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# Characterization of a Metastable, Partially Replicated Dimeric Intermediate of Minute Virus of Mice

EMANUEL A. FAUST* AND GREG GLOOR

*Cancer Research Laboratory, University of Western Ontario, London, Ontario, Canada N6A 5B7*

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Intracellular, replicative-form DNA of minute virus of mice was characterized by agarose gel electrophoresis, velocity sedimentation, electron microscopy, restriction endonuclease digestion, and sensitivity to the single-stranded nuclease S1. This analysis demonstrated the presence in murine cells infected with minute virus of mice of a 10.0-kilobase pair dimer replicative form, a 5-kilobase pair monomer replicative form, as well as a 5-kilobase viral single-stranded DNA species. Two additional viral DNA species that migrated in 0.5% agarose gels with apparent sizes of 8.0 and 5.5 kilobase pairs were also observed. Further investigation indicated that the 8.0-kilobase pair DNA represents a novel class of metastable, partially replicated, dimeric intermediates. This finding has important implications for the mechanism of parvovirus DNA replication.

Minute virus of mice (MVM), a member of the helper-independent parvovirus group (12), contains a linear single-stranded DNA molecule of 5,081 nucleotides (3) that replicates in the S-phase nucleus of susceptible rodent cells grown in tissue culture (10, 12, 15, 17). The termini of MVM single-stranded (SS) DNA are folded in a base-paired hairpin configuration (1, 2, 4, 5) and play an essential role in viral DNA replication. Deletion mutants that retain both terminal self-complementary sequences can be propagated when complemented in *trans* by wild-type virus, despite the loss in these mutants of up to 90% of the internal viral sequences (7).

Infection of rodent cells in tissue culture with MVM leads to the accumulation of linear duplex replicative intermediates that have been characterized as monomeric (5 kilobase pairs [kbp]) or dimeric (10 kbp) replicative forms (RFs) (16). Both monomeric and dimeric RF DNA molecules are capable of instantaneous reannealing. This indicates that viral and complementary strands are covalently linked, an observation which suggests that terminal palindromic sequences serve as primers during the initiation of viral DNA replication.

Dimers consisting of monomeric equivalents oriented in a head-to-head fashion have been observed such that the 3′ and 5′ ends of viral and complementary strands, respectively, are juxtaposed at the junction of the two monomeric units. Discontinuities, located at or near the joint region, are present in dimer RF DNA extracted from infected cells by the method of Hirt (8) and are thought to reflect the in vivo action of an as-yet-unidentified site-specific nuclease involved in processing replicative intermediates during viral DNA replication (14, 16). Such a protein may remain bound to the DNA after endonucleolytic scission since the monomer RF DNA of H-1, a parvovirus related to MVM, exists in the cell in a complex with a protein of unknown origin which is covalently bound to the 5′ terminus of the DNA (9).

We undertook a detailed investigation of the intracellular forms of MVM DNA in an effort to clarify further the role of these DNA molecules in viral DNA replication. In the course of these experiments, we discovered a novel "8.0-kbp" intracellular viral DNA species. The results of a structural analysis of this DNA are presented in this report.

As shown in Fig. 1, when murine cells infected with MVM were extracted by the Hirt procedure (8) to selectively recover low-molecular-weight viral DNA molecules, a variety of intracellular forms of viral DNA were released into the Hirt supernatant. Five discrete DNA species with apparent molecular sizes of 10.0, 8.0, 5.5, 5.0, and 3.5 kbp were detected upon electrophoretic analysis of Hirt supernatant fractions resulting from infections in various host-virus combinations. The 10.0-, 5.0-, and 3.5-kbp viral DNA species were invariably found to be present upon infection of four different susceptible mouse cell lines including Ehrlich ascites (EA) cells, Ltk$^-$ aprt$^-$ cells, $A_9$ cells, and 3T6 cells. Based on their size, as determined both by sedimentation in neutral sucrose gradients and by their mobility during agarose gel electrophoresis, as well as their sensitivity to S1 nuclease and restriction enzyme digestion pattern (see below), it is clear that the 10.0-, 5.0-, and 3.5-kbp viral DNA species represent dimer RF, monomer RF, and viral SS DNA, respectively.

