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Site-specific cleavage/packaging of herpes simplex virus DNA and the selective maturation of nucleocapsids containing full-length viral DNA

(defective viruses/rolling circle/interference/DNA replication)

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ABSTRACT **Defective genomes present in serially passaged herpes simplex virus (HSV) stocks have been shown to consist of tandemly arranged repeat units containing limited sets of the standard virus DNA sequences. Invariably, the HSV defective genomes terminate with the right (S component) terminus of HSV DNA. Because the oligomeric forms can arise from a single repeat unit, it has been concluded that the defective genomes arise by a rolling circle mechanism of replication. We now report on our studies of defective genomes packaged in viral capsids accumulating in the nuclei and in mature virions (enveloped capsids) translocated into the cytoplasm of cells infected with serially passaged virus. These studies have revealed that, upon electrophoresis in agarose gels, the defective genomes prepared from cytoplasmic virions comigrated with nondefective standard virus DNA (M_r 100 × 10^6). In contrast, DNA prepared from capsids accumulating in nuclei consisted of both full-length defective virus DNA molecules and smaller DNA molecules of discrete sizes, ranging in M_r from 5.5 to 100 × 10^6. These smaller DNA species were shown to consist of different integral numbers (from 1 to approximately 18) of defective genome repeat units and to terminate with sequences corresponding to the right terminal sequences of HSV DNA. We conclude on the basis of these studies that (*i*) sequences from the right end of standard virus DNA contain a recognition signal for the cleavage and packaging of concatemeric viral DNA, (*ii*) the sequence-specific cleavage is either a prerequisite for or occurs during the entry of viral DNA into capsid structures, and (*iii*) DNA molecules significantly shorter than full-length standard viral DNA can become encapsidated within nuclear capsids provided they contain the cleavage/packaging signal. However, capsids containing DNA molecules significantly shorter than standard virus DNA are not translocated into the cytoplasm.**

The standard DNA genomes of herpes simplex viruses 1 and 2 (HSV-1 and HSV-2) are approximately 100 × 10^6 in M_r and are organized in two covalently linked components, L and S (1–3). The L component consists of unique sequences U_L (M_r, 67 × 10^6) bounded by the inverted repeats *ab* and *b'a'* (M_r, 5.8 × 10^6). The S component consists of unique sequences U_S (M_r, 9 × 10^6) bounded by the inverted repeats *ac* and *c'a'* (M_r, 4.1 × 10^6). The sequence *a* is present in one or few copies at the L and S termini and at the L–S junctions (3–8). The L and S components invert relative to each other, forming four equimolar isomers (3, 9–11).

In addition to the standard (helper) virus genomes, serially passaged virus stocks also contain variable proportions of defective virus genomes consisting of tandem reiterations of sequences of limited complexity (repeat units). All of the defective HSV genomes characterized to date contain, within their repeat units, DNA sequences derived from the end of the S component of standard virus DNA. However, they fall into two distinct classes on the basis of the additional sequences that they contain: class I defective genomes contain additional DNA sequences from S, whereas class II defective HSV genomes derive the majority of their repeat unit DNA sequences from U_L within map coordinates 0.35 to 0.42 (12–17).

We have recently shown that the transfection of cells with a mixture of standard helper virus DNA and monomeric repeat units (average M_r, 5.5 × 10^6) derived from class I defective genomes of HSV-1 (Justin) results in the regeneration of full-length (M_r, 100 × 10^6) defective genomes, consisting of head-to-tail reiterations of the seed repeat unit (18). These regenerated defective genomes, as well as the original defective virus DNA molecules, terminate at one end with the S-end sequences of standard virus DNA (14, 18).

On the basis of these results we proposed that the limited viral DNA sequences present in the class I defective Justin genomes (corresponding to sequences within map coordinates 0.94 to 1.00 of standard HSV DNA) contain an origin for viral DNA replication as well as a signal specifying the cleavage of viral DNA concatemers at the terminal *a* sequence. In this paper we report on further studies concerning the site-specific maturation-cleavage of defective HSV genomes.

