THE MEASLES VIRUS V PROTEIN

by

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ABSTRACT

The measles virus (MV) V protein has been predicted to arise from an edited phosphoprotein (P) mRNA, in which an additional nontemplated G residue has been inserted, thereby permitting access to another reading frame. The P and V proteins have identical amino-termini but differ in their carboxy-terminal residues. Although an edited P mRNA has been detected in MV-infected cells, no corresponding protein has yet been identified in vivo (Cattaneo *et al.*, 1989).

The present research characterizes the V protein in vivo. Antisera directed against synthetic peptides corresponding to five different regions of the MV V protein were prepared. Three of these regions were located in the amino-coterminal region, while two were located in the unique V carboxyl terminus region. The resulting peptide antisera were used to immunoprecipitate in vitro and in vivo synthesized P and V proteins. V protein (M_r 40,000) was detected in MV-infected Vero cells by immunoprecipitation, and found to be phosphorylated. Immunofluorescence techniques revealed that the V protein was distributed throughout the cytoplasm of infected Vero cells.

RESUME

L'hypothèse a été posée que la protéine V du virus de la rougeole est produite par une version éditée de l'ARNm de la phosphoprotein (P) où un nucléotide G additionnel, ne se trouvant pas dans le génome, a été inséré, rendant accessible un second cadre de lecture. Les protéines P et V partagent donc leurs extrémités N-terminal tandis que leurs extrémités C-terminal diffèrent. Malgré que la version éditée de l'ARNm de la protéine P ait été détectée dans des cellules infectées par le virus de la rougeole, la protéine V n'a toujours pas été identifiée in vivo (Cattaneo et al., 1989).

La présente recherche charactérise la protéine V in vivo. Pour ce fait, des antisera ont été dirigé contre des peptides synthétiques correspondant à cinq régions différentes de la protéine V. De ces peptides, trois se localisent dans l'extrémité N-terminal partagée tandis que les deux autres se trouvent dans l'extrémité C-terminal unique à la protéine V. Les antiséra ont été utilisés pour immunoprécipiter les protéines V et P synthétisées in vitro et in vivo. La protéine V (Mr 40 000) a ainsi pu être détectée dans des cellules de type Vero infectées par le virus de la rougeole. La protéine V possède également un ou des groupements phosphoryles ainsi qu'une distribution cytoplasmique diffuse et accentuée autour du noyau de la cellule, comme il a pu être démontré par immunofluorescence.

INTRODUCTION

1. Measles Virus

Measles virus (MV) infection is still a leading cause of infant mortality in third world countries. Clinical measles is a highly contagious disease in which virus is spread by respiratory secretions. Symptoms of measles include fever, cough, coryza, conjunctivitis, and a specific enanthem (Koplik's spots), followed by a generalized maculopapular eruption (Norrby and Oxman, 1990). In rare cases persistence of MV induces the fatal central nervous system disease subacute sclerosing panencephalitis (SSPE) (ter Meulen *et al.*, 1983). Fortunately, MV infection can be prevented by the adminstration of live attenuated virus vaccine (Enders, 1962; Enders *et al.*, 1962).

i. Classification and Morphology

Measles virus is a member of the Morbillivirus subgroup of the Paramyxoviridae family. Other members of this subgroup are canine distemper virus, rinderpest virus, and pestes des petits ruminants virus. Unlike other Paramyxoviruses, the Morbilliviruses do not encode any detectable neuraminidase

activity and they interact with cellular receptors which are insensitive to neuraminidase treatment (Norrby and Oxman, 1990).

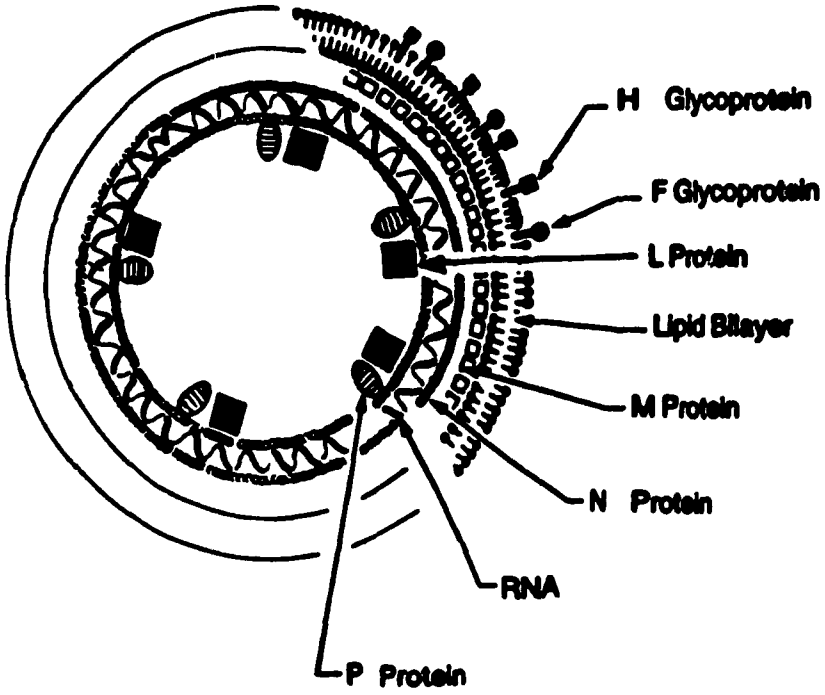
All MV strains studied belong to a single antigenic type. Virions are spherical particles made up of two structural modules: an internal ribonucleoprotein core or nucleocapsid (RNP) surrounded by a lipoprotein envelope (Fig. 1). The particles are pleomorphic, ranging in diameter from 100 to 250 nm with a mean of about 150 nm. The outer envelope is a lipid bilayer of host cellular origin. There are two distinct types of surface projection, or peplomer, inserted within the envelope having a length of 9-15 nm. One of the peplomers is the hemagglutinin (H), which has a conical appearance when viewed by electron microscopy (EM), and the other is the dumbbell shaped fusion (F) protein. Located on the inside of the envelope is the matrix (M) protein, which is thought to interact with the surface peplomers as well as with the nucleocapsid (NP) protein. Helical RNP's are packed within the virus envelopes in the form of symmetrical coils. Free intact RNP is approximately 1 μm in length with a diameter of 17-18 nm.

