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Early Events in Parvovirus Replication: Lack of Integration by Minute Virus of Mice into Host Cell DNA

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Viral DNA sequences were not detected in high-molecular-weight host DNA until well after the onset of viral DNA replication.

The parvoviruses are remarkable for their small size and the restricted coding capacity of their single-stranded DNA genomes (10). Their limited genetic endowment renders them uniquely dependent upon external resources for replication. In the case of the "autonomous" group of parvoviruses, including minute virus of mice (MVM), replication requires host cell events which may occur during S phase (7, 9). The second group of parvoviruses, the adeno-associated viruses, depends upon coinfection with adenovirus for growth. Recent experiments by Berns et al. (1) have shown that adeno-associated viruses can establish a latent state in Detroit 6 cells which allows it to survive in the absence of helper virus. At least some of the adeno-associated virus DNA appears to be integrated into the host genome (5) and could be involved in maintaining a latent viral state. Although there is no very compelling evidence for an analogous latency among the autonomous parvoviruses, we have investigated whether integration of the viral genome plays any role in the lytic infection cycle of MVM. If integration of the viral genome into host cell DNA has a role in the infectious cycle, we would expect integration to occur prior to the onset of viral DNA replication. In a single-step infection, no integration of MVM DNA was detected prior to the onset of viral DNA replication. Therefore it appears unlikely that integration serves any necessary role in lytic infection by MVM.

To study the integration of viral DNA during the replication cycle, we used ^{32}P-labeled viral single-stranded DNA as a probe in a filter hybridization assay. The ^{32}P-labeled hybridization probe showed the expected hybridization kinetics when tested against increasing amounts of in vitro-synthesized double-stranded viral DNA fixed to nitrocellulose filters (Fig. 1).

A denaturing gradient system was used to separate free viral DNA from sequences integrated into host DNA from infected cells. DNA was gently extracted from infected cells by protease K digestion in detergent. The DNA was then denatured and allowed to reanneal to form a network of high-molecular-weight DNA. The network was separated from low-molecular-weight DNA by centrifugation through sucrose. Over 85% of the cellular DNA was recovered in the network. In a reconstruction experiment, in vitro-synthesized, radiolabeled, double-stranded viral DNA was added to the cells at the time of extraction, and approximately 7% of the label was found trapped in the DNA network at this stage. The remaining viral DNA was present in the supernatant (i.e., recovery at this stage is quantitative). The network DNA was resuspended in a denaturing buffer, underlaid with a 10 to 40% alkaline sucrose gradient, and the host DNA was separated from the residual viral sequences by centrifugation. At this step of the procedure, [^{3}H]thymidine-labeled DNA of RT cells sediments mostly as a peak of 60–70S in the alkaline gradient (Fig. 2). When ^{3}H-labeled double-strand viral DNA was added to cells during the protease K extraction, the viral label was located at the top of the alkaline sucrose gradient; no label was detected in the high-molecular-weight host cell DNA region of the gradient (Fig. 3). The high-molecular-weight DNA (fractions 5 to 16, Fig. 3) was pooled, immobilized on nitrocellulose filters, and hybridized against the ^{32}P viral DNA probe. By hybridization, the host cell DNA region was contaminated by 0.27% of the initially added viral DNA sequences. This measure of viral DNA contamination was used to correct the hybridization data obtained from the host cell DNA region of the alkaline gradients. In each infected cell DNA sample, the total amount of viral DNA was measured by hybridization, and 0.27% of this amount was subtracted from hybridization to the pooled high-molecular-weight DNA (Table 1). The combination of the network formation step with alkaline gradient centrifugation allows one to load the DNA of 10^7 cells on a single denaturing gradient with low contamination of the host DNA by viral sequences.

