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Characterization of Capsid and Noncapsid Proteins of B19 Parvovirus Propagated in Human Erythroid Bone Marrow Cell Cultures

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The major capsid and noncapsid proteins of the pathogenic parvovirus B19, propagated in vitro, were detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, immunoprecipitation, and immunoblot of the erythroid fraction of infected human bone marrow cell cultures. There were two capsid proteins of 58 kilodaltons (kDa; the major species) and 84 kDa (the minor species). Newly synthesized capsid viral proteins were present in the supernatants of infected cultures. The major noncapsid protein of 77 kDa was localized to the nucleus.

The parvovirus B19 can cause bone marrow failure in humans; it most commonly causes transient aplastic (erythroblastopenic) crisis in individuals with underlying hemolysis (20). However, the virus also has been associated with significant thrombocytopenia and leukopenia in both clinically and experimentally infected subjects (1, 20). In a patient with underlying immunosuppression, persistent B19 infection has caused transfusion-dependent anemia and severe neutropenia (G. Kurtzman, K. Ozawa, B. Cohen, G. Hanson, R. Oseas, and N. S. Young, N. Engl. J. Med., in press). The tropism of the B19 parvovirus for hematopoietic cells has been used to develop a unique culture system for viral propagation, which employs bone marrow from patients with sickle cell disease (15). B19 parvovirus intermediate replicative forms have been demonstrated in the nuclei of infected erythroid, but not myeloid, bone marrow cells (K. Ozawa, G. Kurtzman, and N. Young, Blood, in press); B19 parvovirus has not been propagated in any cell line (15). The pattern of parvovirus RNA transcription within these cells is distinctive in comparison with that in other parvoviruses (14). In this report we describe the capsid and noncapsid B19 parvovirus proteins generated in the natural target cells of the virus.

Bone marrow mononuclear cells, which were obtained from patients with informed consent, were mixed with 4 μl of serum-containing B19 parvovirus (Minor II; 60 μg of B19 DNA per ml) per 2×10^7 cells in 0.4 ml. Following absorption at 4°C for 2 h, cells were cultured at 2×10^6/ml in the Iscove modification of Dulbecco medium (IMDM) with recombinant erythropoietin (1 U/ml; Amgen Biologicals, Thousand Oaks, Calif.)–20% fetal calf serum as described previously (15). For all experiments, cells were harvested at 40 to 48 h postinoculation. For polyacrylamide gel electrophoresis (PAGE) with and without immunoprecipitation, cells were labeled with [^{35}S]methionine (2). Cells were washed twice with methionine-free IMDM and then incubated at 5×10^6 cells per ml with methionine-free IMDM containing 5% dialyzed fetal calf serum and 1 U of erythropoietin per ml for 30 min at 37°C to deplete intracellular methionine. Labeling was accomplished by the addition of 100 μCi of [^{35}S]methionine (New England Nuclear Corp., Boston, Mass.) per ml for 3 to 4 h. After cells were washed with IMDM-fetal calf serum, they were separated into erythroid and leukocyte fractions by panning with anti-leukocyte antibodies (anti-Leu M1 and anti-Hle-1 [Becton Dickinson and Co., Mountain View, Calif.]) as described by Ozawa et al. (in press). For the study of proteins in culture supernatants, cells were washed on day 1 with methionine-free IMDM and incubated with 1 μg of cold methionine per ml, 10% dialyzed fetal calf serum, 1 U of erythropoietin per ml, and 10 μCi of [^{35}S]methionine per ml; on day 3 the virus from culture supernatants was harvested by ultracentrifugation on 40% sucrose–phosphate-buffered saline at $100{,}000 \times g$ overnight at 4°C. Cells and the particulate fraction of the supernatants were dissolved in lysing buffer containing proteinase inhibitors (1 mM phenylmethylsulfonyl fluoride, 100 U of aprotinin per ml, and 1 mM diisopropylfluorophosphate) and, for direct sodium dodecyl sulfate (SDS)-PAGE (10), diluted with sample buffer prior to application to 8% gels. For immunoprecipitation (2), samples were precleared with nonimmune serum and protein A-Sepharose (Pharmacia Fine Chemicals, Piscataway, N.J. [Div. Pharmacia, Inc.]), treated with antibodies to B19 parvovirus (high-titer human convalescent serum or rabbit anti-capsid or anti-noncapsid recombinant proteins; gifts from S. Cotmore and P. Tattersall) overnight at 4°C, and precipitated with protein A-Sepharose. Immunoblotting was performed with unlabeled proteins by using the same antibodies by a slight modification of a previously published procedure (19); for these experiments, erythroid and leukocyte cells were fractionated into subcellular components by Dounce homogenization (15). For supernatant analysis the viral inoculum was removed by washing at day 1. After SDS-PAGE, the samples were transferred to Gene Screen Plus (New England Nuclear), and specific proteins were detected with ^{125}I-labeled protein A by using the BLOTTO (Bovine Lacto Transfer Technique Optimizer) method (8).

