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Physical Map of the Channel Catfish Virus Genome: Location of Sites for Restriction Endonucleases *Eco*RI, *Hin*dIII, *Hpa*I, and *Xba*I

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The overall arrangement of nucleotide sequences in the DNA of channel catfish virus has been studied by cleavage with four restriction endonucleases. Physical maps have been developed for the location of sites for *Eco*RI, *Hin*dIII, *Hpa*I, and *Xba*I. The sum of the molecular weights of fragments generated by each restriction enzyme indicates a molecular weight of approximately 86 × 10^6 for the channel catfish virus genome. Fragments corresponding to the molecular ends of channel catfish virus DNA have been identified by their sensitivity to exonuclease treatment. The distribution of restriction sites in the genome shows that sequences included in a 12 × 10^6-molecular weight region at one end are repeated with direct polarity at the other end, and that the overall genomic sequence order is nonpermuted.

Channel catfish virus (CCV), a herpesvirus, is the causative agent of a lethal disease occurring in populations of channel catfish fry (8). The genome of this virus is duplex DNA with a mean guanine plus cytosine content of 56% (11). Velocity sedimentation and electron microscopy (Sheldrick, Berthelot, and Chousterman, manuscript in preparation) revealed the genome to be a linear molecule of 84 ± 3 × 10^6 molecular weight, and suggested a nonpermuted arrangement of nucleotide sequences. Extensive inverted repeat sequences, characteristic of some herpesvirus genomes (3-5, 12, 20, 26, 31-33), were not found in CCV DNA, but evidence was obtained for a direct terminal repeat of at least 10 × 10^6.

Those studies could not, however, rigorously discriminate between the type of terminal repeat present in the genome of bacteriophage T5 (17), for example, where sequences within the repeat are not repetitive, and the terminal regions of the *Herpesvirus saimiri* (2) and *Herpesvirus ateles* (9) genomes, which are themselves highly repetitive. The two alternatives may easily be distinguished if restriction enzyme sites can be found that lie within the region in question—the outcome of mapping experiments is clearly different for each case. Here we report experiments permitting the localization of sites in CCV DNA for the restriction endonucleases *Eco*RI, *Hin*dIII, *Hpa*I, and *Xba*I. The present results support our previous conclusions concerning molecular weight and nucleotide sequence arrangement, demonstrate that sequences within the terminal repeats are not highly repetitive, and provide a physical map for future studies on the expression of the CCV genome.

MATERIALS AND METHODS

Cells. BB cells are a continuous fish cell line (35). The cells were grown as monolayers in Eagle medium supplemented with 10% fetal calf serum. For preparation of labeled DNA, infected monolayers in 70-cm^2 plastic flasks were incubated in Eagle medium (phosphate free) containing 70 μCi of carrier-free [^{32}P]orthophosphate (C.E.N., Saclay, France) per ml.

Virus growth and purification. CCV was kindly provided by Pierre de Kinkelin (Institut National de la Recherche Agronomique, Grignon, France). Cell monolayers were infected with CCV at 1 to 5 PFU/cell. After 18 h at 30°C, the lysed culture was frozen and thawed three times and clarified by centrifugation at 1,200 × *g* for 10 min, and the virus was sedimented at 15,000 × *g* for 2 h at 4°C. The resuspended pellet was layered onto 2.5-ml CsCl step gradients (21) in nitrocellulose tubes of an SW41 rotor (Beckman). After centrifugation for 90 min at 30,000 rpm (15°C), the virus band was collected in a syringe by piercing the side of the tube, dialyzed at 4°C against 0.1 M NaCl-1 mM EDTA-0.01 M sodium phosphate (pH 7.5), and stored at 4°C.

DNA extraction. The concentrated virus was lysed with 1% (final concentration) sodium dodecyl sulfate in 0.01 M EDTA and extracted twice with equal volumes of pH 8 (0.1 M PO_4^{3-})-equilibrated phenol. The aqueous phase was directly applied to 11-ml linear 5 to 20% (wt/wt) sucrose gradients (polyallomer tubes) prepared in 1 M NaCl-1 mM EDTA-0.01 M sodium phosphate (pH 7.5) and centrifuged for 2 h at 40,000 rpm in an SW41 rotor at 20°C. DNA-containing gradient fractions (four to five fractions near the center

of a 25-fraction gradient) were pooled and directly precipitated with 2 volumes of ethanol. After centrifugation, the DNA pellet was gently resuspended in 0.01 M Tris-hydrochloride (pH 7.4)-1 mM EDTA, exhaustively dialyzed against the same buffer, and stored at 4°C.

Enzymes. Restriction endonucleases *Hin*dIII and *Eco*RI were kindly provided by P. Yot and M. Guérineau, *Xba*I was the generous gift of N. Wilkie, and *Hpa*I was purchased from New England Biolabs (Beverly, Mass.). Lambda 5'-exonuclease was the kind gift of J. Leboucher. The specific activity of the exonuclease was determined by digestion of ^{3}H-labeled simian virus 40 DNA form III (16).

Restriction endonuclease digestion. All incubations were carried out at 37°C for 1.5 h with sufficient endonuclease to produce a limit digest. Reaction mixtures contained 1 μg of CCV DNA and 100 μg of bovine serum albumin in a total volume of 70 μl. Buffers for the various enzymes were: 100 mM Tris-hydrochloride (pH 7.5)-10 mM $MgSO_4$-30 mM NaCl for *Eco*RI; 6 mM Tris-hydrochloride (pH 7.5)-6 mM $MgCl_2$-50 mM NaCl-1 mM dithiothreitol for *Hin*dIII; 6 mM Tris-hydrochloride (pH 7.5)-6 mM $MgCl_2$-1 mM dithiothreitol for *Xba*I; 10 mM Tris-hydrochloride (pH 7.5)-10 mM $MgCl_2$-6 mM KCl-1 mM dithiothreitol for *Hpa*I. For digestion of ^{32}P-labeled CCV DNA, 0.5 μg of unlabeled lambda DNA was added. Reactions were quenched by the addition of 1/10 volume of a solution containing 0.1 M EDTA, 2% sodium dodecyl sulfate, 70% glycerol, and 0.2% bromophenol blue. When DNA was submitted to double digestion the two restriction enzymes were added together, and in this case the most complete buffer was used. Digestion with lambda 5'-exonuclease was carried out in 67 mM glycinate buffer (pH 9.4)-2.5 mM $MgCl_2$ at 37°C for 30 min; before subsequent treatment with restriction enzymes, the reaction mixture was incubated at 65°C for 5 min and adjusted with the buffer corresponding to the second enzyme.

