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A genetically engineered cell line that produces empty capsids of B19 (human) parvovirus

(transfection/fifth disease/transient aplastic crisis/immunoassay/vaccine)

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ABSTRACT **B19 parvovirus is pathogenic in humans, causing the common childhood exanthem fifth disease and bone-marrow failure, both acute (transient aplastic crisis of hemolysis) and chronic (pure erythrocyte aplasia in immunodeficiency). The virus is tropic for a human red cell progenitor cell, and failure to culture B19 in a cell line has limited its clinical study. We cotransfected the right half of the cloned B19 genome and a minigene derived from the human dihydrofolate reductase gene (*DHFR*) into *dhfr*− Chinese hamster ovary cells and screened selected clones by RNA analysis; after amplification in methotrexate, clones were tested for capsid protein expression. A cell line, designated 3-11-5, stably expressed nearly full-length transcripts for the two capsid proteins. These cells produced the major and minor structural protein species in natural proportions that self-assembled into virion capsids. Capsids from 3-11-5 cells could be separated from virions by sucrose gradient sedimentation and had the density on cesium chloride isopycnic sedimentation of empty parvovirus capsids. Capsid protein was present in both nuclei and cytoplasm on immunofluorescence study but fractionated with the cytosol on purification. Empty capsid production was equal to or greater than virion production by infected bone-marrow cells, 1000–2000 capsids per cell, but cell growth was not diminished by capsid production. This cell line will be useful in developing practical assays for B19 parvovirus antibody and a vaccine for the virus, as well as potentially serving as a packaging cell line for gene therapy.**

B19 parvovirus causes several human diseases (1). In normal children, virus infection leads to fifth disease, a common, measles-like rash illness (2). Adults with fifth disease usually have inflammatory joint symptoms, which may persist in a rheumatoid arthritis-like disease. In persons with underlying hemolysis, B19 causes transient aplastic crisis (3), and in patients with underlying immunodeficiency, persistent infection leads to chronic pure red cell aplasia (4, 5). Fetal infection can result in nonimmune hydrops fetalis and possibly spontaneous abortion early in pregnancy (6). However, the full spectrum of illness due to parvovirus infection is unknown. The virus has very limited tropism, infecting erythroid progenitor cells, for which it is toxic (7). B19 parvovirus has been shown to replicate only in explants of human erythroid bone marrow (8). The few research laboratories that test for virus infection have been forced to rely for antigen on very small quantities of sera known to contain virus.

The fastidious behavior of B19 parvovirus led us to develop a productive cell culture system by using genetic engineering techniques. Molecular analysis of infected bone-marrow cultures showed that the genomic organization of B19 parvovirus resembled that of other *Parvoviridae*, with two capsid proteins encoded by overlapping transcripts that were encoded by the right side of the genome (9, 10). An important early observation was that expression of the left side of the genome was associated with apparent lethality, suggesting that the nonstructural protein of B19 parvovirus was cytotoxic (11). Finally, we used a dihydrofolate reductase minigene, which served to select cells containing transfected genes, to amplify the gene number, and to increase protein expression.

MATERIALS AND METHODS

Cell Culture. The *dhfr*⁻ Chinese hamster ovary (CHO) cell line DG-21 was grown in Iscove's modified Eagle's medium containing 10% fetal calf serum, 0.1 mM glycine, 0.1 mM hypoxanthine, 0.016 mM thymidine (collectively, GHT⁺ medium), nonessential amino acids, and antibiotics at 37°C and 95% humidity (12). The 3-11-5 cell line was maintained in GHT⁻ medium with added 10 μM methotrexate (Lederle Laboratories, Pearl River, NY). Human bone-marrow cells, obtained from normal donors or patients with sickle cell disease, were obtained with informed consent under approved protocol of the National Heart, Lung, and Blood Institute institutional review board. Mononuclear cells were cultured in Iscove's modification of Dulbecco's medium with 20% fetal calf serum and recombinant human erythropoietin at 1 unit/ml (Amgen Biologicals) and infected with parvovirus-containing serum as described (13).

