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"Endless" Viral DNA in Cells Infected with Channel Catfish Virus

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The state of intracellular viral DNA in cells infected with channel catfish virus has been studied by the Hirt selective extraction procedure and by restriction endonuclease digestion. The sedimentation properties and restriction patterns of viral DNA in the Hirt supernatant fraction indicate that the majority, if not all, of the DNA is in the form of linear unit-length ($M_r \cong 85 \times 10^6$) molecules. However, restriction digests of viral DNA in the pellet fraction lacked two fragments corresponding to the molecular ends of unit-length DNA. In addition, there appeared in *Hpa*I digests of pellet DNA a new restriction fragment interpretable as the product of fusion between the ends of unit-length molecules. The size of the new fragment requires that fusion occur in such a way that one copy of the terminally repeated sequences ($M_r \cong 12.3 \times 10^6$) of the unit-length DNA is lost in the process. In pulse-chase experiments, radioactivity flowed from the pellet fraction to the supernatant fraction, suggesting a precursor-product relationship for these DNA species. The results are easily understood if unit-length virion DNA is generated by excision from concatemeric structures.

As extracted from virions, the genome of the herpesvirus channel catfish virus (CCV) is a linear molecule of double-stranded DNA with a molecular weight of approximately 85×10^6 (5, 7). CCV DNA is terminally repetitive; that is, the same nucleotide sequence ($M_r \cong 12.3 \times 10^6$) is present at both ends of the molecule (5). The repeated sequences are mutually oriented with direct polarity, a configuration which permits the formation of circles or concatemers by intramolecular or intermolecular recombination, respectively (4, 17–19). As circles or concatemers are formed in this way from linear DNA, the molecular ends are either lost in the former case, or in the latter case, their frequency relative to a given internal sequence is reduced in proportion to the number of unit-length molecules making up the concatemer.

When linear DNA is digested with restriction endonucleases, the molecular ends generate a subset of fragments in which one end of the fragment is defined by an internal restriction site and the other end by the molecular end itself. For CCV virion DNA, in which the terminal sequences are not altered by permutation (17) or inversion (11), this subset contains two fragments. The size and relative position of these have been determined for the restriction endonucleases *Eco*RI, *Hin*dIII, *Hpa*I, and *Xba*I (5).

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In this communication, we describe experiments which use the Hirt selective extraction technique (12) to fractionate intracellular DNA late in the replicative cycle of CCV. *Hpa*I and *Xba*I digests of CCV DNA in the supernatant fraction contained normal amounts of end fragments, but these were not detectable in digests of viral DNA from the pellet fraction. The data indicate the existence of two forms of intracellular CCV DNA, one of which corresponds to unit-length genomes and the second to "endless" structures such as circles, long concatemers, or possibly some combination of the two. Moreover, pulse-chase experiments suggest that the endless form of CCV DNA is a precursor of the unit-length genomes.

Results similar to those presented here have been previously published by Ben-Porat and Rixon (2) for pseudorabies virus and by Jacob et al. (13) for herpes simplex virus.

(A preliminary report of this work was presented at the Herpes Virus Workshop, Cambridge, England, in August 1978.)

MATERIALS AND METHODS

Cells and virus. The continuous catfish cell line BB (20) was cultivated at 30°C in Eagle minimum essential medium (MEM) supplemented with 5% fetal calf serum. Confluent monolayers in plastic petri dishes (60- or 100-mm diameter containing, respectively, 3×10^6 to 4×10^6 cells and 10×10^6 to 12×10^6 cells) were infected with CCV at a multiplicity of 10 to 20 PFU per cell. Virus adsorption was carried out in 0.2-ml (60-

mm dish) or 0.5-ml (100-mm dish) volumes of MEM containing 10% (vol/vol) dimethyl sulfoxide for 20 min at 22°C. Two milliliters (60-mm dish) or 5 ml (100-mm dish) of MEM without serum was then added, and the infected cells were incubated for the desired time at 22°C under an atmosphere of 95% air–5% CO_2.

Radiolabeling of intracellular DNA. Cultures were labeled with [*methyl*-^{3}H]thymidine (~50 Ci/mmol; Commissariat à l'Energie Atomique, Saclay, France, and New England Nuclear Corp., Boston, Mass.) by adding the isotope to the culture fluid to obtain a final concentration of 5 to 50 μCi/ml, depending upon the experiment. Chases were performed by removal of the radioactive medium, followed by a wash with MEM containing 2 mM thymidine and 0.1 mM deoxycytidine (1), with additional incubation in the same medium.

