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Assembly of Empty Capsids by Using Baculovirus Recombinants Expressing Human Parvovirus B19 Structural Proteins

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Empty parvovirus B19 capsids were isolated from insect cells infected with a recombinant baculovirus expressing parvovirus B19 VP2 alone and also with a double-recombinant baculovirus expressing both VP1 and VP2. That VP2 alone can assemble to form capsids is a phenomenon not previously observed in parvoviruses. The stoichiometry of the capsids containing both VP1 and VP2 was similar to that previously observed in parvovirus B19-infected cells. The capsids were similar to native capsids in size and appearance, and their antigenicity was demonstrated by immunoprecipitation and enzyme-linked immunosorbent assay with B19-specific antibodies.

Little information is available on the three-dimensional structure of parvoviruses except that the nonenveloped capsids have icosahedral symmetry with a diameter of 22 nm (7). Preliminary results of X-ray crystallographic analysis of canine parvovirus suggest a pseudo T = 3 lattice in which approximately 60 copies of the major coat protein would have a tandem repeat of three similar β-barrel folds per subunit arranged as in pseudo T = 3 viruses (13). This agrees with estimates of 60 to 72 molecules per virion (19).

Since small amounts of empty capsids of human parvovirus B19 (1,000 to 2,000 per cell) are formed spontaneously from their components in Chinese hamster ovary cells (12), the possibility that the capsid proteins of parvovirus B19 would form particles when expressed by a recombinant baculovirus in insect cells was explored. The baculovirus expression vector (21) has been successfully used for the synthesis of large amounts of poliovirus particles (22) and bluetongue virus core particles (10). High expression levels would enable the isolation of large amounts of parvovirus B19 capsids for X-ray crystallographic analysis and provide a model system for the study of the mechanism of capsid assembly. Such particles may well be a suitable substrate for diagnostic use and vaccine development. The use of recombinant DNA techniques for the production of capsids is necessitated by the fact that parvovirus B19 only replicates in explants of human erythroid bone marrow (17). Since symptoms of a B19 virus infection occur after viremia (1), large amounts of virus have been isolated from serum only by random screening of blood donors.

The major coat protein of parvovirus B19, VP2 (58 kDa), is an NH_2-terminal-truncated product of VP1 (16) (Fig. 1A) and makes up about 96% of the capsid protein (18). Transcriptional and translational control results in a very low abundance (about 4%) of the minor coat protein, VP1 (84 kDa), in infected cells (15). To determine whether VP2 could self-assemble into capsids in the absence of VP1, we used the recombinant baculovirus containing the VP2 gene under the control of the polyhedrin promoter, AcB19VP2L (3). A double-recombinant baculovirus (AcB19VP12L), expressing VP1 from the p10 promoter and VP2 from the polyhedrin promoter, was constructed for the production of capsids incorporating both VP1 and VP2 which may mimic the authentic B19 capsid. *Spodoptera frugiperda* (Sf9) cells infected with the VP2 recombinant synthesized large amounts of VP2, and cells infected with the double recombinant synthesized large amounts of VP1 and VP2. In both cases, the proteins assembled to form empty capsids, the characterization of which is described here.

Baculovirus expression of B19 virus proteins. Baculoviruses were grown and plaque assayed (5) in monolayers of Sf9 cells (ATCC CRL 1711) in TC-100 medium (GIBCO/BRL) containing 10% fetal calf serum and 50 mg of gentamicin per ml. Infections were done on monolayers of *S. frugiperda* cells at a multiplicity of infection of 20 PFU per cell. The construction of the recombinant baculoviruses AcB19VP1L (VP1 recombinant) and AcB19VP2L (VP2 recombinant), expressing VP1 and VP2, respectively, has been described previously (3).

Since double infections with the VP1 and VP2 recombinants were difficult to control, a double recombinant expressing both VP1 and VP2 was constructed. The VP1 gene was cloned behind the baculovirus late p10 promoter (24) in the vector pAcAS3, as described in the legend to Fig. 1, creating construct pAcAS3.VP1. Plasmid pAcAS3 contains a DNA cassette comprising the *Drosophila melanogaster* heat shock promoter, hsp70, the *lacZ* gene (for identification of recombinants), and the simian virus 40 transcription initiation and termination sequences (25). Cotransfection of AcB19VP2L DNA with pAcAS3.VP1 on monolayers of *S. frugiperda* cells resulted in a double recombinant (AcB19VP12L) with VP2 expressed from the polyhedrin promoter and VP1 expressed from the p10 promoter. The proteins produced by the VP1, VP2, and double recombinants were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 2A). The 58-kDa protein produced by the VP2 and double recombinants and the 84-kDa protein produced by the VP1 and double recombinants migrated at the positions of the B19 virus-specific major and minor capsid proteins, respectively. Western immunoblot analysis (performed as described in reference 3) of the protein components of infected insect cells confirmed the identity of VP1 and VP2 (Fig. 2B). The

