JOURNAL OF VIROLOGY, Mar. 1980, p. 1151-1164
0022-538X/80/03-1151/14$02.00/0

Vol. 33, No. 3

Replication of Herpesvirus DNA

V. Maturation of Concatemeric DNA of Pseudorabies Virus to Genome Length Is Related to Capsid Formation

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The maturation of pseudorabies virus DNA from the replicative concatemeric form to molecules of genome length was examined using nine DNA$^+$ temperature-sensitive mutants of pseudorabies virus, each belonging to a different complementation group. At the nonpermissive temperature, cells infected with each of the mutants synthesized concatemeric DNA. Cleavage of the concatemeric DNA to genome-length viral DNA was defective in all the DNA$^+$ *ts* mutants tested, indicating that several viral gene products are involved in the DNA maturation process. In none of the *ts* mutant-infected cells were capsids with electron-dense cores (containing DNA) formed. Empty capsids with electron-translucent cores were, however, formed in cells infected with six of the nine temperature-sensitive mutants; in cells infected with three of the mutants, no capsid assembly occurred. Because these three mutants are deficient both in maturation of DNA and in the assembly of viral capsids, we conclude that maturation of viral DNA is dependent upon the assembly of capsids. In cells infected with two of the mutants (*ts*N and *ts*IE13), normal maturation of viral DNA occurred after shiftdown of the cells to the permissive temperature in the presence of cycloheximide, indicating that the temperature-sensitive proteins involved in DNA maturation became functional after shiftdown. Furthermore, because cycloheximide reduces maturation of DNA in wild-type-infected cells but not in cells infected with these two mutants, we conclude that a protein(s) necessary for the maturation of concatemeric DNA, which is present in limiting amounts during the normal course of infection, accumulated in the mutant-infected cells at the nonpermissive temperature. Concomitant with cleavage of concatemeric DNA, full capsids with electron-dense cores appeared after shiftdown of *ts*N-infected cells to the permissive temperature, indicating that there may be a correlation between maturation of DNA and formation of full capsids. The number of empty and full capsids (containing electron-dense cores) present in *ts*N-infected cells incubated at the nonpermissive temperature, as well as after shiftdown to the permissive temperature in the presence of cycloheximide, was determined by electron microscopy and by sedimentation analysis in sucrose gradients. After shiftdown to the permissive temperature in the presence of cycloheximide, the number of empty capsids present in *ts*N-infected cells decreased with a concomitant accumulation of full capsids, indicating that empty capsids are precursors to full capsids.

The replication of the DNA of pseudorabies (Pr) virus (a herpesvirus) can be divided into two phases, as follows. During the first round of replication, most of the DNA is in the form of circular molecules, as well as small concatemers (6a, 10), and newly synthesized viral DNA sediments with an S value up to approximately twice that of unit-size viral DNA (6). During the later phase of replication, newly synthesized viral DNA is associated with structures of high sedimentation value (3, 6). These large concatemeric structures are composed of linear, tandem arrays of Pr viral DNA molecules in head-to-tail alignment (4). "Maturation" of viral DNA to unit length does not require DNA synthesis but does require concomitant protein synthesis, indicating that the proteins necessary for viral DNA maturation either are structural proteins present in limiting amounts or are unstable enzymes (3).

The experiments presented in this paper were designed to elucidate the mechanism by which viral DNA matures from the concatemeric forms into unit-length genomes. Several facts concerning the structure of Pr DNA are relevant to these studies, as follows. (i) The genome of Pr virus consists of a linear, noncircularly permuted molecule with a molecular weight of approximately 90×10^6 to 92×10^6 (20, 21). (ii) Upon

entering the host cell, the ends of parental Pr viral DNA are digested by an exonuclease, and circular, as well as concatemeric, forms of viral DNA may be observed (6a, 10; T. Ben-Porat, B. Ladin, and R. A. Veach, *in* D. Schlessinger, ed., *Microbiology—1980*, in press). (iii) DNA replicates semiconservatively (13), and newly synthesized DNA accumulates in intracellular pools, from which it is withdrawn at random to be encapsidated into mature virions (2).

Using several DNA^+ temperature-sensitive (*ts*) mutants of Pr virus, we show here that the maturation of viral DNA is a complex process involving multiple viral gene products and that DNA maturation is dependent on capsid formation. Furthermore, we show that empty capsids are precursors to full capsids.

(A preliminary report of this work was presented at the Fourth Cold Spring Harbor Meeting on Herpesviruses, 28 August–2 September 1979.)

MATERIALS AND METHODS

Viruses and cell culture. The properties of Pr virus and the cultivation of rabbit kidney (RK) cultures have been previously described (11). Temperature-sensitive mutants of Pr virus were isolated by mutagenesis with bromodeoxyuridine, nitrosoguanidine, or UV irradiation (manuscript in preparation).

Media and solutions. EDS: Eagle synthetic medium (8) plus 3% dialyzed bovine serum. ELS + FU: Earle saline + 0.5% lactalbumin hydrolysate and 5% bovine serum, plus 5-fluorouracil (FU) (20 or 50 μg/ml) and thymidine (1 μg/ml). Thymidine medium: EDS containing thymidine (100 μg/ml) and deoxycytidine (25 μg/ml). TBSA: Tris-buffered saline, containing 1% crystalline serum albumin (1). Pronase solution: 0.2 M NaCl, 0.02 M Tris (pH 7.3), and self-digested (nuclease-free) pronase (5 mg/ml). SSC + 2% Sarkosyl: 0.15 M NaCl, 0.015 M sodium citrate (pH 7.4), plus 2% sodium lauryl sarkosinate-97. EDS $\bar{s}$ PO_4: Same as EDS but without PO_4. EDS $\bar{s}$ PO_4 + FU: EDS $\bar{s}$ PO_4 plus FU (20 μg/ml) and thymidine (5 μg/ml). EDS $\bar{s}$ amino acids: EDS without amino acids, plus 3% dialyzed calf serum, as well as 2 mM glutamine and 0.6 mM arginine.

Chemicals and radiochemicals. FU was purchased from Calbiochem. Restriction enzyme *Kpn*I was obtained from New England Biolabs, Beverly, Mass. [*methyl*-^{3}H]thymidine (62 Ci/mmol), [^{14}C]thymidine (57 mCi/mmol), [^{3}H]leucine (65 Ci/mmol), and [^{14}C]leucine (312 mCi/mmol) were purchased from Schwarz/Mann; inorganic ^{32}P (carrier free) was purchased from ICN.

Sedimentation of DNA in neutral sucrose gradients. This DNA sedimentation technique was as described previously (3), except that monolayers were scraped directly in SSC + 2% Sarkosyl (instead of reticulocyte standard buffer + sodium dodecyl sulfate) at a concentration of 4×10^5 cells per ml.

Sedimentation of intranuclear particles in sucrose density gradients. Intranuclear particle sedimentation was performed as described previously (5).

Restriction endonuclease digestion and electrophoresis in agarose gels. ^{32}P-labeled wild-type (WT) or *ts*N viral DNA was digested with restriction endonuclease *Kpn*I. The DNA fragments were separated by electrophoresis in a 0.7% agarose slab gel (14 by 18 by 0.3 cm) at 35 mA for approximately 16 h, dried, and exposed for autoradiography, as described previously (4).

Purification of DNA fragments. Specific ^{32}P-labeled restriction fragments generated by cleavage of either WT or *ts*N viral DNA with *Kpn*I restriction endonuclease were separated on agarose gels as described above. The DNA was excised from the gels and extracted from the agarose by the method of Tapper and DePamphilis (25).