Agarose gel electrophoresis and molecular weight determinations. Restriction endonuclease reaction mixtures were electrophoresed on 0.3%, 0.5%, 1.1%, or 1.5% agarose slab gels (25 by 16 by 0.3 cm) at 45 V for 20 h at room temperature in a buffer containing 40 mM Tris-hydrochloride, 20 mM sodium acetate, and 2 mM Na_2 EDTA (pH 7.8). The gels were stained with ethidium bromide (0.5 μg/ml) and photographed under a 252-nm UV-light source. Molecular weights were estimated relative to *Eco*RI lambda DNA fragments (29) and *Eco*RI and *Hin*dIII herpes simplex virus type 1 (HSV-1 strain A_{44}) DNA fragments (S. Chousterman, unpublished data) used as markers. Within experimental error, the gel patterns and molecular weights of HSV-1 (A_{44}) DNA restriction products were identical to those previously reported (12) for HSV-1 (MP) DNA. The patterns were not affected by 10-fold excess enzyme or by heating the sample to 68°C followed by rapid cooling in ice prior to electrophoresis. Digestion of CCV DNA with 50 μg of preincubated proteinase K per ml did not change the electrophoretic profile of the limit digest. Molecular weights of CCV DNA fragments, obtained by double digestion, or by digestion of a fragment with a second enzyme, were estimated by comparison (in the same gel) with the pattern obtained with each individual enzyme. The molecular weights of fragments larger than 14×10^6 were estimated from the sum of molecular weights of the fragments generated by a second restriction endonuclease. For autoradiography, the gels were air-dried onto Whatman no. 3MM filter paper and placed in contact with Kodak RP-Royal X-Omat film. For determination of molar ratios, the radioactive gels were cut into 1-mm-thick slices. Each slice was suspended in 3 ml of water, and ^{32}P radioactivity was measured by Cerenkov radiation.

For hybridization experiments, the DNA bands were excised from gels and electrophoretically eluted into dialysis bags (23). This step is sufficient for hybridization experiments. However, for digestion with a second enzyme, the eluted DNA was further purified by extraction with a two-phase system consisting of 2-methoxyethanol (Methyl Cellosolve; Merck) and 1.25 M (pH 7.6) phosphate buffer (15). Approximately 50 to 60% of the DNA from gel slices could be recovered by this method.

Hybridization procedures. A restriction enzyme digest containing 10 to 15 μg of CCV DNA was applied to a slab gel (0.5 or 1.1% agarose) with a single slot extending the width of the gel (14 cm), and after electrophoresis the DNA fragments were transferred to nitrocellulose membranes by the method of Southern (24). Briefly, the DNA fragments were denatured by soaking the slab in 0.3 M NaOH-0.6 M NaCl for 1 h and neutralized with 1 M Tris-hydrochloride (pH 7.4)-0.6 M NaCl for 1 h. The DNA was transferred onto a nitrocellulose membrane (Schleicher and Schüll BA 85), using 6× SSC (0.9 M NaCl-0.09 M sodium citrate) as the eluting buffer, for a minimum of 4 h. After transfer, the sheet was baked in a vacuum oven at 80°C for 4 h and stored at room temperature. The membrane was cut into 7- to 10-mm-wide vertical strips, each containing 1 to 1.5 μg of unlabeled DNA fragments. For hybridization, the strips were placed in glass vials containing 2× SSC, 10 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid buffer (pH 7.4), 1 mM EDTA, 0.1% sodium dodecyl sulfate, 50% formamide, and 5 to 25,000 cpm of denatured ^{32}P-labeled CCV DNA in a volume of 3 ml. Hybridization conditions were 37°C for 36 h, after which the strips were thoroughly washed with 2× SSC at 37°C, dried, and subjected to autoradiography.

RESULTS

Molecular weights and molarities of CCV fragments produced by four restriction endonucleases. Electrophoretic profiles of the digestion products of CCV DNA with *Eco*RI, *Hin*dIII, *Hpa*I, and *Xba*I are shown in Fig. 1. The number of observable bands ranged from 20 for *Eco*RI, 9 for *Xba*I, and 7 for *Hpa*I to as few as 4 for *Hin*dIII. The relative amount of DNA in each band was estimated from ^{32}P-labeled fragments electrophoretically separated on agarose gels and is listed for each enzyme in Table 1. Notice that although some bands (such as *Eco*RI [D, E] or *Xba*I [D, E]) exhibit twice the molarity of others, no bands show less than

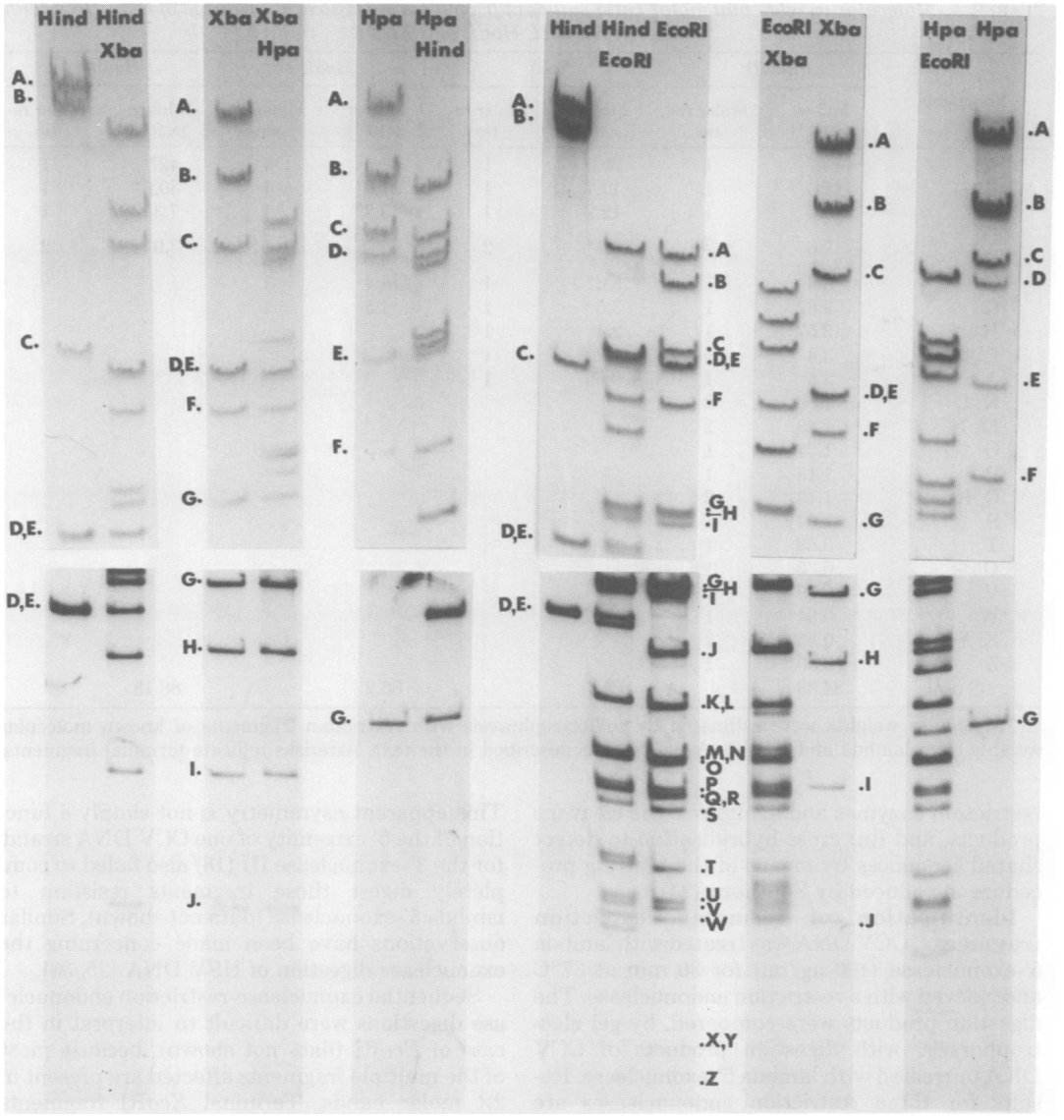

FIG. 1. *Single and double restriction endonuclease digests of ^{32}P-labeled CCV DNA. ^{32}P-labeled CCV DNA was cleaved with one or two (simultaneously) of the following restriction endonucleases: EcoRI, HindIII, HpaI, and XbaI. Digestion products were electrophoresed and autoradiographed as described in the text. The illustrated autoradiograms are composites of separate gels (upper, 0.3% agarose; lower, 1.1% agarose) to more clearly show separations among large and small fragments. Each fragment from single digests has been assigned a letter. The molecular weights and molarities are given in Table 1. The apparent underrepresentation of HpaI fragments D and E depended on the DNA preparation, and may indicate partial heterogeneity in the uncloned virus used to prepare the DNA.*