MATERIALS AND METHODS

Fractionation of Cells and Analyses of Viral DNA. Nuclear and cytoplasmic fractions of infected Vero cells were separated by treatment with 0.5% Nonidet P-40 (Shell) in reticulocyte standard buffer (RSB; 0.01 M Tris·HCl, pH 7.4/1.5 mM $MgCl_2$/0.01 M NaCl). Cytoplasmic and total nuclear virus DNAs were prepared from cytoplasmic and nuclear fractions by proteinase K (250 μg/ml) digestion in RSB containing 0.5% $NaDodSO_4$ followed by CsCl equilibrium density centrifugation (8). The preparation of encapsidated nuclear viral DNA followed the procedure of Gibson and Roizman (19). Briefly, nuclei in RSB were lysed with 0.5% sodium deoxycholate and then treated with pancreatic DNase I (Worthington; 50 μg/ml) for 12 min at 37°C. The resultant extracts were then treated with $NaDodSO_4$/proteinase K followed by CsCl equilibrium density centrifugation as above. Alternatively, the cytoplasmic and nuclear fractions in RSB were sonicated and the resultant extracts were sedimented through 10–50% sucrose gradients in 0.01 M Tris·HCl, pH 7.5/0.15 M NaCl at 4°C for 70 min at 23,000 rpm in a Beckman SW 27 rotor (19). Aliquots of individual gradient fractions were treated with phenol/chloroform/2% isoamyl al-

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Abbreviations: HSV, herpes simplex virus; P3, passage 3; pfu, plaque-forming units.

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cohol and precipitated with ethanol prior to electrophoresis in 0.35% agarose gels. Alternatively, aliquots as above from selected gradient fractions were treated with DNase I (Worthington, 50 μg/ml) or RNase (Worthington, 50 μg/ml) prior to deproteinization, ethanol precipitation, and gel electrophoresis as above.

RESULTS

Size Distribution of Packaged Defective Virus DNA. We analyzed the size distribution of packaged standard and defective virus DNA in the cytoplasmic and nuclear fractions of the infected cells. The following points are pertinent to our experimental design. (*i*) Previous studies (20, 21) have shown that HSV nucleocapsids are assembled in the nucleus and acquire their envelope as they bud out through the inner lamellae of the nuclear membrane. Thus, viral DNA present in the cytoplasm of the infected cells is contained within enveloped virions. Gibson and Roizman (19) reported a procedure for the purification of both full and empty viral capsids from the nuclei of infected cells by lysing the nuclei with deoxycholate, digesting the nuclear extracts with DNase I in order to degrade cellular chromatin, and banding of the capsids by rate centrifugation in sucrose gradients. In the present study we used the deoxycholate lysis/DNase treatment steps in order to prepare encapsidated viral DNA that is protected from the nuclease attack.

(*ii*) As reviewed elsewhere (17), the class I defective HSV genomes are G+C rich. Thus, class I (ρ = 1.732 g/cm^3) defective genomes can be separated from helper virus DNA (ρ = 1.726 g/cm^3) by centrifugation in suitable equilibrium density gradients. In contrast, the class II defective genomes do not differ in their density from standard virus DNA.

The experiments reported below were initially done with passage 3 (P3) of the CO-2 virus series obtained (18) by transfection of cells with standard HSV-1 (F) helper DNA and monomeric repeat units of defective HSV-1 (Justin) genomes. The resultant progeny virus stocks contained the standard F helper virus DNA as well as regenerated full-length Justin defective genomes (18). In the present study, cells infected with CO-2 P3 were fractionated into the cytoplasmic and nuclear fractions. The cytoplasmic fraction and a portion of the nuclear fraction were digested with proteinase K in the presence of $NaDodSO_4$ and were centrifuged to equilibrium in CsCl density gradients in order to prepare the high-density (defective) or low-density (helper) total cytoplasmic and nuclear viral DNAs. The second portion of the nuclear fraction was used to prepare encapsidated DNA by deoxycholate lysis, DNase treatment, proteinase K digestion, and CsCl equilibrium density centrifugation. The

