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Molecular Cloning of Avian Sarcoma Virus Closed Circular DNA: Structural and Biological Characterization of Three Recombinant Clones

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Unintegrated, circular viral DNA, isolated from Prague A avian sarcoma virus (PrA-ASV)-infected quail cells (QT6), was cloned in the lambda vector λgtWES·λB. Three independent lambda-ASV recombinants were identified, and each contained a complete copy of the PrA-ASV genome. The arrangement of the ASV sequences within the recombinants was determined by restriction enzyme analysis and hybridization with labeled ASV-specific complementary DNA. One of the recombinants (λRPA101) resulted from cloning at the *Eco*RI site located within the terminally repeated sequence and therefore was virtually co-linear with PrA-ASV virion RNA. The other two recombinants (λRPA102 and 103) resulted from cloning at the *Eco*RI site located within the viral *env* gene. By restriction enzyme analysis and by measurement of R-loops formed between λRPA101 and PrA-ASV virion 35S RNA, the viral genome was estimated to be 9,100 bases in length. Genome length viral DNA purified from clones λRPA102 and 103 was biologically active. Transfection of chicken embryo cells with viral DNA, in the form of either circles or linear dimers, produced foci of transformed cells within 8 to 10 days. Linear DNA was much less efficient at inducing transformation. Viral DNA from the clone λRPA101 was unable to cause transformation; the basis for this defect is unknown.

Retroviruses represent a unique class of genetic elements which are capable of causing neoplastic transformation of specific tissues in their natural host (2, 13, 29). Retrovirus replication requires the synthesis of a double-stranded viral DNA intermediate, which becomes covalently integrated into the DNA of the host cell. The analysis of avian cells infected with avian sarcoma viruses (ASVs) has shown that replication of viral DNA commences in the cytoplasm of the cell within 1 h after infection and proceeds first via the synthesis of a linear duplex DNA intermediate followed by the formation of a covalently closed circular form, found exclusively in the nucleus (22). Linear viral DNA isolated from the cytoplasm of infected cells contains terminally repeated sequences of about 300 base pairs in length and is derived by contiguous transcription of sequences present at the 5′ end (100 nucleotides) and the 3′ end (200 nucleotides) of the RNA genome (15, 16, 21). Two classes of circular viral DNA have been observed in the nucleus of infected cells. These DNAs differ only in the number of 300-base-pair repeated sequences; smaller circles contain a single copy of the terminal repeat, and larger circles contain two copies of the terminal repeat (15, 21). The integration of viral DNA into the host DNA appears to take place at a unique position within the terminally repeated sequences and results in an integrated viral DNA with a copy of the terminally repeated sequence on either end (4, 9, 16, 20).

The analysis of the structure and the biological activity of viral DNA intermediates has been greatly hampered by the limited amounts of viral DNA that can be obtained by conventional isolation techniques. The development of recombinant DNA technology provides an experimental approach by which individual viral DNA intermediates can be isolated, propagated, and characterized. We have employed molecular cloning techniques to isolate unintegrated viral DNA from cells infected with ASV. Covalently closed circular (form I) viral DNA, purified from quail cells 18 h after infection with Prague A strain of ASV (PrA-ASV) were cloned in the lambda phage vector λgtWES·λB. In this paper we describe the structure and biological activity of three recombinant ASV DNA clones, each containing a complete copy of the ASV genome.

MATERIALS AND METHODS

Cells and virus. Prague A strain of ASV was propagated in chicken embryo cells as described pre-

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viously (9). The lambda vector system was obtained from L. Enquist, National Cancer Institute, Bethesda, Md. The characteristics of this host vector system have been previously described (18, 24).

Restriction endonucleases. Restriction endonucleases were purchased from Bethesda Research Laboratories and New England Biolabs. Digestion of DNA was performed under conditions recommended by the supplier.

Agarose gel electrophoresis, DNA transfer, and hybridization conditions. Restriction fragments were resolved by agarose gel electrophoresis, and, where designated, the DNA was transferred to nitrocellulose filters and hybridized with ^{32}P-ASV complementary DNA (cDNA) as described previously (9, 23).

Preparation of unintegrated ASV DNA. The quail cell line, QT6 (19), was infected with PrA-ASV at a multiplicity of 0.5 to 1.0 focus-forming units per cell, and infected cells were harvested after 18 to 20 h. Low-molecular-weight DNA was purified by the method of Hirt (14). Covalently closed circular DNA (form I DNA) was resolved from linear DNA by centrifugation in cesium chloride propidium diiodide gradients (11). The distribution of ASV-specific DNA within the gradient was determined by subjecting an aliquot of each gradient fraction to agarose gel electrophoresis, transferring the DNA to nitrocellulose filters, and hybridizing with ^{32}P-ASV cDNA. The DNA in fractions containing form I or linear DNA was pooled and ethanol precipitated.