Electron microscopy. The medium was removed from the infected RK cell cultures and replaced with cold 2.5% glutaraldehyde in sodium cacodylate buffer (0.1 M sodium cacodylate buffer containing 3 mM $CaCl_2$) (pH 7.4). The cultures were left standing at room temperature for 15 min. The cells were then carefully scraped into buffered glutaraldehyde solution (see below) and centrifuged at low speed (1,000 × *g* for 5 min). The cells were resuspended in the same solution and left for 1 h at 4°C. The fixed cells were washed three times in sodium cacodylate buffer and postfixed in 1% OsO_4 in sodium cacodylate buffer for 1 h at room temperature. The cells were dehydrated in a series of ethanol solutions and embedded in Spurr's low-viscosity epoxy resin (24).

Thin sections were cut on an LKB ultratome III using a diamond knife. Sections were poststained with uranyl acetate and lead citrate (26) and observed in a Philips 301 electron microscope at 60 kV.

RESULTS

Capsid formation and maturation of viral DNA at the nonpermissive temperature in cells infected with DNA^+ *ts* mutants. Forty *ts* mutants of Pr virus were isolated after mutagenesis of virus stocks by treatment with either nitrosoguanidine, bromodeoxyuridine, or UV irradiation. Of these 40 mutants, 9 were DNA^+; i.e., cells infected with these mutants at the nonpermissive temperature synthesized at least 50% as much viral DNA as did WT virus-infected cells. These nine DNA^+ mutants fall into nine different complementation groups (manuscript in preparation). Since all these mutants are DNA^+, as expected, all synthesize late viral proteins, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, although in some cases some of the viral proteins are unstable at the nonpermissive temperature (manuscript in preparation). We have used these mutants to study the process of viral DNA maturation from its replicative concatemeric form into genome-length mature viral DNA.

Figure 1 illustrates the results of a representative experiment performed to determine whether viral DNA maturation occurs in the *ts* mutant virus-infected cells. As previously de-

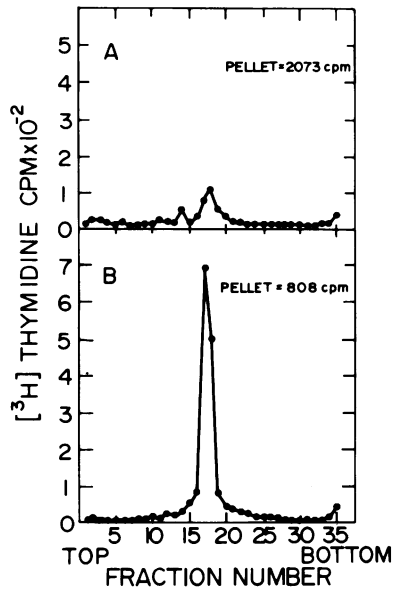

FIG. 1. *Sedimentation in neutral sucrose gradients of DNA synthesized in WT-infected cells during a short labeling period and after a chase. Cells that had been preincubated for 16 h in ELS + FU to inhibit cellular DNA synthesis (12) were infected with WT virus (MOI, 20 PFU/cell) and incubated at 41°C. Between 4 and 5 h postinfection, the cells were labeled with [3H]thymidine (10 μCi/ml). Part of the cultures was harvested immediately thereafter (A), and part was further incubated in thymidine medium for 3 h at 41°C (B). The cells were scraped into SSC + 2% Sarkosyl, heated to 60°C for 15 min, and treated with nuclease-free pronase for 2 h at 37°C. A sample was sedimented in neutral sucrose gradients (5 to 20%) at 12,000 rpm for 16 h in a Beckman SW27 rotor.*

scribed (3), after a 1-h incubation with [3H]thymidine, the labeled viral DNA synthesized by cells infected with WT virus (Fig. 1A) was associated with rapidly sedimenting structures which, under the centrifugation conditions used, sedimented to the bottom of the tube. After a 3-h chase period (Fig. 1B), however, 62% of the labeled DNA sedimented in the position characteristic of mature viral DNA (54S). On the other hand, when the same experiment was performed with cells infected with all nine DNA+ *ts* mutants, the DNA remained associated with the rapidly sedimenting structures even after the chase period; i.e., none of the DNA matured (data not shown).

Quantitative analyses of data obtained in similar experiments are presented in Table 1. In these experiments, the cells were supplied with [3H]thymidine for 4 h during the peak of the viral DNA synthesis, and the amount of radioactivity incorporated both into the viral DNA and into mature (54S) viral DNA was determined. The following points emerged from this experiment. (i) The amount of total viral DNA synthesized by *ts* mutant virus-infected cultures (as measured by the incorporation of thymidine into viral DNA) varied somewhat (between 50 and 143% of the WT value). That [3H]thymidine was incorporated into viral DNA only and not into cellular DNA was shown by the characteristic buoyant density in CsCl of the DNA synthesized (data not shown). (ii) Significant amounts of mature viral DNA were not formed in any of the *ts* mutant virus-infected cells incubated at the nonpermissive temperature. Since each of the mutants tested falls into a different complementation group (i.e., each is probably defective in a different gene function), and since all these functions appear essential to the process of maturation of viral DNA, we conclude that maturation of concatemeric DNA to unit-size genomes is a complex process involving several gene products. It seemed likely, therefore, that maturation of Pr viral DNA might be dependent upon the assembly of a supermolecular structure, such as the viral capsid, as it is in several bacteriophage systems (14). We examined therefore the assembly of capsids in the *ts* mutant virus-infected cells at the nonpermissive temperature.

Assembly of capsids in cells infected with DNA+ *ts* mutants at the nonpermissive tem-

TABLE 1. *Viral DNA synthesis and maturation in WT- and ts mutant-infected cells at the nonpermissive temperature (41°C)*[a]

Virus	[3H]thymidine incorporated[b]	Total radioactivity in viral DNA (% of wt)	Radioactivity in mature viral DNA[c] (% of total)
WT	12.68	100	59
*ts*N	8.84	70	6
*ts*J	10.14	80	2
*ts*1	18.12	143	4
*ts*101	10.76	85	8
*ts*106	9.76	77	3
*ts*109	11.79	93	1
*ts*IE12	6.28	50	5
*ts*IE13	6.32	50	7
*ts*UH3	10.00	71	2

[a] RK cells were incubated in ELS + FU for 16 h before infection to inhibit cellular DNA synthesis (12). The cultures were infected with the appropriate virus stock at 41°C (multiplicity of infection [MOI], 20 PFU/cell) and were incubated with [3H]thymidine between 4 and 8 h postinfection. The cells were then scraped in SSC + 2% Sarkosyl. Part of the sample was taken to determine the amount of [3H]thymidine incorporated into DNA, and part was sedimented in a neutral sucrose gradient, as described in the legend to Fig. 1.

[b] Counts per minute × 10^4 incorporated into viral DNA per 10^6 cells.

[c] Percentage of DNA sedimenting as 54S molecules.

perature. Thin sections of RK cells infected at the nonpermissive temperature with either WT virus or one of the *ts* mutants were examined by electron microscopy, as described in Materials and Methods.

