Evolutionary Variants of Rous Sarcoma Virus: Large Deletion Mutants Do Not Result from Homologous Recombination

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Large deletion (LD) mutants of Prague strain Rous sarcoma virus, subgroup B (PrB), derived by serial undiluted passage through chicken (C/E) cells, were isolated and characterized. Individual LD viruses were initially isolated by cloning in soft agar of infected, chemically transformed quail (QT6) cells. Two regions of the PrB genome were deleted in the formation of the LD virus. This resulted in the junction of *gag* sequences in p12 to *env* sequences in gp37, and in the loss of the *src* gene. DNA restriction analysis of biologically active λ Charon 27-LD recombinant clones indicated that individual LD viruses contained similar but not identical deletion endpoints. Two LD isolates, LD25 and LD85, were further subcloned into pBR322, and the deletion junctions were examined by DNA sequencing. Although the *gag-env* deletion endpoints were identical in the two subclones, heterogeneity was observed across the *src* deletion in that both mutants analyzed had the same 5' endpoint but slightly different 3' endpoints. In all cases, only a single homologous base (always an A residue) was found at the deletion endpoint. S1 nuclease analysis of the RNA from a number of QT6-LD clones gave similar results, indicating that the LD population was composed of viruses with similar but not identical deletion endpoints. Such viruses may have been generated from errors during reverse transcription of the virion RNA with subsequent selection assuring their dominance in the population.

RNA virus genomes commonly undergo rapid mutation during repeated undiluted passage, generating subgenomic deletion mutants (1, 8, 44, 55). These have been observed in virtually every RNA virus examined to date. Those described in non-retrovirus systems have been named Von Magnus or defective interfering (DI) particles (30, 32). In many cases, DI particle RNA is deleted for various amounts of internal information but retains one or both termini. The termini contain sites for replication initiation and for packaging (32), and it has been suggested that DI particle RNA, at least in vesicular stomatitis virus, may be generated by a copy choice mechanism of replication (24). In addition, DI particles require a helper virus for replication, and they interfere with closely related helper viruses (33) by a mechanism that appears to be related to virus replication (2).

Among the retroviruses, subgenomic deletion mutants have also been generated by serial undiluted passage of virus in tissue culture. The most commonly observed deletion in Rous sarcoma virus (RSV) is that of the *src* gene (19). Such mutants are called transformation defective (*td*), since the loss of the *src* gene renders them incapable of transforming cells. Unlike DI particles, *td* RSV remains replication competent since *src* is not essential for viral growth.

More highly deleted viruses have also been observed to arise spontaneously at relatively high frequency in a variety of retroviruses (12, 37, 54; E. Woodland and P. Shank, personal communication). To investigate the evolution of such mutants in cell culture, we previously subjected Prague strain RSV subgroup B (Pr-RSV-B) to 100 cycles of undiluted passage (8). After about 50 passages, a specific replication-defective deletion mutant appeared and soon became the majority component of the virus stock. Like DI particles, the mutant virus required a helper virus for replication, and although the mutant did not interfere with the

replication of the helper virus (as DI particles do), it overgrew the helper to a significant extent. We called the mutant virus LD, for large deletion, since it lacked two large regions (8; S. Voynow, Ph.D. thesis, Tufts University, Boston, Mass., 1984). One deletion involved the 3' portion of *gag*, all of *pol*, and the 5' portion of *env*; the second deletion involved the *src* gene. Both viral termini were retained; these presumably contain signals for replication initiation and packaging as well as for proviral integration. Interestingly, clones containing only the LD virus produced *gag*-related particles that could be observed in an electron microscope, indicating that retention of functional p19^{gag} and p27^{gag} is sufficient for the production of virions in infected cells (49).

Noncoding regions on either side of the *src* gene were also retained, including a 110- to 120-nucleotide direct repeat (r1r2). In Prague strain RSV (PrC), the r1r2 sequence 3' to *src* is interrupted by a 135-nucleotide-long sequence called E to give the structure r1r2-*src*-r1Er2 (5). It has been suggested that loss of *src* may be mediated by homologous recombination between the directly repeated sequences flanking that gene (10, 16, 29, 53). It has also been suggested that one complete copy of the direct repeat is required in *cis* for assembly of genomes into virions (42).

We were interested in utilizing the LD virus to examine the biological overgrowth property and to help elucidate the mechanism of deletion formation in retroviruses. Our data indicate that the deletions that formed the LD virus did not result from homologous recombination. We were unable to find any obvious correlation between deleted genomes and biological overgrowth.

