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# DNA Analysis of Insect Iridescent Virus 6: Evidence for Circular Permutation and Terminal Redundancy

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DNA analysis of small insect iridovirus 6 was performed. Combined exonuclease-restriction endonuclease digestions revealed that all resulting fragments were degraded without preference for any one DNA fragment. Upon denaturation and reannealing of native linear Chilo iridescent virus DNA (158 × $10^6$ daltons), duplex DNA circles of a smaller size (140 × $10^6$ daltons) with protruding tails were formed.

Iridoviruses are icosahedral cytoplasmic DNA viruses which have been isolated from a variety of invertebrate and vertebrate host species, principally from insects. Tinsley and Kelly (20) have suggested a provisional classification scheme whereby isolates are listed in numerical sequence according to their date of isolation.

Iridovirus 6 or Chilo iridescent virus (CIV) was isolated by Fukaya and Nasu from the rice stem borer, *Chilo suppressalis* (*Lepidoptera*) (7); this virus occurs in Japan and the United States (18). CIV might be of agricultural importance, since it has been shown to infect the green rice leafhopper *Nephtotettix cincticeps* and to be lethal for 99% of the leafhopper *Colladonus montanus* (*Homoptera: Cicadellidae*), the vector of a mycoplasma agent of stone fruits (10). Serological studies by Kelly et al. (13) have shown that CIV is not closely related to any one of the 29 iridoviruses isolated so far (2). Bellet and Inman reported on DNA properties of CIV (1) such as G+C content and molecular weight. DNA of frog virus 3 (FV3), a member of the genus *Ranavirus* within the family *Iridovirus*, recently has been shown to be circularly permuted and terminally redundant (8). The genome of fish lymphocystis disease virus (FLDV), another iridovirus, has also recently been shown to be circularly permuted and terminally repetitious (4). This result led us to analyze the DNA of a true insect iridovirus for circular permutation. Here, we report evidence which indicates that the genome of the CIV is both circularly permuted and terminally redundant.

*Choristoneura fumiferana* (spruce budworm) cells were grown at 28°C in plastic Falcon flasks in Grace medium (pH 6.2) containing 10% fetal bovine serum (19), infected with CIV, and incubated for 3 days at 28°C (3). CIV and CIV DNA were harvested and purified as described previously (4).

CIV DNA was prepared for electron microscopy by the cytochrome spreading technique (6). Figure 1 shows electron micrographs of viral DNA molecules. The majority of the molecules were linear double-stranded DNA (Fig. 1A). Twenty-five molecules of sizes above 50 μm were measured. The smaller ones were probably fragments of random sizes. The 13 largest molecules, however, belonged to a distinct size class of 84.9 ± 1.1 μm, corresponding to 238.4 ± 3.1 kilobase pairs (kbp) (157.7 ± 1.3 × $10^6$ daltons). Among 200 molecules scanned in one preparation, 3 double-stranded circular molecules were found (Fig. 1B). The origin of these circles is unknown. They had a size of 75.5 ± 2.0 μm, corresponding to 212.1 ± 5.7 kbp (140.3 ± 3.8 × $10^6$ daltons). The difference in size between the linear and the circular molecules of 26 kbp suggests that the linear form might contain a terminal redundancy of about 12%.

Single-stranded DNA was purified by agarose gel electrophoresis by the method of Koller et al. (14). When the preparation which contained a high percentage of intact single strands was self-annealed, the formation of double-stranded or partially double-stranded circles was observed in about 20% of the reannealed DNA, whereas the rest yielded linear or more complex structures. Most of the circular molecules, as, e.g., the one shown in Fig. 2, displayed single-stranded tails at one or at several positions of variable distances. This might indicate that the circles were formed by more than two single strands with the single-stranded tails representing redundant DNA sequences. Another possibility is that the circles were obtained by the reannealing of two single strands which were terminally redundant, circularly permuted, or both only with the double tails generated by branch migration. The average size of 13 reannealed circles was 76.9 ± 2.6 μm, corresponding to 208.9 ± 6.9 kbp (138.2 ± 6 × $10^6$ daltons). This value is close to the size of the double-stranded circles found in the nondenatured preparation and confirms the presence of a terminal redundancy in the linear DNA genomes.

