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Multiplication of Parvovirus LuIII in a Synchronized Culture System

IV. Association of Viral Structural Polypeptides with the Host Cell Chromatin

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Newly synthesized structural polypeptides of parvovirus LuIII, VP_1 (62,000 daltons) and VP_2 (74,000 daltons), were detected in nuclei of synchronized, infected HeLa cells at 11 to 12 h postinfection, i.e., after cells had passed through the S phase of the cell cycle. At this time, most of intranuclear viral polypeptides were associated with the chromatin acidic proteins. However, 13 to 14 h postinfection, about one-third of intranuclear VP_1 and VP_2 also could be extracted in the fraction containing nuclear sap proteins. According to pulse-chase experiments, VP_1 and VP_2 accumulated in the chromatin with a time lag of 20 to 30 min. About 90% of these chromatin-associated viral polypeptides represented empty viral capsids. In addition, chromatin prepared at 14 h postinfection contained 90 to 95% of the total intranuclear viral 16S replicative-form DNA. Since viral replicative-form DNA and empty viral capsids seem to be associated specifically with cellular chromatin, we assume that this subnuclear structure is the site of the synthesis of progeny viral DNA and the formation of complete virions.

Complete virions of parvovirus LuIII contain a linear single-stranded molecule of DNA (1.6×10^6 daltons) and two structural polypeptides (VP_1 [62,000 daltons] and VP_2 [74,000 daltons]) (6, 19). In addition, a third polypeptide, VP_3 (68,000 daltons), was found in empty viral capsids or in particles containing only parts of the viral genome (6). LuIII virus, like other parvoviruses (18), replicates within the nucleus of the host cell (20, 21). Thus, both the replication of viral DNA and the intranuclear accumulation of viral antigens depend upon cellular events occurring in the late S or early G_2 phase of the cell cycle (20). The molecular natures of the site of viral DNA replication and of virus assembly, however, are still unknown.

In the experiments described below, we investigated whether intermediates in viral replication and, especially, viral polypeptides were associated with specific nuclear structures. For this purpose, the time course of the appearance of viral polypeptides within the fractions containing nuclear sap, histones, and chromatin acidic proteins of synchronized HeLa cells (20) was analyzed. Viral structural polypeptides were found as early as 11 to 12 h postinfection (p.i.) mainly in association with the chromatin acidic proteins. In additional experiments, we studied the specificity of the association of viral polypeptides with the host chromatin. The results showed an accumulation of empty viral capsid structures in the host cell chromatin which, in addition, contained most of the double-stranded viral replicative-form (RF) DNA (21) of parvovirus LuIII.

(A preliminary report of part of this work was presented at the 3rd International Congress of Virology, Madrid, September 1975.)

MATERIALS AND METHODS

Cell cultures and virus. HeLa cells grown to a density of 4×10^6 to 5×10^6 cells per 75-cm^2 plastic flask were synchronized with thymidine (5 mM) for 20 h and then infected with parvovirus LuIII at a multiplicity of infection of 10 as described previously (20).

Labeling of proteins and DNA. Proteins were labeled for 2 h (10 μCi/ml) or for shorter times (100 μCi/ml) with L-[^{3}H]valine and L-[^{3}H]leucine (specific activities, 30 and 60 Ci/mmol, respectively). In some experiments, the 2-h pulses were performed with a mixture of 0.5 μCi of L-[^{14}C]arginine and L-[^{14}C]lysine (specific activity, 0.3 Ci/mmol) per ml and 5 μCi of L-[^{3}H]tryptophan (specific activity, 8 Ci/mmol) per ml. All radioactive amino acids were added to a culture medium containing 1/5 (for 2-h pulses) or 1/10 (for shorter pulses) of the normal concentration of amino acids. For DNA labeling, 40 μCi of [*methyl*-^{3}H]thymidine (specific activity, 60 Ci/mmol) per ml was used. Labeled proteins and

DNA were chased in media containing 100 times the original concentration of L-valine and L-leucine and 10^{-5} M thymidine.

Isolation of nuclei. LuIII-infected or mock-infected monolayer cultures were treated with trypsin, and cells were washed with cold Hanks balanced salts solution and sedimented at 600 × *g* for 10 min at 0°C. All further manipulations were performed at 0°C. For lysis, the cells were suspended at a concentration of 4×10^6 to 5×10^6 cells per ml in a lysing solution containing 0.5% Nonidet P-40, 0.14 M NaCl, 5 mM KCl, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, and 25 mM Tris-hydrochloride (pH 7.4) (28). After 10 min, nuclei were pelleted at 1,000 × *g* for 5 min and washed twice with the above-mentioned solution.

Preparation of crude nuclear lysates. Nuclei were lysed (2.5×10^6 nuclei per ml) in high-salt buffer (0.5 M NaCl, 0.05 M $MgCl_2$, 0.01 M Tris-hydrochloride, pH 7.4) and incubated with DNase I (25 μg/ml) at 37°C for 5 min (16). After addition of EDTA to a final concentration of 30 mM, samples were treated with sodium dodecyl sulfate (SDS; 0.2%, wt/vol) and β-mercaptoethanol (0.1%, vol/vol) at 37°C for 15 min. Unsolubilized material was pelleted at 4,000 × *g*, and the proteins contained within the supernatant were precipitated by the addition of an equal volume of 10% trichloroacetic acid. The precipitate was washed twice with cold acetone and then prepared for gel electrophoresis.

