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# Intracellular DNA of the Parvovirus Minute Virus of Mice Is Organized in a Minichromosome Structure

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Minute virus of mice (MVM) nucleoprotein complexes were leached from infected cell nuclei in the presence of a hypotonic buffer. Detailed biochemical analyses performed on the extracted complexes revealed nucleoprotein complexes sedimenting together with virions at 110S and defective particles sedimenting at 50S. In contrast to the virions, the nucleoprotein complexes were found to be sensitive to treatment with DNase, Sarkosyl, and heparin. They were found to be composed of replicative forms of MVM DNA and cellular histones. After extensive micrococcal nuclease digestion performed on purified nucleoprotein complexes, a viral nucleosome core containing a DNA segment of about 140 base pairs in length was identified. These complexes when visualized by electron microscopy revealed the existence of beaded structures (minichromosomes) having 26 and 52 beads per monomer and dimer molecules, respectively. We suggest that the organization of the intracellular viral DNA in a minichromosome structure is an essential step in the virus growth cycle.

Minute virus of mice (MVM) is a member of the autonomous parvovirus group (25, 37). This group of viruses contains the smallest viruses known and has therefore been used as a model system for studies of DNA replication (1, 36) and RNA transcription (9, 31) in animal cells. The infectious virions are icosahedral particles, with diameters of 18 to 30 nm, and contain linear single-stranded (ss) DNA of about $1.5 \times 10^6$ daltons (37). The defective subgroup (adeno-associated viruses) package plus and minus DNA strands in separate virions and are dependent for their multiplication on coinfection with adenovirus as a helper (1). In contrast to adeno-associated viruses, the autonomous subgroup packages only the minus strand of the viral DNA (1).

Most, if not all, viral proteins are extracted from infected cells as assembled particles, which can be separated into four main classes by physical techniques (22, 37). The first two include particles which band at 1.30 to 1.32 g/cm$^3$ in cesium chloride density gradients and have sedimentation constants of 50 to 70S. These particles are either empty capsids devoid of DNA or particles containing incomplete genomes (6, 37). Infectivity is predominantly associated with particles of the third class, which band at 1.38 to 1.43 g/cm$^3$ in cesium chloride and sediment at approximately 110S. The fourth class of particles, with lower specific infectivity, is found at a density of 1.46 to 1.48 g/cm$^3$ in cesium chloride. These particles appear to be immature forms of infectious virions and have the same sedimentation rate as mature virus. The immature infectious virus consists of two viral capsid proteins: A (83,000 daltons) and B (64,000 daltons). The mature virus consists of three viral capsid proteins: A, B, and C (61,000 daltons), where C is derived by processing of the polypeptide B. When grown in various cell lines, a fourth polypeptide D (~50,000 daltons) inconsistently is associated with the mature infectious particle (20, 33, 34, 35). No histones are found in the viral capsid (33).

The replication process of the autonomous parvoviruses involves formation of double-stranded (ds) replicative intermediates (RIs) that exceed genome length and contain ss regions (1, 40). Optimal yields of MVM and other members of the autonomous parvoviruses are obtained in cultures of actively dividing cells during or immediately after cellular DNA synthesis, in late S or G2 phase (1, 32). Thus, a potential viral DNA replication dependence on host cell factors made during S phase was suggested (32). One of these factors could be cellular histones, since their synthesis is known to be closely coupled to cellular DNA synthesis (28). It is possible that the histones in their nucleosomal beaded structure confer a specific tertiary structure on the intracellular viral DNA which is essential for the proper regulation of its gene expression.

To verify this possibility, DNA protein complexes (DPC) were extracted from isolated nuclei of MVM-infected cells and analyzed by biochemical methods and electron microscopy (EM). We present herein evidence for the occur-

rence of intracellular MVM DNA in the form of minichromosomes possessing beaded structures composed of cellular histones. We suggest that the tertiary organization of the intracellular viral DNA is an essential step in the virus growth cycle.

## MATERIALS AND METHODS

**Cells and virus.** The MVM(T) strain of MVM was plaque assayed in A9 cells as described by Tattersall (32). Low-multiplicity-derived virus was grown in Ehrlich ascites cells as described by Tattersall et al. (34). For single-cycle infections, 5 ml of viral stock containing $5 \times 10^7$ PFU per ml was added to a subconfluent culture of A9 cells at $10^7$ cells per 15-cm petri dish. After 2 h, the virus inoculum was removed and the cells were supplemented with fresh medium (Dulbecco modified Eagle medium with 10% calf serum). Labeling of cells with [*methyl*-$^3$H]thymidine (38.4 Ci/mmol; Nuclear Research Center, Beer Sheva, Negev, Israel) was carried out in 5 ml of medium per culture.

**Preparation of hypotonic extract.** The method described by Jakobovits and Aloni (11) was used. Briefly, MVM-infected cells labeled for 2 h with [$^3$H]thymidine were washed twice with buffer H (50 mM Tris-hydrochloride, pH 7.9, 1 mM $MgCl_2$, and 5 mM 2-mercaptoethanol), harvested with a rubber policeman, and collected by centrifugation at $1,000 \times g$ for 2 min. Nuclei were prepared by suspension of the cell pellet in 5 ml of ice-cold buffer H followed immediately by centrifugation at $1,000 \times g$ for 2 min. The nuclear pellet was resuspended in 1 ml of buffer H per $5 \times 10^7$ to $1 \times 10^8$ cells and incubated at 37°C for 60 min (unless otherwise indicated) with occasional shaking. The suspension was then centrifuged at $1,000 \times g$ for 1 min (in an Eppendorf microfuge), and the supernatant was carefully removed.

**Sedimentation in neutral sucrose gradients.** $^3$H-labeled nuclear extract (0.5 to 1.0 ml) was layered on a 10 to 40% (wt/wt) linear sucrose gradient in buffer H containing 0.1 M $(NH_4)_2SO_4$. Sedimentation was carried out in a Beckman SW41 rotor for 2.5 h at 39,000 rpm at 4°C. Fractions (0.3 ml) were collected from the bottom of the tube, and 0.01-ml samples were assayed for radioactivity.

**Sedimentation in alkaline sucrose gradients.** Purified virus or $^3$H-labeled viral DNA was sedimented through a 5 to 20% (wt/wt) alkaline sucrose gradient (containing 0.3 M NaOH, 0.7 M NaCl, and 0.15% Sarkosyl). Centrifugation was carried out in a Beckman SW50.1 rotor at 48,000 rpm for 4 h at 5°C (35).