Plasmid Construction and Transfection. Plasmids were constructed by using standard methods (14) and information derived from cloning (15) and sequencing (16) of the virus and its expression in human marrow cells (11). The capsid expression vector pCP94 (Fig. 1*A*) was constructed from the nearly full-length B19 parvovirus cloned DNA pYT103c [ref. 15, modified as described in ref. 9] by removal of the 0.1-kilobase (kb) *Eco*RI–*Aat* II fragment (left terminal inverted repeat region) and the 2.5-kb *Xba* I–*Sma* I fragment (left side of the B19 parvovirus genome containing the nonstructural protein sequence). pCP94 therefore included nucleotides (nt) 102–477 (the promoter region) and 2071–5107 (the complete coding region of the overlapping capsid protein genes, VP1 and VP2). Thus, the coding region of the nonstructural protein gene was virtually deleted, although a 50-amino-acid aberrant polypeptide was a theoretical product (the first 15 amino acids of the nonstructural protein are included in nt 102–477 and the last 105 nt of the gene with a frameshift in 2071–5107). The human *DHFR* minigene DM-14 (Fig. 1*B*) was composed of a 1.1-kb *Tha* I–*Bgl* II fragment containing the *DHFR* coding sequences from DM-1 (12), a 0.44-kb *Kpn* I–*Hin*dIII

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Abbreviations: SV40, simian virus 40; CHO, Chinese hamster ovary; nt, nucleotide(s).

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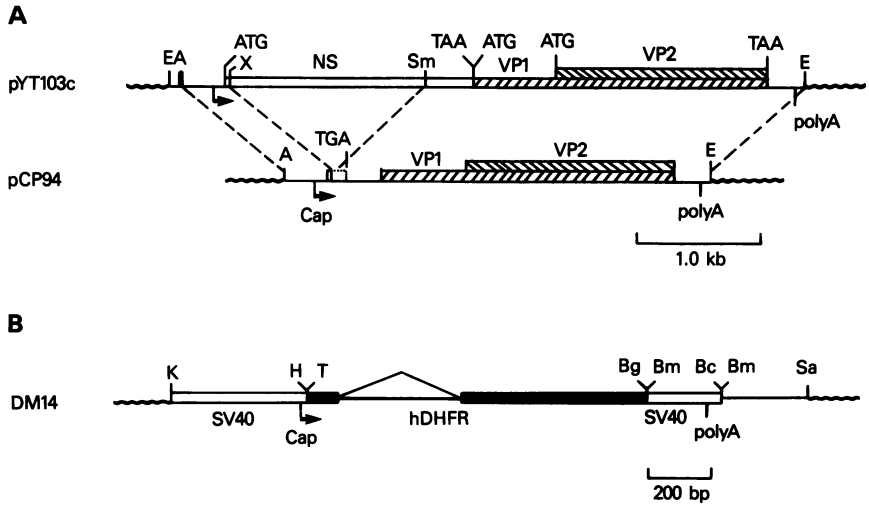

FIG. 1. Structure of expression plasmids. (*A*) Parvovirus capsid expression vector pCP94. pCP94 was derived from the cloned DNA of parvovirus, termed pYT103c (9, 14). Hatched boxes, coding region of the structural proteins (VP1 and VP2); open box, coding region of the nonstructural (NS) protein. Plasmid vector DNA is represented by wavy lines, pAT153 for pYT103c and pLTN1 for pCP94. E, *Eco*RI; A, *Aat* II; *X*, *Xba* I; and Sm, *Sma* I. ATG, position of the translation initial codon; TAA and TGA, termination codons for translation. Dotted box on PCP94, remaining portion of NS protein gene. (*B*) *DHFR* minigene DM-14. Exons 1–6 are shown as solid boxes; exon 1 and intron 1 (thick line) are derived from the functional *DHFR* gene, and exons 2–6 are derived from the intronless *DHFR* pseudogene (12). Open boxes, simian virus 40 (SV40) early promoter/enhancer unit and the SV40 polyadenylylation signal; wavy lines, pUC19 sequences. K, *Kpn* I; H, *Hin*dIII; T, *Tha* I; Bg, *Bgl* II; Bm, *Bam*HI; Bc, *Bcl* I; and Sa, *Sal* I.