To measure the amount of viral DNA inte-

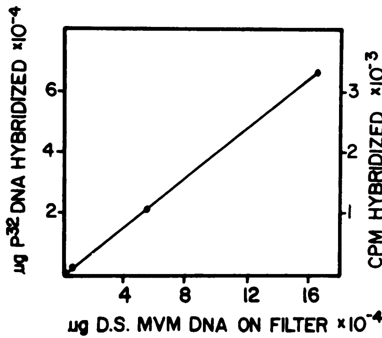

FIG. 1. *Hybridization of ^{32}P-labeled viral DNA to in vitro-synthesized double-stranded viral DNA. MVM particles were isolated by sucrose gradient centrifugation (8), DNase digested (50 µg/ml, 1 h at 37°C, 1 mM $MgCl_2$), and disrupted in alkali (0.1 N NaOH, 30 min, room temperature). Unit-length DNA was isolated by sucrose gradient centrifugation (15 to 30% sucrose, 1 M NaCl, 1 mM EDTA, 10 mM Tris [pH 8.0]; 25,000 rpm, 16 h at 20°C in an SW27 rotor). For synthesis of the ^{32}P-labeled hybridization probe, the single-strand viral DNA was fragmented by sonic disruption and end labeled with [γ-^{32}P]ATP by the method of Maxam and Gilbert (6). Double-stranded MVM DNA was synthesized in vitro by the method of Bourguignon et al. (2). The varying amounts of the double-stranded viral DNA were mixed with 100 µg of calf thymus DNA and were fixed to nitrocellulose filters by the method of Gillespie and Spiegelman (4). When ^{32}P-labeled viral double-stranded DNA was used under these conditions, virtually all (97%) of the label bound and remained on the filters during the hybridization. The filters were hybridized with ^{32}P-labeled single-strand DNA (5 × 10^6 cpm/µg) in 0.3 M NaCl, 0.03 M sodium citrate, and 50% formamide for 24 h at 40°C, according to the method of Dawid (3).*

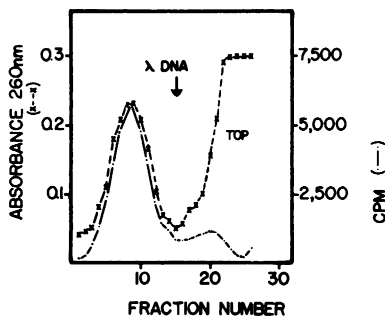

FIG. 2. *Alkaline gradient centrifugation of DNA networks. RT-7 cells (8) were grown in [^3H]thymidine for 2 days. Cells (10^7) were removed from T-flasks in 1 mM EDTA, centrifuged, and resuspended in 0.2 ml of 30 mM Tris (pH 7.5)-5 mM EDTA-100 mM NaCl. Protease K solution (0.3 ml; 6% Sarkosyl, 4 mM EDTA, 50 mM NaCl, 600 µg of protease K per ml, 60 mM Tris, pH 7.5) was added to the cells and incubated for 60 min at 37°C. An equal volume of 5 M NaCl was added, and the sample was incubated for an additional 60 min at 37°C. A 1-ml volume of denaturation buffer (0.5 M NaOH, 80 mM EDTA, 1% Sarkosyl) was added, and the sample was allowed to stand at 4°C overnight. The sample was neutralized by the addition of 0.5 ml of 2 M NaH_2PO_4 and incubated for 4 h at 68°C to allow network formation. The solution was underlaid with 20% sucrose in 5 mM EDTA-30 mM Tris, pH 8.0, and centrifuged for 120 min at 38,000 rpm in an SW40 rotor at 20°C. The supernatant fluid was removed, and 0.6 ml of a denaturing solution (4 M urea, 77% formamide, 10 mM EDTA, 5% Sarkosyl, 0.1 M Tris, pH 8.2) was added and incubated for 30 min at 60°C, followed by the addition of 2 ml of 0.5 M NaOH-80 mM EDTA-1% Sarkosyl solution. The sample was then underlaid with a 10 to 40% sucrose gradient in 0.9 M NaCl-0.1 M NaOH and centrifuged in an SW27 rotor for 5 h at 4°C at 25,000 rpm. Fractions were collected from the bottom of the gradient and assayed for absorbance at 260 nm and for radioactivity.*

grated during the course of infection, rapidly growing RT cells were infected in monolayer with 40 50% tissue culture infective doses per cell (8). At various times (4, 8, 12, and 22 h after infection), samples of 10^7 cells each were processed through to the alkaline gradient centrifu-