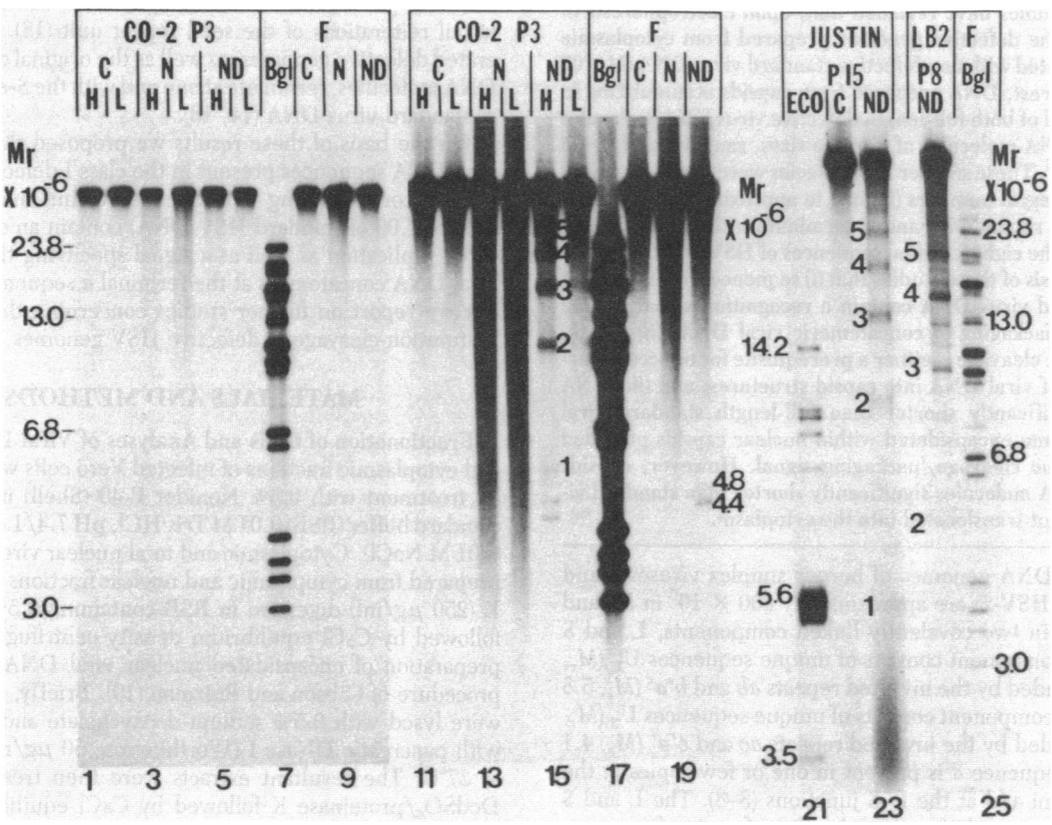

FIG. 1. Size distribution of nuclear and cytoplasmic viral DNA. Vero cells were infected [2 plaque-forming units (pfu)/cell] for 20 hr with plaque-purified HSV-1 strain F, P3 of the CO-2 virus series (18), P15 of the HSV-1 (Justin) series (16), and P8 of the tsLB2 series (16). The Nonidet P-40-separated cytoplasmic (C) and nuclear (N) fractions were deproteinized and centrifuged to equilibrium as described in the text, or the nuclei were disrupted with 0.5% deoxycholate and digested with DNase I (ND) prior to deproteinization and equilibrium density centrifugation. DNA of viral density was electrophoresed in 0.35% agarose gels. In the case of CO-2 P3 infections, the CsCl gradient fractions containing the heavy (H) defective virus DNA and the light (L) helper virus DNA were applied separately to the gel. In the case of the Justin P15 DNA the density fractions containing the heavy and light viral DNA were pooled. Lanes 11–20 are from a longer autoradiographic exposure of lanes 1–10. Lanes 7, 17, and 25 contain *Bgl* II-cleaved HSV-1 (F) DNA as size markers. Lane 21 contains *Eco*RI-cleaved Justin P15 DNA serving as size marker and showing the position of the monomeric (*Eco*RI-cleaved) repeat unit of the Justin defective genomes. Defective genomes in CO-2 P3 and Justin P15 are composed of head-to-tail reiterations of repeat units of M_r 5.4–5.6 × 10^6. The tsLB2 series contains class II defective genomes consisting of three major repeats, each deriving its sequences from U_L (map coordinates 0.34–0.40) and the end of S (16). In the tsLB2 profile (lane 24), the triple "ladder" band pattern corresponds to the three different-sized repeats in the population. Bands labeled 1–5 represent molecules consisting of 1–5 repeats, respectively.