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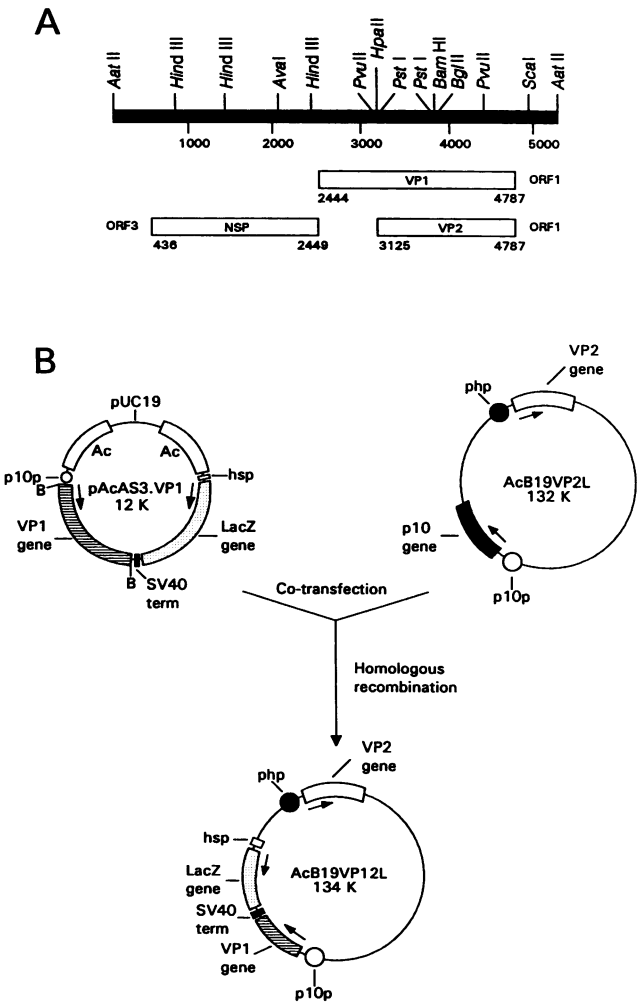

FIG. 1. (A) Genome organization of parvovirus B19. (B) Construction of baculovirus recombinants. The DNA encoding VP1 and VP2 was cloned in the plasmid vector pAcYM1 via plasmids pUC19 and pUC7, and cotransfection with *A. californica* nuclear polyhedrosis virus DNA resulted in the recombinant baculoviruses AcB19VP1L and AcB19VP2L (as described in reference 3). In the same manner, the VP1 gene was cloned in the plasmid vector pAcAS3 and cotransfected with AcB19VP2L DNA. Upon recombination in insect cells, the double-recombinant baculovirus AcB19VP12L was isolated. ORF, open reading frame; Ac, *A. californica* nuclear polyhedrosis virus sequences flanking the p10 gene; p10p, p10 promoter; php, polyhedrin promoter; hsp, *D. melanogaster* hsp70; SV40 term, simian virus 40 termination sequence; B, *Bam*HI site; NSP, nonstructural protein. Arrows show direction of transcription.

level of expression of VP1 and VP2 was estimated by densitometric scanning of a polyacrylamide gel. For VP1, this was approximately 40 mg/10^9 cells, for VP2 it was 50 mg, and for the double recombinant it was 33 mg of VP2 and 13 mg of VP1. For the VP2 and double recombinants, this is sufficient protein to produce approximately 10^7 particles per insect cell, assuming each particle consists of 60 molecules (19). In infected erythroid cells, about 8×10^3 particles are produced per cell (12). The ratio of total VP1/VP2 produced in insect cells was about 1:2.5, and the lower amounts of VP1 produced in this system compared with VP2 were expected to be favorable for capsid assembly.

