JOURNAL OF VIROLOGY, Feb. 1980, p. 845-855
0022-538X/80/02-0845/11$02.00/0

Vol. 33, No. 2

Molecular Cloning of the Harvey Sarcoma Virus Circular DNA Intermediates

II. Further Structural Analyses

HARDY W. CHANG,[1]* CLAUDE F. GARON,[2] ESTHER H. CHANG,[3] DOUGLAS R. LOWY,[3] GORDON L. HAGER,[4] EDWARD M. SCOLNICK,[4] ROY REPASKE,[1] AND MALCOLM A. MARTIN[1]

DNA Recombinant Research Unit, National Institute of Allergy and Infectious Diseases,[1] Laboratory of Biology of Viruses, National Institute of Allergy and Infectious Diseases,[2] Dermatology Branch, National Cancer Institute,[3] and Tumor Virus Genetics Branch, National Cancer Institute,[4] National Institutes of Health, Bethesda, Maryland 20205

Three species of unintegrated supercoiled Harvey sarcoma virus DNA (6.6, 6.0, and 5.4 kilobase pairs) have been molecularly cloned from Harvey sarcoma virus-infected cells. On the basis of restriction enzyme analyses, the 6.6- and 6.0-kilobase pair viral DNAs contain two and one copies, respectively, of a 650-base pair DNA segment which contains sequences present at the 3′ and 5′ termini of the viral genome. R-loop structures formed between Moloney leukemia virus RNA and the cloned Harvey sarcoma virus DNA indicated that about 500 base pairs of the 650-base pair repeating segment was complementary to the 3′ end of the viral RNA. During amplification in the *Escherichia coli* host, some recombinants containing the 6.6- or the 6.0-kilobase pair Harvey sarcoma virus DNA insert acquired or lost the complete 650-base pair DNA segment. These changes occurred in both *recA*$^+$ and *recA*$^-$ *E. coli*.

We have previously reported the cloning of unintegrated supercoiled forms of Harvey sarcoma virus (HaSV) DNA in *Escherichia coli* (6), using the λgtWES.λB vector system (9). Three size classes of infectious HaSV viral DNA inserts were isolated (6.6, 6.0, and 5.4 kilobase pairs [kbp]), which correspond to supercoiled forms of viral DNA found in cells after infection (E. H. Chang et al., manuscript in preparation). Restriction enzyme digestions and electron microscopic analyses of the recombinant phage DNA preparations were consistent with the 6.0-kbp HaSV DNA insert, being identical in size and composition to the linear form of unintegrated viral DNA, which contained terminal repetitions analogous to those reported previously for other retroviruses (5, 7, 14). The smallest HaSV DNA insert (5.4 kbp) apparently lacked the nucleotide sequences corresponding to one of the terminal repeats of linear unintegrated viral DNA, whereas the largest (6.6 kbp) viral DNA insert appeared to contain a tandem reiteration of such terminal sequences.

In this report we have employed several restriction enzymes which cleave HaSV DNA at sites within 500 nucleotides of the 3′ or 5′ termini and have analyzed R-loop structures formed between different cloned HaSV DNA preparations and Moloney leukemia virus (Mo-MuLV) RNA. Such studies indicate that the 5.4-, 6.0-, and 6.6-kbp HaSV DNA inserts contain one, two, and three copies, respectively, of nucleotide sequences derived from the termini of the RNA genome. In both the 6.0- and 6.6-kbp DNA inserts, the repeated terminal sequences are tandemly arranged. The 650-bp reiterated DNA segment contains approximately 500 bp corresponding to sequences present at the 3′ terminus and about 150 bp derived from the 5′ terminus of the RNA molecule.

MATERIALS AND METHODS

Preparation of [^{32}P]HaSV recombinant phage DNA. The construction, propagation, and preliminary characterization of λ-HaSV I (6.6-kbp DNA insert), λ-HaSV II (6.0-kbp DNA insert), and λ-HaSV X (5.4-kbp DNA insert) DNAs have been previously described (6). Overnight cultures (2.0 ml) of *E. coli* DP50 *supF* were grown in Jackson medium, previously treated to lower the phosphate concentration (10), and infected with λ-HaSV recombinant phage at a multiplicity of infection of 0.002. After a 15-min adsorption at room temperature, the infected culture was diluted 50-fold with Jackson medium (with lowered phosphate content) containing carrier-free [^{32}P]orthophosphate (20 μCi/ml). Bacterial lysis usually occurred 8 to 12 h postinfection, at which point chloroform (1.0 ml) was added, and the preparation was centrifuged at 6,000 rpm for 15 min at 4°C in a GSA rotor (Du Pont Instruments) to remove cell debris. Recombinant phage was precipitated with polyethylene glycol and purified by isopycnic centrifugation in CsCl as previously described (6). Phage DNA, prepared from CsCl-banded virus by phenol extraction, was dialyzed exten-

sively against 0.01 M Tris-hydrochloride, pH 8.0, and 0.1 mM EDTA before use. ^{32}P-labeled phage DNA prepared in this manner had a specific activity of approximately 8×10^4 cpm/μg. It has been our experience that other low-phosphate media, such as MOPS (morpholinepropanesulfonic acid) medium, failed to support the efficient growth of *E. coli* DP50 *supF*.

Restriction enzyme digestions. *Eco*RI, *Hpa*II, *Kpn*I, and *Xba*I were purchased from Bethesda Research Laboratory. *Hinc*II, *Hin*dIII, *Sac*I, and *Sma*I were purchased from New England Biolabs. Restriction endonuclease digestions were usually carried out in 0.030-ml reaction mixtures containing the following buffers (*Eco*RI: 100 mM Tris-hydrochloride, pH 8.0, 50 mM NaCl, and 5 mM $MgCl_2$; *Sac*I: 6 mM Tris-hydrochloride, pH 7.4, 20 mM NaCl, 6 mM $MgCl_2$, 6 mM 2-mercaptoethanol, and 100 μg of bovine serum albumin per ml; *Sma*I: 6 mM Tris-hydrochloride, pH 8.0, 20 mM KCl, 6.6 mM $MgCl_2$, 6 mM 2-mercaptoethanol, and 100 μg of bovine serum albumin per ml; *Kpn*I: 10 mM Tris-hydrochloride, pH 7.6, 6 mM NaCl, and 6 mM $MgCl_2$; *Hinc*II + *Hin*dIII: 10 mM Tris-hydrochloride, pH 7.9, 7 mM $MgCl_2$, 60 mM NaCl, 7 mM 2-mercaptoethanol, and 100 μg of bovine serum albumin per ml; *Hpa*II: 10 mM Tris-hydrochloride, pH 7.4, 10 mM $MgCl_2$, 6 mM KCl, 1.0 mM dithiothreitol, and 100 μg of bovine serum albumin per ml; *Xba*I: 6 mM Tris-hydrochloride, pH 7.4, 100 mM NaCl, and 6 mM $MgCl_2$) and incubated at 37°C for various periods of time.