**CsCl density gradients.** The peak fractions of $^3$H-labeled DNA of a neutral sucrose gradient were pooled and brought to a final volume of 4.3 ml with buffer H. CsCl (2.7 g) was added to a final density of 1.41 g/cm$^3$, and the sample was centrifuged in a Beckman SW50.1 rotor at 35,000 rpm at 20°C for 48 h. Fractions (0.150 ml) were collected from the bottom of the tube, 0.025-ml samples were precipitated with 10% trichloroacetic acid, and the radioactivity was determined.

**Polyacrylamide gel electrophoresis.** The sodium dodecyl sulfate-Tris-glycine system of Laemmli (14) was used, electrophoresis being done on acrylamide gels (12%) at 125 V/10 cm for 4 h. Samples were precipitated with 2 volumes of ethanol, suspended in a sample buffer (10% glycerol, 5% 2-mercaptoethanol, 3% sodium dodecyl sulfate, and 80 mM Tris-hydrochloride, pH 6.8), and boiled before loading onto the gel. Gels were stained with Coomassie blue.

**Cloning of MVM DNA.** Replicative-form (RF) MVM DNA was partially digested with *Eco*RI restriction endonuclease (Bio-Labs) and ligated onto *Eco*RI-cleaved pBR322 plasmid DNA. Transfection of *Escherichia coli* HB101 resulted in 12% colonies, all of which contained plasmid carrying fragment A of MVM DNA. Fragment A spans from 20.5 to 68.5 map units of MVM genome (40).

**Nick-translation of MVM DNA.** The plasmid pBR322 containing the *Eco*RI A fragment of MVM DNA was radiolabeled to high specific activity in vitro by the nick-translation procedure as described by Rigby et al. (21). The specific activities of the radiolabeled DNA varied from $3 \times 10^7$ to $5 \times 10^7$ cpm/μg.

**Agarose gel electrophoresis.** After electrophoresis at 20 V/13 cm for 16 h with Tris-acetate running buffer (40 mM Tris-hydrochloride, pH 6.8, 20 mM sodium acetate, and 1 mM EDTA), the agarose (1.4%) gels were stained for 30 min with 5 μg of ethidium bromide per ml in running buffer. For fluorography, the gels were treated with 25% 2,5-diphenyloxazole (PPO) in ethanol. The Southern technique (27) as modified by Wahl et al. (39) was used for blotting the DNA onto a nitrocellulose membrane filter. The nitrocellulose blots were then incubated with $^{32}$P-labeled pBR322 plasmid DNA containing the *Eco*RI fragment A of MVM DNA. At the end of the incubation time, the blots were washed and exposed to Agfa X-ray RP-2 films.

**Micrococcal nuclease digestion.** The 110S peak fractions were pooled. $CaCl_2$ was added to a final concentration of 1 mM, micrococcal nuclease (Sigma Chemical Co.) was added to a final concentration of 0.1 μg/ml (0.01 U/ml), and the mixture was incubated at 37°C for various durations. Digestion was terminated by addition of 0.1 volume of solution containing 50 mM EDTA, 5% sodium dodecyl sulfate, and 1 mg of proteinase K per ml. The solution was incubated at 22°C for 10 h. DNA was extracted with phenol, and electrophoresis was performed in 1.4% agarose gel.

## RESULTS

**Optimization of the extraction conditions.** Infected cells were labeled with [$^3$H]thymidine from 17 to 19 h postinfection (p.i.). At the end of the labeling time the cells were harvested, and viral complexes were leached from the nuclei in a hypotonic buffer (11) for various durations at 4, 25, and 37°C. At all temperatures, radioactivity was continuously released from the nuclei into the extraction buffer with time (Fig. 1). The rate of release of labeled material was highly dependent on the extraction temperature, the radioactivity obtained at 37°C being 1.5 times that found at 25°C and up to 3 to 4 times that found at 4°C. However, to minimize possible degradation, the viral complexes were extracted for only 60 min. Under the standard extraction conditions (60 min 37°C), the yield of extracted labeled DPC represented more than 50% of the

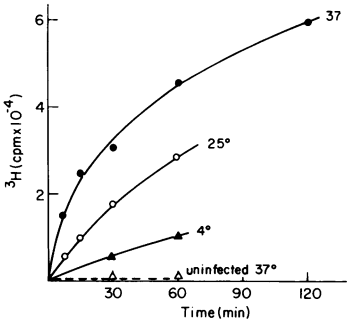


FIG. 1. Rate of leakage of labeled nucleoprotein complexes at various temperatures. Nuclei of infected cells labeled from 17 to 19 h p.i. were suspended in buffer H, and equal fractions were incubated at 4°C (▲), 25°C (○), or 37°C (●). At the indicated times, equal samples were centrifuged at 10,000 × *g* for 1 min at 25°C, and the supernatants were assayed for radioactivity by trichloroacetic acid precipitation.

DNA obtained by the Hirt extraction procedure, as modified by Tattersall et al. (35). Uninfected cell nuclei or infected cell nuclei labeled for 24 h with [$^3$H]thymidine before the infection released no radioactivity in an identical extraction procedure.

The optimal time after infection for the accumulation of labeled DPC was determined by labeling cells for 30 min at various times p.i. followed by a hypotonic extraction of the nuclei and determination of the radioactivity released. No labeled material was leached from the nuclei during the first 12 h p.i. (Fig. 2). Leached labeled DNA then appeared in increasing amounts from 13 to 20 h p.i., after which it remained fairly constant. Based on these results, cells were harvested at 18 to 22 h p.i. in most experiments.

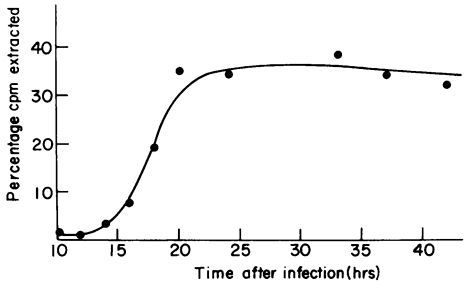


FIG. 2. Percentage of labeled DNA extracted at various times after infection. Nuclei of infected cells labeled for 30 min with [$^3$H]thymidine were suspended in buffer H and incubated at 37°C for 90 min. Samples of the extracted labeled DNA were centrifuged at 10,000 × *g* for 1 min at 25°C, and the supernatant was assayed for radioactivity by trichloroacetic acid precipitation. The total labeled DNA in the nuclei (100%) was determined by trichloroacetic acid precipitation on whole nuclei.