MATERIALS AND METHODS

Bacterial strains, bacteriophage, and plasmids. Plasmids were propagated in *Escherichia coli* K-12 derivative RV200 (*thi*⁺ *lacX74 rpsL200*). Plasmid pATV-8, PrC DNA cloned into the *Hind*III site of pBR322, was constructed by R. A. Katz and R. V. Guntaka (18). Plasmid pRAV-1, RAV-1 DNA

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cloned into the *SalI* site of pBR322, was a gift from J. M. Bishop. Plasmids pLD25 and pLD85 were constructed as described below. Bacteriophage λ Charon 27 (34) was a gift from E. Woodland. λ was grown in *E. coli* K802 (*supE hsr⁻_k hsm⁺_k Met⁻ Gal⁻*) (52), also a gift from E. Woodland.

Eucaryotic cells and viruses. Chicken embryo fibroblasts (C/E; *chf⁻, gs⁻, ev-1*) were prepared from fertilized eggs from SPAFAS (Norwich, Conn.). Turkey embryo fibroblasts (T/BD) were prepared from fertilized eggs from Orlopp Enterprises, Inc. (Arosi, Calif.). QT6 cells are a continuous line derived from a methylcholanthrene-induced fibrosarcoma of Japanese quail (27). Prague strain of RSV subgroup B clone 3H (PrB) was generated by focus assay cloning infected chicken cells. The RAV-0 strain of endogenous avian leukosis virus was harvested from line 100 chicken embryo fibroblasts C9 and was a gift from G. Cooper. Both viruses have been described by Coffin et al. (7). Rous-associated virus (RAV) 1 was a gift from H. Hanafusa; it was used to infect fresh chicken cells from which viral supernatants were harvested and stored at -70°C . C/B chicken cells infected with RAV-3 were received from P. Vogt, and viral supernatants were harvested and stored. LD mutant virus stock was derived by sequential *in vitro* passage of PrB virus (8). Line 100 chicken cells were infected with the passage 99 PrB stock, and the resulting virus was passaged five times through T/BD cells to derive the PrB (passage 99)/RAV-0 mixture (C. Barker, personal communication).

Biological cloning of the LD virus. QT6 cells were infected with the undiluted PrB (passage 99)/RAV-0 virus mixture. After 18 to 20 h, cells were suspended into a semisolid agarose medium and incubated at 37°C . Individual colonies were suspended into growth medium and incubated further. Cells were then tested for virus production by assaying for reverse transcriptase activity (14); clones containing only LD proviruses were identified by Southern blot analysis (43, 50).

Molecular cloning of the LD virus. To purify unintegrated LD DNA for cloning into λ Charon 27, nonproducer clones were superinfected with RAV-3, the supernatants were used to infect chicken cells, and the infected chicken cells were cocultivated at a ratio of 1:10 with uninfected QT6 cells. Since QT6 cells accumulate unintegrated viral DNA beginning about 18 h postinfection (36), after 18 to 20 h, the cells were collected and lysed, and the unintegrated DNA was precipitated by the method of Hirt (15). The DNA was then treated with pancreatic RNase and pronase. To further purify the LD DNA away from the unintegrated helper DNA, as well as from a small amount of contaminating host DNA, the preparation was treated with *HindIII* (which cleaves helper but not LD DNA) and banded in a cesium chloride gradient. The LD-containing fractions were then identified by Southern blot analysis.

Cesium chloride-purified LD DNA was cloned into the unique *EcoRI* site of Charon 27 DNA. The *EcoRI* site was chosen because preliminary Southern blot analysis indicated that *EcoRI* cleaved the LD DNA just twice, once in each long terminal repeat (LTR), generating a unit length molecule. LD DNA (20 ng) and Charon 27 DNA (600 ng) were digested with *EcoRI* and ligated under standard conditions at 14°C for 5 h or at room temperature for 1 to 1.5 h. The recombinant bacteriophage were then packaged *in vitro* into phage particles as previously described (13) and used to infect *E. coli* K802. Molecular recombinants were identified by the plaque hybridization technique (3), using a nick-translated, ^{32}P -labeled RAV-1 probe. After three cycles of

plaque purification on *E. coli* K802, DNA was prepared by the plate lysate method (21), analyzed by restriction enzyme digestion, and tested for biological activity by transfection (see below).

For subcloning viral inserts into the *EcoRI* site of pBR322, recombinant DNA was digested with *EcoRI*; the LD DNA was isolated by agarose gel electrophoresis followed by binding to glass powder (48); and the LD DNA was ligated to phosphatase-treated pBR322 at the unique *EcoRI* site and introduced into *E. coli* RV200. Colonies that arose were screened by the colony hybridization technique (21), using a nick-translated, ^{32}P -labeled RAV-1 probe. DNA was purified by the alkaline lysis procedure (21) and analyzed as described above. This DNA was then used for further mapping and sequencing analyses.

Transfection of eucaryotic cells. Viral inserts were separated from plasmid DNA by digestion with the appropriate restriction enzymes and ligated with T4 DNA ligase to form nonpermuted circular and concatameric molecules. T/BD cells were transfected by either the calcium phosphate method (11) or the DEAE-dextran method (9). Cells were passaged twice and tested for reverse transcriptase activity. Supernatants were passaged 9 to 14 times; virions were then ^{32}P -labeled and the virion RNA was analyzed by T₁ oligonucleotide fingerprinting (6).

