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The genome of frog virus 3, an animal DNA virus, is circularly permuted and terminally redundant

(genome structure/restriction endonuclease mapping/DNA replication)

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ABSTRACT **We examined the structure of the frog virus 3 (FV 3) genome by using electron microscopic and biochemical techniques. The linear FV 3 DNA molecules ($M_r \approx 100 \times 10^6$) formed circles when partially degraded with bacteriophage λ 5′-exonuclease and annealed, but not when the annealing was done without prior exonuclease digestion. The results suggest that the DNA molecules contain direct terminal repeats. The repeated region composed about 4% of the genome. Complete denaturation of native FV 3 DNA molecules followed by renaturation produced duplex circles each bearing two single-stranded tails at different points along the circumference. The tails presumably represent the terminal repeats. The formation of duplex circles suggests that the FV 3 genome is circularly permuted. This is further borne out by (*i*) failure to identify a specific restriction endonuclease fragment containing the label when the molecular ends were radiolabeled by using the polynucleotide kinase procedure, and (*ii*) similarity in the restriction patterns of virion DNA and large concatemeric replicating viral DNA as revealed by endonucleolytic cleavage of both DNAs with *Hin*dIII. From the above data, we conclude that the FV 3 genome is both circularly permuted and terminally redundant—unique features for an animal virus.**

Frog virus 3 (FV 3) is an icosahedral DNA virus that replicates in many different vertebrate cells, including human cells, at low temperature (12–32°C) (reviewed in ref. 1). The genome is a double-stranded linear DNA molecule with a M_r of $\approx 100 \times 10^6$ (2) and a G+C content of 53% (3). A surprisingly large proportion (≈25%) of the cytosine residues in virion DNA are methylated at the 5 position (4). Quantitative and qualitative analyses of FV 3 transcripts have been greatly facilitated by resolution of the viral RNAs into 47 bands on polyacrylamide gel analysis (5). FV 3 messages lack detectable poly(A) (6) but contain methylated cap structures at their 5′ ends (7). Viral transcription occurs in at least three phases, termed immediate early, early, and late (5, 8). Unlike the situation for many other viruses, late transcription is not dependent upon viral DNA replication (9, 10). The expression of about two-thirds of the viral genes is also regulated at the posttranscriptional level (5). The posttranscriptional control in the infected cells is mediated through several viral proteins (11). FV 3-specific protein(s) selectively enhance(s) the translation of late viral messages in a mRNA-dependent *in vitro* protein synthesizing system (R. Raghow and A. Granoff, personal communication).

The replication strategy of FV 3 is novel in that the virus utilizes both the host cell nucleus and cytoplasm for its nucleic acid synthesis but assembles in the cytoplasm (12). Recent data suggest that FV 3 DNA, like bacteriophages T4 (reviewed in ref. 13) and λ (reviewed in ref. 14) DNA, replicates in two stages.* These two stages are distinguished by differences in their temporal appearance during the infection, size of the replicating molecules, and site of synthesis. During the first stage, the smaller replicating DNA molecules (equal to or less than twice the genome length) are synthesized exclusively in the nucleus early in infection (up to 3 hr after infection). During the second stage, extremely large replicating complexes are synthesized only in the cytoplasm and late in infection (after 3 hr after infection). The large replicative complex acts as the precursor for the production of mature viral DNA size molecules.*

To complement these studies on DNA replication and to understand the structural organization of the genome, we have examined virion DNA by electron microscopy and restriction endonuclease analysis. In this paper, we show that the FV 3 genome is terminally redundant and circularly permuted—unique features for an animal virus.

MATERIALS AND METHODS

Cells and Virus. Fathead minnow (FHM) cell monolayers were grown at 33°C in plastic roller bottles with Eagle's minimal essential medium containing 5% fetal calf serum. A clonal isolate of FV 3 was used to infect cells at a multiplicity of 0.1 plaque-forming unit per cell. FV 3 virions were purified as described (12). To minimize nicks and gaps in the viral DNA, crude virus preparations were not sonicated, freeze-thawed, or pelleted.

Extraction of Viral DNA. DNA from purified FV 3 preparations was extracted by the phenol/chloroform procedure (15).