Nonselective extraction of intracellular DNA. The extraction procedure was derived from the method of Gross-Bellard et al. (10). Infected cell monolayers were lysed with 0.5% sodium dodecyl sulfate (SDS) in 10 mM Tris-hydrochloride (pH 7.5)–10 mM EDTA (1 ml per 10^6 cells), and the lysate was incubated in the presence of 100 μg of proteinase K (E. Merck AG, Darmstadt, Germany) per ml for 12 h at 37°C. After a second addition of proteinase (100 μg/ml, 6 h at 37°C), the lysate was adjusted to 1% SDS and extracted twice with an equal volume of phenol previously equilibrated with 0.5 M Tris-hydrochloride (pH 8.3)–10 mM EDTA. The aqueous phase was dialyzed against two changes of 10 mM Tris-hydrochloride (pH 7.5)–1 mM EDTA and stored at 4°C.

Selective extraction of intracellular DNA. Selective DNA extraction by the method of Hirt was performed as previously described (12), except that proteinase K (100 μg/ml) was included in the lysis buffer. The presence of proteinase was found to facilitate subsequent resuspension of ethanol-precipitated DNA (below); otherwise, no essential differences were observed in the velocity sedimentation properties or buoyant densities of supernatant of pellet DNAs if the proteinase was omitted, in agreement with Hirt's original observations (12). The lysate (1 ml of extraction buffer per 1×10^6 to 2×10^6 cells) was incubated at 37°C for 1 h before the addition, with gentle mixing, of 0.25 volumes of 5 M NaCl. After being held for at least 2 h at 4°C, the precipitate was pelleted by centrifugation at 27,000 × *g* for 30 min. For CsCl or sucrose gradient analyses, the supernatant was used directly. For restriction endonuclease analysis, the supernatant was first dialyzed at 4°C against two changes of 100 volumes of 10 mM Tris-hydrochloride (pH 7.5)–1 mM EDTA, adjusted to 0.3 M ammonium acetate, and precipitated with 2 volumes of 95% ethanol. The centrifuged precipitate was then suspended in a small volume of the appropriate restriction endonuclease buffer (see below). The high-salt pellet fraction was gently resuspended (usually 24 h at room temperature without agitation) in the original volume of extraction buffer (10 mM Tris-hydrochloride [pH 7.5]–10 mM EDTA) lacking SDS and proteinase K and reprecipitated with NaCl as described above. Pellet fractions to be subjected to CsCl density gradient centrifugation were first extracted with phenol as indicated for total DNA.

CsCl gradient centrifugation. Solutions of the material to be analyzed were adjusted to a density of 1.7 g/cm^3 by the addition of crystalline CsCl. Centrifugation was carried out at 20°C in polyallomer tubes for 72 h at 35,000 rpm in a 50 Ti (Beckman Instruments, Inc., Fullerton, Calif.) rotor, or for 20 h at 50,000 rpm in a 65 VTi (Beckman) vertical rotor. For radioactivity measurements, samples of gradient fractions were deposited on GF/C filters (Whatman, Inc., Clifton, N.J.) which were sequentially soaked in 10% trichloroacetic acid (10 min) and 70% ethanol (10 min) and then dried.

Sucrose gradient centrifugation. Samples (usually 1 ml) were layered onto 10-ml, 5 to 20% (wt/wt) linear sucrose gradients (in 1 M NaCl–1 mM EDTA–10 mM sodium phosphate [pH 7.5]) and centrifuged in an SW41 (Beckman) rotor at 36,000 rpm for 150 min at 20°C. Measurements of radioactivity were performed as described above.

Restriction endonuclease analyses. All enzymes were purchased from New England Biolabs (Beverly, Mass.). Incubation buffers for *Hpa*I and *Xba*I were as previously described (5).

DNA was digested with an excess (2 to 5 times based on the activity specified by the manufacturer) of enzyme for 2 h at 37°C. Incubation mixtures included 100 μg of bovine serum albumin per ml. Reactions were terminated by adding 1/10 volume of 0.1 M EDTA–2% SDS–70% glycerol–0.2% bromophenol blue, and the digestion products were electrophoresed on 0.5% agarose horizontal slab gels (27 by 10 by 0.4 cm) at 1.5 V/cm for 15 to 20 h at ambient temperature (~22°C). The electrophoresis buffer was 40 mM Tris-hydrochloride–30 mM acetic acid–20 mM sodium acetate (pH 7.8)–2 mM EDTA. Radioactivity was detected by fluorography as described previously by Bonner and Laskey (3), using presensitized Kodak RPX-omat radiographic film (14).

