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Virally Coded Noncapsid Protein Associated with Bovine Parvovirus Infection

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A phosphorylated protein (NP-1) with an M_r of 28,000 has been detected in nuclei of bovine parvovirus (BPV)-infected cells in association with chromatin. No protein in this size range was detected after infection of appropriate cells with several autonomous rodent parvoviruses although the BPV-specific protein is similar in size to noncapsid proteins associated with rabbit parvovirus or adeno-associated virus infection. Structural homology between NP-1 and a BPV capsid protein could be detected by electrophoretic analysis of the products of proteolysis with chymotrypsin. This protein can be detected after in vitro translation of RNA from BPV-infected cells and BPV-specific RNA. Homology between the in vivo- and in vitro-synthesized species was shown by the similarity of the chymotryptic products.

Bovine parvovirus (BPV) is one of the autonomous parvoviruses, which as a group show a requirement for host cell passage through the S phase of the cell cycle for their replication (2). The genome of BPV is single-stranded DNA, 5.5 kilobases in size (1.6×10^6 daltons) (19). The virion contains four polypeptides of M_r 80,000, 72,000, 62,000, and 60,000 (9). The virion proteins have been shown to have amino acid homology by serological methods and by partial proteolysis (9). This type of homology has been noted for minute virus of mice (21) and for the capsid proteins of adeno-associated virus (AAV) (10), one of the group of defective parvoviruses which require helper virus for productive infection.

Noncapsid proteins coded by parvovirus genomes have also been detected. Buller and Rose detected two polypeptides of M_r 25,000 and 15,000 in AAV-infected cells and as in vitro translation products of AAV RNA (4, 5). Mitra et al. (12) found three in vitro translation products of RNA from Kilham rat virus-infected cells of M_r = 38,000, 32,000, and 21,000. Astell et al. (1) reported that Cotmore and Tattersall detected two noncapsid proteins (85,000 and 25,000 daltons) in cells infected with minute virus of mice. Rhode and Paradiso have found an in vitro translation product of H-1 RNA (84,000 daltons), which immunoprecipitates with sera from infected hamsters but not with antivirion antiserum (18). A phosphorylated noncapsid protein of 71,000 daltons from Aleutian disease virus-infected mink cells shows a similar immunoprecipitation pattern (3). Matsunaga and Matsuno (11) have identified two noncapsid proteins of 25,000 and 22,000 daltons in rabbit parvovirus-infected cells which share amino acid homology and, in contrast to the H-1 and ADV noncapsid proteins, were immunoprecipitable by anticapsid antibody. We report here the appearance of a noncapsid protein (NP-1) in BPV-infected cells, its translation with BPV-specific RNA as template, and its structural relationship to BPV capsid proteins.

(A preliminary report of a portion of this work has been presented [J. T. Patton, R. C. Bates, and E. R. Stout, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, S86, p. 232].)

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MATERIALS AND METHODS

Cell culture, virus infection, and radiolabeling. Cells used in this investigation included secondary bovine fetal lung or spleen cells, buffalo lung cells (ATCC CCL 40), bovine turbinate cells (ATCC CRL 1390), NB (SV40-transformed newborn human kidney) cells, and normal rat kidney cells. Cells were cultured and infected with 10 PFU per cell of autonomous parvoviruses as described by Parris and Bates (14). KB cells in suspension culture were infected with adenovirus and AAV as described by Buller and Rose (4). For $^{32}P_i$ labeling, the medium was changed to phosphate-free minimal essential medium containing 10% dialyzed fetal calf serum and 10 μCi of $^{32}P_i$ per ml at 6 h postinfection. For [^{35}S]methionine labeling, the medium was changed to minimal essential medium containing 0.1 the normal amount of methionine and 10% dialyzed fetal calf serum at 6 h postinfection, and [^{35}S]methionine (5 μCi/ml, 1,200 Ci/mmol) was added 10 h postinfection. Cells were harvested 24 to 26 h postinfection.

Preparation of NP-1. Mock- and virus-infected cells were washed twice in a modified phosphate-buffered saline (20), and, for electrophoresis, the pellets were resuspended directly in gel application buffer (8) and sonicated to disrupt DNA.

