JOURNAL OF VIROLOGY, Mar. 1980, p. 1026-1033
0022-538X/80/03-1026/08$02.00/0

Vol. 33, No. 3

Isolation and Characterization of Recombinant DNA Clones of Avian Retroviruses: Size Heterogeneity and Instability of the Direct Repeat

GRACE JU, LAWRENCE BOONE, AND A. M. SKALKA*

Department of Cell Biology, Roche Institute of Molecular Biology, Nutley, New Jersey 07110

Unintegrated proviral DNA of Schmidt-Ruppin B Rous sarcoma virus was cloned in the bacteriophage λ vector Charon 21A. A total of 12 independent recombinant λSRBtd clones which were derived from the transformation-defective component in the viral preparation were analyzed with restriction endonucleases and molecular hybridization techniques. Three classes of clones were observed. Type I clones contained a 5.0-megadalton insert of viral DNA, type II clones contained phage with two size classes of inserts (5.0 and 5.2 megadaltons), and one type III clone contained only a 5.2-megadalton insert. The smaller insert present in type II clones appeared to be derived by deletion of one copy of a directly repeated sequence which was present in the larger insert. Mapping data indicated that the deletion includes all or part of the terminal repeat found in linear double-stranded proviral DNA. Similar results were obtained from λRAV2 recombinant clones derived from Rous-associated virus type 2. Analysis of DNA from type II and type III clones of λSRBtd and λRAV2 revealed limited heterogeneity in the size of the direct repeat.

Replication of the retroviruses involves the production of proviral DNA intermediates before integration into the host cell genome. At least three types of intermediates have been identified in cells after exogenous infection: linear double-stranded DNA and two sizes of covalently closed circular DNA (10). For the avian retroviruses, the linear double-stranded DNA has been shown to contain a direct repeat of approximately 300 base pairs (bp) (0.2 megadalton [Md]) at its termini. This duplication is also present in the larger of the two circular intermediates (16, 25), which may be formed from the linear DNA by intramolecular joining at the termini. Restriction mapping data indicate that the smaller circular species has only one copy of the terminal sequence (25). The circular forms are found exclusively in the nucleus (26), and, as with bacteriophage lambda (9), a circular intermediate of the provirus is thought to be the substrate for integration into the host genome (10, 13). Integrated provirus has also been found to contain terminal redundancies (17, 24), suggesting that the repeated sequences are involved in the integration reaction. The limited quantity of viral DNA found in infected cells has made analysis of these intermediates difficult. However, larger amounts of purified DNA can now be obtained by using recombinant DNA methodology.

Unintegrated circular proviral DNA from chicken embryo fibroblasts infected with Schmidt-Ruppin B Rous sarcoma virus (SR-RSV-B) was isolated and cloned in the bacteriophage λ vector Charon 21A (Ch21A) by using an enzyme known to cut the proviral DNA at only one site. Three types of λSRBtd recombinant DNA clones derived from the transformation-defective (td) component of the virus were isolated. One type contained a viral DNA insert of 5.0 Md, a second contained a larger viral insert of 5.2 Md, and a third contained recombinant molecules with either of these two size classes of inserts. Experiments using clones with two size classes indicated that the smaller insert could arise by deletion of one copy of the repeated sequence from the larger insert during growth within procaryotic host cells. Similar results were obtained with recombinant clones prepared from unintegrated proviral DNA of Rous-associated virus type 2 (RAV-2). Furthermore, in six independent clones examined, the size of the repeated sequences was found to be heterogeneous. The implications of these results for mechanisms of viral replication in vivo are discussed.

MATERIALS AND METHODS

Cells and viruses. Chicken embryo fibroblasts (gs^- chf^-; SPAFAS, Norwich, Conn.) were prepared and propagated as described previously (20). The SR-RSV-B used in these experiments was a recombinant between *NY68*, a temperature-sensitive mutant of SR-RSV-A (subgroup A), and RAV-2 (subgroup B). It

contained the temperature-sensitive mutation of *NY68* in the transformation gene (*src*) and the subgroup B specificity of RAV-2 (18). The virus was obtained as a cloned stock from H. Hanafusa, Rockefeller University. The λ vector Ch21A has been described previously (4). *Escherichia coli* K-12 strains employed were the EK1 hosts ED8767 ($recA^-r_k^-m_k^+$) (22) and C600 ($recA^-r_k^-m_k^-$), which was obtained from R. Davis, Stanford University, and the EK2 host DP50 *supF* ($recA^+$).