Nucleotide sequence analysis. DNA fragments were ^{32}P -labeled at the 3' end with DNA polymerase I large fragment and at the dephosphorylated 5' end with T4 polynucleotide kinase (2). Secondary restriction enzyme digestion generated DNA fragments labeled at one end only, and these were isolated by polyacrylamide gel electrophoresis (23). DNA sequencing was performed by the method of Maxam and Gilbert (23) as previously described (13), and the data were analyzed by a dot matrix program (Coffin, unpublished).

S1 mapping. S1 mapping was performed as previously described (25), with the following exceptions. DNA probes were either dephosphorylated at the 5' end and then labeled with T4 polynucleotide kinase, or 3' end labeled with DNA polymerase I large fragment. (21). Total cellular RNA (15 μg) was hybridized with 1×10^3 to 2×10^3 cpm of denatured probe in 10 μl of S1 hybridization buffer (80% formamide, 50 mM PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid)] [pH 6.4], 0.4 M NaCl, 1 mM EDTA) for 3 h at 50°C . Samples were then diluted into 250 μl of S1 digestion buffer (0.25 M NaCl, 50 mM sodium acetate [pH 4.5], 3 mM ZnSO₄) on ice and digested with 10 U of S1 nuclease at 45°C for 10 min.

Enzymes and reagents. Restriction endonucleases were purchased from New England Biolabs or Bethesda Research Laboratories and used under the conditions suggested by the vendors. T4 DNA ligase and T4 polynucleotide kinase were supplied by New England Biolabs, calf alkaline phosphatase was from Boehringer Mannheim Biochemicals, and S1 nuclease was from Worthington Diagnostics. *E. coli* DNA polymerase I, large fragment (Klenow), as well as sequencing reagents and radioisotopes, were supplied by New England Nuclear Corp.

RESULTS

Isolation of LD virus-infected cell clones. For a study of the properties of the LD virus apart from its helper, a virus mixture containing passage 100 PrB was used to infect QT6 cells. Since QT6 cells are resistant to subgroup B virus, the virus subgroup was first changed to E by pseudotyping with RAV-0 and passaging five times in T/BD cells, to allow overgrowth of the LD virus. At 18 to 20 h postinfection, the

FIG. 1. Detection of LD-infected nonproducer clones. (A) Total cell DNA (10 μ g) from the indicated clones of QT6 cells infected with the LD/RAV-0 phenotypic mixture was digested with *Eco*RI, separated on a 1% agarose gel by electrophoresis, and transferred to a nitrocellulose filter. The filter was hybridized with a 32 P-labeled *td* PrB cDNA probe (representative of the entire genome) for identification of virus-containing fragments. 32 P-labeled, *Eco*RI-digested λ DNA served as a marker. — indicates DNA from uninfected QT6 cells; + indicates DNA from turkey cells infected with the LD/RAV-0 virus mixture. The 1.5-, 2.4-, and 4.3-kb fragments are those predicted for *td* RSV (Fig. 5); the 3.5-kb fragment is LD specific. (B) Total cell DNA (10 μ g) from the QT6-LD25 and QT6-LD85 clones was digested with *Hind*III and analyzed as described above. λ DNAs digested with *Hind*III and *Eco*RI served as markers.

QT6 cells were plated in soft agar medium. Individual clones were isolated and tested for reverse transcriptase activity. LD virus-infected clones were then identified by transfer hybridization of *Eco*RI-digested DNA from reverse transcriptase-negative clones, since preliminary experiments indicated that the LD provirus yielded a single *Eco*RI restriction fragment at about 3.5 kilobases (kb) (Fig. 1A, lane +), whereas helper proviruses yielded three fragments of about 1.5, 2.4, and 4.3 kb. A number of clones (17, 18, 19, 22, 26, and 27) did not contain a provirus; a few clones contained a mixture of helper and LD virus sequences (16, 24, and 29); and the rest of the clones contained only LD sequences (20, 21, 23, 25, 28, and 30). Total cellular DNA from two clones that contained only LD proviruses, 25 and 85, was also digested with *Hind*III. This enzyme does not cleave within the LD sequences (see Fig. 5) and thus generates a unique fragment for each integrated provirus. Hybridization analysis indicated the presence of multiple proviruses in both cases (Fig. 1B).

Analysis of LD virus RNA. Selected QT6 clones identified as containing LD, but not helper, proviruses were then tested for the presence of biologically active virus. Individual nonproducer clones were superinfected with a helper virus (RAV-3), and the virus released in the supernatants was passaged once through turkey cells and analyzed by RNase T1-oligonucleotide fingerprinting. As expected, only RAV-3- and LD-specific oligonucleotides were detected, indicating both the purity of these clones and the biological activity of the LD virus (Fig. 2). Further, LD-specific oligonucleotides were in significant excess over helper-specific oligonucleotides (for example, compare the relative intensities of oligonucleotides 25 and 26). Thus, the rescued

virus contained at least a twofold excess of the defective LD species.