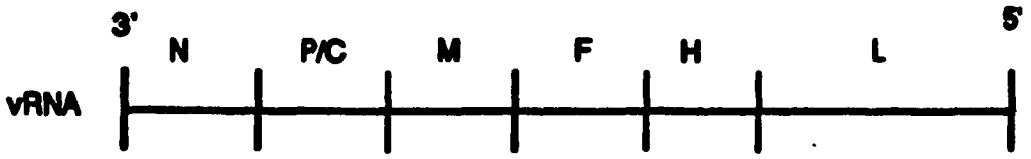
ii. Genome Organization and Virus Specific Proteins

The MV genome is a nonsegmented, linear, negative-sense, single-stranded 50-52 S RNA with an estimated M_r of 4.5 x 10^6 daltons (Baczko *et al.*, 1983; Lund *et al.*, 1984). The complete MV genome has now been sequenced and contains approximately 15,900 nucleotides, the exact number depending on the virus strain (Alkhatib and Briedis, 1986; Bellini *et al.*, 1986; Bellini *et al.*, 1985; Billeter *et al.*, 1984; Blumberg *et al.*, 1988; Cattanec *et al.*, 1989; Crowley *et al.*, 1988; Richardson *et al.*, 1986; Rozenblatt *et al.*, 1985). The RNA genome encodes six structural proteins including NP, the phosphoprotein (P), M, F, H, the large or polymerase protein (L), and two nonstructural proteins, C and V. The C and V proteins are derived from the P gene by mechanisms that will be discussed below. The order of the mRNA transcription from the 3′ end of the genome RNA is as follows: N-P/V/C-M-F-H-L (Fig. 1) (Dowling *et al.*, 1986; Rima *et al.*, 1986; Yoshikawa *et al.*, 1986). Preceding the NP gene at the 3′ terminus of the genome RNA is a 52-53 nucleotide leader sequence which shows a high degree of complementarity with the 40 extragenic nucleotides at the 5′ terminus (Billeter *et al.*, 1984; Crowley *et al.*, 1988). Between the boundaries of adjacent genes there

Figure 1. Schematic diagram of MV virion. The virion is composed of a central core containing a single species of linear RNA. The nucleocapsids (NP), phosphoprotein (P), and the large (L) proteins are tightly associated with viral RNA. The viral envelope consists of lipid (H), fusion (F), and matrix proteins. The order of the mRNA transcription from the 3′ end of the genome RNA is depicted below. Diagram adapted from Ginsberg 1988.

STRUCTURE OF THE MEASLES VIRUS





are consensus nucleotide sequences believed to be involved in transcriptional regulation (Crowley *et al.*, 1988).

Three of the MV structural proteins are complexed with the viral RNA and the other three are involved in the formation of the envelope. The most abundant internal protein is NP. It is tightly associated with the genome RNA and protects it against nucleases. NP is phosphorylated and has an M_r of approximately 60 kDa (Rozenblatt *et al.*, 1985). Other internal virion components include the L ($M_r \approx 200$ kDa) and P (M_r 72 kDa) proteins, which are present in limited quantities. There are about 10-fold more molecules of P than of L associated with each RNP (Kingsbury, 1990). Most information about these two proteins has come from studies with paramyxoviruses other than MV. The RNA-dependent RNA polymerase of negative-strand RNA viruses functions as part of a transcriptase complex composed of template RNA in tight association with the NP, P, and L proteins, the latter two of which are thought to be responsible for polymerase activity (Buetti and Choppin, 1977; Hamaguchi *et al.*, 1983). P and L are believed to act in concert as it was found that neither is capable of catalysing RNA synthesis when added individually to nucleocapsid templates of Newcastle disease virus (Hamaguchi *et al.*, 1983). Furthermore, it was demonstrated by

immunoelectron microscopy that L colocalizes with P in clusters along viral nucleocapsids of Sendai virus (Portner and Murti, 1986; Portner *et al.*, 1988).