size of each of the DNA preparations was determined by electrophoresis in 0.35% agarose gels.

The results of the analyses for the CO-2 P3-infected cells, as well as for HSV-1 (F) standard virus-infected cells, are shown in Fig. 1 and can be summarized as follows. (*i*) The cytoplasmic fractions of both infected cell cultures contained primarily standard genome length (M_r, $\approx 100 \times 10^6$) viral DNA molecules. (*ii*) The total nuclear viral DNA preparations from both the F and CO-2 P3 infections contained genome-length viral DNA molecules as well as smaller viral DNA molecules varying continuously in size. The smaller DNA molecules were represented in both the heavy and light viral DNA fractions. (*iii*) DNase digestion of the HSV-1 (F) nuclear extracts resulted in the removal of 40% of the total nuclear DNA and in significant reduction in the proportion of the viral DNA molecules migrating faster than standard virus DNA. (*iv*) The DNase treatment of the nuclear extracts of the CO-2 P3-infected cells resulted in degradation of approximately 70% of the total viral DNA and in similar reduction in the amount of the faster migrating DNA species. However, the DNase treatment of the CO-2 P3 nuclear extracts made apparent the presence of a series of DNase-protected viral DNA molecules ranging in M_r, in discrete steps, from 5.5×10^6 to 100×10^6. This series of discrete DNA bands was most prominent in the heavy viral DNA preparation. Furthermore, the DNA in adjacent bands differed in M_r by 5.5×10^6—i.e., by the size of repeat units of the predominant defective genomes contained in the CO-2 P3 virus pool. (*v*) Two minor bands, of M_r 4.38×10^6 and 4.80×10^6, were also apparent in the DNase-protected nuclear material of both the F and CO-2 P3 virus-infected cells. The identity of these bands is unclear.

We conclude on the basis of these data that the nuclei of the CO-2 P3-infected cells contained encapsidated viral DNA molecules of discrete sizes shorter than standard genome-length DNA and that capsids containing the shorter viral genomes are not translocated into the cytoplasm.

Similar analyses of the size distribution of DNase-protected nuclear DNA were done with one series (tsLB2) containing class II defective genomes (Fig. 1) and three additional series containing class I defective genomes, including series derived from HSV-1 strain Justin (Fig. 1), CO-1 (18), and HSV-2 strain G (data not shown). In each case the nuclear fractions contained a family of DNase-protected viral DNA molecules corresponding in size to integral multiples of the defective genomes repeat units present in the respective virus stocks. In several instances the smaller discrete DNA bands were detected in the total nuclear DNA preparations, although their presence was most clearly apparent after the elimination of heterogeneous background by the DNase treatment. On the other hand, cytoplasmic fractions from these same infected cells either did not contain the discrete-sized smaller viral DNA molecules or contained these in barely detectable amounts, most likely due to cross contamination between the nuclear and cytoplasmic fractions (e.g., lanes 22 and 23 of Fig. 1). In addition, control treatment of the Nonidet P-40 cytoplasmic lysate with DNase prior to deproteinization did not result in the generation of the smaller DNA molecules (data not shown).