DNA isolation. To prepare proviral DNA, 25 petri dishes (150 mm^2) of chicken embryo fibroblasts were infected at high multiplicity (multiplicity of infection, >1 focus-forming unit per cell). After 48 h, infected cells were lysed with detergent, and high-molecular-weight nuclear DNA was pelleted by the method of Hirt (15). The Hirt supernatant fraction was treated with proteinase K (50 μg/ml) at 37°C for 2.5 h, extracted with phenol, and precipitated with ethanol. The ethanol precipitate was next dissolved, extracted with chloroform–isoamyl alcohol (100:1), and again concentrated by ethanol precipitation. The DNA was then dissolved in TEN buffer (0.01 M Tris, pH 7.4, 0.001 M EDTA, 0.01 M NaCl), treated with RNase A (50 μg/ml) at 37°C for 1 h, extracted with phenol and chloroform–isoamyl alcohol, and then ethanol precipitated. The precipitate was dissolved and covalently closed circular viral DNA was separated from relaxed circles and linear molecules by isopycnic centrifugation in a cesium chloride-propidium diiodide density gradient (27).

Recombinant DNA techniques. All cloning experiments were conducted under the P2-EK2 or P3-EK1 conditions specified by the December 1978 revised National Institutes of Health guidelines.

A portion of the covalently closed proviral DNA preparation of SR-RSV-B was digested with *SalI*, ligated to *SalI*-digested λ Ch21A with T4 ligase (21), encapsulated by in vitro packaging techniques (3), and plated on the $recA^-$ strain ED8767. Approximately 36,000 plaques were screened for viral DNA inserts by using the method of Benton and Davis (2) and a ^{32}P-labeled RAV-2 viral RNA probe. Positive plaques were isolated and purified twice before amplification and extraction of DNA for restriction endonuclease analysis.

Nuclease digestion. Restriction endonucleases *AluI*, *SalI*, *HindIII*, *XhoI*, *XbaI*, *KpnI*, and *PvuI* were purchased from New England Biolabs, Beverly, Mass. *EcoRI* was purchased from Miles Laboratories, Elkhart, Ind. Digestions were performed in 25 or 50 μl of the appropriate buffer.

Gel electrophoresis, DNA transfer, and hybridization. Agarose gel electrophoresis and DNA transfer were performed by the method of Southern (28), as previously described (20). Hybridization procedures have been described previously (20). For molecular weight determinations, parallel lanes of the gels contained *HindIII* digests of ^{14}C-labeled λ DNA.

To determine the size of small DNA fragments (<1,000 bp), digested DNA samples were analyzed by electrophoresis in vertical 6% polyacrylamide (acrylamide/bisacrylamide ratio, 40:1) gels in TBE buffer (0.89 M Tris, pH 8.3, 0.89 M boric acid, 0.01 M EDTA). Fragments of φX174 DNA digested with *AluI* were used as size markers.

RESULTS

Molecular cloning of SR-RSV-B viral DNA. Unintegrated covalently closed circular proviral DNA was purified from chicken embryo fibroblasts infected with SR-RSV-B as described above. The proviral DNA was digested with restriction endonuclease *SalI*, which was found to cleave the circular molecules at a single location (Fig. 1). The cleaved DNA was then ligated to the λ vector Ch21A and packaged into phage particles in vitro. Recombinant clones were screened for virus-specific inserts by using ^{32}P-labeled RAV-2 viral RNA. SR-RSV and RAV-2 are approximately 80 to 90% homologous, as determined by hybridization of SR-RSV 70S RNA to complementary DNA prepared from RAV-2 (14).

Cells infected with avian sarcoma viruses contain nondefective and td DNA molecules, which have sizes of approximately 6.2 and 5 Md, respectively (25). The td molecules are deletion mutants derived from nondefective molecules by loss of all or part of the viral *src* gene, which codes for cellular transformation. In the proviral DNA preparation used for cloning, td molecules represented approximately 50% of the covalently closed circular molecules (data not shown). In this report, recombinant clones containing only td molecules are discussed. The cloning vehicle Ch21A, in combination with the in vitro packaging procedure, selects for inserts with a maximum size of approximately 5.3 Md (8.2 kilobases) (11a). Because of its size limit, the use of Ch21A allowed us to obtain clones with inserts of only single td molecules (Fig. 1 and 2).