Preparation of phage DNA and purification of vector arms. *Escherichia coli* DP50 *supF* or *E. coli* LE-392 were grown in TB broth and infected with phage as described by Sternberg et. al (24). Phage were purified by polyethylene precipitation and banded on CsCl density gradients (24). The phage band was collected and dialyzed against 100 volumes of 10 mM Tris-hydrochloride (pH 7.6)–10 mM $MgSO_4$. DNA was prepared from the phage by digestion with pronase B (100 μg/ml) in the presence of 0.5% sodium dodecyl sulfate, followed by phenol extraction and ethanol precipitation. For the preparation of the *Eco*RI arms of λgtWES·λB, 20 μg of λgtWES·λB DNA was digested to completion with 30 U of *Eco*RI. The DNA was electrophoresed on a 0.7% agarose gel to resolve the left (21.7-kilobase [kb]) and right (14.2-kb) λgtWES arms from each other and from the 4.85-kb internal λB fragment. The individual λ arms were recovered by electroelution. A second digestion with *Eco*RI was performed to ensure complete cleavage of λ arms.

Construction and isolation of recombinant phage containing viral DNA inserts. DNA, recovered from CsCl gradient fractions containing ASV viral form I DNA, was subjected to partial *Eco*RI digestion under conditions which yielded approximately 1 cleavage per molecule (incubation of 1 U of *Eco*RI per μg of DNA at 10°C for 10 min). The partially digested DNA (1 to 5 μg) was ligated to the purified *Eco*RI λgtWES arms (5 μg) at 9°C overnight using T_4 DNA ligase (Biolabs) (26). DNA was packaged in vitro into phage particles by the procedure of Enquist and Sternberg (7). Phage suspensions were titrated on *E. coli* strain DP50 *supF*. Approximately 10^3 phage particles were mixed with DP50 *supF* and plated in a 15-cm dish, and the resultant phage plaques were transferred to nitrocellulose filters as described by Benton and Davis (1). Phage plaques containing viral DNA were identified by hybridization with ^{32}P-ASV cDNA, and virus-specific recombinant phage were plaque purified three times. Single plaques were then amplified in *E. coli* DP50 *supF*. Recombinant phage were grown as described above.

Electron microscopy. The formation of R-loops (25) between the DNA from λRPA101 and 36S PrA virion RNA was carried out in a hybridization mix containing 50 μg of DNA per ml, 50 μg of RNA per ml, 70% formamide, 0.1 M PIPES, [piperazine-*N*-*N*'-bis(2-ethanesulfonic acid), pH 8.0], 0.5 M NaCl, and 10 mM EDTA incubated at 52°C for 16 h. The mixture was diluted 50-fold into 50% formamide–0.1 M Tris-hydrochloride (pH 7.8)–10 mM EDTA. Cytochrome *c* was added to a concentration of 75 μg/ml, and the samples were spread onto a hypophase of distilled water. The hybrid molecules were picked up on Parlodion-coated grids, stained with uranyl acetate, and shadowed with platinum-palladium. Grids were examined in a Jeol 100 cx microscope at 60 kV. R-loop molecules were photographed at magnifications of 5,000 to 10,000 and measured with a Numonics graphic calculator. The lengths of the R-loops were calculated relative to the left and right arms of λgtWES DNA and pBR322 DNA.

Transfection of chicken cells with viral DNA. DNA from λRPA101, 102, and 103 was digested with *Eco*RI (1 U/μg of DNA) in 100 mM Tris-hydrochloride (pH 7.4)–50 mM NaCl–4 mM $MgCl_2$ for 60 min at 37°C to obtain partial restriction products. The partial *Eco*RI-digested DNA was subjected to electrophoresis in a 0.75% agarose gel, and the 6.0-megadalton (Md) virus-specific fragment was recovered by electroelution. The purified viral DNA was adjusted to a final DNA concentration of 20 μg/ml and ligated overnight at 10°C with T_4 DNA ligase. Ligated DNA was again subjected to agarose gel electrophoresis in the presence of 1 μg of ethidium bromide per ml as described above. DNA was recovered from the bands containing linear, form I, and form II/dimeric DNA by electroelution as described above. Transfection of chicken cells with viral DNA was carried out by a modification (6) of the calcium precipitation method (10). Viral DNA was suspended in 1.5 ml of HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid) buffered saline containing 40 μg of calf thymus DNA per ml. The DNA solutions were adjusted to 0.125 M $CaCl_2$ and incubated for 30 min at room temperature, and 0.5 ml was added to triplicate cultures (60 mm) of chicken embryo cells. After 30 min at room temperature, 5 ml of Dulbecco-modified Eagle medium (Flow Laboratories, Inc., Rockville, Md.) supplemented with 10% tryptose phosphate (Difco Laboratories, Detroit, Mich.), 4% calf serum, and 2% chicken serum (Flow Laboratories) and containing 2 μg of polybrene per ml (Aldrich Chemical Co., Milwaukee, Wis.) was added, and the plates were incubated at 37°C. After 8 to 15 h, the media were removed, fresh media without polybrene were added, and the cultures were incubated. Cultures

were transferred every 3 days and monitored for transformation at 7, 14, and 21 days.

