Physical Map of Infectious Baboon Type C Viral DNA and Sites of Integration in Infected Cells

NARAYANA BATTULA* AND GEORGE J. TODARO

Laboratory of Viral Carcinogenesis, National Cancer Institute, Bethesda, Maryland 20205

Three species of unintegrated viral DNAs were found in permissive cells infected with baboon type C virus. The major species was a 9.0-kilobase (kb) linear DNA that was infectious. A restriction endonuclease map of this DNA was constructed and oriented with respect to the viral RNA. The linear DNA had a 0.6-kb sequence repeated at each terminus. These terminal repeat sequences were required for infectivity of the viral DNA. The minor species of the unintegrated viral DNAs were covalently closed circles of 9.0 and 8.4 kb. The smaller circle was in two- to threefold excess over the larger circle. The difference appeared to be that the smaller circle lacked one of the two 0.6-kb repeat sequences found in the larger circle. Restriction endonuclease maps of the integrated viral DNAs were constructed, and the sequences on both viral DNA and cellular DNA that are involved in integration were determined. The integrated viral DNA map was identical to that of the unintegrated infectious 9.0-kb linear DNA. Therefore, a specific site in the terminal repeat sequence of the viral DNA was used to integrate with the host cell DNA. The sizes of the cellular DNA fragments were different from clone to clone but stable with cell passage. Therefore, many sites in the cell DNA can recombine with the viral DNA.

Baboon type C virus was the first primate retrovirus isolated from a species of the Old World monkey (4, 13). The genomes of all baboon cells contain DNA sequences that can code for the type C viral RNA genome and, therefore, the virus was classified as endogenous (12, 37). Several endogenous primate type C viruses have since been isolated, and as a group they have some interesting characteristics (36). For example, these endogenous viruses are helper independent and do not grow in cells of their origin, but grow well in other cells such as human. The cellular genome contains 50 to 150 DNA sequences that are related to the viral RNA genome (6). The structure and function of these multiple endogenous sequences, however, is not understood. Recent advances in technology make it feasible to investigate the organization, regulation, and expression of these multiple copies of endogenous type C viral genetic information in the primate cells.

To pursue these studies, we used the M7 isolate of baboon endogenous type C virus as a model system to study the events in the life cycle of these viruses. We chose to use dog thymus cells (Cf2Th) which are permissive for M7 (37). These cells lack viral DNA sequences related to primate type C viruses, and therefore integration can be studied in the absence of cellular DNA background. We report here on the isolation, characterization, and restriction endonuclease mapping of the unintegrated viral DNAs. We also report on sequences of the viral DNA as well as the sequences of the cellular DNA involved in integration.

We show here that there are three species of unintegrated viral DNAs in M7-infected Cf2Th cells. The major species was a linear DNA with repetition of sequences at each terminus (terminal repeat sequences). The minor species consisted of two covalently closed circular DNAs. The smaller circle contained a single terminal repeat sequence, whereas the larger circle contained a double repeat. The linear unintegrated DNA was infectious, and the terminal repeats were required for infectivity. The integrated DNA was colinear with the unintegrated linear viral DNA, indicating that the sequences near the ends of terminal repeats were used in fusing with cellular DNA. The fusion fragments of cellular and viral sequences were different from clone to clone, indicating that many sites in the cellular DNA allow integration of viral DNA.

MATERIALS AND METHODS

Cells and viruses. Cells were grown in Dulbecco-modified Eagle minimal essential medium, supplemented with 5 or 10% calf serum. Canine thymus cells (Cf2Th) were obtained from Naval Biomedical Research Laboratory, Oakland, Calif., bat lung fibroblasts (Tbl-Lu) were obtained from the American Type Culture Collection, Rockville, Md., and A549, a human lung tumor cell line, was derived in this laboratory

(22). The M7 isolate of baboon endogenous type C virus was cloned on permissive Cf2Th cells by two cycles of endpoint dilution. Clone number 68 (M7 #68) was used in these experiments. A mass culture of Cf2Th cells was infected with M7 #68, and independent clones from this producer culture were isolated by serial dilution and plating in microtiter dishes. Polymerase-positive clones from the highest dilution were used in these expeiments.

Extraction of unintegrated M7 #68 DNA. Subconfluent cultures of Cf2Th cells in plastic roller bottles (Corning Glass Works) were infected at high multiplicity with M7 #68. Forty hours after infection the cells were harvested and washed twice with phosphate-buffered saline, and the unintegrated viral DNA was purified by hydroxyapatite chromatography as described (29). The unintegrated viral DNA eluted in 0.5 M phosphate buffer (pH 6.8) was concentrated on a smaller hydroxyapatite column, and the DNA concentration was determined by absorbance at 260 nm (A_{260}). The A_{260}/A_{280} ratio of this DNA was 1.9, and the yield was less than 0.5% of total cell DNA.

Viral DNA was further purified by centrifugation on cesium chloride-ethidium bromide (EtBr) equilibrium density gradients. Solid cesium chloride was added to the preparation to a density of 1.585 g/cm³, and EtBr was added to a concentration of 100 μg/ml. The samples were centrifuged in a type 65 rotor (Beckman Instruments, Inc.) at 40,000 rpm for 60 h at 20°C. Fractions were collected from the bottom of the gradients, and their densities were determined from refractive indices. A portion of each fraction was used to hybridize with ³H-labeled complementary DNA (cDNA) probe prepared from M7 viral RNA in an endogenous reaction (5). The peak fractions in the covalently closed circular DNA density region and the linear density region were separately pooled, extracted with isopentanol to remove EtBr, dialyzed against 0.1× SSC (SSC = 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0), and used in the assays.