The viral envelope consists of lipid membrane associated with the H, F, and M proteins. The surface glycoprotein H (M_r 70-80 kDa) mediates virus attachment to host cells (Choppin and Scheid, 1980). The H protein is strongly hydrophobic at its amino terminus and it is this domain which serves to anchor the protein within the lipid bilayer of the virus envelope (Alkhatib and Briedis, 1986). Once the virion is attached to the cell, the second surface glycoprotein, F, mediates membrane fusion and cell penetration (Choppin and Scheid, 1980). The F glycoprotein is initially synthesized as an inactive precursor, F_0, which is subsequently cleaved by host proteolytic enzymes to yield two disulfide linked polypeptides, F_1 (M_r 41 kDa) and F_2 (M_r 18-20 kDa) (Graves *et al.*, 1978; Scheid and Choppin, 1974). F_1 contains the carboxyl terminus of F_0, and this domain anchors the protein into the viral membrane. F_2 comprises the amino terminus of F_0 minus the amino terminal signal peptide (Choppin and Scheid, 1980; Scheid and Choppin, 1977; Varsanyi *et al.*, 1985). The M protein (M_r 36-37 kDa) lines the inner surface of the virion envelope (Bellini *et al.*, 1986). As previously mentioned it

is thought that the surface glycoproteins and the RNPs interact with the M protein. In addition to its structural function, M is thought to have a key role in virus maturation, probably mediating both viral assembly and budding from the infected cell membrane.

The functions of the remaining two MV proteins, C and V (M_r 20 kDa and 40 kDa respectively), are unknown. Because the C protein is seen only in virus infected cells it has been termed nonstructural (Norrby and Oxman, 1990). The MV V protein is even more mysterious as only the mRNA corresponding to the V protein, and not the protein itself, have been detected *in vivo* (Cattaneo *et al.*, 1989). Further details of the MV V protein and its expression constitutes the major portion of this thesis.

iii. Measles Virus Replication

All events during MV replication take place within the cytoplasm and the infection is usually lytic. Replication of MV is not associated with restriction of host cell metabolism. However, cellular gene expression is modified, as shown by synthesis of stress proteins and induction of I_a antigens (Massa *et al.*, 1987; Sheshberadaran *et al.*, 1984). Under

certain conditions, MV can cause rapid destruction of cells because of cytopathic effects of virus replication and derangements in cell function that result from the cell-fusion phenomenon (Norrby and Oxman, 1990). Cell fusion causes formation of multinucleate giant cells called syncytia.

The molecular details of MV replication have been poorly defined. The H protein binds to sialic acid residues on host cell receptor molecules at the cell surface. The infecting RNP is then delivered into the cytoplasm by F protein-mediated membrane fusion. Transcription is the next step in virus gene expression after cytoplasmic delivery of the RNP. The genome RNA of negative strand RNA viruses serves as template for transcription of both subgenomic mRNAs and full-length plus sense replicative intermediates. Like those of all the other negative-strand RNA viruses, MV particles contain an RNA-dependent RNA polymerase closely associated with the virion RNA (Seifried *et al.*, 1978).

Transcription of MV-specific genes is believed to occur entirely in the cytoplasm of infected cells and is independent of host cell mRNA synthesis (Kingsbury, 1990). The MV genome is nonsegmented and transcription is believed to occur by a "start and stop" mechanism. The validity of this model is