An indication of the LD genome size (about 3.5 kb) was obtained from fingerprint and restriction enzyme analyses. In addition, analysis of freshly prepared RNA from QT6-LD cell clones indicated the presence of only a 3.6- to 3.7-kb band of virus-specific RNA (data not shown). Thus, the LD provirus seemed to yield only a single transcript, presumably serving both as genome and as mRNA for the truncated *gag* protein found in the QT6-LD cell clones (49).

Cloning of LD virus DNA. For more precise analysis of the DNA, molecular clones of the LD virus were prepared from nonproducer QT6-LD25 and QT6-LD85 clones. To ensure the cloning of DNA derived from rescuable proviruses, QT6-LD clones 25 and 85 were infected with RAV-3 and the virus mixtures were used to infect fresh QT6 cells. Unintegrated LD proviral DNA was cleaved at the unique *Eco*RI site in the LTR and cloned into the *Eco*RI site of λ Charon 27. For examination of the heterogeneity of the cloned DNA, DNA from a number of different λ LD25 and λ LD85 clones were digested with a combination of *Eco*RI plus *Hind*III, and *Eco*RI plus *Xho*I (Fig. 3). Digestion with *Eco*RI plus *Hind*III generated a unit length LD molecule of about 3.2 kb in most recombinant phage, plus three lambda fragments of 2, 21, and 22 kb. The 5.2-kb fragment in the λ LD25 clones was probably an incomplete digestion product composed of the 3.2 and 2.0-kb fragments. Digestion with *Eco*RI plus *Xho*I generated three LD-containing fragments in most recombinant phage, of ca. 2, 0.75, and 0.4 to 0.54 kb. All other fragments belonged to λ DNA.

These data, together with the data from subsequent digestions (not shown), indicated the following. (i) The λ LD

FIG. 2. Rescue of virus-specific RNA from a QT6-LD clone. For an assessment of the biological activity of the cloned LD virus, QT6-LD25 cells were superinfected with RAV-3, and supernatants were passed once through turkey (T/BD) cells. 32 P-labeled virus RNA was prepared, digested with RNase T₁, and analyzed as described previously (7, 8; unpublished data). Only RAV-3 and LD oligonucleotides are present. LD oligonucleotides are underlined.

clones were somewhat heterogeneous in size. For example, the viral DNA of λ LD25H appeared to be slightly smaller, and that of λ LD25J slightly larger, than expected. (ii) There was some variability in the 5' leader region, in that one λ LD clone (25H) had suffered a small (ca. 50-base-pair) deletion and no longer contained the *Pst*I site in that region. (iii) Fragments spanning the *gag-env* deletion, such as the 2-kb *Xho*I fragment, were remarkably similar in size from clone to clone in both λ LD25 and λ LD85. (iv) Heterogeneity was found mainly within fragments spanning the *src* deletion, which usually varied by about 50 base pairs, but occasionally by more (note λ LD85D and λ LD25H, which lacked the 3'

*env Xba*I, *Sst*I, and *Xho*I sites specific for Prague strain viruses). The presence of the *Xho*I site 3' of *env* in most clones indicated that the *src* deletion began within, or 3' to, the r2 region. Since the original QT6-LD clones contained multiple proviruses, the observed heterogeneity around the *src* deletion could be due to different LD proviral deletions; it could represent further deletions suffered during the cloning procedure; or it could be the result of recombination with either the RAV-3 or RAV-0 helper virus (neither of which contains the *Xba*I, *Sst*I, or *Xho*I sites).

This preliminary mapping was then extended by subcloning the LD25E and LD85A DNAs into plasmid pBR322 and

FIG. 3. Heterogeneity of cloned LD DNA. DNA was isolated from λ Charon 27-LD clones, digested with a combination of *Eco*RI and *Xho*I or *Eco*RI and *Hind*III, and separated on a 1% agarose gel. *Hind*III-digested λ DNA and *Eco*RI-digested λ DNA served as size markers. On the left half of the gel, clones B to J were digested with *Eco*RI plus *Hind*III. On the right half of the gel, clones B to J were digested with *Eco*RI plus *Xho*I.

FIG. 4. Restriction map of the LD DNA. Restriction sites were identified in three ways: (i) by digestion of the DNA from pLD25F, (ii) by Southern blot analysis of QT6-LD cell DNAs (not shown), and (iii) by DNA sequence analysis of pLD25E and pLD85A.

analyzing the recombinants by restriction endonuclease digestion. In this way, a restriction map was generated (Fig. 4). A representative map comparing the parental PrB virus with the LD virus, and including both oligonucleotide and restriction data, is shown in Fig. 5.

