Fusion of the Termini of the Murine Cytomegalovirus Genome After Infection

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The genome of murine cytomegalovirus, extracted from extracellular virions, is a linear double-stranded DNA molecule ca. 240 kilobase pairs long. In our initial cloning of subgenomic fragments of the murine cytomegalovirus genome, we obtained a *Hind*III clone which contained fused *Hind*III-terminal fragments. By hybridizing this cloned DNA fragment to infected-cell DNA, we identified an intracellular restriction fragment which was the length of the sum of the two authentic termini. This fusion fragment was not present in virion DNA but could be detected as early as 2 h postinfection and reached its highest level shortly after the onset of DNA replication at 16 h postinfection. The prereplicative increase of fused ends was not inhibited by a level of phosphonoacetic acid which effectively shut off viral DNA synthesis, nor was the early conversion from free to fused ends prevented by inhibitors of protein or RNA synthesis. The results are consistent with the fused state of viral DNA being a replicative intermediate and precursor to DNA synthesis.

An increasing body of data indicates that herpesvirus DNA replication proceeds via circular or concatemeric intermediates or both. Electron microscopy has revealed that termini join during the replication of herpes simplex virus type 1 (HSV-1) (8, 11), human cytomegalovirus (HCMV) (13), and pseudorabies virus (PrV) (1, 12). Circular and concatemeric structures have been observed in viral DNA extracted from infected cells. Restriction enzyme analysis has also shown a loss of free termini and a concomitant increase in fusion or junction fragments for HSV-1 (5, 10, 14), HCMV (15), PrV (2, 3), and channel catfish virus (4). Pulse-chase experiments have shown that these endless molecules are the precursors to mature virion DNA (4, 14).

The status of the termini of herpesviruses may also indicate latent infection. Rock and Fraser (21) recently reported the absence of HSV-1 genomic termini in neural tissue from latently infected mice. Furthermore, Puga et al. (20) found that the size of the HSV-1 terminal fragments is altered in DNA from latently infected trigeminal ganglia of mice, suggesting genomic rearrangements or integration events. In contrast, Wigdahl et al. (23), using an in vitro model system for HSV-1 latency, reported neither a loss of free termini nor an increase in the number of junction fragments.

The genome of murine cytomegalovirus (MCMV), extracted from virions, is a linear double-stranded DNA molecule ca. 240 kilobase pairs long. Unlike HCMV, it does not have invertible segments. In addition, the MCMV genome does not have long stretches of repeated sequences either at the termini or internally (7, 16).

We previously reported the cloning of *Eco*RI and *Hind*III restriction fragments and the ordering of these fragments on the MCMV genome (16). A *Hind*III clone was obtained which contained the *Hind*III-terminal fragments from both ends of the genome. This clone has provided an ideal opportunity to study the MCMV genome during replication. Since MCMV does not have invertible segments, long repeated sequences, or heterogeneity, it can be used as the simplest possible herpesvirus prototype. In this communica-

tion, we report the detection of circular or concatemeric forms of intracellular viral DNA.

MATERIALS AND METHODS

Cells and virus. Mouse embryo cells (MEC) were prepared from 15- to 19-day-old BALB/c mouse embryos, and the cells were passaged and maintained as described previously (16). MCMV was originally obtained from F. Dutko and propagated in MEC at a multiplicity of infection of 0.01 to 0.05.

Infections for time course experiments were carried out on nearly confluent MEC at a multiplicity of infection of 3 to 5. Virus was adsorbed for 2 h, at which time the inoculum was removed and the cells were washed once with phosphate-buffered saline. In some experiments, cells were infected and maintained in the presence of 300 μ g of phosphonoacetic acid (PAA) (Sigma Chemical Co.) per ml or 2 μ g of actinomycin D (Calbiochem-Behring) per ml. In other experiments, cells were pretreated with 200 μ g of cycloheximide (CH) (Sigma) per ml or 100 μ M anisomycin (Sigma) and then infected and maintained in CH (300 μ g/ml) or 100 μ M anisomycin.

Isolation of DNA. Extracellular virion DNA was purified as described by Mercer et al. (16). To obtain intracellular DNA, infected or uninfected cells were harvested with trypsin and pelleted at $2,000 \times g$. Cell pellets were suspended in 0.1 M NaCl-10 mM Tris-10 mM EDTA (pH 8.0) and lysed with 500 μ g of pronase per ml-1% sodium dodecyl sulfate at 37°C for 1 h. The lysate was extracted with phenol-chloroform-isoamyl alcohol (50:48:2), and the aqueous phase was precipitated with ethanol at -20°C.

