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# Characterization of the Sendai Virus V Protein with an Anti-Peptide Antiserum

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**The Sendai virus V protein, which is a fusion of the P and V ORFs of the P gene, was characterized with antisera to a portion of the V ORF and compared to the P protein. The only property found in common with P is that V is also highly phosphorylated, and this is so even when these proteins are expressed independently of the other viral proteins. Otherwise, V was not found in virions, was not strongly associated with viral nucleocapsids like P, and anti-V had no effect on viral RNA synthesis *in vitro* under conditions where anti-P was highly inhibitory. The available evidence suggests that V may play a role in RNA synthesis, but it is not an essential one like that of the P protein.** 

Paramyxovirions contain a helical nucleocapsid (NC) core composed of the minus-strand RNA genome (15–16 kb) and ca. 2000 copies of the nucleocapsid protein NP. In addition, ca. 200 copies of the phosphoprotein P and 50 copies of the large protein L are bound to this structure. The genome is expressed via complementary monocistronic mRNAs and is replicated via a full-length complementary strand, the antigenome. Reconstitution experiments with Newcastle disease virus (NDV) have shown that polymerase activity *in vitro* requires both the P and the L proteins as well as the RNA–NP core (Hamaguchi *et al.*, 1983). The P and L proteins are also found as a complex, both on and off the NC core (Hamaguchi *et al.*, 1983; Portner *et al.*, 1988). By analogy to the rhabdovirus VSV, whose L gene and that of the paramyxoviruses are thought to share a common ancestor (Poch *et al.*, 1989), L is the actual polymerase, and capping and poly(A) formation of mRNAs are thought to map to this protein as well (Banerjee, 1987). The VSV counterpart to the paramyxovirus P protein is NS, which has recently been referred to as P (Herman, 1986; Bilsel *et al.*, 1990), as NS (nonstructural) is a misnomer. The paramyxovirus and rhabdovirus P proteins are not related by sequence homology or size, but are organized similarly. Both are acidic proteins, whose N terminal halfs are also highly phosphorylated (Bell and Prevec, 1985; Vidal *et al.*, 1988) and poorly conserved among related viruses. The C-termini are better conserved and contain the site(s) by which this protein binds to the RNA–NP core (Emerson and Schubert, 1987; Gill *et al.*, 1986; Kolakofsky *et al.*, 1990; Ryan and Portner, 1990).

The paramyxovirus P genes are, however, more complex. Those of the morbilliviruses measles (Bellini *et al.*, 1985) and CDV (Barrett *et al.*, 1985), and the parainfluenza viruses SEN (Giorgi *et al.*, 1983; Shioda *et al.*, 1983) and parainfluenza virus 3 (PIV3) (Galinski *et al.*, 1986; Luk *et al.*, 1986; Sakai *et al.*, 1987; Spriggs and Collins, 1986), which are 568–602 residues long, also contain a 200 aa ORF (called C) overlapping the N-terminus of P. The P and C ORFs are expressed by ribosomal choice (Curran and Kolakofsky, 1988). Those of the other parainfluenza viruses (SV5 (Thomas *et al.*, 1988), mumps virus (Elliott *et al.*, 1990; Paterson and Lamb, 1990; Takeuchi *et al.*, 1990), NDV (Sato *et al.*, 1987; McGiness *et al.*, 1988), PIV2 (Ohgimoto *et al.*, 1990; Southern *et al.*, 1990), and PIV4 (Kondo *et al.*, 1990)) are only 392–398 aa in length and lack the C ORF. However, all paramyxovirus P genes except respiratory syncitial virus (RSV) and PIV1 (see Discussion) also contain an ORF called V and are transcribed into at least two slightly different mRNAs, one for the P and one for the V protein (Fig. 1). In all cases one mRNA is an exact copy of the gene, whereas the other is modified by the addition of either one or two Gs, within a short run of Gs (Thomas *et al.*, 1988; Cattaneo *et al.*, 1989; Vidal *et al.*, 1990a). The G additions are thought to take place by the viral polymerase reiteratively copying a template C residue during mRNA synthesis, by a stuttering mechanism (Vidal *et al.*, 1990b; Pelet *et al.*, 1991). These insertions alter the reading frame in the middle of the gene and allow ribosomes to translate the alternate ORF downstream (Fig. 1).