Figures 2 and 3 show electron micrographs of the nuclei of infected, as well as of uninfected, cells. As expected, the following two predominant types of particles were observed in the nuclei of cells infected with WT virus (Fig. 2A): (i) particles with electron-translucent cores, which have been shown to be devoid of DNA; (ii) particles with electron-dense cores, which contain DNA (17). Capsids with electron-dense cores did not appear in any of the mutant-infected cells. In some of the mutant-infected cells, however, capsids with electron-translucent cores were observed, for example, as in *ts*106-infected cells (Fig. 2B). In cells infected with some of the other mutants, for example, *ts*J (Fig. 3B), viral capsids were not detectable, although virus-induced changes such as margination of the chromatin were observed (compare Fig. 3A and B). Thus, although cells infected with all DNA$^+$ *ts* mutants synthesized all viral proteins, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (manuscript in preparation), capsids were not assembled in some of the mutant-infected cells. A similar finding has been described previously for cells infected with some *ts* mutants of herpes simplex virus (7, 22).

The number of capsids observed in the nuclei of cells infected with each of the *ts* mutants is summarized in Table 2. As mentioned above, capsids with electron-dense cores were not observed in the nuclei of cells infected with any of the DNA$^+$ mutants. This finding is not unexpected, because maturation of viral DNA, presumably a prerequisite for full capsid formation, does not occur in these cells (see Table 1). In six of the nine DNA$^+$ *ts* mutant-infected cells, capsids with a morphology similar to that of the capsids illustrated in Fig. 2B were seen. Thus, despite the fact that all six of these mutants fall into different complementation groups, no differences in the morphology of these structures could be distinguished. Whether there are biochemical differences between these capsids is under current investigation.

In cells infected with three of the mutants, however, no capsids were detectable. Since these three mutants (which, according to our tests, appear to be single mutants [manuscript in preparation]) are defective in both DNA maturation and capsid assembly, we conclude that capsid formation is necessary for viral DNA maturation.

Analysis by sedimentation in sucrose gradients of the particles present in the cells infected with the mutants confirmed the results obtained by electron microscopy. We have previously reported (5) that three types of viral particles can be isolated from RK cells infected with Pr virus: (i) fully enveloped particles; (ii) nucleocapsids containing DNA; (iii) empty capsids. The sedimentation behavior of these three types of particles is illustrated in Fig. 4A.

When extracts of cells infected with the *ts* mutants were similarly sedimented in sucrose gradients, empty capsids could be isolated from the cells in which these capsids had been observed in the electron microscope (Fig. 4B). Viral particles could not be isolated from mutant-infected cells in which capsids had not been observed by electron microscopy. The results of the sedimentation analysis of viral particles present in the *ts* mutant-infected cells thus confirmed those obtained by electron microscopy (see Table 2).

Assembly of full viral particles after temperature shiftdown of mutant-infected cells in the absence of protein synthesis. The following experiments were designed to determine whether in some mutant-infected cells the *ts* proteins that are necessary for viral maturation become functional after shiftdown to the permissive temperature. This was of particular interest because the availability of such mutants could provide a means of showing unequivocally that there is a precursor-product relationship between empty and full capsids in herpesvirus-infected cells, a question that has not been completely resolved to date.

As a first step, we determined whether unit-size, mature DNA would be cleaved from concatemeric DNA after shiftdown of the mutant-infected cells from the nonpermissive to the permissive temperature in the absence of de novo protein synthesis (Table 3).

As expected, after a 1-h labeling period, a maximum of 7% of the labeled DNA isolated from both the WT- and the mutant-infected cells sedimented as mature genomes. After a 3-h chase in the presence of cycloheximide, only 21% of the WT DNA had matured, confirming previous results which showed that the maturation of DNA in Pr virus-infected cells is considerably reduced in the absence of concomitant protein synthesis (3). Under the same conditions, however, a much larger proportion of the DNA in *ts*N- and *ts*IE13-infected cells (58 and 49%, respectively) sedimented as 54S DNA.

To determine whether the DNA that sediments as 54S molecules in *ts*N-infected cells represents mature DNA that had been cut normally from concatemeric DNA, we determined whether the DNA had acquired the unique ends present on mature viral DNA. We have shown

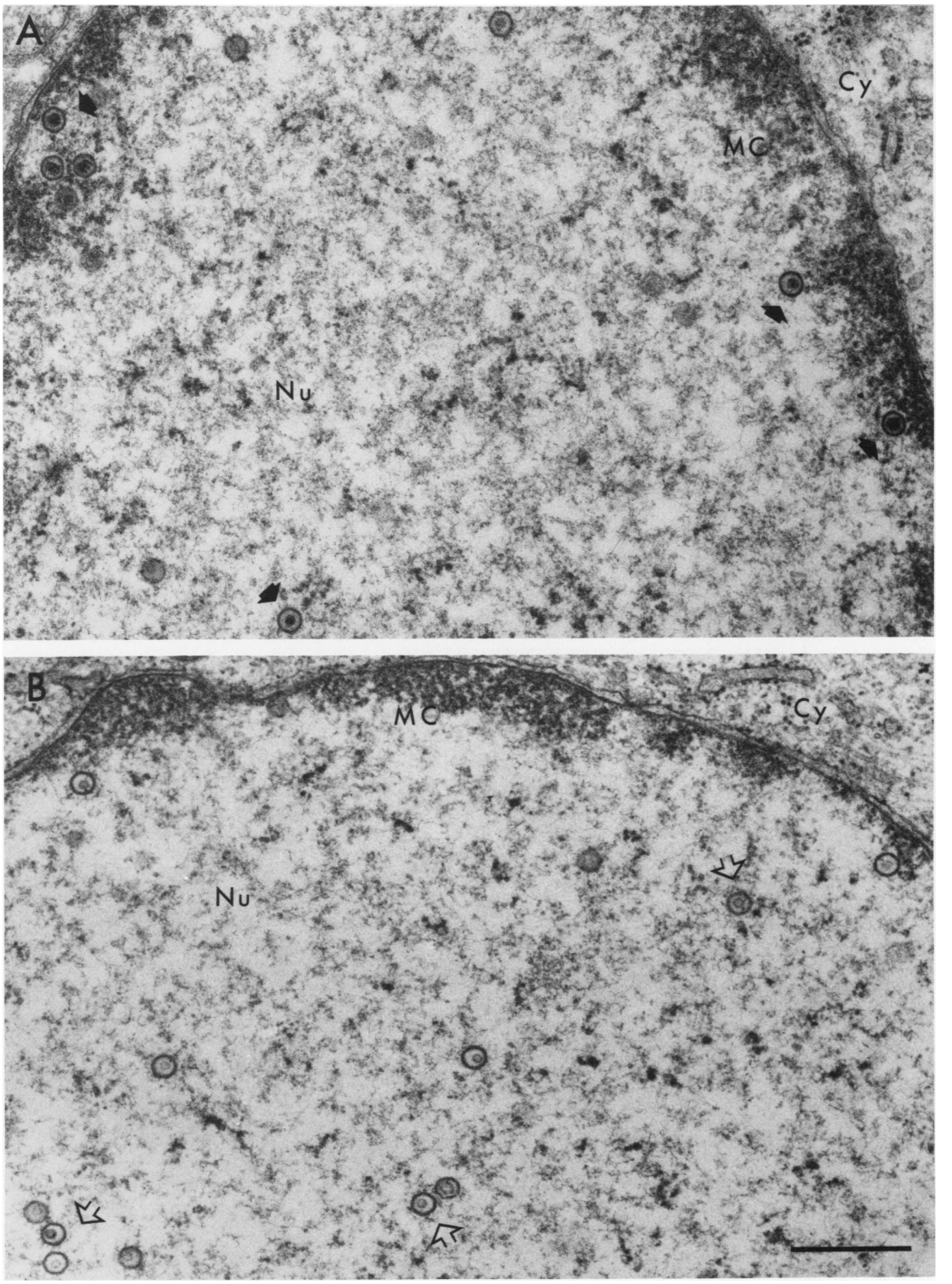

FIG. 2. *Electron micrographs of thin sections of WT and ts106-infected cell nuclei. RK cells were infected either with WT (A) or ts106 (B) and incubated at the nonpermissive temperature (41°C) for up to 6 h postinfection. The infected cells were harvested, and thin sections were prepared for electron microscopy as described in the text. Cy, Cytoplasm; MC, marginated chromatin; Nu, nucleus; open arrow, viral particles containing electron-translucent cores; short closed arrow, viral particles containing electron-dense cores. Bar, 0.5 μm.*