Testing molecularly cloned DNA for biological activity. Since the cloning procedures involved a number of steps, any one of which might have altered the biological activity of the LD virus, it was necessary to show that the recombinant LD genome was still rescuable by helper virus. Several isolates each of recombinant clones λ LD25 and λ LD85 were used to transfect chicken (C/E) cells in the presence of infectious RAV-1 DNA. Transfected cells were passaged several times to ensure virus spread, then the supernatants from the transfected cells were passaged 9 to 14 times through chicken cells, 32 P-labeled, and fingerprinted. In each case, both LD- and RAV-1-specific spots were observed, indicating that the LD virus could be rescued; however, neither the LD25 nor the LD85 virus overgrew the helper virus (data not shown). These cells were also metabolically labeled with [35 S]methionine, and gag-related proteins were examined by immunoprecipitation. Both LD- and RAV-1-specific proteins were observed (data not shown).

As an additional test for the extent of LD replication, chicken cells were infected with the virus mixture derived

from 8 or 14 virus passages after cotransfection of LD and RAV-1 DNA. DNA for these cells was examined by *EcoRI* digestion and transfer hybridization. LD DNA was clearly identifiable (Fig. 6), although it appeared to be significantly less abundant in the population than was the RAV-1 DNA. In addition, a fraction of the virus population appeared to have undergone recombination, since a doublet in the 1.3-kb region represented both 3' RAV-1 sequences (as expected) and 3' LD sequences (derived from recombination with RAV-1). This experiment was repeated with DNA from an infectious clone of RAV-0 (a virus that grew more slowly than the LD in the preliminary passage experiments) as helper, and with the DNA from pLD25 and pLD85; in this case, the viruses were passaged through T/BD cells. Here too, virus was rescued from both LD clones, but neither appeared to overgrow the helper (data not shown).

Characterization of deletion endpoints in the LD virus genome. The deletion endpoints of two plasmid subclones, pLD25E-48 and pLD85A-4, were precisely determined by DNA sequencing (22). For characterization of the gag-env deletion junction, a 3' 32 P-labeled fragment extending from the *Bam*HI site in p12^{gag} to the *Pvu*II site in gp37^{env} was sequenced (Fig. 7). The sequence of this strand was identical in both clones, with the crossover points corresponding to nucleotide 1931 or 1932 in gag, and at 6329 or 6330 in env in

FIG. 5. Comparison of LD and PrB virus genomes. The PrB genome is defined according to T1-oligonucleotide mapping (6) and restriction endonuclease analysis (13). Oligonucleotides in RAV-0 allelic to those in PrB are marked. The LD genome is aligned with PrB, and deletion crossover points defined by oligonucleotide mapping are indicated as boxes containing crosses. Vertical arrows indicate the specific deletion endpoints as defined by DNA sequencing. Horizontal arrows indicate the fragments sequenced and the direction of sequencing; the 5' or 3' designation indicates the 32 P-labeled end.

FIG. 6. Rescue of LD virus by cotransfection. DNA (4 μ g) from λ LD25 or λ LD85 was digested with *Eco*RI, mixed with 2 μ g of *Sst*I-digested pRAV-1 DNA, and used to transfect chicken (C/E) cells. The transfected cells were passaged several times to ensure virus spread, and then supernatants were passaged 5 to 14 times through chicken (C/E) cells. DNA was isolated from infected cells, digested with *Eco*RI, and analyzed by transfer to nitrocellulose and hybridization with a nick-translated RAV-1 probe. (a) LD25. Lanes C, E, and F represent different transfected clones, with virus passaged 8 (vp8) or 14 (vp14) times. (b) LD85. Lanes represent different transfected clones, with virus passaged nine times. The arrow indicates the LD-specific restriction fragment.

the PrC sequence (35). Because this sequence was identical in both clones, and because it could be unambiguously matched to the PrC sequence, confirmatory second-strand sequencing was not done.

For characterization of the *src* deletion, a 3' 32 P-labeled fragment extending from the *Xho*I site in the *env-src* border region to the *Eco*RI in the LTR was sequenced in both clones. The same fragments were also 5' 32 P-labeled at the *Xho*I site and sequenced. The crossover points at this deletion (Fig. 8) varied between the two clones; the clone 25 deletion extended from 7019 or 7020 to 8722 or 8723 in the PrC sequence, whereas the clone 85 deletion extended from 7029 or 7030 to 8737 or 8738. Both sequences exactly matched the corresponding PrC sequence, with the exception of a single A at position 7018 to 7023, missing in clone 85. These data show that the deletion event across *src*, generating the *td* LD virus, did not occur by homologous recombination between the *r1r2* sequences. In computer-generated comparisons of sequences upstream and downstream of these regions in PrC genome, significant direct or inverted homologies of eight or more nucleotides in a row were not found. Neither were significant homologies found between the LD deletion junctions and the corresponding regions of RAV-2 or RAV-0, implying that the heterogeneity observed must be LD-specific and not due to recombination with either helper.