The V ORF is the most highly conserved of all of the sequences of this gene (Thomas *et al.*, 1988) and is a Cys-rich domain similar to other transcriptional cofactors such as zinc-finger proteins (Klug and Rhoads,

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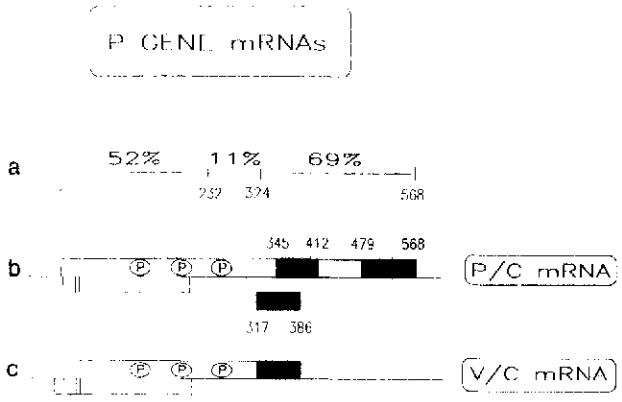



FIG. 1. Schematic representation of the SEN P/C and V/C mRNAs. The P gene mRNAs (lines b and c) are drawn as horizontal lines, with the ORFs indicated as boxes either above (P and V ORFs) or below (C ORF) the line. The vertical lines at the left of the C ORF indicate the multiple start codons. The stipled areas of the P ORF, residues 345–412 and 479–568, indicate the region of this protein involved in attachment to the NC (Ryan and Portner, 1990); the circled Ps indicate the region of phosphorylation. The V ORF is colored in black, and in the P/C mRNA is indicated as an independent black box. The difference between the P/C and V/C mRNAs is the addition of a single guanosine within the codon for amino acid 317, to the V/C mRNA. Line a shows the regions of homology between the P proteins of SEN and PIV1. The percentages refer to residue identity of each region.

1987) and the HIV tat protein (Frankel *et al.*, 1988). It seems plausible then that the SEN V protein, which shares the first 317 residues with P, also acts in RNA synthesis. Actually, the SEN V protein has not as yet been detected; in most strains it is expected to comigrate with NP on SDS gels and could be hidden by the more abundant NP. Only the inserted mRNA has been found, which is half as abundant as that of P (Vidal *et al.*, 1990a). To learn more about this protein, we have prepared an antibody to a V peptide (anti-V).

## MATERIALS AND METHODS

### Cell cultures and virus

BHK cells were subcultured in minimal essential medium (MEM) containing 5% fetal bovine serum. Cells were infected with Sendai virus (20–50 PFU per cell) or vaccinia viruses (5–10 PFU per cell). Infections were maintained in serum free MEM at 33°.

SEN was purified from infected cell medium by first pelleting out cellular debris (5 min at 10,000 *g*) and then loading the supernatant onto a 25% glycerol–TNE (50 m*M* NaCl, 10 m*M* Tris, pH 7.4, 1 m*M* EDTA) cushion. Virus was pelleted by centrifugation at 150,000 *g* for 1 hr in either an SW41 or an SW28 rotor.

### Metabolic labeling and immunoprecipitation

Cellular proteins were pulse labeled with [$^{35}$S]cysteine essentially as outlined previously (Curran and Kolakofsky, 1988, 1989). When labeling with $^{32}PO_4$, cell monolayers on 9-cm dishes were incubated for 3 hr in phosphate free medium prior to addition of 2 mCi of $^{32}PO_4$ at 5 hr p.i. Infections were harvested at 24 hr p.i. by directly solubilizing the monolayer in 500 $\mu$l of RIPA buffer (150 m*M* NaCl, 1% deoxycholate, 1% Triton X-100, 0.1% SDS, 10 m*M* Tris, pH 8.3) containing 2% SDS, 1% 2-mercaptoethanol, 1% aprotinin, and 6 m*M* PMSF. Chromosomal DNA was sheared by sonication.

For immunoprecipitation (Curran and Kolakofsky, 1989), cell extracts were diluted 20-fold in RIPA buffer prior to addition of the antibody and incubated for 2–4 hr at 4° before addition of 50 $\mu$l of a 50% suspension of Protein A–Sepharose 4B (Pharmacia) prepared in 20 m*M* Tris, pH 8.0. Incubation at 4° was then continued for a further 2 hr. The Protein A beads were then washed twice in 150 m*M* NaCl, 50 m*M* Tris, pH 7.8, 5 m*M* EDTA, 0.5% NP-40 and once in 0.5 *M* LiCl, 1% 2-mercaptoethanol, 0.1 *M* Tris, pH 7.8, before being mixed with 30 $\mu$l of 2 × protein sample buffer (6% SDS, 4% 2-mercaptoethanol, 125 m*M* Tris, pH 6.8, 20% glycerol, and 0.2% bromophenol blue). For $^{32}PO_4$-labeled cell extracts, an additional wash in PBS containing 5 $\mu$g/ml DNase and 5 $\mu$g/ml RNase A at 37° for 20 min was carried out.

### Preparation of V polyclonal antibody

The peptide HRREHIIYERDGYIVDESWC, representing amino acids 2–21 in the V ORF (or residues 319–338 of the V protein), was coupled to keyhole limpet haemocyanin via *m*-maleimidobenzoyl *N*-hydroxysuccinimide ester. The coupled peptide was purified on Sephadex G-25. Rabbits were twice injected subcutaneously at a 1-month interval with the equivalent of 200 $\mu$g of peptide and boosted 2 months later with the same amount. Blood was then taken 2 and 3 weeks after the boost. Animals responding to the peptide (as assayed by ELISA against the peptide coupled to BSA) were further boosted at 6-week intervals and blood was taken 2 weeks later.