fragment containing the SV40 early promoter/enhancer unit, and a 0.24-kb *Bam*HI–*Bcl* I fragment containing a SV40 late polyadenylylation signal. Plasmid DNA was cotransfected into *dhfr*$^-$ DG21 CHO cells by calcium phosphate precipitation (15), and cells were selected in GHT$^-$ medium.

Analysis of DNA and RNA. DNA was obtained by standard phenol/chloroform extraction with proteinase K and RNase digestion and, after 1% agarose gel electrophoresis, analyzed by the Southern technique (14). RNA was prepared by guanidinium hydrochloride extraction and analyzed by the Northern (RNA) technique after 1.5% formaldehyde gel electrophoresis and transfer to Nytran (Schleicher & Schuell; ref. 14). For hybridization the full-length pYT103c plasmid was labeled with [α-^{32}P]dCTP in the presence of random oligonucleotide primers by reaction with the Klenow fragment of DNA polymerase I.

Detection of Parvovirus Protein. Proteins were detected by immunoprecipitation, immunoblot, and immunofluorescence. For immunoprecipitation, 3-11-5 cells were plated at 1×10^6 cells per 10-cm-diameter dish and, after 24-hr growth, washed twice and then incubated with 4 ml of methionine-free medium/5% fetal calf serum/10 μM methotrexate for 30 min before adding 50 μCi of [^{35}S]methionine per ml (specific activity 1000 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear) for 6 hr. Bone-marrow cells were labeled similarly at various times after inoculation with virus by using 50 μCi of [^{35}S]methionine per 2.5×10^6 cells in 1 ml. Cell lysates in the presence of protease inhibitors (aprotinin at 100 kallikrein inhibitor units per ml; 1 mM phenylmethylsulfonyl fluoride; pepstatin A at 1 μg/ml; leupeptin hemisulfate at 5 μg/ml) were precipitated with high-titer convalescent-phase human antiserum and subjected to 8% PAGE as described (17). Immunoblot was done with unlabeled cell lysates and human immune serum as described (17). For immunofluorescence, cytocentrifuge preparations of cultured cells were fixed in acetone at −20°C for 30 sec and treated with a monoclonal antibody to B19 capsid protein (1:50 dilution; Chemicon) and goat anti-mouse IgM–fluorescein isothiocyanate (1:20 dilution; Kirkegaard and Perry Laboratories, Gaithersburg, MD).

Sedimentation Analysis. 3-11-5 cells were labeled for 6 hr with [^{35}S]methionine and then cultured in complete medium for another day. Bone-marrow cells were similarly labeled, and the supernatant from cultures was harvested at 3 days after inoculation with virus. Homogenized cells or supernatant were centrifuged at 100,000 × *g* through 40% (wt/vol) sucrose in phosphate-buffered saline to concentrate particles (7). Equilibrium density-gradient sedimentation was accomplished in 50 mM Tris·HCl, pH 8.7/5 mM EDTA and 0.1% Sarcosyl/CsCl at 1.31 g/ml for empty capsid and 1.4 g/ml for intact virus. Peaks of the appropriate density, as determined by refractometry, were subjected to three cycles of banding in CsCl. To show separation of empty and full capsids, purified preparations from 3-11-5 cells and bone-marrow culture supernatants were mixed and subjected to either isopycnic sedimentation in CsCl at 1.35 g/ml (100,000 × *g* for 40 hr) or linear 15–30% sucrose-gradient sedimentation (110,000 × *g* for 3 hr). Virus was localized by scintillation counting of fractions.