expected. In attributing DNA fragments to the various visible bands we adhere to current usage and assign a letter to each fragment, starting with A for the greatest molecular weight. Successive letters in parentheses are used to designate 2× molar bands. The sums of the molecular weights, corrected for molarity differences, of all observable fragments from a given enzyme are listed in Table 1. We took advantage of the small number of fragments generated by *Hind*III and began the analysis with this enzyme. The analyses to be described below were carried out and cross-checked with three techniques: (i) simultaneous or (ii) sequential digestion with two

TABLE 1. *Molecular weights and molar ratio values of fragments generated by cleavage of CCV DNA with EcoRI, HindIII, HpaI, and XbaI*[a]

Fragments	*Eco*RI		*Xba*I		*Hpa*I		*Hin*dIII	
	Mol wt ($\times 10^{-6}$)	Molar ratio	Mol wt ($\times 10^{-6}$)	Molar ratio	Mol wt ($\times 10^{-6}$)	Molar ratio	Mol wt ($\times 10^{-6}$)	Molar ratio
A	13.7	1	29.0	1	31.4	1	42*	1
B	11.5	1	17.7	1	17.0*	1	30.8*	1
C	8.5	1	12.2	1	13.2*	1	7.35	1
D	7.6	2	6.7*	2	11.75	1	3.0	2
E					6.95	1		
F	6.4	1	5.6*	1	4.4	1		
G	3.62	1	3.5	1	1.5	1		
H	3.57	1	2.4	1				
I	3.4	1	1.22	1				
J	2.5	1	0.65	1				
K, L	1.86	2						
M, N	1.38	2						
O	1.35	1						
P	1.18	1						
Q, R	1.13	2						
S	1.04	1						
T	0.83	1						
U	0.73	1						
V	0.69	1						
W	0.64*	1						
X, Y	0.35	2						
Z	0.30*	1						
Total	84.98		85.7		86.2		86.15	

[a] Molecular weights were estimated by coelectrophoresis with restriction fragments of known molecular weights from lambda and HSV-1 (A_{44}) DNAs as described in the text. Asterisks indicate terminal fragments.

restriction enzymes and analysis of the cleavage products, and (iii) cross-hybridization to detect shared sequences by means of the blotting procedure introduced by Southern (24).

Identification of terminal restriction fragments. CCV DNA was treated with lambda 5′-exonuclease (100 μg/ml) for 30 min at 37°C and cleaved with a restriction endonuclease. The digestion products were compared, by gel electrophoresis, with digestion products of CCV DNA untreated with lambda 5′-exonuclease. Results for three restriction endonucleases are shown in Fig. 2. After lambda 5′-exonuclease treatment, certain bands either disappeared from the gel or exhibited a diminished intensity: B and C for *Hpa*I, (D, E) and F for *Xba*I, and A and B for *Hin*dIII. The data indicate that the ends of CCV DNA are not equally susceptible to lambda 5′-exonuclease action, since two terminal fragments of comparable size (e.g., *Hpa*I B [17×10^6] and *Hpa*I C [13.2×10^6]) were lost to different extents during digestion. Continued treatment with exonuclease (data not shown) did not lead to further loss of the resistant fragments, *Hpa*I C and *Xba*I (D, E), suggesting that approximately 30 to 40% (the estimated resistant fraction for both fragments) of a given population of CCV DNA molecules is totally refractory at the one ("left"; see below) end. This apparent asymmetry is not simply a function of the 5′ extremity of one CCV DNA strand, for the 3′-exonuclease III (18) also failed to completely digest those fragments resistant to lambda 5′-exonuclease (data not shown). Similar observations have been made concerning the exonuclease digestion of HSV DNA (25, 33).

Sequential exonuclease-restriction endonuclease digestions were difficult to interpret in the case of *Eco*RI (data not shown), because most of the multiple fragments affected are present in 2× molar bands. Terminal *Eco*RI fragments were therefore identified by the Southern (24) hybridization procedure. ^{32}P-labeled terminal fragments *Hpa*I B and C and *Xba*I (D, E) hybridized to filter-immobilized *Eco*RI bands (D, E), (K, L), and (M, N) (Fig. 3b and c). In a reciprocal experiment (Fig. 4), ^{32}P-labeled *Eco*RI (D, E) hybridized strongly to *Xba*I bands (D, E) and F, and weakly to *Xba*I A. Thus, at least one fragment from each of the above *Eco*RI 2× molar bands lies in the terminal regions of the CCV genome and, as will be shown later, both fragments from each band are in fact included within the terminal redundancy.

Mapping the molecular center of the CCV genome. From the above data, the two large fragments *Hin*dIII A and B contain the genome termini; therefore *Hin*dIII C and (D, E) must be

grouped at the center of the molecule. To determine their relative order, ^{32}P-labeled *Hin*dIII C and (D, E) were separately hybridized to cold *Eco*RI fragments. Figure 3a shows that *Hin*dIII C (7.35×10^6) hybridized to *Eco*RI I (3.4×10^6), C (8.5×10^6), and J (2.5×10^6), whereas *Hin*dIII (D, E) (3×10^6 each) hybridized to *Eco*RI B (11.5×10^6) and J (2.5×10^6). Since *Eco*RI B and C are both larger than *Hin*dIII C or (D, E), they must be external. Since *Eco*RI J hybridized to *Hin*dIII C and (D, E), the order is

-B-J-I-C-	*Eco*RI
-D-E-C-	*Hin*dIII

In the case of *Xba*I, fragment H hybridized to *Eco*RI J and fragment G to *Eco*RI I and J, as shown in Fig. 3c. So the order is

-B- J - I -C-	*Eco*RI
-H-G-	*Xba*I

Completing the catalog of centrally located fragments, *Hpa*I A (31.4×10^6) hybridized to the four *Eco*RI fragments B, J, I, and C (Fig. 3b).

Alignment of *Hpa*I fragments. From the above experiments, the order (putting *Hpa*I C on the "left") for the *Hpa*I fragments is: C-----A-----B. *Hpa*I A also hybridized to *Eco*RI F and to the *Eco*RI (M, N), O band (Fig. 3b). *Eco*RI digestion of fragment *Hpa*I A gave *Eco*RI B, E, I, J, O, and two new fragments (2.15×10^6 and 0.71×10^6), absent from either *Eco*RI or *Hpa*I digests of total CCV DNA (Table 2). The two new fragments must be derived from the ends of *Hpa*I A; the *Hpa*I A/*Eco*RI 2.15×10^6 fragment can be generated only by the cleavage of *Eco*RI fragments larger than itself, from *Eco*RI J (2.5×10^6) to *Eco*RI A (13.7×10^6). On the other hand, the fragments *Eco*RI B, C, I, and J can be eliminated as sources of the 2.15×10^6 fragment because they are already part of *Hpa*I A. *Eco*RI G hybridized to the terminal fragment *Hpa*I B (Fig. 3b) and was not cleaved by *Hpa*I. According to the hybridization data in Fig. 3b, the new 2.15×10^6 fragment must come from *Eco*RI F since the fragment is cleaved by *Hpa*I and in addition hybridizes to *Hpa*I A, whereas *Eco*RI A does not.

