In Vitro and In Vivo Studies of Bovine Parvovirus Proteins

MURIEL LEDERMAN,* ROBERT C. BATES, AND ERNEST R. STOUT

Department of Biology, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061

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Total cytoplasmic RNA from bovine parvovirus (BPV)-infected cells or BPV-specific RNA selected by hybridization to cloned BPV genomic sequences were translated in a message-dependent rabbit reticulocyte lysate. Immunoprecipitation, using immunoglobulin G from rabbits injected with purified BPV, resulted in the detection of [35S]methionine-labeled polypeptides with Mr's of 80,000, 72,000, 62,000, and 60,000. These in vitro translation products had the same mobility on sodium dodecyl sulfate-polyacrylamide gels as that of the four proteins found in purified virions. The three largest polypeptides had amino acid sequence homology, as judged by serological methods and partial proteolysis with Staphylococcus aureus V8 protease. Additional noncapsid proteins with Mr's of 25,000, 27,000, and 31,000 were also detected as translation products of these RNAs. All of the above species were immunoprecipitated by immunoglobulin G from a calf which was naturally infected with BPV. All four capsid proteins but only one of the lower-molecular-weight polypeptides were detected after the immunoprecipitation of BPV-infected cells. The results presented here indicate that the BPV genome codes for four capsid proteins and a noncapsid protein which may be structurally related to the capsid proteins.

The paroviruses are among the smallest DNA-containing viruses that infect eucaryotic cells. They are divided into two groups, defective and autonomous, on the basis of a helper virus requirement for productive infection. The autonomous paroviruses, which do not need helper virus, have a requirement for host cell passage through the S phase of the cell cycle for the production of progeny viruses. The genome of the autonomous paroviruses is a single-stranded DNA of $1.4 \times 10^6$ to $1.6 \times 10^6$ daltons; predominantly minus strand is encapsidated, and there is no evidence for transcription from the plus strand (26).

Autonomous rodent parovirus and defective adeno-associated virus contain three capsid proteins (24), whereas autonomous rabbit parovirus contains four capsid proteins (15). The capsid proteins of minute virus of mice have considerable amino acid sequence homology, as determined by tryptic peptide mapping (25), as have the virion proteins of adeno-associated virus (13). These data, taken in conjunction with studies for both autonomous and defective paroviruses which map the bodies of the major RNA species to the right half of the genome, suggest that only a portion of the genome is used to code for the capsid proteins (Carter et al., in K. Berns (ed.), The Paroviruses, in press).

In vitro translation of unseparated viral RNA or individual RNA species can be used to detect and characterize the protein products of paroviral genomes, including possible noncapsid proteins which may be involved in replication. Buller and Rose (4) demonstrated the in vitro synthesis of adeno-associated virus capsid proteins and two protein species in the 15,000- to 25,000-dalton range. Rhode and Paradiso (22) detected a nonstructural protein as an in vitro translation product of H-1 virus RNA by immunoprecipitation with sera from H-1 virus-infected hamsters, although it did not precipitate with anticapsid antibody.

In the analysis reported here of the translation of total cytoplasmic RNA from cells infected with autonomous bovine parovirus (BPV) and of BPV-specific RNA, we demonstrated the synthesis of four proteins apparently identical to the virion structural proteins. We also demonstrated the synthesis of three lower-molecular-weight species. All of these translation products were immunoprecipitated with either antibody to purified virions or antibody from an infected calf. The capsid proteins and one of the lower-molecular-weight polypeptides were immunoprecipitated from infected cell lysates.

MATERIALS AND METHODS

Cell culture and virus propagation. Mock-infected and BPV-infected parasynechronous bovine fetal lung cells were used exclusively in these studies. Culture conditions were as described by Parris and Bates (18).
Infection was at 10 PFU/ml. For the production of [35S]methionine-labeled virus, cells were infected in medium containing 10% the normal amount of methionine in the absence of serum. After 1 h, dialyzed fetal bovine serum (FBS) was added to 10%, and 100 μCi of [35S]methionine (1,200 Ci/mmole) was added per roller bottle at 10 h postinfection (p.i.). After 24 h, an equal volume of regular medium containing 10% dialyzed FBS was added.