Studies of the subcellular distribution of NP-1 followed a modification of the fractionation protocol of Tremblay et al. (22). Cells were fractionated into nuclei and cytoplasm as described by Pritchard et al. (16). The nuclei were resuspended in 50 mM Tris-chloride (pH 7.5)–5 mM $MgCl_2$–25 mM KCl–3 mM DTT and lysed by addition of an equal volume of 0.2% Sarkosyl. The suspension was layered over 4 ml of 15% sucrose over 40% sucrose in the same buffer and centrifuged in an SW27 rotor at 25,000 rpm for 60 min at 4°C. The nuclear membrane (the band forming at the interface), the chromatin (pellet), and the nucleoplasm (material remaining at the top of the gradient) were electrophoresed on 7 to 15% linear gradient sodium dodecyl sulfate (SDS)-polyacrylamide gels, and the distribution of NP-1 was determined from densitometric tracings of autoradiograms.

Electrophoresis and autoradiography. SDS-polyacrylamide gel electrophoresis was carried out by the method of Laemmli (8). Gels containing ^{32}P were exposed to Kodak XAR-5 X-ray film either directly or with a Cronex Lightning-Plus intensifier screen at −80°C. Gels containing [^{35}S]me-

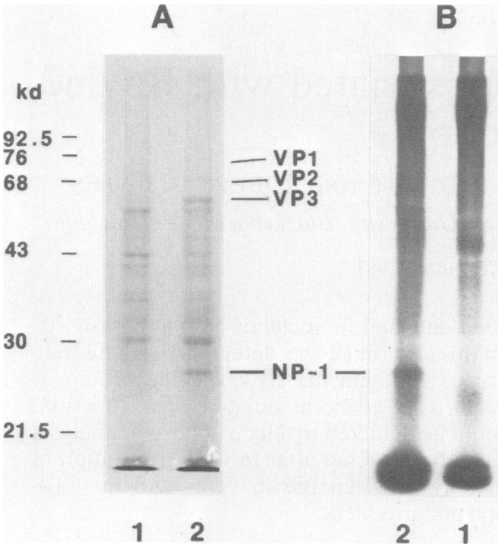

FIG. 1. Detection of NP-1 in BPV-infected bovine fetal lung cells. (A) Coomassie brilliant blue R-stained 10% SDS-polyacrylamide gel of ^{32}P-labeled lysates of mock-infected (lane 1) or BPV-infected (lane 2) cells. The positions of the molecular weight markers phosphorylase b (92,500 daltons), human transferrin (75,000 daltons), bovine serum albumin (68,000 daltons), ovalbumin (43,000 daltons), carbonic anhydrase (30,000 daltons), and soybean trypsin inhibitor (21,500 daltons) are indicated. (B) Autoradiogram of the gel shown in A. The position of NP-1 is indicated. VP1, VP2, and VP3 mark the positions of the capsid proteins.

thionine were fluorographed with En^{3}Hance (New England Nuclear Corp., Boston, Mass.) and exposed with an intensifying screen. Gels were stained with 0.2% Coomassie brilliant blue R (Sigma Chemical Co., St. Louis, Mo.) in 50% methanol–10% acetic acid and destained in 30% methanol–10% acetic acid.

In vitro translations. RNA from BPV-infected cells was translated in a message-dependent rabbit reticulocyte lysate as previously described (9). BPV-specific RNA was prepared by hybridizing 2 mg of infected cell RNA to 50 μg of BPV viral DNA covalently bound to 500 mg of epoxycellulose (13). Binding, hybridization, and elution were done according to the recommendations of the supplier (Bethesda Research Laboratories, Gaithersburg, Md.). Sequential immunoprecipitation of in vitro translation reactions with preimmune rabbit immunoglobulin G (IgG) and then with rabbit anticapsid IgG was done as previously described (6, 9).