These findings confirm the interpretation of a previous characterization of intracellular forms of MVM DNA by Ward and Dadachanji (16). In addition to these viral DNA species, infections of EA and 3T6 cells yielded a novel 8.0-kbp viral DNA species which had not been characterized previously. An Ltk$^-$ aprt$^-$ cell line yielded a 5.5-kbp viral DNA species, which has also been observed on a number of separate occasions upon MVM infection of EA cells; in such DNA preparations the 8.0-kbp species was invariably absent. Thus, the 5.5- and 8.0-kbp viral DNA species appear to be mutually exclusive.

To investigate the structure of the 8.0-kbp DNA species further, total intracellular viral DNA labeled in vivo with [$^3$H]thymidine was extracted from infected EA cells and sedimented in a neutral sucrose gradient (Fig. 2). The location of each MVM DNA species in the gradient was then determined by agarose gel electrophoresis of portions taken from each gradient fraction. As expected, viral SS DNA sedimented relatively rapidly under neutral, high-ionic-strength conditions with a peak at 20S (4). The monomer RF (15S) and the dimer RF DNA species also sedimented as expected according to their relative sizes. However, the apparent 8.0-kbp DNA sedimented at approximately 20S,

* Corresponding author.

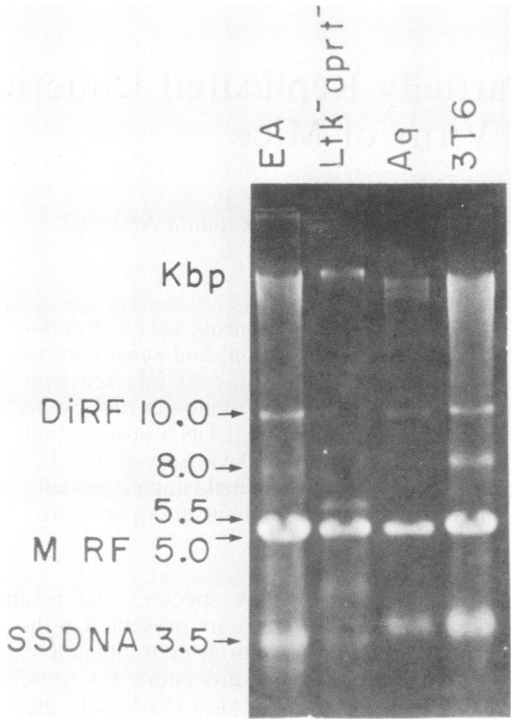


FIG. 1. Analysis of intracellular forms of MVM DNA by agarose gel electrophoresis. Murine cell lines were infected with wild-type MVM virions which had been purified by CsCl equilibrium density gradient centrifugation as described previously (7). Cells were grown in monolayer in 125-cm$^2$ plastic tissue culture flasks (Corning) in Dulbecco minimal essential medium supplemented with 10% fetal bovine serum. When cells reached 50% confluence they were infected with 100 hemagglutination units of virus per 125-cm$^2$ flask. One hemagglutination unit was taken to be equivalent to $5 \times 10^5$ PFU, assuming a particle-to-PFU ratio of 200. To prepare intracellular viral DNA, infected cells were trypsinized at 21 h postinfection and lysed in 50 mM Tris-hydrochloride buffer, pH 8.0, containing 0.15 M NaCl, 1 mM EDTA, and 0.6% sodium dodecyl sulfate. Subsequent steps were carried out as described by Hirt (8). Approximately 5% of the total DNA preparation was analyzed by electrophoresis in a 0.5% horizontal agarose slab gel as described previously (6). The gel was stained in a 1 μg/ml solution of ethidium bromide, and the DNA was visualized by illuminating the gel with short-wavelength UV light.

more rapidly than its electrophoretic mobility alone would predict if this DNA species were simply a linear 8.0-kbp double-stranded DNA molecule. Since electron microscopic analyses of electrophoretically purified 8.0-kbp DNA did not reveal any circular molecules (unpublished data), this anomalous sedimentation behavior could most likely be explained if the 8.0-kbp DNA possessed extensive regions of SS DNA as part of a partially replicated dimeric intermediate. This interpretation is supported by the results of the restriction endonuclease and S1 nuclease digestion patterns presented below.