Restriction endonuclease analysis of λCh21A SR-RSV-B td (λSRBtd) recombinant clones. A total of 12 independently derived clones containing viral DNA sequences were isolated and plaque purified. DNA from each λSRBtd clone was digested with *SalI* and analyzed by agarose gel electrophoresis. Digestion with *SalI* generated the two arms of Ch21A

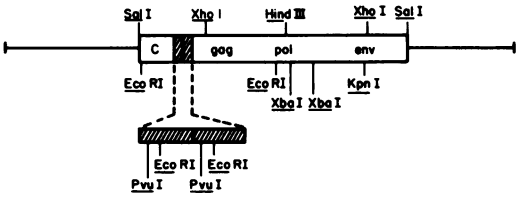

FIG. 1. *Restriction endonuclease map of λSRBtd recombinant clones. Cleavage sites of seven restriction endonucleases in the larger (5.2-Md) viral insert of λSRBtd clones are indicated. The insert is shown in orientation r with a direct repeat (shaded region). The relative order of the viral genes in the viral RNA genome is: (5') gag, pol, env, C (3'). A jagged line indicates the site of joining of the repeated sequences.*

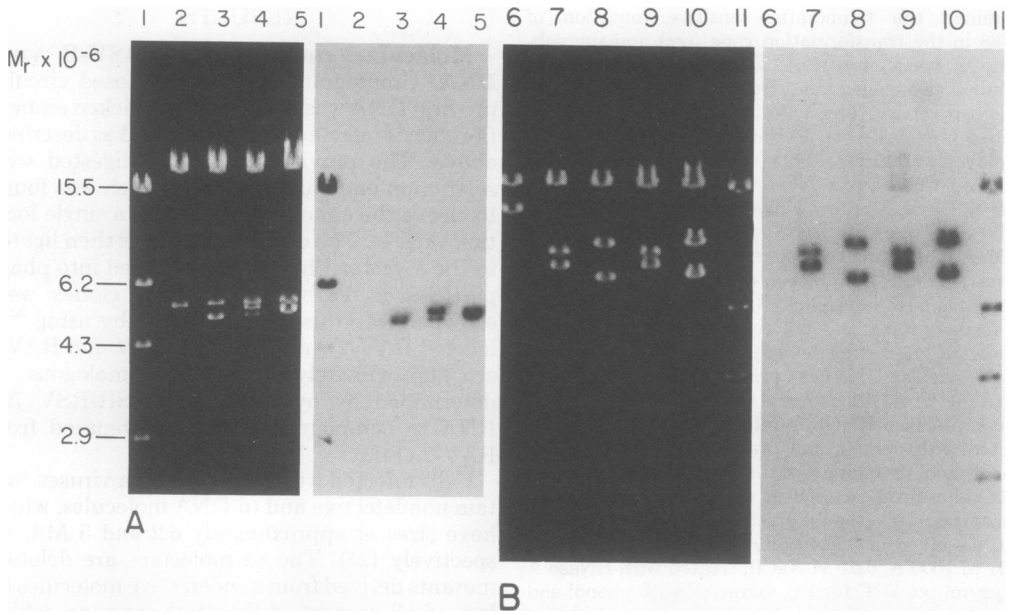

FIG. 2. *SalI and HindIII digestion patterns of λSRBtd clones. DNA from phage of λSRBtd clones was extracted, digested, analyzed by electrophoresis in 0.7% agarose gels, and transferred to nitrocellulose filters as previously described (20). Bands containing viral sequences were detected after hybridization with ^{32}P-labeled RAV-2 RNA (specific activity, ~2 × 10^7 to 4 × 10^7 Cerenkov cpm/μg), prepared, and utilized as described previously (20). The left panels are ethidium bromide visualizations of digested DNA from λSRBtd clones, and the right panels are autoradiograms of the gels after transfer and hybridization. (A) SalI digestion patterns. Lane 1, marker ^{14}C-labeled DNA cleaved with HindIII; lane 2, Ch21A; lane 3, λSRBtd-1; lane 4, λSRBtd-2; lane 5, λSRBtd-7. (B) HindIII digestion patterns. Lane 6, Ch21A; lane 7, λSRBtd-1 (orientation l); lane 8, λSRBtd-5 (orientation r); lane 9, λSRBtd-2 (l); lane 10, λSRBtd-7 (r); lane 11, ^{14}C-labeled λ DNA cleaved with HindIII.*

and the insert fragment(s) in each recombinant clone. Three types of clones could be distinguished by differences in their digestion patterns (Fig. 2A and Table 1). Digestion of type I clones (8 of 12) generated an insert fragment of 5.0 Md. Type II clones (3 of 12) contained two different sizes of insert DNA (5.0 and 5.2 Md). One type III clone (λSRBtd-7) contained an insert of 5.2 Md.

Several λSRBtd clones were further characterized by digestion with a number of restriction endonucleases. Figure 1 is a composite map of the larger viral insert in Ch21A, showing the cleavage sites for seven restriction enzymes. Although we observed differences in the number and location of some of the *Hin*dIII and *Xba*I sites, our map is in general agreement with the restriction maps reported for other avian sarcoma virus strains (16, 25).