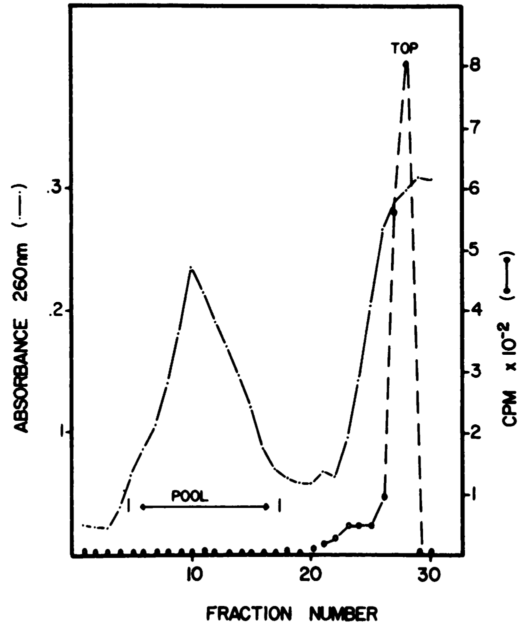

FIG. 3. *Reconstruction experiment measuring contamination of host DNA by viral DNA sequences after alkaline gradient centrifugation. Double-stranded MVM DNA (3 × 10^4 cpm) synthesized in vitro was added to RT cells at the end of the protease K digestion step. Extraction was carried out through alkaline centrifugation as described in the legend to Fig. 2. Gradient fractions were assayed for absorbance to locate the host DNA and for radioactivity to locate the viral DNA sequences.*

TABLE 1. *Integration of MVM DNA into infected RT-7 cells*[a]

DNA	Hours	cpm hybridized per 10^7 cells[b]	Viral contamination (cpm)[c]	Genomes of MVM DNA per cell
Integrated sequences[d]	4	0	3	0
	8	0	12	0
	12	89	94	0
	22	623	495	0.53
Total viral DNA[e]	4	1,250		5.2
	8	4,450		18.6
	12	34,950		104
	22	183,200		766

[a] RT-7 cells were infected at a high multiplicity (40 50% tissue culture infective doses per cell). At 4, 8, 12, and 22 h, 10^7 cells were removed and processed for high-molecular-weight DNA as described in the legend to Fig. 2. A portion of total DNA was removed from each sample at the network formation step. This DNA and the high-molecular-weight DNA collected from alkaline sucrose gradients were fixed to nitrocellulose filters and hybridized with ^{32}P-labeled single-stranded MVM DNA.

[b] The background of a blank filter with 100 μg of denatured calf thymus DNA was subtracted from each sample (137 cpm).

[c] The number of counts per minute (cpm) above background that is expected in the high-molecular-weight DNA due to contaminating viral DNA produced during the infection (0.27% of the total viral DNA).

[d] Hybridization of ^{32}P-labeled viral DNA to high-molecular-weight DNA.

[e] Hybridization of ^{32}P-labeled viral DNA to total DNA.

gation step. The high-molecular-weight DNA peak from each gradient was pooled, attached to nitrocellulose filters, and hybridized against ^{32}P-labeled viral DNA (Table 1). During the course of the infection, the amount of total viral DNA present in the samples increased logarithmically, and viral DNA synthesis was clearly well underway at 8 h after infection. However, in the high-molecular-weight DNA fraction from the alkaline gradients, no viral sequences were detected by hybridization until 12 h postinfection. The level of viral sequences in the host DNA fraction did not exceed the expected contamination by free viral DNA until 22 h postinfection, and then only by a marginal amount.

These experiments do not rule out the possibility that some viral DNA may become integrated into host DNA late in infection. Such integration could play a role in establishing a latent state in cells that survive the infection. However, since significant levels of integration are not detected prior to the onset of viral DNA synthesis, it seems unlikely that integration is an obligatory step in lytic infection by MVM.

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