### Immunofluorescense

BHK cells seeded at low density on Lab-Tek 8-chamber glass slides (Miles Scientific) were infected with Sendai virus at a multiplicity of 10–20 PFU/cell. At 18 hr postinfection, mock and infected cells were washed with PBS (without $Ca^{2+}$, $Mg^{2+}$) and fixed with 3% formaldehyde in PBS. After fixation, cells were permeabilized with acetone for 3 min at −20° and then blocked with 1% BSA, 0.1% Triton X-100 in PBS for 1 hr at 4°. The primary anti-P monoclonal antibody was diluted 1/100 in blocking solution and incubated for 1 hr at 4°. The cells were then washed twice in PBS

before incubation with the second antibody, goat anti-mouse conjugated to fluorescein (diluted 1/50 in blocking buffer: Nordic, Switzerland), for 1 hr at 4°. After two washes with PBS, an anti-V rabbit antibody diluted in blocking solution (1/100) was applied for 1 hr at 4°. The cells were then washed two times in PBS before incubation for 1 hr at 4° with a 1/50 dilution of the secondary antibody, goat anti-rabbit coupled to rhodamine (Nordic, Switzerland). Chambers were then washed two times in PBS before viewing in a Nikon Optiphot microscope.

The specificity of both the primary and the secondary antibodies was carefully controlled. The anti-P and anti-V primary antibodies produced no detectable signals on mock-infected cells and no cross-reactivity was observed between the anti-mouse and anti-rabbit secondary antibodies.

### Isolation of Sendai virus RNP

Infected cell extracts were prepared by three methods.

1. Lysolecithin extracts were prepared as described by Carlson *et al.* (1985). Cells were disrupted either in TN buffer (30 m*M* NaCl, 10 m*M* Tris, pH 7.6) or in RSB (10 m*M* KCl, 10 m*M* Tris, pH 7.5, 1.5 m*M* $MgCl_2$).
2. Cells were scraped into cold PBS, pelleted, and resuspended in 150 m*M* NaCl, 50 m*M* Tris, pH 7.4, 0.6% NP-40. Nuclei were pelleted from the extract (5 min at 10,000 *g*), and the supernatant was adjusted to 6 m*M* EDTA and stored on ice.
3. Cell pellets were resuspended in ice-cold RSB buffer, and the cell membrane was broken by 10 strokes in a Dounce homogenizer. Nuclei and membranes were pelleted by centrifugation.

Viral RNP was pelleted through 50% (v/v) glycerol–TN buffer onto a 500-μl cushion composed of 68% (w/w) sucrose in $D_2O$–TN buffer (43,000 rpm for 90 min at 20°) in an SW60 roter. Gradient fractions (0.5 ml) were collected and analyzed either by Western ELISA when the cell extracts were unlabeled or by immunoprecipitation when labeled with [$^{35}$S]cysteine.

### Sendai virus RNA synthesis *in vitro*

Purified SEN and DI-H4 (19 μl) were incubated in a reaction mix (100 μl) as described previously (Carlsen *et al.*, 1985), but with 20 μ*M* [$^{32}$P]CTP instead of [$^{3}$H]-UTP and with the addition of RNasin (1 U/ml) and 10% glycerol. The soluble protein fraction was prepared by sedimenting the extract at 50,000 rpm for 90 min at 4° in the SW55 rotor. The samples were incubated in the absence or presence of various Protein A–Sepharose CL-4B (Pharmacia)-purified antibodies, as indicated in the text, for 30 min at 4° and then for 2 hr at 30°. The RNA products were isolated and analyzed by electrophoresis on acid–urea–1.5% agarose gels.