With each of the clones, digestion with *Hin*dIII, which cleaves close to the center of the insert DNA (Fig. 1) and once within the λ vector left arm, produced two large fragments containing virus-specific sequences. The sizes of the two large fragments differed slightly in the various clones (Fig. 2B), as expected if the proviral DNA was inserted in either one of the two possible orientations. The orientations suggested by the *Hin*dIII digestion patterns were verified by analysis with other restriction endonucleases. Inserts incorporated such that the avian sarcoma virus genetic map is colinear with λ late genes (tran-

TABLE 1. *Recombinant DNA clones of λSRBtd*

Type	λSRBtd clone	Size of insert (Md)[a]	Orientation[b]
I	1	5.0	*l*
	3	5.0	*l*
	4	5.0	*l*
	5	5.0	*r*
	6	5.0	*r*
	8	5.0	*r*
	11	5.0	*r*
	12	5.0	*r*
II	2	5.0 and 5.2	*l*
	9	5.0 and 5.2	*r*
	10	5.0 and 5.2	*r*
III	7	5.2	*r*

[a] Determined by *SalI* digestion.
[b] Determined by *Hin*dIII digestion.

scribed to the right) are designated as being in the *r* orientation (Table 1). Inserts in the *r* orientation yielded virus-specific fragments of 8.65 and 8.0 Md when digested with *Hin*dIII, and inserts in the left (*l*) orientation produced fragments of 9.2 and 7.45 Md after digestion with *Hin*dIII (Fig. 2B). Results of *Hin*dIII digestions of individual clones also indicated that none of the clones contained multiple tandem inserts.

Smaller inserts in type II clones arise by deletion of larger inserts. Because of the size limitations of the Ch21A vector and the data from restriction analysis, it was clear that each recombinant phage chromosome contained only one proviral genome. However, two sizes of inserts were detected in DNA from type II clones. It seemed possible, therefore, that the 5.0-Md inserts in type II clones might arise by one of the two following mechanisms: (i) the original plaque contained two or more different recombinant phage particles; or (ii) deletion of part of the larger insert generated the smaller one during passage and amplification of the original plaque-purified clone.

To distinguish between these two possibilities, one type II clone (λSRBtd-2) was replated at low density, and phage from 16 independent plaques were isolated and amplified. *Sal*I restriction endonuclease analysis of DNA from the subclones revealed that eight were identical to the original parental clone and also contained phage with the same two sizes of inserts. The relative proportions of the larger and smaller inserts were variable among these clones, as shown by the four representative examples in Fig. 3, lanes 3 through 6. The other eight subclones contained only the smaller 5.0-Md insert, as shown by the four clones in Fig. 3, lanes 7 through 10. None of the 16 subclones contained only the 5.2-Md insert.

If the type II clones had contained a mixture of particles, then the subsequent plaque purification should have produced clones that contained either the larger or the smaller insert. Since no subclones contained only the larger insert and 50% of the subclones contained two sizes of inserts, it seemed most likely that the smaller insert arose from the larger one by deletion. Thus, we conclude that each type II clone probably originated from a recombinant molecule which contained a single unique insert of 5.2 Md. A subsequent specific deletion of approximately 0.2 Md during passage of each clone generated the mixture of molecules. The variation in the relative proportion of the two sizes of inserts in subclones of λSRBtd-2 (Fig. 3) indicated that the deletion event probably occurred at a different time for each subclone. Although the deletion of a 0.2-Md fragment occurred frequently, no additional deletions were detected in any of the λSRBtd clones or λSRBtd-2 subclones.

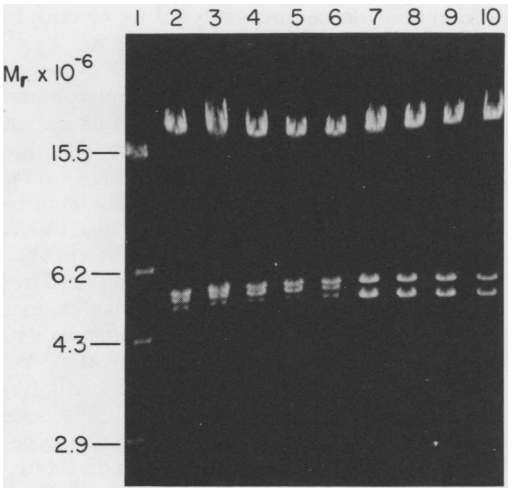

FIG. 3. *SalI digestion patterns of subclones of λSRBtd-2. The type II clone λSRBtd-2 was plaque purified on strain ED8767. Phage from independent plaques were isolated and amplified. DNAs from these subclones were analyzed by SalI digestion and electrophoresis in 0.7% agarose gels. This figure shows results with the parent clone λSRBtd-2 and eight representative subclones. Fragments were visualized after ethidium bromide staining. Lane 1, marker λ DNA cleaved with HindIII; lane 2, parent clone λSRBtd-2; lane 3, λSRBtd-2.3; lane 4, λSRBtd-2.6; lane 5, λSRBtd-2.7; lane 6, λSRBtd-2.11; lane 7, λSRBtd-2.4; lane 8, λSRBtd-2.14; lane 9, λSRBtd-2.15; lane 10, λSRBtd-2.16.*