Electrophoresis of DNA. DNA preparations were analyzed by electrophoresis in either 0.5% agarose (Seakem) gels buffered (pH 7.8) with 40 mM Tris-acetate, 5 mM sodium acetate, and 1 mM EDTA, or 3% polyacrylamide–0.5% agarose composite gels buffered (pH 7.2) in 40 mM Tris-acetate, 20 mM sodium acetate, and 1 mM EDTA. Electrophoresis through horizontal agarose slab gels (25 by 20 cm) was carried out at 150 V for 3 h. Samples were analyzed in composite vertical slab gels (25 by 20 cm) at 100 V for 5 h. DNA bands were visualized by UV transluminination after a 20-min staining with ethidium bromide (0.5 μg/ml).

DNA was transferred from agarose slab gels onto nitrocellulose filters and hybridized to a ^{32}P-labeled DNA probe as outlined by Southern (16). λ-HaSV DNA was labeled in vitro with ^{32}P-labeled nucleoside triphosphates by nick translation (specific activity, 8×10^7 cpm/μg) of appropriate recombinant phage DNA (12). Dried gels or nitrocellulose filters were exposed to Kodak XR-2 film at −70°C in the presence of a Du Pont Cronex Lightning Plus intensifying screen.

Purification of HaSV DNA inserts from λ-HaSV recombinant DNA. ^{32}P-labeled λ-HaSV phage DNA was incubated at 42°C for 1 h in 100 mM Tris-hydrochloride, pH 8.0, and 10 mM $MgCl_2$ to facilitate the annealing of the cohesive termini of λ-DNA. Dithiothreitol (10 mM) was then added, and the preparation was digested with *Eco*RI at 37°C for 2 h. Complete cleavage was verified by electrophoretic analysis of a portion of the reaction mixture in 0.5% agarose. Approximately 10 μg of the restricted λ-HaSV DNA was then applied to a 10 to 40% (wt/vol) sucrose gradient containing 1 M NaCl, 20 mM Tris-hydrochloride, pH 8.0, and 10 mM EDTA and centrifuged at 25,000 rpm for 16 h and 4°C in an SW27 (Spinco) rotor, or at 40,000 rpm for 7.5 h and 4°C in an SW41 (Spinco) rotor. Fractions (0.5 ml each) were collected by puncturing the bottom of the centrifuge tube, and radioactivity was monitored by Cerenkov counting. Fractions containing the HaSV DNA inserts were pooled, ethanol-precipitated, and subjected to a second cycle of centrifugation through 10 to 40% sucrose followed by dialysis against 10 mM Tris-hydrochloride, pH 7.5. HaSV DNA isolated in this manner was less than 10% contaminated with λgtWES.λB vector DNA.

Plating of recombinant phage on *recA*$^-$ *E. coli*. *E. coli* Y mel and Y mel *recA*$^-$ were gifts of L. Enquist. The *recA*$^+$ and *recA*$^-$ genotypes were verified by their UV sensitivity as well as the ability of the bacterial cells to support the growth of *gamma*$^-$ lambda phage (19). λ-HaSV recombinant phage was doubly plaque purified on Y mel *recA*$^-$ cells and then propagated in the same cells for the preparation of phage DNA.

Electron microscopy. R-loops were formed between intact recombinant DNA molecules and Mo-MuLV RNA at 52°C for 2 h in a solution containing 70% formamide, 0.1 M tricine buffer, pH 8.0, 0.25 M NaCl, and 0.01 M EDTA. Samples were mounted for electron microscopy by spreading the mixture described above or a dilution of the same mixture with no additions other than cytochrome *c* over a distilled water hypophase. In some instances RNase A (Worthington, catalog no. WRAF, in 2X SSC [1X SSC is 0.15 M NaCl plus 0.015 M sodium citrate] preheated to 90°C for 10 min) was added to R-loop hybridization mixtures to a final concentration of 10 μg/ml and incubated at 37°C for 1 min immediately before spreading. Grids were rotary shadowed with platinum-palladium and examined in a Siemens Elmiskop 101 at 40-kV accelerating voltage. Electron micrographs were taken on Kodak electron image plates at magnifications of 4,000 to 8,000. Magnification was calibrated with a grating replica (E. F. Fullam, catalog no. 1000), and contour lengths were measured with a Numonics Digitizer interfaced to a Wang 2200 computer.

RESULTS

Restriction enzyme digestions of HaSV recombinant phage DNA. We have previously reported the molecular cloning and the preliminary characterization of HaSV DNA (6). Restriction endonuclease (*Eco*RI, *Hin*dIII, and *Xba*I) digestions as well as electron microscopic analyses of R-loop structures formed between three different sized inserts of HaSV DNA and HaSV RNA were compatible with the presence of zero, one, and two copies of a 650-bp DNA segment in recombinants λ-HaSV X, λ-HaSV II, and λ-HaSV I, respectively (6). Since the circular HaSV DNAs had been cloned at the unique *Eco*RI site, which cleaves the unintegrated linear viral DNA a single time near the middle of the molecule (Fig. 1) (6), the cloned DNAs were permuted with respect to the linear viral DNA and the HaSV RNA genome. The cloned DNAs

were found to contain the same sequences as the linear viral DNA and the viral RNA, and the 650-bp segment was localized to the junction between the 3′ and 5′ ends of the viral RNA on the R-loops and the termini of the linear viral DNA (6). Digestion with *Xba*I, which cleaves the linear unintegrated HaSV DNA at a site corresponding to sequences located within 500 bp of the two termini, generated a restriction fragment of approximately 650 bp with λ-HaSV II and λ-HaSV I DNAs but not with λ-HaSV X DNA; this result was consistent with the existence of different amounts of "terminal" sequences in the three cloned DNAs. Our previous examination of restricted HaSV DNA was limited to blot hybridization analyses of λ-HaSV DNAs using a HaSV complementary DNA probe which was unsuitable for the precise quantitation of reiterated "terminal" sequences.