To place the sequencing results of the two cloned LD DNAs in a framework relative to the deletions present in the LD population as a whole, the RNA from a number of QT6-LD clones was analyzed by S1 nuclease mapping. Probes were prepared from pATV-8 and labeled at the 5' or 3' ends with 32 P, as appropriate.

The *gag-env* deletion junction was probed at its 5' end with a fragment extending from the *Bgl*II site in p27 to the *Eco*RI site in p15 (probe 1), and at its 3' end with a fragment extending from the *Bcl*II site in gp37 to the *Hind*III site in gp85 (probe 2) (Fig. 9). The *src* deletion was probed at its 5' end with a fragment extending from the *Xba*I site in gp37 to the leftmost *Sma*I site in *src* (probe 3), and at its 3' end with a fragment extending from the *Eco*RI site in the LTR to the rightmost *Sma*I site in *src* (probe 4) (Fig. 9). The hybrids

were treated with S1 nuclease to digest all single-strand regions and were subjected to polyacrylamide gel electrophoresis. RNA from *td* PrB-infected cells was used as a control for sequence divergence between the PrC probe and PrB-derived LD virus. Uninfected QT6 cells were used as a control for random hybridization. The results are shown in Fig. 10.

Among the panel of clones tested, the S1 nuclease digestion results were consistent from clone to clone; in all experiments, however, multiple bands were observed. Subsequent DNA blot analysis indicated that some of the QT6 clones contained multiple LD proviruses (Fig. 1). Taken together with the sequencing results of the two cloned DNAs, the complexity in the S1 patterns is probably a reflection of multiple RNA species from multiple genomes. Analysis of the *gag-env* deletion from the 5' end (probe 1) showed that a fragment of about 300 bases was the primary product protected by the RNA from LD clones; this corresponds to a 5' deletion endpoint of about 1930 on the PrC map, in agreement with the sequencing data. At the 3' end (probe 2), fragments protected by LD RNA ranged from 230 to 280 nucleotides; from the sequencing results, a protected fragment of about 244 nucleotides was expected. Thus, across the *gag-env* deletion, the S1 mapping is in good agreement with the DNA sequencing data; however, some minor variability in the apparent deletion endpoints within a single QT6-LD clone was observed.

Analysis of the *src* deletion from the 5' end (probe 3) indicated protected fragments ranging from 147 to 175 bases. DNA sequencing indicated different 5' deletion endpoints in the two cloned DNAs, corresponding to protected fragments of about 158 and 165. S1 nuclease results across the 3' side of the *src* deletion (probe 4) were the most variable, with protected fragments ranging from 200 to 600 nucleotides in length. The predominant protected fragment was about 500 bases long; this is in agreement with the DNA sequencing results, which predicted fragments of about 502 and 516. It has been suggested that transformation-defective viruses are generated by a recombination event across the repeated (*r1r2*) sequences flanking the *src* gene (10, 53). However, all of the 3' *r1r2* region and most of the 5' *r1r2* region seemed

FIG. 7. Sequence of the *gag-env* deletion junction. A 3' ³²P-labeled fragment extending from the *Bam*HI site in p12^{gag} to the *Pvu*II site in gp37 was sequenced by the method of Maxam and Gilbert (22) and analyzed on a 20% polyacrylamide denaturing gel. A computer-generated comparison of the clone 25 and clone 85 sequence with the PrC sequence is also included. □ and □ indicate ambiguity in the origin of the base at the crossover point.

to be conserved in the major RNA species of LD clones 23, 25, 52, and 85 (Fig. 10). Thus, the major *src* deletion in the LD clones was generated by an event that apparently ignored the r1r2 homology.

DISCUSSION

Rapid appearance of mutant RNA virus genomes is commonly observed upon repeated, undiluted virus passage. Subgenomic deletion mutants described in nonretrovirus systems have been named defective interfering particles. DI particles contain RNA lacking various amounts of internal information but retaining one or both termini; they require a wild-type helper virus for their own replication; and they overgrow that helper to a significant extent, actually interfering with its replication (32).

The evolution of retrovirus genomes was examined by a similar approach. Serial, undiluted passage of a cloned stock of Pr-RSV-B through chicken cells generated a mixed population of *td* virus mutants, composed of both helper and LD viruses (8). The LD component had sustained two large deletions which removed over half of the genome, yet it was capable of being rescued, implying that it contained the sequences required in *cis* for genome replication and packaging. In addition, the LD virus overgrew the helper component significantly but did not interfere with the replication of the helper (8).