Digestion of DNA with λ 5′-Exonuclease. The procedure of Grafstrom *et al.* (16) was used with some modifications. The reaction mixture (total volume 0.1 ml) containing DNA at 10 μg/ml, enzyme (New England BioLabs) at 160 units/ml, bovine serum albumin at 50 μg/ml, 1 mM 2-mercaptoethanol, 3 mM $MgCl_2$, 67 mM glycine buffer (pH 9.6) was incubated for 30 min at 23°C. The reaction was stopped by cooling to 4°C and adding 1/10th vol of 3 M NaCl/0.3 M sodium citrate. The partially digested DNA was annealed at 75°C for 30 min, then incubated at room temperature for 12 hr. The samples were then prepared for electron microscopy. The extent of digestion was monitored by determining the trichloroacetic acid-soluble radioactivity after exonuclease treatment, using [^{14}C]thymidine-labeled FV 3 DNA as substrate.

Denaturation and Renaturation. Ten microliters of DNA (0.2 μg) was mixed with 50 μl of pH 12 buffer (0.6 M NaCl/0.1 M $Na_3PO_4 \cdot 12H_2O$) and the mixture was held at 22°C for 10 min. The sample was brought to neutral pH by the addition of 10 μl of solution containing 1.0 M $NaH_2PO_4 \cdot H_2O$ (pH 4.27). The sample was then mixed with 50% (vol/vol) formamide/0.1 M Tris/0.01 M EDTA (pH 8.5), and kept at 34°C for 1 hr.

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Abbreviation: FV 3, frog virus 3.

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Radiolabeling of the 5′ Ends of DNA by the Polynucleotide Kinase Procedure. The 5′ termini of FV 3 or λ DNA were phosphorylated by sequential enzymatic treatment with phosphate and polynucleotide kinase (17). Phosphate groups at 5′ ends were removed by bacterial alkaline phosphatase (Collaborative Research, Waltham, MA). The reaction mixture (total volume 0.5 ml), containing 1 mM DNA, 0.1 M Tris·HCl (pH 8.0), and the enzyme at 2 units/ml, was incubated at 37°C for 60 min. The reaction was stopped by phenol extraction (twice) of the DNA, followed by extensive dialysis against 0.05 M Tris·HCl (pH 7.6). The free OH groups of the phosphatase-treated DNA were radiolabeled with [γ-^{32}P]ATP by polynucleotide kinase (Collaborative Research). The reaction mixture (total volume 0.3 ml), containing 5 μmol of DNA, 0.01 M $MgCl_2$, 0.01 M 2-mercaptoethanol, 0.05 M Tris·HCl (pH 7.6), 5 nmol of [γ-^{32}P]ATP (specific activity 4100 Ci/mmol, ICN; 1 Ci = 3.7×10^{10} becquerels), and 150 units of polynucleotide kinase, was incubated at 10°C for 12 hr. Free [γ-^{32}P]ATP was removed by extensive dialysis against buffer containing 0.5 M NaCl, 0.01 M Tris·HCl, and 1 mM EDTA (pH 8). The efficacy of 5′-terminal labeling was measured by determining the acid-precipitable radioactivity. Reaction endonuclease (*Hin*dIII) digestion and gel electrophoresis of the digested DNA was performed as described below.

Restriction Enzyme Digestion. *Hin*dIII enzyme digestions were performed at a ratio of 4 units of enzyme per μg of DNA according to the directions of the supplier (Bethesda Research Laboratories). DNA samples (0.5–1 μg) were electrophoresed on a 0.7% agarose gel submerged in F buffer (40 mM Tris, pH 7.8/20 mM sodium acetate/2 mM EDTA) for 18 hr at 50 V. Gels containing [^{3}H]DNA were impregnated with EN^{3}HANCE autoradiography enhancer (New England Nuclear) before drying and exposing to Kodak XAR-5 film at −70°C for 2–3 days. Gels with [^{32}P]DNA were fixed (twice) in 1% lanthanum acetate/1% acetic acid for 1 hr, dried, and exposed to Kodak XAR-5 film at −70°C in the presence of a Du Pont Cronex intensifying screen for 3–5 days.