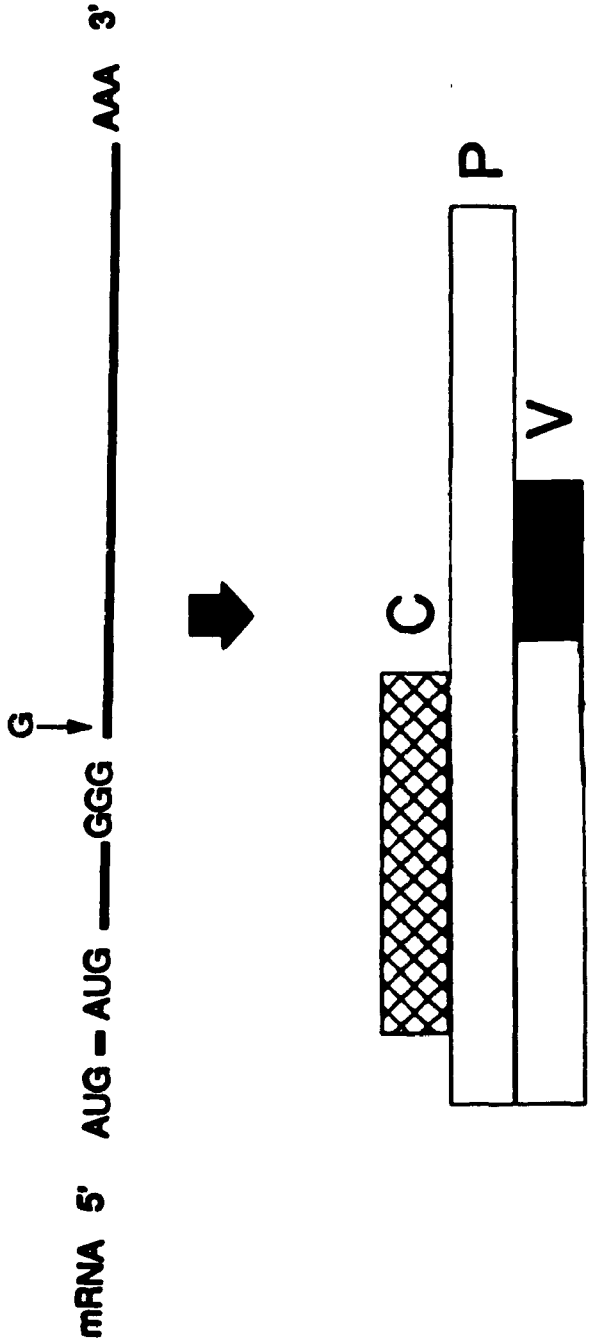
supported by the presence of consensus nucleotide sequences at gene boundaries (Cattaneo *et al.*, 1987; Crowley *et al.*, 1988). These sequences appear to be analogous to those known to be involved in signalling initiation and termination of transcription encoded in genes of eukaryotic cells as well as of many DNA viruses (Kingsbury, 1990). At least nine to ten distinct species of mRNA which are capped at the 5′ end and have poly (A) tails at their 3′ ends have now been identified (Hasel *et al.*, 1987; Udem and Cook, 1984; Yoshikawa *et al.*, 1986). Not all of these have been shown to be functional.

Both mRNA and replicative intermediate plus sense RNA synthesis initiate at the 3′ end of the leader RNA template. Transcriptional regulation of mRNA abundance is determined by gene order along with an attenuating role of the transcriptional regulatory sequences. The efficiency of transcription of a gene declines as its distance from the 3′ end of the genome increases (Kingsbury, 1990). The relative abundance of each protein reflects its position in the genome, with the exception of the proteins encoded by the P/C and P/V genes. Therefore there are about 100 fold more NP than L protein molecules.

All MV genes are monocistronic except for the P gene. Both C and V are derived from the P gene (Fig. 2). The P gene contains an additional reading frame overlapping that of P, allowing the synthesis of the shorter C protein (Bellini *et al.*, 1985). The first 5′ proximal AUG of the P/V/C mRNA sequence initiates the P open reading frame (ORF) while the second AUG, located 19 nucleotides downstream, initiates the C ORF. The mechanism of initiation for the C protein is unclear but there is evidence that initiation may occur by direct internal ribosome binding (Alkhatib and Briedis, 1988). The V protein is derived from the P gene by a mechanism termed RNA editing. The V protein is translated from mRNAs in which a nontemplated G residue is inserted after three genomically encoded G's at position 751 (Cattaneo *et al.*, 1989). The added G nucleotide causes a switch in the reading frame such that the V protein is translated with the same amino acid terminus as P but with a distinct carboxyl terminus.

Initial RNA synthesis is referred to as "primary transcription". Translation of the resulting MV-specific mRNAs is essential for the onset of genomic RNA synthesis. Replication of the genome involves duplicating the negative stranded RNA in its entirety. Therefore, the sequences not expressed in mRNA must be incorporated into the antigenome

Figure 2. Coding strategies of the P gene. The first 5' proximal AUG of the P/V/C mRNA sequence initiates the P ORF while the second AUG initiates the C ORF. The V protein is derived from an edited P mRNA in which one G residue has been inserted after 3 genomically encoded G's at position 751. The P and V proteins are amino coterminal (white box) but V has a unique carboxy terminus (black box). The C protein has no amino acids in common with P (hatched box).


mRNA 5'
AUG
AUG
GGG
G
AAA 3'
C
V
P

(positive-strand RNA) that serves as an intermediate template for the synthesis of progeny negative-strand genomes. The antitermination mode necessary for this is achieved by accumulation of a critical concentration of NP protein which then changes the way the RNA polymerase complex copies its template (Blumberg *et al.*, 1983; Carlsen *et al.*, 1985). The exact mechanism of this switch is unknown.

RNP's are assembled in the cytoplasm. The NP structural units combine with the genomic and antigenomic RNA to form helical ribonucleoprotein templates and then the auxiliary nucleocapsid-associated protein P and L are added to the template. The presence of the 3'-terminal leader sequence distinguishes between RNAs to be encapsidated and RNAs to be translated (Castaneda and Wong, 1990). Assembly of the virus envelope occurs at the cell surface. The MV-specific glycoproteins, which have been modified by glycosylation during their transport through the endoplasmic reticulum and the Golgi apparatus, replace most endogenous cellular proteins in the plasma membrane lipid bilayer (Norrby and Oxman, 1990). The M protein molecules are aggregated at the inner surface of the nascent envelope, presumably by noncovalent interactions with the glycoproteins and/or lipid bilayer (Kingsbury, 1990).