All recombinant phage particles were originally grown and amplified in ED8767, a *recA*$^-$ host. To determine whether the Rec function could accelerate deletion formation, type I, type II, and type III clones were also grown in a second *recA*$^-$ mutant (derived from C600) and a *recA*$^+$ (DP50 *supF*) host. No significant differences were observed in the restriction patterns of the DNAs isolated in any of these preparations (data not shown).

Size heterogeneity of the direct repeat. The size difference between the larger and smaller inserts (0.2 Md) in type II clones corresponds to the size of the direct repeat present in vivo in the larger circular DNA species and absent in the smaller one (16, 25). The direct repeat contains a centrally located *Eco*RI site (Fig. 1) (16, 25). Therefore, digestion with this enzyme should produce a fragment equal in size to the repeat unit. To determine whether the putative deletion from the larger insert in type II clones involved loss of one copy of the repeated sequence, DNAs from type I, type II, and type III clones were digested with *Eco*RI restric-

tion endonuclease and analyzed by electrophoresis in 6% polyacrylamide gels (Fig. 4).

DNAs from type II and type III clones did produce an *Eco*RI fragment of approximately 300 bp (0.2 Md). DNAs from type I clones and from subclones of λSRBtd-2 (type II) which contained only the 5.0-Md insert did not contain this *Eco*RI fragment. Similar results were obtained when *Pvu*I was used, which also cleaves within the direct repeat (data not shown).

Analysis of three λRAV2 clones prepared from RAV-2 proviral DNA (Boone and Skalka, manuscript in preparation) yielded analogous data. Two type II clones, λRAV2-2 and λRAV2-12 (Fig. 4, lanes 9 and 10), produced small fragments when digested with *Eco*RI. No small *Eco*RI fragment was detected when a type I clone, λRAV2-1 (Fig. 4, lane 4), was digested.

Unexpectedly, the size of the small *Eco*RI fragment in λSRBtd and λRAV2 clones was heterogeneous. Digestion of six different type II and type III clones with *Eco*RI yielded fragments that ranged in size from 200 to 340 bp (Table 2). This size heterogeneity was probably not the result of instability during passage of the recombinant phage particles, because each of the eight subclones of λSRBtd-2 which contained the larger insert produced a 340-bp *Eco*RI fragment identical in size to that of the parent clone. Three discrete sizes of this fragment were observed in the six clones examined: 200, 250, and 340 bp. Therefore, the size heterogeneity of the small *Eco*RI fragment appears to be limited.

DISCUSSION

Using unintegrated proviral DNA of SR-RSV-B and the λ vector Ch21A, we generated, isolated, and characterized 12 recombinant clones of λSRBtd. Analysis of the 12 clones by restriction endonuclease digestion revealed three classes of recombinant clones, all derived from the td component of the viral DNA. DNA from type I clones contained a viral insert of 5.0 Md, type II clones contained phage DNA with two sizes of inserts (5.0 and 5.2 Md), and the type III clone contained phage DNA with only the larger insert (5.2 Md). A restriction endonuclease map of the SR-RSV-B td insert showed extensive homology with the maps previously described for proviral DNA of the Prague (subgroups A, B, and C) and B77 strains of RSV (16, 25).

It was of interest to determine the relationship between the 5.0- and 5.2-Md inserts, as well as the origin of the three classes of clones. For the analysis of the λSRBtd clones, two assumptions were made. First, each recombinant molecule was assumed to contain only a single insert at the time of conception and throughout passage. The possibility of tandem incorporation of two or more viral inserts was unlikely, because of the size limit imposed by the use of Ch21A and because of the results of restriction analysis.