Electron Microscopy. The DNA (1–2 μg/ml) in 50% (vol/vol) formamide/0.1 M Tris/0.01 M EDTA (pH 8.5) was prepared for electron microscopy as described (18). The DNA was examined with a Philips 301 electron microscope. Contour lengths of the molecules were measured with a Numonics 1224 graphic digitizer. Native and denatured PM2 bacteriophage DNAs were used as size standards.

RESULTS

Evidence for the Presence of Direct Terminal Repeats. The genome of FV 3 is a linear duplex molecule with a M_r of 100×10^6 (2, 19). In linear bacteriophage genomes, the presence of direct terminal repeats (identical sequences at both ends) has been demonstrated by the classic experimental approach of Thomas and colleagues (20). Limited digestion of DNA molecules with either a 3′ or a 5′-specific exonuclease exposes single strands at the ends of the molecules. If the exposed ends are complementary, renaturation of these molecules leads to circle formation. We have done this experiment with FV 3 DNA, using λ 5′-exonuclease. FV 3 DNA was partially digested (7.3–10.6%) with λ exonuclease, annealed for 12 hr at 22°C, and examined in the electron microscope. Of the 297 molecules observed in three samples, 80–85% were linear duplexes with single-stranded ends and 15–20% were duplex circles each con-

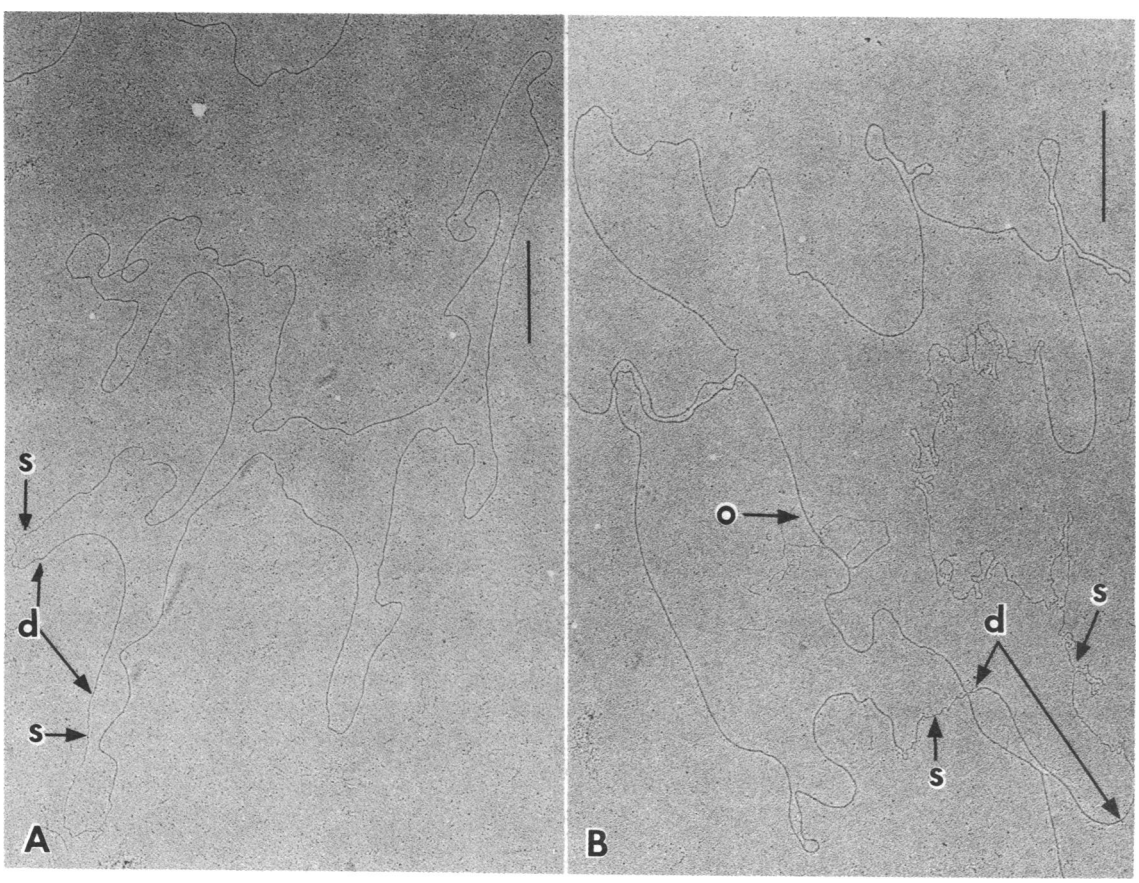

FIG. 1. Electron micrographs of FV 3 DNA molecules digested with λ 5′-exonuclease and annealed for 12 hr at 22°C. The two circles illustrated (*A* and *B*) each contain a duplex region (d) flanked by single-stranded gaps (s). An overlapping linear molecule (o) is present in *B*. Bars equal 1 μm.