Fractionation of nuclear proteins into nuclear sap proteins, histones, and non-histone chromosomal proteins. All operation steps were carried out at 0°C. To obtain the nuclear sap proteins, a constant number of isolated nuclei (4×10^6 to 5×10^6) were washed twice with 1 ml of 0.15 M NaCl–0.01 M Tris-hydrochloride (pH 8.0) (2, 23, 29) and twice with 1 ml of 0.35 M NaCl–0.01 M Tris-hydrochloride (pH 8.0) (4, 10). After each wash, the nuclei were pelleted at 1,000 × *g* for 5 min. The combined supernatants represented the nucleoplasmic fraction F_1. Histones were extracted from crude chromatin with 1 ml of 0.4 N H_2SO_4 (1, 7, 12) for 8 h; this step was repeated once. After centrifugation at 4,000 × *g* for 5 min, histones were precipitated from the supernatants with 5 volumes of cold acetone (fraction F_2) and washed once with acetone. The "dehistonized" chromatin was washed twice with cold acetone, and the residual non-histone chromosomal proteins were extracted with 2% (wt/vol) SDS-1% (vol/vol) mercaptoethanol-0.01 M phosphate buffer (pH 7.0) at 50°C for 2 h, followed by heating to 100°C for 2 min (fraction F_3). Samples were loaded directly onto acrylamide gels.

Preparation of chromatin under controlled conditions. All samples were kept at 0°C. The hypotonic method used for the isolation of HeLa cell chromatin was essentially the same as described elsewhere (15, 24) but with the following modifications. Constant numbers (4×10^6 to 5×10^6) of isolated nuclei were washed twice with 1 ml of 0.15 M NaCl-0.01 M Tris-hydrochloride (pH 8.0) and pelleted as indicated above. The supernatants were combined and designated as fraction F_{1a}. Nuclei were then lysed by the addition of 1 ml of distilled water. The chromatin was allowed to swell until a gel was formed. Any shearing forces were avoided. Centrifugation of the swollen chromatin gel samples at 4,000 × *g* for 15 min resulted in a clear nonviscous supernatant. It was removed carefully from the denser chromatin gel and collected as fraction $F_{1b(1)}$. Successive fractions $F_{1b(2-5)}$ were extracted similarly from crude chromatin by repeated resuspension of the dense chromatin gel in 1 ml of distilled water. Thereafter, the chromatin was washed with 0.35 M NaCl–0.01 M Tris-hydrochloride (pH 8.0) and sedimented at 4,000 × *g* for 5 min. This step was repeated four times. The supernatants represented fraction $F_{1b(6-9)}$. The remaining sediment was defined as the chromatin fraction.

Isolation of viral particles from the chromatin. Chromatin prepared from infected or mock-infected cells under controlled conditions was dissociated in 3.5 M CsCl. The solution was centrifuged to equilibrium in a Beckman SW50.1 rotor at 35,000 rpm for 42 h at 15°C. Fractions were collected from the top of the gradients, and samples were counted in Instagel (Packard Instruments). Appropriate fractions were pooled, dialyzed against a solution of 0.35 M NaCl, 0.01 M Tris-hydrochloride (pH 8.0), and 1 mM EDTA, and sedimented in linear 10 to 30% (wt/wt) sucrose gradients (14 ml) containing 0.35 M NaCl, 0.01 M Tris-hydrochloride (pH 8.0), and 1 mM EDTA (Beckman SW27.1 rotor, 25,000 rpm, 5 h, 4°C). The gradients were formed on the top of a 1.5-ml cushion of 60% (wt/wt) sucrose. The radioactivity of each fraction was measured in a toluene-scintillator solution containing 33% (vol/vol) Triton X-100.

Extraction of DNA from the nuclear fractions. Fraction F_{1b} or chromatin was mixed with 1/10 volume of Pronase (Calbiochem B grade, nuclease free, 5 mg/ml in 0.01 M Tris-hydrochloride [pH 7.4], self-digested at 37°C for 1 h) and 1/10 volume of Sarkosyl (Serva, Sarkosyl NL-97, 10% [wt/vol] in 0.01 M Tris-hydrochloride [pH 7.4]) and then incubated at 37°C for 16 h. The digest was directly used for DNA analysis.

Sedimentation analysis of DNA. Linear 5 to 20% sucrose gradients (14 ml) containing 1 M NaCl, 0.01 M Tris-hydrochloride, 0.01 M EDTA, 0.1% (wt/vol) Sarkosyl, and 0.01 M sodium citrate (pH 8.2) were layered onto a 1.5-ml cushion of 40% sucrose. Centrifugation was performed in a Beckman SW27.1 rotor at 23,000 rpm and 4°C for 14 h. Gradients were calibrated with DNA from phage λ, and S values were calculated by the method described previously (5).

SDS-polyacrylamide gel electrophoresis. Proteins of the various extracts were precipitated by addition of an equal volume of 10% (wt/vol) trichloroacetic acid, washed twice with cold acetone, and suspended in 2% SDS-1% mercaptoethanol-0.01 M phosphate buffer (pH 7.0). The technique for SDS-polyacrylamide slab gel electrophoresis, as well as the determination of molecular weights, was described previously (5).

In the figures, the positions of the viral polypeptides VP_1, VP_2, and VP_3 are given by arrows. The respective molecular weights were determined previously to be 62,700, 73,500, and 68,200, respectively, with standard deviations of ≦2%.

RESULTS

Viral polypeptides in nuclear lysates of infected cells. By means of immunofluorescent staining, antigens of parvovirus LuIII were demonstrated within the nuclei of synchronized HeLa cells as early as 10 to 12 h p.i. (20). According to these results, virus-infected or mock-infected cell cultures were labeled from 11 to 12 h p.i. with [^{3}H]leucine and [^{3}H]valine. Nuclear lysates were prepared and analyzed on SDS-acrylamide slab gels (Fig. 1). The nuclear lysate of infected cells contained two polypeptides with the same molecular weights as the viral structural polypeptides VP_1 and VP_2, as well as a polypeptide in the electrophoretic position of VP_3. The ratio of $VP_1/VP_2/VP_3$ was identical to that found in purified, incomplete virus particles (6).