Restriction Enzyme Analyses of Total and Protected Nuclear Viral DNA. In order to determine whether the nuclear family of discrete-sized small DNA molecules shown in lanes 15 and 23 of Fig. 1 represented defective genomes differing in the number of repeat units, we used restriction enzymes to analyze the DNA contained in the five fastest migrating bands as well as genome length DNA (M_r, $\approx 100 \times 10^6$) which were each extracted from a gel containing DNase-protected high-density viral DNA prepared from nuclei of cells infected with P15 of the HSV-1 (Justin) series. The results (Fig. 2) showed that the protected discrete-sized DNA molecules were indeed composed of defective genome repeat units. Furthermore, each of the various oligomeric forms of the defective genomes contained one unique terminus corresponding to the S-end segment of standard HSV DNA.

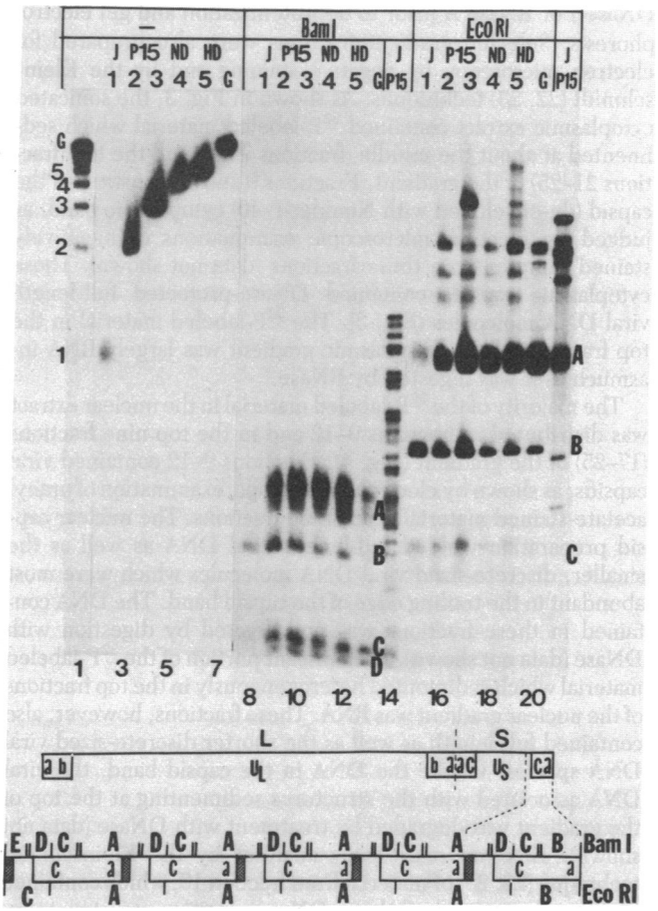

FIG. 2. Restriction enzyme analyses of the smaller DNase-protected nuclear viral DNA molecules. (*Upper*) DNA from the discrete low molecular weight DNA bands (1–5) as well as from the genome size DNA (M_r, 100×10^6) band (G) was eluted from the respective fractions of the gel shown in lane 1 and reelectrophoresed without treatment (lanes 2–7) or after *Bam* I (lanes 8–13) or *Eco*RI (lanes 15–20) digestion. Lanes 14 and 21 contain a preparation of Justin P15 DNA cleaved with *Bam* I and *Eco*RI, respectively. (*Lower*) Schematic representation of the *Bam* I and *Eco*RI cleavage sites within a pentameric defective genome. Note that (*i*) *Bam* I fragment B and *Eco*RI fragment B are generated from the *ac* terminus of each oligomeric molecule and *Bam* I fragment E and *Eco*RI fragment C are generated from the other (U_S) terminus of each oligomeric molecule, whereas the *Bam* I fragments A, C, and D and *Eco*RI fragment A arise from internal positions of the oligomers (14). The relative ratios between the terminal and internal fragments vary in accordance with the number of repeat units in the oligomeric species which are digested with the restriction enzymes. (*ii*) As reported (14), the Justin defective genomes contain several types of repeat units ranging in M_r from 5.3×10^6 to 5.6×10^6 and containing variable amounts of the U_S sequences located within the *Bam* I fragment A (dark area). *Bam* I fragment A and *Eco*RI fragment A therefore are heterogeneous in size. *Eco*RI fragment C, constituting the U_S terminus of the oligomeric defective genomes, is similarly heterogeneous in size due to the presence of the variable-sized repeat units in the population. The corresponding (U_S terminus) *Bam* I fragment E is expected to be M_r 0.7×10^6 and therefore to run off the gel. (*iii*) In the experiment shown here, the *Eco*RI pattern also contains partial digestion products.