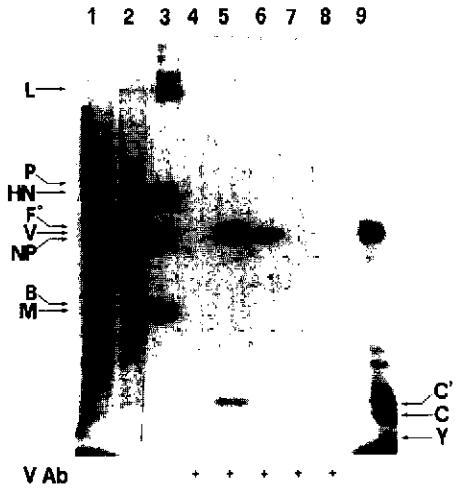



FIG. 2. Detection of V protein in SEN-infected cells but not in purified virus. Cells infected with either SEN or vaccinia viruses were labeled with 10 μCi/ml of [$^{35}$S]cysteine at 16–20 hr p.i. Cell monolayers, or purified virions, were solubilized in RIPA buffer and immunoprecipitated with anti-V. Proteins were resolved on 12.5% polyacrylamide–SDS gels and visualized by fluorography. Lanes: 1, mock-infected BHK cells; 2, SEN-infected BHK cells; 3, purified SEN; 4, purified SEN immunoprecipitated with anti-V; 5, SEN-infected cells immunoprecipitated with anti-V; 6, vv–SEN–V recombinant-infected cells immunoprecipitated with anti-V; 7, wild-type vv-infected cells immunoprecipitated with anti-V; 8, mock-infected cells immunoprecipitated with anti-V; 9, *in vitro* translation of RNA transcribed from SP65 V/C DNA in a reticulocyte lysate in the presence of [$^{35}$S]-methionine.

## RESULTS

### Sendai virus V protein is nonstructural

To more easily detect the V protein, we used the Z strain of SEN, in which NP migrates slightly faster on gels than in the other strains, and radiolabeled the infections with [$^{35}$S]Cys. When the cell extracts were separated on SDS gels (lane 2, Fig. 2) and compared to the uninfected control (lane 1), three closely spaced virus-specific bands of 55–65 kDa are seen. The top and bottom bands were found to represent Fo and NP, respectively, using monoclonal antibodies (mAbs) (not shown). The middle band could be specifically immunoprecipitated with anti-V from infected (lane 5) but not uninfected cells (lane 7), as well as from cells infected with a recombinant vaccinia virus (vv) carrying the P gene with the precise 1G insertion (vv–SEN–V, lane 6), but not from cells infected with nonrecombinant vv (lane 8). The V proteins made *in vivo* also comigrated with V made *in vitro* from a plasmid-generated mRNA (lane 9). The V band in lane 2 appears twice as strong as that of P, and this is consistent with the relative Cys

content of these proteins and the abundance of their mRNAs (V contains 10 Cys and P contains 3 Cys, but there are twice as many P as V mRNAs).

Virions purified from the culture medium, however, did not contain detectable amounts of V when examined directly (lane 3). Neither could this protein be immunoprecipitated from these samples (lane 4), even though eight times more material was used than was analyzed directly in lane 3. V is clearly underrepresented in virions relative to infected cells, in contrast to structural proteins such as P. We find less V in virions than the C proteins (not shown) which are considered as nonstructural proteins. By this criterion, V is then also a nonstructural protein. This is consistent with the description of the SV5 (Peluso *et al.*, 1977) and mumps virus (Herrler and Compans, 1982) V proteins as nonstructural proteins. However, a recent study has found considerable amounts of V in mumps virions (Takeuchi *et al.*, 1990). This variability may be due to the difficulty in purifying mumps virus, which grows to very low titers.

## Intracellular location of the V protein

To examine whether V was associated with NCs, cytoplasmic extracts were centrifuged on sucrose gradients, and equal amounts of each fraction were immunoprecipitated with a mixture of anti-V and a mAb to the C-terminus of P. As shown in Fig. 3, the majority of the P protein, as expected, was found in the bottom fraction where the NCs have sedimented. The selection of the L protein as well from this fraction is presumably due to a P–L complex resistant to the detergent conditions used. The V protein, in contrast, was found only in the top two fractions. In this experiment the extract was prepared with the nonionic detergent NP-40 in normal salt, but identical results were obtained with the lysolecithin protocol, or when the extract was prepared by dounce homogenization without detergent, and in low salt. Moreover, similar results were obtained when the gradients were examined by Western blotting rather than precipitation (not shown). This latter result appears to rule out the possibility that the absence of V in the NC fraction was due to the masking of the anti-V epitope on the NC with another protein. When fraction 1 of Fig. 3 was examined for RNA synthesis, it was found to be as active as a comparable amount of purified virions (not shown). It remains possible that V is weaky associated with NCs, but is removed during the centifugation, even though the gradient solutions contained only 30 m*M* NaCl. Nevertheless, it is clear that the SEN V protein is not associated with NCs in a stable manner as is P.