To circumvent this problem and to more definitively characterize cloned HaSV DNA preparations, recombinant phage were labeled with [^{32}P]orthophosphate during their propagation in *E. coli* DP50 *supF*, and phage DNA was prepared as described above. In the first group of experiments to be described, λ-HaSV DNA preparations were digested with restriction enzymes (*Sac*I, *Sma*I, *Kpn*I, and *Xba*I) that cleave unintegrated linear HaSV DNA at sites located within 500 nucleotides of either termini (Fig. 1) (6; Chang et al., manuscript in preparation). When ^{32}P-labeled λ-HaSV I and λ-HaSV II DNAs were restricted with *Sac*I (which does not cleave the λgtWES.λB "arms"), six fragments were generated, the two largest of which represent HaSV DNA joined to λgtWES.λB DNA (Fig. 2, lanes AI and AII). The four smaller bands represent HaSV DNA fragments 650, 520, 350, and 220 bp in size (calculated from *Hinc*II + *Hin*dIII simian virus 40 DNA marker fragments). The *Sac*I digest of λ-HaSV X DNA generated all of the cleavage products obtained with λ-HaSV I and λ-HaSV II DNAs except the 650-bp fragment (Fig. 2, lane AX). A similar result was observed when ^{32}P-labeled λ-HaSV I, λ-HaSV II, and λ-HaSV X DNAs were digested with other restriction enzymes that cleave sequences located near the terminal regions. *Sma*I, *Kpn*I, and *Xba*I digestions of λ-HaSV I and λ-HaSV II DNAs invariably generated the 650-bp cleavage product (Fig. 2, lanes BI and BII, CI and CII, DI and DII, respectively) which was not present in digests of λ-HaSV X DNA (Fig. 2, lanes BX, CX, and DX, respectively).

The autoradiogram presented in Fig. 1 also shows that the intensity of the 650-bp band, relative to the other cleavage products, was always greater in digests of λ-HaSV I than in λ-HaSV II. Densitometer tracings of the bands generated after digestion of the three recombinant phage DNAs with *Sac*I and *Sma*I indicated that λ-HaSV I DNA contained approximately twice as much of the 650-bp fragment as did λ-HaSV II DNA (Fig. 3). These data are consistent with the presence of two and three copies of terminal sequences in HaSV II and HaSV I DNAs, respectively.

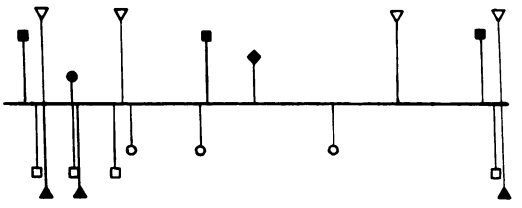

FIG. 1. *Restriction endonuclease map of the 6-kbp, unintegrated linear HaSV DNA isolated from infected mouse cells (6). The 5′ portion of the viral RNA is located to the left; the 3′ portion of the viral RNA is on the right. Symbols:* ▽, *Sma*I; ■, *Xba*I; ◆, *Eco*RI; ●, *Bam*HI; ○, *Hin*dIII; □, *Sac*I; ▲, *Kpn*I.

We were also interested in determining whether the three cloned HaSV DNAs differed in regions other than that corresponding to the terminal segments. ^{32}P-labeled HaSV DNA inserts were purified from recombinant phage DNA after *Eco*RI digestion as described above and digested with restriction enzymes that cleave the viral DNA at multiple sites. *Hinc*II and *Hin*dIII digestion of HaSV I, HaSV II, and HaSV X DNAs generated a restriction pattern that differed only in the size of a single fragment which presumably contained decreasing amounts of terminal sequences (Fig. 4, lanes AI, AII, and AX). No prominent differences were observed following digestion of the HaSV DNA inserts with *Hpa*II (Fig. 4, lanes BI, BII, and BX) except that the intensity of a 300-bp DNA fragment appeared to be greater in λ-HaSV I than in λ-HaSV II or in λ-HaSV X.

Double digestion of HaSV I and HaSV II DNAs with *Xba*I and *Sac*I (which cleave near the termini) gave rise to a 630-bp fragment not detected with HaSV X DNA (Fig. 4, lanes CI, CII, and CX). A *Kpn*I plus *Sma*I cleavage of the same HaSV DNA preparations generated a fragment approximately 640 bp in size that was absent in HaSV X DNA. These data indicate that: (i) in the terminal segment, the *Xba*I and *Sac*I cleavage sites are about 20 nucleotides apart and the *Kpn*I and *Sma*I sites are separated by approximately 10 nucleotides; and (ii) aside from sequences corresponding to the two ends of the HaSV genome, the cloned HaSV DNAs were indistinguishable.

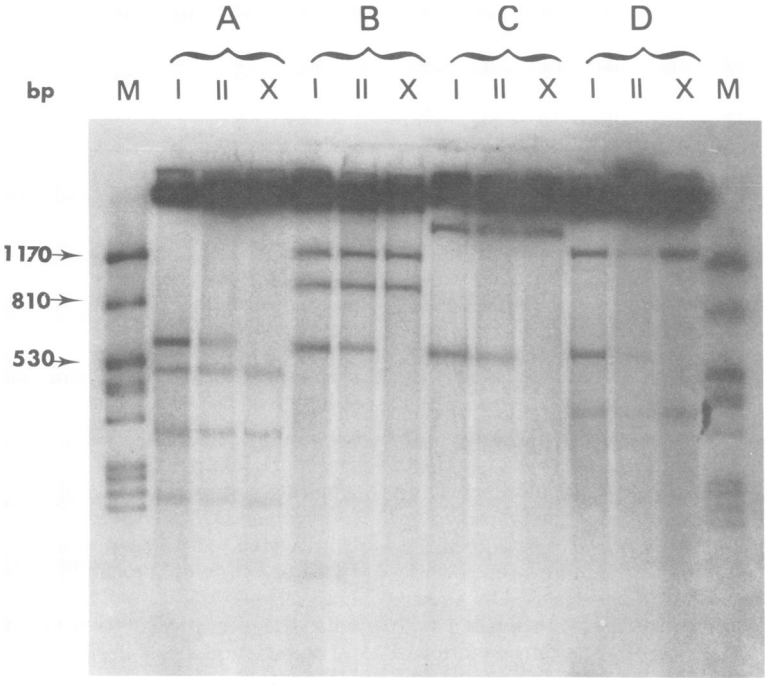

FIG. 2. *Restriction enzyme analyses of ^{32}P-labeled HaSV DNAs. ^{32}P-labeled HaSV recombinant phages were prepared, cleaved with restriction enzymes, and electrophoresed in a 3% polyacrylamide–0.5% agarose composite gel as described in the text. (M) HincII + HindIII digest of ^{32}P-labeled simian virus 40 DNA. (A, B, C, and D) SacI, SmaI, KpnI, and XbaI digests of (I) λ-HaSV I, (II) λ-HaSV II, and (X) λ-HaSV X DNAs, respectively. HaSV DNA is inserted in the same orientation in the three isolates.*