A few single strands formed stem loop structures as shown in Fig. 3. Four such loops were measured. They had sizes of between 33 and 44 kilobases (kb). Occasionally a smaller deletion or substitution loop of a size around 3.4 kb was observed. These exceptional structures may be the result of anomalous events in the replication or recombination of the viral DNA.

To test for a circularly permuted DNA structure, a combined exonuclease-restriction enzyme digestion of CIV DNA was performed (16). Figures 4A and B present the resulting DNA cleavage patterns which were obtained after first degrading CIV DNA with either the 5′ lambda exonuclease or the 3′ *Escherichia coli* exonuclease III, and then by digesting it with various restriction enzymes. It is evident that the DNA cleavage patterns do not show a preferential digestion of a particular CIV DNA fragment; instead a gradual decrease in the intensities of all viral DNA fragments was observed, consistent with a circularly permuted DNA

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FIG. 1. Electron micrograph of double-stranded DNA molecules isolated from CIV virus. (A) Linear molecule. The ends are marked by stars. (B) Circular molecule. The DNA was spread with cyanogen bromide-cleaved cytochrome from 30% formamide–0.1 M Tris-hydrochloride (pH 8.5)–1 mM EDTA onto a hypophase of 0.005% octyl glucopyranoside (Sigma Chemical Co.). The small circles are PM2 DNA molecules added as a length standard.