The intracellular location of V relative to P was also examined by double label immunofluoresence studies,

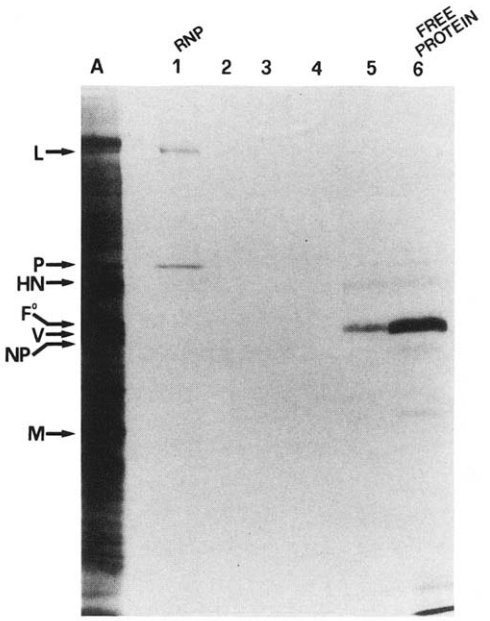
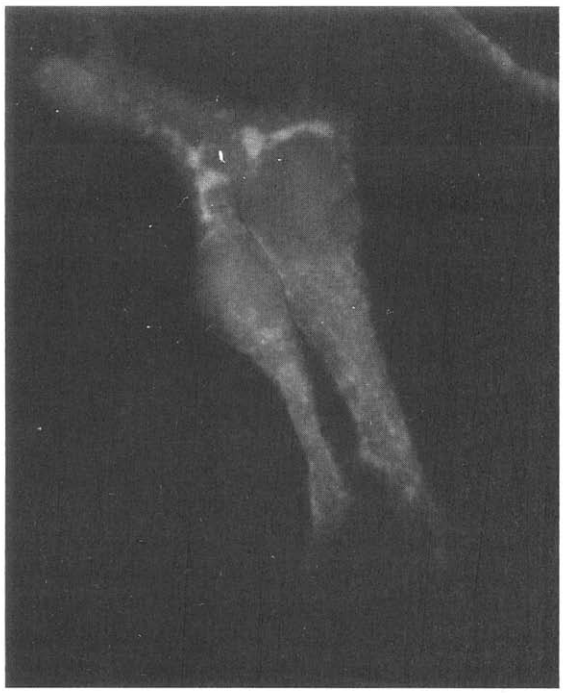


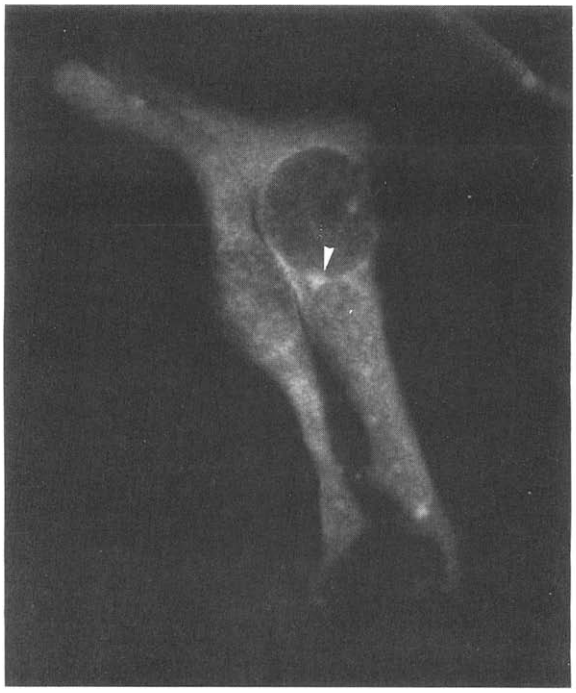

FIG. 3. Analysis of viral proteins which cosediment with RNP. An NP-40 cytoplasmic extract of SEN-infected cells labeled with [$^{35}$S]-cysteine (10 $\mu$Ci/ml) at 16–20 hr p.i. was layered onto a step gradient composed of a 500-$\mu$l cushion of 68% (w/w) sucrose in $D_2O$–TN and 2.5 ml of 50% glycerol–TN formed in an SW60 centrifuge tube. The gradient was spun at 43,000 rpm for 90 min at 20°. Samples of each fraction were immunoprecipitated with anti-V and a MAb to P, and the proteins resolved on a 12.5% SDS–polyacrylamide gel. Lane A: [$^{35}$S]cysteine-labeled infected cell extract. Lanes 1–6: fractions from step gradient.

using a mAb to P (1180, which reacts with the C-terminus of P) and the anti-V peptide antiserum (Materials and Methods). We chose cells which give a punctate anti-P staining in the cytoplasm, with relatively large patches as well (Fig. 4), as found previously (Kristensson and Oervell, 1983). The same pattern is obtained with an anti-NP mAb (not shown), and these are probably areas of NC concentration. These large patches are mostly near the nucleus and to one side of it (Fig. 4). The anti-V fluoresence is generally also found in the areas where P is most intense, but in $\frac{1}{4}$ of the cells examined, there were clearly areas where V was found in the relative absence of P (arrow, Fig. 4). In addition, there is often a ring of anti-V fluorescence around the nucleus which is not seen with anti-P, and the anti-V fluoresence tends to be more granular and evenly distributed in the cytoplasm than that of P. In contrast, we were unable to find any differences in P and NP localization in similar experiments (not shown). Uninfected cells treated identically on adjacent chambers of the slide showed no fluoresence under these conditions (Fig. 4). Thus, whereas the vast majority of P colocalizes with NCs, there appears to be a significant frac-