RESULTS

Kinetics of thymidine incorporation during CCV infection. Wolf and Darlington (20) estimated the length of the CCV infectious cycle at 22°C to be approximately 12 h. In the present study, we wished to place our experiments at a time in the infectious cycle near the maximum level of viral DNA synthesis, so we first determined the kinetics of thymidine incorporation into CCV DNA. Confluent BB monolayers at 22°C were infected with 10 to 20 PFU of CCV per cell, and, after a 20-min adsorption period, were subjected hourly to 30-min pulses of [^{3}H]thymidine. Immediately after the labeling period, total DNA was extracted and analyzed by CsCl gradient density centrifugation as described above. The relative proportion of label incorporated into cellular DNA (p = 1.700 g/cm^3) and viral DNA (p = 1.717 g/cm^3 [9]) at various times after infection is shown in Fig. 1. Label associated with CCV DNA was first detectable in the pulse beginning at 2 h postinfection (p.i.) and became maximal within the interval defined by the 3 h-p.i. and 5 h-p.i. pulses. In the experiments described below, we therefore used 30-min labeling periods beginning at 3.5 h p.i. Dixon and Farber (7) obtained kinetics for CCV DNA synthesis at 30°C which are similar to those reported here.

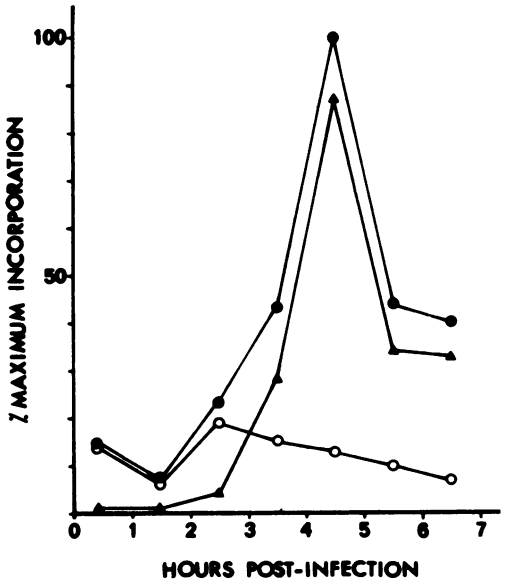

FIG. 1. Kinetics of [^{3}H]thymidine incorporation into CCV-infected cellular DNA. Infected cellular DNA was extracted and analyzed by CsCl density gradient centrifugation as described in the text. Results are expressed as the viral (▲) and cellular (○) components of the total (●) incorporation. The maximum total incorporation was assigned a value of 100%. The proportion of counts in viral and cellular DNAs was estimated from density gradient profiles similar to those of Fig. 3.

Fractionation of ^{3}H-labeled DNA by Hirt extraction. Hirt (12) developed a selective precipitation procedure permitting the separation of low-molecular-weight polyoma virus DNA ($M_r \cong 3 \times 10^6$) from high-molecular-weight cellular DNA ($M_r > 100 \times 10^6$). Since its introduction, the technique has been extended to studies of viruses with genomes of considerably higher molecular weight than that of polyoma virus, notably pseudorabies virus ($M_r \cong 90 \times 10^6$ [1]) and herpes simplex virus ($M_r \cong 100 \times 10^6$ [15]). In applying the Hirt procedure to CCV-infected cells, we observed that successive extractions continued to liberate labeled material into the supernatant fraction in decreasing amounts (Fig. 2). Of the total radioactivity recovered in the supernatants of four serial extractions, ~50% was released in the first extraction, ~25% in the second, ~10% in the third, and ~5% in the fourth; further extractions failed to release significant amounts of radioactivity from the pellet. In a representative experiment, ~30% of the total label incorporated in a 30-min pulse was found in the combined supernatants, and ~70% remained pellet associated. When pulse-labeled uninfected BB cells were extracted in the same way (data not shown), >95% of the incorporated radioactivity remained associated with the pellet.

To investigate the partitioning of radioactivity between viral and cellular DNAs, successive supernatants and the exhaustively extracted pellet were centrifuged to equilibrium in CsCl gradients. Roughly 90% of the radioactivity in each supernatant banded at the density of viral DNA (Fig. 3A), whereas about 60% of the pellet radioactivity had the density of viral DNA (Fig. 3B). The supernatants and pellet were also characterized by velocity sedimentation in 5 to 20% sucrose gradients (Fig. 4). Sedimentation profiles of radioactivity in successive supernatant fractions were typically heterodisperse (Fig. 4A and B) and covered a range of sedimentation coefficients from ~50S to ~20S. Unit-length CCV DNA extracted from virions sediments at ~53S (P. Sheldrick, N. Berthelot, and S. Chousterman, unpublished data). Similar experiments with the final pellet fraction (not shown) revealed that 80% of the radioactivity sedimentd to the bottom of the gradient.