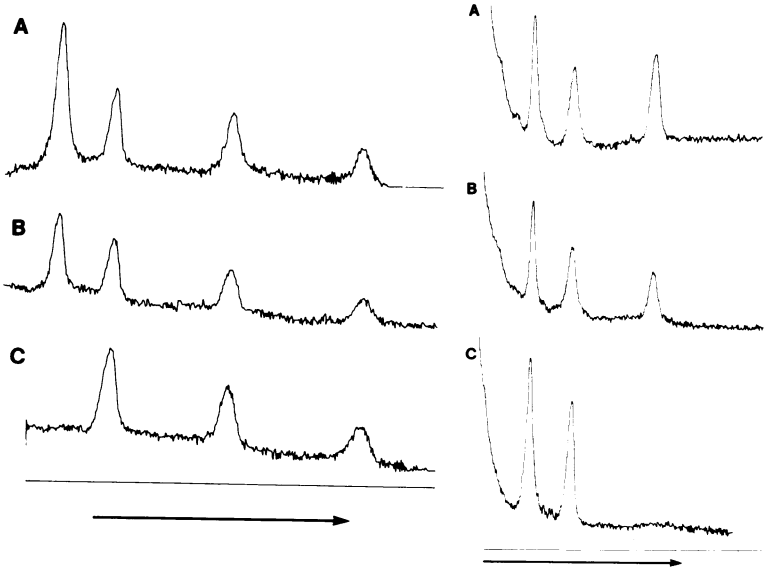

FIG. 3. *Densitometer tracings of some of the fragments produced by SacI or SmaI digestion of λ-HaSV I, λ-HaSV II, and λ-HaSV X DNAs. ^{32}P-labeled HaSV recombinant DNAs were cleaved with SacI or SmaI and electrophoresed in a 3% polyacrylamide–0.5% agarose composite gel as described in Fig. 1. Autoradiograms were scanned using a Joyce-Loebl densitometer. The densitometer tracings shown are for SacI (left) and SmaI digests (right) of (A) λ-HaSV I, (B) λ-HaSV II, and (C) λ-HaSV X DNAs, respectively.*

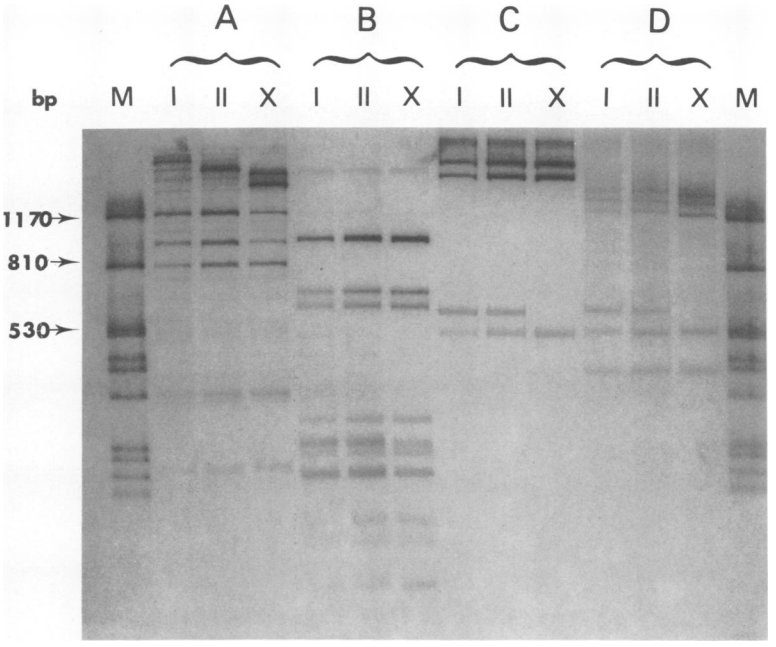

FIG. 4. *Restriction enzyme analysis of ^{32}P-labeled HaSV DNAs. ^{32}P-labeled HaSV DNA inserts were purified from recombinant phage DNA after EcoRI digestion as described in the text. (M) HincII + HindIII digest of ^{32}P-labeled simian virus 40 DNA. (A, B, C, and D) HincII + HindIII, HpaII, SacI + XbaI, and SmaI + KpnI digests of (I) HaSV I, (II) HaSV II, and (X) HaSV X DNAs, respectively.*

Electron microscopy of R-loop structures formed between Mo-MuLV RNA and cloned HaSV DNA preparations. To further characterize the three different forms of supercoiled HaSV DNA cloned in the λgtWES.λB vector, R-loops were formed between Mo-MuLV RNA and each of the viral DNA inserts and analyzed by electron microscopy. Mo-MuLV RNA was a particularly useful reagent in this regard, since it contains approximately 1,000 nucleotides at its 3′ terminus that are also present at the 3′ end of the HaSV RNA genome (15; N. Davidson, personal communication). R-loops which appear subsequent to the annealing of Mo-MuLV RNA to the different forms of HaSV DNA would locate the position and number of sequences corresponding to the 3′ terminus of the viral genome. λ-HaSV I, λ-HaSV II, and λ-HaSV X DNAs were digested with *Eco*RI, the viral DNA inserts were purified, and R-loops were formed as described above. HaSV X, HaSV II, and HaSV I DNAs contained one, two, and three displacement loops, respectively, after annealing to Mo-MuLV RNA (Fig. 4). To facilitate more accurate measurements of the sizes of the displacement loops, R-loop structures formed with Mo-MuLV RNA were digested with pancreatic RNase, and the position of the displacement loop was determined for each of the three HaSV DNA samples (Fig. 5 through 8). In the case of HaSV I DNA, one large and two smaller R-loops were visualized (Fig. 5C and Fig. 6), whereas HaSV II DNA contained one large and one small R-loop (Fig. 5B and Fig. 7). A single R-loop was identified with HaSV X DNA (Fig. 5A and Fig. 8). Contour length measurements indicated that the duplex segment opposite the largest displacement loop present in HaSV X, HaSV II, and HaSV I DNAs contained approximately 908 (±77), 870 (±60), and 840 (±80) bp (Fig. 9). The smaller displacement loops spanned duplex segments 499 (±60) to 574 (±100) bp in length and were joined by short, double-stranded DNA bridges containing 197 (±70) to 261 (±60) bp (Fig. 9). Our interpretation of these results is that the large R-loop observed with all three HaSV DNA preparations represents the annealing of the 3′ terminus of Mo-MuLV RNA with homologous sequences (approximately 870 bp) conserved in HaSV DNA. The additional displacement loops visualized with HaSV II and HaSV I DNAs reflect the annealing of only a portion of the sequences shared by Mo-MuLV and reiterated terminal segments present in cloned HaSV DNA. These additional R-loops are smaller than the entire 650-bp reiteration, presumably because of the presence in the terminal repeat of nucleotides derived from the 5′