Extraction of high-molecular-weight cell DNA. Cells were harvested from roller bottles or T150 flasks (Corning) with 0.05% trypsin and 1 mM EDTA. The cells were washed twice with phosphate-buffered saline and resuspended in 1× SSC at a concentration of approximately 10⁷ cells/ml. Sodium dodecyl sulfate (SDS) and proteinase K (E. Merck) were added to a final concentration of 0.5% and 100 μg/ml, respectively, and incubated for 2 h at 37°C. The digests were extracted twice with phenol and once with a chloroform-isoamyl alcohol mixture (24:1), and the DNA was collected by spooling (2). The spooled DNA was air dried, dissolved in 1× SSC, and digested sequentially with 100 μg of RNase per ml for 1 h and 100 μg of proteinase K per ml for 2 h. The aqueous phase was extracted once with phenol and once with a chloroform-isoamyl alcohol mixture, and the DNA was collected by spooling. The DNA was sterilized by immersion in 70% ethanol for a few hours, dissolved in sterile 0.1× SSC, and stored at 4°C. The A_{260}/A_{280} ratio of the DNAs was 1.8 to 1.9. The size of the DNA was greater than 30 × 10⁶ daltons as determined by its migration through agarose gels.

Assay of infectious viral DNA. Dulbecco minimal essential medium supplemented with 5% calf serum was used throughout the transfection experi-

ments. Recipient Cf2Th cells were plated at 2 × 10⁵ cells per 35-mm dish the day before transfection. Overnight medium on the recipient cultures was replaced with 2 ml of fresh medium on the day of transfection. DNA in the transfection assay samples was maintained at 40 μg/ml by adding carrier DNA extracted from uninfected Cf2Th cells. The DNAs were sheared through a 20-gauge syringe needle 10 times, and the samples were prepared for transfection by the calcium phosphate coprecipitation procedure (14, 33). CaCl₂ was added to a final concentration of 0.13 M, and the samples were left at room temperature for 20 min. Then 0.25-ml portions of each sample were added directly to the medium in 35-mm dishes and incubated at 37°C for 4 h. The cultures were washed once with medium, and each plate was treated with 0.2 ml of 25% dimethyl sulfoxide (DMSO) for 2 min. DMSO was removed; then the cultures were washed twice and replaced with 2 ml of medium. This concentration of DMSO showed no significant cytopathic effect on the recipient cultures.

Two days after transfection the cells were transferred from 35-mm to 60-mm dishes, the medium was changed every 3 to 4 days, and the cells were split 1:10 at weekly intervals. Between 3 and 5 weeks after transfection, the media were assayed for virus production by testing for sedimentable DNA polymerase activity using polyriboadenylate-oligodeoxythymidylate [poly(rA)·oligo(dT₁₂₋₁₈)] template-primer (4, 37). Incorporation of 1 pmol or more (40,000 cpm/pmol) of [³H]TMP into the polydeoxythymidine product in a 60-min reaction at 37°C constitutes a polymerase-positive result. Quantitation of infectivity in the DNA samples was determined by endpoint dilution of donor DNA (11). A specific infectivity of 5 ID₅₀ units/μg was calculated for the cell DNAs of clones 34 and 35, and a specific infectivity of 10⁵ ID₅₀ units/μg was calculated for unintegrated M7 DNA (1 ID₅₀ unit = amount required to infect half the cultures). Transfection assays could be carried out on either bat lung fibroblasts, A549 cells, or Cf2Th cells.

Digestion of DNAs with restriction endonucleases. Restriction endonucleases used were purchased either from New England Biolabs, Beverly, Mass., or Bethesda Research Laboratories, Rockville, Md., and digestions were carried out according to the supplier specifications. The completeness of the digestions was monitored by including phage lambda DNA in the incubation mixture. Double-enzyme digestions were done sequentially. Control DNA samples were mock incubated in the absence of enzyme. The digestion reactions were stopped by adding EDTA to a final concentration of 0.02 M and incubating at 65°C for 5 min. Before electrophoresis, the DNA samples were brought to a final concentration of 10% glycerol and 10 μg of bromophenol blue per ml.

Gel electrophoresis and transfer of DNA. Agarose (SeaKem) gels (0.8%) were poured to 0.6-cm thickness on to a horizontal slab gel unit (Bethesda Research Laboratories, model H1) and electrophoresed in Tris-acetate buffer, pH 7.9, at room temperature (27). After electrophoresis, the DNA was denatured in situ and blotted onto a 0.45-μm nitrocellulose membrane (Schleicher & Schuell Co., Keene, N.H.), according to the procedure described (31).

Preparation of viral RNA and cDNA Probes.