P Ab

V Ab

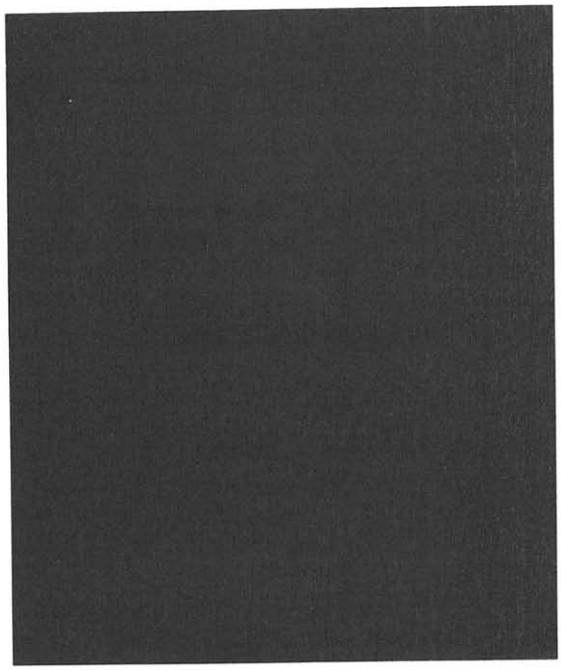


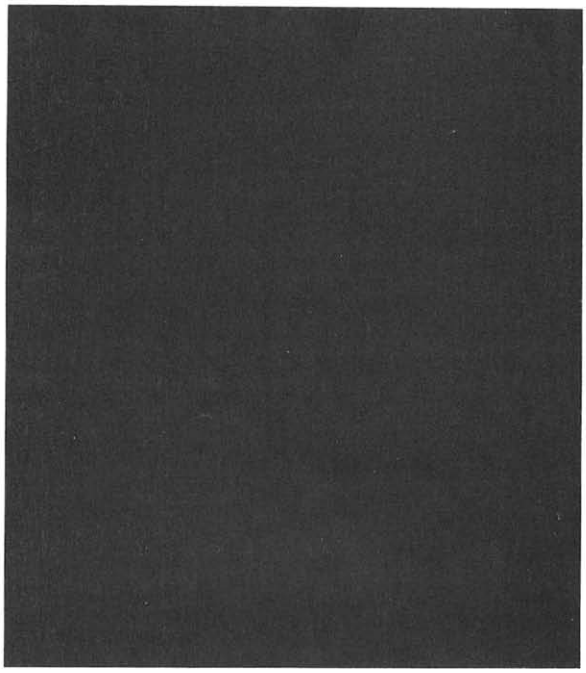


INFECTED

P Ab

V Ab





MOCK

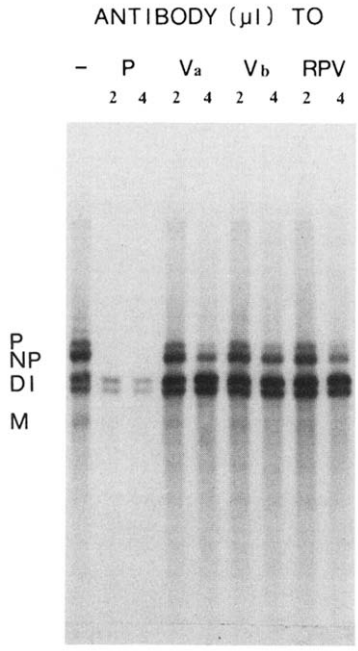



FIG. 5. Effect of anti-V peptide antibody on *in vitro* RNA synthesis. Purified virions including DI-H4 particles were incubated in a reaction mix in the presence of [$^{32}$P]CTP (Materials and Methods) and antibodies as indicated at the top of the figure. All reactions contained soluble protein supernatant from uninfected cells and either no antibodies (left lane) or 2 or 4 μl of anti-P, anti-V from two separate rabbits ($V_a$ and $V_b$), or rabbit anti-rabbit poxvirus as an unrelated control (RPV). Total RNA was isolated and analyzed by acid–urea–agarose gel electrophoresis. The various bands are indicated on the left side. DI indicates the doublet of (+) and (–) strands which separate in this system; P, NP, and M, refer to these viral mRNAs.

tion of V not associated with NCs, some of which is distributed in areas of high density (arrow, Fig. 4).

## Effect of anti-V on RNA synthesis

A possible role for the V protein in viral RNA synthesis *in vitro* was next examined. For these studies, we used a mixture of purified SEN and a DI particle (H4, Kolakofsky, 1976) which, when supplemented with a soluble supernatant of infected cells, carries out mRNA synthesis from the SEN genome (predominantly the NP mRNA) and replicates the DI genome as well (Baker and Moyer, 1988) (left lane, Fig. 5).

