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Chromosomal Organization of the Herpes Simplex Virus Genome during Acute Infection of the Mouse Central Nervous System

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After corneal inoculation, herpes simplex virus type 1 replicates in the mouse eye, trigeminal ganglia, and brainstem, producing first an acute and then a latent infection. Previous work from this laboratory focused on the structure of the viral DNA in this system. We have now examined the structure of the viral genome at the chromosome level by using micrococcal nuclease digestion. Studies with disaggregated cell preparations made from the brainstems of acutely infected mice show that the majority of the viral DNA is in a nonnucleosomal form; however, a nucleosomelike fraction was also consistently detected. A similar result was obtained for viral DNA in herpes simplex virus type 1-infected C1300 (clone NA) neuroblastoma cells (a neuronal cell line).

Eucaryotic chromatin has a repeating structure generated by the folding of DNA around a core of histone proteins to form nucleosomes (8, 10, 12). One way to demonstrate this structure is by digesting chromatin with micrococcal nuclease; the histones provide a degree of protection against the nuclease, so that during a partial digestion only the DNA linking the nucleosomes is cut. Consequently, when DNA from partially digested chromatin is run on an agarose gel, a series of fragment sizes is seen, each an integral multiple of the nucleosomal repeat unit.

The chromosome structures of several herpesviruses have been examined, and there is some variation between them. Shaw et al. (19) showed that the latent, episomal Epstein-Barr virus (EBV) DNA in Raji cells has a nucleosomal structure, whereas EBV DNA in the producer cell line P3HR-1 is predominantly nonnucleosomal. Nucleosomes have also been demonstrated on episomal EBV DNA in B95-8 cells, only 0.5% of which are in the productive cycle (2). For both human cytomegalovirus (22) and herpes simplex virus type 2 (7), there is evidence for the existence of viral nucleosomes in the nuclei of infected tissue culture cells. For herpes simplex type 1 (HSV-1) in tissue culture cells, the available data suggest that a nucleosomal form is either absent (15) or, at most, a minor component (9). In addition, replicating HSV-1 DNA in Vero cells is more accessible to trimethylpsoralen photobinding than is cellular DNA (20), consistent with a nonnucleosomal structure.

As part of our study of HSV-1 infections of the nervous system, we investigated the chromosomal structure of the viral genome in vivo, specifically in acutely infected mouse brains and also in C1300 (clone NA) neuroblastoma cells (isolated by R. J. Klebe and F. H. Ruddle [13]), which display several properties of differentiated neurons, including the formation of neurites, the presence of neuronal enzymes, and the synthesis of neurotransmitters. We found that the viral DNA is predominantly nonnucleosomal, as shown by its susceptibility to random cleavage by micrococcal nuclease. However, a nucleosomal fraction was also detected.

To investigate the structure of specific DNA sequences in central nervous system tissue, dispersed cell preparations were made from mouse brainstems, followed by isolation of cell nuclei and incubation with micrococcal nuclease. The method used for cell dispersal was modified from that developed for rat brains by Farooq and Norton (3). Brainstems were incubated for 1 h at 37°C in cell isolation medium (10 mM potassium phosphate, 8% glucose, 5% fructose [pH 6.0]) containing 0.1% acetylated trypsin and 0.25% collagenase and then for 10 min on ice with cell isolation medium containing 0.1% soybean trypsin inhibitor. The tissue was washed with cell isolation medium and then aspirated through a 1.5-mm-diameter nozzle and passed through a 420-μm-mesh nylon filter. Cells were pelleted by centrifugation at 700 × *g* for 10 min at 4°C and suspended in 10 mM Tris hydrochloride (pH 7.5)–10 mM NaCl–3 mM $MgCl_2$ (reticulocyte standard buffer [RSB]) plus 0.1 M sucrose for isolation of nuclei. After 10 min on ice, they were Dounce homogenized (loose pestle) and centrifuged (1,000 × *g* for 5 min at 4°C). The pellet was resuspended in RSB plus 0.1 M sucrose, and Nonidet P-40 was added to 1% to lyse unbroken cells. The nuclei were pelleted as described above and resuspended in RSB plus 0.1 M sucrose. An equal volume of RSB plus 0.25 M sucrose was added, and the nuclei were centrifuged through an equal volume of 10 mM Tris hydrochloride (pH 8.0)–5 mM $MgCl_2$–0.33 M sucrose (500 × *g* for 10 min at 4°C). The supernatants obtained after Dounce homogenization and Nonidet P-40 treatment were pooled and are referred to as the cytoplasmic fraction.