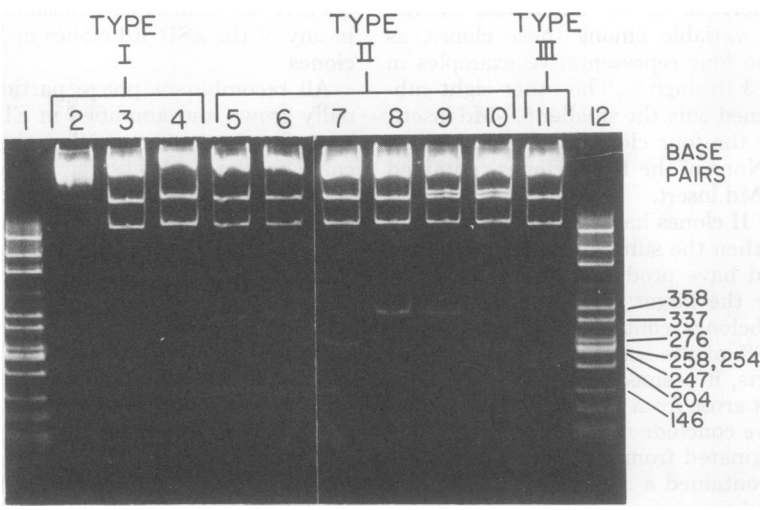

FIG. 4. *Size of repeat unit in λSRBtd and λRAV2 clones. After digestion with EcoRI, DNAs from recombinant clones were analyzed by electrophoresis in 6% polyacrylamide gels. Restriction fragments were visualized by ethidium bromide staining. All λSRBtd clones and λRAV2-1 were derived by cleavage of proviral DNA with SalI before insertion into Ch21A. λRAV2-2 and λRAV2-12 were derived by cleavage of proviral DNA with HindIII before insertion into Ch21A. Lanes 1 and 12, marker φX174 DNA cleaved with AluI; lane 2, λSRBtd-1; lane 3, λSRBtd-2.4; lane 4, λRAV2-1; lane 5, λSRBtd-2; lane 6, λSRBtd-2.2; lane 7, λSRBtd-9; lane 8, λSRBtd-10; lane 9, λRAV2-2; lane 10, λRAV2-12; lane 11, λSRBtd-7.*

TABLE 2. *Size heterogeneity of the repeated sequence*

Clone	Type	Size of small (<1,000-bp) *Eco*RI fragment (bp)
λSRBtd-1	I	NP[a]
λSRBtd-5	I	NP
λSRBtd-8	I	NP
λSRBtd-2	II	340
λSRBtd-9	II	250
λSRBtd-10	II	340
λSRBtd-7	III	200
λSRBtd-2.4[b]	I	NP
λSRBtd-2.10	I	NP
λSRBtd-2.2	II	340
λSRBtd-2.3	II	340
λSRBtd-2.6	II	340
λSRBtd-2.7	II	340
λSRBtd-2.8	II	340
λSRBtd-2.9	II	340
λSRBtd-2.11	II	340
λSRBtd-2.13	II	340
λRAV2-1	I	NP
λRAV2-2	II	340
λRAV2-12	II	250

[a] NP, Not present.

[b] Clones λSRBtd-2.2 to λSRBtd-2.13 are subclones of λSRBtd-2.

Second, we assumed that SR-RSV-B is homologous to the other RSV strains in its *Eco*RI digestion pattern. Therefore, the small (<1,000-bp) fragment that appeared after *Eco*RI digestion presumably contained sequences from the direct repeat, and the size of this fragment corresponded to the size of the repeat unit in each clone. Analogous results from *Pvu*I digestion supported this assumption.

From the data in Fig. 2 and the assumptions made, we conclude that each λSRBtd clone originated from a recombinant molecule that contained a single insert of either 5.0 or 5.2 Md. During plaque purification and amplification, the type II clones with 5.2-Md inserts lost a portion of the viral DNA and generated some molecules with a 5.0-Md insert. This deletion does not seem to occur more rapidly in *recA*$^+$ hosts. It is not known whether the φ80 general recombination function (RED), which is carried on the Ch21A vector, plays a critical role in this event.

The deletion apparently involved the loss of all or part of one of the redundant sequences. The DNA molecules with 5.2-Md inserts yielded a small fragment upon digestion with *Eco*RI. This fragment is the size of the terminal redundancy of linear proviral DNA (~300 bp or 0.2 Md). Type I clones with 5.0-Md inserts did not contain this *Eco*RI fragment. Although deletion of this small repeat apparently occurred at a high frequency (in 50% of subclones of λSRBtd-2), the 5.0-Md insert appeared to be stable. No other deletions were detected in either the λ or the viral portion of the hybrid molecule. The high frequency of deletion of the repeated sequence in the larger insert suggests that the smaller circular DNA found in vivo may be generated by the loss of this redundant sequence from the larger circular species. Whether this deletion is of biological significance in the viral replicative cycle is unclear. Preliminary data from DNA transfection experiments indicate that the virus-specific inserts from both type I and type II clones have biological activity. Studies are underway to determine the efficiency of transfection and integration of viral DNA inserts with and without the repeated sequence.