Electron Microscopy. To demonstrate viral capsids, purified fractions from CsCl isopycnic sedimentation were negatively stained with 2% (wt/vol) phosphotungstic acid, pH 6, and examined with a transmission electron microscope at 80 kV.

RESULTS

Production of the 3-11-5 Clone. In preliminary experiments six different plasmids containing B19 genes were constructed, including full-length plasmids and those with the left or right side of the genome deleted or mutated. After cotransfection with the *DHFR*-containing plasmid DM14 into CHO cells, none of the selected colonies expressed B19 proteins as determined by immunofluorescence. To assess B19 transcripts in these cells, RNA from 137 colonies was subjected to Northern analysis: in 28 colonies no B19 RNA was detected, in 68 colonies aberrant patterns were present, and in 45 colonies the expected pattern was found. These latter colonies were subcloned and exposed to increasing concentrations of methotrexate to amplify the transfected genes. In general, cells expressing the left side of the parvovirus genome did not grow well, and none showed nonstructural protein after amplification. Some clones containing only the right side of the B19 genome did show structural protein

expression when screened by immunofluorescence; because of its very high intensity of specific fluorescence, a clone termed 3-11-5 was chosen for further characterization.

For Southern analysis, DNA from the 3-11-5 cell line was digested with the enzyme *Hpa* I; the two enzyme sites within the transfected parvovirus capsid protein sequence of 3.4 kb would result in an expected excised fragment of 1.7 kb (Fig. 2 *Lower*). After hybridization with a radioactively labeled, full-length B19 parvovirus probe, DNA from 3-11-5 cells showed a single fragment of the expected 1.7 kb, which was greatly amplified (>10-fold) by growth in 10 μM methotrexate (Fig. 2 *Upper Left*). The presence of a heterogeneous pattern of higher-molecular-weight DNA fragments that hybridized with the parvovirus probe suggested random integration of the vector as expected.

On Northern analysis, two discrete B19 transcripts were present, of slightly smaller dimension than the capsid RNA species present in infected bone-marrow cells. This result was anticipated because a region upstream of the coding sequences of the structural proteins, including the normal splice acceptor sites at nt 1908 and 2028 (9), was deleted during plasmid construction (Fig. 2 *Lower*). The number of parvovirus RNA transcripts also was greatly increased after exposure to methotrexate (Fig. 2 *Upper Right*). (The 1.7-kb RNA probably represents hybridization with abundant cellular ribosomal RNA, which behaves anomalously on electrophoresis due to its very high concentration; this common artifact disappears after purification of polyadenylylated RNA.)

In comparison to either the parental CHO cell line or transformed CHO cells, the growth of 3-11-5 cells was normal (data not shown). Cells have retained amplified B19 DNA after ≈50 serial passages.

Expression of B19 Protein. Both the major 58-kDa and minor 83-kDa capsid species were detected in lysates of 3-11-5 cells (Fig. 3). The 58-kDa capsid protein was ≈6-fold more abundant than the 83-kDa protein, comparable to the ratio in infected erythroid cells of 10–20:1. As determined by immunoblot of lysates of cells from mature cultures, accumulation of capsid proteins in the cell line was superior to that from infected normal bone-marrow culture, although not as great as seen after infection of predominantly erythroid cells in marrow from a patient with sickle cell disease (Fig. 3*A*). By comparison with human serum from infected patients, the genome copy number of an average 3-11-5 cell was calculated to be 1–2 × 10^3, in comparison with 8 × 10^3 copies in an average infected erythroid cell. However, kinetic analysis of capsid production after pulse labeling of cultures showed that