**Characterization of the extracted complexes.** MVM-infected cells were labeled with [$^3$H]thymidine for various lengths of time, and the labeled DNA was leached from the nuclei and analyzed by sedimentation through sucrose gradients. At the shortest labeling time (5 min), only a 50S peak was recognized (Fig. 3). At the 10-min pulse an additional 110S peak appeared, and

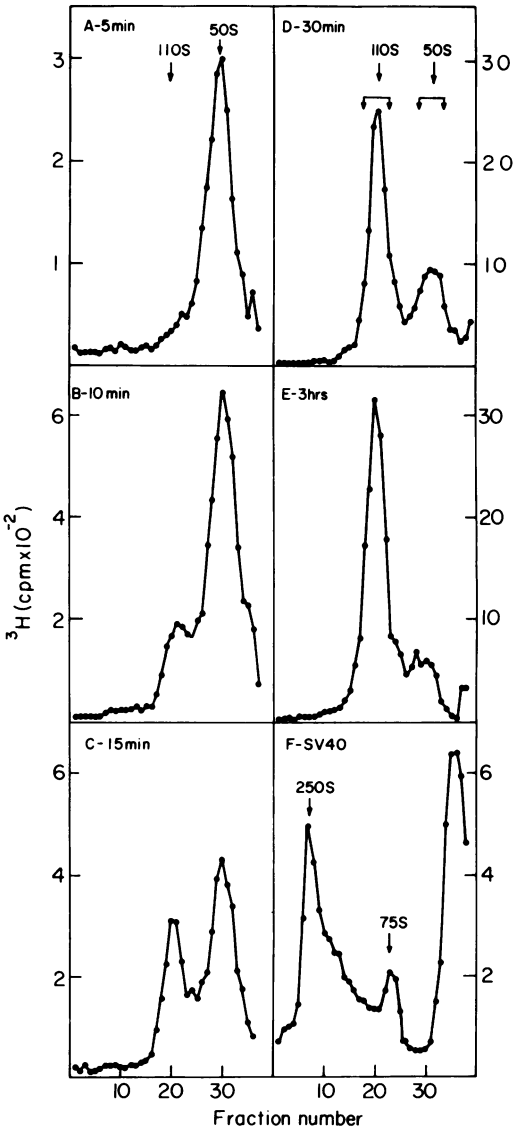


FIG. 3. Sedimentation profiles in sucrose gradients of $^3$H-labeled DPC. MVM-infected cells were labeled with [$^3$H]thymidine for various times as indicated and harvested at 22 h p.i. The extracts were centrifuged through 5 to 30% (wt/wt) sucrose gradients in a Beckman SW41 rotor at 32,000 rpm at 4°C for 2 h. Panel F shows the positions of SV40 minichromosomes (75S) and virions (250S) (11) used as sedimentation markers.

it became the major peak at longer labeling times. Fractions of the two peaks obtained after a 30-min pulse were pooled (Fig. 3D) and analyzed on CsCl density gradients (Fig. 4) and by sedimentation through alkaline sucrose gradients (Fig. 5).

Almost all of the labeled material present in the 110S peak banded at a density of 1.46 g/cm$^3$ (Fig. 4A), indicating the presence of immature infectious virus (1, 20, 37). It is worth noting that in several experiments a fraction of the labeled material was found at the bottom of the tube at a density more appropriate to that of free DNA. The source of this DNA was identified in the following experiments. The labeled material present in the 50S peak when banded on a CsCl density gradient yielded a major peak at a density of 1.31 g/cm$^3$ and a heterogeneous population of particles banding at higher densities (Fig. 4B), corresponding to the densities of defective particles containing DNA shorter than genome length. Some of the defective particles contain as little as 5% of the genome sequence and they are poorly resolved from empty particles (6). Figure 5 shows the sedimentation profiles of the DNA present in the 110S and 50S peaks. The 110S peak contained mainly ss DNA of genome length (15S), a shoulder of a peak (~20S) presumably containing dimer ss DNA, and some smaller DNA molecules. The 50S peak contained mainly small DNA. It has been previously shown that under the denaturing conditions of the sucrose gradients, RF DNA (nicked near both 3′ and 5′ ends) and RI DNA (with large gaps) are converted to unit-length ss DNA molecules as well as to ss DNA molecules of various sizes smaller than unit length, whereas a hairpin monomer duplex is denatured into a dimer ss DNA sedimenting at ~20S (26, 29). We therefore suggest that the 110S peak contained in addition to ss virion DNA a certain proportion of

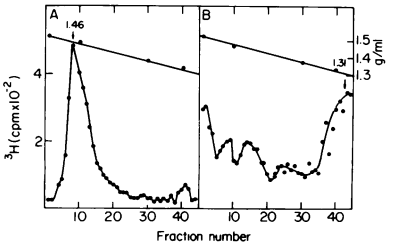


FIG. 4. CsCl density gradient of the material present in the 110S and 50S peaks (Fig. 3D). (A) Analysis of the 110S peak; (B) analysis of the 50S peak. Centrifugation was carried out in a Beckman SW50.1 rotor at 35,000 rpm for 48 h at 20°C. Analysis of radioactivity was carried out as in Fig. 1. The recoveries of labeled material in the density gradients were about 50%.

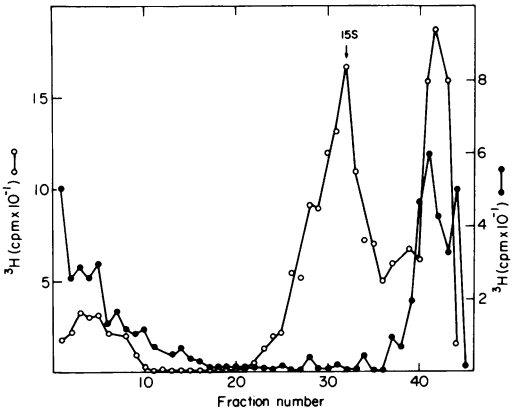


FIG. 5. Alkaline sucrose gradient analysis of the 110S and 50S peaks (see Fig. 3D). Centrifugation was carried out in a Beckman SW50.1 rotor at 48,000 rpm for 4 h at 5°C. Analysis of radioactivity was carried out as in Fig. 1. Symbols: ○, Material present in the 110S peak; ●, material present in the 50S peak.

RF and RI molecules. To further characterize the non-encapsidated DPC in the 110S peak, pooled fractions were subjected to digestion with DNase I and treatment with 0.1 and 0.25% Sarkosyl followed by sedimentation analyses through sucrose gradients. After DNase I digestion, the 110S peak was resolved into two peaks, one sedimenting at the original position and a new peak sedimenting at the top of the sucrose gradient (Fig. 6B).