Appearance of viral polypeptides in the chromatin acidic protein fraction and the nuclear sap fraction. To measure the time course of the appearance of viral polypeptides in the subnuclear fractions with respect to the cell cycle, synchronized HeLa cells were infected with LuIII virus and labeled with [^{3}H]tryptophan, as well as with [^{14}C]arginine and [^{14}C]lysine, for 2 h at various times after infection. Nuclei were then isolated and fractionated into nuclear sap proteins (fraction F_1), histones (fraction F_2), and non-histone chromosomal proteins (fraction F_3). The rate of histone synthesis was determined by calculating the ratio of [^{14}C]arginine and [^{14}C]lysine to [^{3}H]tryptophan incorporated into acid-soluble proteins of fraction F_2 (Fig. 2). This parameter was used to estimate the position of the cells with respect to the S phase. Since [^{3}H]tryptophan was incorporated into nuclear proteins of both infected and uninfected cells in comparable amounts, it can be concluded that synchronized cells synthesize histones in similar proportions, whether they are virus infected or mock infected.

When the incorporation of [^{3}H]tryptophan into fractions F_1 and F_3 was measured, differences between incorporation rates in virus-infected and mock-infected cells became evident as early as 11 to 12 h p.i. At that time, both fractions F_1 and F_3 of infected cells contained at least 1.5 times the amount of [^{3}H]tryptophan-labeled polypeptides present in the corresponding fraction of the controls. Analysis of

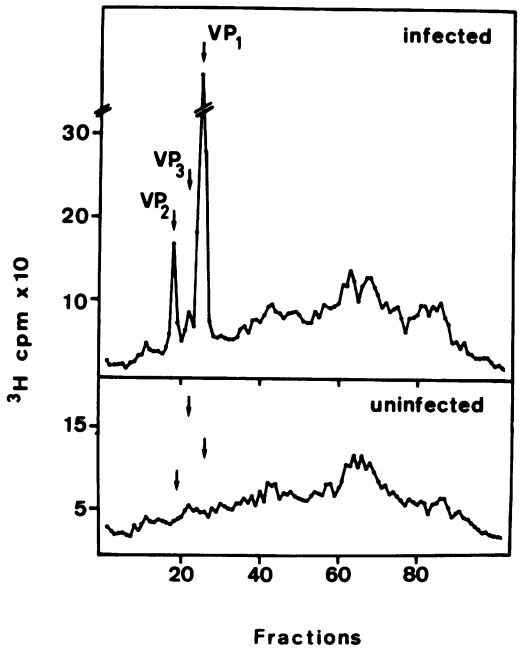

FIG. 1. *Presence of parvovirus LuIII structural polypeptides in a crude nuclear lysate. HeLa cells were synchronized with thymidine (5 mM) and infected at a multiplicity of infection of 10 concomitantly with release from the synchronization block. From 11 to 12 h after infection they were labeled with [^{3}H]leucine (5 μCi/ml) and [^{3}H]valine (5 μCi/ml). Crude nuclear lysates of both infected and mock-infected cells were prepared, and samples were run on 7.5% SDS-acrylamide slab gels. Arrows indicate the position of viral polypeptides VP_1, VP_2, and VP_3.*

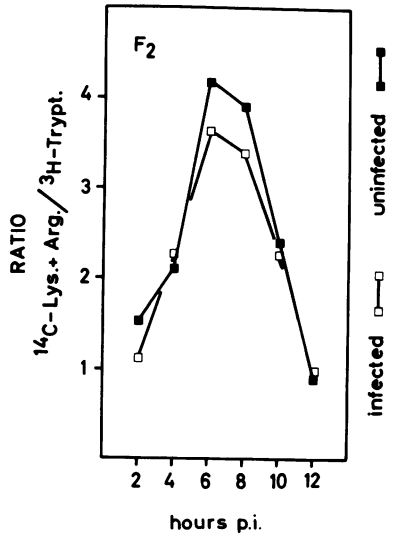

FIG. 2. *Rate of histone synthesis in LuIII virus-infected and mock-infected synchronized HeLa cells. Cells were synchronized, infected, and subsequently labeled for regular 2-h intervals at various times after infection with [^{14}C]lysine (0.25 μCi/ml), [^{14}C]arginine (0.25 μCi/ml), and [^{3}H]tryptophan (5 μCi/ml). The rate of histone synthesis was determined by the ratio of [^{14}C]lysine and [^{14}C]arginine to [^{3}H]tryptophan incorporated into acid-soluble nuclear proteins (fraction F_2).*

[^{3}H]tryptophan-labeled polypeptides on SDS-acrylamide slab gels revealed that, 11 to 12 h p.i., F_1 proteins of infected cells contained only traces of a polypeptide migrating in the position of VP_1 (Fig. 3). On the other hand, 5 to 8% of the total radioactivity of fraction F_3 was found to be associated with two polypeptides having the same molecular weights as VP_1 and VP_2 (Fig. 4A). These newly synthesized polypeptides are considered to be the structural polypeptides VP_1 and VP_2 since, in addition to the appropriate molecular weights, they could be found neither in mock-infected cells nor in cells infected with empty viral capsids. Thus, at the end of the S phase or in the early G_2 phase, over 90% of VP_1 and VP_2 present within the nuclei could be extracted with the chromatin acidic proteins.