Centrifugation Analyses of Nuclear and Cytoplasmic Extracts. In order to determine directly whether the protected small oligomers were present within viral capsids, Nonidet P-40-separated cytoplasmic and nuclear fractions of cells infected with Justin P17 were sonicated and then centrifuged in 10–50% sucrose gradients. Aliquots from individual gradient fractions were deproteinized, precipitated with ethanol, and then subjected to electrophoresis in agarose gels or were treated with DNase I or RNase A prior to deproteinization and gel electrophoresis. Selected gradient fractions were also prepared for electron microscopy by negative staining and by the Kleinschmidt (22, 23) techniques. As shown in Fig. 3, the sonicated cytoplasmic extract contained ^{32}P-labeled material which sedimented at about the middle (fractions 9–10) and the top (fractions 21–25) of the gradient. Fractions 9 and 10 constituted the capsid (de-enveloped with Nonidet P-40) cytoplasmic band, as judged by electron microscopic examinations of negatively stained samples from these fractions (data not shown). These cytoplasmic capsids contained DNase-protected full-length viral DNA molecules (Fig. 3). The ^{32}P-labeled material in the top fractions of the cytoplasmic gradient was largely RNA inasmuch as it was digested by RNase.

The majority of the ^{32}P-labeled material in the nuclear extract was distributed in fractions 9–12 and in the top nine fractions (17–25) of the gradient (Fig. 4). Fractions 9–12 contained viral capsids, as shown by electron microscopic examination of uranyl acetate-stained material from these fractions. The nuclear capsid preparation yielded full-length viral DNA as well as the smaller, discrete-sized viral DNA molecules which were most abundant in the trailing edge of the capsid band. The DNA contained in these fractions was not affected by digestion with DNase (data not shown). A significant portion of the ^{32}P-labeled material which sedimented heterogeneously in the top fractions of the nuclear gradient was RNA. These fractions, however, also contained full-length as well as the shorter discrete-sized viral DNA species. Unlike the DNA in the capsid band, the viral DNA associated with the structures sedimenting at the top of the gradient was degraded by treatment with DNase (data not shown). Electron microscopy analyses by the Kleinschmidt technique (22, 23) of material from fraction 19, which contained high concentrations of these DNase-sensitive structures, revealed the presence of beaded nucleosome-like entities which most likely represented sonicated cellular chromatin (Fig. 4). Because a deproteinized HSV DNA marker which was included in a separate gradient in the same centrifugation experiment banded in fraction 24 (data not shown), it appears from these results that the nuclei of infected cells contained approximately full-length viral DNA as well as the small, discrete-sized viral DNA molecules in complexes that were sensitive to DNase and that cosedimented with cellular chromatin. These structures were absent from the cytoplasmic extracts of the same infected cells.