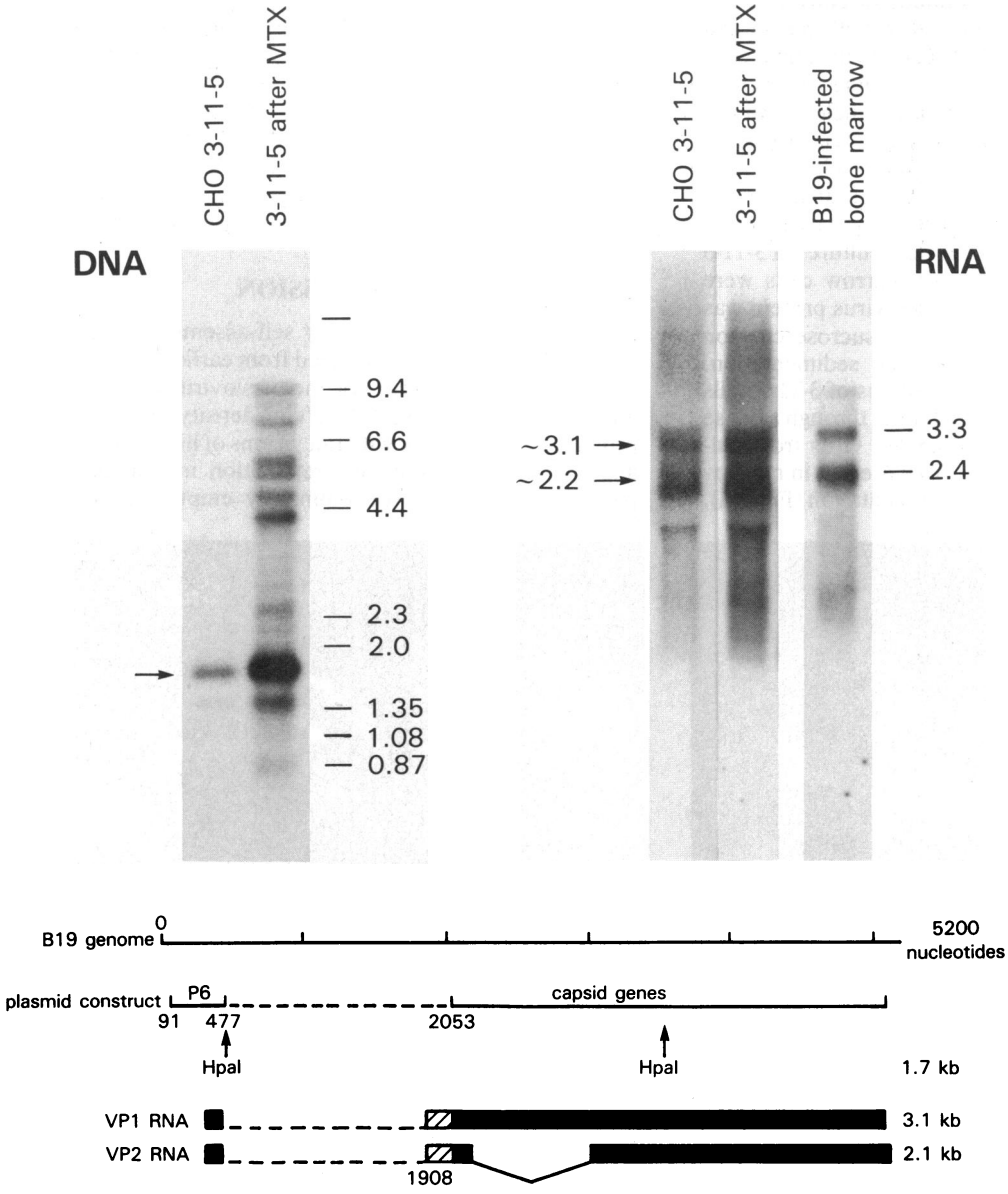

FIG. 2. Parvovirus DNA and RNA of 3-11-5 cells. DNA (*Upper Left*) and RNA (*Upper Right*) extracted from 3-11-5 cells harvested before and after exposure to methotrexate (MTX) at 10 μg per well was subjected to Southern or Northern analysis with a B19-specific full-length probe (pYT103c) for hybridization. (*Lower*) Diagram of the B19 parvovirus genome and plasmid construction containing the right side of the B19 genome.

