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Detection of Bovine Parvovirus Proteins Homologous to the Nonstructural NS-1 Proteins of Other Autonomous Parvoviruses

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Two nonstructural proteins of bovine parvovirus (BPV) with apparent molecular sizes of 75,000 and 83,000 daltons have been detected. The proteins were immunoprecipitated from lung cells infected with various isolates of BPV and from in vitro translations of infected cell mRNA. These proteins were expressed as nuclear phosphoproteins and were synthesized early in infection, before the peak of capsid protein synthesis. Early in infection, the 75-kilodalton-size species could be resolved into two bands of equal intensity, but later in infection, the lower-molecular-size form predominanted. Antibodies directed against bacterial fusion proteins encoding amino acid sequences from a highly conserved region of the NS-1 polypeptides of two other parvoviruses, minute virus of mice and the human virus B19, gave specific nuclear fluorescence with BPV-infected cells, although the antibodies failed to immunoprecipitate any viral proteins. The noncapsid proteins appear to be homologous to the previously characterized NS-1 proteins of other autonomous parvoviruses.

Bovine parvovirus (BPV), one of the autonomous parvoviruses, is known to code for three capsid proteins (13) and for NP-1 (14), a phosphorylated, nuclear, nonstructural protein with an apparent molecular size of 28,000 daltons. Other members of the *Parvoviridae* are known to code for a much larger nonstructural protein, usually designated NS-1, which is a nuclear phosphoprotein (3, 9, 17, 18) implicated in both the replication of viral DNA (11, 24; M. Merchlinsky, Ph.D. dissertation, Yale University, New Haven, Conn., 1984) and *trans*-activation of a viral promoter controlling synthesis of the capsid proteins (20). Minute virus of mice (MVM) also codes for another nonstructural protein, NS-2, approximately the size of NP-1 (7), as do adeno-associated virus and lapine parvovirus (4, 15). The overall genomic organization of these viruses appears to be similar (5), and it is known that the NS-1 polypeptides of MVM (7, 8) and H-1 (21) and B19 (6, 22) viruses are encoded in a large open reading frame (ORF) which occupies most of the left half of the genome. The protein encoded by this ORF contains a sequence of about 60 amino acids which is highly conserved among the different viruses (23) and which is thought to be a purine nucleoside-triphosphate-binding site since it contains the required consensus sequence (10). The genome of BPV also has a large ORF in the left half of the genome, but there is little overall homology between its putative protein and that of other parvoviruses, because the similarity coefficients are 0.13, 0.15, and 0.17 between BPV and MVM, adeno-associated virus, and B19 virus, respectively (5). The BPV ORF contains an appropriately placed consensus nucleoside-triphosphate-binding site located in a region of conserved sequence (5), suggesting that it, too, probably encodes a protein(s) physically and functionally homologous to the NS-1 polypeptides of the other viruses. In this report, we describe the identification of two such proteins.

Parasynchronous bovine fetal lung (BFL) cells, grown as described by Parris and Bates (19), were infected with one of three different isolates of BPV: the HADEN strain (1); strain 4, identified as 71-1-20 W (2); and a strain (designated strain Y) which was derived from the strain 4 stock TC2 A13 (the gift of Jeffrey Leary) but was repeatedly passaged in embryonic bovine tracheal cells at Yale University. Cells were labeled with 7.5 μCi of [^{35}S]methionine (1,000 Ci/mmol; New England Nuclear Corp., Boston, Mass.) per ml in the presence of 10% dialyzed fetal calf serum, extracted by the procedure of Bloom et al. (3), omitting the second preclearing step, and immunoprecipitated with antisera prepared against purified BPV capsids (rabbit 0118) or from a calf experimentally infected and immunized with disrupted (frozen and thawed) BPV-infected cells (calf 86). Immunoprecipitation products were collected with Formalin-fixed *Staphylococcus aureus* cells (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) and electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gels (12) which were treated with Amplify (New England Nuclear) and exposed at −80°C with an intensifying screen. While serum 0118 specifically precipitated the viral structural proteins (VP-1, 80 kilodaltons [kDa]; VP-2, 72 kDa; and VP-3, 62 kDa) from each infected cell extract, calf 86 serum also recognized three additional major polypeptide species: a low-molecular-size moiety of around 28 kDa which may be the previously described NP-1 molecule and two higher-molecular-size polypeptides of 75 and 83 kDa (Fig. 1A). The relative proportions of the structural and nonstructural proteins precipitated by calf 86 serum differed somewhat with the infecting strain. Differential expression of the genes for the structural and nonstructural proteins was seen more dramatically in the in vitro translation products of mRNA extracted from two different infections (Fig. 1B). Cytoplasmic RNA from BPV (strain 4)-infected BFL cells programmed the synthesis of structural proteins and NP-1 as detected by immunoprecipitation with rabbit 0118 serum but gave only faint bands at the positions of the 75- and 83-kDa nonstructural proteins when analyzed with calf 86 serum or with serum from a rabbit (rabbit 8) which had been injected with partially purified BPV (8). In contrast, the translation products of RNA from embryonic bovine tracheal cells