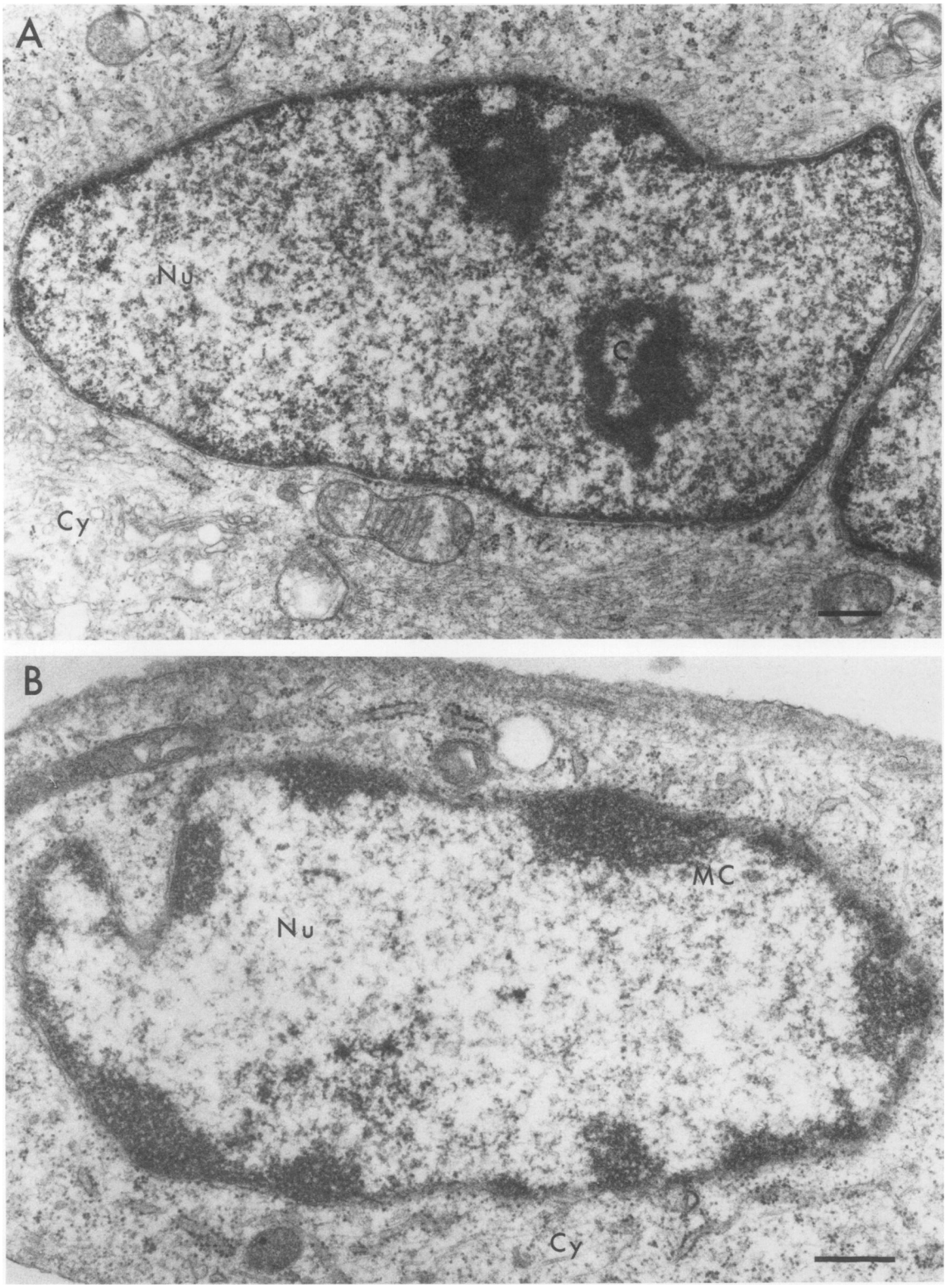

FIG. 3. *Electron micrographs of thin sections of uninfected and tsJ-infected cell nuclei. Uninfected RK cells (A) or RK cells infected with tsJ (B) were incubated at 41°C for up to 6 h postinfection. The cultures were harvested, and thin sections were prepared for electron microscopy as described in the text. C, Chromatin; Cy, cytoplasm; MC, marginated chromatin; Nu, nucleus. Bar, 0.5 μm.*

TABLE 2. *Assembly of capsids in cells infected with DNA⁺ ts mutants at the nonpermissive temperature*[a]

Virus	No. of capsids per nucleus		Capsid assembly[b]	
	Electron-translucent cores	Electron-dense cores	Empty	Full
WT	9	9	+	+
*ts*N	40	0	+	−
*ts*J	0	0	−	−
*ts*1	0	0	−	−
*ts*101	31	0	+	−
*ts*106	20	0	+	−
*ts*109	0	0	−	−
*ts*IE12	36	0	+	−
*ts*IE13	43	0	+	−
*ts*UH3	34	0	+	−

[a] RK cells were infected (MOI, approximately 20 PFU/cell) either with WT or with one of the *ts* mutants and incubated at 41°C. The infected cells were collected at 6 h postinfection and prepared for electron microscopy, as described in the text. Capsids with electron-translucent cores are defined as those capsids containing a core with an electron-translucent center (as in Fig. 2B), as opposed to capsids in which the entire core is electron dense (as in Fig. 2A). The number of capsids in 20 nuclei was determined; the results are expressed as the average number of capsids per nucleus. The range of the number of capsids per nucleus was 11 to 98 in the *ts* mutant-infected nuclei and 7 to 37 in WT-infected nuclei.

[b] Analysis of capsid assembly by velocity centrifugation in sucrose gradients was performed as described in the legend to Fig. 4B.

previously (4) that in restriction enzyme digests of newly synthesized Pr viral DNA, the fragments comprising the unique ends of mature DNA are underrepresented and that, instead, a new fragment consisting of the two end fragments joined together appears; i.e., that the DNA is in concatemeric structures consisting of tandem arrays of the viral genome. After maturation, the DNA acquires its unique ends, and the fragments consisting of the joined end fragments disappear. That this is also the case for *ts*N-infected cells after shiftdown in the presence of an inhibitor of protein synthesis is shown by the experiment illustrated in Fig. 5.