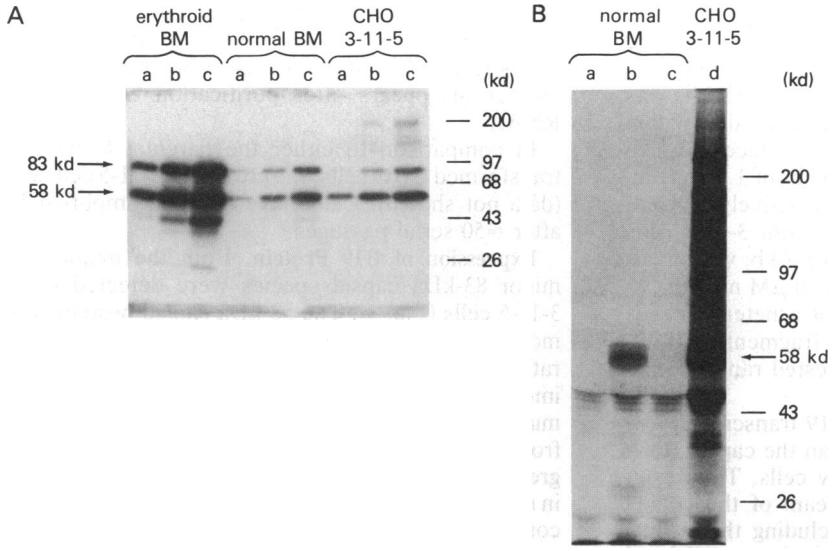

FIG. 3. Capsid protein production by 3-11-5 cells. (*A*) Immunoblot analysis of lysates of 3-11-5 cells, normal cells, and erythroid bone marrow (BM) cells. Cells were harvested after 48 hr of logarithmic-phase growth (3-11-5) or 48 hr after inoculation with virus (bone marrow); lanes a, 1×10^5 cells; lanes b, 3×10^5 cells; lanes c, 1×10^6 cells. (*B*) Immunoprecipitation of 3-11-5 cells and normal bone-marrow cells exposed for 6 hr to [^{35}S]methionine before harvest. Normal bone-marrow cells were exposed for 6 hr at 0 hr (lanes a), 24 hr (lanes b), or 48 hr (lanes c) after inoculation; 3-11-5 cells (lanes d) were in logarithmic-phase growth. kd, kDa.

58-kDa protein synthesis was 30- to 40-fold higher in 3-11-5 cells than in total normal bone-marrow cells at the height of virus protein production (Fig. 3*B*). By immunofluorescence with either a specific monoclonal antibody or convalescent-phase serum, B19 protein was detected in both nuclei and cytoplasm of virtually every 3-11-5 cell, with some cells showing characteristic localization of staining in nucleoli (Fig. 4). However, extraction of separated nuclear and cytosol fractions resulted in the bulk of capsid protein appearing in the cytosol (data not shown), possibly the result of leakage of proteins with swelling of the nuclear membrane.

Capsid Assembly. To determine whether parvovirus protein products assembled into intact capsids, cultures of 3-11-5 cells and virus-infected erythroid bone-marrow cells were labeled with a radioactive amino acid, and virus protein was subjected to partial purification through a sucrose cushion followed by density-gradient or isopycnic sedimentation. More than 90% of the labeled capsid proteins of 3-11-5 cells were present in the pellet after centrifugation through a dense sucrose cushion (data not shown). Labeled virus from cultures could be separated from intact virus present in marrow cultures by sucrose density-gradient sedimentation (Fig. 5*A*). In CsCl, [^{35}S]methionine-labeled capsid protein sedimented with the expected density of empty parvovirus capsids (1.31 g/ml) compared with intact virions from infected cultures (1.42 g/ml) (Fig. 5*B*). Particles of the appropriate appearance and dimension for parvovirus were detected in the peak fraction by transmission electron microscopic study of negatively stained preparations (Fig. 5*B Inset*).