Evidence for the presence of VP_3 was obtained in fraction F_3 when infected cultures were labeled 11 to 12 h p.i. with [^{3}H]leucine and [^{3}H]valine instead of [^{3}H]tryptophan (Fig. 4B). However, a polypeptide with similar electrophoretic characteristics also became labeled in fraction F_3 from mock-infected cultures. The VP_3-like polypeptide proved to be associated strictly with the chromatin. When in a subsequent labeling period, i.e., from 13 to 14 h p.i., cells were labeled with [^{3}H]leucine and [^{3}H]valine, 35 to 40% of the total of intranuclear VP_1 and VP_2 was recovered within the nuclear sap protein fraction (Fig. 5), yet there was no indication of the presence of VP_3.

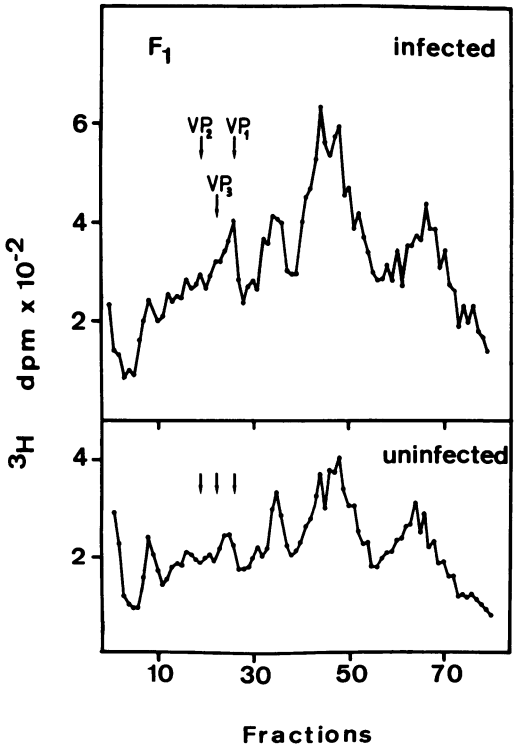

FIG. 3. *[^{3}H]tryptophan-labeled polypeptides in the nuclear sap proteins (fraction F_1) 11 to 12 h after infection. HeLa cells were synchronized, infected, and labeled as described in the legend of Fig. 2. Nuclear sap proteins were extracted and run on SDS-acrylamide slab gels. The positions of VP_1, VP_2, and VP_3 are indicated by arrows.*

Viral polypeptides are not random contaminants of the chromatin. At 11 to 12 h p.i. most of the viral polypeptides VP_1 and VP_2 had been demonstrated to be within the chromatin acidic protein fraction. Two hours later, when the synthesis of viral proteins had increased, they also could be isolated with the nuclear sap proteins. To exclude the possibility that the isolation of viral polypeptides with the host cell chromatin represented nothing but a contamination of the chromatin fraction by VP_1 and VP_2 originally present in the nuclear sap, the cellular chromatin was first isolated under controlled conditions. For this purpose, synchronized HeLa cells were mock infected or infected with LuIII virus and at 14 h p.i. were labeled with either [^{3}H]leucine and [^{3}H]valine or [^{3}H]thymidine for 1 h. Nuclei were isolated by the Nonidet P-40 procedure, and subnuclear fractions F_{1a}, $F_{1b(1-9)}$, and chromatin were prepared as described in Materials and Methods.

During the stepwise extraction of labeled proteins with either water or 0.35 M NaCl, the recovered radioactivity in the supernatants dropped to about 5% of the initial value, irrespective of whether the chromatin was prepared from mock-infected or from LuIII virus-infected cells (Fig. 6, top). Nearly identical amounts of labeled proteins were released by both the water and 0.35 M NaCl extraction steps. However, the water fractions contained 27 to 32% of the total viral polypeptides present in the nuclei at 14 h p.i., whereas the 0.35 M NaCl extracts almost exclusively yielded cellular proteins. Since 35 to 40% of the intranuclear VP_1 and VP_2 had been isolated already with the nuclear sap, 32 to 37% of VP_1 and VP_2 still remained in strict association with the isolated chromatin. They could be recovered in the non-histone chromosomal protein fraction.

The lower panel in Fig. 6 represents the results of a similar extraction experiment performed with [^{3}H]thymidine-labeled cells. Less than 1% of the total labeled DNA was isolated from control cells with water and 0.35 M NaCl, indicating that only minimal degradation of the chromatin occurred during the extraction process. In contrast, large amounts of newly synthesized DNA were recovered from the crude chromatin of infected cells. These molecules sedi-