After the nucleocapsids arrive at the cell surface, progeny virions are formed by budding from the cell membrane.

2. RNA Editing

There are a number of mechanisms identified as having a role in the processing or modification of initial RNA transcripts to yield mature mRNAs. The most common process in eukaryotic cells is splicing of primary RNA transcripts (Padgett *et al.*, 1986). Modifications introduced by splicing include alternative splicing, such that exons are excluded from some, but not all, of the mature mRNA, giving rise to sequence diversity, and trans splicing, in which two independently transcribed RNAs are ligated to form mature RNAs (Breitbart *et al.*, 1987; Konarska *et al.*, 1985; Solnick, 1985). In this type of modification the conversion of a primary transcript to the mature, translatable mRNA is felt to be controlled by sequence information contained in the pre-mRNA.

Another mechanism of RNA modification is the phenomenon termed RNA editing, in which sequences are found in the mature mRNA that are not encoded in the gene. RNA editing was first described for mitochondrial genes of trypanosomes. This is characterized by the addition or deletion of U residues at

specific sites in the mature mRNA that are not encoded by the gene (Benne *et al.*, 1986; Feagin *et al.*, 1987; 1988; Shaw *et al.*, 1988). Other examples of RNA editing soon followed including: the mammalian apolipoprotein B, some plant mitochondrial genes, and, most recently, for the V protein in paramyxoviruses (Cattaneo *et al.*, 1989; Chen *et al.*, 1987; Covello and Gray, 1989; Ohgimoto *et al.*, 1990; Paterson and Lamb, 1990; Powell *et al.*, 1987; Southern *et al.*, 1990; Takeuchi *et al.*, 1990; Thomas *et al.*, 1988; Vidal *et al.*, 1990).

i. Trypanosomes

Trypanosomatids are parasitic protozoa including Trypanosoma, Leishmania, and Crithidia. Trypanosomes have a single mitochondrion, or "kinetoplast", which contains a large catenated network or minicircles (0.7-1.0 kb, 5-20,000 copies) and maxicircles (20-38 kb, 25-50 copies) (Englund *et al.*, 1982; Simpson, 1986). The maxicircles encode mitochondrial genes and the minicircles are of unknown function. The maxicircle coding capacity is unusual because many encoded genes contain frameshifts and/or lack initiation codons, and other genes normally encoded in mitochondrial DNA are not detected (reviewed in Benne, 1989; Simpson and Shaw, 1989).

RNA editing was first reported by Benne *et al.* (1986) when they found that the primary cytochrome oxidase II (CO II) transcript was edited by co- or posttranscriptional insertion of four U residues. RNA editing in trypanosome mitochondria based on insertion and excision of U residues was soon extended by the additional examples of the CO III subunit, cytochrome b (Cy b), and maxicircle unidentified reading frames 2 and 3 (MURF-2 and MURF-3) transcripts (Decker and Sollner-Webb, 1990; Feagin *et al.*, 1987; 1988; Shaw *et al.*, 1988). Editing can be quite extensive, as in the CO III transcript of T. brucei, where over 50% of the transcript sequence is created by RNA editing (Feagin *et al.*, 1988).

The observation that RNA editing likely proceeded in a 3′ to 5′ direction along the primary transcript seemed to rule out co-transcriptional editing and also tended to exclude the ribosome as part of the editing machinery (Abraham *et al.*, 1988). In addition, attempts to detect either a DNA or RNA template using oligonucleotide probes were unsuccessful (Maizels and Weiner, 1988). Now the discovery of a new class of RNAs called "guide" RNAs (Blum *et al.*, 1990) has led to detailed proposals for a novel RNA editing mechanism that

would convert cryptogene transcripts into functional mRNA sequences.

These "guide" RNA molecules are encoded in the intergenic regions of the mitochondrial maxicircle DNA and contain sequences that represent precise complementary versions (if G:U base pairing is permitted) of the mature mRNAs within the edited regions. There is as yet no experimental evidence that guide RNAs participate in the editing process but the 5′ portions of several "guide" RNAs could in principle form hybrids with mRNAs just 3′ of the predicted region. Blum *et al.* (1990) have proposed a model whereby the partial hybrid formed between the "guide" RNA and preedited mRNA is a substrate for multiple cycles of cleavage, addition or deletion or uridylates, and relegation, eventually resulting in a complete hybrid between guide RNA and the mature edited mRNA.