taining two single-stranded gaps. Examples of the latter are shown in Fig. 1 *A* and *B*. The circles had a mean (±SD) contour length of 48.10 ± 6.0 μm, a value slightly lower than that of the native linear molecules (52.00 ± 5.60 μm) (19). The discrepancy is presumably due to errors in the measurement of less-stretched circular molecules.

No circles were observed when annealing was performed without prior digestion with exonuclease; thus it is very unlikely that FV 3 contains "cohesive" ends (short, complementary single strands on opposite strands at opposite ends) as found in bacteriophage λ (21). The result establishes that circularization of the FV 3 DNA molecule depends on the exposure of complementary strands by the exonuclease.

The length of direct terminal repeats, in principle, can be determined by measuring the amount of partial digestion required to give the maximum frequency of circularization after renaturation. However, an accurate determination of length by this approach requires synchronous digestion of DNA molecules. Digestion by the enzyme was not uniform in this study because the single-stranded ends of linear duplex molecules and the single-stranded gaps in circular molecules were of heterogeneous length (compare the single-stranded gaps in Fig. 1 *A* and *B*). The reasons for this variable digestion are not clear, but similar results have been reported for herpesvirus DNA (16).

Another method of determining the length of the terminal repeat is to measure the short duplex region (d in Fig. 1) flanked by the two single-stranded gaps (s in Fig. 1) in the circle; the single-stranded gaps in these circles most likely arise by digestion of molecules beyond the terminal repeats. The measurement of the duplex region between the single-stranded gaps in 10 molecules gave a mean length of 2.32 ± 0.33 μm. By using PM2 DNA as a size standard (length, 3.38 μm; M_r, 6.4×10^6), the moleculear weight of the terminal repeat was calculated to be $4.38 \pm 0.62 \times 10^6$ or about 4% of the size of the native FV 3 genome.

Evidence for Circular Permutation. *(i) Circle formation.* The main evidence that FV 3 DNA is an ensemble of circularly permuted molecules was obtained by the denaturation–renaturation experiment (20). Native, duplex linear FV 3 DNA molecules were completely denatured in an alkaline buffer, and the sample was then neutralized and allowed to renature in a buffer containing 50% formamide (see *Materials and Methods*). Under these experimental conditions, about 70% (152/218; 74/109) of the DNA molecules were renatured, yielding two types of molecules: (*i*) linear duplexes with single-stranded ends (Fig. 2*A*) and (*ii*) duplex circles containing two single-stranded tails