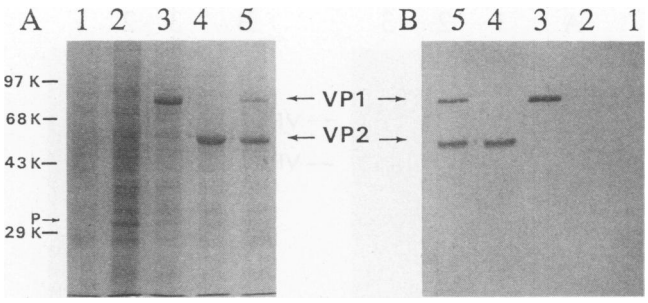

FIG. 2. Expression of parvovirus B19 structural proteins VP1 and VP2 in insect cells by recombinant baculoviruses. (A) Fast green-stained SDS-polyacrylamide gel of protein components of insect cells that were mock infected (lane 1), *A. californica* nuclear polyhedrosis virus infected (lane 2), VP1 recombinant infected (lane 3), VP2 recombinant infected (lane 4), or double recombinant infected (lane 5). Cells were harvested 72 h postinfection. P, Polyhedrin. (B) Western immunoblot of panel A with human serum containing B19 virus-specific IgG. K, kilodaltons.

Detection and purification of capsids expressed in insect cells. To determine whether insect cells expressing B19 VP2 or both VP1 and VP2 could synthesize particles, we infected *S. frugiperda* monolayers with the VP2, double, or VP1 recombinant. Cells were harvested 3 days postinfection, washed with phosphate-buffered saline (PBS), and lysed by sonication in PBS. Cell debris was removed by low-speed centrifugation, and the supernatants were layered on a 40% (wt/wt) sucrose cushion and centrifuged for 2.5 h at 100,000 $\times$ *g*. The pellets were resuspended in PBS and analyzed in a Philips CM12 electron microscope after negative staining with 0.05 M uranyl acetate (pH 3.6). Since particles could be seen in both the VP2 and double-recombinant preparations, the pellets were banded in a linear sucrose gradient (15 to 30% [wt/wt]) by centrifugation for 2.5 h at 110,000 $\times$ *g*. The pellet from the VP1 recombinant was also run in the sucrose gradient. An opalescent band could be seen for the VP2 and double recombinants which corresponded to a protein peak as monitored at 280 nm. The VP1 recombinant preparation contained no visible band and did not show a protein peak. Sucrose gradient fractions corresponding to the banded material for the VP2 and double recombinants, and the same fraction of the VP1 gradient were analyzed by SDS-PAGE, and the proteins were detected by silver staining of the gel (14). The fraction from the VP2 recombinant contained mainly VP2, that from the double recombinant contained chiefly VP1 and VP2, and the fraction from the VP1 recombinant contained no detectable protein (Fig. 3A). The identity of the proteins was confirmed by Western immunoblot analysis (Fig. 3B). The copurification of VP1 with VP2 in the double recombinant suggests that VP1 is incorporated in the particle.

The sucrose gradient fractions were analyzed in the electron microscope as described above. Large numbers of particles could be seen in both the VP2 (Fig. 4A) and double-recombinant (Fig. 4B) preparations. The corresponding fraction from the VP1 gradient did not contain particles. Both types of particles had an appearance similar to that of native B19 virus particles and a diameter of approximately 22 nm. Most of the particles were isometric and thus intact. Similar results were obtained with CsCl gradients (data not shown).

Sucrose gradient-purified particles from the VP2 and double recombinants were shown to be empty by hybridization

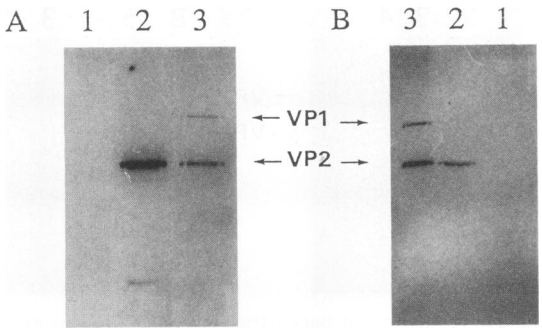

FIG. 3. Analysis of protein components of purified capsids. (A) Silver-stained SDS-polyacrylamide gel of sucrose gradient fractions prepared from VP1 recombinant-infected cells (lane 1), VP2 recombinant-infected cells (lane 2), and double-recombinant-infected insect cells (lane 3). (B) Western immunoblot of panel A with human serum containing B19 virus-specific IgG.

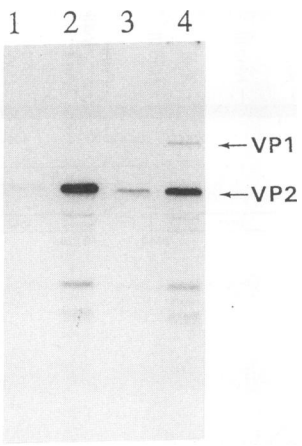

FIG. 5. Immunoprecipitation with human serum of [^{35}S]methionine-labeled purified capsids isolated from insect cells expressing VP2 or both VP1 and VP2. Lanes: 1, precipitation of VP2 capsids with B19 virus-specific IgG-negative serum; 2, precipitation of VP2 capsids with B19 virus-specific IgG-positive serum; 3, precipitation of VP1-VP2 capsids with B19 virus-specific IgG-negative serum; 4, precipitation of VP1-VP2 capsids with B19 virus-specific IgG-positive serum.

with parvovirus B19 and *Autographa californica* nuclear polyhedrosis virus DNA (data not shown).