M7 viral 70S RNA was prepared from freshly harvested and banded virus by disrupting at room temperature in a solution containing 1% SDS and 0.2% (vol/vol) diethylpyrocarbonate in 10 mM Tris-hydrochloride, pH 7.2, and centrifuging in a 15 to 30% sucrose gradient as described (24). Polyadenylate [poly(A)]-containing viral RNA used to synthesize the cDNA complementary 3' end of viral RNA was prepared by partial degradation of the 70S RNA at 95°C for 5 min in 50 mM Tris-hydrochloride (pH 7.8) and sedimenting in a sucrose gradient. Fractions of 12 to 18S were pooled, and the poly(A)-containing RNA was selected by two cycles of chromatography on oligo(dT)-cellulose (type 1, Collaborative Research, Inc., Waltham, Mass.) as described (28). rRNA was prepared from Cf2Th cells (1), and the peak fractions of 28 and 18S rRNA on the sucrose gradients were used to make the cDNA probes.

Single-stranded [^{32}P]cDNA's were synthesized in a reaction catalyzed by avian myeloblastosis virus (AMV) DNA polymerase and primed with oligomers of calf thymus DNA (35). Briefly, 5 μg of viral 70S RNA was added to a 0.25-ml reaction consisting of 50 mM Tris-hydrochloride (pH 8.0), 1 mM dithiothreitol, 8 mM MgCl$_2$, 50 mM NaCl, 150 μg of actinomycin D per ml, 0.1 mM each of dATP, dGTP, and dTTP, 0.2 mCi of [^{32}P]dCTP (400 Ci/mmol; New England Nuclear Corp., Boston, Mass.), 200 μg of calf thymus fragments, and 50 U of AMV DNA polymerase. The reaction was incubated for 60 min at 37°C and extracted with an equal volume of phenol saturated with 50 mM Tris-hydrochloride (pH 8.0). The aqueous phase was chromatographed on a Sephadex G-50 column to remove unincorporated [^{32}P]dCTP. Peak fractions were pooled and treated with 0.5 N NaOH at 37°C for 2 h, neutralized, and precipitated with ethanol. [^{3}H]cDNA was made in an endogenous reaction from detergent-disrupted virions as previously described (5).

Hybridization procedure. The nitrocellulose membranes containing the blotted DNAs were baked at 80°C in vacuum for 2 h, prehybridized (38) in a solution consisting of 50% formamide, 3× SSC, 0.1% (wt/vol) each of bovine serum albumin, polyvinylpyrrolidone, and Ficoll 400, 50 mM sodium phosphate (pH 6.5), 1% glycine, and 25 μg of sonicated, denatured salmon sperm DNA per ml, and incubated at 42°C for at least 2 h. The membranes were then hybridized in the same solution containing 2 × 10^6 cpm of [^{32}P]cDNA per ml for 16 to 18 h at 42°C. The filters were washed (38), air dried, and exposed to X-ray films at −70°C by using two Du Pont Lightning Plus intensifying screens (34).

RESULTS

Unintegrated M7 linear DNA is infectious. Unintegrated DNA prepared from permissive Cf2Th cells 40 h after infection was separated into a linear DNA fraction and a covalently closed circular DNA fraction by equilibrium centrifugation on CsCl-EtBr density gradients. The DNA fractions were electrophoresed through agarose gels and transferred to nitrocellulose membranes, and the virus-specific DNA was identified by annealing to labeled viral cDNA.

The linear DNA fraction contained a single species of linear viral DNA with a size of 9.0 kb. The linear DNA fraction was assayed for infectivity by transfecting Cf2Th cell recipients by the calcium phosphate precipitation method (14, 33). Virus production was detected by assaying the cell supernatants for sedimentable DNA polymerase activity (4, 37). All of the cultures transfected with the linear M7 DNA produced infectious virus. The progeny virus had the same host range properties and, as determined by competitive radioimmunoassays, the same major group-specific protein p30 of M7.

Specific infectivity of the viral DNA was determined by assaying serial threefold dilutions of the linear DNA preparation for virus production. The amount of virus-specific DNA in the preparation was calculated by nucleic acid hybridization using the [^{32}P]cDNA probe. We estimate that there were 10^5 ID$_{50}$ units of viral DNA per μg; similar values have been reported for unintegrated DNAs of other retroviruses (16, 30).

Restriction map of the infectious unintegrated linear DNA. Figure 1 shows the sizes of the viral DNA fragments generated after digestion of the 9.0-kb linear DNA with different restriction enzymes. *Pvu*I had not altered the size of the DNA, indicating that this enzyme lacks recognition sequences on the viral DNA. *Eco*RI generated fragments of 5.0 and 4.0 kb, *Hind*III generated fragments of 6.2 and 2.8 kb, and *Sal*I generated fragments of 7.4 and 1.6 kb. The sum of the sizes of the two fragments in each of the digests equaled the size of the undigested DNA. This is consistent with the interpretation that the enzymes *Eco*RI, *Hind*III, and *Sal*I each make one cut in the linear DNA.

Digestion of the DNA with *Bgl*II produced fragments of 4.7, 3.1, and 1.2 kb; the combined size of these fragments equals the unit size of viral DNA, showing that this enzyme makes two cuts in the viral DNA. Digestion of the DNA with *Kpn*I produces three fragments of 4.2, 2.9, and 1.3 kb, which do not add up to the size of viral DNA, indicating that this enzyme makes a minimum of three cuts in the viral DNA.