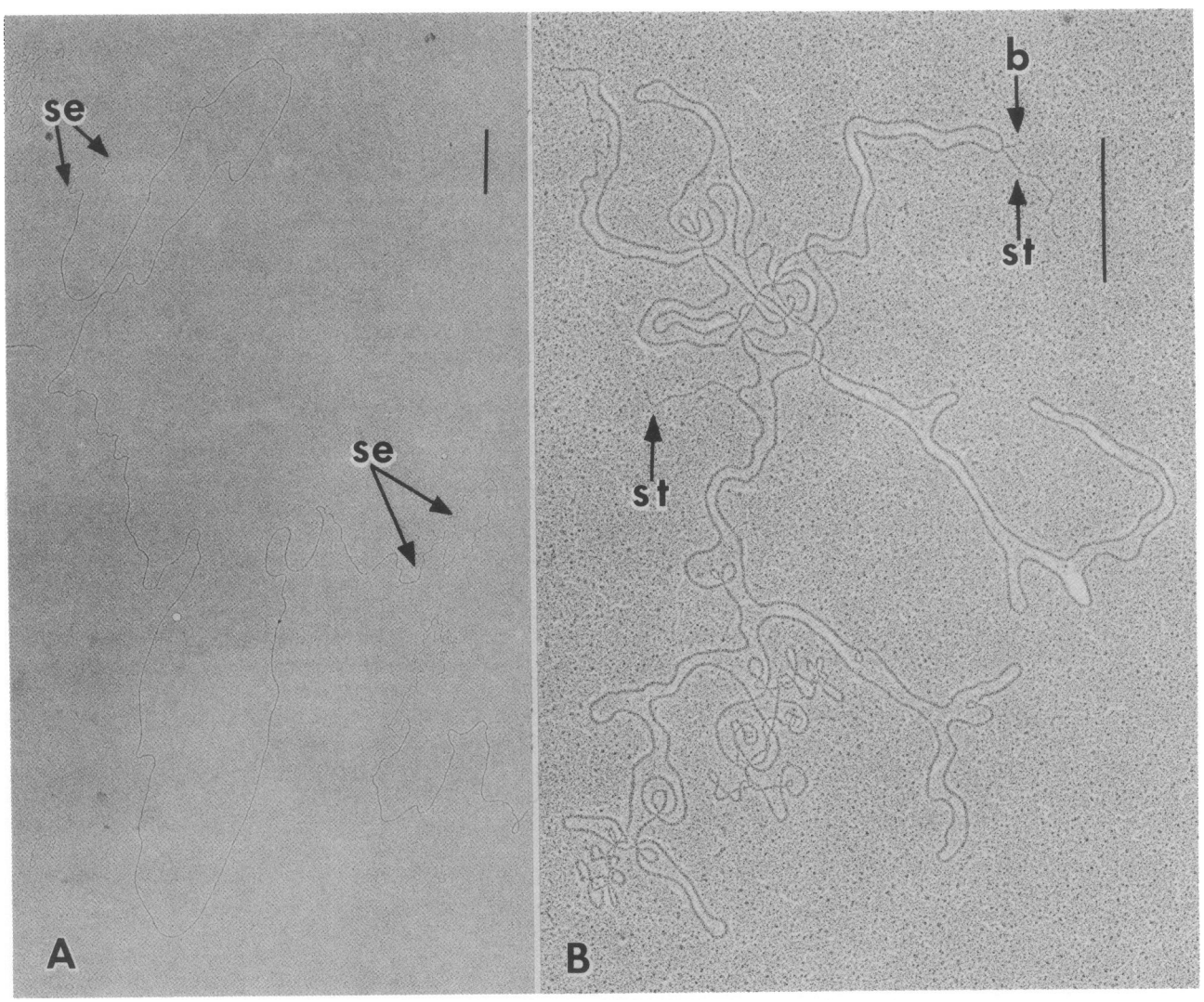

FIG. 2. Electron micrographs of FV 3 DNA molecules denatured with alkali and renatured for 1 hr in 50% formamide at 34°C. (*A*) Linear duplex molecule containing single-stranded ends (se). (*B*) Circular molecule containing two single-stranded tails (st). b represents an end that has undergone branch migration. Bars equal 1 μm.

attached at different points along the circumference (Fig. 2*B*). The first type of molecules occurred with a frequency of 60–70%. The length of the single-stranded ends in these molecules varied between 1% and 25% of the total contour length. Some of these molecules might have failed to circularize because of short (less than the length of the terminal repetition) complementary regions, while others with long single-stranded ends were unable to circularize due to kinetic reasons (22).

The second type of molecules, duplex circles with single-stranded tails, occurred with a frequency of 30–40%. Most of these circles had single-stranded tails attached at two different points along the circumference (st in Fig. 2*B*). The tails represent terminal repetitions in the native DNA (23, 24). The lengths of the tails measured in 20 molecules varied between 0.50 and 5.84 μm, with a mean of 3.37 $\pm$ 0.65 μm. This corresponds to 6.4% of the length of the native FV 3 genome. The wide variation in tail length possibly reflects the imprecision inherent in measuring the collapsed single-stranded region; however, the size of the terminal repeat, as estimated by this method, is in reasonable agreement with that obtained from the exonuclease experiment (see previous section). Occasionally, two single-stranded tails originated from the same point along the circumference of the circle (b in Fig. 2*B*), and these presumably represent single repetition ends that have undergone branch migration as a result of hybridization-competition (22).