**Purification of virus.** Virions were purified from cells and medium by high-speed centrifugation and treatment of the suspended cell pellet in 50 mM Tris-chloride (pH 8.0)–5 mM MgCl2; with 50 μg of DNase I and 100 μg of RNase A per ml for 30 min at 37°C, followed by incubation with 0.1% N-lauroylsarcosine for 10 min at 37°C. All solutions used to this point contained 1 mM phenylmethylsulfonyl fluoride. The virus was repelletted and layered over a cesium chloride-sucrose step gradient in 50 mM Tris-chloride, pH 8.0. The gradient contained 2 ml of 40% CsCl, 2 ml of 35% CsCl, and 2.8 ml of 1 M sucrose. Centrifugation was for 18 h at 34,000 rpm in an SW41 rotor. The virus band was collected, dialyzed, and rerun on a 5 to 30% sucrose gradient in 50 mM Tris-chloride–5 mM EDTA (pH 8.7) at 41,000 rpm in an SW41 rotor for 2 h.

**Polyacrylamide gel electrophoresis.** Electrophoresis was carried out by the method of Laemmli (12). Gels were stained with 0.2% Coomassie brilliant blue in 50% methanol–10% acetic acid and destained in 30% methanol–10% acetic acid. Proteolysis with Staphylococcus aureus V8 protease during electrophoresis was done by the procedure described by Cleveland et al. (6), except that the resolving gel was an 8 to 15% gradient from which sodium dodecyl sulfate (SDS) was omitted.

**Production of antibody.** Purified BPV was administered to rabbits as an immunogen in the presence of an equal volume of Freund complete adjuvant. Individual virion proteins were prepared by electrophoresis on 10% polyacrylamide gels. One track was stained with Coomassie brilliant blue and the appropriate areas of the adjacent tracks were cut out. The protein in the slice was eluted into 50 mM Tris-chloride (pH 8.0) containing either 1 mM phenylmethylsulfonyl fluoride or 0.8 U of aprotinin per ml (Sigma Chemical Co.) and dialyzed against 20 mM NaPO4, (pH 7.2) before injection with an equal volume of Freund complete adjuvant. Purity was assayed by reelectrophoresis, followed by silver staining (17). A 2-month-old calf was orally infected with 5 × 106 PFU of BPV prepared in bovine fetal lung cells. Serum was harvested during the convalescent phase of the infection for this experimentally infected calf and for a calf infected in the field. The titer of the antiscapsid antibodies was 1/10,000, and that of the calf antibodies was 1/128 by hemagglutination inhibition. Immunoglobulin G (IgG) (IgG) fractions of sera were prepared by using DEAE-Affigel blue (Bio-Rad Laboratories) following the directions of the supplier. Ouchterlony double diffusion was performed in 0.75% agarose in 20 mM NaPO4 (pH 7.2)–0.1 M NaCl–0.02 M iodoacetamide (10). Bands were observed after a minimum of 16 h of incubation at 37°C.

**Preparation of RNA.** RNA was extracted from BPV-infected bovine fetal lung cells at 16 to 20 h p.i. Cells were washed in 0.14 M NaCl–1.5 mM MgCl2–10 mM Tris-chloride (pH 8.0), broken in a hypotonic buffer (10 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid], 5 mM KCl, 0.5 mM MgCl2, 0.5 mM diithiothreitol [pH 7.4]) with a Potter-Elvehjem homogenizer, and brought to 0.25 M sucrose, and a cytoplasmic fraction was prepared by centrifugation at 5,000 × g for 5 min. The cytoplasmic fraction was adjusted to 1 g of CsCl and 0.2 g of N-lauroylsarcosine per ml and layered over 5.7 M CsCl containing 0.1 M K-EDTA and 2% Sarkosyl, and the RNA was pelleted by centrifugation in an SW41 rotor at 34,000 rpm for 18 to 24 h. The RNA was suspended, ethanol precipitated, extracted with chloroform-isooamyl alcohol, and precipitated two more times.