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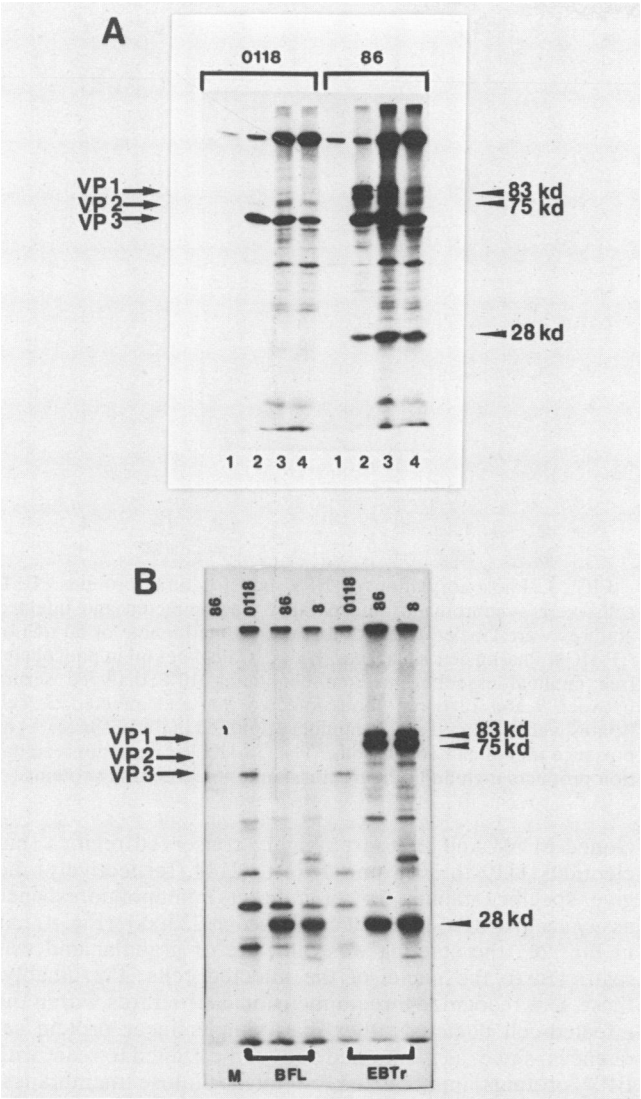


FIG. 1. (A) Presence of BPV nonstructural proteins in cells infected with various strains of BPV. Cells were labeled with [^{35}S]methionine as described in the text in the absence of virus (lane 1) or after infection with the HADEN strain (lane 2) (1), BPV 4 (lane 3), or the Y strain (lane 4). Lysates of these cells were immunoprecipitated with either anticapsid antibody (rabbit 0118 antibody) or calf 86 antibody. (B) Presence of structural and nonstructural proteins of BPV as in vitro translation products of BPV RNA. Cytoplasmic RNA from mock-infected and strain 4-infected BFL cells and Y strain-infected embryonic bovine tracheal (EBTr) cells was translated as described by Lederman et al. (14). Reactions were immunoprecipitated with rabbit 0118 anticapsid antibody, calf 86 antibody, and rabbit 8 antibody. This last antibody, supplied by J. Leary, was prepared to partially purified BPV. The capsid proteins are indicated by arrows, and the noncapsid proteins are indicated by arrowheads.