The restriction endonuclease *Eco*RI cleaves 5.0-kbp MVM monomer RF DNA twice to yield three unique DNA fragments of 2.4, 1.5, and 1.0 kbp; the latter two fragments map to the 5′ and 3′ termini of the viral genome, respectively (3, 5, 16). The *Eco*RI digestion patterns of electrophoretically purified 10.0-kbp dimer RF DNA and 8.0-kbp DNA, as well as sucrose gradient-purified 5.0-kbp monomer RF DNA, were compared in the Southern blot analysis depicted in Fig. 3.

Upon *Eco*RI digestion, the 10.0-kbp dimer RF DNA species yielded fragments of 2.5, 2.2, 1.5, and 1.0 kbp (Fig. 3, lane d), whereas 5.0-kbp monomer RF DNA yielded fragments of 2.5, 1.5, and 1.0 kbp, as expected (Fig. 3, lane f). The extra 2.2-kbp fragment derived from the 10.0 kbp dimer RF DNA species is believed to represent a dimer bridge fragment (16) and is consistent with the arrangement of two monomeric units in a head-to-head configuration (i.e., with juxtaposed 3′ termini). Cleavage of the 8.0-kbp DNA species yielded fragments of 2.5, 1.5, and 1.0 kbp, which were indistinguishable in their mobility from the *Eco*RI fragments derived from monomer RF DNA. In addition,

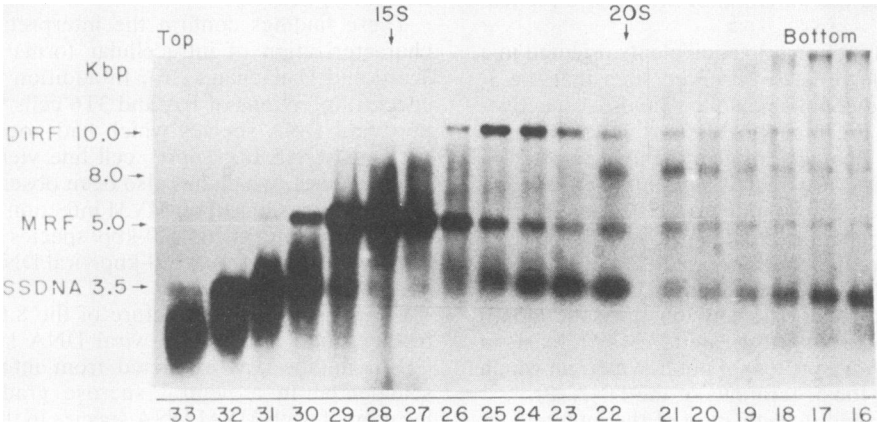


FIG. 2. Velocity sedimentation analysis of intracellular viral DNA. Virus was grown in EA mouse cells in the presence of 20 μCi of [$^3$H]thymidine per ml (30 Ci/mmol; New England Nuclear). Radiolabeled intracellular viral DNA was applied to a 5 to 20% neutral sucrose gradient made up in 50 mM Tris-hydrochloride buffer, pH 8.0, containing 1.0 M NaCl and 1 mM EDTA. The gradient was centrifuged at 4°C for 20 h at 28,000 rpm in a Beckman SW40 rotor. Portions (50 μl) of each 250-μl gradient fraction were analyzed by electrophoresis in a 0.5% horizontal agarose slab gel. To visualize the radiolabeled viral DNA, the gel was soaked for 1 h in agarose gel running buffer containing 1.0 M sodium salicylate. After a second 1.0 M sodium salicylate soak in fresh buffer, the gel was dried and exposed to X-ray film at −70°C in the presence of a Dupont Lightning Plus intensifying screen.

there was some viral DNA in these digests which migrated as a relatively broad band between 4.0 and 4.5 kbp. The possible significance of this material is discussed below. Virtually none of the 8.0-kbp DNA remained in the *Eco*RI-treated DNA samples, indicating quantitative cleavage of the 8.0-kbp DNA species. Thus, the 8.0-kbp DNA structure contains the entire sequence of MVM DNA in a double-stranded form which can be cleaved by *Eco*RI.