BPV-specific RNA was isolated from cytoplasmic RNA by hybridization to a mixture of pBR322 containing the 4.21-kilobase EcoRI A fragment of BPV and pBR322 containing the 4.0-kilobase PstI A fragment of BPV, each covalently bound to diazobenzyloxymethylcellulose. The plasmids were constructed by J. Leary, Yale University, New Haven, Conn. Hybridization and elution conditions were those of Goldberg et al. (9). In vitro translation. Cytoplasmic RNA at a concentration of 300 to 400 μg/ml was translated either in a commercially available rabbit reticulocyte lysate (Bethesda Research Laboratories) according to the directions of the supplier or in a lysate prepared by the method of Ranu and London (20) and made dependent on exogenous RNA by using micrococcal nuclease (19). Incubation conditions were as follows: 50 mM HEPES (pH 7.7), 1 mM diithiothreitol, 80 mM KAc, 1 mM MgAc2, 0.2 mM spermidine, 0.05 mM concentrations of each of 18 amino acids except cysteine and methionine, 50 μg of phenol-extracted calf liver rRNA per ml (Boehringer Mannheim Corp.), 10 mM creatine phosphate, 80 μg of creatine phosphokinase per ml, 1 mM ATP, 0.1 mM GTP, and 320 μCi of [35S]methionine per ml (1,200 Ci/mmole). Incubation was for 90 min at 30°C in a reaction volume of 25 μl.

**Immunoprecipitation.** In vitro protein synthesis reactions were immunoprecipitated with either preimmune IgG and a 10% suspension of Formalin-fixed S. aureus cells (Bethesda Research Laboratories) as a source of protein A or immune IgG from rabbits injected with purified virions and the protein A-containing cells by the method of Conboy and Rosenberg (7). Alternatively, a single in vitro reaction was diluted in RIPA buffer (0.15 M NaCl, 20 mM Tris-chloride [pH 7.4], 0.1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100) precipitated first with preimmune IgG and then with immune IgG by the method of Cepko et al. (5). When cells were labeled for the immunoprecipitation of in vivo-synthesized proteins, the medium was changed to low-methionine medium containing 10% dialyzed FBS at 4 h p.i. At 10 h p.i., 5 μCi of [35S]methionine per ml was added, and the cells were harvested at 24 h p.i. and washed twice in modified phosphate-buffered saline (23). The cells were lysed in RIPA buffer, sonicated briefly, and immunoprecipitated by the method of Cepko et al. (5).

Bovine IgGs were prepared by using DEAE-Affigel blue as described above for rabbit IgG. Carrier virus was included in all immunoprecipitations. The final precipitates were eluted with electrophoresis application buffer, and the gels were fluorographed (3) and exposed with Kodak XAR-5 film and a Cronex Light-
RESULTS

Products of in vitro translation of RNA from BPV-infected cells. The in vitro translation of RNA (300 to 400 μg/ml) extracted from BPV-infected cells resulted in a 10-fold stimulation of the incorporation of [35S]methionine into total acid-insoluble material compared with a parallel incubation without added RNA after 90 min of incubation at 30°C (data not shown). After immunoprecipitation of the translation products of cytoplasmic RNA from BPV-infected cells with immune IgG from rabbits injected with purified BPV, bands corresponding to proteins with molecular weights of 80,000, 72,000, 62,000, and 60,000 were observed after SDS-polyacrylamide gel electrophoresis. Three lower-molecular-weight species with $M_r$s of 25,000, 27,000, and 31,000 were also seen. These lower-molecular-weight species showed cross-reactivity with rabbit preimmune IgG (Fig. 1A, lane 3).