Of the four remaining *Hpa*I fragments, only *Hpa*I D and E hybridized to *Eco*RI A and therefore must be contiguous (see Table 4). Furthermore, *Hpa*I E (6.95×10^6) hybridized only to *Eco*RI A and was not cleaved by *Eco*RI, so *Hpa*I E must be contained in *Eco*RI A. On the other hand, *Hpa*I D (11.75×10^6) hybridized to *Eco*RI A, F, and S as well as to the *Eco*RI P, (Q, R) band (Table 2). *Hpa*I/*Eco*RI double digestion experiments (Fig. 1) showed that *Eco*RI S and at least one of the *Eco*RI P, (Q, R) fragments are located in *Hpa*I D, since *Eco*RI

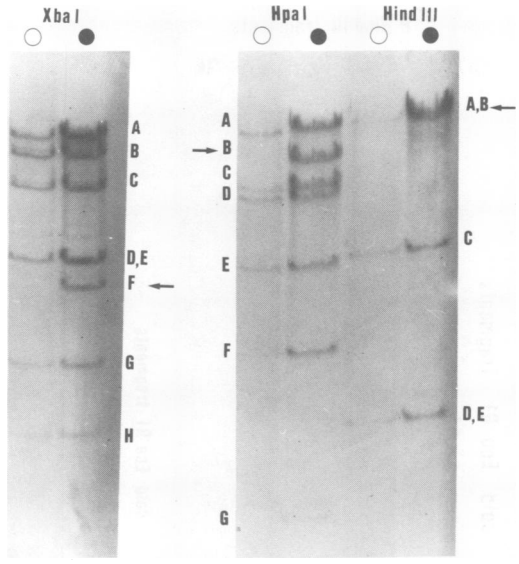

FIG. 2. *Lambda 5'-exonuclease-restriction endonuclease digest of CCV DNA: identification of terminal fragments. CCV DNA was digested with lambda 5'-exonuclease as described in the text, limit digested with three restriction endonucleases, and submitted to electrophoresis in 0.5% agarose. Arrows identify the exonuclease-sensitive fragments. The presence of faint bands in the XbaI gels is due to incomplete restriction.*

S, P, and (Q, R) were not cleaved by *Hpa*I. Here again, two new fragments (5.25×10^6 and 4.35×10^6) were generated (Fig. 1). These fragments are not part of *Hpa*I B, C, or A (Fig. 5a, Table 2). Both are larger than *Hpa*I G (1.5×10^6) and are absent from the *Hpa*I F/*Eco*RI cleavage products. Hence, they must be located at the ends of *Hpa*I D and can arise only from *Eco*RI A and *Eco*RI F, respectively. As discussed above, one end of *Hpa*I A hybridized to the 2.15×10^6 fragment from *Eco*RI F (6.4×10^6), so there remains 4.25×10^6 of *Eco*RI F, which must correspond to one end of *Hpa*I D. This shows that *Hpa*I D adjoins *Hpa*I A. The arrangement is thus -----E-D-A-----, and the order of *Eco*RI fragments in this region must be: ---A-P (or Q or R) -S-B-J-I-C---.

The next step was to locate *Hpa*I F and G. Both hybridized to *Xba*I A (29×10^6) and must be at the same end of *Hpa*I A (31.4×10^6). Fragment *Xba*I A was cleaved into four fragments by *Hpa*I: F, G, and two new fragments of 11.4×10^6 and 11.6×10^6 (see Table 3). Therefore *Hpa*I F adjoins *Hpa*I G, and both are bracketed by the two new fragments. On the other hand, *Hpa*I B cleaved by *Xba*I also produced two new fragments (5.6×10^6 and 11.4×10^6) (Table 2). As can be seen from the double digestion pattern of *Hpa*I/*Hba*I (Fig. 1), the 5.6×10^6 fragment

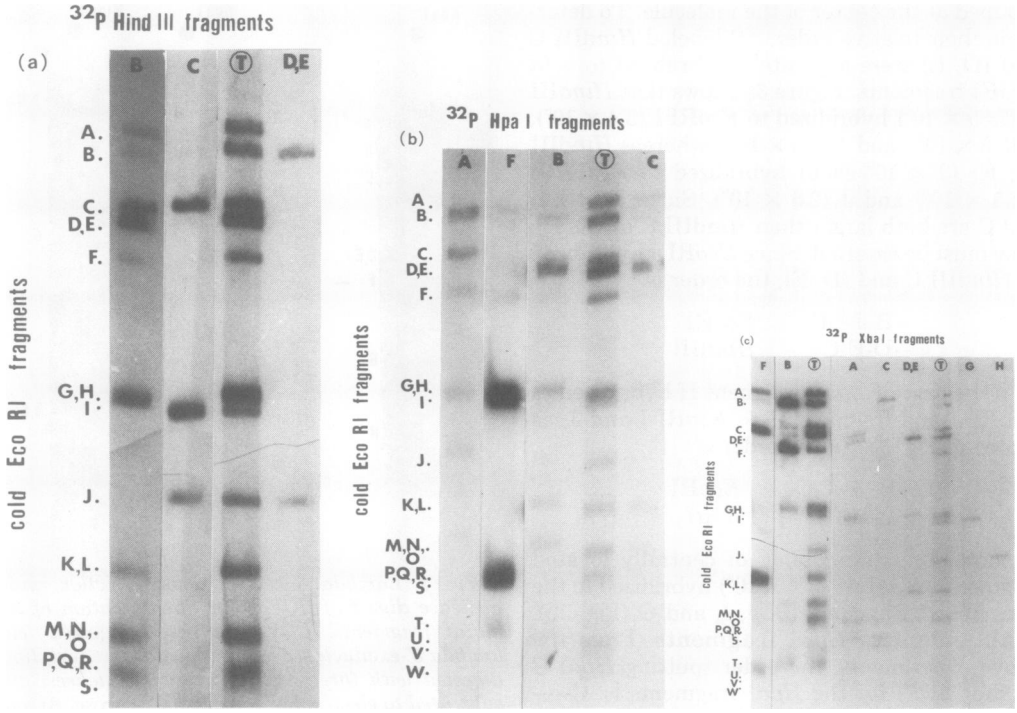

FIG. 3. *Blot hybridization of individual ^{32}P-labeled restriction fragments to filter-bound EcoRI fragments: (a) HindIII, (b) HpaI, (c) XbaI. The technique was that described by Southern (24), and details are given in the text. Ⓣ indicates a nitrocellulose membrane strip hybridized with ^{32}P-labeled CCV DNA to locate all EcoRI fragments. As observed by other authors (32, 34), seemingly pure isolated fragments are often contaminated by sequences from other regions of the genome. Therefore in the hybridization experiments, as well as in the further cleavage of isolated fragments, we only considered bands of high intensity on the autoradiograms.*

corresponds to *Xba*I F. Thus the terminal fragments *Hpa*I B and *Xba*I F reside at the same end of the genome.