Nuclei were incubated with micrococcal nuclease (50 U/ml) at 37°C, as described by Spiker et al. (21), followed by incubation with proteinase K (100 μg/ml) for 3 h at 37°C. DNA was isolated as previously described (4).

The feasibility of this approach for the determination of chromatin structure in brain cells was tested by examining the mouse β-major globin gene. DNA samples, prepared from micrococcal nuclease-digested nuclei, were electrophoresed on 1.5% agarose gels for 16 h at 2 V/cm, transferred to nitrocellulose, and hybridized with ^{32}P-labeled nick-translated pMBΔ2 DNA (11, 23), which is specific for this gene (Fig. 1a). The pattern of hybridization (1a, B) was clearly nucleosomal and coincided with the bands seen after ethidium bromide staining (1a, A). The success of this technique for analysis of a gene present at two copies per cell indicated its usefulness for studying the HSV genome in acutely infected brainstems.

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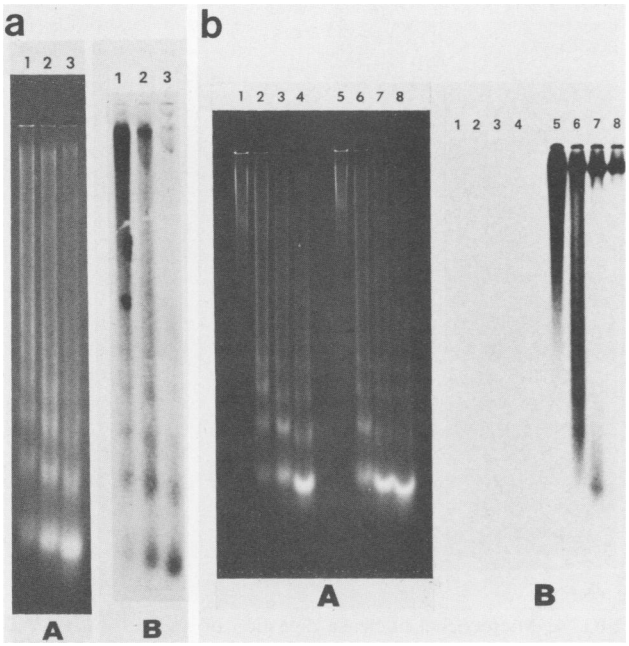

FIG. 1. Micrococcal nuclease digestion of nuclei from mouse brain tissue. Nuclei were isolated from the brainstems of uninfected mice (a and lanes 1 to 4 of b) and from the brainstems of acutely infected mice (b, lanes 5 to 8). They were incubated with micrococcal nuclease for various times, after which the DNA was extracted, electrophoresed (30 μg of each sample), stained with ethidium bromide (A), and then transferred to nitrocellulose and probed with ^{32}P-labeled nick-translated DNA (B). (a) Digestion times: 1 min (lane 1), 2 min (lane 2), and 5 min (lane 3). The probe was pMBΔ2 for hybridization to globin sequences. (b) Digestion times: 0.5 min (lanes 1 and 5), 1 min (lanes 2 and 6), 2 min (lanes 3 and 7), and 5 min (lanes 4 and 8). The probe was HSV-1 virion DNA.