The amount of both types of particles produced was estimated to be approximately 10 mg/10^9 cells, which is equivalent to about 2×10^6 particles per insect cell assuming that each particle consists of 60 molecules (19).

Electron microscopy of ultrathin sections of insect cells expressing VP1, VP2, or both VP1 and VP2 showed that the proteins were localized to the cytoplasm and indicated that VP1 and VP2 associate in the cells in the case of the double recombinant (data not shown).

Antigenicity of intact particles. Antigenicity of intact particles was investigated by immunoprecipitation of sucrose gradient-purified [^{35}S]methionine-labeled capsids with B19 virus-specific immunoglobulin G (IgG) (Fig. 5). Proteins were labeled in vivo (21), and immune complexes were bound to Pansorbin (Calbiochem) as described in reference 23. VP2 was specifically precipitated with serum containing B19 virus-specific IgG from the VP2 capsid preparation (Fig. 5, lane 2), and both VP1 and VP2 were precipitated with B19 virus-specific IgG from the capsid preparation isolated from cells infected with the double recombinant (Fig. 5, lane 4). This again indicates that capsids synthesized from the double recombinant contain VP1. Serum containing no B19 virus-specific IgG precipitated very low levels of protein as shown in Fig. 5, lane 1, for VP2 particles and Fig. 5, lane 3, for VP1-VP2 particles. Precipitation of the capsids by B19 virus-specific antibodies demonstrates that both types of capsid are antigenic.

To determine the stoichiometry of the VP1-VP2 particles, we had to exclude the possibility that particles isolated from insect cells infected with the double recombinant consist of a mixed population of VP2 particles and particles containing both VP1 and VP2. This was investigated by using a VP2-specific monoclonal antibody (MAb) (Biosoft, Paris, France)

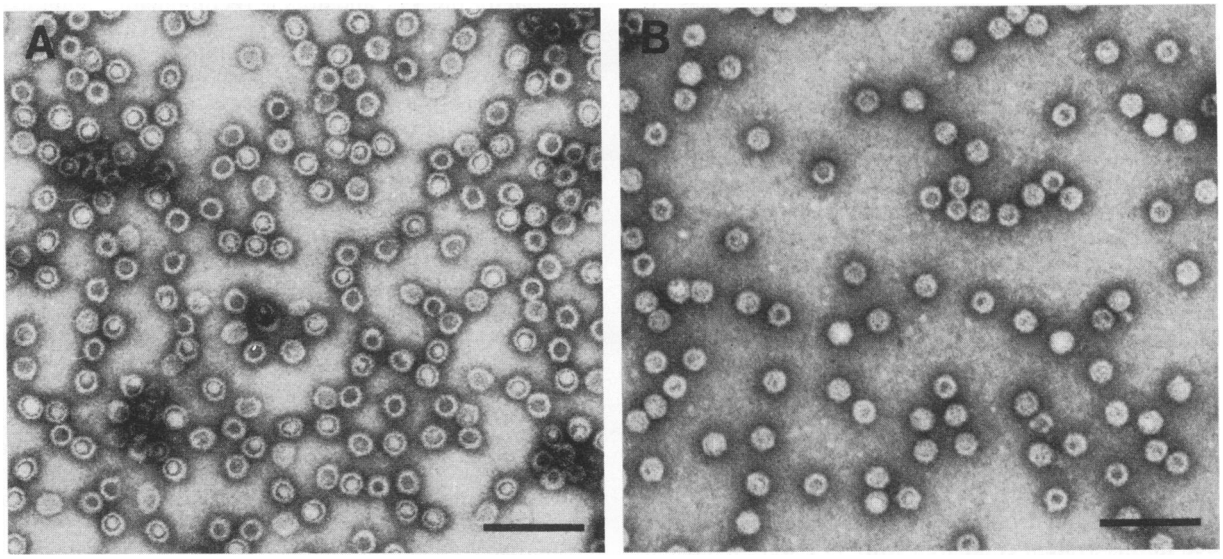

FIG. 4. Electron micrographs of sucrose gradient-purified particles. (A) VP2 particles; (B) VP1-VP2 particles. Bar, 100 nm.