Partial proteolytic cleavage of NP-1. Partial proteolysis of in vitro [^{35}S]methionine-labeled NP-1, in vivo [^{35}S]methionine-labeled NP-1 and BPV capsid protein, or ^{32}P-labeled NP-1 was carried out by the method of Cleveland et al. (7). Stained proteins, cut from a preparative gel of a lysate of BPV-infected cells, were stored frozen overnight in buffer containing 1.3 U of aprotinin per ml (Sigma). In vitro translation reactions (50 μl) containing RNA from infected cells were immunoprecipitated as described above. Eluates of the immune precipitates were electrophoresed, and areas of the gel corresponding to marker NP-1 were cut out and processed as described above.

RESULTS

Detection and distribution of NP-1. When lysates of bovine parvovirus-infected bovine fetal lung cells prepared 24 h postinfection were electrophoresed on SDS-polyacrylamide gels and compared with a parallel lysate of uninfected cells, a protein (NP-1) of apparent M_r of 28,000 was detected in infected cell material by staining (Fig. 1A). This protein was in high concentration in infected cells (about 250 μg/10^7 cells as estimated by the intensity of Coomassie brilliant blue R staining) and was as prominent as the major capsid protein, VP3. Although no phosphorylation of BPV capsid proteins was detected after electrophoresis on gels of different acrylamide percentages (data not shown), NP-1 was found to be phosphorylated, as shown by the comigration of $^{32}P_i$ incorporated in vivo between 6 and 24 h postinfection (Fig. 1B). NP-1 was detected after BPV infection of bovine fetal spleen cells, buffalo lung cells, and bovine turbinate cells. To determine the cellular localization of this protein, infected cells were fractionated as described above. At least 95% of the ^{32}P label associated with NP-1 was found in a nuclear pellet, and greater than 85% of the nuclear NP-1 was associated with chromatin (Table 1).

No increase in mass of a protein in this molecular weight range was detected in nuclear lysates of appropriate host cells infected with the other autonomous parvoviruses LuIII, H-1, or Kilham rat virus (data not shown). No in vivo incorporated $^{32}P_i$ was detected in the 28,000-dalton range in nuclear lysates of Kilham rat virus-infected normal rat kidney cells as compared with uninfected cells. However, NP-1 had a similar electrophoretic mobility to a protein originally detected by Buller and Rose (4, 5) which appeared in KB cells after coinfection with human adenovirus and AAV type 2 (data not shown).

Proteolytic analysis of the relation of NP-1 to BPV capsid proteins. To detect possible structural homology between NP-1 and BPV capsid proteins, the partial proteolysis products of NP-1 and both the 80,000- (VP1) and 62,000-dalton (VP3) capsid proteins were compared. Previous studies on amino acid homology among the BPV capsid proteins had used *Staphylococcus aureus* V8 protease (9). However, only two species very close in molecular weight were detected as proteolysis products of NP-1 with this enzyme. The action of chymotrypsin on NP-1 gave numerous bands, two of which were identical in migration to bands obtained after treatment of VP1 with this enzyme (Fig. 2). No homologous bands between NP-1 and VP3 were detected with this enzyme (data not shown).

Determination of the genome coding for NP-1. The appearance of NP-1 after viral infection and its amino acid homology to a capsid protein suggested that this protein was coded by the viral genome. We have previously detected three polypeptides in the M_r range of 25,000 to 30,000 as immunoprecipitation products of in vitro translation of cytoplasmic RNA from BPV-infected cells with either anticapsid IgG or IgG from BPV-infected calves and of BPV-specific RNA

TABLE 1. Intracellular distribution of ^{32}P-labeled NP-1 in BPV-infected bovine fetal spleen cells

Subcellular fraction	% Total[a]
Cytoplasm	<5
Nuclei	>95
Nucleoplasm	<2
Nuclear envelope	<3
Chromatin	>90

[a] Percentages were obtained from densitometric scans of autoradiograms of electropherograms of cell fractions prepared as described in the text.