Mitochondrial editing also occurs in an unrelated slime mold Physarum polycephalum, where a reading frame is created by insertion of single cytidines into the primary transcript (Weiner and Maizels, 1990). Characterization of the Physarum system has only begun so it has yet to be determined whether

the cytidine editing in Physarum are mechanistically or ancestrally related to the uridine editing in Trypanosomes.

ii. Apolipoprotein B

Two closely related forms of apolipoprotein B (apo-B) circulate as obligatory constituents of plasma lipoprotein. In humans, apo-B100 is synthesized in the liver and apo-B48 is synthesized by the intestine. Apo-B48 is so designated because it is roughly half the size of apo-B100 using the centile system. It was determined by cDNA cloning that there was a single C to U conversion in the apo-B mRNA in intestinal cells, but not in liver cells, thus generating a termination codon and a truncated version of the protein (Chen *et al.*, 1987; Powell *et al.*, 1987). The mechanism of editing is unknown but the base change may be caused by a deaminase recognizing some structural feature of the mRNA much as in transfer RNA modification in both prokaryotes and eukaryotes.

iii. Plant Systems

It had previously been proposed that in plant mitochondria there was a departure from the universal genetic code, with CGG specifying tryptophan instead of arginine (Fox and Leaver,

1981). This was because CGG codons were often found in plant mitochondrial genes at positions corresponding to those encoding conserved tryptophans in other organisms. The apparent coding anomalies were explained when an editing phenomena that involves the conversion of cytidine to uridine at multiple positions in the mRNA of plant mitochondrial genes was discovered (Covello and Gray, 1989; Gualberto *et al.*, 1989; Schuster *et al.*, 1990). A deamination mechanism, similar to that for apo-B48, may be responsible for edited forms of mRNA in plant mitochondria.

iv. Paramyxoviruses

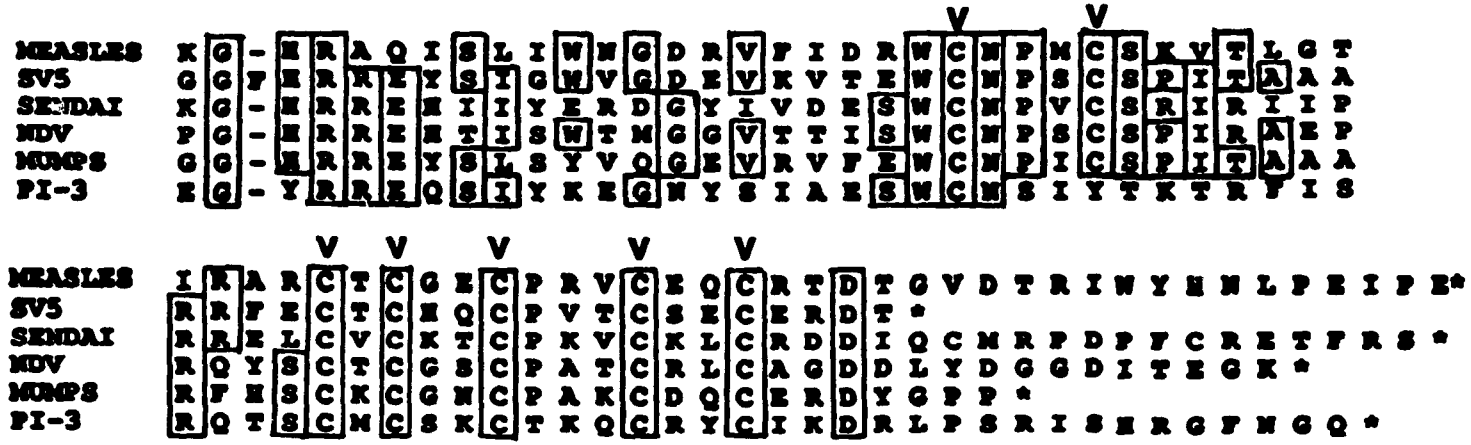
Evidence of RNA editing in paramyxoviruses was found first for simian virus 5 (SV5) by Thomas *et al.* (1988) and then later for MV (Cattaneo *et al.*, 1989). They found that RNA transcripts derived from the P gene were altered to allow for the synthesis of more than one distinct mRNA species that differ from one another in the number of G residues at a specific insertion point in the RNA. The addition of nontemplated G residues causes a switch in the reading frame such that two amino co-terminal proteins are synthesized, one the P protein and the other a cysteine rich protein, termed V. Moreover, amino acid sequences of overlapping open reading

frames of the P gene in different paramyxoviruses indicated that a cysteine rich protein could be made by Sendai virus, Newcastle disease virus (NDV), canine distemper virus (CDV), mumps virus and parainfluenza virus-3 (PIV-3) (Fig. 3). It is interesting that the amino acid sequence of this region is more highly conserved among paramyxoviruses than is the amino acid of the P protein region encoded by the same nucleotides in a different reading frame. This suggests that the V protein may have an important biological function.

The MV V protein mRNA was first identified by Cattaneo *et al.* (1988) when they performed in vitro transcription/translation analysis of cDNA clones generated from mRNA from the brains obtained at autopsy of patients dying from subacute sclerosing panencephalitis (SSPE). Only half of the P gene-specific clones generated P proteins of the expected M_r, 70-85 kDa, whereas the other half yielded a 46 kDa product. By sequencing it was found that there was an additional nontemplated G insertion at position 751, following three G residues corresponding to three genomic C residues (Cattaneo *et al.*, 1989). This causes a switch in the reading frame replacing the last 277 amino acids of the P ORF with 68 amino acids, 7 of which are cysteines, of the V ORF. No genomic RNA with this modification was detected. Using synthetic RNAs,

Figure 3. Amino acid sequence homology between the cysteine rich region of the V protein in different paramyxoviruses. A dash indicates that a gap was placed in the alignment, the arrowhead above the sequence shows the seven conserved cysteine residues. From Thomas *et al.*, 1988.