Hybridizations. Agarose gel electrophoresis, purification of restriction fragments from SeaPlaque agarose gels, transfer of DNA to nitrocellulose paper, preparation of 32 P-labeled DNA by nick translation, hybridization and washing conditions, and autoradiography were all performed as described by Tamashiro et al. (22).

RESULTS

Identification of a terminal fusion fragment in intracellular DNA. We previously reported the isolation of an MCMV

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*Hind*III cloned insert, *Hind*III-E', which contained sequences from both ends of the genome (16). The insert was determined to be a fusion of the two *Hind*III-terminal fragments E and Q. A restriction map of the genome and the orientation of *Hind*III-E' are shown in Fig. 1. It was improbable that *Hind*III-E' was a cloning artifact (i.e., that fragments E and Q fortuitously ligated into the same plasmid), since this particular clone was derived from *Hind*III-cleaved viral DNA which had been fractionated by size on an agarose gel before ligation. *Hind*III-E and -Q were widely separated on our gel system, and therefore *Hind*III-Q should not have been present in the high-molecular-weight fraction cut from the gel. If this fragment was not the result of the *in vitro* ligation and cloning procedure, then it had to be derived from viral DNA with fused ends. We initially tested this possibility with DNA extracted from MCMV-infected MEC.

Figure 2 shows the results of a time course experiment in which DNA samples extracted at 8, 16, 24, and 44 h p.i. were hybridized with a 32 P-labeled 1.9-kilobase-pair *Eco*RI-*Hind*III subfragment of *Hind*III-E'. This subfragment, *Hind*III-E'-1.9, is composed of the two terminal fragments, *Eco*RI-c and *Hind*III-Q (see Fig. 1). *Hind*III-E'-1.9 instead of the entire *Hind*III-E' insert was used as a probe in all of the experiments described here to simplify analysis of the hybridizations.

At 16 h p.i. (Fig. 2A), the only bands visible were fragments which had the expected mobility of fused termini; i.e., 5 kilobase pairs in the *Eco*RI digest (the sum of the lengths of *Eco*RI fragments X and c), and 1.9 kilobase pairs in the double digest (the sum of *Hind*III-Q and *Eco*RI-c). At 24 and 44 h p.i., the fusion fragments were still present, but substantial quantities of free termini could be seen. This result indicated that clone *Hind*III-E' was derived from an authentic intracellular form of viral DNA. Therefore, the virion DNA preparation which was the source of this clone was probably contaminated with intracellular DNA.

In the second blot (Fig. 2B and C; the exposure time was 10 times longer in B), the DNA is from the same experiment

as shown in panel A, but three times more of the 8-h sample of DNA was loaded onto the gel to increase the sensitivity for this time point. The fusion fragments were visible at 8 h p.i. in the longer exposure (Fig. 2B), and the molecules with fused ends predominated over those with free ends. The relative abundance of fused and free ends at 8 and 16 h can be estimated by comparing the two exposures. When the fusion fragments at 8 and 16 h were approximately the same intensity on the autoradiograms, free ends were visible at 8 h but not at 16 h. The first three lanes of this blot have increasing amounts of *Eco*RI-digested virion DNA. No fusion fragment could be detected in these lanes, in accord with our previous results (16). It is clear, however, that our virion DNA preparations varied with respect to contamination with intracellular DNA, since our purification protocol did not include a virion banding step.

Terminal fusion fragment formed in the absence of viral DNA synthesis. The last experiment (Fig. 2) demonstrated the presence of fused molecules as early as 8 h p.i., which is at or before the onset of viral DNA synthesis (17, 19; unpublished data). To clarify whether the number of fusion fragments observed at 8 h required viral DNA replication, we performed similar time course experiments but on a shorter time scale. DNA was extracted from infected cells at 2, 4, 6, and 8 h p.i., cleaved with *Eco*RI, blotted onto nitrocellulose, and hybridized with the 32 P-labeled fusion fragment *Hind*III-E'-1.9 (Fig. 3). The fusion fragment was detectable at the earliest time point examined (2 h p.i.), and the ratio of fused-end to free-end molecules increased during the course of the experiment.

Although this experiment indicated that formation of the fused terminal fragment was independent of viral DNA synthesis, it was possible that some viral DNA synthesis occurred which was below the limits of our detection. Therefore, we repeated the experiment with DNA extracted from MCMV-infected cells in the presence of 300 μ g of PAA per ml. It has been reported that PAA at 200 μ g/ml will inhibit virtually all HCMV DNA replication in permissive fibroblasts (9). We also found that PAA was an effective

FIG. 1. Restriction endonuclease map of the MCMV genome. (A) Positions of the *Eco*RI and *Hind*III sites on the linear genome. *Eco*RI fragments shown in parentheses were mapped as internal fragments of the *Hind*III fragments under which they are placed but were not ordered. The scale shows map units. (B) Expanded representation of the fusion fragment, *Hind*III-E', and the *Eco*RI subfragment *Hind*III-E'-1.9. The genome is drawn in the head-to-tail fused configuration, with the point of fusion labeled as the termini.