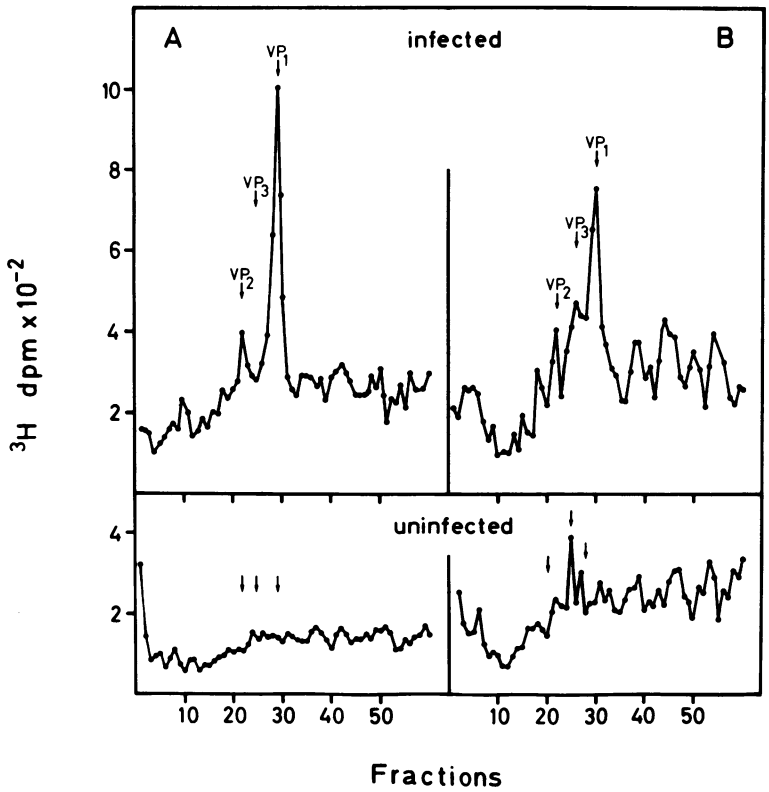

FIG. 4. *Demonstration of viral polypeptides in the non-histone chromosomal protein fraction (F_3) 11 to 12 h after infection. F_3 proteins were prepared from synchronized LuIII-infected or mock-infected HeLa cells and were run on SDS-acrylamide slab gels. (A) F_3 proteins labeled with [^{3}H]tryptophan (5 μCi/ml); (B) F_3 proteins labeled with [^{3}H]leucine (5 μCi/ml) and [^{3}H]valine (5 μCi/ml) for 2 h. The position of viral proteins is indicated by arrows.*

mented mainly at 8S (data not shown). (A detailed presentation of their characteristics will be the subject of a later paper.)

Finally, the probability of the contamination of the chromatin by viral polypeptides was tested further in a reconstruction experiment. Nuclear sap from infected cells containing labeled VP_1 and VP_2 (Fig. 5) was incubated with unlabeled, salt-washed nuclei of either LuIII-infected or control cells for 1 h at 0°C. The nuclei were then lysed and the chromatin was allowed to swell for 4 h at 0°C. The extraction procedure was exactly the same as described above. Ninety to 95% of the total radioactive input could be recovered in the water fractions [$F_{1b(1-5)}$]. The remaining 5 to 10% of the labeled proteins was found in the isolated chromatin. As electrophoretic analysis showed, this 5 to 10% contained only 3 to 5% of the previously added viral polypeptides. Thus, the degree of contamination of chromatin by VP_1 and VP_2 was the same irrespective of whether viral polypeptides had been added to nuclei from virus-infected or mock-infected cells.

VP_1 and VP_2 accumulate mainly as capsid structures in the chromatin. Data concerning the appearance of viral polypeptides in the chromatin acidic protein fraction at 11 to 12 h p.i. suggested that these polypeptides accumulated first in this fraction and, with a certain time lag, also in the nuclear sap. To see whether VP_1 and VP_2 accumulated in the chromatin mainly as free polypeptides or as capsid-like structures, we investigated whether the ratio of VP_1 to VP_2 and VP_3 was changing during the time of accumulation. LuIII virus-infected or mock-infected cell cultures were pulse-labeled 14 h p.i. with [^{3}H]leucine and [^{3}H]valine for 10, 20, or 40 min. In parallel, cultures pulse-labeled for 10 min were chased for 30 or 60 min, and the labeling patterns of the chromatin acidic proteins were analyzed on acrylamide gels.

After a 10-min pulse, neither VP_1 nor VP_2 could be detected in association with the chromatin acidic proteins, and samples from cultures pulse-labeled for 20 min contained only traces of VP_1 and VP_2. However, after a pulse

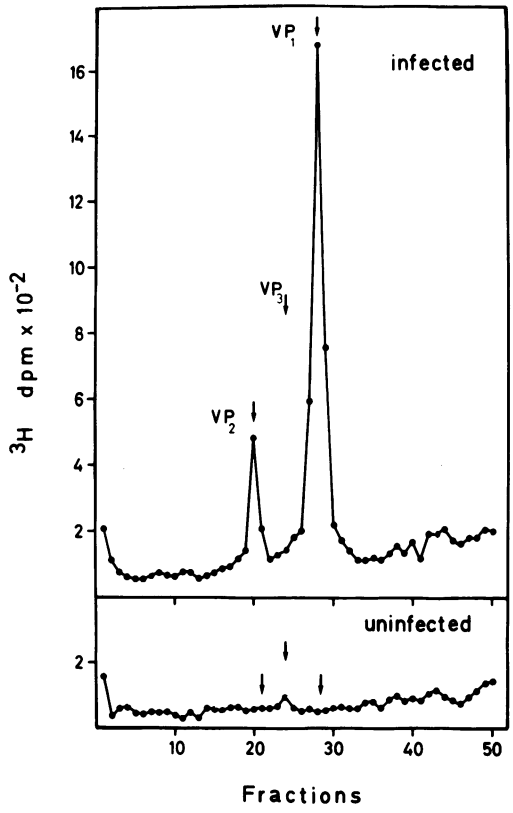

FIG. 5. *Viral polypeptides VP_1 and VP_2 in the nuclear sap protein fraction 13 to 14 h after infection. Synchronized LuIII virus-infected or mock-infected HeLa cells were labeled with [^{3}H]leucine (5 μCi/ml) and [^{3}H]valine (5 μCi/ml) for 2 h. Nuclear sap proteins were extracted from isolated nuclei, and samples were analyzed on 7.5% SDS-acrylamide slab gels. Arrows indicate the position of viral proteins.*