HOMOLOGY



with and without an additional G residue, Cattaneo et al. (1989) were able to translate in vitro protein products of the expected molecular weights. Moreover, using polyA mRNA from MV-infected cells they produced proteins of the expected size by in vitro translation. Evidence for the possible in vivo production of a 40 kDa MV P-related protein was obtained by Rima *et al.* (1981) using partial protease digestion. Cattaneo *et al.* (1989) have suggested that the 46 kDa protein they detected in vitro corresponds to the 40 kDa P-related protein despite migration differences in their gel systems. However, no strong signal at 40-46 kDa has been found in MV-infected cells after immunoprecipitation with antibodies directed against P (Bellini *et al.*, 1985), possibly because the paramyxovirus P protein carboxyl terminus is much more immunogenic than the rest of the protein (Vidal *et al.*, 1988).

Evidence now exists that in addition to MV and SV5, a V protein is derived from a P-related transcript in Sendai virus, PIV-2, and mumps virus infected cells (Ohgimoto *et al.*, 1990; Paterson and Lamb, 1990; Southern *et al.*, 1990; Takeuchi *et al.*, 1990; Vidal *et al.*, 1990). There are two slightly different coding strategies for P and V among paramyxoviruses. In MV and Sendai virus, the P proteins are encoded by the genomic sequence and it is the synthesis of V protein that

requires a G insertion (Cattaneo *et al.*, 1989; Vidal *et al.*, 1990). SV5, mumps, and PIV-2, on the other hand, use the original genomic sequences for encoding the V and the edited mRNAs with insertion of two G residues for synthesis of the P protein (Ohgimoto *et al.*, 1990; Paterson and Lamb, 1990; Southern *et al.*, 1990; Takeuchi *et al.*, 1990; Thomas *et al.*, 1988; Vidal et *al.*, 1990). The relative frequency of mRNAs containing G insertions has been estimated to be 50% for MV (Cattaneo *et al.*, 1990), 45% for SV5 (Thomas *et al.*, 1988), 31% for Sendai virus (Vidal *et al.*, 1990), and 40-50% for mumps (Takeuchi *et al.*, 1990).

Paterson and Lamb (1990) have reported another protein, I (M_r 19 kDa) to be synthesized from a different species of edited mumps virus P mRNA. In this case, four G residues are added to the insertion site to gain access to the I reading frame. Of 54 mRNA clones examined 34 were unedited (encoding V), ten had two G's inserted (encoding P), seven had three G's inserted, two had five G's inserted, and only one had four G's inserted (encoding I). Takeuchi *et al.* (1990) have also found a single cDNA clone with four G's inserted. Also, they immunoprecipitated a 24 kDa protein using anti-peptide antisera directed against the amino terminus of P. They did not determine the origin of this protein but postulated that

it could be produced by the mRNA with four G's inserted. Others have reported misinsertions of the number of G residues, at a frequency of 6 to 10%, in MV and Sendai virus but no corresponding proteins have yet been identified (Cattaneo *et al.*, 1989; Vidal *et al.*, 1990). No variation was reported at the insertion site for SV5 or PIV-2 but the number of clones examined may have been insufficient to detect this (Ohgimoto *et al.*, 1990; Thomas *et al.*, 1988).

v. Possible Mechanisms for RNA Editing in Paramyxoviruses

The exact mechanism responsible for the addition of nontemplated G residues in the P protein mRNA is still unknown. Neither is it known whether this is a cotranscriptional or posttranscriptional process. The favoured theory is that of a stuttering mechanism similar to the one which probably occurs during polyadenylation. In negative strand RNA viruses, the virus-encoded RNA-dependent RNA polymerase is responsible for the polyadenylation of virus-specific mRNAs. Polyadenylation is thought to occur by a "slippage" or "stuttering" mechanism at a stretch of U residues located at the end of each gene. Nontemplated G residues are added to P transcripts at a position where the template vRNA has a run of 3 to 7 G's, depending on the virus.

Thus it is possible that the polymerase stutters while copying this region. Thomas *et al.* (1988) have noted that immediately upstream of the four C residues on the SV5 genomic RNA is a sequence which resembles the putative polyadenylation signal found at the end of SV5 genes and is in fact identical to the sequence at the end of the SV5 HN gene. Identical sequences are found on the genomes of mumps virus and PIV-2 (Southern *et al.*, 1990).