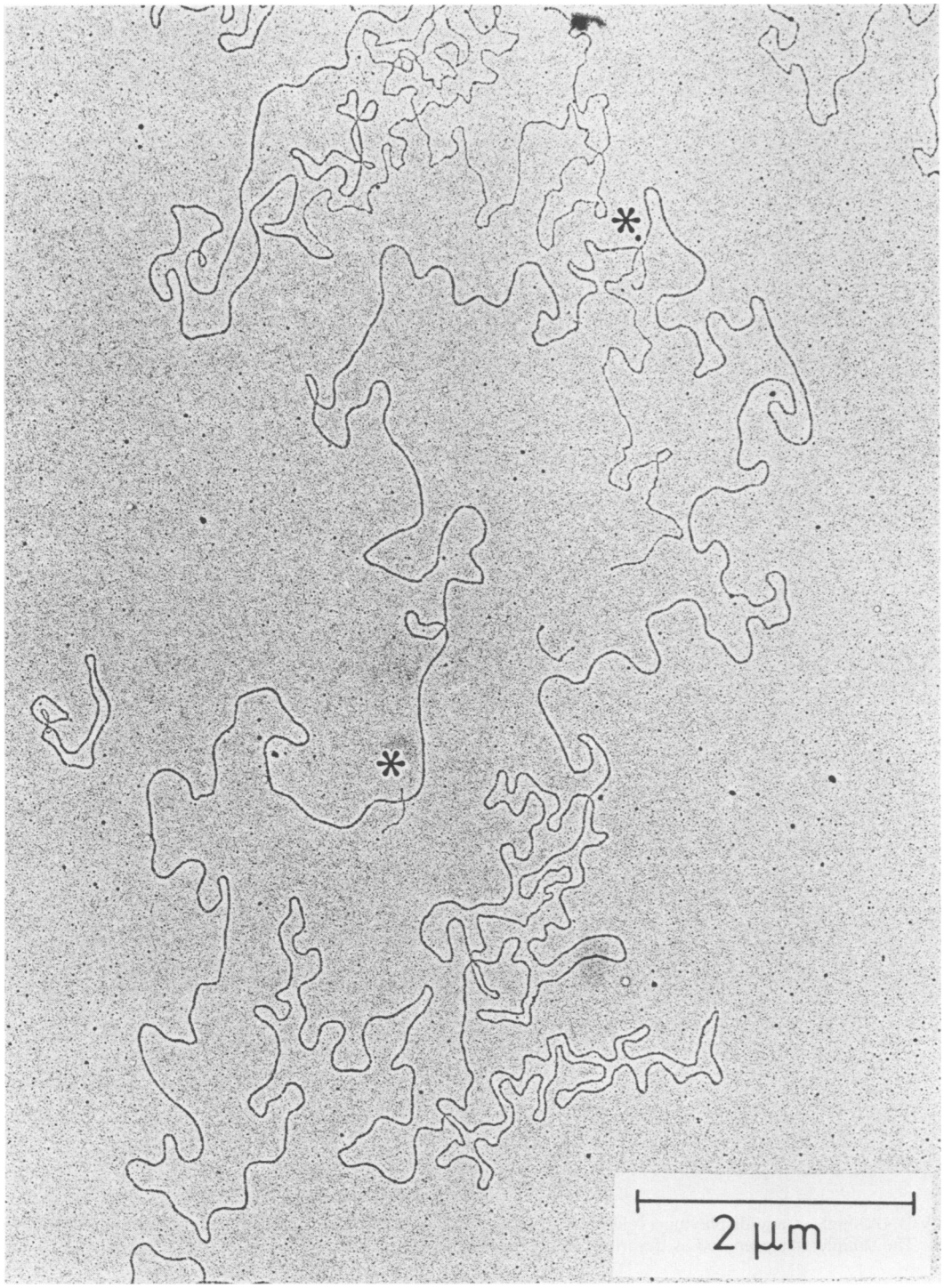


FIG. 2. A circular double-stranded molecule formed by self-annealing of denatured CIV DNA. The stars indicate positions of single-stranded tails on the circle. The CIV DNA (50 μl of 2 μg/ml in 10 mM Tris-hydrochloride [pH 7.4]–1 mM EDTA–60% formamide) was denatured by immersing the tube into boiling water for 90 s. After addition of 6 M CsCl to a final concentration of 0.6 M, the sample was incubated for 60 min at 32°C. An aliquot of the DNA was spread as described in the legend to Fig. 1.