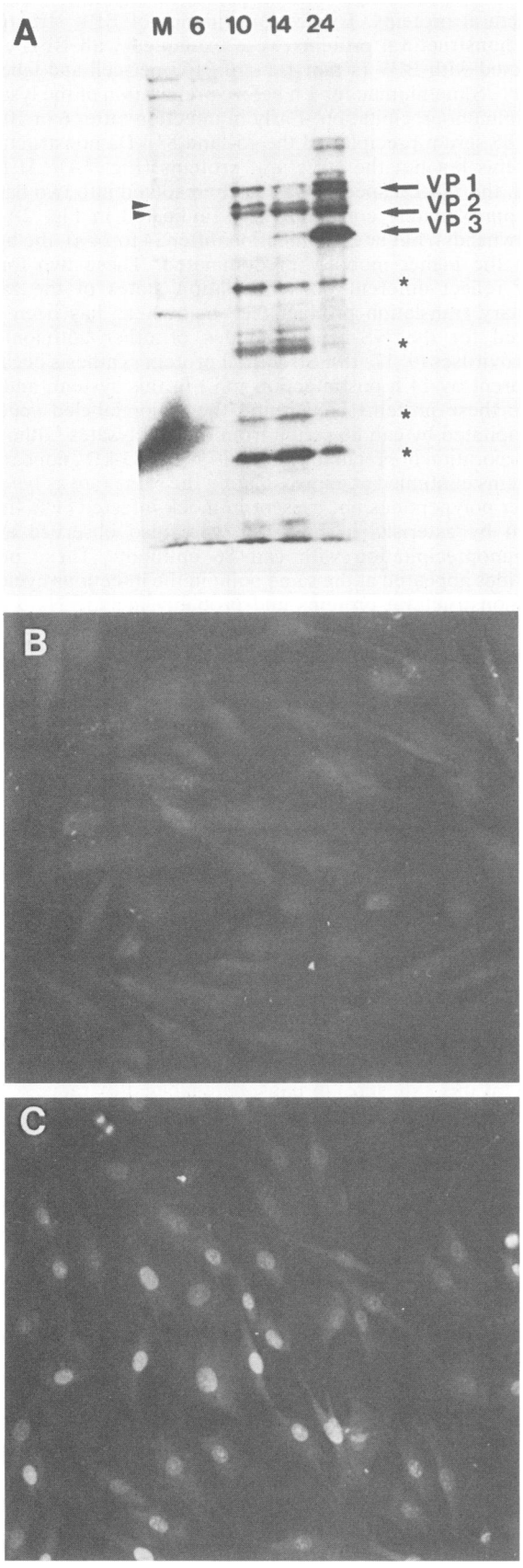
infected with strain Y gave relatively large amounts of the 75- and 83-kDa species when precipitated with either calf 86 or rabbit 8 serum.