When the products of a single translation reaction were immunoprecipitated first with preimmune IgG and then with rabbit immune IgG, the virion proteins were detected in approximately the same proportions as those that were observed in virions labeled in vivo with [35S]methionine (Fig. 1B, lanes 1 and 2; Table 1). The three lower-molecular-weight species were still observed, as was a minor component at ca. 45,000 daltons. Increasing the volume of preimmune IgG in the first step to two- or fourfold that which is normally used did not specifically decrease the precipitation of these species with immune IgG, nor did Formalin-fixed S. aureus cells alone specifically precipitate these lower-molecular-weight species (data not shown). None of these protein species was detected after the immunoprecipitation of translation reactions from which RNA was omitted (Fig. 1A, lanes 1 and 2; Fig. 1C, lane 1) or in translation reactions in which cytoplasmic RNA from mock-infected cells was translated (Fig. 1C, lane 2).

These proteins were also observed as translation products of BPV-specific RNA (Fig. 1D,
TABLE 1. Distribution of $^{[35S]}$methionine between BPV capsid proteins labeled in vivo or in vitro

<table>
<thead>
<tr>
<th>Protein component (daltons)</th>
<th>% Total$^a$ In vivo</th>
<th>% Total In vitro</th>
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<tbody>
<tr>
<td>80,000</td>
<td>6.8</td>
<td>7.1</td>
</tr>
<tr>
<td>72,000</td>
<td>4.6</td>
<td>14.3</td>
</tr>
<tr>
<td>62,000</td>
<td>76.8</td>
<td>61.3</td>
</tr>
<tr>
<td>60,000</td>
<td>11.8</td>
<td>17.3</td>
</tr>
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</table>

$^a$ Percentages were integrated and computed from densitometric tracings of autoradiograms of fluorographed SDS-polyacrylamide gels of virion proteins from in vivo $^{[35S]}$methionine-labeled virus purified as described in the text and from densitometric tracings of an in vitro translation reaction programmed with cytoplasmic RNA from BPV-infected cells with $^{[35S]}$methionine as label, which was sequentially immunoprecipitated as described in the text.

lane 2). BPV-specific RNA was prepared by hybridization to a mixture of plasmids containing inserts which spanned ca. 85% of the BPV genome. These plasmids were covalently bound to diazobenzyloxyethylcellulose. The recovery of BPV-specific RNA after this procedure was 0.05 to 0.1% of the $^{[3]H}$uridine-labeled input RNA.

Characterization of BPV capsid proteins. Since four proteins were observed in full virions of BPV isolated from cells labeled with $^{[35S]}$methionine (Fig. 1B, lane 2) and in empty virions (data not shown), other autonomous parvoviruses were purified as described above and electrophoresed on 10% SDS-polyacrylamide gels to determine whether these viruses contained any additional capsid proteins. None were detected in H-1 virus, LuIII virus, minute virus of mice, or Kilham rat virus (Fig. 2).

Since tryptic peptide mapping for autonomous and defective parvoviruses indicated amino acid sequence homology among the capsid proteins, the extent of the homology of the three largest BPV capsid proteins was investigated. Antibody was produced to these proteins after separation on and extraction from SDS-polyacrylamide gels. Double-diffusion tests showed that antibody to each individual capsid protein cross-reacted with its own immunogen and with the other capsid proteins (data not shown). The homology among these capsid proteins was also shown by electrophoretic analysis of the cleavage products generated by proteolysis with S. aureus V8 protease (6) (Fig. 3). The capsid proteins were separated on 10% SDS-polyacrylamide gels and stored frozen overnight in the presence of 0.8 U of aprotinin per ml to prevent spontaneous proteolysis. Lanes 1, 2, and 3 of Fig. 3 show the 80,000-, 72,000-, and 62,000-dalton BPV capsid proteins, which had been separated, stored as described above, and re-electrophoresed without added protease. Lanes 4 through 9 show the capsid proteins treated identically except for the addition of various amounts of S. aureus V8 protease to each well of the gel during the second electrophoresis. Polypeptides with apparently identical molecular weights (indicated by arrows) can be seen among the digestion products of all three capsid proteins, indicating homology of amino acid sequences as judged by the linear distribution of substrate sites for S. aureus V8 protease.