SDS-PAGE of radioactively labeled cells showed a prominent band at 58 kilodaltons (kDa) in infected but not in control cultures (Fig. 1). By densitometry, the 58-kDa protein, which corresponded to the major capsid protein, constituted 10% of the total cell protein synthesized and 15% of the nonglobin protein (>20 kDa) synthesized. The 58-kDa species was observed only in the erythroid cell fraction and not in the leukocyte fraction.

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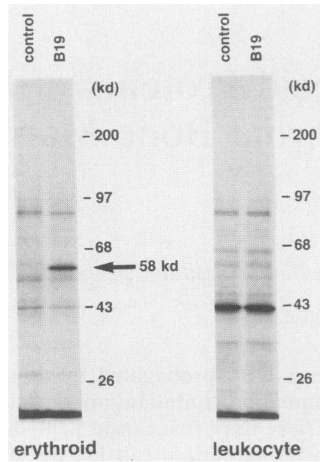

FIG. 1. SDS-PAGE of [^{35}S]methionine-labeled proteins from infected (B19) and uninfected (control) cultures that were harvested at 40 h and labeled with radioisotope for 4 h. Cells were fractionated into erythroid and leukocyte fractions by panning.

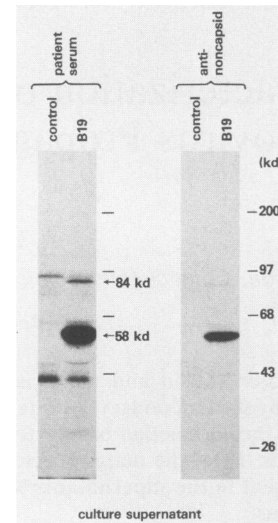

FIG. 3. Detection of newly synthesized B19 capsid proteins in culture supernatants. The particulate fractions of supernatants were immunoprecipitated after metabolic radioisotope labeling. (Aggregation of capsid proteins during incubation with antiserum led to the appearance of bands at 58 and 84 kDa in some experiments.)

B19 capsid and noncapsid proteins were resolved by immunoprecipitation with B19 parvovirus-specific sera of infected erythroid cell lysate proteins; no B19 proteins were observed in leukocyte fractions of infected cultures. The major 58-kDa capsid protein (96% of total capsid protein by densitometry) and the minor 84-kDa capsid species (4% of capsid protein) were detected with a specific anti-capsid rabbit serum and serum from a patient with a high titer of anti-B19 immunoglobulin G (IgG) (Fig. 2). Three putative noncapsid proteins were detectable with a rabbit anti-noncapsid protein serum sample and, more faintly, also with a serum sample from a patient. The major 77-kDa protein has been predicted to be encoded by a long open reading frame from the left side of the B19 genome; by employing transfection of vectors derived from portions of the cloned B19 genome and their mutants, the 77-kDa protein has been shown to be the product of a 2.3-kilobase unspliced transcript from the left side (T. Shimada, K. Ozawa, J. Ayub, H. Yu-Shu, G. Kurtzman, and N. Young, unpublished data). The shorter species of 52 and 34 kDa were reproducibly observed, but appropriate transcripts for proteins of these sizes have not been detected in infected erythroid bone marrow cells (14); these proteins might represent degradation products or a posttranslational modification of a single large noncapsid protein. Radioactively labeled B19 capsid proteins were detected as well in the particulate fraction of supernatants, indicating the release of newly synthesized intact virions from infected cells (Fig. 3); as expected, nonstructural protein was not detected in culture supernatants.