This result may, as well, demonstrate a difference in the concentration of mRNA for structural and nonstructural proteins in the translated RNA, reflecting the point of the infectious cycle at the time of harvest. Molitor et al. (17) showed that expression of a major nonstructural protein (NS-1) of porcine parvovirus preceded expression of the structural proteins. Expression kinetics of BPV structural and nonstructural proteins were examined with BFL cells infected with BPV (strain 4) at 10 PFU per cell and labeled with [^{35}S]methionine for 1 h before preparation of the lysates for immunoprecipitation. Early in infection (after 6 or 10 h), calf 86 serum precipitated the 75- and 83-kDa nonstructural proteins but not the structural proteins (Fig. 2A). At that time, the 75-kDa species was easily resolved into two bands of approximately equal intensity (indicated in Fig. 2A by arrowheads), but later in infection (after 14 to 24 h), the band with the higher mobility predominated. These two forms may reflect different phosphorylation states of the same primary translation product (see below), as has been observed for the NS-1 polypeptides of other autonomous parvoviruses (9, 17, 18). Structural protein synthesis became apparent by 14 h postinfection (p.i.) in this system, and by 24 h, these proteins constituted the major labeled species precipitated by calf 86 serum from the cell lysates, although incorporation of precursor into the 75- and 83-kDa noncapsid proteins continued at approximately the same rate as before. Other polypeptides not present in mock-infected cells (indicated by asterisks in Fig. 2A) were also observed after immunoprecipitation with calf 86 antibody. These polypeptides appeared at the same point in the infectious cycle as the nonstructural proteins and probably include NP-1 and additional species expressed from the left half of the viral genome, as has been described for adeno-associated virus (16).

Indirect immunofluorescence studies with cells harvested at 7 h p.i. confirmed the early synthesis of the nonstructural proteins. As seen in Fig. 2B, anticapsid antibody (rabbit 0118 serum) failed to give positive fluorescence at that time, but calf 86 serum (Fig. 2C) gave intense punctate or granular nuclear staining similar to that seen for the NS-1 proteins of the rodent viruses (23).

The 75- and 83-kDa proteins of BPV are heavily phosphorylated in vivo, as are their rodent virus counterparts. Figure 3 shows parallel immunoprecipitates from $^{32}P_i$- or [^{35}S]methionine-labeled cells extracted at 12 h p.i. after a 3-h pulse of isotopically labeled precursor. Although both the structural and nonstructural proteins were being synthesized at that time in the infected cells (as shown by the species immunoprecipitated from methionine-labeled material with calf 86 serum), only the nonstructural proteins were detectable (at this exposure) in phosphate-labeled extracts.

Since these proteins appear to be homologous to the NS-1 polypeptides of other parvoviruses, we used monospecific antisera raised against purified bacterial fusion proteins expressing specific regions of the MVM (7) and B19 virus (6) nonstructural proteins in an attempt to analyze this relationship in more detail. The expressed regions of the MVM proteins were the common amino-terminal peptide of NS-1 and NS-2 (nucleotides 225 to 534), part of the highly conserved region of NS-1 which contains the putative ATP/GTP-binding site (nucleotides 1110 to 1638), and the carboxy-terminal half of NS-2 (nucleotides 2075 to 2291). To assess the relationship with B19 virus, we used antibodies directed against a larger contiguous region of the NS-1 polypeptide (nucleotides 1072 to 2144) which encompassed the whole of the highly conserved nucleoside-triphosphate-binding site. None of these antisera immunoprecipitated BPV proteins from radiolabeled cell lysates or from in vitro translations primed by RNA from infected BFL cells or embryonic bovine tracheal cells (6), nor did they stain immunoblots (data not shown). However, the antisera to the