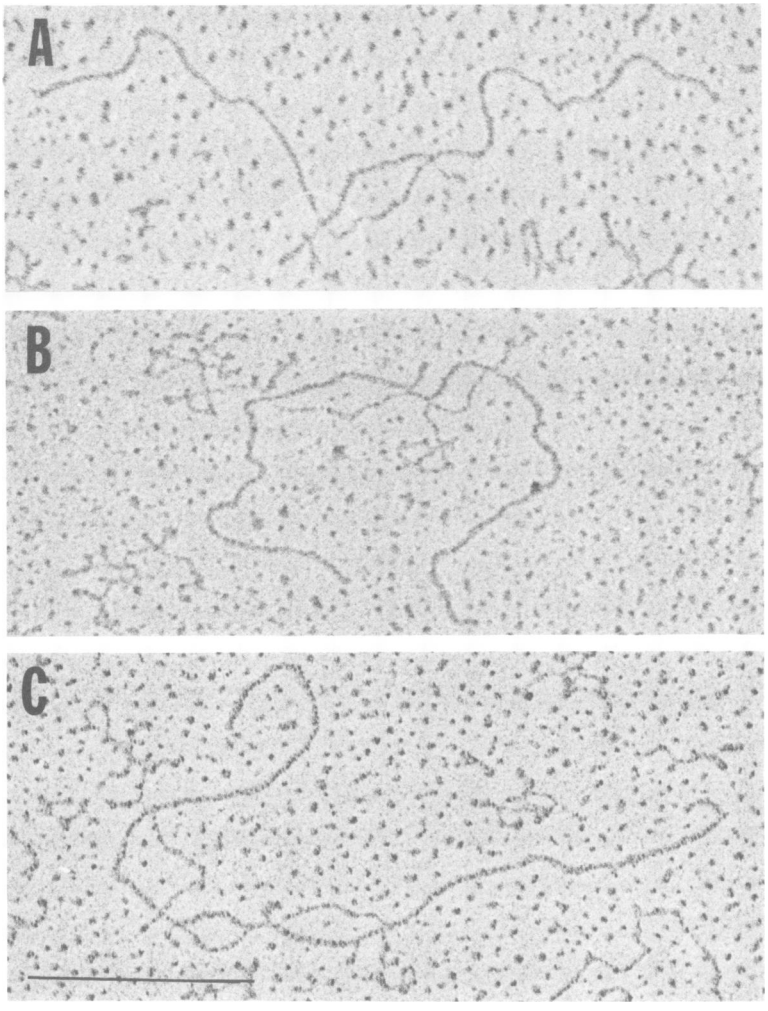

FIG. 5. *Electron microscopic analysis of R-loop structures formed between Mo-MuLV RNA and EcoRI-cleaved (A) HaSV X, (B) HaSV II, and (C) HaSV I DNA inserts. HaSV DNA preparations were restricted with EcoRI and incubated with Mo-MuLV RNA for 2 h at 52°C as described in the text. Samples were mounted for microscopy in 70% formamide over a distilled water hypophase. Grids were rotary shadowed with platinum-palladium. The bar represents 0.5 μm.*

terminus of the viral genome which are not homologous with Mo-MuLV RNA (Fig. 9). These contour length measurements suggest that the length of the sequences comprising the tandem repeats in HaSV II and HaSV I DNAs involves about 600 to 700 bp of which approximately one-third are derived from the 5′ terminus and two-thirds are from the 3′ terminus.

Stability of λ-HaSV phage during propagation in *recA*$^+$ and *recA*$^-$ *E. coli*. We have previously reported that recombinant HaSV phage, originally isolated from a single plaque and which contain two or three copies of the terminal segments, gave rise to progeny particles containing various amounts of these terminal viral DNA sequences (6). In addition to the predominant species present, HaSV DNA containing one to four copies of terminal sequences were also detected in the progeny of a single plaque isolate (6). Since HaSV DNA had been propagated as a component of a recombinant phage in *recA*$^+$ *E. coli* DP50 *supF*, the most likely explanation for the appearance of DNA molecules containing various amounts of terminal sequences is homologous recombination.

To ascertain whether the accumulation or loss of terminal sequences during the propagation of HaSV I and HaSV II indeed reflected *recA*-mediated recombination, λ-HaSV I was plaque purified twice on *E. coli* Y mel (*recA*$^-$) cells and

then propagated in the same *recA*$^-$ background. Phage DNA was isolated and analyzed by electrophoresis in a 0.5% agarose gel. In addition to the major DNA insert (6.6 kbp) typical of HaSV I (Fig. 10), minor species (7.2 and 6.0 kbp in size) of HaSV DNA were also present in the progeny recombinant phage preparation. Thus, the addition and deletion of tandemly duplicated segments of HaSV DNA in *E. coli* occurs regardless of whether the host cell employed was *recA*$^+$ or