The *Kpn*I fragments H and D (which are end fragments) were missing from viral DNA that accumulated at the nonpermissive temperature in *ts*N-infected cells. *Kpn*I band B, on the other hand, was overrepresented. Figure 6 (track A) shows that DNA in band B excised from digests of concatemeric DNA hybridized to bands B, D, E, and H. (Fragments E and H both originate from the inverted repeat, and therefore sequences complementary to end fragment H are also complementary to E.) DNA in band B excised from digests of mature DNA, on the other hand, hybridized only to band B (track B). Thus, band B in concatemeric DNA contains both the B fragment normally present in mature virion DNA and the joined D and H end fragments. In digests of viral DNA of *ts*N-infected cells obtained after shiftdown in the presence of

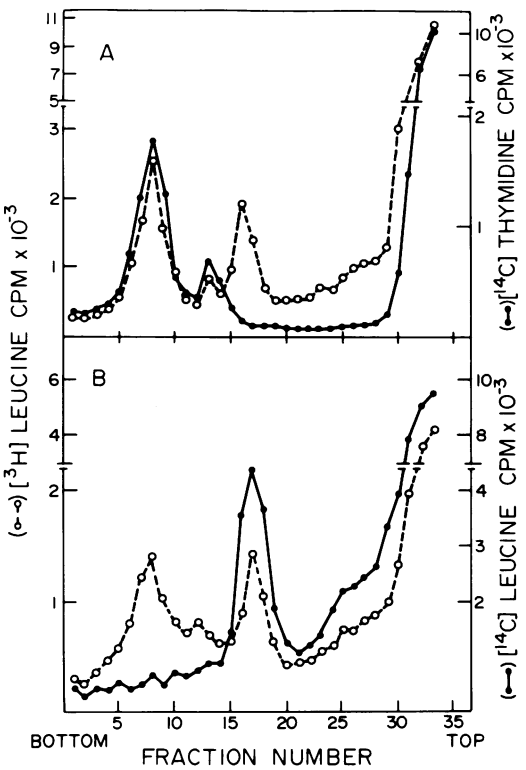

FIG. 4. *Isolation of viral particles from WT- and tsN-infected cells. (A) RK cells were infected with WT virus, incubated at 37°C, and labeled with [³H]leucine (30 μCi/ml) and [¹⁴C]thymidine (0.5 μCi/ml) from 4 to 8 h postinfection. The infected cells were harvested in the culture fluid, the cells were disrupted by freezing and thawing, the cell debris was removed by low-speed centrifugation (1,000 × g, 5 min), and the viral particles were isolated as described previously (5). Briefly, viral particles were collected by centrifugation (60 min, 13,500 rpm) on a sucrose cushion (30%). The pellet containing the viral particles was suspended in TBSA, layered onto a 15 to 30 TBSA sucrose gradient, and centrifuged for 70 min at 15,000 rpm in a Beckman SW27 rotor. Fractions (1 ml) were collected from the bottom of the gradient, and a portion of each fraction was acid precipitated. (B) RK cells were infected with either WT or tsN virus and incubated at 41°C. Between 4 and 8 h postinfection, WT-infected cells were labeled with [³H]leucine (30 μCi/ml), and tsN-infected cells were labeled with [¹⁴C]leucine (0.5 μCi/ml). The WT- and tsN-infected cells were harvested and mixed, and the viral particles were sedimented in sucrose gradients, as described for (A).*

TABLE 3. *Maturation of viral DNA synthesized at the nonpermissive temperature by cells infected with ts mutants after shiftdown to the permissive temperature in the presence of cycloheximide*[a]

Virus	Radioactivity in mature DNA (% of total)	
	Before shiftdown	After shiftdown
WT	6.0	21.0
*ts*N	6.0	58.0
*ts*J	3.0	6.0
*ts*1	3.1	3.3
*ts*101	7.0	7.3
*ts*106	3.0	1.5
*ts*109	1.0	1.3
*ts*IE12	5.0	3.7
*ts*IE13	3.0	48.8
*ts*UH3	2.0	3.2

[a] RK cells, which had been pretreated with ELS + FU for 16 h, were infected either with WT virus or with one of the *ts* mutants (MOI, 20 PFU/cell) and incubated at 41°C. The infected cultures were labeled between 6 and 7 h postinfection with [^{3}H]thymidine (10 μCi/ml). Part of the culture was harvested immediately thereafter, and part was shifted down to 32°C and incubated in thymidine medium containing cycloheximide (100 μg/ml) for 3 h. The infected cells were collected, lysed, and treated with pronase, and the DNA was analyzed in neutral sucrose gradients, as described in the legend to Fig. 1.

cycloheximide, both the D and H fragments were present and the amount of band B was reduced concomitantly. Since the normal ends appeared after *ts*N-infected cells had been shifted from the nonpermissive temperature to the permissive temperature in the presence of cycloheximide, we conclude that the concatemeric DNA had been cut normally. Thus, although concomitant protein synthesis is necessary for cleavage of DNA in WT-infected cells, it is not necessary for the maturation of *ts*N and *ts*IE13 DNA after shiftdown of the mutant-infected cells from the nonpermissive to the permissive temperature. It seems, therefore, that a protein which is normally limiting in WT infections has accumulated at the nonpermissive temperature in the mutant-infected cells. Also, it is clear that a *ts* protein essential for the process of DNA maturation can regain an active configuration after temperature shiftdown in *ts*N and *ts*IE13-infected cells.

To study the process of DNA maturation in *ts*N-infected cells in greater detail, the kinetics of DNA maturation in the presence or absence of an inhibitor of protein synthesis was determined (Fig. 7). As described previously (3), concurrent protein synthesis is necessary for continued maturation of viral DNA in WT-infected cells. In the particular experiment illustrated in Fig. 7, by 3 h after the beginning of the chase period approximately 20% of the labeled viral DNA in WT-infected, cycloheximide-treated cultures and 60% of the DNA in untreated cultures had matured. In the presence of cycloheximide, maturation was complete within the first hour after shiftdown. In similarly treated *ts*N-infected cells, however, mature viral DNA was formed throughout the 3-h chase period when more than 60% of the viral DNA had matured

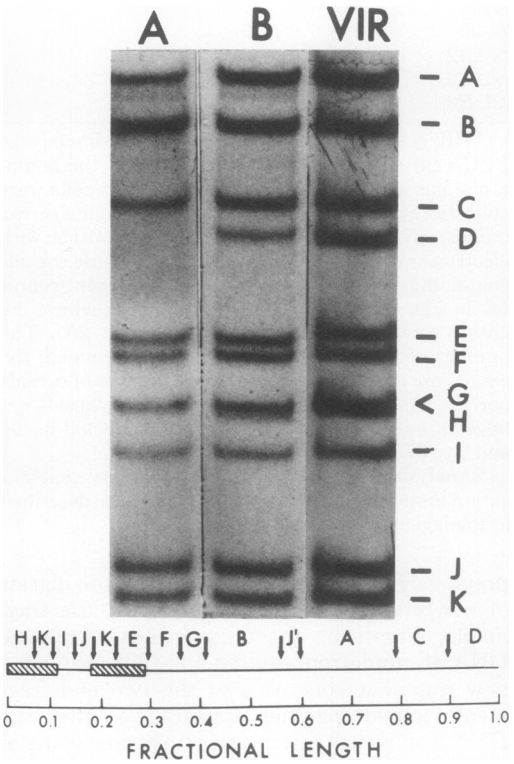

FIG. 5. *Autoradiogram of KpnI-digested concatemeric and "mature" tsN DNA. RK cells were washed with EDS $\bar{s}$ PO_4 and incubated in this medium for 24 h. The cells were then further incubated for 16 h in EDS $\bar{s}$ PO_4 + FU (20 μg/ml) + thymidine (5 μg/ml) to inhibit cellular DNA synthesis (12) and for 24 h in the same medium containing $^{32}PO_4$ (100 μCi/ml). The cells were then infected with tsN and incubated in the same ^{32}P medium at 41°C up to 6 h postinfection, when part of the cultures was harvested and part was shifted to 32°C and incubated in the presence of cycloheximide for 2 h. The viral DNA was isolated by isopycnic centrifugation in CsCl, limit-digested with KpnI, and electrophoresed as described in the text (4). (A) Viral DNA obtained from tsN-infected cells incubated at 41°C for 6 h; (B) viral DNA obtained from tsN-infected cells incubated at 41°C for 6 h and further incubated at 32°C in the presence of cycloheximide for 2 h; (Vir) viral DNA obtained from mature virions.*