Immunoprecipitation of in vivo- and in vitro-synthesized BPV proteins with various antisera. Experiments were carried out to determine whether the lower-molecular-weight translation products were present during the course of BPV infection. Translation reactions were sequentially immunoprecipitated first with FBS and then with IgG from either a calf infected in the field (Fig. 4, lane 3) or a calf infected with BPV grown in tissue culture cells (Fig. 4, lane 4). The pattern of proteins observed was the same as that found in vivo.
when IgG from rabbits injected with purified virions was used for immunoprecipitation (Fig. 4, lane 2). This result showed that the three species with \( M_s \)s of ca. 30,000 cross-reacted with an antibody produced in the host animal during a natural infection, presumably to a protein synthesized during the course of the disease. These species do not specifically immunoprecipitate with FBS (data not shown).

To determine whether these 30,000-dalton species were synthesized in vivo during infection, mock-infected and BPV-infected cells which had been labeled in vivo with \( ^{35}S \)methionine were immunoprecipitated with IgG from a rabbit injected with purified virions (Fig. 5, lanes 1 and 2). IgG from a calf infected in the field (Fig. 5, lanes 3 and 4), IgG from an experimentally infected calf (Fig. 5, lanes 5 and 6), a mixture of IgGs prepared to the separated capsid proteins (Fig. 5, lanes 7 and 8), IgG to the 62,000-dalton capsid protein (Fig. 5, lanes 9 and 10), IgG to the 72,000-dalton capsid protein (Fig. 5, lanes 11 and 12), and IgG to the 80,000-dalton capsid protein (Fig. 5, lanes 13 and 14). With all antisera, only one of the three lower-molecular-weight species (27,000 daltons) was immunoprecipitated. However, additional species with \( M_s \)s of >100,000 and ca. 45,000 which are not major in vitro translation products were immunoprecipitated and remain to be characterized. These results also showed that antibody produced against any one capsid protein also cross-reacted with the other capsid proteins and the lower-molecular-weight polypeptide. In contrast, antibodies to the individual capsid proteins precipitated the three 30,000-dalton species from an in vitro translation reaction containing RNA from BPV-infected cells (data not shown).

**DISCUSSION**

We showed that purified virions of BPV, an autonomous parvovirus, contain four polypeptide components with molecular weights of 80,000, 72,000, 62,000, and 60,000. The largest and the two smallest of these correspond to the A, B, and C polypeptides described by Bates et al. (1). The polypeptides of HADEN virus described by Johnson and co-workers (10) and Johnson and Hoggan (11) correspond to those identified here, assuming that the techniques used by those investigators did not resolve the two smallest polypeptides. We suggest that a nomenclature be adopted for BPV such that the four capsid proteins are identified as VP1, VP2, VP3, and VP4 in order of decreasing size.

**FIG. 4.** Immunoprecipitation of in vitro translation reactions with various antisera. (Lane 1) no RNA, preimmune IgG. followed by antivirion IgG; (lane 2) cytoplasmic RNA from BPV-infected cells, preimmune IgG, followed by antivirion IgG; (lane 3) cytoplasmic RNA from BPV-infected cells, FBS, followed by IgG from a calf infected in the field; and (lane 4) cytoplasmic RNA from BPV-infected cells, FBS, followed by IgG from a calf infected with BPV grown in tissue culture.