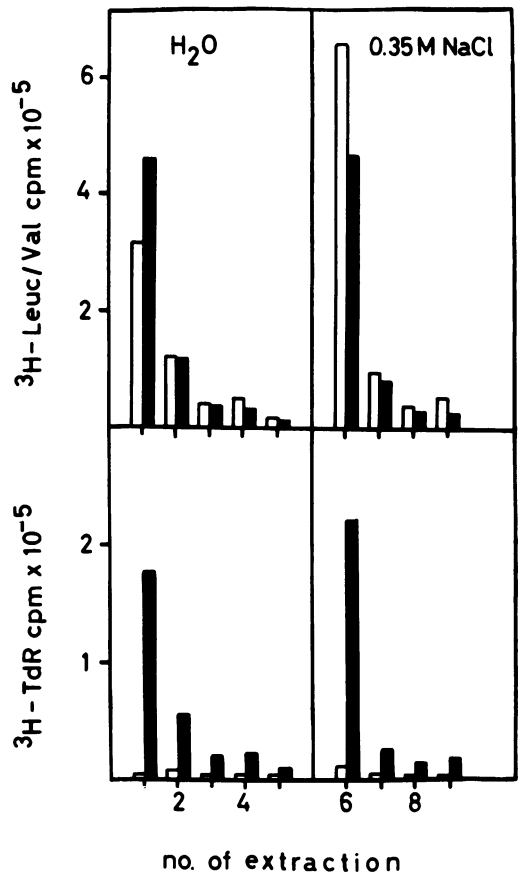

FIG. 6. *Recovery of labeled proteins (top) and DNA (bottom) in the water and 0.35 M NaCl supernatants (fraction F_{1b}) during extraction of chromatin under controlled conditions. LuIII-infected or mock-infected HeLa cells were labeled 14 h after infection with either [^{3}H]leucine (50 μCi/ml) and [^{3}H]valine (50 μCi/ml) or [^{3}H]thymidine (40 μCi/ml) for 1 h. After the nuclear sap was removed from isolated nuclei by a wash with 0.15 M NaCl, chromatin was extracted stepwise with water (extractions 1 through 5) and 0.35 M NaCl (extractions 6 through 9). Each extraction step was followed by sedimentation of the crude chromatin at 4,000 × g. The radioactivity recovered in the supernatants is given as mean value of two individual extractions from virus-infected (filled columns) and mock-infected cells (empty columns).*

period of 40 min (Fig. 7A) or after a 10-min pulse followed by either a 30- or 60-min chase period (Fig. 7B), about equal amounts of labeled VP_1 and VP_2 were detected in the respective fractions of the chromatin acidic proteins. In all of these samples, VP_1 and VP_2 were present in a constant ratio of about 5.5/1, which previously had been determined for isolated viral particles (6). On the other hand, the polypeptide in the position of VP_3, like other nonhistone chromosomal proteins, was no longer detectable after a chase of 60 min. These results strongly suggest that VP_1 and VP_2 become associated with the chromatin in the form of capsid-like structures after a time lag of about 20 to 30 min.

In further experiments, virus-infected or mock-infected cells were labeled 14 h p.i. for 40 min with [^{3}H]leucine and [^{3}H]valine. Chromatin was then isolated, dissociated in 3.5 M CsCl, and analyzed in buoyant density gradients. Figure 8 shows a clear peak at 1.31 g/ml, which corresponds to the density of empty viral capsids (19). When the radioactivity was chased for 90 min, additional material accumulated at the same density. The structures banding in the range of 1.29 to 1.37 g/ml were analyzed further by sedimentation in 10 to 30% sucrose gradients containing 0.35 M NaCl (Fig. 9). Most of the

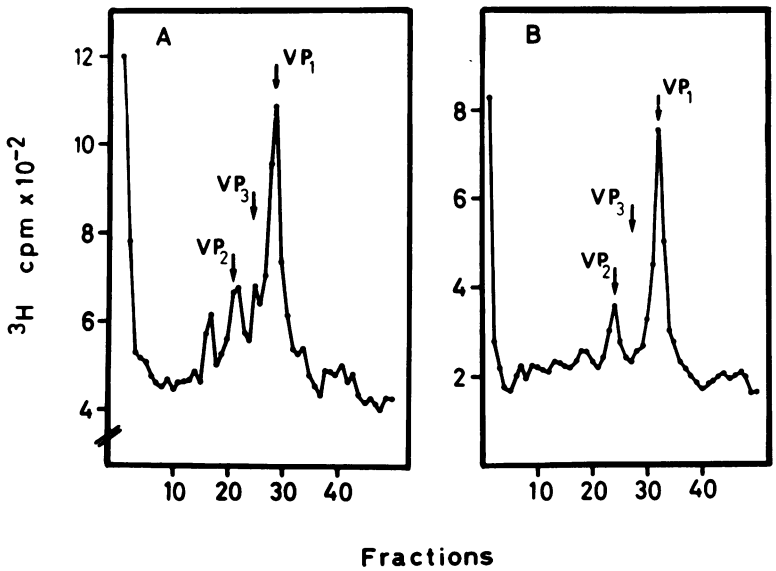

FIG. 7. *Labeling pattern of viral polypeptides accumulating in the chromatin acidic protein fraction. LuIII virus-infected or mock-infected HeLa cells were labeled 14 h after infection with [^{3}H]leucine (50 μCi/ml) and [^{3}H]valine (50 μCi/ml). Chromatin was isolated as indicated in the legend of Fig. 6, and chromatin acidic proteins (fraction F_3) were prepared and analyzed on SDS-acrylamide slab gels. (A) F_3 polypeptide pattern of LuIII virus-infected cells labeled for 40 min. (B) F_3 polypeptide pattern of LuIII virus-infected cells labeled for 10 min and chased for 60 min in the presence of a 100-fold excess of unlabeled leucine and valine. Arrows show the position of viral proteins.*