We next examined the behavior of pulse-labeled supernatant and pellet DNA during a 4-h chase with excess nonradioactive thymidine. The results (Fig. 5) show that as the chase progressed, radioactivity was lost from the pellet and accumulated with similar kinetics in the supernatant. In control experiments not presented, the presence of excess thymidine from 3.5 h p.i. on did not measurably affect the yield of infectious virus from a full growth cycle (measured at 24 h p.i.). Velocity sedimentation analy-

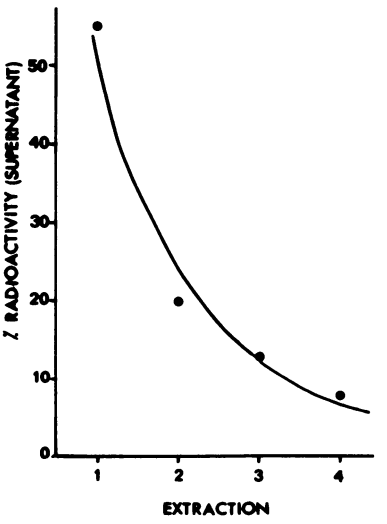

FIG. 2. Successive Hirt extractions of CCV-infected cells. Infected cells were labeled with [^{3}H]thymidine for 30 min at 3.5 h p.i. and extracted as described in the text. Results are expressed as the percentage of total radioactivity extracted.

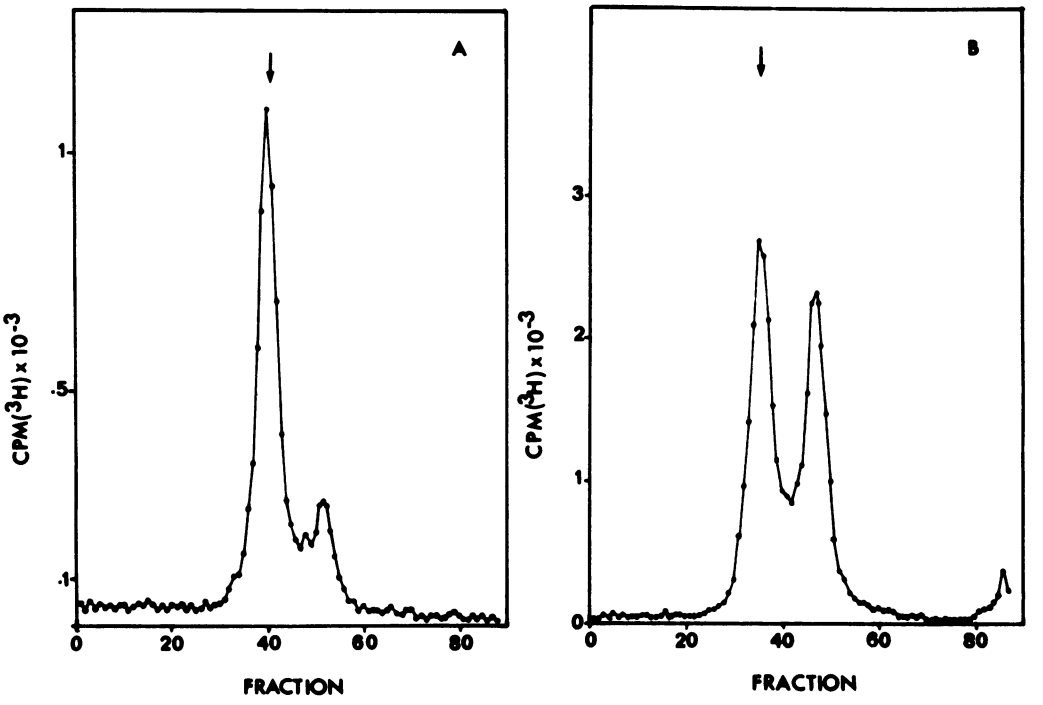

FIG. 3. Buoyant density gradients of supernatant and pellet DNAs. (A) Combined supernatant fractions from the experiment shown in Fig. 2. (B) Pellet fraction after four successive extractions (Fig. 2). As a buoyant density reference, [^{14}C]thymidine-labeled CCV virion DNA (arrows) was added to the gradients. The gradient bottoms are to the left.