The two populations of DPC present in the 110S peak were also resolved by the 0.1% Sarkosyl treatment (Fig. 6C). It is known that virions are stable in this concentration of Sarkosyl (17). Treatment at a higher concentration (0.25%) resulted in only one peak sedimenting at the top of the gradient, indicating that virions are sensitive to this Sarkosyl concentration. We conclude that the 110S peak contained, in addition to DNase-resistant and 0.1% Sarkosyl-resistant virions, DPC that were sensitive to these treatments.

**Identification of MVM minichromosomes.** Heparin treatment of minichromosomes removes the nucleosomes from the DNA (10; E. B. Jakobovits and Y. Aloni, unpublished data). To verify whether the DNase- and Sarkosyl-sensitive material of the 110S peak was MVM DNA complexed with nucleosomes in a minichromosome structure, the material present in the 110S peak was treated with heparin and rerun through a sucrose gradient. The 110S peak contained, in addition to heparin-resistant material (II in Fig. 7), heparin-sensitive material appearing as a new peak of labeled DNA at the top of the gradient (III in Fig. 7). Figure 8 shows

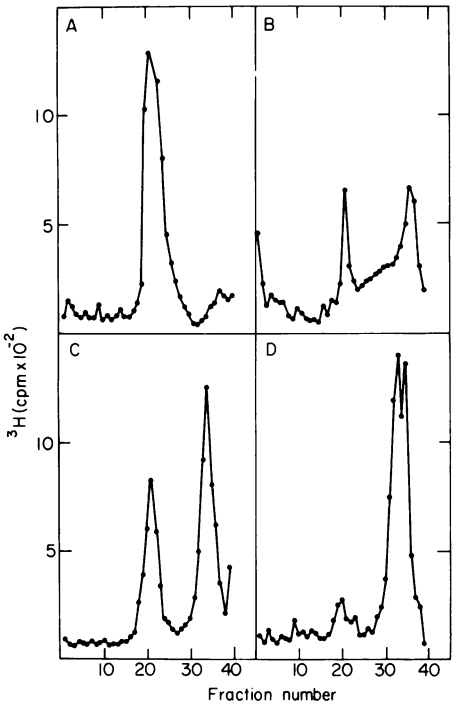


FIG. 6. Sensitivity of the material present in the 110S peak to DNase and Sarkosyl. (A) Rerun of the pooled fractions 18 to 22 of Fig. 3E; (B) rerun after treatment with DNase I (100 μg/ml) for 30 min at 0°C; (C) rerun after treatment with 0.1% Sarkosyl (incubated for 30 min at 0°C); (D) rerun after treatment with 0.25% Sarkosyl (incubated for 30 min at 0°C). Centrifugation was as in Fig. 3.

an analysis of the proteins present in the 110S peak before and after the heparin treatment. Before the treatment, in addition to the viral capsid proteins A and B (present in immature infectious virus), histones were also resolved (lane I). Although the 110S peak still contained the capsid proteins (lane II) after heparin treatment, a substantial portion of the histones was found in the fractions of the upper peak (lane III). These results indicate that the heparin-sensitive material present in the 110S peak was DNA complexed with cellular histones. The evidence that non-encapsidated DNA is of viral origin was obtained from the following experiment. Portions of the 110S peak fraction were electrophoresed on two agarose gels. At the end of the run, one gel was treated with PPO in ethanol, dried, and exposed to X-ray film, and the other was blotted onto a nitrocellulose filter and hybridized with a $^{32}$P-labeled MVM DNA probe (fragment A; see Materials and Methods). Figure 9 shows the results of the fluorography (lanes A and B) and hybridization (lanes C and D). It is evident that the 110S peak contained labeled DNA that migrated in the gel as monomer ds MVM DNA (RF) that hybridized with the $^{32}$P-labeled viral probe, as well as larger DNA or intact virions. These results suggest that the DNA associated with histones in the 110S peak pertains to RIs of MVM.

Micrococcal nuclease digestion can provide evidence for the nucleosome structure of the DPC present in the 110S peak (7, 15). This enzyme has a strong preference for intercore (spacer) DNA fragments (7, 15). It should be noted that the enzyme has about 100 times preference for ss DNA over ds DNA. After micrococcal nuclease digestion of the DPC present in the 110S peak, the resistant DNA was sized by electrophoresis on 1.4% agarose gels and stained with ethidium bromide. Simian virus (SV40) DNA fragments were used to calibrate

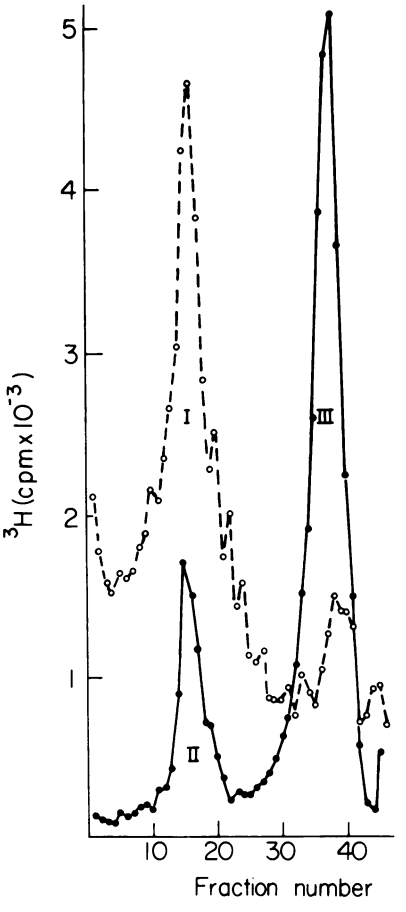


FIG. 7. Sensitivity of the material present in the 110S peak to heparin. Symbols: ○, Rerun of the pooled fractions 18 to 22 of Fig. 3E; ●, pooled fractions incubated with heparin (1 mg/ml) for 10 min at 37°C before centrifugation. Centrifugation was as in Fig. 3.

