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

Received 5 July 1983/Accepted 10 October 1983

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-identified 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, A9 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 [3H]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.
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 EcoRI 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 EcoRI 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 EcoRI 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 EcoRI fragments derived from monomer RF DNA. In addition, 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.

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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 EcoRI-treated DNA samples, indicating quantitative cleavage of the 8.0-kbp DNA species. Thus, the 8.0-kbp DNA species contains the entire sequence of MVM DNA in a double-stranded form which can be cleaved by EcoRI.

Although the intensity of the various bands in Fig. 3 was not accurately quantified, it appears that in general the various EcoRI 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 2:1:2 stoichiometry of these respective fragments in 10.0-kbp dimer RF DNA. Similarly, in the EcoRI 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, and therefore the data remain consistent with the proposed structure.

This structure is also consistent with the results of EcoRI digestion. Thus, the 8.0-kbp DNA structure could yield a 2.5-kbp EcoRI 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 EcoRI fragment present in these digests is presumably derived from the 5.0-kbp break-
demonstrated lanes population kbp DNA EcoRI terminal replicated down unit-length monomer kbp DNA of within this intermediates 5.0-kbp parallel. Untreated samples: kbp DNA; lanes (see the legend as species, indicating was not of the RF; and is of the parental strand; viral strand. Moreover, 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.

We thank D. Denhardt for critically reading the manuscript and for many helpful suggestions in regard to these experiments. We are indebted to George Chaconas, Janet Miller, and Aileen Hogan for their advice and assistance in spreading DNA samples for electron microscopy. The valuable technical assistance of Mary Clement and Randy Nagy is gratefully acknowledged. We thank Dale Marsh and Linda Bonis for typing the manuscript.

This work was supported by grants from the Medical Research Council of Canada and the National Cancer Institute of Canada.

LITERATURE CITED


7. Faust, E. A., and D. C. Ward. 1979. Incomplete genomes of the 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 DNA breakdown of the 8.0-kbp 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 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.

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 ZnSO4, 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. 6). Unaltered 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.

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.