The nature of nucleotide sequences and the mechanisms involved in the integration of retroviruses are not understood. In procaryotic systems, integration of translocatable elements by illegitimate recombination is associated with direct or inverted repeated DNA sequences. Insertion elements and translocatable antibiotic resistance elements contain inverted complementary repeats at their termini (6). In addition, insertion and translocatable antibiotic resistance elements and the bacteriophage Mu generate small duplications of host sequences at the site of insertion (1, 7, 12, 19).

Recently, translocatable elements in eucaryotic organisms have been described. These elements, which have been isolated from yeast (8) and from *Drosophila* (23, 29), are strikingly similar to the retroviruses in several features. Like the retroviruses, these elements contain direct repeats of 0.25 to 0.5 kilobases at their termini, code for an abundant polyadenylic acid-containing RNA, and can integrate at many sites in the host genome. The presence of short repeated DNA sequences at the termini of procaryotic and eucaryotic translocatable elements suggests that the direct repeats found in the proviral DNA may be essential for integration and possibly translocation.

Deletions are also frequently associated with procaryotic translocatable elements. Translocatable antibiotic resistance and insertion elements and bacteriophage Mu generate *recA*-independent deletions of nucleotide sequences (6). One endpoint of the observed deletions occurs at the

repeated terminus of the translocatable element. However, cloned inserts of repeated nontranslocatable eucaryotic DNA sequences have also been observed to undergo deletion and other rearrangements when propagated in *recA*$^-$ procaryotic hosts (5, 11, 30). Thus, it is not clear that the instability of the direct repeat in the λSRBtd clones is related to its presumed role in recombination and integration into the host genome.

Analysis of the repeat fragment generated by *Eco*RI digestion of DNA from λSRBtd and λRAV2 clones revealed some heterogeneity in the size of the repeat unit. Of six clones examined, each had a redundancy of a characteristic size, ranging from 200 to 340 bp (Table 2). This size difference did not seem to be due to instability during passage, since subclones of a particular clone (λSRBtd-2) had a redundancy of the same size as the parent clone. Because we observed the same sizes of the redundant sequences in λRAV2 clones and in λSRBtd clones, it is unlikely that the size heterogeneity of the direct repeat is peculiar only to SR-RSV-B td molecules.

At least three discrete sizes of the repeat unit seem to be present in proviral DNA. The type III clone λSRBtd-7 had a larger insert that was apparently stable and did not generate molecules with 5.0-Md inserts. This clone also had the smallest *Eco*RI fragment (200 bp). It is possible that a repeat unit of minimal size may result in a more stable redundant sequence.

The size heterogeneity of the small *Eco*RI fragment is of interest. It implies that the sequences of the terminal repeat in the proviral DNA are variable. Thus, the termination event(s) which generates the end of the terminal redundancy and/or the recombination event which results in covalent closure to form circular molecules may not be site specific. Analysis of the small *Eco*RI fragment from several clones should allow us to determine whether any sequences are common at the recombination junction.

LITERATURE CITED

1. **Allet, B.** 1979. Mu insertion duplicates a 5 base pair sequence at the host inserted site. Cell **16**:123–129.
2. **Benton, W. D., and R. W. Davis.** 1977. Screening λgt recombinant clones by hybridization to single plaques *in situ*. Science **196**:180–182.
3. **Blattner, F. R., A. E. Blechl, K. Denniston-Thompson, H. Faber, J. E. Richards, J. L. Slightom, P. W. Tucker, and O. Smithies.** 1978. Cloning human fetal γ globin and mouse α-type globin DNA: preparation and screening of shotgun collections. Science **202**:1279–1284.
4. **Blattner, F. R., D. O. Kiefer, D. D. Moore, J. R. de Wet, and B. G. Williams.** 1978. Application for EK2 certification of a host-vector system for DNA cloning, supplement 9, Data on Charon 21A.
5. **Brutlag, D., K. Fry, T. Nelson, and P. Hung.** 1977. Synthesis of hybrid bacterial plasmids containing highly repeated satellite DNA. Cell **10**:509–519.
6. **Bukhari, A. I., J. A. Shapiro, and S. L. Adhya (ed.).** 1977. DNA insertion elements, plasmids, and episomes. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.
7. **Calos, M. P., L. Johnsrud, and J. H. Miller.** 1978. DNA sequences at the integration sites of the insertion element IS*1*. Cell **13**:411–418.
8. **Cameron, J. R., E. Y. Loh, and R. W. Davis.** 1979. Evidence for transposition of dispersed repetitive DNA families in yeast. Cell **16**:739–751.
9. **Campbell, A.** 1971. Genetic structure, p. 13–44. *In* A. D. Hershey (ed.), The bacteriophage lambda. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.
10. **Coffin, J. M.** 1979. Structure, replication, and recombination of retrovirus genomes: some unifying hypotheses. J. Gen. Virol. **42**:1–26.
11. **Cohen, A., D. Ram, H. O. Halvorson, and P. C. Wensink.** 1978. Deletions within *E. coli* plasmids carrying yeast rDNA. Gene **3**:135–147.