Biological and physical containment. All experiments were carried out under P2-EK2 containment as specified by the National Institutes of Health guidelines.

RESULTS

Isolation and characterization of recombinant DNA clones. The objective of our cloning experiments was to obtain recombinant DNA clones containing a complete copy of the ASV genome. The experimental approach used was to isolate unintegrated, covalently closed circular viral DNA from QT6 cells infected with PrA-ASV, cleave this DNA at a single site with *Eco*RI to generate linear viral DNA, and clone this DNA at the *Eco*RI site in the lambda cloning vector λgtWES·λB. Since circular viral DNA contains three *Eco*RI sites (four sites in viral DNA containing two copies of the terminal repeat, Fig. 2A), we employed partial *Eco*RI digestion to generate genome length (6.0 to 6.2-Md) linear DNA. Partial *Eco*RI digestion of circular DNA preparations resulted in 60 to 80% of the viral DNA being cleaved to a 6.0-Md linear DNA species, as determined by agarose gel electrophoresis, Southern blotting, and hybridization with a ^{32}P-ASV cDNA (data not shown). This DNA was then ligated with purified *Eco*RI arms of λgtWES DNA and packaged in vitro (7), and the resulting phage was plaqued on the *E. coli* host DP50 *supF*. Recombinant phage plaques containing virus-specific DNA inserts were identified by hybridization with ^{32}P-ASV cDNA (1). Three recombinant DNA phages identified in this manner were picked and plaque purified, and the phage DNA was isolated. The recombinant phage were designated λRPA101, -102, and -103, respectively.

Covalently closed circular viral DNA contains either three or four *Eco*RI sites; therefore partial *Eco*RI digestion produces genome length (6.0- to 6.2-Md) linear DNA which is circularly permuted. For this reason, it was necessary not only to establish that the three recombinant DNA clones contained a complete copy of the viral DNA genome, but also to determine the arrangement of viral DNA sequences within each recombinant. This was accomplished by comparing the known restriction enzyme patterns of the λ vector and viral DNA isolated from PrA-ASV-infected QT6 cells with the restriction enzyme patterns obtained for the individual recombinant clones. Covalently closed circular DNA from PrA-ASV-infected QT6 cells and λRPA101 DNA were digested with the enzymes *Eco*RI, *Bam*HI, *Hin*dIII, and *Sac*I. The DNA was fractionated by agarose gel electrophoresis and transferred to nitrocellulose filters, and virus-specific DNA fragments were identified by annealing with ^{32}P-labeled ASV cDNA (Fig. 1). A comparison of the viral DNA fragments generated by digestion of the DNAs with *Eco*RI (Fig. 1, lanes 3 and 4) revealed that all of the viral fragments present in circular PrA DNA were also present in λRPA101 DNA. We did not observe the small 300-base-pair *Eco*RI fragment which would have been generated from a viral DNA insert containing more than a single copy of the terminally repeated sequence (data not shown). These data indicated that λRPA101 contained a complete copy of the viral genome. To determine the linear arrangement of the *Eco*RI fragments within the cloned viral DNA insert (and hence the relative orientation of viral DNA sequences within the vector), recombinant DNA and circular viral DNA were digested with *Bam*HI and *Sac*I. *Bam*HI digestion yielded two virus-specific fragments common to both insert and circular viral DNA (Fig. 1, lanes 5 and 6). Previous analysis has shown that the 1.1-Md *Bam*HI fragment spans the junction of the 1.5- and 2.5-Md *Eco*RI fragments; therefore, these two *Eco*RI fragments must be contiguous in the cloned insert. We have established that *Sac*I cleavage of integrated proviral DNA yielded a 4.2-Md internal fragment containing sequences from the 1.5-, 2.5-, and 2.0-Md *Eco*RI fragments (4). Since digestion of both λRPA101 DNA and circular PrA DNA yielded an identical 4.2-Md *Sac*I fragment (Fig. 1, lanes 9 and 10), we concluded that the *Eco*RI fragments of λRPA101 DNA must be arranged in the order 1.5-2.5-2.0 (Fig. 2B). The orientation of the viral insert within the λ vector was determined relative to the known positions of the *Bam*HI and *Hin*dIII cleavage sites in the left and right arms of λgtWES. Digestion of λRPA101 DNA with these enzymes gave virus-specific fragments which were not present in the restriction pattern of viral DNA circles (marked with an asterisk in Fig. 1) and therefore represented insert-vector DNA junctions. The size of the fragments enabled us to orient the viral insert within the λ vector.