Although the intensity of the various bands in Fig. 3 was not accurately quantified, it appears that in general the various *Eco*RI fragments were present in close to the expected stoichiometric quantities. For example, the 2.2-kbp dimer bridge fragment is less intense than either the 5′ terminal (1.5 kbp) or internal (2.5 kbp) fragments in lane d of Fig. 3, consistent with the expected 1:2:2 stoichiometry of these respective fragments in 10.0-kbp dimer RF DNA. Similarly, in the *Eco*RI digests of 8.0-kbp DNA, an approximate 1:1 stoichiometry for 5′ terminal (1.5 kbp) and internal (2.5 kbp) fragments was also observed (see below).

In an attempt to further clarify its structure, electrophoretically purified 8.0-kbp DNA was treated with S1 nuclease. After S1 nuclease treatment, the DNA was again analyzed by agarose gel electrophoresis to determine whether the mobility of the DNA had been affected. Undigested 8.0-kbp DNA was analyzed in parallel. Dimer RF and monomer RF DNA were included in the experiment as controls and for purposes of comparison. The results are depicted in Fig. 4. Although monomer and dimer RF DNA samples remained largely unaffected by S1 nuclease digestion, the 8.0-kbp DNA species was no longer present in the S1 nuclease-treated samples, revealing the presence of one or more S1 nuclease-sensitive regions of SS DNA in the 8.0-kbp DNA structure. Moreover, quantification of the 5.0-kbp monomer RF band by densitometric analysis of autoradiograms revealed a 2.5-fold increase in the intensity of this band after treatment of 8.0-kbp DNA with S1 nuclease. Therefore, the 8.0-kbp DNA species can be converted by S1 nuclease to a 5.0-kbp DNA species which comigrates with bona fide 5.0-kbp monomer RF DNA.

It is evident upon examination of the data in Fig. 3 and 4 that electrophoretically purified 10.0-kbp dimer RF and 8.0-kbp DNA preparations contain some 5.0-kbp monomer RF DNA. The 8.0-kbp DNA preparation also contains some unit-length viral SS DNA. (This species is no longer present in S1 nuclease-treated samples; compare lanes b and e in Fig. 4.) A possible interpretation of these observations is that both the 10.0-kbp dimer RF DNA and 8.0-kbp DNA are metastable and break down to yield the viral DNA species observed when electrophoretically purified DNA preparations are examined by agarose gel electrophoresis.

The experimental evidence presented in this paper suggests the existence in MVM-infected murine cells of a novel class of partially replicated dimeric intermediates consisting of an unreplicated portion as well as a fully replicated portion. This partially duplex structure (Fig. 5) appears to reconcile the mobility of the 8.0-kbp DNA in agarose gels with its sedimentation behavior, although it is difficult to predict precisely how such a molecule would behave in either case based only on a comparison with either duplex or SS molecules as standards. Other structures which do not behave like linear duplexes when their sedimentation properties are compared with their mobility in agarose gels include open circular or supercoiled structures or rolling circle intermediates. However, only linear molecules were observed when electrophoretically purified preparations of 8.0-kbp DNA were examined in the electron microscope,