The efficient circularization of the DNA upon denaturation and renaturation and the presence of two single-stranded tails on the duplex circles establish that the native FV 3 molecules are circularly permuted and terminally redundant.

(ii) Specificity of the ends. In non-circularly permuted genomes, the ends of the DNA molecules are identical. Therefore, radiolabeling of the ends with [γ-^{32}P]ATP by sequential exposure to phosphatase and polynucleotide kinase followed by restriction endonuclease digestion and analysis of restriction fragments on agarose gels can be used to identify the two end fragments containing the label (25). The ends of circularly permuted genomes will be labeled by the above procedure, but because their position within a population of DNA molecules varies, the labeled fragments will be very heterogeneous in size. Hence, no specific fragment should be radiolabeled, and the radioactivity should become part of the background upon gel analysis.

In a phosphatase/polynucleotide kinase reaction, radioactivity was incorporated into FV 3 DNA, but we were unable to detect any labeled restriction fragments (data not shown). The failure to radiolabel the end fragments was not due to some anomaly in FV 3 DNA, because digestion of FV 3 DNA with restriction endonuclease *Hin*dIII followed by polynucleotide kinase reaction labeled all the restriction fragments. Furthermore, the end fragments of phage λ DNA were radiolabeled by this procedure under identical conditions. Thus, our inability to detect the end fragments after polynucleotide kinase reaction is consistent with the conclusion that FV 3 DNA is circularly permuted.

(iii) Restriction endonuclease analysis of virion DNA compared with concatemeric DNA. When large concatemers produced from non-circularly permuted genomes are cleaved by restriction endonucleases, the results are strikingly different from the cleavage patterns of mature viral DNA. (*i*) the quantity of end fragments is substantially reduced and (*ii*) a new fragment, possessing the combined molecular weights of the end fragments, appears (26). In circularly permuted genomes, by contrast, the pattern of restriction fragments generated from large concatemers should be identical to that produced from the mature viral DNA. The reasons for this identical cleavage pattern are twofold: (*i*) the end fragments of circularly permuted genomes are not detectable, so that their loss cannot be documented, and (*ii*) in a large collection of circularly permuted genomes, sequences at the end of some molecules should be linked in other molecules (23); the latter molecules would behave as concatemers in the restriction endonuclease analysis.

In late stages of FV 3 infection (4 hr and thereafter) about 70% of the newly synthesized progeny DNA (15-min pulse with [^{3}H]thymidine) sediments as large concatemers.* This DNA was cleaved with restriction endonuclease *Hin*dIII, and the profile of the restriction fragments was compared with that of mature viral DNA. As shown in Fig. 3, the restriction fragments produced from these two kinds of molecules were similar, suggesting that mature FV 3 DNA molecules represent circularly permuted DNA sequences. Parenthetically, it should be mentioned that the topmost band obtained from mature viral DNA was extremely diffuse and therefore represents DNA fragments of heterogeneous sizes. Heterogeneity in a specific band has

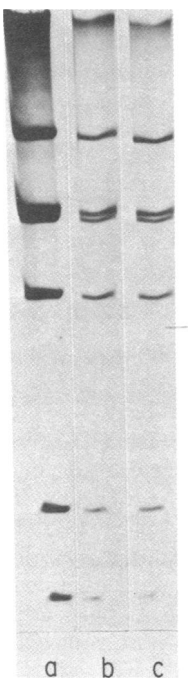

FIG. 3. Comparison of restriction fragments produced from concatemeric and mature FV 3 DNA. FV 3-infected cells (10 plaque-forming units per cell) were labeled with [^{3}H]thymidine (10 μCi/ml) at 4.0 or 5.0 hr after infection for 15 min. After the labeling period, cells were washed three times with phosphate-buffered saline and directly lysed by 0.5% Sarkosyl solution in hypotonic buffer (10 mM/KCl/1.5 mM $MgCl_2$/10 mM Tris·HCl, pH 7.8). The samples were heated at 60°C for 15 min and incubated at 37°C for 2 hr after mixing with an equal volume of Pronase solution (2 mg/ml). An aliquot was then layered on a neutral sucrose gradient composed of a 2-ml sucrose cushion in 1.0 M NaCl/0.003 M EDTA/0.05 M Tris/0.1% Sarkosyl, and 35 ml of a 5–20% linear gradient of sucrose in 1.0 M NaCl/0.003 M EDTA/0.005 M Tris/0.1% Sarkosyl. The gradients were centrifuged in a Beckman SW 27 rotor for 12 hr at 10,000 rpm. Under these conditions, most of the concatemeric DNA accumulated at the cushion, whereas mature viral DNA remained at the top third of the gradient. The concatemeric DNA was extensively dialyzed against 15 mM NaCl/1.5 mM sodium citrate, digested with restriction endonuclease *Hin*dIII, and electrophoresed in a 0.7% agarose gel. Lane a, DNA extracted from purified FV 3 particles; lanes b and c, concatemeric DNA extracted at 4 or 5 hr after infection, respectively.