Figure 2 summarizes the above data as well as data obtained from a similar analysis of λRPA102 and -103. The viral insert of λRPA101 is co-linear with the viral RNA genome as a result of cleavage of circular viral DNA at the *Eco*RI site located within the terminally repeated sequence (Fig. 2A). This site corresponds to a sequence in the viral RNA genome 54 nucleotides from the polyadenylic acid addition site (30; Schwartz and Gilbert, personal com-

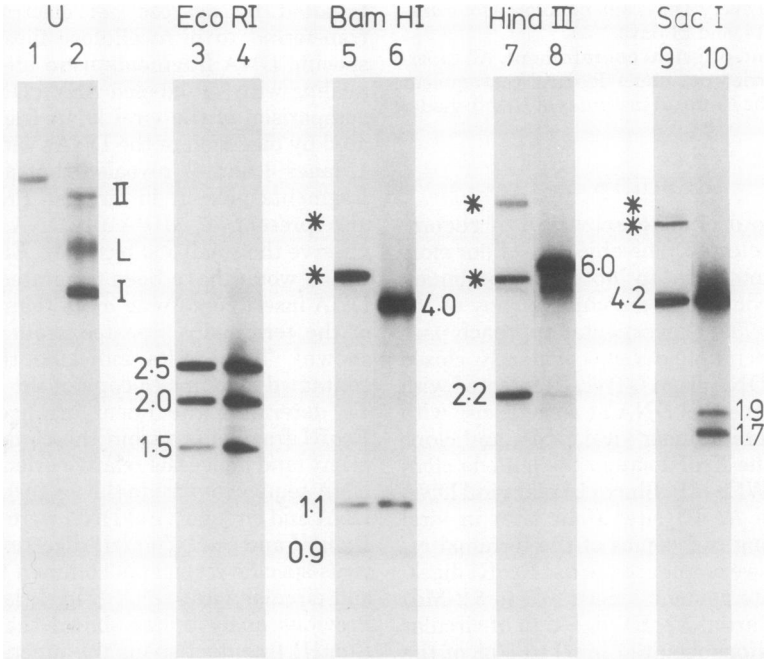


FIG. 1. *Comparison of λRPA101 DNA and viral DNA isolated from PrA-infected QT6 cells. DNA was digested by restriction enzymes (Eco*RI, *Hin*dIII, *BamHI, and Sac*I), *and the DNA fragments were resolved by agarose gel electrophoresis and transferred to nitrocellulose filters as described in the text. The virus-specific restriction fragments were detected by hybridization with* ^{32}*P-ASV cDNA (10 pmol/ml; specific activity, 5 × 10*^{8} *to 8 × 10*^{8} *dpm/μg). Restriction fragments containing only ASV-specific sequences are designated in Md; fragments containing both ASV and λ sequences are designated by an asterisk. Lane 1, uncut λRPA101 DNA. Lane 2, uncut DNA isolated from PrA-infected QT6 cells; L, linear DNA (6.0 Md), I, form I DNA (3.5 Md), and II, form II DNA (10 to 12 Md). Lanes 3, 5, 7, and 9, λRPA101 DNA; lanes 4, 6, 8, and 10, PrA-ASV DNA from infected QT6 cells. Molecular masses were determined by parallel electrophoresis of HindIII digested λ DNA.*

munication). Therefore, sequences corresponding to the 3′ end of the RNA genome are present at the 5′ end of the viral insert. The viral inserts of λRPA102 and -103 have resulted from the cleavage of circular viral DNA at the *Eco*RI site located in the *env* gene (Fig. 2A).

Digestion of λRPA102 DNA with either *Eco*RI or *Pvu*I yielded a 340-base-pair fragment, consistent with this clone containing two copies of the terminally repeated sequences (data not shown). A similar analysis of λRPA101 and -103 DNA did not yield a 340-base-pair fragment, and therefore these clones contain a single copy of the terminally repeated sequence (Fig. 2B).

Figure 3 summarizes the restriction enzyme map of the viral DNA insert of λRPA101 with 14 different restriction enzymes. With the exception of *Hin*dIII, all of the restriction enzyme sites present in the viral DNA insert of λRPA101 were also present in the viral DNA inserts of λRPA102 and λRPA103.