Moreover, the new 11.4 × 10^6 fragment *Hpa*I B/*Xba*I must be derived from *Xba*I A, so the fragments situated at the right end of the CCV DNA molecule are ordered as follows:

*Hpa*I	---A-(F,G)-B
*Xba*I	---A F

from which a partial linkage of HpaI fragments can be deduced: C-E-D-A-(F,G)-B. The relative order of F and G will be discussed with the mapping of *Eco*RI sites (below), inasmuch as these two fragments are not cleaved with *Hin*dIII or *Xba*I.

Alignment of *Xba*I fragments. From previous results we know that fragments *Xba*I G and H are part of *Hpa*I A. Two other *Xba*I fragments bracketing them hybridize to *Hpa*I A: *Xba*I A and *Xba*I B (Fig. 4). Fragment *Xba*I B also hybridizes to *Hpa*I D, and from the preceding section *Xba*I A is known to be located next to *Xba*I F at the right end of the genome. Since *Xba*I F is at the right end, the other terminal fragment *Xba*I D (or E) must of course be located at the left end, and the arrangement at this step is: D---B---H-G---A-F. *Xba*I (D, E) fragments (6.7 × 10^6 each) are not cleaved by *Hpa*I (Fig. 1). On the other hand, *Xba*I (D, E) behave in the hybridization experiments as a unique end fragment: they hybridized to the same set of *Eco*RI fragments (Fig. 3c) as did *Hpa*I C. We conclude that *Xba*I D and E are contiguous. *Xba*I C (12.15 × 10^6) hybridized strongly to *Hpa*I D and E (Table 3) and weakly to *Hpa*I B and C. Since *Xba*I C is not an end fragment, the hybridization to *Hpa*I B and C can be due either to a contamination with *Xba*I (D, E) or to the presence in *Xba*I C of a sequence also present in the end fragments, but currently we are not able to distinguish between these possibilities. *Xba*I C is cleaved by *Hpa*I (Table 3) and thus must span the *Hpa*I D–*Hpa*I E fragments. Moreover, *Xba*I I (1.22 × 10^6) hybridizes only to *Hpa*I E (Table 3). Since fragment *Xba*I J (0.65 × 10^6) is not cleaved by *Hpa*I and is found as a unique fragment in the double digestion pattern *Hpa*I/*Xba*I (Fig. 1), the 0.65 × 10^6 fragment found in *Hpa*I E cut with *Xba*I

(Table 2) must be *Xba*I J. Since *Xba*I I and J, as well as a part of *Xba*I C, are located in *Hpa*I E, they must be at the left end of *Hpa*I E and must be contiguous. The relative order of *Xba*I I and J could not be determined because they are not cleaved by *Hin*dIII and *Eco*RI. However, partial digestion experiments with *Xba*I (data not shown) indicate that the order is: ---E-I-J-C---. Thus the order of all *Xba*I fragments is: D-E-I-J-C-B-H-G-A-F.

Alignment of *Eco*RI fragments. (i) Terminal fragments. The previous finding (Sheldrick et al., manuscript in preparation) that CCV DNA is terminally redundant means that some, and in certain cases all, sequences of those restriction fragments identified as terminal (above) will lie within the redundancy. Depending on the location of cleavage sites with respect to the redundant sequences, digestion experiments may have three possible outcomes, as follows.

(a) If there is no site within the redundancy, then each terminal fragment may be of any size exceeding that of the redundant region. This is the case for *Hpa*I B (17×10^6) and C (13.2×10^6).

(b) If there is one site within the redundancy, then the sizes of the terminal fragments will be complementary and the sum of the two will be just the size of the redundant region. This is the case for *Xba*I D (6.7×10^6) and F (5.6×10^6): the sum 12.3×10^6, which defines the size of the redundancy, agrees well with the $\simeq 10 \times 10^6$ estimated by electron microscopy.

(c) If there are multiple sites within the redundancy, then fragments may be of any size smaller than the redundant region, and if there are n sites, then at least (n-1) bands with two identical (in size and sequence) fragments will be generated. As we show in the following analysis, this is the case for *Eco*RI.

It will be recalled that several *Eco*RI terminal fragments are present in 2× molar bands: (D, E), (K, L), and (M, N). The terminal fragments *Xba*I (D, E) and F were used in ordering these, and adjoining, *Eco*RI fragments. *Xba*I F (5.6×10^6) hybridizes only to *Eco*RI (D, E) (7.6×10^6 each) and *Eco*RI (K, L) (1.86×10^6 each) (Fig. 3c). Since the fragments constituting *Eco*RI (D, E) are larger than *Xba*I F, *Eco*RI (K, L) must be located to the right of *Eco*RI (D, E) and *Eco*RI (M, N) (1.38×10^6 each) to their left (for *Eco*RI double fragments present in the terminal redundancy, the first letter is assigned to the left of the genome). *Eco*RI cleavage of *Xba*I (D, E) generates *Eco*RI K, M, X, and Z and three new fragments (5×10^6, 2.65×10^6, and 1.75×10^6) (Table 3). Since the *Eco*RI D fragment is known

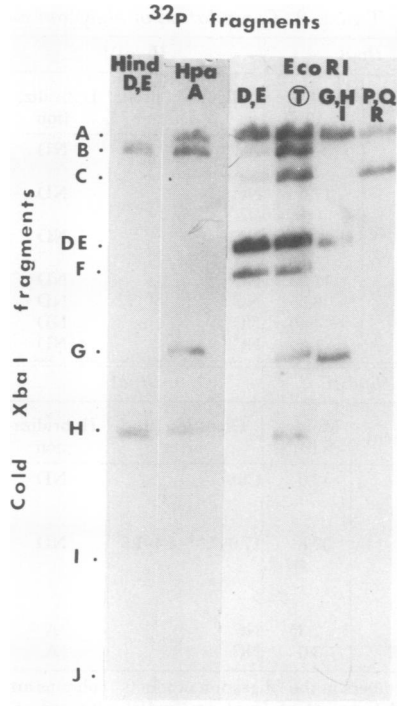

FIG. 4. *Blot hybridization of individual ^{32}P-labeled restriction fragments to filter-bound Xba*I *fragments. See legend to Fig. 3.*

to lie within the region defined by the contiguous fragments *Xba*I (D, E), the 5×10^6 and 2.65×10^6 fragments must define an *Xba*I site in *Eco*RI D, and the 1.75×10^6 fragment must therefore arise from the right extremity of *Xba*I E. On the other hand, *Xba*I F cleaved by *Eco*RI (Fig. 6b; Table 3) gives *Eco*RI L, W, Y, and a new fragment of 2.65×10^6; the last derives from *Eco*RI E and is situated at the left end of *Xba*I F. Since the *Eco*RI fragments D, K, and M (E, L, and N) are already ordered, we conclude that *Eco*RI X and Y are located to the right of *Eco*RI (D and E), but at present we are unable to determine their position relative to *Eco*RI (K and L) (in the map of Fig. 2, we have arbitrarily placed them to the right). Finally, *Eco*RI W and Z must be extreme terminal fragments (1× molar fragment), and the order at the ends of the genome is: Z-M-D-K,X----------N-E-L,Y-W.