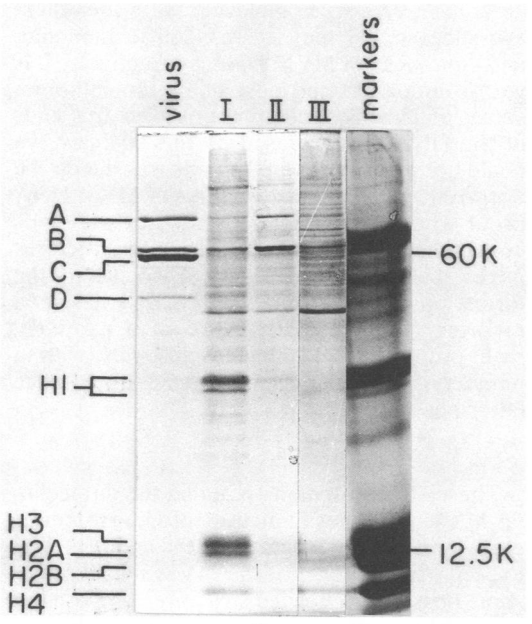


FIG. 8. Analysis of the proteins present in the three fractions of Fig. 7 by polyacrylamide gel electrophoresis. The lane "virus" represents proteins of light full-purified virions (33). Lane I is peak I of Fig. 7 (fractions 12 to 21); lane II is peak II of Fig. 7 (fractions 12 to 17); lane III is fraction 37 of peak III of Fig. 7. Molecular weight markers: Cytochrome *c*, 12,500; catalase, 60,000.

the gels. The SV40 DNA fragments span a molecular weight range of 1,768 to 215 base pairs (bp). The inverse relationship between the logarithm of the molecular weight and the distance of migration is linear in these gels from 2,600 to 200 bp (16). A ladder of bands corresponding to a nucleosome core (~140 bp) and multimer fragments was evident (Fig. 10A). Moreover, after 20 min of digestion a major DNA fragment of about 140 bp was observed. It is also apparent that the micrococcal digestion reduced the amount of monomer and oligomer ds forms of MVM DNA. Essentially no ds RF MVM DNA remained after 20 min of digestion. The ss viral DNA was unaffected because the viral capsid provided protection from nuclease digestion. However, ss DNA of the RFs, if complexed with or free of nucleosomes, should preferably be cleaved by the enzyme. To substantiate the viral origin of the nucleosome core, the DNA present in the lower part of the gel was blot transferred to diazobenzyloxymethyl paper (39) and hybridized to $^{32}$P-labeled MVM-specific probe (fragment A). The results shown in Fig. 10B indicate the presence of viral DNA sequences. After 5 and 20 min of digestion, a core of viral DNA of about 140 bp was evident. The high level of hybridization with the viral DNA probe obtained after only 30 s of digestion was presumably due to the preferential digestion of the ss DNA of the RFs. The ss digestion products hybridized efficiently with the $^{32}$P-labeled probe but were poorly stained with ethidum bromide. Because no similar viral DNA band was obtained when RFs of MVM DNA were subjected to micrococcal nuclease digestion, when the RFs of MVM DNA were added to the hypotonic extract and then digested with the nuclease (not shown), or when the sample was treated with RNase before electrophoresis, these results indicated that the 140-bp viral DNA pertained to a nucleosome core. Viral DNA core of similar length was obtained when the micrococcal nuclease digestion was carried out on nuclei isolated from MVM-infected cells (results not shown).

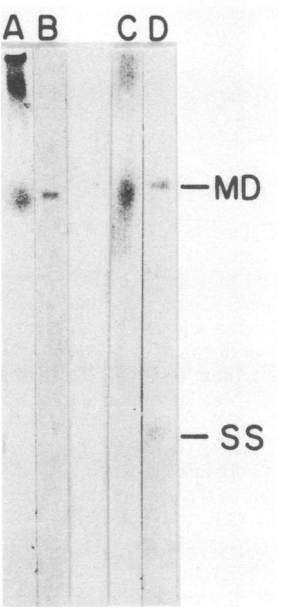


FIG. 9. Analysis of the DNA present in the 110S peak by agarose gel electrophoresis and by blot hybridization. Sodium dodecyl sulfate (final concentration, 0.5%) was added to the pooled fractions of the 110S peak, and electrophoresis was performed on two agarose gels (1.4%). One gel was fluorographed. (A) Position of labeled DNA present in the 110S peak; (B) position of ds MVM DNA (MD) used as a marker; (C) after electrophoresis the second gel was blotted onto a nitrocellulose filter and hybridized with $^{32}$P-labeled MVM DNA (fragment A); (D) positions of double-stranded (MD) and single-stranded (SS) MVM DNA. Single-stranded DNA is not evident in either the fluorography (A) or hybridization (C) because apparently the viral DNA was not completely released from the virions. Broken virions remain at the origin of the gel (see lanes A and C).

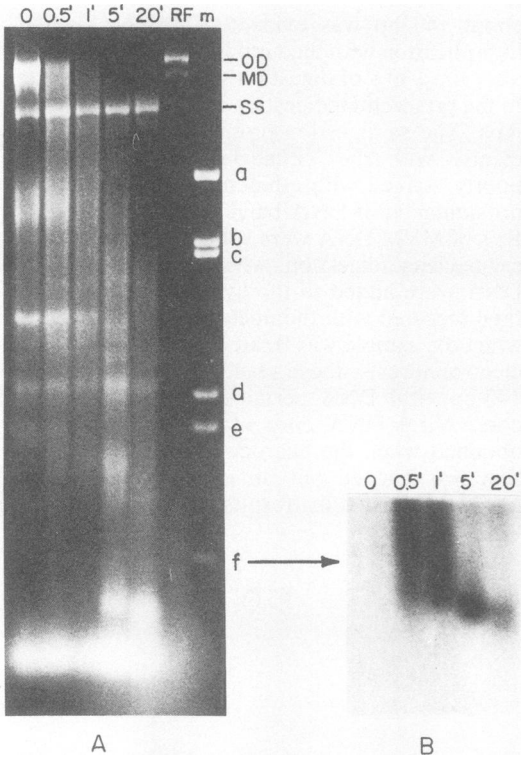


FIG. 10. Electrophoresis of DNA fragments produced by micrococcal nuclease digestion of the DPC present in the 110S peak. (A) DPC present in the 110S peak were digested (see Materials and Methods) for the times indicated and DNA extracted, and electrophoresis was performed on a 1.4% agarose gel and stained with ethidium bromide. RF, Replicative forms of MVM DNA (MD, monomer duplex; OD, oligomer duplex); m, *Hin*dIII digest of SV40 DNA used as size marker. a = 1,768 bp; b = 1,169 bp; c = 1,115 bp; d = 526 bp; e = 447 bp; and f = 215 bp. (B) Autoradiogram of the lower part of the gel blotted onto diazobenzyloxymethyl paper (39) and hybridized with $^{32}$P-labeled MVM DNA (fragment A). The autoradiogram is aligned with the portion of the gel that was blotted onto the diazobenzyloxymethyl paper. The arrow indicates the position of fragment f in the autoradiogram.