Capsids from 3-11-5 cells were not infectious in human bone marrow *in vitro*, assayed by inhibition of erythroid colony formation in progenitor assays (ref. 18; data not shown). Parvovirus DNA was not detected by dot-blot hybridization of purified capsid preparations (data not shown).

DISCUSSION

Formation of empty capsids by self-assembly of parvovirus structural proteins was predicted from earlier studies of other species of *Parvoviridae*. For the parvovirus minute virus of mice, empty capsids of 1.42 g/cm^3 density were seen to be produced concurrently with full virions of higher density (19), and a temperature-sensitive replication mutant of rat H-1 parvovirus produced predominantly empty capsids at the

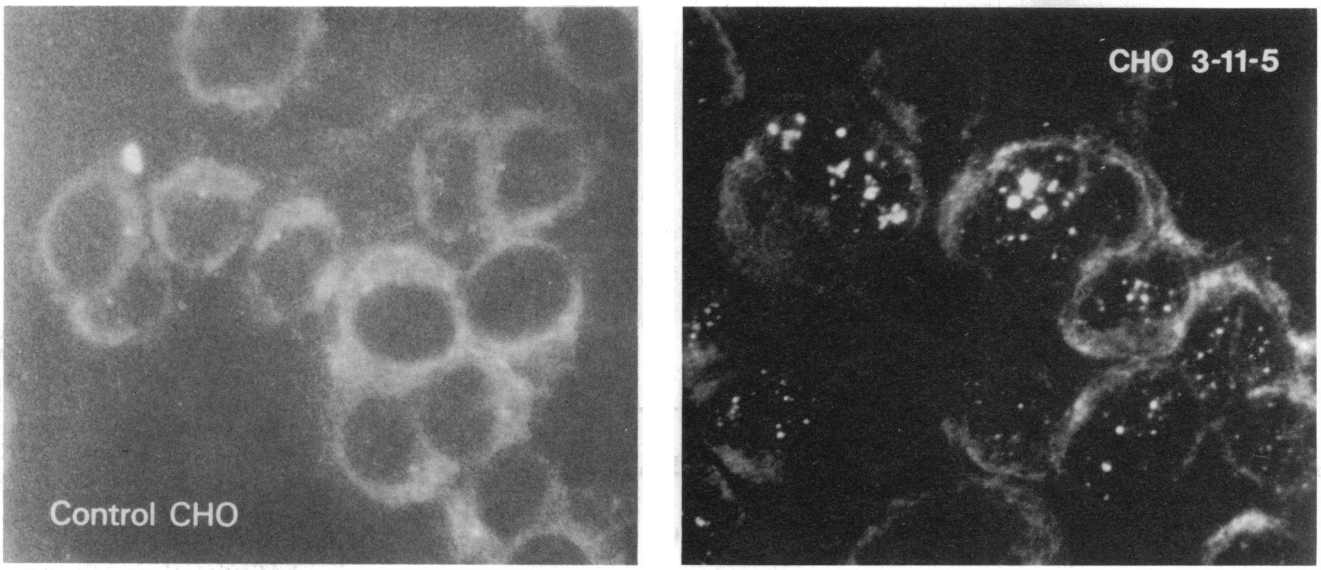

FIG. 4. Immunofluorescence of 3-11-5 and control CHO cells. A pattern of specific punctate nuclear and diffuse cytoplasmic fluorescence was seen in 3-11-5 cells. Control cells were photographed by using automatic exposure; exposure of control preparations for an equivalent period of time as 3-11-5 cells would result in a totally dark photograph. (×1500.)