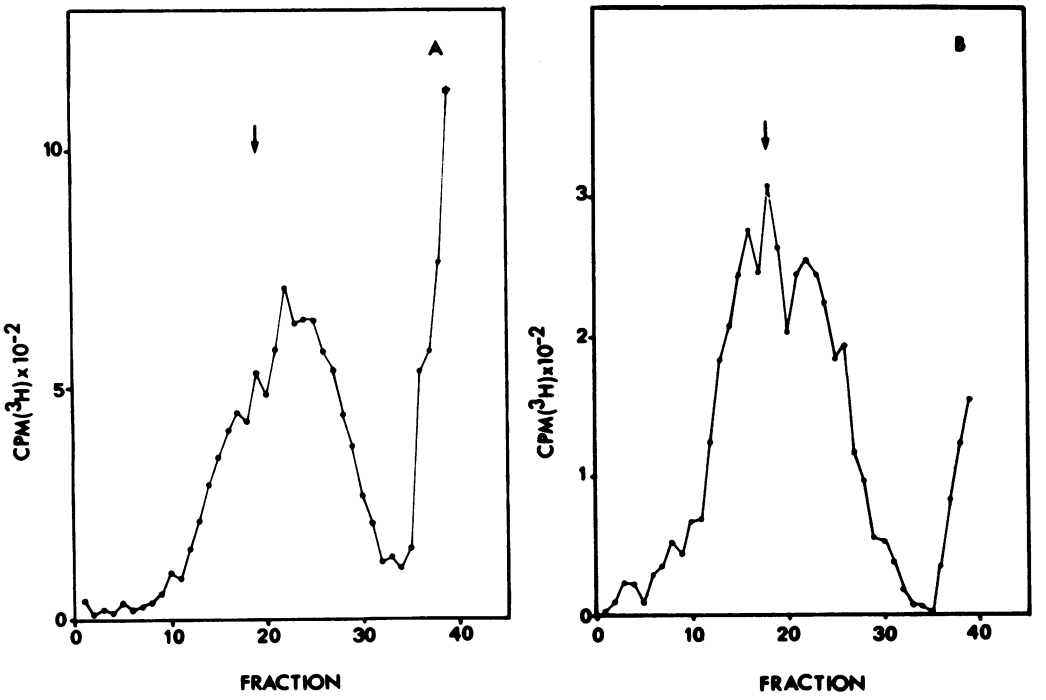

FIG. 4. Velocity sedimentation of supernatant DNA. Sucrose gradients (5 to 20%) of the first (A) and second (B) successive supernatant fractions shown in Fig. 2. Arrows indicate the position of ^{14}C-labeled CCV virion DNA added to the gradients. Sedimentation was from right to left.

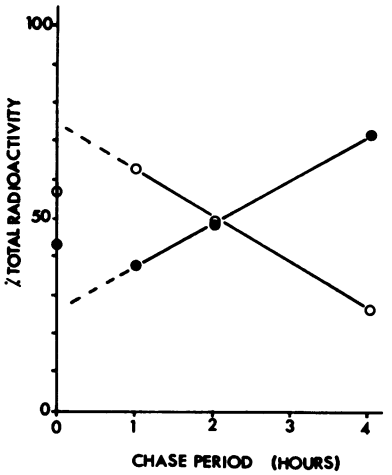

FIG. 5. Partitioning of radioactive CCV DNA between supernatant and pellet fractions during a pulse-chase. Infected cells were pulse-labeled as in the legend to Fig. 2 and incubated for the indicated times in the presence of excess thymidine (see text). Radioactivity in CCV DNA was determined by density gradient centrifugation (as in Fig. 1). Values for each time point were obtained from four (combined) supernatant fractions (●) and from the final pellet fraction (○).

sis of the supernatant fraction prepared at various times during the chase (Fig. 6) showed that the major part, if not all, of the accumulating radioactivity sedimented at ~53S, the position of unit-length CCV DNA.

Restriction endonuclease analysis of supernatant, pellet, and nonselectively extracted DNAs. In restriction endonuclease digests of linear DNA, the ensemble of fragments contains a subset produced on the one hand by internal restriction sites and on the other hand by the molecular ends of the DNA. For unit-length CCV DNA, two fragments appeared in this subset. The present experiments were carried out with *Hpa*I, whose recognition sites lie outside the terminally repeated sequences, and with *Xba*I, one of whose recognition sites lie within these sequences (see Fig. 9 and reference 5).

CsCl gradient-purified CCV DNA from the supernatant and pellet fractions was digested with each of these enzymes, and the resulting fragments were resolved by agarose gel electrophoresis (see above). The fragment profiles of restricted supernatant DNA are indistinguishable from those of CCV virion DNA digested with the respective enzymes (Fig. 7A through D), supporting our conclusion that this fraction contains principally unit-length viral genomes. With pellet-fraction DNA, however, quite different results were obtained. The profile of *Xba*I digests lacked band F altogether, and, as estimated from densitometer tracings of photographic negatives, the intensity of the 2× molar band (D, E) relative to bands, C, G, and H diminished to the equivalent of ~1.1 molar (Fig. 7 G and H). Similarly, in gels of *Hpa*I digests, bands B and C were no longer detectable (Fig. 7E and F).