Female BALB/cByJ mice (4 to 6 weeks old) were infected with 10^7 PFU of HSV-1 (strain F) per eye after corneal scarification. Brainstem nuclei from acutely infected mice (7 days postinfection [p.i.]) and from uninfected mice were incubated with micrococcal nuclease for various times (Fig. 1b, legend). Figure 1b shows an ethidium bromide-stained gel of the resulting DNA fragments (A) and hybridization with labeled HSV-1 virion DNA (B). Since the ratio of cellular to viral DNA in the brainstem at the acute stage of infection is about 10,000 to 1 (17), the nucleosomal bands in panel A represent digested cellular DNA. Unlike the cellular DNA, most of the viral DNA was cut randomly to produce a smear of fragment sizes (B, lanes 5 to 8). A minority of the viral DNA produced a ladder of fragments coincident with the cellular DNA, which is indicative of a nucleosomal form. The nucleosomal hybridization was not due to cross-hybridization of the viral probe to cellular DNA, as shown by the result for uninfected brainstem DNA (lanes 1 to 4).

Some of the viral DNA was completely resistant to nuclease action, perhaps due to packaging into nucleocapsids. If so, then cytoplasmic viral DNA should also be resistant to digestion, as it represents viral genomes which have been encapsidated and enveloped (14). To test this possibility, a cytoplasmic extract of brainstem cells was prepared and incubated with micrococcal nuclease (Fig. 2, lanes 4 to 6). Since the composition of the cytoplasmic fraction differed from that of the nuclease digestion buffer (21), sucrose and $CaCl_2$ were added to final concentrations of 0.25 M and 1 mM, respectively, before digestion. To demonstrate that the enzyme was active under these conditions, a reconstituted mixture of nuclei and cytoplasm was also incubated with micrococcal nuclease (Fig. 2, lanes 1 to 3). Viral DNA in cell nuclei was digested at the same rate, whether in digestion buffer or the modified cytoplasmic fraction. However, there was no digestion of cytoplasmic virion DNA. We therefore conclude that encapsidated viral DNA is protected from micrococcal nuclease digestion, in agreement with the results of Leinbach and Summers (9) for cytoplasmic virion DNA in Vero cells.

To rule out the possibility of an inherent periodicity to micrococcal nuclease cleavage of HSV-1 DNA, purified virion DNA was digested with nuclease (15 U/ml) and the hybridization pattern was determined after electrophoresis and Southern blotting (Fig. 3, lanes 5 to 7). The smear of hybridization with a virion DNA probe (lane 5) indicates that naked HSV-1 DNA was cut randomly by micrococcal nuclease. At longer digestion times, the virion DNA was completely digested (lanes 6 and 7). Migration of the digested virion DNA was not affected by mixing it, before electrophoresis, with DNA from a micrococcal nuclease digest of uninfected mouse brain nuclei (Fig. 3, lanes 1 to 3). So the viral nucleosome bands found with acutely infected mouse brain DNA were not caused by the trapping of a fraction of the viral DNA within the cellular nucleosomal bands during electrophoresis.

We also investigated the HSV-1 genome structure in infected C1300 (clone NA) neuroblastoma cells. C1300 nu-