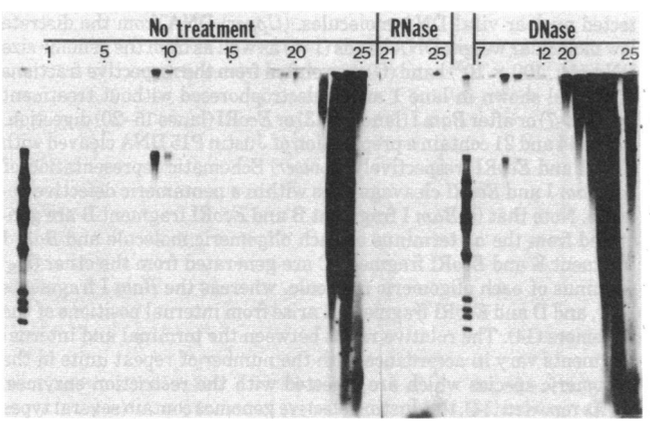

FIG. 3. Velocity gradient sedimentation of sonicated cytoplasmic extracts. P17 Justin-infected cells (2 pfu/cell) were labeled with [^{32}P]orthophosphate from 3.5 to 10 hr after infection. Following separation of the cytoplasmic and nuclear fractions by Nonidet P-40 treatment, the cytoplasmic fraction was sonicated (30 sec) and centrifuged in a 10–50% sucrose gradient. Equal aliquots of the gradient fractions shown were either deproteinized, ethanol precipitated, and electrophoresed in the gel (no treatment) or were first treated with RNase or DNase, as described in the text. Fraction 1, bottom; fraction 25, top.

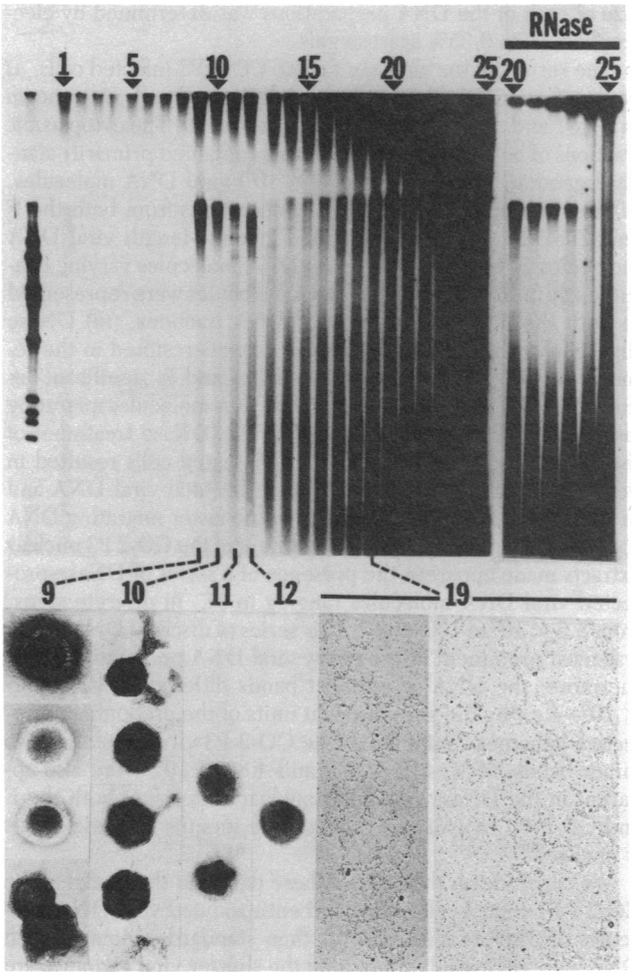

FIG. 4. Velocity gradient sedimentation of sonicated nuclear extracts. (*Upper*) The Nonidet P-40-separated nuclear fraction was sonicated (60 sec) and then sedimented in 10–50% sucrose gradients. Aliquots of each gradient fraction were deproteinized, concentrated by ethanol precipitation, and electrophoresed in an agarose gel along with a *Bgl* II-digested tsLB2 P17 DNA size marker (leftmost lane), which includes the *Bgl* II-resistant defective genomes. The lanes under the bar show the result of treating aliquots from gradient fractions 20–25 with RNase A prior to deproteinization and electrophoresis. (*Lower*) Capsid structures and nucleosomal-like beaded structures observed in negatively stained material from fractions 9–12 and from material spread by the Kleinschmidt technique from fraction 19.