Clone λRPA101 contained an additional *Hin*dIII site, absent from clones λRPA102 and -103, mapping 6.1 kb from the 5′ end of the viral genome (Fig. 3). Digestion of λRPA101 DNA with *Hin*dIII yielded a 2.2-Md internal virus-specific DNA fragment (Fig. 2, lane 7). *Hin*dIII digestion of circular PrA-ASV DNA yielded a major virus-specific DNA fragment of 6.0-Md and a minor fragment of 2.2-Md (Fig. 2, lane 8). This observation suggests that the viral insert of λRPA101 DNA was derived from a viral DNA species present in the original circular viral DNA preparation. λRPA102 DNA contains a *Hin*dIII site within the terminally repeated sequence (Fig. 2). Double digestion with either *Hin*dIII and *Eco*RI or *Hin*dIII and *Pvu*I showed that this *Hin*dIII site was close to or at the junction of the two terminally repeated sequences (data not shown).

To confirm that the cloned viral DNA sequences represented a complete copy of the viral RNA genone without detectable deletions or rearrangements, DNA from λRPA101 was annealed with 36S PrA virion RNA, and the resultant R-loop structures were visualized in the electron microscope. Figure 4 shows a representative molecule in which the left vector arm (L) and the right vector arm (R) can be distinguished along with a R-loop of about 9,100 base

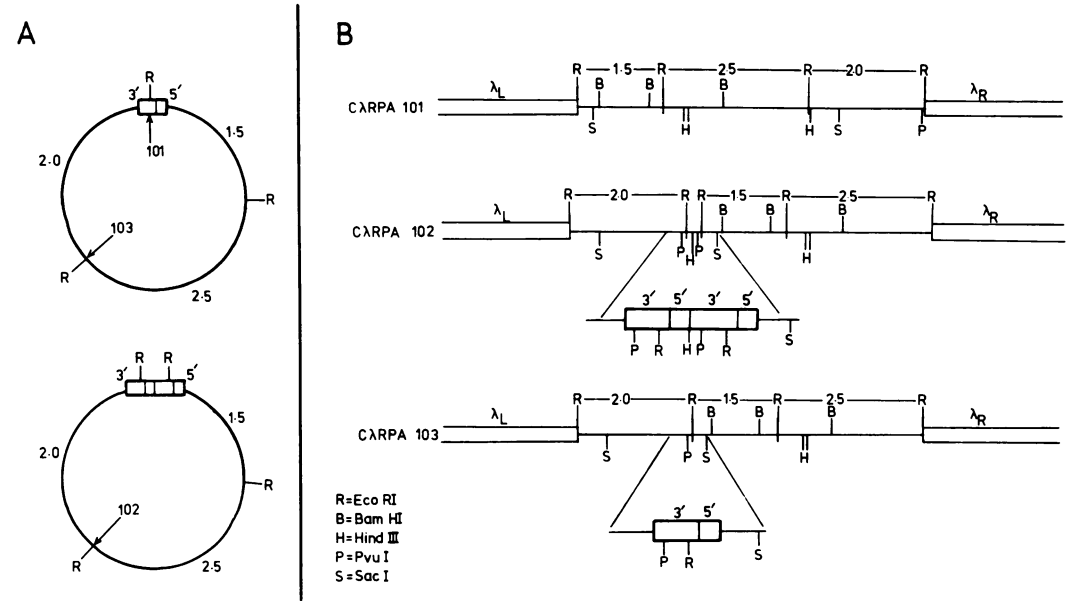


FIG. 2. *Comparison of unintegrated ASV DNA circles and the ASV recombinant clones. (A) Map of circular ASV DNA: —□— designates the terminally repeated sequences; R designates the sites of EcoRI cleavage. The individual restriction fragments are designated by their size, in Md. The arrows indicate the EcoRI site cleaved to generate the individual recombinants. (B) Map of the ASV recombinants: λ_L and λ_R, left and right λ vector arms, respectively. The viral insert is designated by a single, solid line. The terminally repeated sequences have been presented in expanded form. The ASV-specific EcoRI restriction fragments are designated by their molecular masses in Md.*