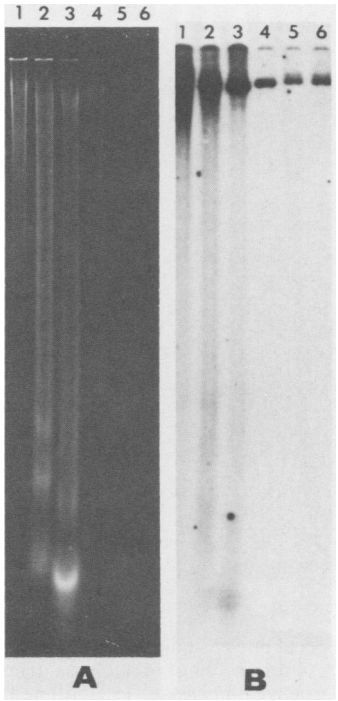

FIG. 2. Micrococcal nuclease digestion of brainstem cell cytoplasm from acutely infected mice. Cytoplasmic and nuclear fractions were prepared from brainstems at 7 days p.i. The cytoplasmic fraction and a reconstituted mixture of the cytoplasmic and nuclear fractions were digested with micrococcal nuclease for 0 to 5 min. DNA was then extracted and electrophoresed. (A) Ethidium bromide staining. (B) Hybridization with ^{32}P-labeled virion DNA. Lanes: 1 to 3, nuclear plus cytoplasmic DNA (50 μg); 4 to 6, cytoplasmic DNA (6, 3, and 6 μg, respectively). Digestion times: 0 min (lanes 1 and 4), 1 min (lanes 2 and 5), 5 min (lanes 3 and 6).

clei were prepared at various times p.i. and incubated with micrococcal nuclease (Fig. 4). At 3 h p.i., the viral DNA was resistant to nuclease digestion, presumably because it was still encapsidated. At all later times there was also a smear of hybridization, indicating that throughout the infection most of the unpackaged progeny viral DNA could be randomly cut by micrococcal nuclease and therefore was not nucleosomal. The faint ladder of hybridization which can be seen superimposed on the smear, particularly in lane 6, was not due to cross-hybridization of the probe with nucleosomal cellular DNA, since it would then be visible in the 3-h samples (lanes 1 and 2). This suggests that a small fraction of the viral DNA may be arranged in a nucleosomal form during an infection of C1300 (clone NA) cells.

Possible explanations for our results, both in vivo and in vitro, include (i) the existence of a few copies of the viral genome in a nucleosomal form; (ii) a nucleosomal arrangement for part of each genome copy; and (iii) the formation of complexes between viral DNA and nonhistone, perhaps viral, proteins that do not protect against micrococcal nuclease digestion as completely as do histones. At present, we cannot distinguish among these possibilities. However, electron microscope studies of chromatin from infected RC-37 African green monkey kidney cells (16) suggest that nucleosomal and nonnucleosomal forms of HSV-1 (strain ANG) chromatin can coexist on the same DNA molecule.

The genome of EBV can also adopt a nucleosomal or nonnucleosomal form. In the latent state, it has a

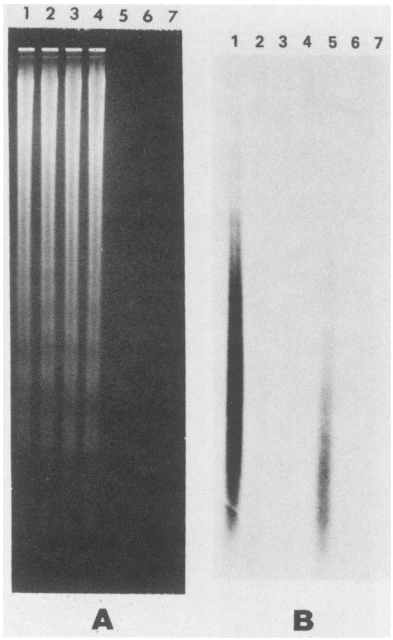

FIG. 3. Micrococcal nuclease digestion of purified HSV-1 virion DNA. Virion DNA (4 μg) in 100 μl of nuclease digestion buffer was incubated with micrococcal nuclease (15 U/ml) at 37°C. Samples (2 μl) were taken at various times and added either to 10 mM Tris hydrochloride (pH 7.4)–10 mM EDTA containing 40 μg of DNA from a 1-min micrococcal nuclease digestion of uninfected mouse brain nuclei (lanes 1 to 3) or to 10 mM Tris hydrochloride (pH 7.4)–10 mM EDTA alone (lanes 5 to 7). The samples were electrophoresed and stained with ethidium bromide (A) and then transferred to nitrocellulose and hybridized with ^{32}P-labeled virion DNA (B). Digestion times: 1 min (lane 1), 2 min (lane 2), 4 min (lane 3), 1.5 min (lane 5), 2.5 min (lane 6), 4.5 min (lane 7). Lane 4 contains 40 μg of the digested mouse brain DNA with no added virion DNA.