Digestion of the viral DNA with *Xho*I reduced its size by approximately 0.6 kb. To determine if *Xho*I cuts at one or both ends of the linear viral DNA, double digestion of the linear DNA with *Xho*I and with single-cut enzymes *Eco*RI and *Hind*III were performed. Double digestion of the DNA with *Xho*I and *Eco*RI showed that the size of each of the two *Eco*RI fragments was reduced by approximately 0.3 kb. Similarly, double digestion of the viral DNA with *Xho*I and
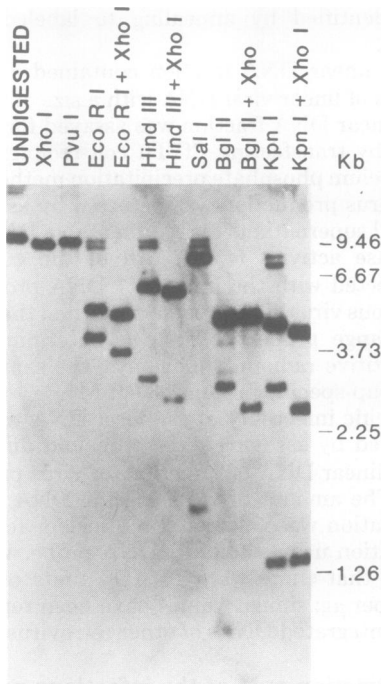
FIG. 1. *Restriction enzyme mapping of M7 linear DNA. Unintegrated viral DNA was prepared from Cf2Th cells 40 h after infection with M7 virus. The DNA was separated into a linear and a covalently closed circular fraction by isopycnic banding on CsCl-EtBr gradients. The linear DNA fraction was digested with the indicated enzymes and subjected to electrophoresis in 0.8% agarose gels. DNA fragments were transferred to 0.45-μm nitrocellulose membrane by the method of Southern (31). Virus-specific DNA was detected by autoradiography after annealing with cDNA representing the entire viral genome. Molecular weights were determined from HindIII digest of bacteriophage lambda DNA and HpaI digest of φX174 DNA run in parallel lanes. Kb represents the size in kilobase pairs. The light doublet band seen in different lanes represents contamination of linear DNA with form II DNA. Accurate determination of the DNA fragment size below 1.26 kb could not be made in this gel. However, from other experiments using low-molecular-weight markers, the size of the BglII fragment was determined to be approximately 1.2 kb.*

HindIII showed that the size of each of the two HindIII fragments was reduced by approximately 0.3 kb. This reduction of size in each of the two EcoRI fragments and in each of the two HindIII fragments shows that XhoI has a recognition sequence approximately 0.3 kb from each end of the linear DNA. This result was confirmed by the digestion of the circular viral DNA species with XhoI (see Fig. 4). We show later that the linear viral DNA has a redundant

sequence of 0.6 kb at each terminus (terminal repeats) and XhoI has a recognition sequence at each end.

Since XhoI cuts near the ends of viral DNA, it was used in double-digestion experiments to distinguish terminal fragments from central fragments. Double digestion of viral DNA with BglII and XhoI compared with digestion with BglII alone showed that the size of the 4.7-kb fragment remained unchanged, whereas the size of each of the 3.1- and 1.2-kb fragments decreased. This indicates that the 4.7-kb fragment is the central fragment and the other two are terminal fragments. Similarly, double digestion of the linear DNA with KpnI and XhoI showed that the 4.2- and 2.9-kb fragments of KpnI are the terminal fragments.

To orient the ends of viral DNA with respect to the 3' and 5' ends of viral RNA, viral restriction products were hybridized to a 3'-specific [^{32}P]cDNA probe. The 5.0-, 6.2-, and 7.4-kb fragments of EcoRI, HindIII, and SalI, respectively, were preferentially hybridized (Fig. 2), indicating that they are homologous to the 3' end of the viral RNA. In longer exposures, low levels of hybridization to other restriction fragments were also detected. These could represent hybridization due to terminal repeat sequences in the 3' probe, as has been reported for other retroviruses (17). Similar hybridizations with a 3'-specific probe using the digest of BglII and KpnI showed the 1.2-kb fragment of BglII and the 4.2-kb fragment of KpnI to be the 3' ends. By using these data and additional double-digestion experiments, a restriction map of the infectious linear DNA of the M7 virus was constructed (Fig. 3).

Circular species of unintegrated M7 DNA. Electrophoresis of the covalently closed circular DNA preparation in agarose gels showed two closely migrating species moving faster than the 9.0-kb linear DNA (Fig. 4). The faster-migrating species was in two- to threefold excess (compared by their relative intensities) over the more slowly migrating species. Storage of this DNA preparation at either −20 or 4°C generated four additional bands; two migrated in the region of approximately 14 kb, and two migrated in the region of 9.0 kb, indicating that these products resulted from the degradation of the faster-moving species.

Digestion of the DNA with EcoRI resulted in the disappearance of the fast- and slow-migrating doublet bands with concomitant appearance of two bands of 9.0 and 8.4 kb. We conclude from these observations that the fast-moving viral DNA bands represent the covalently closed supercoiled DNAs (form I), the slow-moving viral

DNAs represent the nicked circular DNAs (form II), and the 9.0- and 8.4-kb DNAs are linear molecules (form III). The two minor bands at 5.0 and 4.0 kb in the EcoRI digest are a result of EcoRI digestion of the linear DNA contaminating the circular DNA preparation. These bands correspond to the EcoRI fragments of linear DNA shown in an adjacent lane of the gel. Similar results were obtained by digestion of the circular DNA preparation with another one-cut enzyme, HindIII.