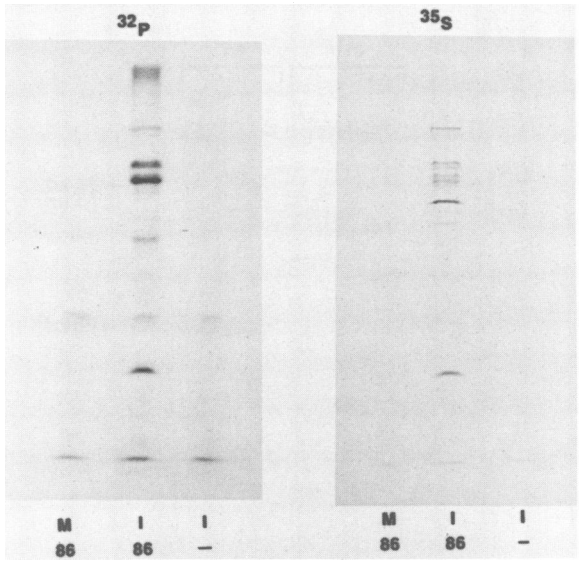


FIG. 3. Phosphorylation of BPV nonstructural proteins. BFL cells were synchronized, and parallel mock-infected and infected cultures were labeled with 7.5 μCi of [^{35}S]methionine or 50 μCi of $^{32}P_i$ (ICN Pharmaceuticals, Inc., Irvine, Calif.) per ml in phosphate-free minimal essential medium containing 10% dialyzed serum between 9 and 12 h p.i. Mock-infected (M) and infected (I) cell lysates were prepared and immunoprecipitated in the absence (−) or presence (86) of calf 86 antibody. The ^{35}S-labeled immunoprecipitation products include both structural and nonstructural proteins.

cloned MVM and B19 virus highly conserved regions (nucleotides 1110 to 1638 and 1072 to 2144, respectively) did give specific staining in an indirect immunofluorescence assay against BPV-infected cells fixed at 22 h p.i. (Fig. 4). As in Fig. 2C, the staining was punctate or granular and was restricted to the nuclei of the infected cells. Presumably, these sera recognize three-dimensional structures within the infected-cell nucleus rather than simple linear protein sequences, since, as mentioned above, they failed to react with BPV proteins immobilized on nitrocellulose membranes. The fact that we consistently saw stronger immunofluorescence with the anti-B19 virus serum than with the anti-MVM serum probably reflected the length of the expressed fragment rather than any particular conserved cluster of amino acids. Although we do not know for certain which proteins in BPV-infected cells reacted with these sera, the 75- and 83-kDa species are the most likely candidate molecules, given the size of the ORF in which this conserved sequence is located (5). Nevertheless, as in adeno-associated virus

FIG. 2. Time course of synthesis of BPV nonstructural proteins. (A) Analysis by immunoprecipitation of radiolabeled cell lysates. Cells were harvested at the indicated time p.i. (lanes 6, 10, 14, and 24) and immunoprecipitated with calf 86 antibody. A mock-infected culture (lane M) was treated in parallel with the infected cultures. The positions of the structural proteins are indicated by arrows, and the two forms of the 75-kDa species are indicated by arrowheads. *, Other polypeptides observed. (B and C) Analysis by indirect immunofluorescence. BFL cells grown on cover slips were synchronized, infected with BPV 4, and harvested at 7 h p.i. The cells were fixed with −20°C methanol, treated with undiluted rabbit 0118 antibody (B) or calf 86 antibody (C) as first antibody and either fluorescein isothiocyanate–goat anti-rabbit IgG (Cooper Biomedical, West Chester, Pa.) or dichlorotriazinyl fluorescein–goat anti-bovine IgG (Jackson Immunoproducts) as second antibody.