Individual biologically active LD viruses were isolated in QT6 cells. These contained rescuable virus that possessed the same genome and growth characteristics as the species found in the high passage mixture. Two clones chosen for further study (LD25 and LD85) were free of helper provi-

FIG. 8. Sequence of the *src* deletion endpoints. The nucleotide sequence of fragments spanning the *src* deletion junction was determined as described in the legend to Fig. 5. $\square$ and $\square$ indicate ambiguity in the origin of the base at the crossover point.

uses, and although they contained multiple integrated LD proviruses, subsequent analyses of the RNA, DNA, and protein contents of these and other QT6-LD isolates revealed that the LD mutants were a relatively homogeneous population. Not only were the genomic deletions similar from clone to clone, but the protein products (of which essentially only a single species was observed in each clone) were almost identical (49). In addition, the QT6-LD cell clones shed particles observable in an electron microscope; these contained a protein indistinguishable by immunoprecipitation from its intracellular counterparts (49).

To facilitate further analysis, unintegrated DNA of the LD virus rescued from two QT6-LD clones was molecularly cloned (pLD25 and pLD85). Curiously, although the pLD genomes were biologically active, as ascertained by the rescue of LD genomes from cells cotransfected with plasmids containing LD and helper virus DNAs, overgrowth of the LD virus relative to the helper was not observed after transfection. The meaning of this result is unclear, since the method chosen for cloning the LD DNA should have preferentially selected the most transcriptionally active genomes.

Structure of the deletions. Preliminary oligonucleotide mapping revealed that the LD mutant had sustained two large deletions. One deletion began in the 3' portion of the *gag* gene and continued into the 3' region of the *env* gene; the other deletion involved the *src* gene and was indistinguishable from the deletion that gives rise to *td* RSV (19).

Restriction endonuclease analysis of the recombinant DNA from clones of LD25 and LD85 indicated that there was little variation in the *gag-env* deletion, but that the *src* deletion was heterogeneous. S1 mapping of the RNA from QT6-LD clones gave the same result, implying that the multiple proviruses in each clone had the same structure in *gag* and *env* but varied more at the 3' end. Nucleotide sequencing across the deletion junctions in pLD25 and pLD85 DNA indicated that the *gag-env* junction, which had appeared similar, was in fact identical in the two cloned genomes, but that the *src* deletion junction differed in the two clones.

These results, together with the oligonucleotide mapping data, suggest the following. Since the characteristic *src* oligonucleotides (spots 5 and 6) were lost within the first 10 passages of PrB (8), the *src* deletion must have occurred first. The *gag-env* deletion must have occurred subsequently and was probably amplified by recombination. Those viruses containing the two deletions were probably then selected within the population by a growth advantage.

Mechanisms for deletion formation. During the course of the passaging and cloning experiments, RAV-0 and RAV-3 were introduced as helper viruses for pseudotyping the LD mutant. It was important, therefore, to exclude the possibility that the apparent *src* deletions were actually due to recombination between the LD virus and either helper. Exhaustive computer searches were made for homologous DNA sequences, where available, in RAV-2 (4) and RAV-0 (46), which might have been capable of serving as crossover

FIG. 9. Deletion junctions in RNA from QT6-LD clones. Total cell RNA (10 μ g) from nonproducer QT6 clones was hybridized to either (A) a 3' 32 P-labeled PrC DNA probe extending from the *Bgl*II site in p12^{src} to the *Eco*RI site in p15^{src}, (B) a 5' 32 P-labeled PrC DNA probe extending from the *Bcl*I site in gp37 to the *Hind*III site in gp85, (C) a 3' 32 P-labeled PrC DNA probe extending from the *Xba*I site in gp37 to the leftmost *Sma*I site in *src*, or (D) a 5' 32 P-labeled PrC DNA probe extending from the *Eco*RI site in the LTR to the rightmost *Sma*I site in *src*. Hybrids were treated with S1 nuclease and analyzed by electrophoresis in a 5% polyacrylamide denaturing gel. Lanes in all experiments are in the same order. Lanes 1 and 11, *Hpa*II-digested pBR322 served as a size marker; lane 2, probe alone; lane 3, uninfected QT6 cells; lane 4, LD23; lane 5, LD25; lane 6, LD30; lane 7, LD52; lane 8, LD60; lane 9, LD85; lane 10, *td* PrB-infected chicken cells. Arrows indicate the position predicted by nucleotide sequencing (in panel C, the upper arrow indicates the predicted position for LD85, and the lower arrow indicates that for LD25).

points for the *gag-env* and the *src* deletions. No RAV-2- or RAV-0-specific homologies were detected in the region of the *src* deletion, obviating the worry that crossovers generated during the cloning procedures were being observed.