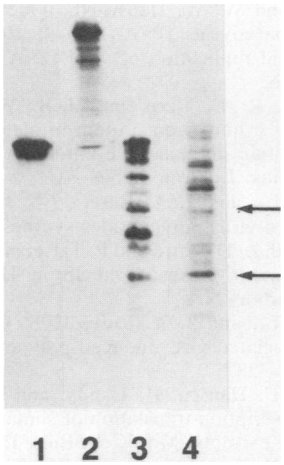

FIG. 2. Homology between NP-1 and a BPV capsid protein (VP1) labeled with [^{35}S]methionine. Lanes 1 and 3: NP-1. Lanes 2 and 4: 80,000-dalton capsid protein. Lanes 1 and 2: no treatment. Lanes 3 and 4: treated with 100 μg of chymotrypsin. Arrows indicate proteolysis products of the same apparent electrophoretic mobility.

with anticapsid IgG (9). To determine whether one of these low-molecular-weight species might be NP-1, RNA from BPV-infected cells was translated in vitro in a message-dependent rabbit reticulocyte lysate, the reaction immunoprecipitated first with preimmune IgG and then with anticapsid IgG. An eluate of the immune precipitate was electrophoresed (Fig. 3, lane 2) in parallel with a [^{35}S]methionine-labeled lysate of BPV-infected cells (Fig. 3, lane 1). One of the three low-molecular-weight in vitro translation products had the same electrophoretic mobility as NP-1. To

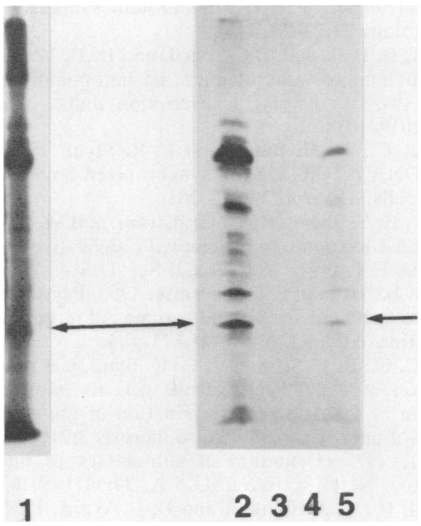

FIG. 3. Comparison of electrophoretic mobility of NP-1 with translation products of various RNAs. Lysate of [^{35}S]methionine-labeled BPV-infected BFL cells (lane 1). Eluates of sequential immunoprecipitation with preimmune IgG and anticapsid IgG of translation mixes containing cytoplasmic RNA from BPV-infected cells (lane 2), no RNA (lane 3), RNA which does not hybridize to virion DNA (lane 4), and BPV-specific RNA (lane 5). Autoradiogram of 10% SDS-polyacrylamide gel of lysates or eluates of immune precipitates. The position of NP-1 is indicated by the arrows.

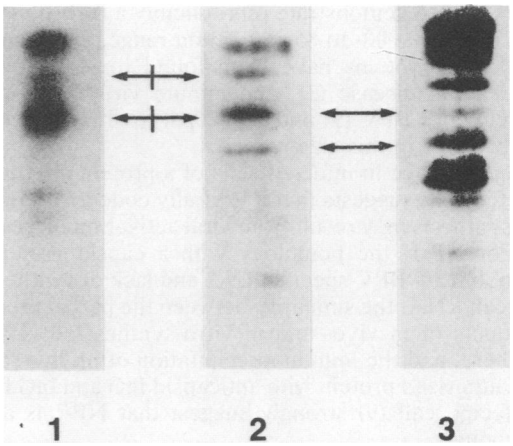

FIG. 4. Comparison of chymotryptic proteolysis products of [^{35}S]methionine-labeled in vitro translation product with those of NP-1 labeled in vivo with either [^{35}S]methionine or $^{32}P_i$. Lane 1, ^{32}P-labeled NP-1. Lane 2, [^{35}S]methionine-labeled in vitro translation product. Lane 3, [^{35}S]methionine-labeled NP-1. Arrows indicate bands of homology between in vitro translation product and in vivo NP-1, both labeled with [^{35}S]methionine. Crossed arrows indicate homology between proteolysis products of in vitro-translated and ^{32}P-labeled NP-1. Proteins were digested with 100 μg of chymotrypsin.

determine the genome coding for NP-1, a portion of the same RNA preparation was hybridized to BPV virion DNA which had been covalently attached to epoxycellulose (13). Of the input RNA, 0.5% bound to this material. The nonhybridizing RNA (presumably bovine cell RNA) and the BPV-specific RNA were also translated and immunoprecipitated as described above for total cytoplasmic RNA. The polypeptide corresponding to NP-1 was detected as a translation product of BPV-specific RNA (Fig. 3, lane 5) but not as a product of RNA which did not hybridize to BPV DNA (Fig. 3, lane 4). No polypeptides were observed in a translation reaction from which RNA was omitted (Fig. 3, lane 3).