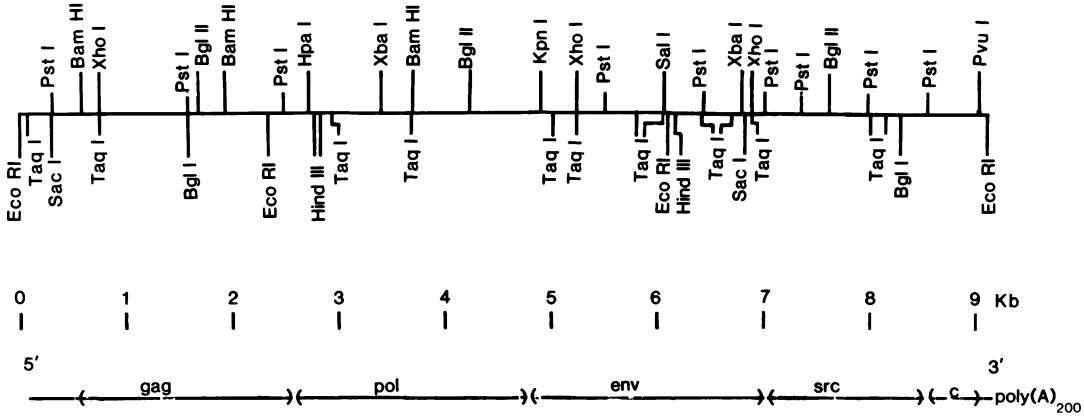
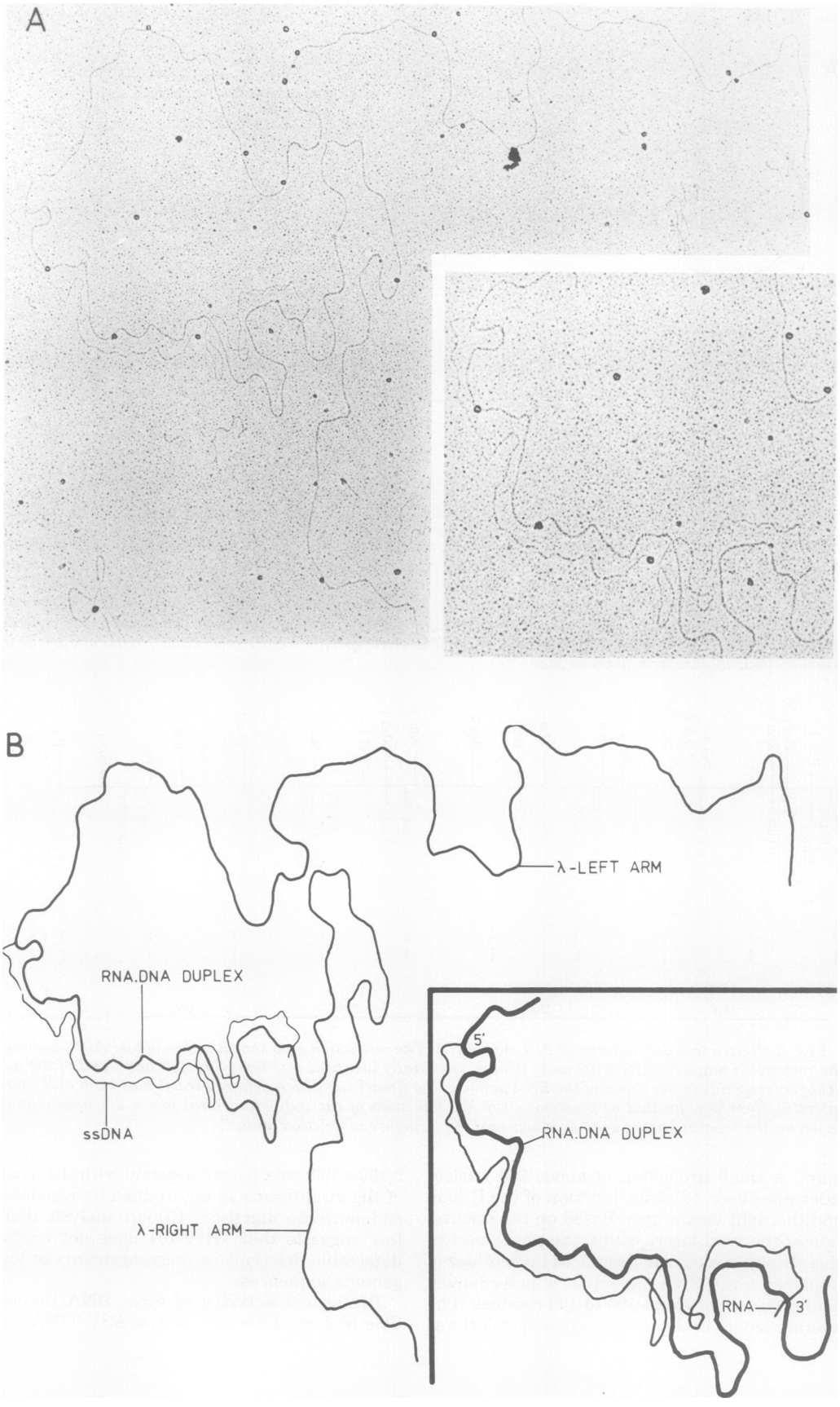


FIG. 3. *Restriction enzyme map of PrA-ASV DNA. The restriction map shown is that of λRPA101, although the restriction maps for λRPA102 and -103 are essentially identical (see text). The left-hand end of the map (0 kb) corresponds to the 5' end of the RNA genome; the EcoRI site which defines the left-hand end of the viral insert is, therefore, located at position −0.06 kb. The sizes of the individual viral genes are approximate, based on the sizes of known gene products and the structure of deletion mutants.*

pairs. A small projection of about 200 nucleotides was observed at the junction of the R-loop and the right vector arm. Based on the orientation of the viral insert within the phage vector, this structure likely represents 54 nucleotides of unpaired viral RNA linked to a polyadenylic acid sequence of about 100 to 150 residues. The average length of the R-loops (20 molecules) was 9,100 ± 630 base pairs consistent with the length of the viral inserts as determined by restriction endonuclease digestion. R-loop analysis therefore suggests that λRPA101 does not contain detectable deletions or rearrangements of viral genome sequences.