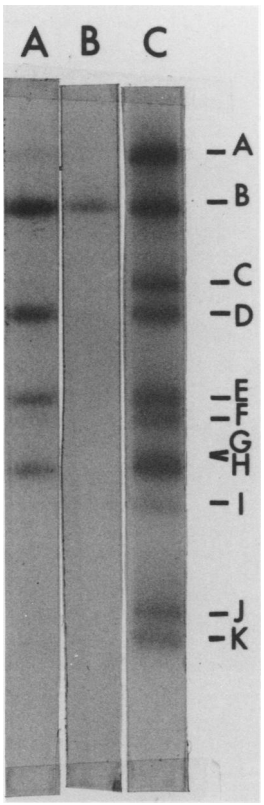

FIG. 6. *Hybridization of KpnI B fragments present in digests of mature virion DNA and concatemeric tsN DNA to filters to which fragments of KpnI-digested mature viral DNA had been fixed.* ^{32}*P-labeled tsN concatemeric or mature viral DNA was limit-digested with KpnI and electrophoresed in agarose gels. The KpnI B band was excised from each of the gels, purified as described in the text, and annealed to filter strips to which restriction fragments of mature Pr DNA generated by KpnI digestion had been fixed according to the method of Southern (23). The filters were washed and autoradiographed as described in the text. (A) Band B obtained from tsN concatemeric DNA; (B) band B obtained from mature virion DNA; (C) total mature virion DNA.*

to unit size molecules. Furthermore, maturation of viral DNA was faster in *ts*N-infected (both cycloheximide-treated and untreated) cells than it was in untreated WT-infected cells. These results confirm our previous conclusion that a nonfunctional protein necessary for DNA maturation had accumulated in *ts*N-infected cells at the nonpermissive temperature and had regained a functional configuration after shiftdown to the permissive temperature, thereby allowing viral DNA maturation to occur.

Since the *ts* protein that accumulates at the nonpermissive temperature is able to regain an active configuration after shiftdown and thereby allow correct maturation of viral DNA, we analyzed, both by electron microscopy and by sedimentation in sucrose gradients, the type of viral particles that accumulate in the cells after shiftdown to the permissive temperature. These experiments were designed to determine (i) whether there is a correlation between maturation of DNA and formation of full capsids and (ii) if so, whether a precursor-product relationship could be established between empty and full capsids.

Figure 8 shows an electron micrograph of *ts*N-infected cells before and after shiftdown to the permissive temperature in the presence of cycloheximide. As discussed above, at the nonpermissive temperature only capsids with electron-translucent cores were observed (Fig. 8A). After shiftdown in the presence of cycloheximide, however, capsids with electron-dense cores appeared in the nucleus (Fig. 8B), and enveloped nucleocapsids were extruded into the cytoplasm. Thus, formation of complete virions occurred in

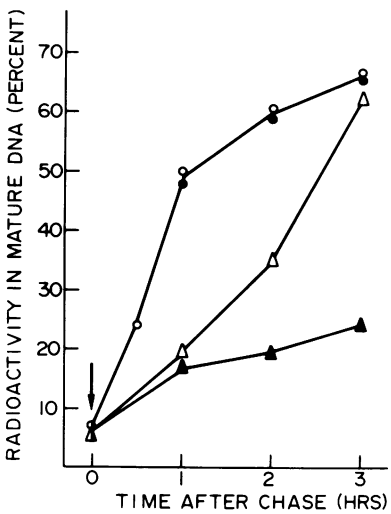

FIG. 7. *Maturation of viral DNA in WT- and tsN-infected cells upon shiftdown in the presence or absence of cycloheximide. RK cells were pretreated with ELS + FU, infected with either WT or tsN (MOI, 20 PFU/cell), and incubated at 41°C. Infected cultures were pulse-labeled between 7 and 7.5 h postinfection with [*^{3}*H]thymidine (10 μCi/ml). Part of the culture was harvested immediately thereafter, and part was shifted to the permissive temperature and incubated in thymidine medium with or without cycloheximide (100 μg/ml) for various periods of time. The cultures were harvested, and the DNA was sedimented in neutral sucrose as described in the legend to Fig. 1. The arrow indicates the end of the labeling period. (○) tsN-infected cells, no cycloheximide; (●) tsN-infected cells plus cycloheximide; (△) WT-infected cells, no cycloheximide; (▲) WT-infected cells plus cycloheximide.*