**EM visualization of MVM minichromosomes.** EM visualization can further verify whether DPC present in the 110S peak are organized in minichromosome structures. Figure 11A, a representative field of structures present in the 110S peak as visualized in the EM (5), clearly shows intact virions and beads-on-a-string structures (minichromosomes) as well as two major classes of minichromosomes. Figures 11B and 11C show selected molecules of the two classes, and Fig. 12 is a histogram of the number of nucleosomes on the minichromosomes. Two main populations of minichromosomes with an average of 26 and 52 beads per molecule were identified. We suggest that they correspond to monomer and dimer RFs of MVM DNA, respectively. It is worth noting that in most of the minichromosomes analyzed we could not observe free ends of the DNA string. Based on this analysis, we could not determine whether this was due to the occurrence of peculiar structures of MVM DNA (2) or whether the minichromosomes were RFs containing ss DNA tails, resulting in structures having a collapsed appearance. Evidence for the formation of nucleosomes on ss DNA has been reported (18). The occurrence of a relatively high proportion of minichromosomes with a number of beads between 26 and 52 supports the latter possibility.

## DISCUSSION

These studies demonstrate that the intracellular MVM DNA has minichromosome structures corresponding in length to RFs and RIs that exceed genome length. The MVM minichromosome possesses a beaded structure composed of cellular histones and viral DNA in a molecular complex which is very similar to that of cellular chromatin.

For the extraction of MVM DPC, we used a mild procedure which is based on an observation made with SV40-infected cells showing that viral complexes can be leached from infected cell nuclei in the presence of hypotonic buffer (11, 30). This procedure has been shown to yield viral complexes in the complete absence of both salt and detergent, two factors implicated in causing disruption of the fine structure inherent in the viral DPC (3, 8, 38). As in the case of SV40, the extract contains intact virions and viral minichromosomes as well as virions containing incomplete genomes. Based on kinetics studies (Fig. 3), it appears that the encapsidation rate of the defective particles exceeds that of the full particles. This observation is consistent with the existence of an independent pool of defective particles containing all of the critical recognition sites necessary for replication of MVM DNA (6).

Somewhat surprising was the observation that the viral minichromosomes sedimented in sucrose gradients at about 110S together with the virions, whereas SV40 minichromosomes with somewhat larger DNA sediment under similar conditions at 75S (11). It is worth noting, however, that we consistently found that the unit-length minichromosomes were more abundant at the tailing side of the peak, whereas the larger minichromosomes were more abundant at the leading side of the peak. The faster sedimentation rate of MVM minichromosomes as compared with that of SV40 minichromosomes is presumably due to its more compact structure

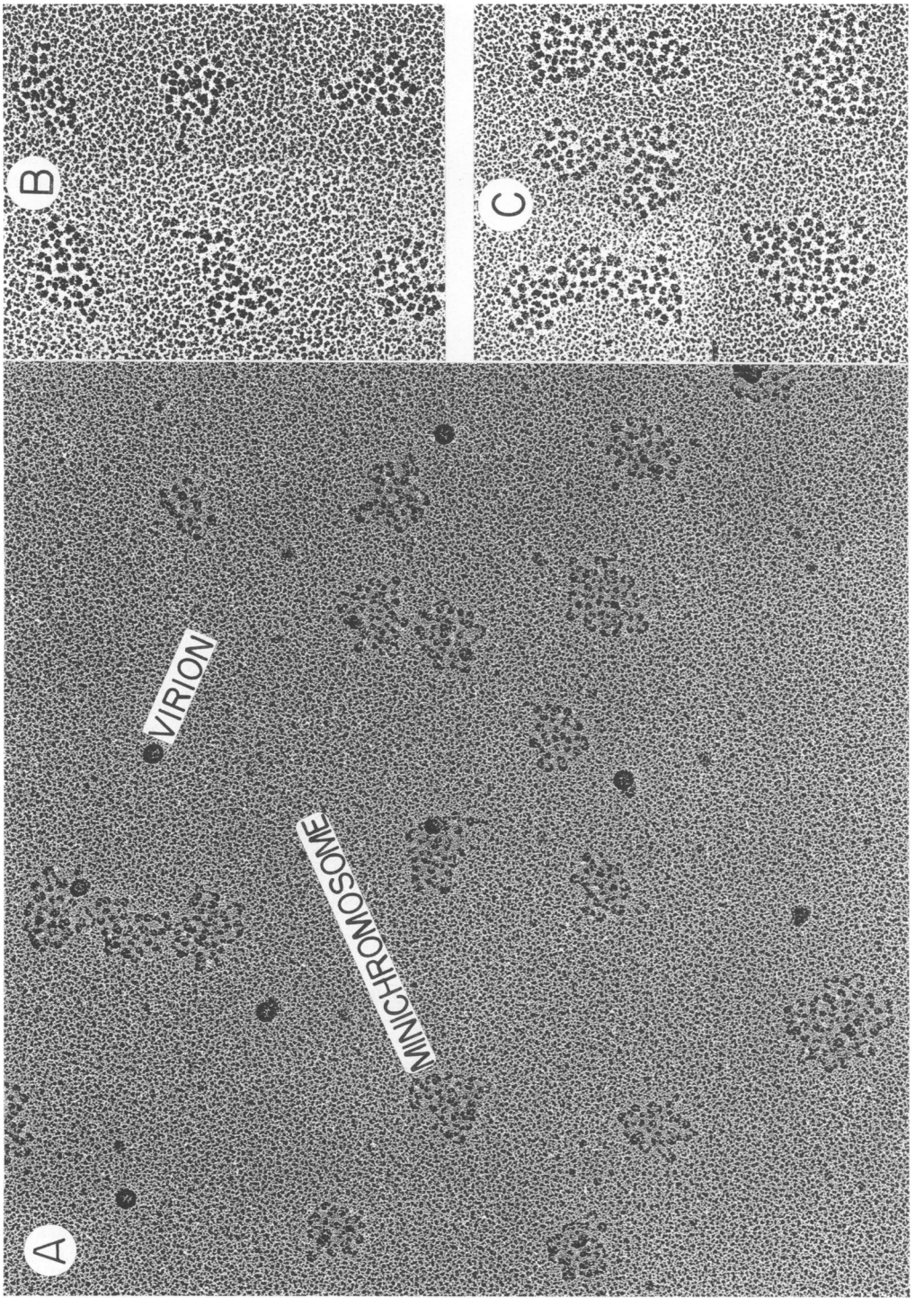


FIG. 11. EM visualization of DPC present in the 110S peak. (A) A sample of fraction 19 of Fig. 3D was mounted on carbon membrane-coated grids activated by glow discharge (5). The grids were stained with 2% uranyl acetate and rotary shadowed with Pt/Pd (80:20). Molecules were visualized with a Philips 400 EM, and photographs were taken at a magnification of ×42,000. (B) Representative molecules of a monomer minichromosome; (C) representative molecules of a dimer minichromosome.