Visual inspection of the gels permitted us to

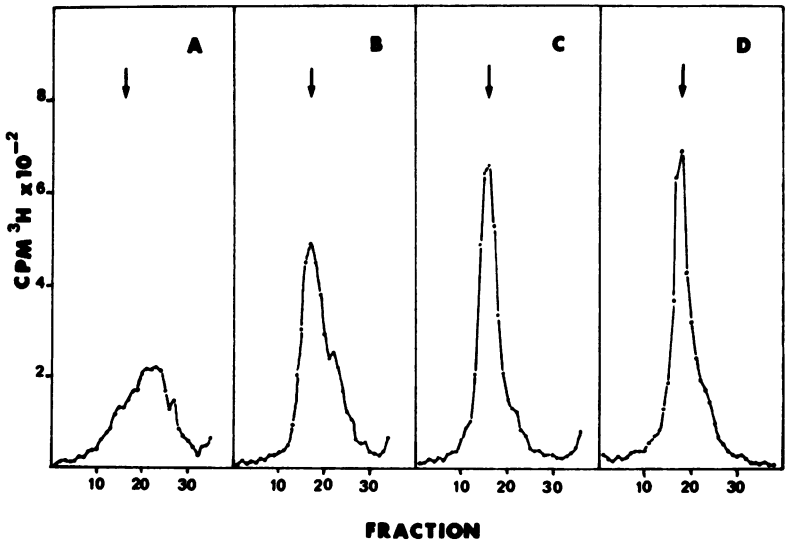

FIG. 6. Velocity sedimentation of supernatant DNA during a pulse-chase. The first supernatant fractions of a 30-min pulse at 3.5 h p.i. (A) and after chases of 1, 3, and 4 h (B, C, and D, respectively) were sedimented in 5 to 20% sucrose gradients. Arrows and direction of sedimentation are as in Fig. 4.

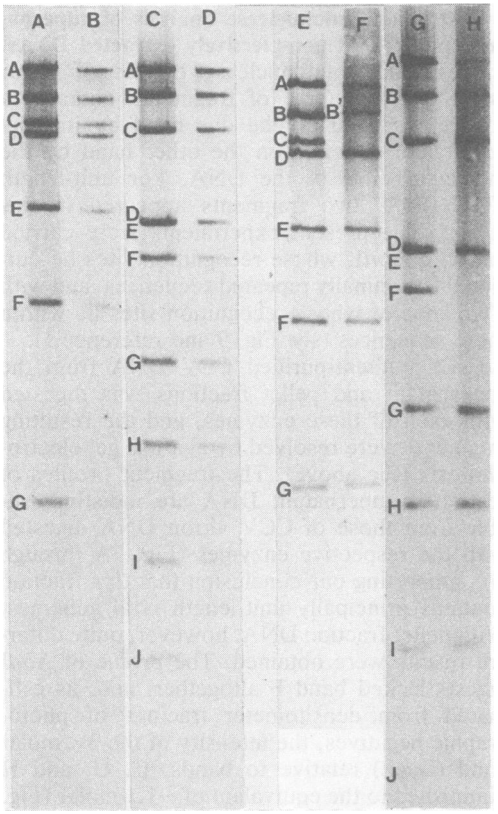

FIG. 7. Restriction endonuclease digests of viral DNA from a Hirt extraction of CCV-infected cells. Infected cells were pulse-labeled (30 min) at 3.5 h p.i., and viral DNA was selectively extracted and purified by CsCl density gradient centrifugation. Supernatant (lanes B and D) and pellet (lanes F and H) DNAs were digested with *Hpa*I (lanes B and F) and *Xba*I (lanes D and H). Digests of ^{14}C-labeled CCV virion DNA with the respective enzymes (*Hpa*I, lanes A and E; *Xba*I, lanes C and G) were included for reference. Conditions for electrophoresis and autoradiography are given in the text.