been rationalized as the consequence of limited circular permutation in bacteriophage P22 (27).

DISCUSSION

FV 3 genome is a linear double-stranded DNA molecule with a M_r of 100×10^6 (2, 19). We now show the presence of direct terminal repeats in the FV 3 genome by circularization of DNA molecules after limited 5'-exonuclease digestion and by the occurrence of two single-stranded tails in the circles formed after complete denaturation followed by renaturation. The estimated size of terminal repeats was about 4% of the total genome length, or 6 kilobases. The variation in the size of terminal repeats was considerable. At least in part this variation reflects technical limitations inherent in the procedure. However, it is also possible that the observed heterogeneity is due to the imprecise mechanism of "headful" packaging of DNA (22). A variation in the size of direct terminal repeats has also been found in bacteriophages T2 (23), P22 (28), and 15 (22).

It is very unlikely that FV 3 DNA possesses cohesive ends as observed in lambdoid phages (29), because when the DNA was renatured either without prior exonuclease digestion or after complete denaturation, no circular molecules were observed.

Complete denaturation of DNA in alkaline buffer followed by slow renaturation resulted in efficient circularization of linear DNA molecules—a characteristic feature of circularly permuted genomes (20). The conclusion that FV 3 DNA is circularly permuted was further substantiated by our inability to radiolabel any specific restriction fragment by the polynucleotide kinase procedure and by the identity of *Hin*dIII restriction fragment profiles generated from mature viral DNA and the large concatemeric DNA.

The generation of circularly permuted and terminally redundant phage T4 DNA molecules was elegantly explained by Streisinger *et al.* by headful packaging of DNA (30). Subsequently, several investigators have provided extensive experimental evidence confirming the essential features of this model (31, 32). According to this model, mature viral DNA is produced by the cleavage of a large concatemer, in lengths that will fill the phage heads—i.e., one headful. The length of the DNA accommodated in the T4 phage head is slightly more than one complete set of genes. Thus, this headful packaging of DNA by cutting the concatemer either sequentially or randomly will produce circularly permuted and terminally redundant DNA molecules. Consistent with this model is the recent evidence that concatemeric DNA is synthesized during FV 3 DNA replication.* Another ramification of headful DNA packaging is that it suggests a specific pathway of virus assembly—i.e., packaging of DNA into preformed heads. Whether FV 3 makes use of a similar pathway of virus assembly remains to be seen.

The conclusion that FV 3 DNA molecules are circular permutations of each other leads to another question: Is circular permutation completely random, as in T4 phage (30), or limited to certain regions of the genome, as in P22 phage (33)? Data on the partial denaturation mapping of FV 3 genome, presented elsewhere (19), indicate that circular permutation is limited so that the ends of FV 3 DNA molecules fall within 20–28% of each other.

The genomes of several bacteriophages—e.g., T-4 (20), P22 (33), and 15 (22)—are circularly permuted and terminally redundant. Several animal viruses, such as herpesviruses (16) and retroviruses (34), contain direct terminal repeats. However, FV 3 is unique among animal viruses in that its genome is both circularly permuted and terminally redundant. To our knowledge, no other animal virus genome exhibiting these structural features has yet been discovered. African swine fever virus, another member of the Iridoviridae family, has a genome that is crosslinked at the termini—a characteristic feature of poxvirus genomes (35)—but evidently is not circularly permuted or terminally redundant (36). It would be interesting to know whether the genome of any other member of the Iridoviridae family is circularly permuted and terminally redundant.

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