Biological activity of viral DNA inserts. The biological activity of the ASV DNA con-


A
B
λ-LEFT ARM
RNA.DNA DUPLEX
ssDNA
λ-RIGHT ARM
5'
RNA.DNA DUPLEX
RNA
3'

tained within clones λRPA101, -102, and -103 was tested in the following manner. Recombinant phage DNA was subjected to partial *Eco*RI digestion and fractionated by agarose gel electrophoresis, and the genome length (6.0-Md) viral DNA was recovered by electroelution. Genome length DNA obtained from each clone was ligated with T4 DNA ligase, and the products were resolved by agarose gel electrophoresis (Fig. 5). DNA, having the mobility of form I DNA (3.5 Md), linear DNA (6.0 Md), and dimeric or form II DNA (12 to 14 Md) were recovered by electroelution and used to transfect secondary cultures of chicken embryo cells (6, 10). Table 1 summarizes the results of transfection experiments carried out with viral DNA isolated from clones λRPA101, -102, and -103 as well as linear ASV DNA purified from PrA-infected QT6 cells. In all experiments carried out to date, viral DNA recovered from λRPA101 (either form I, linear DNA, or dimeric/form II DNA) was unable to induce transformation of chicken cells. In contrast, form I viral DNA of λRPA102 and λRPA103 readily transformed chicken cells, with foci of transformed cells appearing in 8 to 10 days. Dimeric and form II DNAs from both λRPA102 and -103 were also able to transform chicken cells, but no transformation was observed with linear DNA of either clone 102 or 103. These data demonstrate that the viral DNAs contained in clones λRPA102 and -103 are capable of inducing cellular transformation in chicken cells, whereas the viral DNA of λRPA101 is defective in this ability to induce cellular transformation. The exact reason(s) for the defectiveness of λRPA101 is presently not known.

TABLE 1. *Transfection with viral DNA from λRPA101, −102, and −103 and linear ASV DNA from PrA-infected QT6 cells*

Source of viral DNA[a]	No. of cultures transformed by viral DNA[b]	
	Expt 1	Expt 2
λRPA101		
Linear	0/3	0/3
Form I	0/3	0/3
Form II/dimeric	0/3	0/3
λRPA102		
Linear	0/3	0/3
Form I	3/3	3/3
Form II/dimeric	3/3	3/3
λRPA103		
Linear	0/3	0/3
Form I	3/3	3/3
Form II/dimeric	3/3	3/3
QT6 linear viral[c]	3/3	3/3

[a] Viral DNA (form I, linear, and form II/dimeric DNA) was purified as described in the text. The fraction designated form II/dimeric DNA contained both nicked circular DNA and dimers of linear DNA.

[b] Chicken cell cultures (about 5×10^5 cells per 60-mm dish) were transfected with approximately 50 ng of viral DNA as described in the text. Results are expressed as the number of cultures transformed (cultures containing more than five foci of transformed cells) divided by the number of cultures originally transfected with viral DNA. All cultures were maintained for at least 21 days as described in the text.

[c] Linear virus-specific DNA was purified from QT6 cells infected with PrA-ASV as described in the text. Since this fraction was contaminated with fragmented cellular DNA, the optimal amount of DNA required for focus formation was determined by titration on chicken embryo cells.

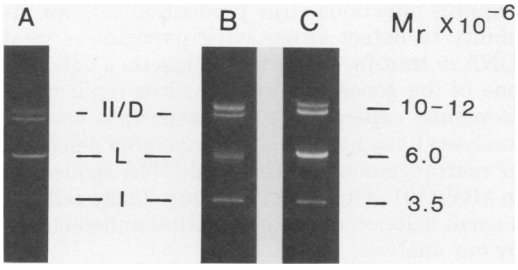


FIG. 5. *Agarose gel electrophoresis of the ligation products of the viral DNA inserts of λRPA101, -102, and -103. The viral DNA inserts (0.5 μg) of λRPA101, -102, and -103 were incubated with 0.01 U of T4 DNA ligase as described in the text and subjected to electrophoresis on a 0.85% agarose gel containing 1 μg of ethidium bromide per ml. Lanes A, B, and C contain ligated DNA of λRPA101, λRPA102, and λRPA103, respectively. I, form I DNA; L, linear DNA; II/D, form II and dimeric DNA. Molecular masses were determined as in Fig. 1.*