Digestion of the two circular DNA species with XhoI, which only cut in the terminal repeats, produced a single common fragment of 8.4 kb. This fragment corresponded in size to that produced from digestion of the 9.0-kb linear DNA with XhoI. This indicates that the two circular forms differ in their terminal repeat sequences. In addition to the 8.4-kb common fragment, another of approximately 0.6 kb was also detected in the XhoI-digested circular
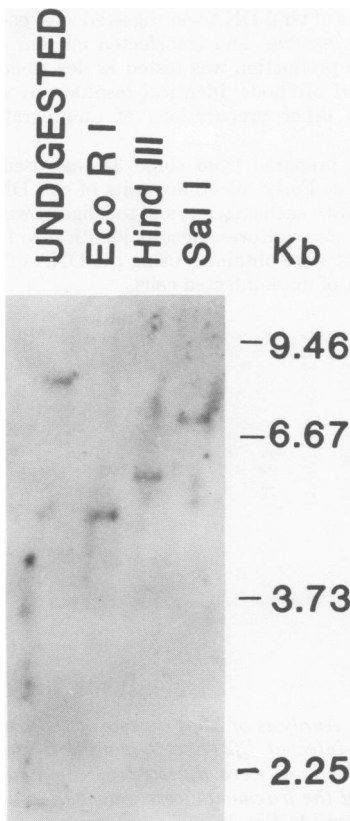

FIG. 2. Detection of 3' end of linear viral DNA. Linear M7 DNA was digested with the enzymes indicated. The resultant DNA products were electrophoresed in 1% agarose gels. Viral DNA identified by annealing with [^{32}P]cDNA$_3$' made from poly(A)-containing 12 to 18S viral RNA.
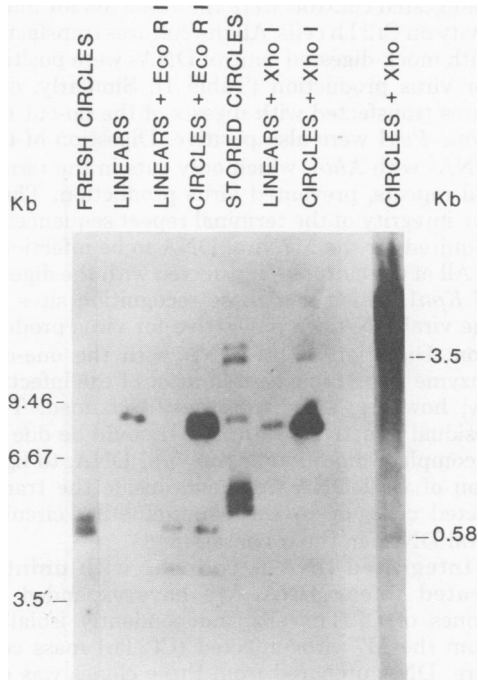

FIG. 4. Circular forms of unintegrated viral DNA. Total unintegrated viral DNA prepared from virus-infected Cf2Th cells was separated into a circular and a linear fraction by isopycnic banding on CsCl-EtBr gradients. The circular DNA fraction was digested with the indicated enzyme. The resultant products were electrophoresed in 0.8% agarose. The gel was treated with 0.25 N HCl before alkali treatment, and the virus-specific DNA was detected as described in the legend to Fig. 1. The autoradiogram was deliberately overexposed to show the weak bands in lanes 2 and 3. In short exposures, the 9.0- and 8.4-kb EcoRI bands in lane 4 were distinct.
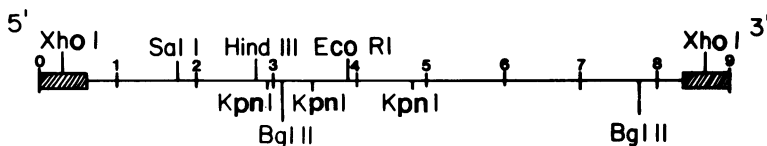

FIG. 3. Restriction endonuclease cleavage sites in linear M7 DNA. The slashed squares at the termini indicate the terminal repeat sequences. The numbered bars show calibration in kilobase pairs from 5' to 3' with respect to viral RNA.

DNAs. We conclude from these results that the larger form has two terminal repeat sequences, and therefore two XhoI sites, and the smaller has one terminal repeat, and therefore a single XhoI site.

Terminal repeat sequences are required for infectivity. Both the unintegrated linear DNA and the DNA extracted from the virus-infected cells were infectious. These infectious DNAs were digested separately with PvuI, XhoI, KpnI, and EcoRI. The digested DNAs and the undigested controls were then assayed for infectivity on Cf2Th cells. All the cultures transfected with mock-digested control DNAs were positive for virus production (Table 1). Similarly, cultures transfected with digests of the no-cut enzyme PvuI were also positive. Digestion of the DNAs with XhoI, which only cuts in the terminal repeats, prevented virus production. Thus, the integrity of the terminal repeat sequences is required for the M7 viral DNA to be infectious.