As a control, we first examined the effect of a mAb to the C-terminus of P in this system. The addition of anti-P inhibited the majority of DI RNA replication, and transcription from the wild-type virus, as expected (Fig. 5), confirming earlier observations (Deshpande and Portner, 1985; Vidal *et al.*, 1988; Moyer and Horikami, 1990). In contrast, anti-V from two separate rabbits and control heterologous rabbit antibody (RPV, Fig. 5) had little effect on either type of RNA synthesis. With the higher levels of antibodies, slightly less mRNA is found, but we suspect that this is due to residual RNase in the antibody preparations. Similar results were found when infected cell extracts rather than virions were used for RNA synthesis (not shown).

We appreciate that the conclusions that can be drawn from this study are limited, both because our anti-V has no effect and because we have used only a single anti-peptide antiserum. Other peptides from the 68-codon-long V ORF were tried but did not produce suitable sera, and attempts to produce a fusion protein in *Escherichia coli* have failed in large part because of the overlapping P ORF. Our anti-V, then, is the only SEN antisera reported to date which does not also react with P. This serum cannot inhibit RNA synthesis even though it is as effective in immune selection as our anti-P.

## The phosphorylation of the V protein

Purified virions have long been known to contain kinase activity, which phosphorylates P and NP (Roux and Kolakofsky, 1974; Lamb, 1975). Unexpectedly, the kinase is tightly associated with the NC core and not the envelope (Vidal *et al.*, 1988) and prefers the P and NP proteins to model kinase substrates such as histones and protamine (unpublished). Moreover, Einberger *et al.* (1990) have recently suggested that the L protein is in fact the P and NP kinase. The P protein is phosphorylated at sites contained within the N-terminal half of the protein, a region that is shared with V.

To further study this phosphorylation, cells infected with either the natural Z strain (Fig. 6A) or vv-recombinants expressing the V or P proteins (Fig. 6B) were labeled with $^{32}PO_4$, and cell extracts were precipitated with specific antibodies. We found that V was indeed phosphorylated, and apparently to the same extent as P during the natural infection. Moreover, when these proteins were expressed in isolation of the other SEN proteins via the vv-recombinants, they were phosphorylated to the same relative extent as during the natural infection.

The kinase responsible for the majority of the phosphorylation of P and V is then unlikely to be the L pro-

FIG. 4. Immunolocalization of the P and V proteins. The top micrographs show BHK-infected cells double-labeled with a monoclonal anti-P Ab (left side) and a polyclonal anti-V Ab (right side) as described under Materials and Methods. Note the punctate staining of P antigen in the cytoplasm with occasional large patches and the perinuclear localization of V antigen (arrow). The bottom micrographs show uninfected cells treated identically. The cells in all the panels were processed simultaneously and photographed under identical conditions. Objective = 60X.

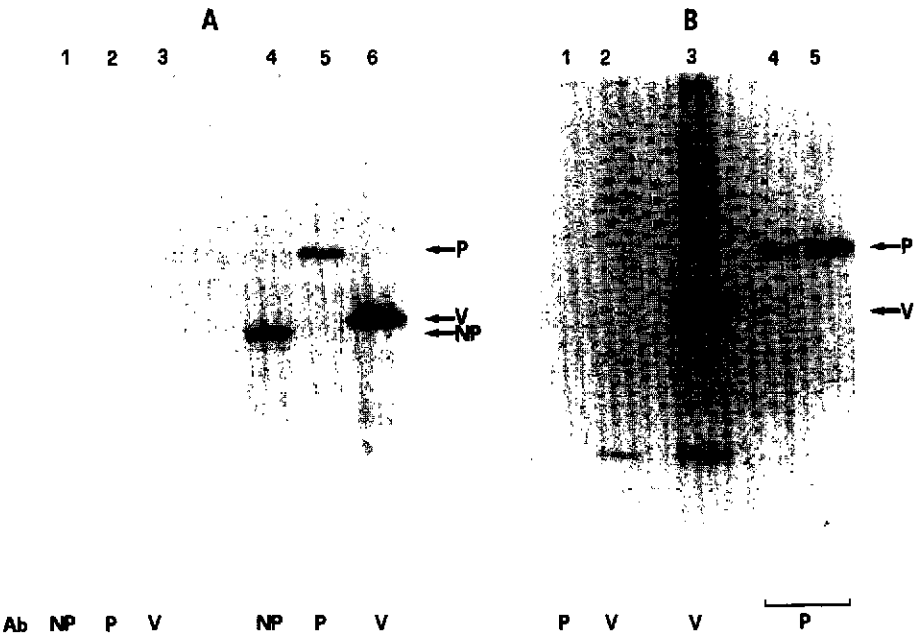



FIG. 6. Detection of viral phosphoproteins. BHK cells infected with either SEN (A) or vv-recombinants expressing either the SEN P or V proteins (B) were labeled with $^{32}PO_4$. Samples were immunoprecipitated with either anti-V or mAbs to the P and NP proteins as indicated below. Proteins were analyzed on a 12.5% SDS–polyacrylamide gel. (A) lanes 1–3, precipitations of uninfected cell extracts; lanes 4–6, precipitations of SEN-infected cell extracts. (B) lanes 1 and 2, wild-type vv-infected cell extracts; lane 3, extract prepared from a vv-recombinant expressing the V protein; lanes 4 and 5, extracts prepared from vv-recombinants expressing the P protein.