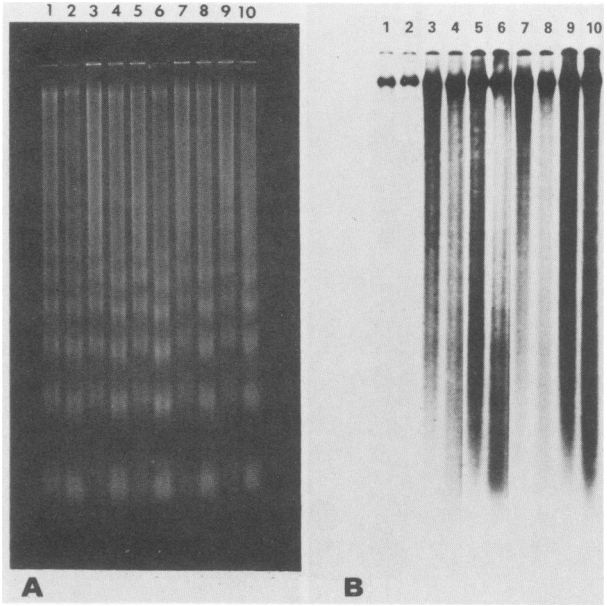

FIG. 4. Micrococcal nuclease digestion of nuclei from HSV-1-infected C1300 cells. C1300 monolayers were infected with HSV-1 at a multiplicity of infection of five, and the inoculum was removed after 1 h (time zero). Nuclei were isolated at various times p.i. and incubated with micrococcal nuclease for 1 to 2 min. DNA was then extracted and electrophoresed. (A) Ethidium bromide staining. (B) Hybridization with ^{32}P-labeled virion DNA. Hours p.i.: 3 (lanes 1 and 2), 5 (laned 3 and 4), 8 (lanes 5 and 6), 12.5 (lanes 7 and 8), 16.5 (lanes 9 and 10). Digestion times: 1 min (lanes 1, 3, 5, 7, and 9), 2 min (lanes 2, 4, 6, 8, and 10). Exposure times: 48 h (lanes 1 to 4), 16 h (lanes 5 and 6), 2 h (lanes 7 to 10).

nucleosomal structure (2, 19). After induction of viral replication by superinfection, the newly synthesized DNA is not nucleosomal (19). Shaw (18) showed that this corresponds with a switch from EBV DNA synthesis by a cellular polymerase in latently infected cells to synthesis by the viral polymerase in productively infected cells.

Why is a structural change necessary? It may be a reflection of increased transcription from the viral genome after induction. While not universally true, there is evidence that many actively transcribed cellular genes do not have the canonical nucleosome structure (1, 24). Thus, one might not expect the EBV genome to be nucleosomal during a productive infection or, indeed, expect the HSV-1 genome to be nucleosomal during the acute phase of infection in the mouse central nervous system.

In summary, we have found that the majority of HSV-1 DNA in brainstems of acutely infected mice is not arranged in a nucleosomal form. However, a small fraction does appear to have a nucleosomelike structure; experiments using cloned regions of the genome as probes should determine whether specific parts of the viral genome are nucleosomal. It will also be interesting to see whether, during the course of infection, there is a change in the proportion or regions of viral DNA in a nucleosomal form, particularly as this may shed some light on the nature of latent infections. Results from immunofluorescence (6) and in situ hybridization experiments (5; preliminary data from this laboratory) suggest that expression from latent HSV genomes is limited to a subset of those genes expressed during an acute infection and this may be reflected in the chromosomal structure of those genes.

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