All of the cultures transfected with the digests of KpnI, which had three recognition sites on the viral DNA, were negative for virus production. Digestion of the DNA with the one-cut enzyme EcoRI inactivated most of the infectivity; however, some infectivity remained. The residual infectivity with EcoRI could be due to incomplete digestion of the viral DNA, to ligation of viral DNA fragments inside the transfected cells, or to the contaminating circular viral DNAs in the preparation.

Integrated DNA is colinear with unintegrated linear DNA. We have expanded 14 clones of Cf2Th cells, independently isolated from the M7 virus-infected (Cf2Th) mass culture. DNA prepared from these clones was digested with different restriction enzymes, and the resulting fragments were analyzed to determine the sites of viral and cellular DNA involved in integration.

To determine how much of the viral genome in the infected cells is intact, we used XhoI, which cuts only in the terminal repeats. Figure 5 shows the virus-specific bands obtained from the DNA of representative clones. In each of the DNAs analyzed, an 8.4-kb internal viral fragment was present. This 8.4-kb fragment corresponded in size to that generated from XhoI digestion of unintegrated viral DNA run in an adjacent well. There was no band corresponding to this size in the DNA extracted from uninfected Cf2Th cells. This result shows that the arrangement of the integrated viral sequences in these clones was similar to that of the unintegrated linear viral DNA within the large internal fragment bounded by XhoI sites. The viral DNA integrated with at least part of the terminal repeats intact, and the junction of the viral and

TABLE 1. Transfection by baboon viral DNA

Enzyme	No. of polymerase-positive cultures	
	Unintegrated viral DNA[a]	Infected-cell DNA[b]
Control	4	4
PvuI	4	4
XhoI	0	0
KpnI	0	0
EcoRI	1	1

[a] Unintegrated viral DNA was prepared from Cf2Th cells 40 h after infection with M7. Forty infectious units of viral DNA was digested with each of the indicated enzymes and transfected on four cultures, and virus production was tested as described in Materials and Methods. Identical results were obtained with two other preparations of unintegrated viral DNA.

[b] DNA prepared from clone 34 was used in this experiment. Forty infectious units of cell DNA were digested with each enzyme, and the digests were transfected on four cultures. Results identical to the DNA of clone 34 were obtained, using the DNA of clone 35 and DNA of mass-infected cells.
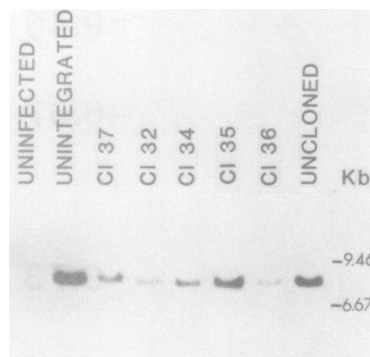

FIG. 5. Analysis of XhoI digests of DNA prepared from M7-infected Cf2Th cell clones. DNAs of the indicated clones were digested to completion with XhoI, and the fragments were detected as described in the legend to Fig. 1.

cell DNA occured outside of XhoI sites. The intensity of the 8.4-kb band was different in different clones, indicating that the number of integrated viral DNA copies is different in these clones.

To examine more closely how extensively the viral DNA sequences in the integrated state were retained, double digestions of the DNAs were carried out. Double digestion with EcoRI and XhoI produced only two bands of approximately 4.7 and 3.7 kb (Fig. 6) from the DNA of each clone. These two bands corresponded in size to those generated by similarly digesting unintegrated linear viral DNA. The DNA of
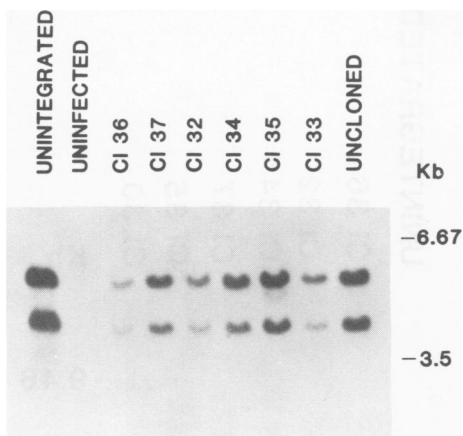
FIG. 6. *Analysis of combined EcoRI and XhoI digests of DNA prepared from virus-infected Cf2Th cell clones. The DNAs of the clones were digested, and the resultant products were electrophoresed in 0.8% agarose gels. The virus-specific fragments were analyzed as described in the legend to Fig. 1.*

clone 35, however, was an exception in that it produced an additional light band of approximately 4.4 kb. Double digestion of the DNAs with *BglII* and *XhoI* showed three bands of 4.7, 2.8, and 0.9 kb from each clone, and they corresponded in size to those produced by similar digestion of the unintegrated linear DNA (not shown). Again, the DNA of clone 35 was an exception in that it produced an additional band of 4.4 kb. There were no bands corresponding to this size in the DNA from uninfected cells. We conclude from these experiments that the integrated M7 DNA is colinear with the unintegrated infectious linear M7 DNA. Similar results were obtained with the DNAs prepared from independently derived clones of M7 virus-infected bat cells and human cells.