tein and is either of host origin or both proteins are themselves kinases. That P and V are auto-kinases, however, seems unlikely, as their common N-terminus is very poorly conserved among related viruses. It would appear that there is a NP and P kinase(s) of cellular origin, which is tightly associated with NCs in virions, but which can also function independently of viral NCs *in vivo*. These results do not rule out that a fraction of the phosphorylation is due to the L protein, as suggested for the VSV P protein (Chattopadhyah and Banerjee, 1987). If phosphorylation of the N-terminus of P modulates its activity as is widely believed, we would then expect that the activity of V would be similarly modulated.

## DISCUSSION

Attention has recently been focused on the paramyxovirus V proteins because of the unusual mechanism involved in generating their mRNAs. Moreover, the C-termini of these proteins are cysteine rich and highly conserved even among very different paramyxoviruses, in contrast to the P proteins which are poorly conserved. The conserved Cys residues of V are reminiscent of other transcription factors such as the HIV tat protein and zinc-finger proteins, which interact with nucleic acids and modify transcription. Since P and V are generated from a single transcriptional unit and share N-terminal sequences, the obvious assumption has been that V would also play a role in viral RNA synthesis.

The work presented here, however, suggests that the situation is not a simple one. In contrast to P, we have been unable to find V in virions or intracellular NCs that are transcriptionally active. This is consistent with previous data. Ryan *et al.* (1991) have found that only two blocks of P, both in the C-terminal half, are neccessary and sufficient for P binding to NCs *in vitro*. The absence of both of these binding sites in V would explain the lack of a stable association of V with NCs and why it is not selectively packaged in virions. There are, however, as many as 200 copies of P per NC, and our data cannot exclude that a few copies of V are present. The reason for so many P proteins on each NC is enigmatic, and it remains possible that unlike P, only very small amounts of V are required for RNA synthesis. However, the only anti-V-specific antibody available so far has no effect on RNA synthesis *in vitro*. We have thus been unable to provide evidence that, like P, V is required for RNA synthesis. In this respect, the recently determined sequence of the PIV1 P gene (Matsuoka *et al.*, 1991) is germane.

PIV1 and SEN are the most closely related pair of paramyxoviruses (Matsuoka *et al.*, 1991), except for bovine and human PIV3. This is also true of their P proteins, although there is only 52% sequence identity

between them. Until PIV1 P was determined, the next closest relative to SEN P was bPIV3 (only 24% identity), making structural comparisons ambiguous. The SEN/PIV1 alignment, in contrast, is unambiguous and shows that the protein is divided into N- and C-terminal parts, with a hypervariable region in between (Fig. 1). One measure of the relatedness of PIV1 and SEN is that PIV1 can replicate a SEN DI genome (Curran and Kolakofsky, 1991). Remarkably, whereas the P and C proteins of SEN and PIV1 are well conserved (residues 75–204 of C are 88% identical), the V ORF in PIV1 is interrupted by nine stop codons, and there is no semblance of an editing site in the vicinity. Barring the possibility that the V function is expressed from an area of the PIV1 genome which has not as yet been elucidated, this would indicate that V is not required for RNA synthesis.

However, even though PIV1 (and RSV) do not appear to code for a V protein, all the other paramyxoviruses do so within the P gene, and the V-specific sequences are the most highly conserved of the three P gene ORFs among these viruses. This conservation, when V is expressed, suggests that it does play an important role in virus replication. We have recently developed an *in vivo* system in which DI genome replication is dependent on cloned viral genes expressed from plasmids (manuscript in preparation). In this system, P expression is essential for RNA synthesis, whereas V expression is not. Moreover, coexpression of V with P inhibits RNA synthesis in a dose-dependent manner. These studies suggest that V is a negative regulator of RNA synthesis. If so, one can more easily reconcile the absence of V in PIV1.

In the particular case of measles virus (Ballart *et al.*, 1990), it will be of interest to determine what effect abrogation of V function has in this system. It has generally been assumed that all gene products of RNA viruses represent essential functions, because of the limited size of RNA genomes. However, Schwarz *et al.* (1990) have recently found that a nonstructural protein (NS2) is not essential for coronavirus replication in cell culture, and Huang *et al.*, (1990) have shown that the nonstructural proteins of influenza virus (NS1 and NS2) are not essential for the replication of an artificial genome.

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