roughly estimate an upper limit for the relative concentration of the missing fragments. For example, in the *Xba*I profile, fragment F, with a molecular weight of 5.6×10^6, was not detectable under the conditions of film exposure used, whereas fragment J, at 0.65×10^6, was clearly visible (Fig. 7H). Since the molecular weights (and supposedly the amount of [^{3}H]thymidine) of these fragments differ by a factor of 10, the concentration of *Xba*I fragment F must be less than 1/10 that of *Xba*I fragment J; otherwise, the band would be visible. Moreover, in virion DNA one copy of *Xba*I fragment J is present per genome, which means that the concentration of ends (strictly speaking, one end for *Xba*I) per genome equivalent in pellet DNA is more than 10-fold lower than in virion (or supernatant) DNA. A similar estimate can be obtained by comparing *Hpa*I fragments B and C ($M_r = 17 \times 10^6$ and 13.2×10^6, respectively) with *Hpa*I fragment G ($M_r = 1.5 \times 10^6$). In addition to the above changes, a new band (B′) appeared in profiles of *Hpa*I digests for pellet DNA (Fig. 7F). The molecular weight ($M_r \cong 18 \times 10^6$) of this new fragment is precisely that expected from a fusion (via overlap of the terminal repeats) of the *Hpa*I fragments B and C of virion DNA (see Fig. 9). This can be calculated by the following equation: fragment B (17×10^6) plus fragment C (13.2×10^6) minus the terminal repeat sequence (12.3×10^6) equals 17.9×10^6.

For another project, we have recently cloned fragment B′ in the *Hpa*I site of cosmid pHC 79. Restriction endonuclease site maps (M. Lacasa and J. Cébrian, unpublished data) agree completely with the present assignment.

The fusion fragment *Hpa*I B′ is more conveniently observed in digests of intracellular DNA

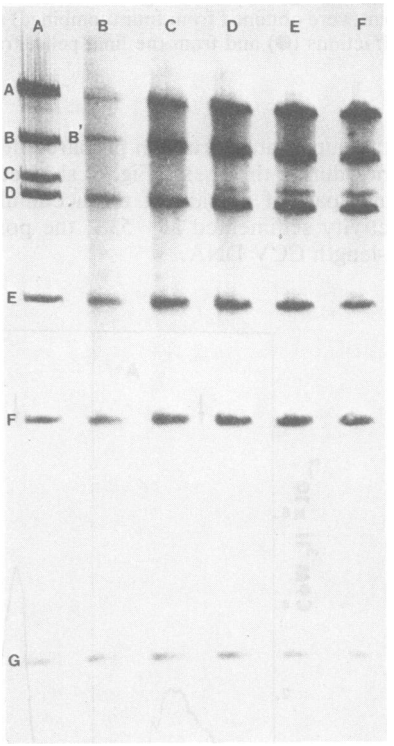

FIG. 8. Restriction endonuclease digests of total DNA from nonselective extraction of CCV-infected cells. *Hpa*I digests of DNA from a nonselective extraction (see text) of infected cells pulse-labeled (30 min) at 3.5 h p.i. (lane B) and chased with excess thymidine for 1 (lane C), 2 (lane D), 3 (lane E), and 4 h (lane F). *Hpa*I-digested, ^{14}C-labeled virion DNA was included as a marker (lane A).

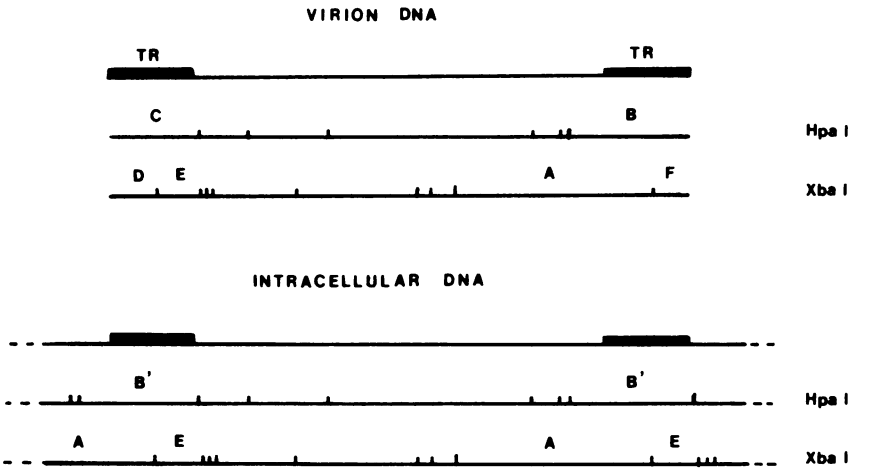

FIG. 9. Restriction endonuclease site map of virion and intracellular CCV DNAs. Thick lines represent the $M_r = 12.3 \times 10^6$ terminal repeat sequences referred to in the text. Only the restriction fragments relevant to the present study are lettered.

from a nonselective extraction (see above), since it and fragment *Hpa*I B form a doublet in gel profiles (Fig. 8B). A chase with nonradioactive thymidine was also incorporated into the experiment shown in Fig. 8, and, as the figure shows, the gel profiles evolved during the course of the chase. In a 30-min pulse, only band B' (as in pellet DNA) was apparent, but as the chase proceeded its intensity decreased whereas the intensities of bands B and C increased. These results are in agreement with the previous suggestion that pellet DNA is a precursor to supernatant DNA.