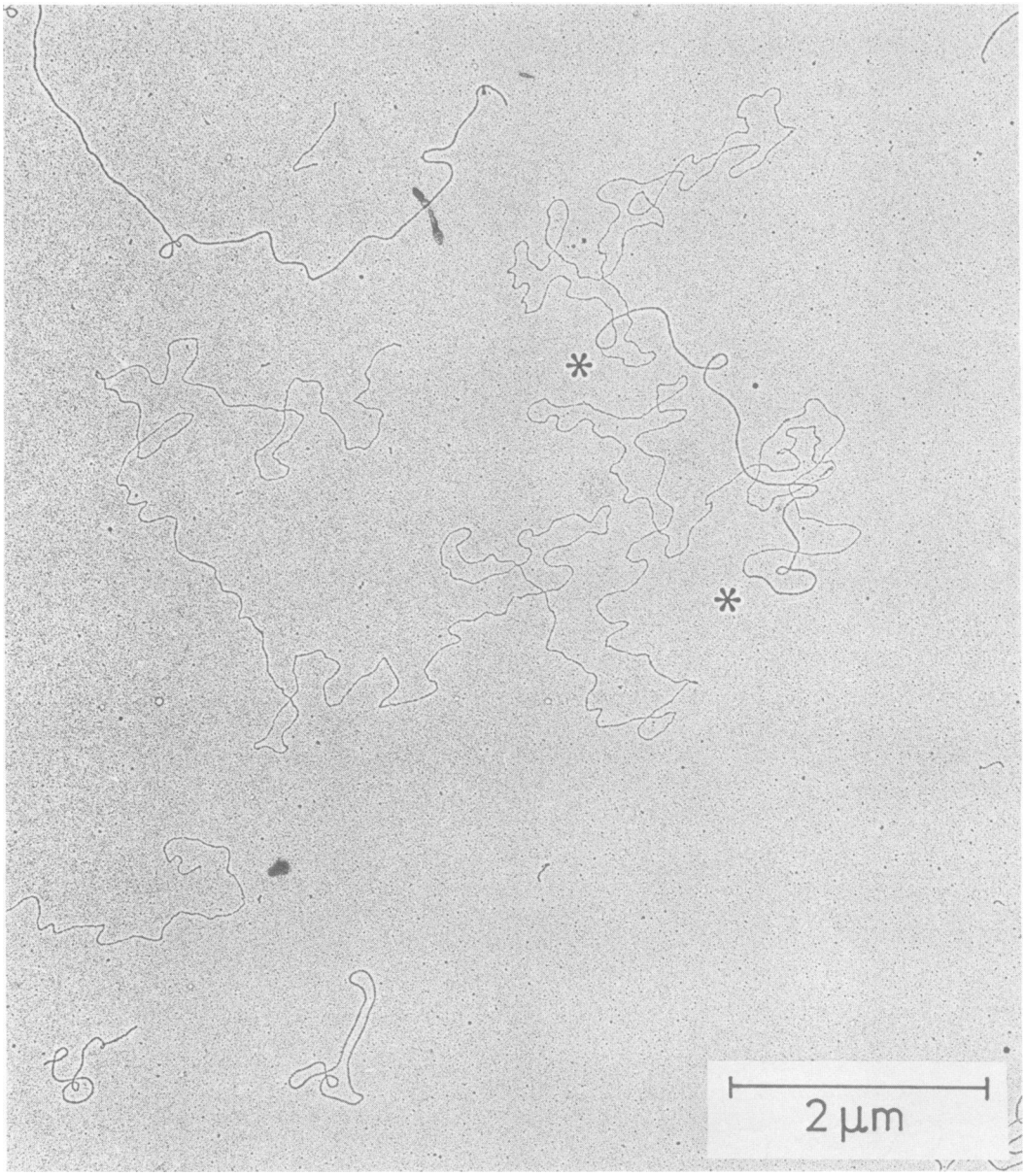


FIG. 3. A CIV DNA single strand displaying a fold-back structure. The stars mark the ends of the double-stranded region. The loop has a size of ca. 33 kb. The sample was prepared as described in the legend to Fig. 2.

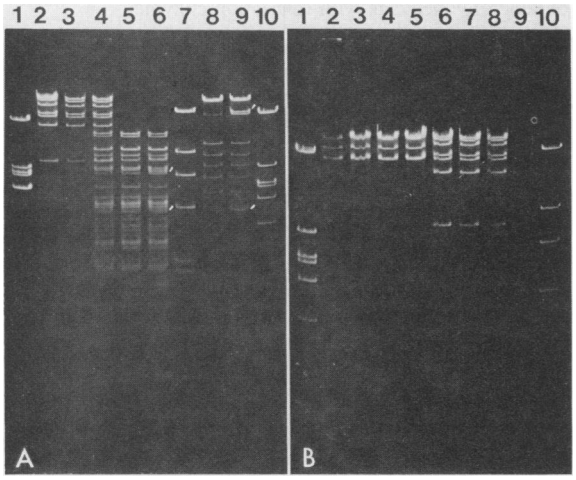


FIG. 4. Restriction endonuclease and exonuclease-restriction enzyme digests of CIV DNA. Agarose (0.6%) slab gel electrophoresis of CIV DNA (1 μg) were treated with lambda 5′ exonuclease, 3′ exonuclease III, or both for 10 min at 37°C (A) and subsequently cleaved with a given restriction enzyme and run under the same conditions in comparison to untreated DNA. (A) Lane 1, lambda DNA digested with *Bam*HI (marker); lane 2, CIV DNA digested with *Bam*HI; lane 3, CIV DNA digested with lambda exonuclease (1.6 U) and thereafter with *Bam*HI; lane 4, mixture of CIV DNA and linear THV DNA digested with lambda exonuclease and *Bam*HI; lane 5, THV DNA digested with lambda exonuclease and *Bam*HI; lane 6, THV DNA digested with *Bam*HI; lane 7, lambda DNA, digested with *Hin*dIII (marker); lane 8, THV DNA digested with lambda exonuclease and *Hin*dIII; lane 9, THV DNA digested with *Hin*dIII; lane 10, lambda DNA digested with *Eco*RI. White small arrows mark terminal fragments of THV DNA. (B) The exonuclease treatments were performed for 12 h, and electrophoresis was run for 16 h. Lane 1, lambda DNA digested with *Eco*RI; lane 2, CIV DNA digested with lambda exonuclease and *Sal*I; lane 3, CIV DNA digested with 3′ exonuclease III (25 U) and *Sal*I; lane 4, CIV DNA, untreated in exonuclease buffer and digested with *Sal*I; lane 5, CIV DNA, digested with *Sal*I; lane 6, CIV DNA digested with *Bam*HI; lane 7, CIV DNA untreated in lambda exonuclease buffer and digested with *Bam*HI; lane 8, CIV DNA digested with 3′ exonuclease and *Bam*HI; lane 9, CIV DNA digested with lambda exonuclease and *Bam*HI; lane 10, lambda DNA digested with *Hin*dIII.