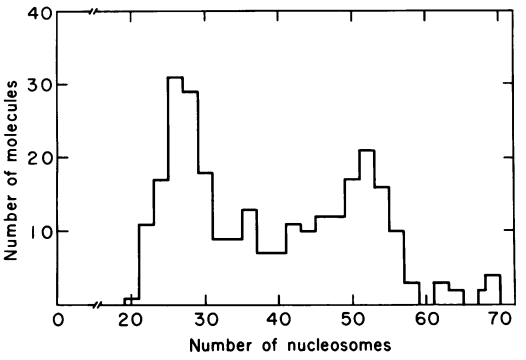


FIG. 12. Histogram of the number of nucleosomes on MVM minichromosomes. The nucleosomes were counted in structures as shown in Fig. 11B and 11C.

resulting from the larger number of nucleosomes per genome (see below). It was possible to identify the minichromosomes in the 110S peak because of their sensitivity to DNase, Sarkosyl, and heparin treatments and from their appearance in the EM. Based on protein analysis of the material present in the 110S peak, it appears that most of the intracellular virions were immature because they contained only protein A and B, in contrast to mature virions that contain, in addition, proteins C and D (20, 33, 34). No histones were detected in the mature virions (see Fig. 8). It appears, therefore, that the histones found in the 110S peak pertained to MVM minichromosomes. It should be noted that a full complement of histones was revealed, including histone H1. Mention should be made of the fact that the ratio between the extracted virions and minichromosomes varied from experiment to experiment and was dependent on the stock of virus used and on the time after infection.

It has been proposed that the bulk of the DNA in eucaryotic chromatin is associated with nucleosomes, each consisting of approximately 200 bp of DNA folded around an octamer of histones (7, 15). The nucleosomal DNA is divided into a region of constant size containing 140 bp of core DNA and a region of variable size containing 10 to 100 bp of linker DNA (7, 15).

Our EM measurements show that the monomer minichromosome consists of an average of 26 nucleosomes. Having an RF of about 5,000 bp of DNA (2, 6; C. R. Astell, personal communication), each repeating unit therefore contains an average of about 192 bp. It is most likely that the nucleosome of the MVM minichromosome consists of a core containing a DNA fragment of about 140 bp associated with approximately 52-bp DNA fragment more susceptible to nuclease attack. Micrococcal nuclease digestion supports this conclusion (Fig. 10). It appears that most of the nucleosomes on the MVM minichromosome are in close contact. This is like cellular chromatin but unlike the SV40 minichromosome, which consists both of nucleosomes lying close together and of nucleosomes connected by segments of DNA of irregular lengths (24). It is difficult to determine from our analysis whether a fraction of the MVM minichromosome population contains a gapped region that is devoid of nucleosome (12, 23).

The replication process of MVM involves formation of ds RIs that contain regions of ss DNA (1, 40). The relatively high proportion of minichromosomes containing between 26 (monomer) and 52 (dimer) nucleosomes may indicate that the ss DNA also has a beaded structure. In this respect, it should be mentioned that ss DNA can form in vitro a complex with the histones which very closely resembles that which exists with ds DNA (18). It has been suggested that the nucleosomal histones have been specifically designed to recognize both ds and ss DNA (18).

MVM is known to encapsidate specifically the minus DNA strand (1). It is possible that the attachment of the nucleosomes, during the encapsidation process, to the plus DNA strand is a step in a mechanism that determines the strand-specific encapsidation. The progress of MVM minichromosomes through replication, deposition of the histone proteins, including H1, and final encapsidation of ss DNA will require further analysis.

Evidence for the involvement of histones in the transcription (7, 13, 15) and replication (7, 15) processes of chromosomes is accumulating, and several aspects of the two processes appear to be similar. In neither process do histones appear to be released from DNA, but altered chromatin structure seems to be a feature of both, resulting in enhanced nuclease susceptibility of active gene and nascent chromatin (7, 15). MVM, like SV40, utilizes mainly the cellular replication and transcription machinery. However, whereas SV40 is capable of stimulating cellular DNA and histone synthesis (4), a potential viral DNA replication dependence on host cell factors made during the S phase has been suggested for MVM (32). It is interesting to note that six enzymes of DNA synthesis were found to be in different cellular compartments, depending on the stage of the cell growth. It has been suggested that the enzymes form a complex termed "replitase" and migrate from the cytoplasm to the nucleus when DNA replication is taking place (19). The association of the replitase with the viral minichromosome or with the nuclear matrix could be the factor that is made during the S phase and is needed for MVM replication.