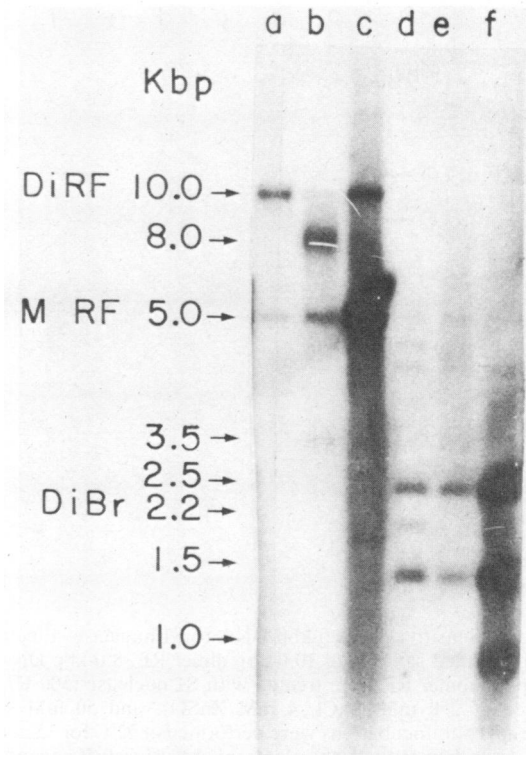


FIG. 3. *Eco*RI digestion of electrophoretically purified 8.0-kbp DNA. Dimer RF DNA and 8.0-kbp DNA were electroeluted from a preparative 0.7% agarose slab gel according to the method of Yang et al. (18). The purified DNA was extracted twice with phenol and once with diethyl ether and then precipitated in 70% ethanol in the presence of 10 to 20 μg of tRNA. The ethanol precipitate was redissolved in 10 mM Tris-hydrochloride buffer, pH 7.4, containing 0.1 mM EDTA and treated with *Eco*RI (2 U/μg of DNA) for 1 h at 37°C in the presence of 0.1 M Tris-hydrochloride, pH 7.4, 5 mM $MgCl_2$, and 6 mM dithiothreitol. The enzyme digests were analyzed by electrophoresis in a horizontal 0.5% agarose slab gel. Untreated samples were analyzed in parallel. After electrophoresis, the DNA in the gel was transferred to a nitrocellulose filter which was then incubated with a nick-translated MVM DNA probe by the method of Southern (11). The $^{32}P$-labeled DNA hybridized to the nitrocellulose filter was visualized by autoradiography. Nick-translated viral DNA was prepared by incubating 2 μg of monomer RF DNA with 10 U of *Escherichia coli* DNA polymerase I and 10 μCi of [α-$^{32}P$]dATP in the presence of 50 mM Tris-hydrochloride, pH 7.4, 10 mM β-mercaptoethanol, 5 mM $MgCl_2$, 50 μg of bovine serum albumin per ml, 1.5 μM each of dATP, dCTP, dGTP, and TTP, and 0.1 ng of DNase I per ml. The mixture was incubated for 1 h at 15°C. Nick-translated DNA was extracted once with phenol and purified by gel filtration on Sephadex G-100. Untreated samples: lane a, 10.0-kbp dimer RF; lane b, 8.0-kbp DNA; lane c, 5.0-kbp monomer RF, sucrose gradient purified. *Eco*RI-treated samples: lane d, 10.0-kbp dimer RF; lane e, 8.0-kbp DNA; lane f, 5.0-kbp monomer RF, sucrose gradient purified.

and therefore the data remain consistent with the proposed structure.

This structure is also consistent with the results of *Eco*RI digestion. The fully replicated duplex portion of the 8.0-kbp DNA structure could yield a 2.5-kbp *Eco*RI internal fragment as well as a 1.5-kbp 5′ terminal fragment (i.e., with respect to the V strand) and these were indeed observed in approximately stoichiometric amounts as expected (Fig. 3, lane e). The 1.0-kbp 3′ terminal *Eco*RI fragment present in these digests is presumably derived from the 5.0-kbp break-