structure. This held true both for exonucleases and for various restriction enzymes even after prolonged exonuclease digestion (Fig. 4A and B). Linear DNA of tupaia herpesvirus (THV) of molecular weight 131 × $10^6$ (5) was used as a control (Fig. 4A, lanes 7 to 9).

CIV DNA was analyzed by digesting it with restriction endonucleases and separating the resulting DNA fragments electrophoretically on agarose slab gels as described previously (4, 17). Nine enzymes were found to cut CIV DNA into a number of DNA fragments suitable for constructing physical maps of the viral genome. Figure 4 and Table 1 show single digestions of CIV DNA with *Apa*I, *Sma*I, *Bam*HI, *Sal*I, and *Bgl*I, all of which produce a limited number of cleavage fragments, whereas most of the other enzymes tested gave a large number of DNA fragments. It is noteworthy that the DNA fragments produced by enzymes which cut CIV DNA only once or three times (*Apa*I and *Sma*I, respectively) migrate as characteristically broad and diffuse DNA bands in agarose (Fig. 5 and Fig. 4, lane 4). This observation is to be expected from a circularly permuted DNA with terminal redundancy but not from a nonpermuted

TABLE 1. Number of fragments produced by digesting CIV DNA with restriction endonucleases and subsequent electrophoretic separation on agarose gels

| Restriction enzyme | Recognition sequence | Apparent no. of DNA fragments |
|---|---|---|
| *Apa*I | GGGCC/C | 2 |
| *Xho*I | C/TCGAG | 2–3 |
| *Mst*I | TGC/GCA | 2–3 |
| *Sac*II | CCGC/GG | 2–3 |
| *Pst*I | CTGCA/G | 2–3 |
| *Xor*II | CGAT/CG | 2–3 |
| *Sma*I | CCC/GGG | 3 |
| *Bgl*I | GCC(N)$_4$/NGGC | 5 |
| *Sal*I | G/TCGAC | 5 |
| *Bst*EII | G/GTNACC | 6 |
| *Bal*I | TGG/CCA | 7 |
| *Bam*HI | G/GATCC | 7 |
| *Ava*I | C/PyCGPuG | 8 |
| *Sac*I | GAGCT/C | 8 |
| *Hae*II | PuGCGC/Py | 10 |
| *Sph*I | GCATG/C | 11 |
| *Pvu*II | CAG/CTG | 13 |
| *Xba*I | T/CTAGA | >20 |
| *Eco*RV | GATAT/C | >20 |
| *Eco*RI, *Bgl*II, *Bcl*I, *Hin*dIII, *Cla*I, *Msp*I, *Hpa*II, *Hin*dII, *Ava*II, *Hpa*I, *Nci*I | | >50 |

DNA like THV DNA (4), provided that CIV DNA was initially intact.