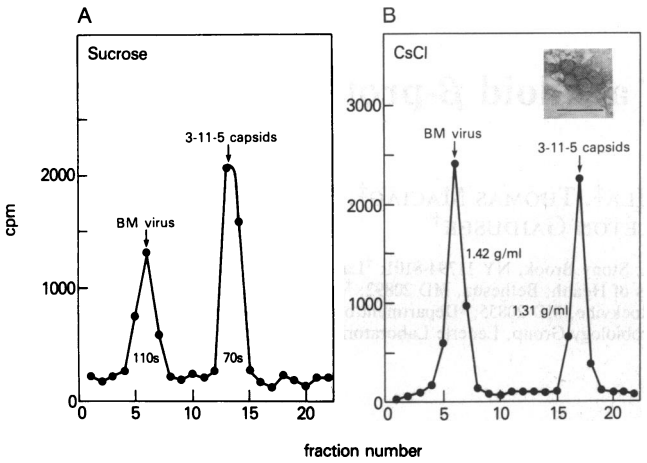

FIG. 5. Separation of labeled empty capsids of 3-11-5 cells from intact virus obtained from the supernatant of infected human erythroid bone-marrow cultures. Parvovirus proteins were labeled metabolically by growth of cells in [^{35}S]methionine. Lysates were centrifuged over a sucrose cushion and then subjected to either sucrose density-gradient sedimentation (*Left*) or CsCl isopycnic sedimentation (*Right*). (*Inset*) Transmission electron micrograph of polygonal shaped negatively stained particles from the peak fraction after isopycnic sedimentation. (Bar = 0.1 μm.)

restrictive temperature (20). More recently, expression of murine parvovirus structural proteins by bovine papillomavirus-minute virus of mice chimera also led to empty capsid production, but the transformed cells were genetically unstable (21). Our results support the conclusions of other authors (19–21) that empty capsid formation may precede DNA insertion to form intact infectious virions. In our stably transformed cells, empty capsid production had no deleterious effect on cell growth, which is further evidence for the toxic role of the nonstructural protein of B19 parvovirus (10). In addition, attempts to use cotransfection of the *DHFR* minigene and plasmid constructs containing the nonstructural protein gene failed to produce cells that expressed nonstructural protein. Both the nonstructural proteins and capsid proteins of parvoviruses have been shown to bind to parvovirus DNA, and both remain candidates as packaging factors for B19 parvovirus. The higher proportional production of the minor capsid protein in 3-11-5 cells compared with that in infected bone-marrow cells and the equivalent quantities of the two structural protein RNAs suggest that the relative amounts of capsid proteins in 3-11-5 cells are mainly translationally regulated by upstream AUG codons (22).

This cell line will have great practical usefulness because it will provide an unlimited supply of antigen for the development of clinical anti-B19 parvovirus antibody assays. In preliminary experiments, crude lysates of 3-11-5 cells that had been fractionated only by a single passage through sucrose could completely replace virus-containing serum in an ELISA for IgG and IgM directed to virus (L. Anderson, Centers for Disease Control, Atlanta, personal communication), although its use in capture immunoassays may depend on the specific monoclonal antibody used (K. E. Brown, Central Public Health Laboratory, London, personal communication). In addition to availability, the noninfectious nature of 3-11-5 lysate will also protect medical workers, who are susceptible to laboratory infection with serum samples (23). Capsids from 3-11-5 cells are also of potential utility in development of a vaccine to prevent parvovirus infection in susceptible pregnant women and in patients with hemolytic disease, as well as in the development of specific immune serum for the treatment of patients with chronic parvovirus infection and bone-marrow suppression. Cats and dogs are successfully immunized with attenuated strains of their respective parvoviruses (24). Development of an attenuated B19 parvovirus mutant may be not only difficult but also dangerous, because of the possibility of extension of the range of tissue tropism beyond erythroid progenitor cells (25).

The 3-11-5 cell line should also prove useful in more basic investigations of parvovirus function. For example, it may be possible to pseudotype adenoassociated virus DNA in B19 capsids and to determine the packaging signals for B19 encapsidation. Studies of the kinetics of B19 parvovirus protein production, trafficking, and release of the cell-surface receptor for B19 parvovirus and of the specific role of nonstructural protein during cell infection will also be facilitated. Elucidation of these aspects of parvovirus biology may allow the use of this cell line for packaging of human genetic material for specific delivery to bone-marrow erythroid progenitor cells as gene therapy.

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