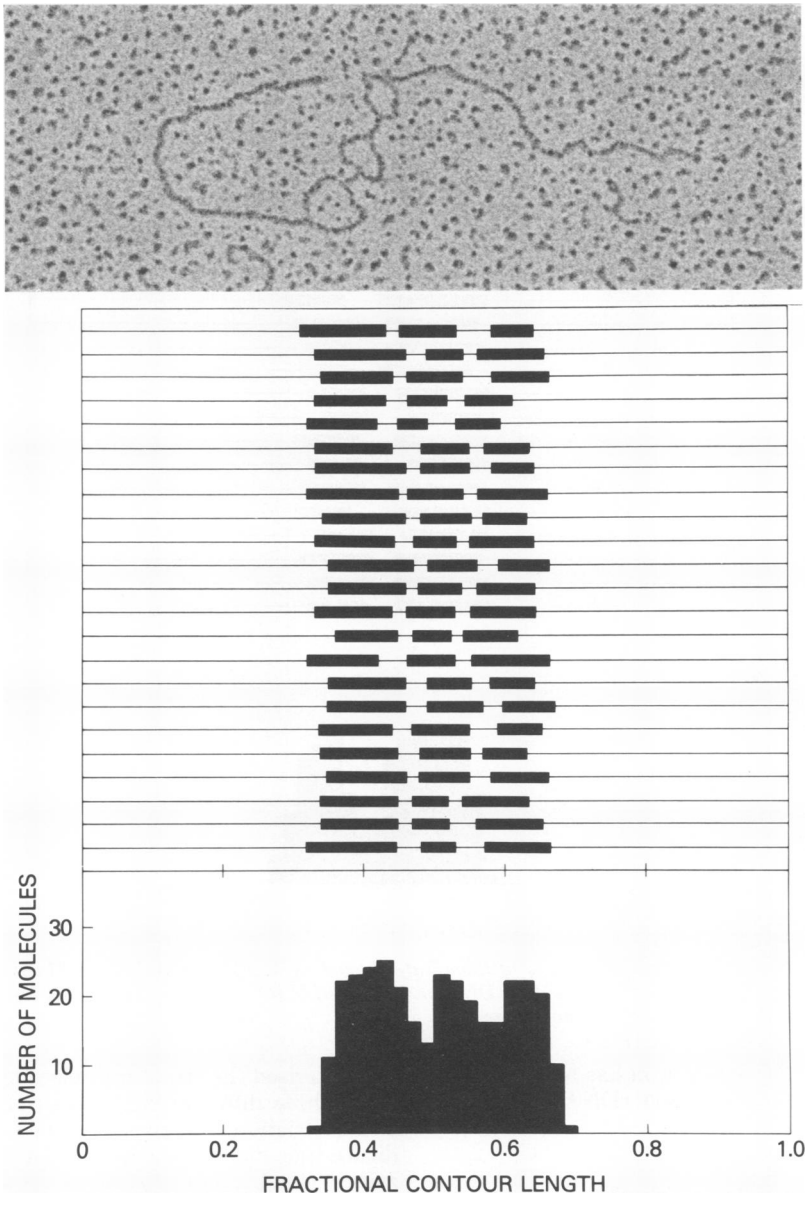

FIG. 6. *Histogram showing the regions of homology shared by Mo-MuLV RNA and HaSV I DNA. The HaSV I DNA insert was purified from recombinant phage DNA and hybridized to Mo-MuLV RNA as described in the text. DNA-RNA hybrids were treated with RNase (10 μg/ml) for 1 min at 37°C before mounting for microscopy. Control experiments did not reveal any changes in R-loop structure even after 5 h of incubation. Molecules were mounted for microscopy in 70% formamide over a distilled water hypophase. The grids were rotary shadowed with platinum-palladium. A schematic representation of individual molecules is also presented. Bars represent hybridized regions of the molecule.*

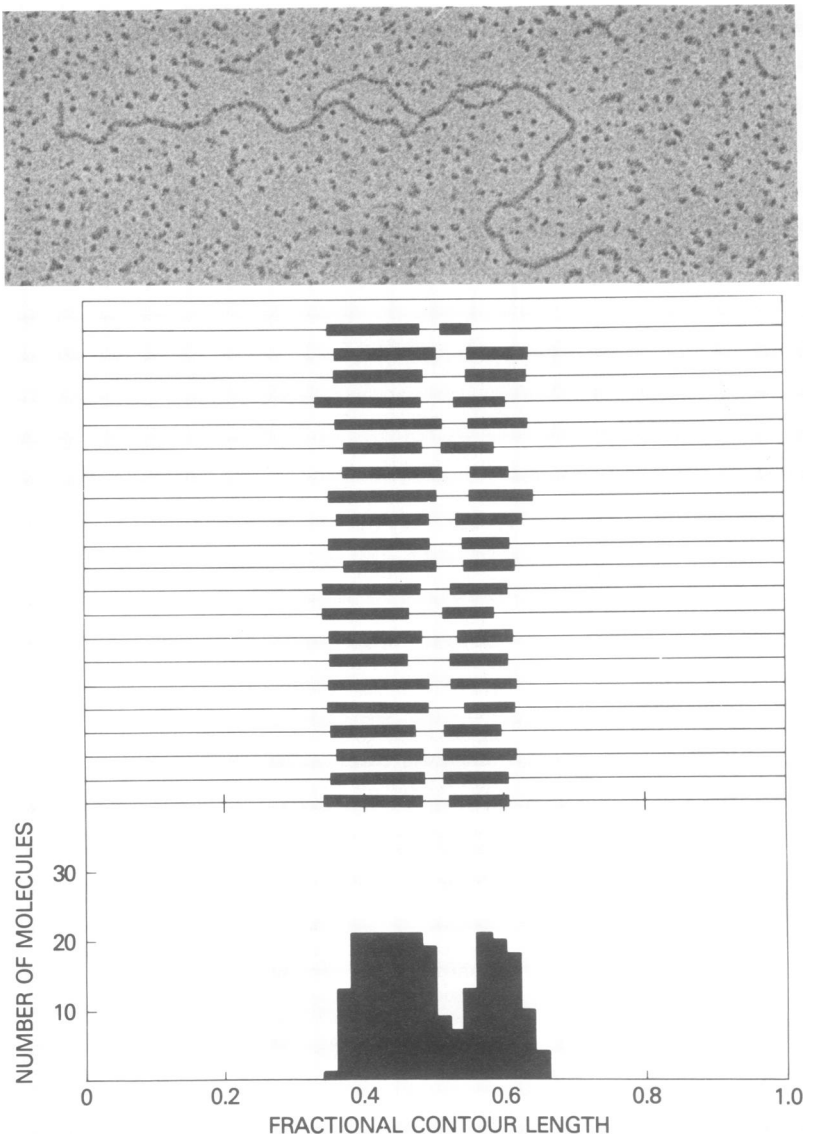

FIG. 7. *Histogram showing the regions of homology shared by Mo-MuLV RNA and HaSV II DNA. R-loops were formed between purified HaSV II DNA and Mo-MuLV RNA as described in the legend to Fig. 5. Bars represent hybrid regions of the molecules.*

recA$^-$. A similar observation has been reported during the cloning of yeast rDNA in recombinant plasmids (3).

DISCUSSION

We have previously reported the molecular cloning of the supercoiled circular forms of HaSV from newly infected mouse cells. Three different size classes of HaSV molecules were isolated, and DNA from all three induced foci in NIH 3T3 cells (6). In the current study, we have characterized the structural relationship among these three different size classes of molecules. The restriction endonuclease digestion data indicate that digestion with each of the four enzymes (*Sac*I, *Xba*I, *Kpn*I, and *Sma*I), which cleave within the terminal 500 bp of the unintegrated linear viral DNA isolated from mouse cells, yields a 650-bp fragment after digestion of HaSV I and HaSV II DNAs which is missing in the HaSV X clone; no other differences in the three species of HaSV DNA could be detected. Densitometer tracing of the restricted DNA

preparations clearly indicated that HaSV X, HaSV II, and HaSV I DNAs contain zero, one, and two copies, respectively, of a 650-bp segment that contains sequences present at the 3′ and 5′ termini of genome RNA.