(ii) Alignment of *Eco*RI fragments to the left of center. Fragment *Hpa*I C is cleaved by *Eco*RI (Table 2) to known *Eco*RI fragments and a new fragment of 1.55×10^6 which must be situated at the right end of *Hpa*I C. This fragment is also found in *Eco*RI A/*Hpa*I sequential digests (Table 4). There is only one 1.55×10^6 fragment present among the *Hpa*I/*Eco*RI dou-

TABLE 2. *Compilation of digestion and hybridization data for HpaI and HindIII fragments*[a]

*Hpa*I		*Hin*dIII		*Xba*I		*Eco*RI	
Fragment	Mol wt ($\times 10^{-6}$)	Digestion products	Hybridization	Digestion products	Hybridization	Digestion products	Hybridization
A	31.4	10.2*-7.9*-7.35-3.0-3.0	ND	13.4*-11.6*-3.5-2.4[b]	A-B-G-H	11.5-8.5-3.4-2.5-2.15*-1.35-0.71*[b]	B-C-I-J-O
B	17.0	NC	ND	11.4*-5.6	ND	7.6-3.57-1.86-1.38-0.64-0.40*	D,E-A-G,H-K,L-M,N
C	13.2	NC	ND	6.7-6.7[b]	ND	7.6-1.86-1.55*-1.38-0.35-0.30	D,E-K,L-M,N
D	11.75	NC	ND	7.5*-4.25*	ND	Cut	A-F-P-S
E	6.95	NC	ND	4.75*-1.22-0.65*	ND	NC	A
F	4.40	NC	ND	NC	ND	2.65*-1.13[b]	G,H-Q,R-T
G	1.50	NC	ND	NC	ND	0.69-0.8*	G,H-Q,R

*Hin*dIII		*Hpa*I		*Xba*I		*Eco*RI	
Fragment	Mol wt ($\times 10^{-6}$)	Digestion products	Hybridization	Digestion products	Hybridization	Digestion products	Hybridization
A	42.0	Cut	ND	ND	ND	13.7-8.0*-7.6-6.4-1.86-1.38-1.18-1.04-0.38-0.30	ND
B	30.8	17.0-7.9*-4.4-1.5	ND	ND	A-F-D,E	7.6-5.6*-3.62-3.57-1.86-1.38-1.35-1.13-0.83-0.73-0.69-0.64-0.35	C-D,E-G-H-O-K,L-M,N-Q,R
C	7.35	NC	A	3.75*-3.5	ND	3.4-2.9*-1.0*	C-I-J
D, E	3.0	NC	A	3.0-2.25*[b]	B-H	3-1.4*-0.57*[b]	B-J

[a] Numbers in the "digestion products" columns are molecular weights ($\times 10^6$) of fragments obtained by further cleavage of an isolated fragment of *Hpa*I or *Hin*dIII with one of the three other endonucleases. Letters in the "hybridization" columns correspond to unlabeled fragments to which a given ^{32}P-labeled *Hpa*I or *Hin*dIII fragment hybridizes. ND, Not done; NC, not cleaved, as shown on the total double digest patterns of Fig. 1. Asterisks (*) refer to new fragments (absent from single digest patterns).

[b] Sum of molecular weights inferior to expected value (see text).

ble digestion products, and since *Hpa*I G (1.5×10^6) is cleaved by *Eco*RI, this new fragment must be common to *Eco*RI A and *Hpa*I C, thus constituting the left end of *Eco*RI A. We estimate that a small fraction of *Eco*RI A sequences lies in the terminally redundant region; *Hpa*I C (13.2×10^6) less 12.3×10^6 (the redundant region) is 0.9×10^6, and the difference between 1.55×10^6 and 0.9×10^6, namely 0.65×10^6, is the amount of *Eco*RI A included in the terminal redundancy. Fragments *Hpa*I D and *Xba*I C both hybridize to *Eco*RI P and S (Fig. 3b and c; Tables 2 and 3). The poor separation of these three bands in the blots does not permit a direct assignment, but evidence that hybridization is with P and not Q or R is derived from *Xba*I C/*Eco*RI digestion (Fig. 6b). The relative order of *Eco*RI P and S cannot be established since they are not cleaved with the restriction enzymes used here; the order is arbitrarily written as P-S. From the mapping of *Hpa*I sites above, *Eco*RI F is located to the right of *Eco*RI P-S. Proceeding to the right, *Xba*I B hybridizes to *Hpa*I A and D (Fig. 4; Table 3) and to *Eco*RI B and F, and in addition is cleaved by *Eco*RI (Fig. 6a; Table 3) to *Eco*RI F (6.4×10^6) and a new fragment of 11.2×10^6. Since *Eco*RI A is not in *Xba*I B, the 11.2×10^6 fragment can arise only from *Eco*RI B. *Eco*RI B and F are therefore contiguous, and the order is Z-M-D-K,X-A-P,S-F-B-J-I-C.

(iii) Alignment of *Eco*RI fragments to the right of center. Fragment *Eco*RI C hybridizes to *Hpa*I A and is not cleaved by *Hpa*I or *Xba*I (Table 4). It remains to locate two fragments produced by *Eco*RI cleavage in *Hpa*I A: 1.35×10^6 and 0.71×10^6, the former arising from the (M, N), O band. *Eco*RI (M, N) fragments belong to the redundant end regions, so the 1.35×10^6 fragment is in fact *Eco*RI O. Therefore, the 0.71×10^6 fragment must be located at the right end of *Hpa*I A.

*Hpa*I F and G follow *Hpa*I A on the right of the map. *Hpa*I F is cleaved by *Eco*RI to *Eco*RI Q (1.13×10^6) and a new fragment of 2.65×10^6 (Table 2). This fragment can only be produced by the *Eco*RI H/*Hpa*I cleavage; all other *Eco*RI fragments larger than 2.65×10^6, except *Eco*RI G and H, are accounted for, and only *Eco*RI H is cleaved by *Hpa*I (Fig. 1). The sum of 2.65×10^6 plus 0.71×10^6 (the *Hpa*I A/*Eco*RI new fragment) most probably corresponds to *Eco*RI H, and the order is:

*Eco*RI C O H
*Hpa*I A ↑ F

*Hpa*I F also contains *Eco*RI Q (above), and since it, and not *Hpa*I G, hybridizes to *Eco*RI T (Table 2), we might conclude that *Eco*RI T, being cleaved by *Hpa*I (Fig. 1), is located at least partly in *Hpa*I F. We have not been able to identify the products of *Eco*RI T cleaved by *Hpa*I, however, and so direct evidence for the precise map position of *Eco*RI T is unavailable at present. Proceeding to the right, *Hpa*I G is cleaved by *Eco*RI into two fragments: *Eco*RI V (0.69×10^6) and a new fragment of 0.8×10^6 very likely shared by *Hpa*I G and B (Table 2). Indeed, *Hpa*I B, cleaved by *Eco*RI, produces several fragments, among which are found *Eco*RI G and U and a new 0.4×10^6 fragment, located at the *Hpa*I B-G junction (Fig. 5, Table 2). The sum of the new fragments, 0.4×10^6 and 0.8×10^6 (from *Hpa*I G and B), corresponds to the size of *Eco*RI R, which is shown to be cleaved by *Hpa*I (Fig. 1). Thus the order for this region is:

*Eco*RI	H	Q	T	V	R	U	G
*Hpa*I	A↑	F	↑	G	↑		B

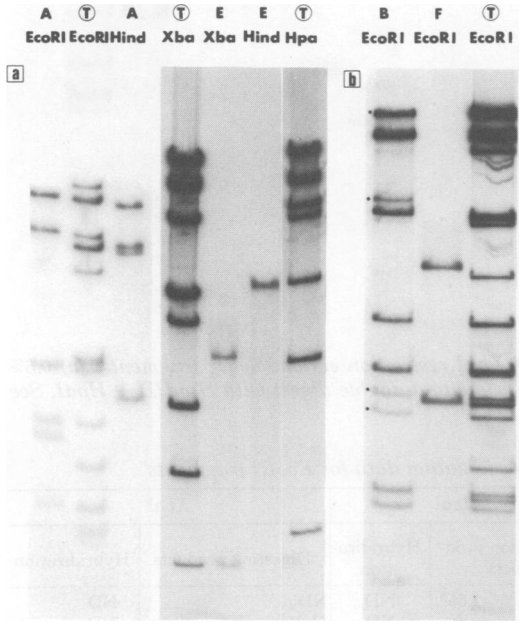

FIG. 5. *Sequential digestion of individual ^{32}P-labeled HpaI restriction endonuclease fragments: (a) 0.5% agarose, (b) 1.1% agarose. Ⓣ refers to single cleavage patterns of total CCV DNA. Molecular weights of new fragments are in Table 2. (●) Bands due to incomplete cleavage and/or contamination.*

Alignment of *Hin*dIII fragments. The final question to be dealt with is the relative location of *Hin*dIII A and B, and thus the location of the *Hin*dIII sites. The strongest evidence identifying *Hin*dIII B as the right end of the genome is available in the digestion of *Hin*dIII B with *Hpa*I. *Hin*dIII B is cleaved by *Hpa*I (Table 2) into four fragments: *Hpa*I B (17×10^6), F (4.4×10^6), and G (1.5×10^6), and a new fragment of 7.9×10^6 located at the *Hin*dIII B-C junction. Fragments *Hin*dIII (D, E) cleaved by *Eco*RI (Fig. 7; Table 2) produce three fragments: *Hin*dIII D, located in *Eco*RI B (Table 4), and two new ones (1.4×10^6 and 0.57×10^6). Conversely, *Eco*RI B cleaved by *Hin*dIII generates three fragments, one of which (0.57×10^6) is included in *Hin*dIII E. Since *Hin*dIII (D, E) fragments hybridize to *Eco*RI B and J, the other new fragment, *Hin*dIII (D, E)/*Eco*RI (1.4×10^6), must be located in *Eco*RI J. However, the sum of the molecular weights of the two visible fragments from *Eco*RI cleavage of *Hin*dIII E (3×10^6) was 10^6 less than expected. A similar result was obtained for the digestion of *Hin*dIII E by *Xba*I (one detectable fragment), where 0.5×10^6 is unaccounted for. The "lost" molecular weight in these cases could not have been in fragments greater than 0.3×10^6, the lower size limit de-

TABLE 3. *Compilation of digestion and hybridization data for HbaI fragments*[a]

Fragment	Mol wt ($\times 10^6$)	*Hin*dIII		*Hpa*I		*Eco*RI	
		Digestion products	Hybridization	Digestion products	Hybridization	Digestion products	Hybridization
A	29	25.2*-3.7*	ND	11.6*-11.4*-4.4-1.5	A-B-G-F	8.5-5.0*-3.62-3.57-1.38-1.13-1.13-0.83-0.75*-0.73-0.69	C-D,E-G,H-O-Q,R
B	17.7	14.4*-3.0[b]	ND	13.4*-4.35*	A-D	11.2*-6.4	B-F
C	12.2	NC	ND	Cut	D-E	10.0*-1.18-1.04	A-P
D, E	6.7	NC	ND	NC	B-C	5.0*-2.65*-1.86-1.75*-1.38-0.35-0.30	D,E-G,H-K,L-M,N
F	5.6	NC	ND	NC	ND	2.65*-1.86-0.64-0.35	D,E-K,L
G	3.5	NC	ND	NC	A	2.65*-0.8*	I-J
H	2.4	2.25[b]	ND	NC	A	1.6*[b]	J
I	1.22	NC	ND	NC	E	NC	ND
J	0.65	NC	ND	NC	ND	NC	ND

[a] See footnote *a*, Table 2.
[b] Sum of molecular weights less than expected (see the text).

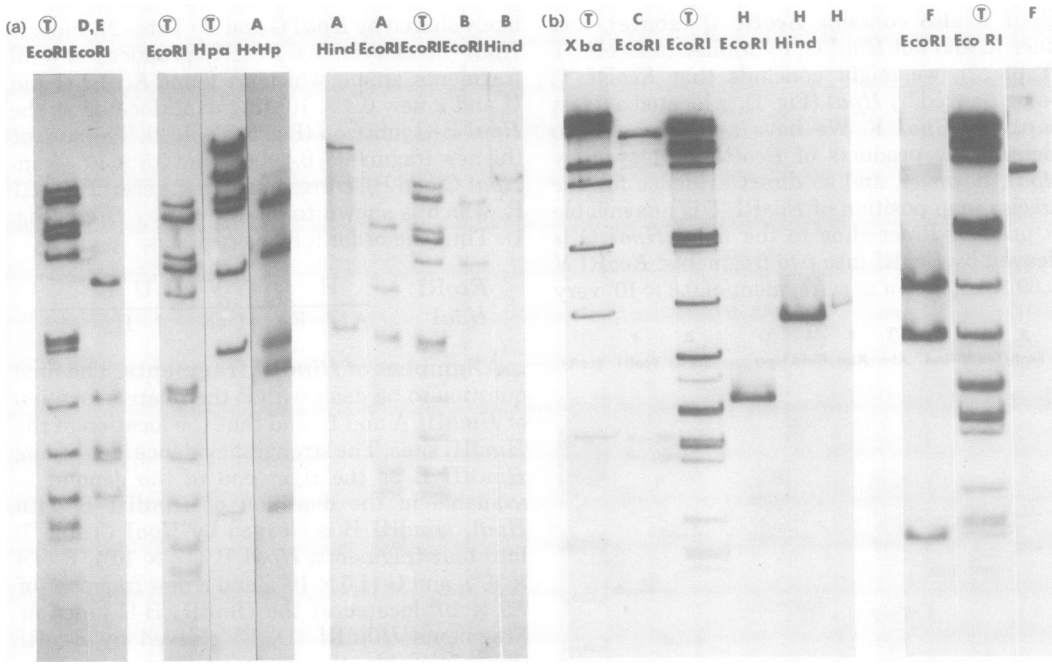

FIG. 6. *Sequential digestion of individual ^{32}P-labeled XbaI restriction endonuclease fragments: (a) 0.5% agarose, (b) 1.1% agarose. See legend to Fig. 5. H + Hp refers to a double digest with HindIII + HpaI. See also Table 3.*

TABLE 4. *Compilation of digestion and hybridization data for EcoRI fragments*[a]

Fragment	Mol wt (×10^6)	*Hin*dIII		*Hpa*I		*Xba*I	
		Digestion products	Hybridization	Digestion products	Hybridization	Digestion products	Hybridization
A	13.7	NC	ND	6.95–5.25*–1.55*	ND	ND	ND
B	11.5	8.0*–3.0–0.7*	ND	ND	ND	11.2*[b]	ND
C	8.5	5.6*–2.9*	ND	NC	A	ND	ND
D, E	7.6 × 2	ND	ND	ND	ND	5.0* × 2–2.65* × 2	A-D,E-F
F	6.4	ND	ND	ND	A-D	ND	ND
G, H, I	3.62–3.57 3.40	NC	ND	ND	ND	3.62–3.57–2.65*–0.75*	A-G-D,E
J	2.50	ND	ND	ND	A	ND	ND
K, L	1.86 × 2	ND	ND	ND	B-C	ND	ND
M, N	1.38 × 2	ND	ND	ND	ND	ND	ND
O P Q, R	1.35 1.18– 1.13 × 2	ND	ND	ND	ND	ND	A-C
S	0.83	ND	ND	ND	ND	ND	C

[a] See footnote *a*, Table 2.
[b] Sum of molecular weights less than expected (see the text).

tectable in our gels. Then if the loss is real, restriction sites for these two enzymes may be clustered at one end of the *Hin*dIII E fragment; this is indicated by unlettered fragments on the map (Fig. 8).