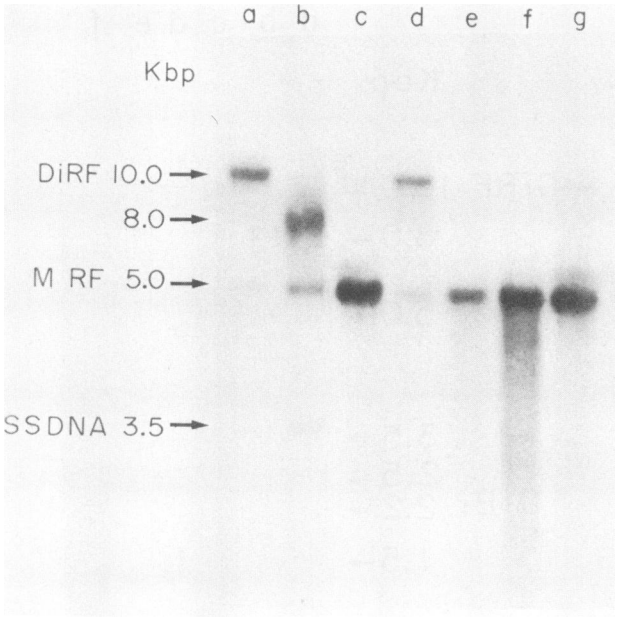


FIG. 4. Sensitivity of 8.0-kbp DNA to S1 nuclease. Electrophoretically purified samples of 10.0-kbp dimer RF, 8.0-kbp DNA, and 5.0-kbp monomer RF were treated with S1 nuclease (500 U) in the presence of 250 mM NaCl, 4 mM $ZnSO_4$, and 50 mM sodium acetate, pH 5.0. Incubations were performed at 22°C for 15 min. The digests were then analyzed by electrophoresis in a 0.5% agarose slab gel followed by Southern blot hybridization and autoradiography (see the legend to Fig. 3). Untreated DNA samples were analyzed in parallel. Untreated samples: lane a, 10.0-kbp dimer RF; lane b, 8.0-kbp DNA; lanes c and g, 5.0-kbp monomer RF. S1 nuclease-treated samples: lane d, 10.0-kbp dimer RF; lane e, 8.0-kbp DNA; lane f, 5.0-kbp monomer RF.

down products of the 8.0-kbp DNA species. A discrete 3′ terminal *Eco*RI fragment attached to a 5-kilobase SS DNA molecule is not readily apparent in *Eco*RI digests of the 8.0-kbp DNA species, indicating that termination sites in partially replicated dimers may be somewhat heterogeneous. This notion was supported by the presence of a heterogeneous DNA population of 4.0 to 4.5 kbp in *Eco*RI digests of 8.0-kbp DNA that was not found in undigested DNA samples (Fig. 3, compare lanes b and e).

Treatment of 8.0-kbp DNA with the SS nuclease S1 clearly demonstrated the presence of regions of SS DNA within this structure. Moreover, removal of these SS DNA regions resulted in conversion of the 8.0-kbp DNA to a 5.0-kbp DNA species. This interpretation is consistent with the results of the *Eco*RI digestion, which also indicated that a unit-length monomer RF DNA constituted a portion of the

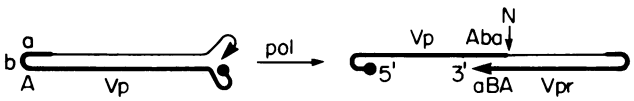


FIG. 5. Structure and proposed origin of partially replicated dimeric intermediates of MVM parvovirus. The structure for 8.0-kbp DNA as deduced from the results of this study is diagrammed on the right and is depicted as a product of hairpin-primed replication of 5.0-kbp monomer RF (see text). N denotes a hypothetical site-specific telomere nuclease. Complementary sequences are designated by upper- and lower-case letters. Abbreviations: pol, DNA polymerase; Vp, parental viral strand; Vpr, progeny viral strand.

8.0-kbp DNA. The metastable properties of 8.0-kbp DNA have suggested in addition that unit-length viral SS DNA forms part of the 8.0-kbp DNA structure since such molecules can be derived together with 5.0-kbp monomer RF DNA by spontaneous breakdown of the 8.0-kbp DNA species. Moreover the results also indicate the presence of a nick (or a small gap) at or near the axis of symmetry in the dimer-length strand as indicated in Fig. 5. Although electrophoretically purified 10.0-kbp dimer RF DNA was also observed to be metastable, the breakdown products included only the 5.0-kbp monomer RF DNA species—as expected from the fully duplex nature of the parent DNA molecule.