FIG. 2. Southern blot analysis of MCMV-infected MEC DNA extracted at 8 through 44 h p.i. DNA was cleaved with *Eco*RI plus *Hind*III (lanes a) or *Eco*RI (lanes b), electrophoresed through 0.8% agarose, blotted onto nitrocellulose, and hybridized with 32 P-labeled *Hind*III-E'-1.9. (A) Lanes contained 5 μ g of infected-cell DNA each. The fusion fragments, X+c and E'-1.9, and the authentic termini, *Eco*RI-X and -c and *Hind*III-Q, are shown. On this exposure, the 16-h DNA sample had only fusion fragments, but at longer exposures free ends were visible. (B and C) A 24-h and 2.5-h exposure, respectively, of one blot with DNA from the experiment shown in panel A. DNA was used at 15 μ g for the 8-h and 5 μ g for the 16-h samples, per lane. The three virion DNA lanes have increasing amounts of *Eco*RI-digested extracellular virion DNA. The electrophoresis time was such that *Hind*III-Q does not appear on these autoradiograms.

inhibitor of MCMV viral DNA synthesis in MEC. There was no increase in MCMV-specific sequences in infected MEC during a 48-h incubation period with 300 μ g of PAA per ml (data not shown). In another experiment, DNA was extracted at 2, 4, 6, and 8 h p.i. from MEC maintained in the presence of 300 μ g of PAA per ml (Fig. 4) and analyzed as described above for Fig. 3. The kinetics of fusion (i.e., the rate at which free termini were converted to the fused fragment) were the same with and without PAA in the medium, indicating that the initial level of and degree of subsequent conversion to fused molecules up to 8 h p.i. were independent of viral DNA synthesis.

Formation of the fusion fragment during inhibition of protein and RNA synthesis. Immediate-early and early proteins are expressed before the onset of DNA replication. Therefore, a de novo-synthesized protein might be involved in the fusion process. To test this possibility, time course experiments similar to those discussed above were performed, using the protein synthesis inhibitor CH or anisomycin in the culture medium. Figure 5 shows Southern blots of *Eco*RI-digested DNA extracted from CH-treated cells and hybridized with *Hind*III-E'-1.9. Again, samples were taken at 2-h intervals, beginning at 2 h p.i. The fusion fragment was present at 2 h p.i. with either CH or anisomycin (data not shown), and the conversion of free to fused ends still occurred in the presence of the inhibitors. The rate of conversion to fused ends was slower than in the control experiment (Fig. 3). CH and anisomycin gave the same result, indicating that the conversion to fused ends observed was probably not due to any inefficiency of the inhibitor in allowing a low level of translation.

The transcription of immediate-early RNAs would not have been inhibited under the conditions described above. To test whether such transcriptional activity might play a role in the fusion of the genomic termini, an analogous experiment was performed with 2 μ g of actinomycin D per ml in the medium. The results (data not shown) were indistinguishable from those obtained when protein synthesis was inhibited, indicating that transcription probably does not play a role in prereplicative fusion.

DISCUSSION

A cloned fragment which contains both ends of the MCMV genome was used to investigate the linkage of the termini before and during replication in MEC. Southern blot hybridizations revealed a fragment which was the length of

FIG. 3. Southern blot analysis of infected-cell DNA extracted at 2 through 8 h p.i. DNA was digested with *Eco*RI, electrophoresed, blotted, and hybridized as described in the legend to Fig. 2. For each time point, 20 μ g of DNA was loaded onto the gel. Virion DNA lanes have, from left to right, 0.5, 1, and 2.6 ng of *Eco*RI-digested extracellular virion DNA.

FIG. 4. Southern blot analysis of DNA extracted at 2-h intervals through 8 h p.i. from cells infected and maintained in the presence of 300 µg of PAA per ml and treated as described in the legend to Fig. 3.

the sum of the two authentic virion termini in both *EcoRI* and *EcoRI-HindIII* digests of infected-cell DNA. These fusion fragments were not present in detectable amounts in extracellular virion DNA preparations.

The fusion fragment was detectable at 2 h p.i., which was the length of our standard adsorption period. There was a gradual increase in the ratio of molecules with fused ends to those with unlinked ends through 8 h p.i. This increase in the ratio took place without a detectable increase in the overall amount of hybridization, indicating that it occurred in the absence of viral DNA synthesis. This result is consistent with the onset of MCMV viral DNA synthesis in MEC at 8 to 12 h p.i. (17, 19; unpublished data). By 8 h p.i., more than half of the total hybridization with our end-specific probe was to the fusion fragment. That the fusion fragment was initially present and that conversion from free to fused ends occurred in the absence of viral DNA replication were confirmed by adding PAA to the culture medium. With an effective concentration of PAA, the appearance of the fusion fragment followed the same kinetics from 2 through 8 h p.i.