DISCUSSION

This paper describes the structure and biological activity of three lambda-ASV recombinant DNA clones, each of which contains a complete copy of the PrA-ASV genome. These recombinants each have a unique arrangement of the ASV DNA within the λ cloning vector. Clone λRPA101 results from cleavage of circular viral DNA at the *Eco*RI site within the terminally

FIG. 4. *R-loop structures of λRPA101 and PrA-ASV 36S virion RNA. (A) Electron micrograph of an R-loop formed between PrA-ASV 36S virion RNA and λRPA101 DNA (magnification, 10,000×). The inset shows the R-loop region of the molecule at higher magnification (16,000×). (B) Schematic drawing of the hybrid molecule shown in A. The heavy line represents double-stranded nucleic acid and the thin line represents single-stranded nucleic acid.*

repeated sequence. The other two clones (λRPA102 and 103) both result from cleavage at an *Eco*RI site within the *env* gene; λRPA102 contains two copies of the terminally repeated sequence, whereas λRPA103 contains only one copy of this sequence. The size of the viral inserts as determined by restriction enzyme analysis and R-loop analysis (about 9,100 base pairs) clearly shows that these recombinants contain a complete copy of the ASV genome.

The viral DNA sequences present in the three recombinant DNA clones appear to be very similar. Each of the viral inserts contains identical cleavage sites for the various restriction enzymes tested to date (see Fig. 3). *Hin*dIII is the only enzyme for which we have observed differences. Clone λRPA101 has a *Hin*dIII site 6.1 kb from the 5′ end of the genome, which is not present in λRPA102 and -103. The *Hin*dIII digestion pattern of circular DNA isolated from PrA-infected QT6 cells reveals a minor population of viral DNA molecules having *Hin*dIII sites identical to the viral insert of λRPA101 (e.g., a 2.2-Md fragment). Shank et al. (21) have reported that linear viral DNA isolated from cells infected with either PrA- or PrC-ASV contain *Hin*dIII sites mapping at similar positions.

Clone λRPA102 contains a unique *Hin*dIII site which maps very close to or at the junction of the terminally repeated sequences. It is unlikely that this site represents a *Hin*dIII recognition sequence present at either the 5′ or 3′ end of the viral RNA, since current models for retrovirus replication would predict that such a sequence in the viral RNA would result in a *Hin*dIII site which would be present in both copies of the terminal repeat (8). However, the *Hin*dIII site observed in λRPA102 could have been generated during propagation of the recombinant DNA in *E. coli*. Terminally repeated sequences of RNA tumor viruses have been reported to undergo recombination and rearrangement during propagation in lambda phage vectors (3, 12, 17, 27). Therefore, it is possible that such an alteration generated the *Hin*dIII site in the terminal repeat of λRPA102. Alternatively, the *Hin*dIII site may result from the aberrant recombination of the two ends of a linear viral DNA molecule during the formation of viral circles in vivo. The proximity of the *Hin*dIII site to the junction of the terminally repeated sequences is consistent with this latter possibility.

In our experiments, we have observed little, if any, instability of the terminally repeated sequences of λRPA102 upon propagation of this recombinant phage. This is in contrast to Ju et al. (17), who have cloned transformation defective Schmidt-Ruppin ASV DNA into the lambda vector Charon 21A and found that recombinants containing two copies of the terminal repeat were unstable. These recombinants were always found in a mixed population of molecules containing either one and two copies of the terminal repeat.

Viral DNA from λRPA102 and -103 is biologically active. Transfection of chicken cells with either form I viral DNA or linear dimers (plus unresolved form II DNA) produced foci of transformed cells within 8 to 10 days. Viral DNA preparations from λRPA102 and -103 appeared to transform cells with equal efficiency, approximately 10^3 to 5×10^3 focus-forming units per μg of viral insert (T. Gilmer, unpublished data). Linear DNA, on the other hand, proved to be considerably less efficient (less than 50 focus-forming units per μg) at inducing transformation. We attribute this defectiveness to an inability to form complete circular viral DNA upon transfection, probably due to the susceptibility of the termini to degradation either during the transfection process or within the cell itself. Viral DNA from λRPA101, either as form I, linear dimers, or as linear monomers, was unable to induce transformation of chicken cells. Cells transfected with λRPA101 viral DNA and maintained in culture for 4 weeks did not make detectable amounts of virus particles, nor did they contain viral DNA sequences as assayed by Southern blot analysis (T. Gilmer and J. T. Parsons, unpublished data). The reason(s) for the defectiveness of λRPA101 DNA is not clear. Since focus formation in this transfection assay requires infectious virus production (5), our inability to detect either virus particles or viral DNA in transfected cultures suggests a defect in one of the genes required for virus replication. R-looping experiments and restriction enzyme analyses have not revealed detectable deletions or rearrangements of the viral DNA sequences in λRPA101. The defect therefore likely reflects a small deletion or point mutation undetectable by our analysis.

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