Mo-MuLV RNA represented a convenient probe to characterize the relative contribution of the 3′ and 5′ ends of the viral RNA to the 650-bp segment, since the Mo-MuLV sequences of HaSV RNA are apparently confined to about 900 bp of the 3′ end of the viral RNA and to a small stretch of sequences (less than 200 bp) at

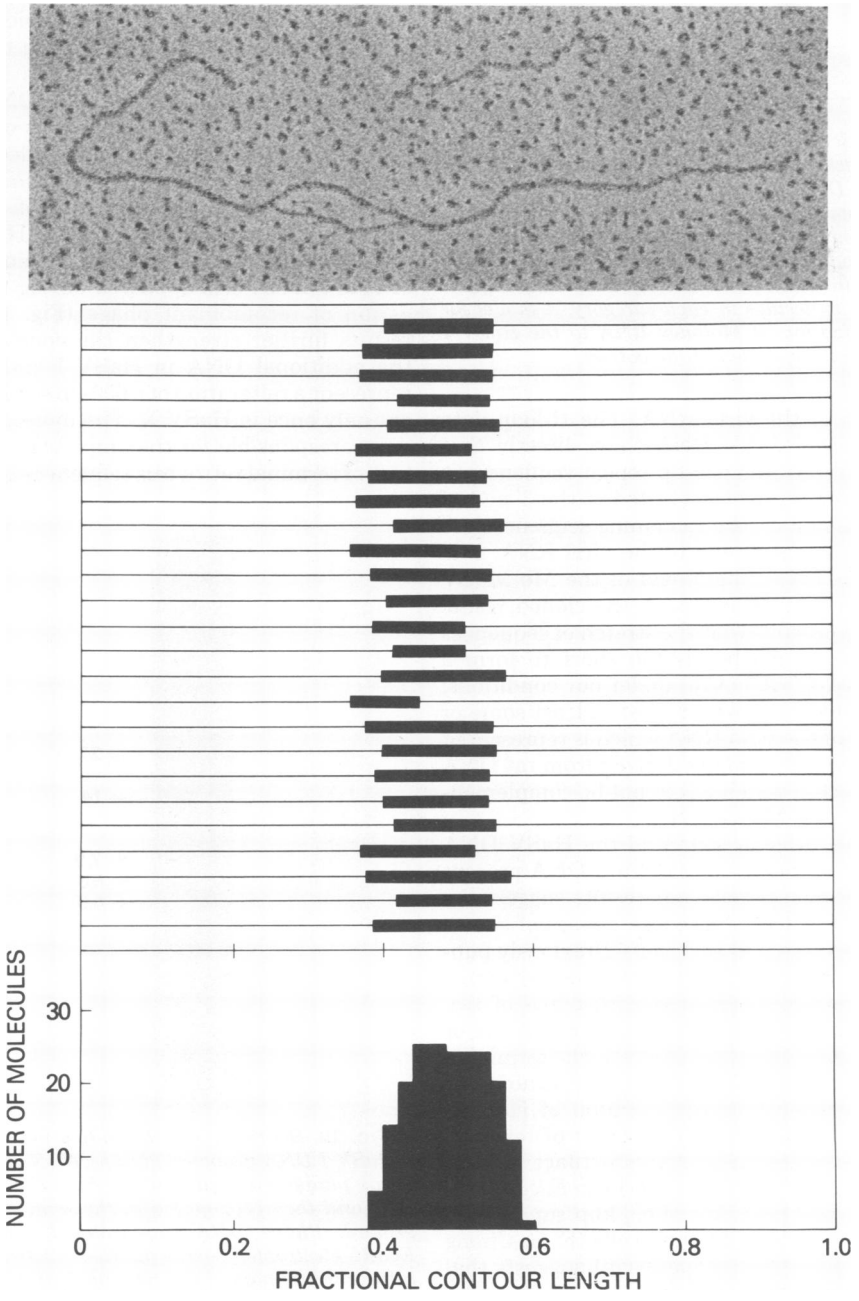

FIG. 8. *Histogram showing the regions of homology shared by Mo-MuLV RNA and HaSV X DNA. R-loops were formed between purified HaSV X DNA and Mo-MuLV RNA as described in the legend to Fig. 5. Bars represent hybrid regions of the molecules.*

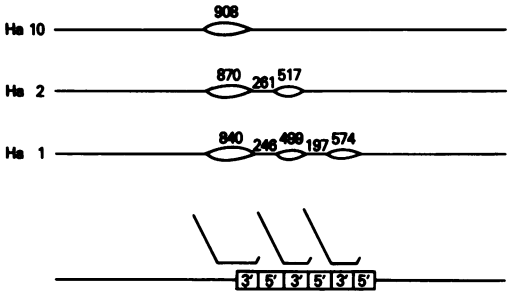

FIG. 9. *Schematic representation of regions of homology between Mo-MuLV RNA and the three different HaSV DNA inserts. The numbers presented in this diagram represent the calculated number of base pairs present in duplex segments opposite the displaced R-loop in each preparation. These values are based on the mean contour lengths of these regions in 20 to 25 molecules in each category. A model for the hybridization of Moloney RNA to the HaSV I DNA insert is presented at the bottom.*

the 5′ end of the viral RNA. The R-loop data using the Mo-MuLV RNA show directly that about 500 bp of the 650-bp repeat sequences is derived from sequences located at the 3′ end of the viral genome. The remaining sequences are derived from the 5′ end of the viral RNA. It is not yet clear why the 5′ end of the Mo-MuLV did not form an R-loop with the cloned HaSV DNA. It is possible that the stretch of sequences (about 150 to 200 bp) is too short to form a stable RNA-DNA hybrid under our conditions; it is more likely, however, that at least some of the 5′ end of the viral RNA which is represented in the 650-bp sequence is derived from rat DNA sequences (15), which would not be complementary to the Mo-MuLV RNA.

Since the original source of the HaSV DNA was closed circular forms of viral DNA present in infected mouse cells, our results suggest the presence of at least three size classes of supercoiled HaSV DNA in such cells. Previously published analyses of avian and murine retrovirus systems have pointed to the existence of two species of supercoiled DNA that contain one or two copies of sequences located at the termini of the RNA genome (2, 5, 7, 13, 18). Although it could be argued that the isolation of HaSV I DNA, which contains three copies of terminal sequences, represents a "cloning artifact" involving homologous recombination in an *E. coli* host, the recent identification of 6.6-kbp supercoiled viral DNA in infected mouse cells (E. H. Chang et al., manuscript in preparation) suggests that such molecules are one of the many unintegrated retroviral DNA intermediates that appear following infection. Furthermore, repeated passaging of λ-HaSV X in either *recA*$^+$ or *recA*$^-$ *E. coli* did not result in the conversion of λ-HaSV X into λ-HaSV II. Therefore, the presence of two copies of terminal sequences in λ-HaSV II DNA could not arise from a recombinant phage containing a single copy of these sequences during its propagation in *E. coli*.