Proteolytic analysis of the relation of in vivo-labeled NP-1 to in vitro translation product. To confirm the identity of the in vitro product with NP-1 synthesized in infected cells, the chymotryptic peptides of these species were compared (Fig. 4). There are several bands in common (arrows) between the [^{35}S]methionine-labeled protein isolated from a gel of a lysate of infected cells (Fig. 4, lane 3) and the [^{35}S]methionine-labeled in vitro translation product (Fig. 4, lane 2). There are also bands (crossed arrows) with the same mobility as proteolysis products of in vivo ^{32}P-labeled NP-1 (Fig. 4, lane 1) and [^{35}S]methionine-labeled in vitro translation product (Fig. 4, lane 2).

DISCUSSION

A phosphorylated protein (NP-1) with an apparent M_r of 28,000 has been detected in nuclei of BPV-infected cells. Within the nucleus, this protein is associated with chromatin. No protein in this size range increased substantially in mass after infection of appropriate cells with the autonomous rodent parvoviruses we have tested or with LuIII. Astell et al. (1) report that Cotmore and Tattersall have detected a 25,000-dalton noncapsid protein after infection with minute virus of mice. Noncapsid proteins of this size are associated with infection with the autonomous rabbit parvovirus (11) and with the defective AAV (4, 5). We have

not been able to demonstrate reproducibly a BPV noncapsid protein in the 70,000- to 85,000-dalton range, although such virus-specific proteins have been found for H-1 (17, 18), Aleutian mink disease (3), and minute virus of mice (1). However, both BPV (9) and rabbit parvovirus (11) contain capsid proteins in this size range.

The appearance in infected cells of a protein absent from uninfected cells suggests that it is virally coded. Alternatively, the synthesis may result from viral activation of a cellular gene. For NP-1, the homology with a capsid protein, its translation from BPV-specific RNA and lack of synthesis by bovine cell RNA, the similarity between the partial proteolysis products of in vivo- and in vitro-synthesized NP-1, as shown here, and the immunoprecipitation of in vivo- and in vitro-synthesized protein with anticapsid IgG and IgG from a field-infected calf (9) strongly suggest that NP-1 is a viral gene product.

From the data presented, it cannot be determined whether NP-1 is a cleavage product of the 80,000-dalton capsid protein which is post-transcriptionally modified by phosphorylation or whether NP-1 is coded by a unique mRNA. Translation of cytoplasmic RNA from BPV-infected cells fractionated by size on a sucrose gradient (M. Lederman, unpublished data) suggests that NP-1 might be coded by a BPV-specific RNA with a size of about 1.1 kilobases. S1 nuclease analysis of RNA from BPV-infected cells reveals a spliced molecule of 1.05 kilobases. Transcription mapping of this RNA with restriction endonuclease fragments of the BPV genome (P. R. Burd, J. T. Patton, R. C. Bates, and E. R. Stout, manuscript in preparation) shows that it is transcribed from the left half (3′ end) of the genome and overlaps the region of the genome coding for the putative mRNAs for the capsid proteins by about 250 bases. The 80 amino acids in common would be sufficient to account for the observed amino acid homology and antigenic cross-reactivity.

The determination of the RNA coding for NP-1 is being investigated by hybrid-arrested translation and translation of mRNAs selected by hybridization to restriction fragments covering the left and right halves of the BPV genome. If NP-1 is coded by the 1.05-kilobase RNA, this organization of the BPV genome, i.e., noncapsid proteins being coded from the left half of the genome and the capsid proteins being coded by the right half of the genome, would be in agreement with the arrangement of other parvovirus genomes (15, 18; B. J. Carter, C. A. Laughlin, and C. J. Marcus-Sekura, *in* K. Berns, ed., *The Parvoviruses*, in press), although the number and sizes of transcripts and capsid and noncapsid proteins differ between individual viruses.

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