The extra band of 4.4 kb in the DNA of clone 35 can be explained by hypothesizing a deletion of approximately 0.3 kb in the viral DNA. The intensity of this additional band corresponded to that of a similar fragment produced from the DNA of a clone containing a single copy of M7 DNA. Supporting evidence for the existence of a deletion in the DNA of clone 35 was obtained from additional double digestions with *XhoI* and *BglII* and with *KpnI* and *BglII* and are not shown here. From these results, we conclude that one of the five M7 DNA copies in the DNA of clone 35 has a deletion of approximately 0.3 kb and is located in the 3.0-kb fragment bound by *KpnI* and *BglII* restriction sites on the right end of the viral DNA.

Viral DNA can integrate at many sites in cellular DNA. The most direct approach to determine the cellular sites of integration would be to digest the M7-infected cell DNA with *PvuI*, which does not cleave the viral DNA but cleaves the cell DNA. The resulting digestion products contained complete viral DNA sequences and some cellular DNA sequences that flank the viral DNA. Determination of the size of these fragments would give the minimum number of integration sites in the DNA analyzed. Unfortunately, *PvuI* cuts Cf2Th cell DNA very infrequently, generating fragments of an average size of 30 kb. DNA fragments of this size range are not resolvable with the presently available agarose gel electrophoretic system, and therefore we have not followed this approach.

*Eco*RI makes a single cut on the unintegrated viral DNA, and therefore digestion of the DNAs from virus-infected cell clones produced only fusion fragments consisting partially of viral and partially of cellular sequences. Results from representative clones presented in Fig. 7 show that the DNA from each clone exhibited unique patterns of virus-cell fusion fragments. They ranged from relatively simple patterns, as in clones 30, 32, and 33, to more complex patterns, as in clones
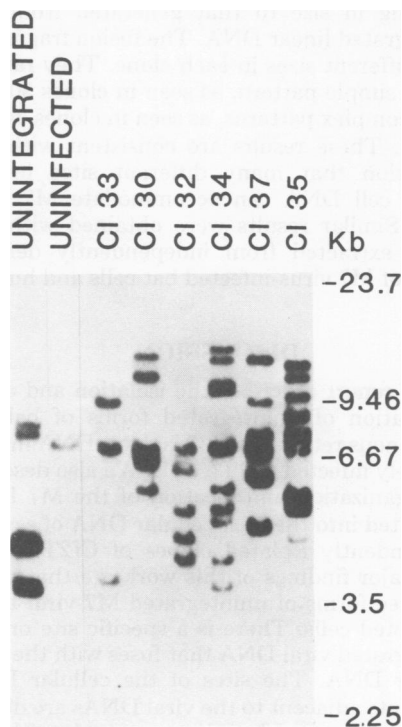

FIG. 7. *Analysis of EcoRI digests of DNAs prepared from M7-infected Cf2Th cell clones. The DNA of each clone was digested to completion with EcoRI, and the DNA fragments were detected as described in the legend to Fig. 1.*

34, 35, and 37. Each fusion fragment was larger than the smallest fragment of EcoRI-digested unintegrated viral DNA. The common band found at approximately 8.0 kb was also found in the DNA of uninfected cells and was due to contamination of the labeled probe with ribosomal DNA. From the above results, we make the following conclusions. Many different sites in the cellular DNA can accommodate viral DNA. The viral DNAs are not integrated in a tandem manner. The cellular DNA is not significantly contaminated with unintegrated viral DNA. Similar analysis was carried out with another single-cut enzyme, HindIII, and the results obtained were similar to those obtained with EcoRI.

The conclusions were confirmed by analysis of the DNAs using BglII. Digestion of the unintegrated linear DNA with BglII produced a large central fragment and a 5'- and a 3'-terminal fragment; BglII digestion of DNAs from the infected cell clones should produce a common internal viral band from each clone and fusion fragments which depend on the site of integration. The DNAs analyzed (Fig. 8) showed a common internal viral band of 4.7 kb, corresponding in size to that generated from the unintegrated linear DNA. The fusion fragments were different sizes in each clone. They ranged from a simple pattern, as seen in clones 30 and 36, to complex patterns, as seen in clones 34, 35, and 37. These results are consistent with the conclusion that many different sites in the Cf2Th cell DNA can accommodate M7 viral DNA. Similar results were obtained with the DNAs extracted from independently derived clones of M7 virus-infected bat cells and human cells.

DISCUSSION

This report describes the isolation and characterization of unintegrated forms of baboon endogenous retrovirus (M7 isolate) DNA in productively infected Cf2Th cells. We also describe the organization and location of the M7 DNA integrated into the host cellular DNA of several independently isolated clones of Cf2Th cells. The major findings of this work are that there are three forms of unintegrated M7 viral DNA in infected cells. There is a specific site on the unintegrated viral DNA that fuses with the host cellular DNA. The sites of the cellular DNA fragments adjacent to the viral DNAs are different from clone to clone but are stable with cell passage.