We previously reported that λ-HaSV recombinants containing two or three copies of terminal sequences were unstable during their propagation in *E. coli recA*$^+$ host cells (6). In the present study, λ-HaSV I (6.6-kbp DNA insert), which contains three 650-bp copies of terminal sequences, was doubly plaque purified in *recA*$^-$ cells and then grown in *recA*$^-$ *E. coli*. Although the 6.6-kbp insert characteristic of λ-HaSV I remained the predominant form of HaSV DNA after propagation in *recA*$^-$ cells, minor species of HaSV DNA also appeared during the propagation of recombinant phage (Fig. 10). These results further strengthen the conclusion that the additional DNA in HaSV I and HaSV II represent a reiteration of a 650-bp sequence present only once in HaSV X. The molecular mechanism responsible for the amplification or deletion of terminal retrovirus sequences is presently

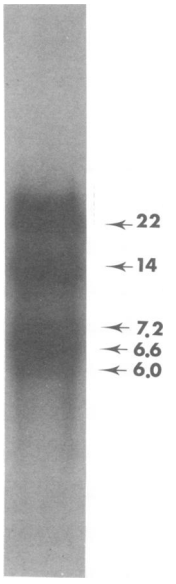

FIG. 10. *Southern blot hybridization analysis of λ-HaSV I DNA propagated in recA*$^-$ *E coli. λ-HaSV I was plaque purified twice in E. coli Y mel recA*$^-$ *cells and then propagated in the same recA*$^-$ *background. Phage DNA was isolated, cleaved with EcoRI, electrophoresed in agarose gel, transferred to nitrocellulose paper, and annealed with* ^{32}*P-labeled λ-HaSV I DNA as described in the text. The sizes of the restriction enzyme fragments are given in kilobase pairs. The 22- and 14-kbp DNA fragments represent the left and right arms of λgtWES.λB DNA.*

unclear but it is apparently *recA* independent. One possibility is that the 650-bp segments, located at each end of unintegrated linear retroviral DNA molecules (2, 5, 7, 14, 18), have properties similar to IS sequences present in a transposon such as Tn*9*, which bears direct repeats at its termini (11). In this regard, it should be noted that retrovirus DNA, bounded by the terminal repeating segment, can be inserted into the mammalian chromosomal DNA at any one of a number of sites (8, 13, 16). Furthermore, some revertants of murine sarcoma virus-transformed cells which have lost the transformed phenotype do not contain detectable viral DNA sequences (1, 4). The acquisition and loss of murine sarcoma virus genomes in these biological systems is quite similar to those described for transposable elements. The behavior of the 650-bp DNA segment in *recA*$^-$ *E. coli* makes such an analogy even more attractive. Studies are currently in progress to further evaluate the properties of retroviral terminal sequences in procaryotic as well as eucaryotic host cells.

LITERATURE CITED

1. **Bensinger, W., K. Robbins, J. Greenberger, and S. Aaronson.** 1977. Different mechanisms for morphologic reversion of a clonal population of murine sarcoma virus-transformed nonproducer cell. Virology **77**:750–761.
2. **Coffin, J., T. Hageman, A. Maxam, and W. Haseltine.** 1978. Structure of the genome moloney murine leukemia virus: a terminally redundant sequence. Cell **13**: 761–773.
3. **Cohen, A., R. Danella, H. Halvorson, and P. Wensink.** 1978. Deletions within *E. coli* plasmids carrying yeast rDNA. Gene **3**:135–147.
4. **Frankel, A., D. Haapala, R. Neubauer, and P. Fichinger.** 1976. Elimination of the sarcoma genome from murine sarcoma virus transformed cat cell. Science **91**: 1264–1266.
5. **Gilboa, E., S. Goff, A. Shields, F. Yoshimura, S. Mitra, and D. Baltimore.** 1979. *In vitro* synthesis of a 9 kbp terminally redundant DNA carrying the infectivity of Moloney murine leukemia virus. Cell **16**:863–874.
6. **Hager, G. L., E. H. Chang, H. W. Chang, C. F. Garon, M. A. Israel, M. A. Martin, E. M. Scolnick, and D. R. Lowy.** 1979. Molecular cloning of the Harvey sarcoma virus closed circular DNA intermediates: initial structural and biological characterization. J. Virol. **31**: 795–809.
7. **Hsu, T. W., J. L. Sabran, G. E. Mark, R. V. Guntaka, and J. M. Taylor.** 1978. Analyses of unintegrated avian RNA tumor virus double-stranded DNA intermediates. J. Virol. **28**:810–818.
8. **Hughes, S., P. Shank, D. Spector, H.-J. Kung, J. M. Bishop, and H. E. Varmus.** 1978. Proviruses of Avian Sarcoma Virus are terminally redundant, co-extensive with unintegrated linear DNA and integrated at many sites. Cell **15**:1397–1410.
9. **Leder, P., D. Tiemeier, and L. Enquist.** 1977. EK2 derivative of bacteriophage lambda useful in the cloning of DNA from higher organisms: the λgt.WES system. Science **1961**:75–177.
10. **Lin, S. and A. D. Riggs.** 1972. *lac* repressor binding to non-operator DNA. Detailed studies and a comparison of equilibrium and rate competition methods. J. Mol. Biol. **72**:671–690.
11. **MacHattie, L. A., and J. B. Jackowski.** 1977. Physical structure and deletion effects of the chloramphenicol resistance element Tn*9* in phage lambda, p. 219–228. *In* A. I. Bukhari, J. A. Shapiro, and S. L. Adhya (ed.), DNA insertion elements, plasmids, and episomes. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
12. **Rigby, P., M. Dieckman, C. Rhodes, and P. Berg.** 1977. Labelling deoxyribonuclease acid to high specific activity *in vitro* by nick translation with DNA polymerase I. J. Mol. Biol. **113**:237–251.
13. **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.
14. **Shank, P., S. Hughes, H.-J. Kung, J. Major, N. Quintrell, R. Guntaka, J. M. Bishop, and H. Varmus.** 1978. Mapping unintegrated Avian Sarcoma virus DNA: termini of linear DNA bearing 300 nucleotides present once as or twice in two species of circular DNA. Cell **15**:1385–1395.
15. **Shih, T. Y., D. R. Williams, M. O. Weeks, J. M. Maryak, W. C. Vass, and E. M. Scolnick.** 1978. Comparison of the genomic organization of Kirsten and Harvey sarcoma viruses. J. Virol. **27**:45–55.
16. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **38**:503–517.
17. **Steffen, D., and R. Weinberg.** 1978. The integrated genome of Murine Leukemia Virus. Cell **15**:1003–1010.
18. **Yoshimura, F., and R. Weinberg.** 1979. Restriction endonuclease cleavage of linear and closed circle murine leukemia viral DNAs: discovery of a smaller circular form. Cell **16**:323–332.
19. **Zissler, J., E. Signer, and F. Shaefer.** 1971. The role of recombination in growth of bacteriophage lambda. I. The gamma gene, p. 455–468. *In* P. D. Hershey (ed.), The bacteriophage lambda. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.