To exclude the possibility that homologous recombination within the PrB virus had generated the LD deletions, the DNA sequence of the highly related PrC virus (35) was also examined. Very little homology was detectable at the crossover site, and sequence stretches of PrC DNA containing homologies of eight base pairs or more were not observed either upstream or downstream of the *gag-env* or *src* deletion endpoints. In fact, the only observed homology was the single base (an A residue in all three cases) at the crossover point, which was therefore slightly ambiguous. In addition, splice consensus sites were not observed in this region of *gag* or of *env*. Several *td* PrC mutants have recently been found to contain shorter direct repeat sequences in the vicinity of the *src* deletion (29), and two *td* mutants of SR-A have also been sequenced through the *src* deletion junctions and found not to be flanked by direct repeats (51).

Interestingly, a sequence comparison of the deletion endpoints of one of the *td* PrC mutants (pATV-6) (29) with those of the LD mutants showed that although the 3' *src* deletion endpoint in pATV-6 is 30 nucleotides upstream of

pLD85 (and 15 nucleotides upstream of pLD25), the 5' *src* deletion endpoints of both pATV-6 and pLD85 are identical. In addition, as with pLD25 and pLD85, significant homology was not detected at the *src* deletion junction of pATV-6.

The preservation through 99 virus passages (probably 200 to 300 viral replication cycles) of virtually the entire direct repeat that originally flanked the *src* gene was a striking and unexpected outcome. Analyses of the genomes of virus at early passages (8; manuscript in preparation) and of molecular clones of such virus (13) have shown that, at least on the basis of population, *src* deletions occur fairly rapidly and in a stepwise fashion. We would have expected to see crossovers that preserved only a single copy of the repeated sequence if the presence of such a long directly repeated sequence influenced deletion formation by homologous recombination (as is the usual case) and if there were no selective advantage to a virus with two copies of the repeated sequence. The sequence that is repeated contains the origin of plus-strand DNA synthesis (26, 40) as well as a sequence that is highly conserved in RSV-related retroviruses (31, 41) and is essential to the virus life cycle (42). However, its single presence in most naturally occurring avian leukosis virus strains (4), in recombinant viruses (46), and in deliberately constructed deletion mutants (16) implies a lack of selective advantage for the presence of two copies.

FIG. 10. Deletion endpoints of LD transcripts in QT6-LD clones. The results from the experiment shown in Fig. 9 are mapped. The genome has been drawn, and coding regions of structural genes located along the genome are shown. $\backslash$ indicates a gap in the genome. The probes are aligned on the genome; asterisks next to the restriction sites indicate the labeled end of the probe. RNA is drawn as a straight line, with hatch marks indicating RNA endpoints. Multiple hatch marks along the RNA from a single LD isolate indicate the presence of RNA molecules with multiple endpoints; asterisks above the hatchmarks indicate the predominant protected fragment (when one was observed). Arrows mark the positions of the deletion endpoints determined by nucleotide sequencing of clone 25 and clone 85 DNA. (A) *gag-env* deletion. (B) *src* deletion.

Thus, contrary to previous speculation, we conclude that such long directly repeated sequences probably are not involved in deletion formation.

Clearly, some other mechanism for deletion formation must be involved here, such as aberrant rearrangements of the genome or breakage-reunion or copy-choice mechanisms of replication. Aberrant rearrangements involving the LTRs have been detected in unintegrated circular forms of retroviral DNA. These include insertions, deletions, duplications, and inversions (17, 38, 39, 45, 47). Such variation is evidently tolerated in the LTR region, since viruses exhibit-

ing each of these aberrations are viable; however, it is difficult to explain the structure of the LD deletions on the basis of aberrant rearrangements, since the genome structure contains no major detectable rearrangements.

In the breakage-reunion model, on the DNA level, the DNA could conceivably be nicked in the cytoplasm at segments in the minus strand that corresponded to unlinked regions in the plus strand; once entering the nucleus, the DNA could be religated intact or, possibly, religated in such a way that one nicked segment was effectively deleted. On the RNA level, an incorrectly spliced mRNA could be encapsidated along with an intact vRNA. Subsequent infection of a cell with such a virion would generate a deleted provirus. Evidence for the encapsidation and subsequent provirus formation of *env* mRNA has been obtained in the mouse mammary tumor virus (20). Aberrant splicing is also an unlikely possibility, since consensus splice signals were not observed in the sequences surrounding the deletion junctions in pLD25 and pLD85.

We feel that a copy choice mechanism of replication is more likely to be responsible for the generation of deletion mutants, assuming that the length requirement for homologous pairing at nicks is very small. Such a model has also been suggested for the generation of vesicular stomatitis virus DI particles (24) and for some types of *env* deletions in RSV (28). In this model, minus-strand DNA is copied from both strands of the infecting RNA dimer, with reverse transcriptase guiding the nascent DNA back and forth between the templates, each time a nick is encountered. Movement between the templates could require only a minimal base pairing of the nascent DNA with a homologous sequence in the RNA, if the major driving force were affinity of reverse transcriptase for its substrate rather than formation of base pairs. These deleted viruses would then be selected upon subsequent infection, and over the course of many generations of undiluted virus passage, highly deleted viruses would be observed to take over the virus population.

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