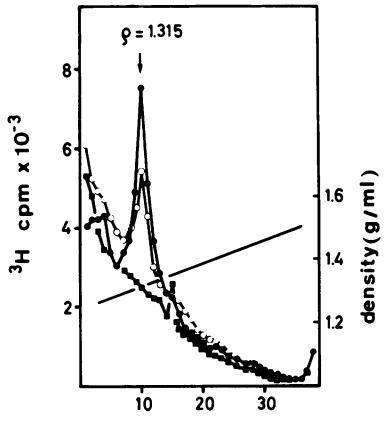

FIG. 8. *CsCl buoyant density gradient centrifugation of chromatin extracted from LuIII-infected and mock-infected HeLa cells. Cells were labeled with [^{3}H]valine and [^{3}H]leucine (50 μCi/ml each) 14 h after infection. Chromatin was prepared as described in the legend of Fig. 6, dissociated in CsCl solution having an average density of 1.39 g/ml, and centrifuged to equilibrium in a Beckman SW50.1 rotor at 35,000 rpm for 42 h at 15°C. Symbols: ■, chromatin of mock-infected cells labeled for 40 min; ○, chromatin of LuIII virus-infected cells labeled for 40 min; ●, chromatin of LuIII virus-infected cells labeled for 40 min and chased for 90 min in the presence of a 100-fold excess of unlabeled leucine and valine.*

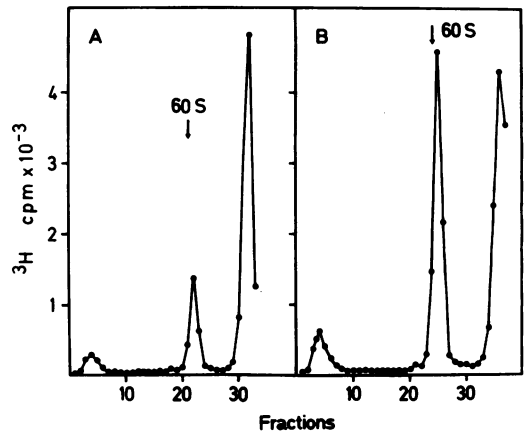

FIG. 9. *Sedimentation in 10 to 30% sucrose gradients of the structures banding at 1.29 to 1.37 g/ml in CsCl (Fig. 8). (A) Structures labeled during a 40-min pulse; (B) structures labeled during a 40-min pulse and chased for 90 min.*

pulse-labeled material sedimented either as a sharp peak at 55 to 60S or remained at the top of the gradient (Fig. 9A). After the chase period, the accumulated labeled material again sedimented predominantly with 55 to 60S, corresponding to the sedimentation value of empty capsids (Fig. 9B). The 55 to 60S, as well as the fast-sedimenting peak, represented essentially

labeled VP_1 and VP_2. Preliminary experiments showed the fast-sedimenting material to consist mainly of viral capsid-DNA structures aggregated under the ionic conditions in the gradient (data not shown).

Based on the data from buoyant density gradients, sedimentation analysis, and gel electrophoresis, it is concluded, therefore, that VP_1 and VP_2 accumulate in the cell chromatin mainly as empty capsid structures. As indicated by the density gradient profiles shown in Fig. 8 and 10, they account for more than 90% of the chromatin-associated labeled virus particles. Only less than 10% of them contained DNA. Thus, particles banding between 1.30 and 1.38 g/ml (Fig. 10) sedimented in sucrose gradients in the range of 65 to 95S, whereas those in the density range of 1.38 to 1.45 g/ml cosedimented preferentially with mature virions at 110S.

Detection of viral RF DNA in the chromatin. Assuming that the association of viral capsids with the cell chromatin represents an intermediate step in the process of viral replication, it may be speculated that their accumulation will take place on some specific site related to the replication of viral DNA. Therefore, experiments were performed to detect viral RF DNA within the chromatin of infected cells. Cells were labeled 14 h p.i. with [^{3}H]thymidine for 1 h. DNA was then extracted from the isolated chromatin by means of a Sarkosyl-Pronase treatment (see Materials and Methods) and was sedimented in neutral 5 to 20% sucrose gradients. DNA from control chromatin sedimented essentially as a broad peak around 25S, whereas DNA present within the chromatin of infected cells contained, in addition, a DNA species sedimenting predominantly at 16S (Fig. 11). These 16S molecules could also be isolated selectively from the chromatin fraction by the method of Hirt (9) and, as described previously (21), were characterized as viral RF DNA. The molecules appeared to be enriched 10- to 20-fold in the isolated chromatin over the total fraction F_{1b}.

