and supercoiled plasmid DNA. Gene Anal. Tech. **2**:89–84.
60. **Zasloff, M., G. D. Ginder, and G. Felsenfeld.** 1978. A new method for the purification and identification of covalently closed circular DNA molecules. Nucleic Acids Res. **5**:1139–1152.