Retroviruses replicate through a DNA intermediate (for a review, see reference 7). The single linear M7 DNA intermediate found in
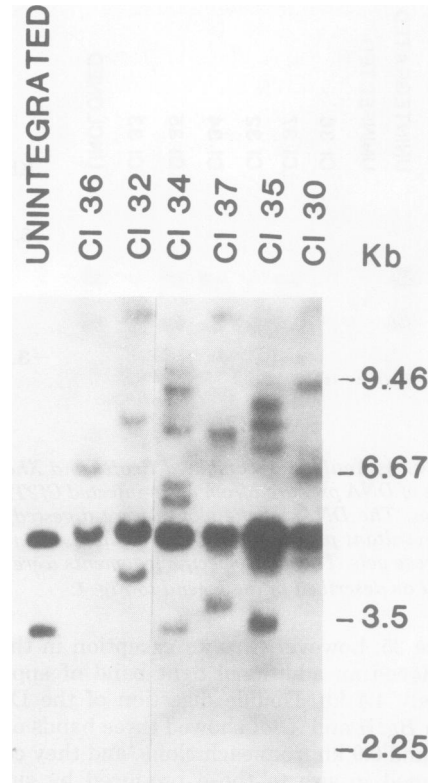
FIG. 8. Analysis of BglII digests of DNA prepared from M7-infected Cf2Th cell clones. DNA from different clones was digested to completion with BglII, and the DNA fragments were detected as described in the legend to Fig. 1.

virus-infected cells was approximately 9.0 kb and was infectious, indicating that all the information required for virus production resides in this molecule. There were two species of covalently closed circular DNA intermediates. The large circular DNA intermediate contained a direct tandem duplication of the terminal repeat sequences and was the same mass as the linear DNA. The other circular DNA intermediate contained a single copy of the terminal repeat sequence and was smaller by 0.6 kb. The linear and covalently closed circular M7 viral DNA intermediates were characterized by restriction endonuclease cleavage analysis, by electrophoretic mobilities, and by their buoyant density in CsCl-EtBr gradients. Several recent reports described the existence of linear and circular viral DNA intermediates containing terminal repeats of various length from 0.3 to 1.2 kb in cells infected with different retroviruses (8, 18, 25, 26, 28, 39). The formation of linear and circular forms of viral DNA intermediates appears to be

a common phenomenon of retroviruses in general.

The origin and relationship between the linear and the covalently closed circular viral DNA intermediates are not understood. The generation of the larger circle can be visualized by joining the two ends of the terminal repeats of the linear form with no substantial loss of sequences in the ligation process. This is consistent with the production of a 0.6-kb fragment after XhoI digestion of circular viral DNA. The size of this fragment is equal to the sum of the terminal repeat sequence fragments generated by XhoI digestion of the linear DNAs. The 0.6-kb fragment therefore includes the joining site on the ends of the linear DNA. Comparison of sequences of 0.6-kb fragment and the 0.3-kb fragments of the linear DNA will be useful in determining the circularization events. In M7-infected cells there was no linear DNA which corresponded to the size of the smaller circular form. The formation of the small circle can be explained by intramolecular recombination of the linear viral DNA containing two terminal repeats with a deletion of one repeat length (25). In M7-infected cells the smaller circle was found in two- to threefold excess over the larger circle. This could be due to higher frequency of formation or greater stability of this molecule.

M7 virus-infected dog, bat, and human cells contain viral DNA integrated into their cellular DNA sequences. Analysis of the restriction endonuclease cleavage fragments of the integrated viral DNA showed that most of the viral DNA sequences were intact, and a common site within the terminal repeat sequence of the unintegrated viral DNA was utilized to recombine with the cellular DNA sequences. Therefore, the integrated viral DNA copies do not occur as circular permutations but rather are colinear with the unintegrated M7 DNA. The integration specificity of M7 DNA resembles that of the avian (3, 10, 19, 20, 23) and murine (9, 32) retroviruses but differs from that of the DNA tumor viruses like simian virus 40 (21), where many sites on the viral DNA can recombine with the host cellular DNA sequences.

Analysis of the restriction endonuclease-cleaved virus-cell fusion fragments of different M7 virus-infected cell clones indicated that the flanking sequences in each clone were unique. Therefore, many sites on the host genome can recombine with the viral DNA. The pattern of primate retroviral DNA integrations in virus-infected cells is consistent with the recent reports on the sites of integrations of the avian (3, 10, 19, 20, 23) and the murine (9, 32) retroviral DNAs.

The unintegrated viral DNA precursor involved in recombination with host cellular DNA is not known. In M7 virus-infected cells there are two species of unintegrated viral DNA whose structure is identical with that of the integrated viral DNA sequences. These are the 9.0-kb linear DNA and the larger covalently closed circular DNA with the same mass as the linear form. Either of these two forms of M7 DNA potentially can serve as the proximal precursor for integration. There is no direct evidence on which of these is the immediate precursor for integration. Indirect evidence suggests that in the case of avian retroviruses circular DNA serves as the immediate precursor (15).

Both the unintegrated M7 linear DNA and the M7 DNA integrated into the infected dog and human cells are infectious and contain both of the terminal repeat sequences. Excision of a portion of the terminal repeat sequences by digestion with XhoI abolished the infectivity of both the unintegrated and integrated M7 viral DNA, indicating that two copies of the terminal repeat sequences are required for the infectivity. Digestion by XhoI retains the equivalent of a single terminal repeat sequence distributed on the ends of M7 viral DNA. These single terminal repeat-containing molecules, if infectious, are less than 2% as efficient as those containing two terminal repeat sequences. We have recently cloned M7 viral DNA containing single and double terminal repeat sequences in bacteriophage lambda and are using these reagents to further investigate the mechanism of viral DNA integrations.

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