The molecular weight of CIV DNA was calculated from the sizes of individual DNA fragments obtained after electrophoretic separation of CIV DNA digested with various restriction enzymes. The results (Table 2) gave a value of 138 × $10^6$ daltons, which is in agreement with our own contour length measurements of circular CIV DNA. To determine

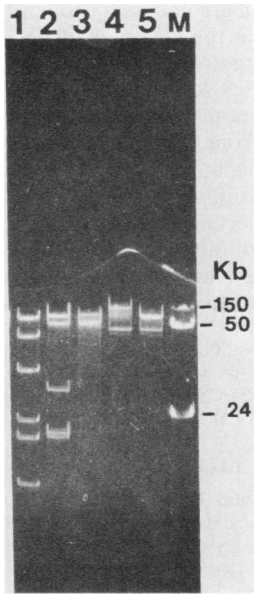


FIG. 5. Restriction endonuclease digestion of CIV DNA (1 μg) and subsequent electrophoretic separation on a 0.45% agarose gel. Running time was 48 h. Lanes: 1, *Bam*HI; 2, *Sal*I; 3, *Sma*I; 4, *Apa*I; 5, *Bgl*I. M are markers herpes simplex virus DNA and lambda DNA + lambda *Hin*dIII fragment A.

TABLE 2. Sizes of insect iridescent virus 6 DNA fragments obtained by cleavage with restriction enzymes

| Enzyme | Mol wt × $10^6$ of fragment | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | A | B | C | D | E | F | G | H | I | J | K | L | M | N |
| *Pvu*II | 33 | 30 | 17.5 | 11.2 | 9.9 | 9.0 | 7.6 | 7.3 | 6.4 | 5.5 | 4.3 | 3.4 | 2.3 | 0.5 |
| *Kpn*I | 44 | 22 | 13 | 13 | 11.2 | 8.6 | 6.8 | 6.2 | 5.4 | 3.1 | 1.55 | | | |
| *Apa*I | 111 | 29 | | | | | | | | | | | | |
| *Sma*I | 78 | 33 | 29 | | | | | | | | | | | |
| *Bam*HI | 48 | 29 | 19 | 13.5 | 12.5 | 10.0 | 5.4 | 0.4 | | | | | | |
| *Sal*I | 62 | 33 | 15 | 13 | 12.8 | | | | | | | | | |
| *Bam*HI-*Apa*I | 33 | 29 | 19 | 13.5 | 13 | 12.5 | 10.0 | 5.4 | 2.9 | | | | | |
| *Bam*HI-*Sal*I | 35 | 18 | 14 | 12.8 | 10.0 | 9.4 | 8.3 | 7.5 | 5.4 | 4.6 | 3.9 | 3.8 | | |
| *Sph*I | 23 | 19 | 18.8 | 17 | 15.5 | 13.0 | 11.5 | 8.1 | 7.1 | 3.65 | 1.75 | 0.92 | | |
| *Bst*EII | 26.5 | 26 | 25.5 | 22 | 13.5 | 12.5 | 11.5 | | | | | | | |

the 5′-terminal fragments of CIV DNA, a labeling experiment with T4 polynucleotide kinase and [$\gamma$-$^{32}$P]ATP was performed followed by digestion with different restriction enzymes and subsequent electrophoretic separation on agarose gels. It was found upon autoradiography that radioactive phosphate was completely distributed over the lane without preference for any one DNA fragment, indicating that almost all termini are randomly distributed over the genome. A combination of this result and of the restriction enzyme-exonuclease analysis with the data obtained by electron microscopy can best be interpreted by assuming a circular permutation and terminal redundancy for CIV DNA.

There are now three eucaryotic viruses, the DNAs of which have in common the property of being circularly permuted and terminally redundant. Frog virus, FLDV, and CIV belong to the same family of iridoviruses, but to different genera (15). This might indicate that iridoviruses have permuted DNA structures. Some of the published evidence on restriction enzyme analyses of iridovirus DNA are consistent with this generalization (9, 11). However, more DNA analyses of iridovirus genomes with respect to circular permutation are necessary to verify this assumption. It is noteworthy that the genomes of FV3, FLDV, and CIV do not have homologous sequences as shown by DNA-DNA reassociation kinetics for CIV and FV3 DNA (11) and by Southern hybridization techniques between FV3 and FLDV DNA (4). Judging from these DNA homology studies (4, 11, 12) and also on the basis of the known viral polypeptide patterns (13), the iridoviruses seem to be a rather diverse family. However, certain DNA parameters, such as genome size, circular permutation, and terminal repetitions, indicate that they also possess unifying features. It will be of interest to learn more about this genome organization in detail.

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