B19 capsid and noncapsid proteins were localized to

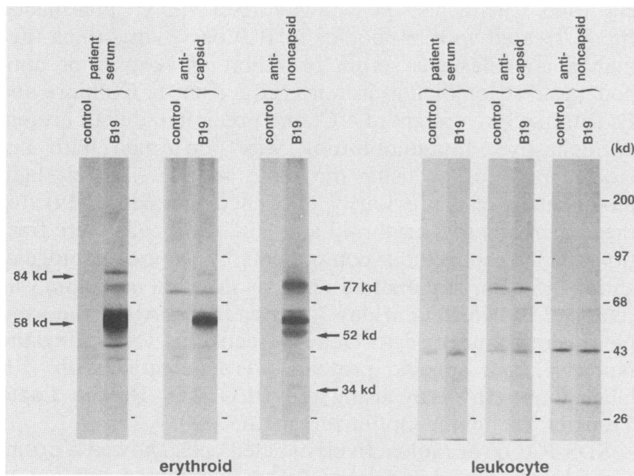

FIG. 2. Immunoprecipitation of ^{35}S-labeled B19 proteins in infected cells. Cells were treated as described in the legend to Fig. 1 and were lysed and reacted to antisera to B19: patient serum with a high titer of anti-B19 IgG or rabbit sera prepared to capsid and noncapsid proteins produced in procaryotes (4). No B19 proteins were detected in control or leukocyte fractions from infected cultures.

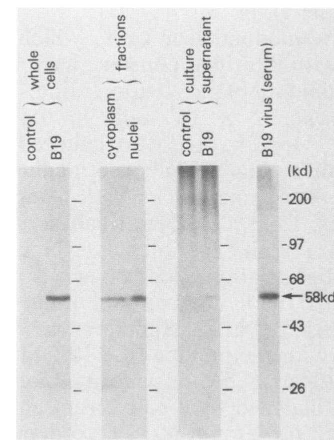

FIG. 4. Immunoblot analysis for capsid protein in the erythroid fraction. Shown are whole cells, subcellular fractions, culture supernatants, and virions by using serum from patients. Capsid protein was present in cells, cytoplasm, nuclei, and supernatants as well as virions. Capsid proteins were not detected in the leukocyte fraction (data not shown).

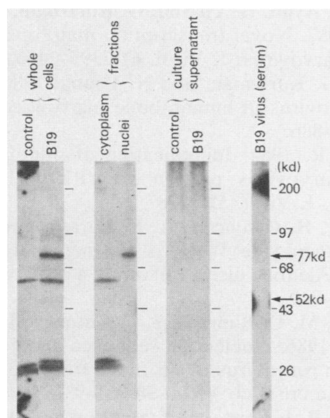

FIG. 5. Immunoblot analysis for noncapsid protein by using anti-recombinant noncapsid protein serum. Data for the erythroid fraction are shown; noncapsid proteins were not detected in the leukocyte fraction. Noncapsid protein distribution was restricted to nuclei of infected erythroid cells.