Our results described above are summarized in the restriction maps presented in Fig. 8.

DISCUSSION

The restriction endonuclease site maps developed in the present study strengthen several conclusions, independently derived from velocity sedimentation and electron microscopic investigations (Sheldrick et al., in preparation), concerning CCV genome structure. One concordant result is that, for each restriction enzyme, the sum of fragment molecular weights (Table 1) agrees well with our prior estimates of 84 ± 3 × 10^6 for the molecular weight of CCV DNA. This, and the absence from gel patterns (Fig. 1) of "minor bands" due to fragments in

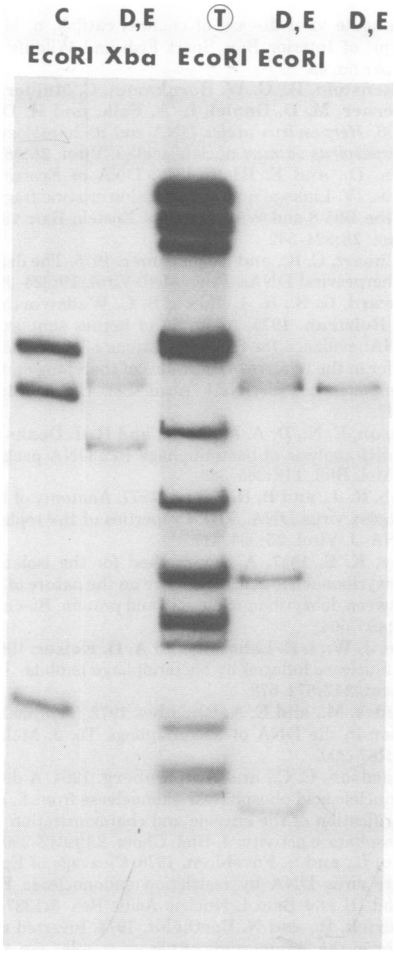

FIG. 7. *Sequential digestion of individual ^{32}P-labeled HindIII restriction endonuclease fragments. See legend to Fig. 5. See also Table 2.*

less than molar amounts (a possible result of sequence inversion; see ref. 12, 33), support the view that CCV DNA has no counterpart to the inverted repeat sequences in the DNAs of HSV (20, 31), equine abortion virus (Sheldrick and Berthelot, unpublished data), pseudorabies virus (26; Powell, Clements, and Wilkie, personal communication), and bovine mammillitis virus (3, 4).

The result that only certain restriction fragments are susceptible to exonuclease digestion (Fig. 2), and are therefore considered to be terminal, taken with the general absence of minor bands, argues strongly against circular permutation of base sequence order—either complete, as in T-even bacteriophage DNA (28), or partial, as in bacteriophage P22 DNA (30)—in a description of the CCV genome. Instead, a common sequence order must be shared by essentially all molecules in the population. This conclusion is pertinent to the mechanism by which viral genomes are packaged into virions. Concatemeric forms of DNA may be intermediates in this process for CCV (Bucchini, Cébrian, and Sheldrick, unpublished data), as they may also be for pseudorabies virus (1) and HSV (14, 22). If unit-length CCV genomes are cut from concatemers, then length determination must be based on a site-specific mechanism, as it is for bacteriophage lambda (6, 7), rather than on the "headful" mechanisms of bacteriophages T4 (27) and P22 (13, 30).

The physical maps presented here provide more precise estimates than hitherto available for the extent and nature of the terminally repeated regions. Thus, the combined size of the terminal fragments generated from a single *Xba*I cleavage site within the repeat (Fig. 8) is 12.3 × 10^6, the extent of terminal repetition. The fact

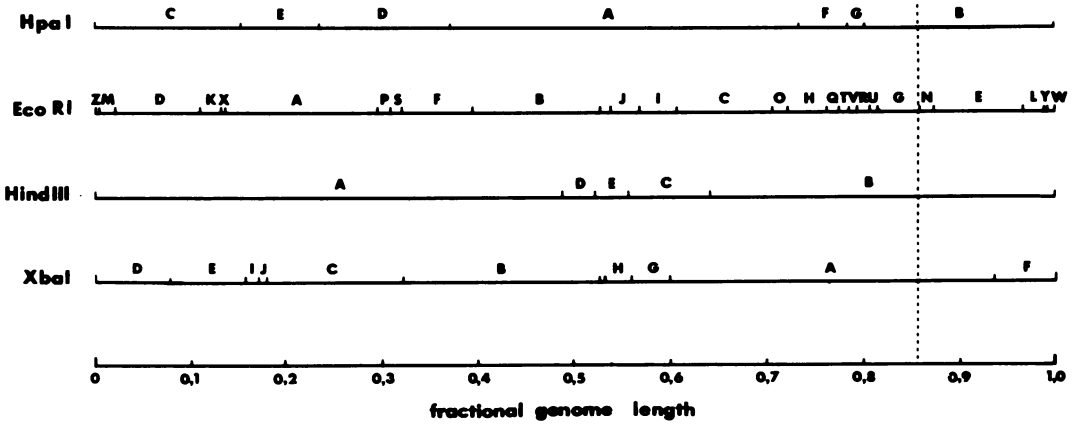

FIG. 8. *Physical maps for the arrangement of restriction endonuclease cleavage sites in CCV DNA. The unlettered regions (at map positions 0.53 to 0.55) contain the putative "clustered" sites referred to in the text. The dashed vertical line indicates the boundary of the terminal redundancy.*

that there is but a single *XbaI* site, and the nonperiodic distribution of *Eco*RI sites in the repeated regions (Fig. 8), exclude the type of highly repetitive terminal sequences observed in the genomes of *H. saimiri* (2) and *H. ateles* (9). The very nature of restriction endonuclease mapping does not allow us, of course, to exclude the possibility of sequence reiteration within an interval defined by adjacent restriction sites. From the maps of Fig. 8 it is obvious that there are many such intervals, and that without further mapping we cannot rule out, for instance, the kind of local sequence reiteration recently found in Epstein-Barr virus DNA (10, 19). Nevertheless, the genomes of CCV and Epstein-Barr virus differ sharply in that the (small) terminal repeats of the latter appear to be internally reiterated (10).

The CCV genome, being ostensibly devoid of inverted repeated nucleotide sequences, and having large but not highly repetitive terminal repeats, is a new structural type of herpesvirus genome. The physiological basis for the varied structural patterns of these genomes is not known, so future studies of how members of the herpesvirus group regulate the expression of their genetic information are bound to hold considerable interest.

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