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LITERATURE CITED

1. **Berns, K. I., and W. W. Hauswirth.** 1978. Parvovirus DNA structure and replication, p. 13–32. *In* D. C. Ward and P. Tattersall (ed.), Replication of mammalian parvoviruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
2. **Bratosin, S., O. Laub, Y. Tal, and Y. Aloni.** 1979. Mechanism of circularization of linear DNAs: circular parvovirus MVM DNA is formed by a "noose" sliding in a "lasso"-like DNA structure. Proc. Natl. Acad. Sci. U.S.A. **76**:4289–4293.
3. **Christiansen, G., and J. Griffith.** 1977. Salt and divalent cations affect the flexible nature of the natural beaded chromatin structure. Nucleic Acids Res. **4**:1837–1851.
4. **Das, G. C., and S. K. Niyogi.** 1981. Structure, replication, and transcription of the SV40 genome, p. 187–241. *In* W. Cohn (ed.), Progress in nucleic acid research, vol. 25. Academic Press, Inc., New York.
5. **Dubochet, J., M. Ducommon, M. Zollinger, and E. Kellenberger.** 1971. A new preparation-method for dark-field electron microscopy of biomolecules. J. Ultrastruct. Res. **35**:147–167.
6. **Faust, E., and D. C. Ward.** 1979. Incomplete genomes of the parvovirus minute virus of mice: selective conservation of the genome termini including the origin for DNA replication. J. Virol. **32**:276–292.
7. **Felsenfeld, G.** 1978. Chromatin review article. Nature (London) **271**:115–122.
8. **Fernandez-Munoz, R., M. Coca-Prados, and M.-T. Hsu.** 1979. Intracellular forms of simian virus 40 nucleoprotein complexes. I. Methods of isolation and characterization in CV-1 cells. J. Virol. **29**:612–623.
9. **Green, M. R., R. M. Lebovitz, and R. G. Roeder.** 1979. Expression of the autonomous parvovirus H1 genome: evidence for a single transcriptional unit and multiple spliced polyadenylated transcripts. Cell **17**:967–977.
10. **Groner, Y., G. Monroy, M. Jaquet, and J. Hurwitz.** 1975. Synthesis of RNA containing a methylated blocked 5′ terminus by HeLa nuclear homogenates. Proc. Natl. Acad. Sci. U.S.A. **72**:2930–2934.
11. **Jakobovits, E. B., and Y. Aloni.** 1980. Isolation and characterization of various forms of simian virus 40 DNA-protein complexes. Virology **102**:107–118.
12. **Jakobovits, E. B., S. Bratosin, and Y. Aloni.** 1980. A nucleosome-free region in SV40 minichromosomes. Nature (London) **285**:263–265.
13. **Jakobovits, E. B., S. Saragosti, M. Yaniv, and Y. Aloni.** 1980. *Escherichia coli* RNA polymerase *in vitro* mimics simian virus 40 *in vivo* transcription when the template is viral nucleoprotein. Proc. Natl. Acad. Sci. U.S.A. **77**:6556–6560.
14. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
15. **Lilley, D. M., and J. F. Pardon.** 1979. Structure and function of chromatin. Annu. Rev. Genet. **13**:197–233.
16. **McDonell, M., M. Simon, and R. Studier.** 1977. Analysis of restriction fragments of T7 DNA and determination of molecular weights by electrophoresis in neutral and alkaline gels. J. Mol. Biol. **110**:119–146.
17. **Müller, A. P., M. Gautschi, and G. Siegl.** 1978. Defective particles of parvovirus Lu III, p. 231–240. *In* D. C. Ward and P. Tattersall (ed.), Replication of mammalian parvoviruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
18. **Palter, K. B., V. E. Foe, and B. M. Alberts.** 1979. Evidence for the formation of nucleosome-like histone complexes on single-stranded DNA. Cell **18**:451–467.
19. **Reddy, G. P. V., and A. B. Pardee.** 1980. Multienzyme complex for metabolic channeling in mammalian DNA replication. Proc. Natl. Acad. Sci. U.S.A. **77**:3312–3316.
20. **Richards, R., P. Linser, and R. W. Armentrout.** 1978. Maturation of minute virus of mice particles in synchronized rat-brain cells, p. 447–458. *In* D. C. Ward and P. Tattersall (ed.), Replication of mammalian parvoviruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
21. **Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg.** 1978. Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick-translation with DNA polymerase I. J. Mol. Biol. **113**:237–251.
22. **Rose, J. A.** 1974. Parvovirus reproduction, p. 1–61. *In* H. Fraenkel-Conrat and R. R. Wagner (ed.), Comprehensive virology, vol. 3. Plenum Publishing Corp., New York.
23. **Saragosti, S., G. Moyne, and M. Yaniv.** 1980. Absence of nucleosomes in a fraction of SV40 chromatin between the origin of replication and the region coding for the late leader RNA. Cell **20**:65–74.
24. **Shelton, E., P. M. Wasserman, and M. L. DePamphilis.** 1978. Structure of simian virus 40 chromosome in nuclei from infected cells. J. Mol. Biol. **125**:491–514.
25. **Siegl, G.** 1976. The parvoviruses. Virol. Monogr. **15**:1–109.
26. **Siegl, G., and M. Gautchi.** 1978. Purification and properties of replicative forms and replicative intermediate DNA molecules of parvovirus Lu III, p. 315–325. *In* D. C. Ward and P. Tattersall (ed.), Replication of mammalian parvovirus. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
27. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments by gel electrophoresis. J. Mol. Biol. **98**:503–517.
28. **Stein, G. S., J. L. Stein, W. D. Park, S. Detke, A. C. Lichtler, E. A. Shephard, R. L. Jansing, and I. R. Phillips.** 1978. Regulation of histone gene expression in HeLa $S_3$ cells. Cold Spring Harbor Symp. Quant. Biol. **42**:1107–1120.
29. **Straus, S. E., E. D. Sebring, and J. A. Rose.** 1978. Self-primed replication of adeno-associated-virus DNA, p. 243–255. *In* D. C. Ward and P. Tattersall (ed.), Replication of mammalian parvoviruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
30. **Su, R. R., and M. L. DePamphilis.** 1976. *In vitro* replication of simian virus 40 DNA in nucleoprotein complex. Proc. Natl. Acad. Sci. U.S.A. **73**:3466–3470.
31. **Tal, J., D. Ron, P. Tattersall, S. Bratosin, and Y. Aloni.** 1979. About 30% of minute virus of mice RNA is spliced out following polyadenylation. Nature (London) **279**:649–651.
32. **Tattersall, P.** 1972. Replication of the parvovirus MVM I. Dependence of virus multiplication and plaque formation on cell growth. J. Virol. **10**:586–590.
33. **Tattersall, P.** 1978. Parvovirus protein structure and virion maturation, p. 53–72. *In* D. C. Ward and P. Tattersall (ed.), Replication of mammalian parvoviruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.
34. **Tattersall, P., P. J. Cawte, A. J. Shatkin, and D. C. Ward.** 1976. Three structural polypeptides coded for by minute virus of mice, a parvovirus. J. Virol. **20**:273–289.
35. **Tattersall, P., L. V. Crawford, and A. J. Shatkin.** 1973. Replication of the parvovirus MVM. II. Isolation and characterization of intermediates in the replication of the viral deoxyribonucleic acid. J. Virol. **12**:1446–1456.
36. **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.
37. **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.
38. **Varshavsky, A. J., V. V. Bakayev, P. M. Chumackov, and G. P. Georgiev.** 1976. Minichromosome of simian virus 40: presence of histone H1. Nucleic Acids Res. **3**:2101–2113.

39. **Wahl, G. M., M. Stein, and G. R. Stark.** 1979. Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl paper and rapid hybridization using dextran sulfate. Proc. Natl. Acad. Sci. U.S.A. **76**:3683–3687.

40. **Ward, D. C., and D. K. Dadachanji.** 1978. Replication of minute virus of mice DNA, p. 297–313. *In* W. C. Ward and P. Tattersall (ed.), Replication of mammalian parvoviruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.