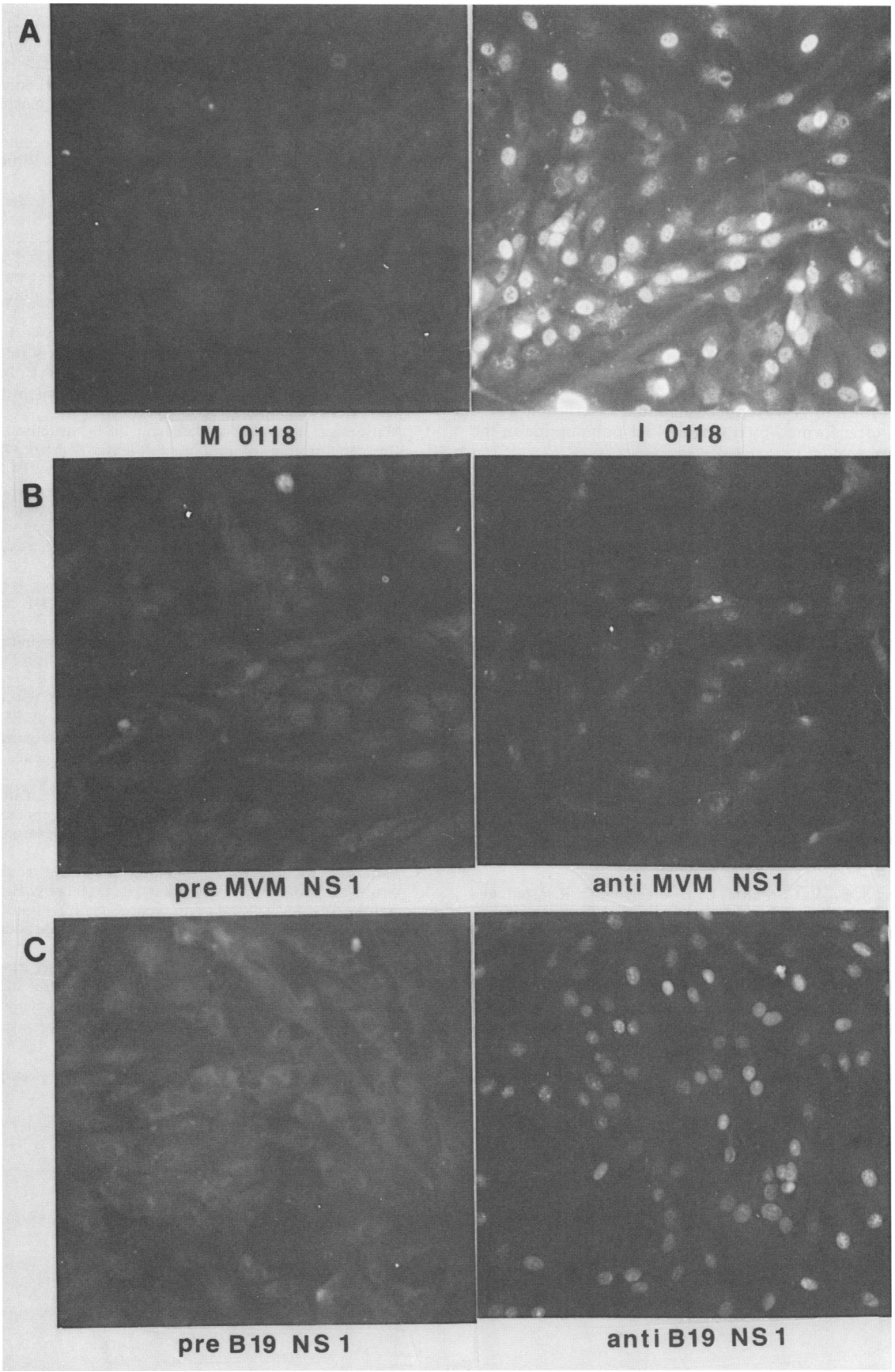


FIG. 4. Immunological cross-reactivity of BPV-infected cells with rabbit antisera prepared against fusion proteins encoding the homology regions of the left ORFs of MVM and B19 parvoviruses. (A) Parasynchronous BFL cells, either mock infected (M) or infected (I) with strain 4 of BPV, were harvested at 22 h p.i., fixed, and exposed to anticapsid antibody. (B and C) Infected cells were also exposed to preimmune serum and the corresponding immune serum containing antibody to the fusion protein expressing the left ORF homology regions.

(16), some of the smaller noncapsid polypeptides seen in Fig. 1A and 2A may also carry these determinants. Antisera against the other regions of the MVM NS-1 and NS-2 proteins failed to give specific staining.

The data presented in this report indicate that BPV encodes at least two high-molecular-weight nonstructural proteins which can be precipitated by sera from animals that have been immunized with infected cells or crude virus preparations. Like the NS-1 proteins of other parvoviruses, these molecules are synthesized early in infection, are heavily phosphorylated, and are confined to the cell nucleus. Since both the 75- and 83-kDa molecules are present in the in vitro translation products of infected-cell RNA, they are likely to be primary translation products arising from different mRNAs rather than being differently processed forms of the same precursor. Nonetheless, size constraints in the BPV genome suggest that the proteins must share much of their amino acid sequences, and it will be of considerable interest to see how they are encoded and whether they share the various roles in virus replication and transcription which appear to be performed by a single NS-1 polypeptide in the rodent viruses.

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