TABLE 1. Reactivity of two B19 virus-specific MAbs with sucrose gradient-purified particles in an ELISA

MAb	Extinction (OD_{492})[a]			
	VP2 particles		VP1-VP2 particles	
	PBS	pH 9.5 buffer[b]	PBS	pH 9.5 buffer[b]
VP2 specific	1.72	NT[d]	0.31	NT
VP1 specific[c]	0.17	0.16	0.69	1.29

[a] Average extinction from two experiments. OD_{492}, Optical density at 492 nm.
[b] 25 mM $NaHCO_3$, pH 9.5.
[c] Produced against a β-galactosidase–B19 virus fusion protein (20).
[d] NT, Not tested.

TABLE 2. Reactivity of 11 human serum samples with baculovirus-expressed B19 antigens in an ELISA

Extinction (OD_{492})	Antigen		
	VP1[a]	VP2[b]	VP1-VP2[b]
≤0.5[c]	3	3	3
0.5–1.0	2	0	0
1.0–2.0	5	0	0
>2.0	1	8	8

[a] VP1 purified from SDS-PAGE.
[b] Sucrose gradient-purified capsids.
[c] Cutoff value for negative sera is 0.5.

in an enzyme-linked immunosorbent assay (ELISA) (Table 1). Particles were coated onto microtiter plates in PBS at a concentration of 200 ng per well, and the MAb was diluted 1:200. Detection of bound antibody was with a 1:8,000 dilution of peroxidase-conjugated rabbit anti-mouse IgG (Dakopatts, Glostrup, Denmark), and the substrate was *o*-phenylenediamine. The MAb reacted strongly with the VP2 particles and showed only background reactivity with particles containing both VP1 and VP2. This indicated that the particles isolated from the double recombinant all contained VP1 and allowed their stoichiometry to be determined. Densitometric scanning of autoradiographs of immunoprecipitated VP1-VP2 particles from two experiments showed a ratio of 1:11 VP1/VP2, which is similar to the ratio of 1:10 to 20 found in erythroid cells (12). In addition, these results suggested that there are differences in the structure of the two types of particles.

Both types of capsids were also tested with a 1:4,000 dilution of a VP1-specific MAb (a gift from J. Middeldorp), produced against a β-galactosidase-B19 virus fusion protein (20), after coating in PBS or 25 mM $NaHCO_3$ (pH 9.5) (Table 1). Detection was as described above. The MAb showed background reactivity with the VP2 particles coated in either PBS or 25 mM $NaHCO_3$ (pH 9.5). When the VP1-VP2 particles were coated in the pH 9.5 buffer, the MAb showed an almost 100% increase in reactivity compared with that against particles coated in PBS, suggesting that more VP1 protein was available to react at high pH. This result can be explained assuming that the unique NH_2 terminus of VP1 is situated on the inside of the capsid and that the capsids are unstable at high pH. It is known that native parvovirus particles are unstable above pH 9.0, and it has previously been suggested that the N-terminal region of VP1 is located internally (6, 8).

The reactivity of the purified capsids with human serum samples containing B19 virus-specific IgG was further tested in an ELISA (Table 2). As a positive control, VP1 that had been purified from a polyacrylamide gel was used. Protein (200 ng) was coated in PBS and incubated with a 1:100 dilution of eight serum samples positive for B19 virus-specific IgG and three negative serum samples, the status of which had been determined in an immunofluorescence assay (4). Bound antibody was detected with a 1:8,000 dilution of peroxidase-conjugated rabbit anti-human IgG (Dakopatts), and the substrate was *o*-phenylenediamine. All positive serum samples reacted with purified VP1 and both types of capsids, while the negative serum samples did not react. Again, this is confirmation of the antigenicity of the capsids and shows that both types of capsids form a very suitable substrate for an ELISA for B19 virus antibodies. Seven of the positive serum samples gave a higher extinction with the capsids than with purified VP1. This may be due to the fact that the capsids more closely resemble native virus than purified VP1, which is in a more or less denatured form, and thus react better with virus-specific antibodies.

The availability of an unlimited supply of particles of this difficult-to-culture human pathogen not only will facilitate the development of a diagnostic test and a vaccine but will also make it possible to perform X-ray crystallographic studies and to elucidate the mechanism of parvovirus B19 particle formation. Information on the three-dimensional structure, in combination with MAbs, will in turn make possible the localization of antigenic determinants. By replacing these determinants with foreign epitopes, such particles may then be used as a carrier for epitope presentation, as has already been described for poliovirus (9, 11) and hepatitis B virus core antigen (2).

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