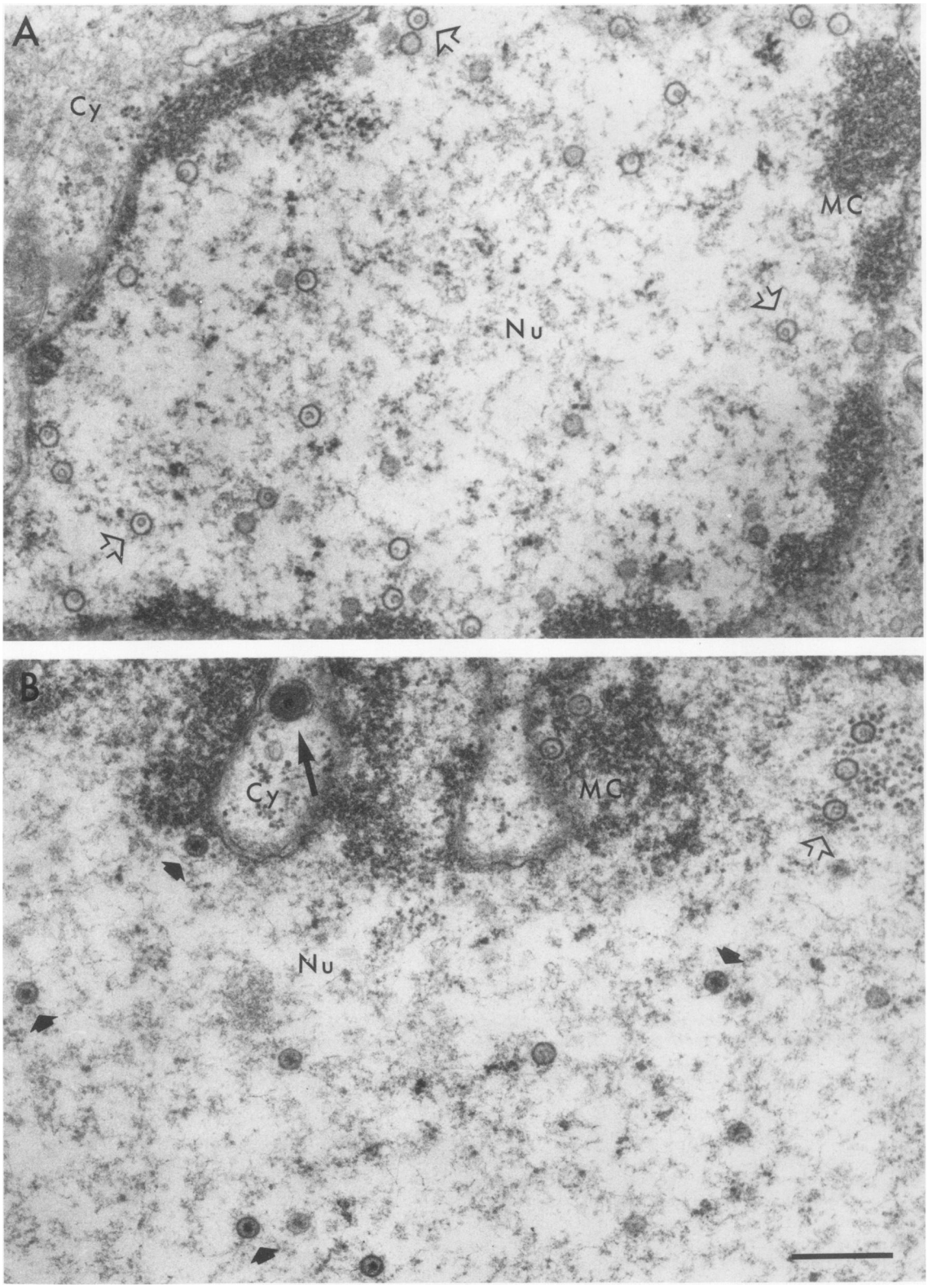

FIG. 8. *Formation of full viral particles after shiftdown of tsN-infected cells in the presence of cycloheximide. RK cells were infected with tsN and incubated at 41°C for up to 6 h postinfection. Part of the infected cultures was then harvested (A), and part of the cultures was shifted to the permissive temperature and incubated in the presence of cycloheximide for 2 h (B). The samples were prepared for electron microscopy as described in the text. Cy, Cytoplasm; MC, marginated chromatin; N, nucleus; open arrow, viral particle containing electron-translucent cores; long closed arrow, mature enveloped viral particles in cytoplasm. Bar, 0.5 μm.*

*ts*N-infected cells after shiftdown in the presence of cycloheximide.

The number of empty and full capsids present in the infected cells before and after shiftdown in the presence of cycloheximide is given in Table 4. Before shiftdown, no full capsids were present in *ts*N-infected cell nuclei. After shiftdown in the presence of cycloheximide, the number of capsids with electron-translucent cores decreased significantly, and instead capsids with electron-dense cores appeared. There was a reduction in the total number of capsids in the nuclei, probably due to the extrusion of full enveloped capsids into the cytoplasm (see Fig. 8B). The reduction in the number of capsids with electron-translucent cores and the concomitant appearance of capsids with electron-dense cores indicate, but do not prove, that capsids devoid of DNA have been converted to capsids containing DNA in *ts*N-infected cells after shiftdown from the nonpermissive to the permissive temperature in the presence of cycloheximide.

To provide conclusive proof, the relative number of empty and full capsids in *ts*N-infected cells before and after shiftdown in the presence of cycloheximide was determined by sedimentation analysis in sucrose gradients. RK cells were infected with *ts*N at the nonpermissive temperature, and the proteins were labeled with [^{3}H]leucine between 4 and 5 h postinfection. The label was then removed, and the cells were further incubated for 2 h at the nonpermissive temperature in the presence of an excess of unlabeled leucine to allow the labeled proteins to assemble into capsids. (Assembly of labeled proteins into empty capsids was observed up to 2 h of chase, but not thereafter.) Part of the cultures was then shifted down to the permissive temperature in the presence of cycloheximide, and the remainder of the cultures was further incubated at the nonpermissive temperature in the presence of the inhibitor. At various times thereafter, the infected cells were harvested, and the relative amount of label associated with empty and full viral particles was determined. The results of a representative experiment are illustrated in Fig. 9, which shows that the amount of radioactivity associated with the peak of empty capsids was decreased after shiftdown, with a concomitant increase of label in the peak of full enveloped capsids. (Few, if any, full nonenveloped particles were detectable, and it is probable that the DNA was lost from these particles during purification.)

Table 5 summarizes the results of three other similar experiments. It is clear from these data that empty particles were stable in cycloheximide-treated cells incubated at the nonpermissive temperature. After shiftdown to the permissive temperature, there was, however, a signifi-

TABLE 4. *Capsids with electron-translucent and electron-dense cores in WT- and tsN-infected cells before and after shiftdown to the permissive temperature in the presence of cycloheximide*[a]

Experimental conditions	Total no. of capsids	Empty capsids	Full capsids	Full capsids (% of total)
WT, 41°C	19	10	9	47
WT → 32°C + cycloheximide	11	7	4	36
*ts*N, 41°C	40	40	0	0
*ts*N → 32°C + cycloheximide	28	15	13	46

[a] RK cells were infected either with WT virus or *ts*N (MOI, 20 PFU/cell) at 41°C. At 6 h postinfection, part of the cultures was harvested, and part of the cultures was shifted to the permissive temperature and incubated in the presence of cycloheximide for 2 h. The samples were prepared for electron microscopy as described in the text. The number of capsids in 20 nuclei was counted, and the average number of capsids per nucleus was calculated.

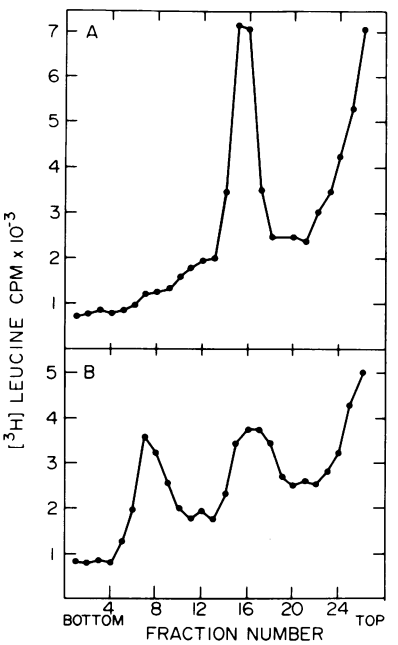

FIG. 9. *Sedimentation analysis of viral particles present in tsN-infected cells incubated at 41°C as well as after shiftdown to the permissive temperature in the presence of cycloheximide. RK cells were infected with tsN, incubated at 41°C, and labeled with [^{3}H]leucine (50 μCi/ml) between 4 and 5 h postinfection. The label was removed, and the infected cells were further incubated for 2 h at 41°C in the presence of an excess of unlabeled leucine. Part of the cultures was then harvested, and part was shifted down to the permissive temperature, further incubated in the presence of cycloheximide for 6 h, and then harvested. The viral particles were collected and then sedimented in sucrose gradients as described in the legend to Fig. 4 and in the text.*

TABLE 5. *Conversion of empty to full capsids in tsN-infected cells after shiftdown to the permissive temperature, as determined by velocity centrifugation in sucrose gradients*[a]

Expt no.	Time after chase (h)	cpm × 10^3						Full (%)
		No shiftdown (41°C)			Shiftdown (32°C)			
		Empty	Full	Total	Empty	Full	Total	
1	0	28.1	0	28.1				
	3	32.0	0	32.0	22.4	10.4	32.8	32
	6	28.6	0	28.6	18.4	10.8	29.2	35
2	0	32.8	0	32.8				
	6	36.8	0	36.8	17.6	13.2	30.8	43
3	0	33.3	0	33.3				
	17	ND[b]		ND	16.8	13.2	30.0	44

[a] RK cells were infected with *ts*N (MOI, 20 PFU/cell) and incubated at 41°C in EDS. The infected cells were labeled with [^{3}H]leucine (50 μCi/ml) in EDS s amino acids between 4 and 5 h postinfection. At 5 h postinfection, the label was removed, and the cells were incubated further at 41°C in EDS containing twice the normal amount of unlabeled amino acids. At 7 h postinfection, part of the cultures was harvested; part of the cultures was further incubated at 41°C in the presence of cycloheximide (100 μg/ml), and part of the cultures was shifted to 32°C in the presence of cycloheximide. The cells were harvested at various times thereafter, and the viral particles were isolated as described in the text and the legend to Fig. 4.