11a. **de Wet, J. B., D. L. Daniels, J. L. Schroeder, B. G. Williams, K. Denniston-Thompson, D. D. Moore, and F. R. Blattner.** 1980. Restriction maps for twenty-one Charon vector phages. J. Virol. **33**:401–410.

12. **Grindley, N. D. F.** 1978. IS*1* insertion generates duplication of a nine-base pair sequence at its target site. Cell **13**:419–426.
13. **Guntaka, R. V., B. W. J. Mahy, J. M. Bishop, and H. E. Varmus.** 1975. Ethidium bromide inhibits appearance of closed circular viral DNA and integration of virus-specific DNA in duck cells infected by avian sarcoma virus. Nature (London) **253**:507–511.
14. **Hayward, W. S., and H. Hanafusa.** 1973. Detection of avian tumor virus RNA in uninfected chicken embryo cells. J. Virol. **11**:157–167.
15. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. **26**:365–369.
16. **Hsu, T. W., J. L. Sabran, G. E. Mark, R. V. Guntaka, and J. M. Taylor.** 1978. Analysis of unintegrated avian RNA tumor virus double-stranded DNA intermediates. J. Virol. **28**:810–818.
17. **Hughes, S. H., P. R. Shank, D. H. Spector, H.-J. Kung, J. M. Bishop, H. E. Varmus, P. K. Vogt, and M. L. Breitman.** 1978. Proviruses of avian sarcoma virus are terminally redundant, co-extensive with unintegrated linear DNA and integrated at many sites. Cell **15**:1397–1410.
18. **Kawai, S., and H. Hanafusa.** 1972. Genetic recombination with avian tumor virus. Virology **49**:37–44.
19. **Kleckner, N.** 1979. DNA sequence analysis of Tn10 insertions: origin and role of 9 bp flanking repetitions during Tn10 translocation. Cell **16**:711–720.
20. **McClements, W., H. Hanafusa, S. Tilghman, and A. Skalka.** 1979. Structural studies on oncornavirus-related sequences in chicken genomic DNA: two-step analysis of *Eco*RI and *Bgl*I restriction digests and tentative mapping of a ubiquitous endogenous provirus. Proc. Natl. Acad. Sci. U.S.A. **76**:2165–2169.
21. **McClements, W., and A. M. Skalka.** 1977. Analysis of chicken ribosomal RNA genes and construction of lambda hybrids containing gene fragments. Science **196**:195–197.
22. **Murray, N. E., W. J. Brammer, and K. Murray.** 1977. Lambdoid phages that simplify the recovery of *in vitro* recombinants. Mol. Gen. Genet. **150**:53–61.
23. **Potter, S. S., W. J. Brorein, Jr., P. Dunsmuir, and G. M. Rubin.** 1979. Transposition of elements of the *412*, *copia* and *297* dispersed repeated gene families in Drosophila. Cell **17**:415–427.
24. **Sabran, J. L., T. W. Hsu, C. Yeater, A. Kaji, W. S.**

Mason, and J. M. Taylor. 1979. Analysis of integrated avian RNA tumor virus DNA in transformed chicken, duck, and quail fibroblasts. J. Virol. **29**:170–178.

25. **Shank, P. R., S. H. Hughes, H.-J. Kung, J. E. Majors, N. Quintrell, R. V. Guntaka, J. M. Bishop, and H. E. Varmus.** 1978. Mapping unintegrated avian sarcoma virus DNA: termini of linear DNA bear 300 nucleotides present once or twice in two species of circular DNA. Cell **15**:1383–1395.
26. **Shank, P. R., and H. E. Varmus.** 1978. Virus-specific DNA in the cytoplasm of avian sarcoma virus-infected cells is a precursor to covalently closed circular viral DNA in the nucleus. J. Virol. **25**:104–114.
27. **Sogo, J. M., M. Greenstein, and A. Skalka.** 1976. The circle mode of replication of bacteriophage lambda: the role of covalently closed templates and the formation of mixed catenated dimers. J. Mol. Biol. **103**:537–562.
28. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
29. **Strobel, E., P. Dunsmuir, and G. M. Rubin.** 1979. Polymorphisms in the chromosomal locations of elements of the *412*, *copia*, and *297* dispersed repeated gene families in Drosophila. Cell **17**:429–439.
30. **Suzuki, Y., and Y. Ohshima.** 1977. Isolation and characterization of the silk fibroin gene with its flanking sequences. Cold Spring Harbor Symp. Quant. Biol. **42**: 947–957.