**FIG. 3.** Electrophoresis of proteolytic digests of BPV capsid proteins. Lanes 1, 2, and 3: untreated 80,000-, 72,000-, and 60,000-dalton proteins. Lanes 4, 5, 6: proteins listed above treated with 10 ng of \( S. aureus \) V8 protease. Lanes 7, 8, and 9: proteins listed above treated with 100 ng \( S. aureus \) V8 protease. Arrows indicate proteolysis products of the same mobility.
We showed that VP1, VP2, and VP3 bear amino acid sequence homology in two ways: by demonstrating that antibody to VP1, VP2, or VP3 will immunoprecipitate all the capsid proteins and by detecting common polypeptides in VP1, VP2, and VP3 after partial proteolysis with *S. aureus* V8 protease. All four capsid proteins are in vitro translation products of RNA extract ed from BPV-infected cells and of BPV-specific RNA selected by hybridization to cloned BPV genomic sequences. These characteristics differentiate VP2 from a polypeptide of similar size with DNA polymerase activity which can copurify with BPV during the early stages of virus purification procedures different from those used in this study (1).

BPV has one more capsid protein than do the autonomous rodent parvoviruses. However, VP2 does not have the same properties as several recently reported species which may be DNA-binding proteins of parvoviruses. It has been detected in both full and empty virions and is immunoprecipitated both with serum against purified virions and with serum from an infected animal. This is in contrast to the H-1 virus (21) and Aleutian disease virus (2) noncapsid proteins, which do not show amino acid sequence homology with capsid proteins and immunoprecipitate only with sera from infected animals. Other studies on the H-1 virus binding protein (21) have shown that it remains associated with DNA after treatment with urea and detergent at 100°C for 2 min, whereas VP2 is released from full virions after treatment with SDS and β-mercaptoethanol at 100°C for 3 min.

The three polypeptides of ca. 30,000 daltons are translation products of cytoplasmic RNA from BPV-infected cells and of BPV-specific RNA. It is not likely that they are coded by truncated defective interfering particles (8), since it has been shown for AAV that these particles do not distort transcription (14). They may result from the translation of degraded RNA molecules, but if so, the breaks must be at highly specific sites since these species have been observed after the translation of every RNA we have prepared. In vitro translation of BPV RNA separated by size on sucrose gradients indicates that these proteins are coded by RNAs of ca. 1.1 kilobases (M. Lederman, unpublished observations). S-1 nuclease analysis of BPV RNA (P. R. Burd, J. T. Patton, R. C. Bates, and E. R. Stout, submitted for publication) has identified two RNA species of 1.05 kilobases which map to the 3′ end of the genome.
The cross-reactivity of the 30,000-dalton species with rabbit preimmune IgG may be a result of the presence in the animals of antibody to rabbit parvovirus (16). Several virus-specific low-molecular-weight species have been detected in tissue culture cells infected with rabbit parvovirus (15). If low-molecular-weight species are important during parvoviral infection, immunological determinants on these proteins may be conserved between different viruses, resulting in the cross-reactivity noted above. Immunoprecipitation of the low-molecular-weight species with antcapsid IgG should not be attributed to replication of BPV in rabbits, with subsequent generation of antibody to additional proteins, since the host range of this virus is restricted to bovine species (G. Siegl, in K. Berns (ed.), The Parvoviruses, in press).

Only one of these three low-molecular-weight species (27,000 daltons) detected after in vitro translation was observed after the immunoprecipitation of lysates of BPV-infected cells. The other two may be artifacts of in vitro translation, such as premature terminations or physiologically important precursors which do not accumulate in vivo. The cross-reactivity of this species with all the antisera tested strongly suggests that it is synthesized, at least in part, from the same portion of the genome as the capsid proteins. This protein has been shown to be NP-1, a phosphorylated protein which accumulates in nuclei of BPV-infected cells and is not found in cells infected with several rodent parvoviruses of LuIII (M. Lederman, J. T. Patton, E. R. Stout, and R. C. Bates, submitted for publication). Further work is required to elucidate the relationship among the capsid proteins and the newly detected noncapsid proteins to determine which regions of the genome code for these proteins and to define their role in the viral infectious cycle.

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LITERATURE CITED