[b] ND, Not done.

cant reduction in the number of empty capsids (up to 40%). Since at the nonpermissive temperature the capsids remained stable, it is unlikely that the decrease in empty capsids observed after shiftdown to the nonpermissive temperature is due to nonspecific breakdown. Furthermore, since full capsids appeared under those conditions, we conclude that the empty capsids had been converted to full capsids and that a precursor-product relationship between these two forms of capsids exists.

Our results thus indicate that: (i) the maturation of viral concatemeric DNA to genome length is correlated with the appearance of full capsids; (ii) empty capsids are precursors to full capsids.

DISCUSSION

The data in this paper bear on two aspects of the maturation of the nucleocapsids of Pr virus: (i) the mechanism by which the concatemeric replicative form of Pr viral DNA is cleaved to genome-size mature DNA; (ii) the precursor relationship between empty and full capsids.

Mechanism of cleavage of concatemeric DNA. Nine DNA$^+$ *ts* mutants, each belonging to a different complementation group, were studied. In none of the cells infected with these mutants at the nonpermissive temperature did the concatemeric forms of the replicative DNA mature to genome size. It is clear, therefore, that maturation of the DNA is a complex process involving several viral gene products. Analysis by electron microscopy and by velocity gradient centrifugation showed that in cells infected with six of the mutants, capsids with electron-translucent cores (empty capsids) accumulated. In cells infected with three of the mutants, however, capsids did not accumulate. Since these three mutants were defective both in capsid assembly and in viral DNA maturation, we conclude that cleavage of concatemeric Pr viral DNA to unit size is dependent on a mechanism related to capsid assembly, as it is in several bacteriophage systems (14).

We have shown previously (3) that maturation of wild-type Pr concatemeric DNA to genome-size mature DNA requires concomitant protein synthesis and that, therefore, either the proteins required for DNA maturation are catalytic, unstable proteins, or else structural proteins, which are present in limiting amounts in the cells, are required for maturation. Since in *ts*N- and *ts*IE13-infected cells maturation of DNA occurs in the presence of an inhibitor of protein synthesis after shiftdown to the permissive temperature, we conclude that a protein, which is normally limiting during the course of infection with WT virus, had accumulated in the mutant-infected cells during the incubation period at the nonpermissive temperature. Thus, during the normal course of infection a viral protein(s) necessary for DNA maturation is present in limiting amounts.

Relationship between empty and full particles. The problem of whether the different types of capsids present in infected cells represent different stages in the process of virion assembly has not been fully elucidated. Gener-

ally, the following two methods have been used to study this question: (i) electron microscopic examination of thin sections of infected cells; (ii) isolation of the different types of capsids from infected cells, which had been subjected to various protocols of pulse-labeling with amino acids, followed by chase periods.

Electron microscopic studies have revealed the presence, within the nuclei of herpesvirus-infected cells, of capsids containing cores of various electron densities (17). Nii et al. (15) were the first to postulate that these different capsid structures represent various stages in the development of the mature virions. However, electron microscopic observations alone of WT-infected cells, although suggestive, cannot provide conclusive proof about the precursor-product relationships between the different capsid forms, because the disappearance of one type of capsid and the appearance of another type cannot be related quantitatively. Nevertheless, Friedman et al. (9) described a synchronous progression in the appearance of capsids with electron-dense punctate bodies to capsids with electron-dense cores, after release of herpes simplex virus-infected cells from hydroxyurea block, a result which indicated that a precursor-product relationship may exist between these two types of capsids.

One of the major difficulties with the interpretation of results of a pulse-chase experiment in which attempts are made to follow the movement of label from one type of capsid to another is that assembly of protein into capsids is a relatively slow process and that unassembled structural proteins synthesized at a given stage of infection continue to be assembled thereafter. Thus, when cells infected with WT Pr virus are pulse-labeled and chased by the addition of an excess of unlabeled amino acids, and the types of particles isolated from the cells are determined, the following results are obtained. Immediately after a pulse, only labeled empty particles are present; after the chase, labeled proteins become associated both with empty capsids and with capsids containing DNA. However, we could not demonstrate a decrease in the radioactivity associated with full particles because labeled viral proteins, which had not already been consumed in the assembly of viral particles, continued to be utilized for the synthesis of empty viral particles during the chase period. Consequently, the possibility could not be eliminated that the capsids being assembled during the chase period were the full ones (T. Ben-Porat, unpublished data). The problem is compounded by the fact that some of the capsid structures are unstable and cannot be isolated in intact form from the infected cells (9; T. Ben-Porat, unpublished data). It is therefore difficult to establish clearly a precursor relationship between different types of capsids. Despite these difficulties, Randall, O'Callaghan, and collaborators (16, 19) have reported results that indicate that capsids containing partial cores (I particles) are precursors to virions, whereas empty capsids (L particles) are not. The virtual disappearance from the cells of the I particles after a chase period indicated that these particles are indeed on the pathway of assembly of mature virions.

The advantages of *ts* mutants in the analysis of the steps involved in capsid assembly are obvious. The usefulness of such mutants in the study of the process of assembly has been demonstrated for several bacteriophage systems (14). In the experiments described in this paper, we have used one *ts* mutant to demonstrate that there is transfer of labeled viral proteins from empty capsids to full, enveloped virions. We have done this by allowing all the structural viral proteins labeled during a pulse, which assemble into capsids, to do so during a chase period at the nonpermissive temperature. Since viral DNA maturation does not occur at this temperature, an accumulation of capsids devoid of DNA occurred under these conditions. After shiftdown in the presence of an inhibitor of protein synthesis, we could demonstrate clearly that empty capsids disappeared and that full capsids appeared instead, indicating that the empty capsids are assembled first and that the viral DNA is inserted into the capsids thereafter. This sequence of events has been proposed also by Friedman et al. (9) and Perdue et al. (18).

General conclusion. On the basis of the results presented in this paper, we consider it probable that the following chain of events, for which there are precedents in other systems (14), occurs during the assembly of the Pr viral nucleocapsids. The capsid proteins are first assembled into empty shells, into which viral DNA is encapsidated thereafter. The empty capsids, as well as possibly other noncapsid viral proteins, are responsible for cutting the viral concatemeric DNA to genome length. The availability of different mutants which are blocked at different steps in the formation of Pr viral nucleocapsids should make it possible to study in greater detail the various stages of herpesvirus assembly.

ACKNOWLEDGMENT

This work was supported by Public Health Service grant AI-10947 from the National Institutes of Health.

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