subcellular fractions by immunoblotting (Fig. 4 and 5). The 58-kDa protein was present in nuclei, cytoplasm, and supernatants of cultures (the 83-kDa protein was detected with a longer exposure of the filters; data not shown). By immunofluorescence with the same antibodies, capsid protein also was localized to the cytoplasm (predominantly) and nuclei of a minority of infected cells in culture (Ozawa et al., in press). In contrast, the noncapsid protein was present only in nuclei of infected cells and was absent from cytoplasm and culture supernatants as well as from intact virions in sera. Possible signals for nuclear transport of this protein are present in the nucleotide sequence (Lys-Lys-Pro-Arg, nucleotides 964 to 975; Lys-Lys-Cys-Gly-Lys-Lys, nucleotides 1381 to 1398; 17a), as described previously for other parvovirus proteins (11). The nuclear localization of noncapsid protein is compatible with its predicted role in the regulation of viral replication.

The likely relationship between B19 proteins and the corresponding transcripts (14) is shown in Fig. 6. B19 capsid proteins of 58 and 84 kDa have been detected by immunoblotting in the sera of infected patients (N. Young and K. Ozawa, *in* J. R. Pattison, ed., *Parvoviruses and Human Disease* in press) and fetal tissue of a spontaneous abortus (4). These proteins are similar in number, size, and relative proportions to capsid proteins of other animal parvoviruses (7, 8, 12, 17, 18). In particular, the major capsid species (VP2) weighed 58 kDa, which is similar to those of the dominant capsid proteins of several adeno-associated viruses, feline panleucopenia virus, LuIII virus, minute virus of mice, and Kilham rat virus. Like Cotmore et al. (4), we observed three noncapsid proteins, although ours had somewhat different molecular masses. The 77-kDa protein is close in size to the predicted product of 86 kDa from the open reading frame on the left side (4). The noncapsid proteins of parvoviruses are highly conserved, and others of this size have been reported for adeno-associated virus (13), Aleutian disease virus (3, 9), H1 (9, 16), and minute virus of mice (5, 6, 9). The origin of the other two minor noncapsid species is uncertain; in contrast to fetal tissue, autolysis under our experimental conditions is unlikely. Conversely, we were unable to detect lower molecular weight proteins that would be predicted from transcript length.

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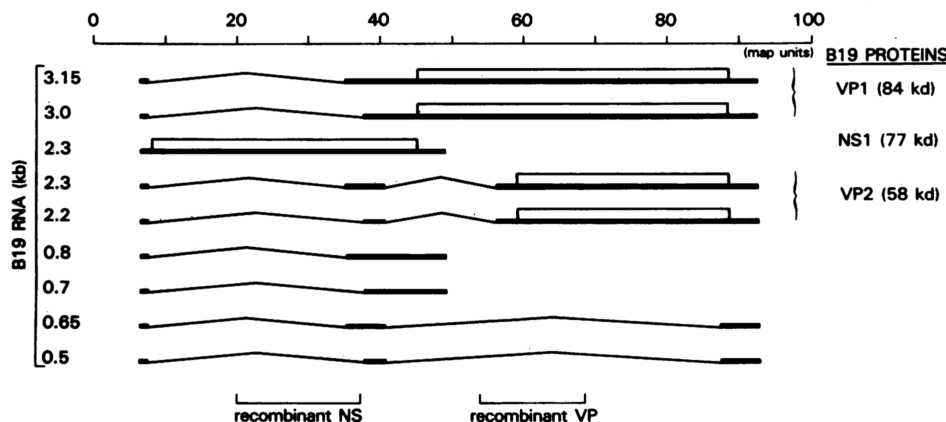

FIG. 6. Functional map of the B19 genome (14). Solid lines show RNA, and the exons are emphasized; open boxes show protein-coding regions. By convention, capsid viral proteins (VP) and nonstructural proteins (NS) of the *Parvoviridae* are numbered in descending order of molecular mass. The locations of the recombinant capsid and noncapsid proteins which were used to raise antibodies (4) are indicated at the bottom of the figure.

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