DISCUSSION

It is well established that specific cellular helper functions are necessary for the replication of parvoviruses such as Kilham rat virus, H-1 virus, minute virus of mice, and LuIII virus within the nuclei of the host cells. These helper events occur in the late S or early G_2 phase of infected cells (8, 17, 20, 26, 27). So far, however,

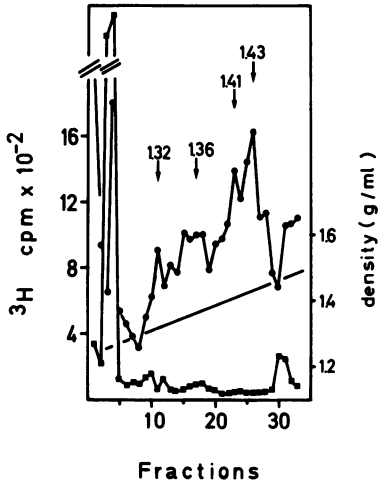

FIG. 10. *CsCl buoyant density gradient centrifugation of chromatin extracted from nuclei of LuIII-infected or mock-infected HeLa cells. At 14 h after infection, cells were labeled with [^{3}H]thymidine (30 μCi/ml) for 40 min, followed by a chase of 90 min in the presence of 10^{-5} M thymidine. The extraction and centrifugation conditions for chromatin were the same as described in the legend of Fig. 8. Symbols: ■, chromatin of mock-infected cells; ●, chromatin of LuIII-infected cells. Buoyant densities related to different viral particle species (19) are indicated by arrows.*

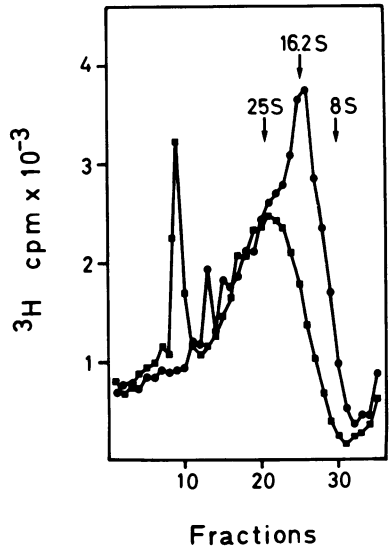

FIG. 11. *Sedimentation analysis of DNA isolated from the chromatin of LuIII-infected (●) and mock-infected (■) cells. HeLa cells were labeled with [^{3}H]thymidine (40 μCi/ml) for 1 h at 14 h after infection. Chromatin was extracted as described in the legend of Fig. 6. DNA was prepared from the chromatin fraction by Sarkosyl-Pronase treatment (see text), and samples were sedimented on neutral 5 to 20% sucrose gradients in a Beckman SW27.1 rotor at 23,000 rpm for 14 h at 4°C.*

it is not known whether the helper functions are provided by cellular enzymes involved in the virus replication machinery or whether, in addition, a distinct cellular structure is needed as a matrix for viral DNA replication and virus assembly.

In our first experiments, we investigated the time-dependent appearance of viral structural polypeptides in subnuclear fractions. The first evidence for the presence of newly synthesized VP_1 and VP_2 was obtained in the chromatin acidic protein fraction at 11 to 12 h p.i. (Fig. 4). At that time, the rate of histone synthesis (Fig. 2) suggested that both virus-infected and mock-infected cultures had arrived at the end of the S or in the early G_2 phase of the cell cycle. One to 2 h later, VP_1 and VP_2 also could be detected in significant amounts in the nuclear sap proteins (Fig. 5). The histone fraction, on the other hand, always proved free from virus-specific, acid-soluble polypeptides. Comparable to these observations, the structural polypeptides VP_1 and VP_2 of parvovirus H-1 were shown to appear in nuclear lysates of synchronized human NB kidney cells 13 to 14 h p.i. (11). Pulse-chase experiments, buoyant density studies, and sedimentation analysis revealed that over 90% of the chromatin-associated VP_1 and VP_2 represented empty viral capsid structures (Fig. 8 through 10). Although one might argue that viral capsids, originally present in the nuclear sap, had aggregated unspecifically with the chromatin during the isolation procedure, this seems unlikely for several reasons. (i) Following the time course of the appearance of viral polypeptides within the nuclear components, the nucleus-associated viral polypeptides were detected within the chromatin fraction before they could be identified clearly in the nuclear sap fraction (Fig. 3 through 5). (ii) The extraction procedure, outlined in Fig. 6, selectively released only 1/3 of the intranuclear VP_1 and VP_2 from the crude chromatin into the water supernatants. Again, the majority of them sedimented at 55 to 60S, like the empty viral capsids (M. Gautschi and G. Siegl, Experientia 31:738, 1975). Subsequent treatment of the chromatin with 0.35 M NaCl, however, removed no additional viral polypeptides. According to Johns and Forrester (10), this would indicate that capsids remaining in association with the chromatin under these conditions might be strongly bound to uncovered phosphate groups of DNA molecules. (iii) When chromatin was prepared from unlabeled infected or uninfected cells in the presence of labeled nuclear sap from infected cells (Fig. 5), only negligible amounts of labeled viral polypeptides were found as contaminants within the isolated chromatin. Therefore, these results suggest that the cellular chromatin obviously represents the subnuclear structure, in which mainly empty viral capsids accumulate specifically in a late stage of virus replication. This conclusion is also supported by the results of Singer and Toolan (22), who, by ultrahistological techniques, found empty capsids as well as complete virions of parvovirus H-1 preferentially situated on extranucleolar chromatin fibers during virus replication.

Besides empty capsids and DNA-containing viral particles, the chromatin fraction of infected cells contained almost all of the intranuclear double-stranded RF DNA (21) of parvovirus LuIII (Fig. 11). Although a specific and high binding affinity of a distinct fraction of viral particles for components of the host cell chromatin cannot be ruled out, it is more likely that synthesis of viral DNA and formation of complete virions are structurally related processes: RF DNA is specifically bound to a defined section of the cellular chromatin and, successively, becomes complexed with empty viral capsids. This point of view is also supported by studies with simian virus 40 (13, 14), adenovirus (25), and herpesvirus (3), where there is good evidence for viral DNA being packaged into preformed capsids. Moreover, the association of replicating DNA-capsid complexes with the cellular chromatin in the late S or early G_2 phase might emphasize the dependence of parvovirus replication on cellular helper events.

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