No de novo-expressed viral function was necessary for the initial fusion (seen immediately after adsorption at 2 h p.i.) or for the conversion to fused ends which took place before replication. Protein synthesis inhibitors (CH and anisomycin) and an RNA transcription inhibitor (actinomycin D) did not prevent the initial fusion or later conversion. These results are in accord with those of Harper and Ben-Porat, who found that CH did not prevent the accumulation of circular or concatemeric forms of PrV (personal communication). Although no virus-coded protein needs to be expressed for prereplicative fusion, the virus might carry into the cell a protein that mediates the rapid joining of ends. Preliminary results from our laboratory, and from Harper and Ben-Porat with PrV (personal communication), indicate that if such a protein exists, it is probably not tightly linked to the ends of the viral DNA.

Prereplicative fusion has also been seen with HCMV and PrV. Circles and concatenes of intracellular PrV and HCMV DNA extracted before the onset of replication have been observed by electron microscopy (12, 13). In addition, restriction enzyme analysis of prereplicating PrV revealed a fusion fragment, the length of which was consistent with joined termini (3). However, LaFemina and Hayward (15) did not find, by restriction enzyme analysis, any loss of HCMV termini either before replication in permissive cells or at any time in nonpermissive cells. A possible explanation for this difference is, as LaFemina and Hayward suggested, that only a small percentage of the input virus might have entered the replicative pool. If this were the case, they would not have detected the small percentage of molecules which would have fused. If our data do not represent a true functional difference between MCMV and HCMV, then the above explanation would indicate that our MCMV stock had a much lower percentage of defective particles.

The level of the fusion fragment seen throughout the infection was entirely consistent with the idea that this form of the DNA is a replicative intermediate. The intracellular ratio of fused to free ends was lowest immediately after infection. By the onset of DNA replication fused molecules predominated, and when a high rate of replication was occurring they comprised approximately 90% of the intracellular MCMV DNA. Later in the infection, when viral DNA was being packaged and virions were being released, the percentage of free termini increased.

A question which our data do not address is the physical form of the molecules that have fused termini. We cannot distinguish between circular and concatemeric forms, but on the basis of electron microscopy data for other herpesviruses, we presume that both forms exist. Whether prereplicative fusion fragments occur only as circles is unclear.

Whatever the form of the genome, viral DNA molecules undergo rapid and specific linking of the termini. By 8 h p.i.,

FIG. 5. Southern blot analysis of DNA extracted from cells infected and maintained in the presence of CH. Cells were pretreated with 100 µg of CH per ml and infected and maintained in the presence of 200 µg of CH per ml. DNA was treated as described in the legend to Fig. 3.

most of the inoculum is found in the head-to-tail fused form, and there are no detectable molecules that might have head-to-head or tail-to-tail joinings. There are several mechanisms by which this fusion might be mediated, none of which can presently be excluded.

If homology in the form of direct repeats exists at the ends of a DNA molecule (e.g., HSV, HCMV, and channel catfish virus), the pathway for fusion could involve either a recombination event or an exonucleolytic digestion of each end, followed by base pairing of the exposed complementary regions. Either mechanism involving direct repeats results in a molecule that is shortened by one repeat. Cebrian et al. (4) have found an intracellular fusion fragment of channel catfish virus that is the sum of the lengths of the two termini minus the length of one repeat (molecular weight 12×10^6).

Alternatively, a complementary overhang at the termini, analogous to the sticky ends of phage lambda, which could facilitate ligation might be present in virion DNA. Mocarski and Roizman (18) have found a 1-base overhang at the ends of HSV-1 which could be fused during replication and then cut in a staggered fashion to regenerate the overhang.

Evidence from other herpesviruses indicates that extensive homology between the ends of the genome is not essential for replication. Davison and Wilkie (6) have isolated intertypic recombinants of HSV-1 and HSV-2 which derive one terminus from each strain. Lack of extensive homology between the terminal repeats from HSV-1 and HSV-2 makes base pairing or recombination unlikely, yet these recombinant viruses are able to grow. Davison and Wilkie (6) reported similar findings with PrV in which sequences at the end of the long unique region have no homology with the repeats bracketing the short unique segment.

Our data support an end-to-end ligation for MCMV since we did not detect any difference between the length of the MCMV fusion fragment and the sum of the lengths of the two termini. A comparison between the sequences present in the fusion fragment and the termini is necessary to clarify the processes involved in this antecedent to replication.

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