Certain bands other than those just discussed were also found to have modified intensities in gel profiles of pellet and total DNA. These were *Xba*I band A ($M_r = 29 \times 10^6$) and *Hpa*I band A ($M_r = 31 \times 10^6$), both of which are clearly underrepresented relative to virion and supernatant DNAs in the gels of Fig. 7 and 8. We have investigated this more closely with *Bgl*II, which gives smaller fragments ($M_r < 15 \times 10^6$; S. Chousterman and M. Lacasa, personal communication) in the genomic regions covered by *Xba*I band A and *Hpa*I band A. In this case (not shown), no reduction in the intensities of the corresponding bands was observed. We therefore think it likely that this effect is due to nonspecific breakage of intracellular DNA during the isolation procedures used (see above). Larger fragments would be affected to a greater extent by such breakage.

DISCUSSION

By applying the selective extraction procedure of Hirt (12) to CCV-infected cells, we have obtained evidence for the existence of two classes of intracellular viral DNA which differ in several important respects. DNA released into the supernatant fraction sedimented at the same rate (~53S) as did DNA extracted from CCV virions (Fig. 4). This result and the fact that restriction endonuclease digest profiles (Fig. 7) were indistinguishable from profiles of virion DNA indicate that supernatant DNA consists of unit-length CCV genomes.

Pellet DNA, on the other hand, besides the fact that it does not enter the supernatant under the extraction conditions, differed from supernatant DNA in that it sedimented to the bottom of gradients in which virion DNA sedimented approximately halfway. In addition, the *Xba*I and *Hpa*I fragments that are diagnostic of the molecular ends of the unit-length CCV genome were not detectable (<1/10 the expected concentration) in digests of pellet DNA (Fig. 7). By this latter criterion, therefore, pellet DNA is endless.

The presence of a new "fusion" fragment (B') which replaced the missing end fragments in *Hpa*I digests of pellet DNA (Fig. 7) is important for two reasons. First, it effectively eliminates the possibility that end fragments are missing from pellet DNA because they are attached to, for instance, a protein which could prevent them from entering the gel. This phenomenon has been found to apply under certain conditions to the end fragments of the adenovirus genome (16). Second, the size of the fusion fragment ($M_r \cong 18 \times 10^6$) requires that the fusion leading to loss of the molecular ends take place through a mechanism in which one copy per genome equivalent of the terminal repetition of 12.3×10^6 is also lost, rather than by a "blunt end" fusion in which case a new fragment with a

molecular weight of 30×10^6 (*Hpa*I fragment B plus *Hpa*I fragment C) would be formed. In *Xba*I digests of pellet DNA (Fig. 7), no fusion fragment is expected, because a restriction site lies within the terminal repeat (Fig. 9).

Fusion with loss of a copy of the terminal repetition could occur by genetic recombination between repeats (4, 17, 18) or by exonucleolytic removal of complementary strands from two repeats with subsequent annealing of the unpaired strands to regenerate a single repeat (e.g., reference 19). Whatever the precise mechanism, fusion of intragenomic repeats would lead to unit-length (less one terminal repeat) circle formation whereas concatemeric structures (including multimeric circles) would result from intergenomic fusion. Subsequent encapsidation of unit length genomes could then proceed via a replicative mechanism of the type proposed for bacteriophage T7 (19) whereby the terminal repeats are "restored" to virion DNA.

Either unit-length circles or concatemers could account for our results. However, unless held in place by a proteinase K-resistant linkage, unit-length circles would not be expected to remain in the pellet fraction. Nor have we been able to detect circular molecules by direct electron microscopic examination of CsCl gradient-purified pellet DNA (N. Berthelot, personal communication). Admittedly, these are not absolutely irrefutable arguments because of possible trapping effects in high-molecular-weight cellular DNA, but they do lead us to favor the idea that pellet DNA consists principally of concatemeric forms (or "rolling circle" forms [8], which would not be distinguishable from concatemers in the present experiments) of the CCV genome. Similar conclusions derived from restriction enzyme analysis have been reached for the replicative forms of DNA in pseudorabies virus (2) and herpes simplex virus (13) infections and for the intracellular DNA of bacteriophage SPO1 (6).

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