DISCUSSION

Previous studies from this and other laboratories have supported the hypothesis that the replication of defective virus genomes occurs by a rolling circle mechanism (14, 17, 18, 24). The studies reported here have several implications concerning

the cleavage and packaging of the resultant concatemeric defective virus DNA molecules. The first conclusion of these studies is that the *ac* sequences present in repeat units located at any interval along the defective genome concatemers can be specifically recognized for cleavage/packaging. This cleavage/packaging yields the family of encapsidated defective virus genomes composed of different numbers of repeat units and invariably terminating at one end with the *ac* end sequences of standard virus DNA. Because the junctions between adjacent repeat units in the various types of defective genomes that we have studied (16) share only the terminal portions of the *ac* sequences (maximal map coordinates 0.98–1.00 of standard HSV DNA), it is apparent that these terminal *ac* sequences contain a sufficient signal for the cleavage/packaging of viral DNA concatemers. The documentation of this specific cleavage during the maturation of defective HSV genomes provides further evidence for the operation of such specific cleavage during the maturation of concatemeric intermediates of standard HSV DNA, as previously suggested (3, 25). Furthermore, because all the defective HSV genomes analyzed to date contain the *ac* (S end) rather than the *ab* terminus (L end) (12–17), it seems plausible to suggest that the *ab* sequences do not contain packaging or cleavage signals. Thus it is possible that the *ab* sequences constitute the other terminus of the standard virus genomes because they are located adjacent to the *ac* sequences (which become cleaved) in the S–L junctions of the standard virus DNA concatemers.

The second conclusion from the studies reported here is that only properly terminated (i.e., *ac* terminated) defective virus genomes are found in DNase-protected capsids. This finding strongly suggests that the specific cleavage of viral DNA concatemers at *ac* sequences is either a prerequisite to or occurs during the entry of progeny virus DNA into viral capsids. The nature of the infected-cell gene products that are involved in this specific maturation of viral DNA is unknown. Ladin *et al.* (26) have recently shown that the cleavage of pseudorabies virus DNA concatemers appears to depend on the expression of multiple virus cistrons and concluded that the cleavage is coupled to virion morphogenesis. Our finding of the slow-sedimenting, DNase-sensitive, nuclear structures which contain genome length as well as the shorter discrete-sized defective virus DNA molecules may be relevant to this question. However, further studies are required in order to determine whether these structures are indeed intermediates in the process of encapsidation or whether they represent breakdown products of viral nucleocapsids that released their nucleoprotein structures during the sonic treatment of the infected cell extracts.

A third conclusion from our data is the existence of a novel type of virion maturation control which allows the complete maturation (envelopment and translocation into the cytoplasm) only of those viral capsids that contain full-size (M_r, $\approx 100 \times 10^6$) DNA molecules. This observation extends previous data of Roizman and coworkers (21) showing that empty capsids that accumulate in the nuclei of infected cells are not transported into the cytoplasm. Although the mechanism underlying the selective transport of capsids containing full-size viral DNA is unknown, the phenomenon provides an explanation for the generalized observation that defective HSV genomes that are present in mature cytoplasmic virions are indistinguishable in size from standard virus DNA (reviewed in ref. 17).

Finally, the results of our studies might be of relevance to the previous observations that infections of cells with certain virus stocks containing defective HSV genomes, as well as virus stocks containing defective genomes of other herpesviruses, result in significant reduction in virus particle production, in reduced and delayed cleavage of helper virus DNA concatemers, and, in some cases, in increased production of incomplete forms of virus capsids (27–31). Thus, the defective virus genomes containing multiple copies of packaging and cleavage signals could efficiently compete with helper virus DNA molecules on limiting cleavage machinery or on limiting capsid constituents produced in the serially passaged virus-infected cells. If so, the obligatory presence within defective virus genomes of the *ac* recognition sites specifying cleavage/packaging of virus DNA could constitute a specific pathway of defective genome interference with homologous standard virus replication.

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