The existence of partially replicated dimers among the intracellular forms of parvovirus DNA has a number of important implications for the mechanism of parvovirus DNA replication. The partially replicated dimer structure is a likely replicative intermediate since it can be envisioned to arise simply by hairpin-primed synthesis of monomer RF from viral SS DNA followed by conversion to the partially replicated dimer by displacement of the parental viral strand (Fig. 5). According to this scheme, which has been considered in detail by Tattersall and Ward (14), partially replicated dimers would arise if DNA synthesis terminated prematurely at or near the parental 3′ terminal V strand hairpin sequence (abA) covalently positioned at the axis of symmetry in the dimer-length strand. The existence of such strong-stop intermediates may be a consequence of the recognition of the telomeric viral sequence abA in the dimer-length strand by a site-specific telomere nuclease. Interactions between this protein(s) and the DNA may arrest DNA synthesis at or near the site of nuclease action, possibly as part of the mechanism for segregating progeny viral SS DNA.

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## LITERATURE CITED

1. **Astell, C. R., M. Smith, M. B. Chow, and D. C. Ward.** 1979. Structure of the 3′ hairpin termini of four rodent parvovirus genomes: nucleotide sequence homology at origins of DNA replication. Cell **17**:691–703.
2. **Astell, C. R., M. Smith, M. B. Chow, and D. C. Ward.** 1979. Sequence of the 3′ terminus of the genome from Kilham rat virus, a nondefective parvovirus. Virology **96**:669–674.
3. **Astell, C. R., M. Thompson, M. Merchlinsky, and D. C. Ward.** 1983. The complete DNA sequence of minute virus of mice, an autonomous parvovirus. Nucleic Acids Res. **11**:999–1018.
4. **Bourguignon, G. J., P. Tattersall, and D. C. Ward.** 1976. DNA of minute virus of mice: self-priming, nonpermuted single-stranded genome with a 5′-terminal hairpin duplex. J. Virol. **20**:290–306.
5. **Chow, M. B., and D. C. Ward.** 1978. Comparison of the terminal nucleotide structures in the DNA of nondefective parvoviruses, p. 205–217. *In* D. C. Ward and P. Tattersall (ed.), Replication of mammalian parvoviruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
6. **Faust, E. A., and C. D. Rankin.** 1982. *In vitro* conversion of MVM parvovirus single-stranded DNA to the replicative form by DNA polymerase α from Ehrlich ascites tumour cells. Nucleic Acids Res. **10**:4181–4201.
7. **Faust, E. A., and D. C. Ward.** 1979. Incomplete genomes of the

parvovirus minute virus of mice: selective conservation of genome termini, including the origin of DNA replication. J. Virol. **32**:276–292.
8. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. **26**:365–369.
9. **Revie, D., B. Y. Tseng, R. H. Grafstrom, and M. Goulian.** 1979. Covalent association of protein with replicative form DNA of parvovirus H-1. Proc. Natl. Acad. Sci. U.S.A. **76**:5539–5543.
10. **Rhode, S. L., III.** 1973. Replication process of the parvovirus H-1. I. Kinetics in a parasynchronous cell system. J. Virol. **11**:856–861.
11. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments by gel electrophoresis. J. Mol. Biol. **38**:503–517.
12. **Tattersall, P.** 1972. Replication of the parvovirus MVM. I. Dependence of virus multiplication and plaque formation on cell growth. J. Virol. **10**:586–590.
13. **Tattersall, P., and D. C. Ward.** 1978. The parvoviruses: an introduction, p. 3–12. *In* D. C. Ward and P. Tattersall (ed.), Replication of mammalian parvoviruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
14. **Tattersall, P., and D. C. Ward.** 1976. The rolling hairpin: a model for the replication of parvovirus and linear chromosomal DNA. Nature (London) **263**:106–109.
15. **Tennant, R. W., K. R. Layman, and R. E. Hand, Jr.** 1969. Effect of cell physiological state on infection by rat virus. J. Virol. **4**:872–878.
16. **Ward, D. C., and D. K. Dadachanji.** 1978. Replication of minute virus of mice DNA, p. 297–313. *In* D. C. Ward and P. Tattersall (ed.), Replication of mammalian parvoviruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
17. **Wolter, S., R. Richards, and R. W. Armentrout.** 1980. Cell cycle-dependent replication of the DNA of minute virus of mice, a parvovirus. Biochim. Biophys. Acta **607**:420–431.
18. **Yang, C., J. Lis, and R. Wu.** 1979. Elution of DNA from agarose gels after electrophoresis. Methods Enzymol. **68**:176–182.