Vidal *et al.* (1990) have provided evidence that the activity that alters the mRNA is likely to be encoded by the virus. They found that G insertions took place during RNA synthesis in vitro using purified Sendai virions as source of polymerase and template. Furthermore, when the Sendai P mRNA was expressed in vivo via a vaccinia virus recombinant, in the presence or absence of other Sendai virus proteins, no insertions occurred. This indicated that virus encoded proteins are essential for this process and that they cannot act in *trans*, presumably because the insertions take place co-transcriptionally.

The mechanism whereby the virus RNA polymerase could stutter at a precise location for a precise number of nucleotides is unknown. There is a weak but clear consensus sequence, 5′

AARRRGGG_CAXRG (the space indicates the insertion site), among paramyxoviruses. However, this sequence is not uniformly conserved and suggests that it has a limited role. Therefore, it is likely that determinants other than linear sequence play a role in specifying exact sites of insertion. For SV5, Thomas *et al.* (1988) noted that the insertion site could theoretically be found as a loop within a potential stem loop structure in the template, but similar structures are absent in MV or Sendai virus sequences (Vidal *et al.*, 1990). Thomas *et al.* (1988) and Vidal *et al.* (1990) observed spurious bands during dideoxy-NTP sequencing of the insertion region of genomic clones, that were absent from clones which had insertions. Vidal *et al.* (1990) have suggested that Klenow or T7 polymerase is stopping or pausing at this site in genomic clones because they sense a structural constraint, which is alleviated by G insertions.

vi. Function of the Paramyxovirus V Protein

The function of the V protein is still unknown. The V protein is likely a structural protein as it has been found to be associated with purified virions of SV5, PIV-2, and mumps virus (Ohgimoto *et al.*, 1990; Takeuchi *et al.*, 1990; Thomas *et al.*, 1988). A possible role for the V protein is as a factor involved in transcription and/or replication of the vRNA,

since the cysteine-rich region is reminiscent of a zinc finger domain, a motif that has been identified in nucleic acid binding regulatory proteins (reviewed in Klug and Rhodes, 1987; Moran and Matthews, 1987). In these metal binding proteins, it is thought that the binding of metal ions by the cysteine rich region plays an important role in either binding of nucleic acid by the protein, mediating protein-protein interactions, or stabilizing oligomeric forms of a protein. It is interesting that in both SV5 and mumps virus the P and V coprecipitated with the NP in some instances (Thomas *et al.*, 1988; Takeuchi *et al.*, 1990). This is probably because the L, NP, P, and V exist in a nucleocapsid complex (Randall *et al.*, 1987; Thomas *et al.*, 1988).

Since the P and V proteins are amino acid co-terminal, it is interesting to look at potential functional domains of these proteins. The precise role of P is unknown but it is clear that P plays a critical role in RNA synthesis (Hamaguchi *et al.*, 1983). In Sendai virus and vesicular stomatitis virus (VSV) it has been found that a carboxyl terminal segment interacts directly with the RNA template (Chattopadhyay and Banerjee, 1987a; Ryan and Kingsbury, 1988; Ryan and Portner, 1990). In contrast, the amino terminal region of P is highly phosphorylated and may interact electrostatically with the

polymerase (Chattopadhyay and Banerjee, 1987b; Vidal *et al.*, 1988). Ohgimoto *et al.* (1990) found that the V protein of PIV-2 was phosphorylated but they did not determine the site(s) of phosphorylation.

3. Use of Antibodies Directed Against Synthetic Peptides

The use of synthetic peptides to prepare antibodies specific for previously uncharacterized proteins has become popular as more DNA sequences and their corresponding protein sequences have become known (reviewed in Lerner, 1982; 1984; Walter, 1986). Peptides are normally synthesized using solid-phase techniques pioneered by Merryfield (1963). Peptides may not be immunogenic on their own due to their small size. To elicit an antibody response directly, they must contain all of the features of any immunogen. Specifically, they must have an epitope for B-cell binding and a site for class II-T cell receptor binding. Therefore, synthetic peptides are purified and coupled to carrier proteins (commonly keyhole lympet hemocyanin (KLH) or bovine serum albumin (BSA)), and these conjugates are then used to immunize animals. The peptides serve as haptens with the carrier proteins providing good sites for class II-T cells receptor binding.

Since the region in the protein with which antipeptide antibodies react is known in advance the antibodies can be said to be of predetermined specificity (Lerner, 1982). Antibodies with a predetermined specificity are in principle very different from those made against intact protein. During an immune response to a native protein, antibody reactivity is confined to only a few antigenic sites of the molecule. The number of antigenic sites is limited and on average there is one antigenic site for each 5000 daltons of protein (Lerner, 1984). Antigenic determinants are also dependent on tertiary conformations often composed of discontinuous regions of the protein. Antibodies induced by intact proteins are therefore generated by largely ordered arrays of atoms and tested against more or less ordered protein targets. On the other hand, antibodies of predetermined specificity are made against a more ordered protein target (Lerner, 1984). Antibodies of predetermined specificity will bind well to denatured proteins, but they may or may not recognize the native protein (Harlow and Lane, 1988). However, some studies have shown that peptide antigens elicit some antibodies reactive against regions of the native protein which immunization with the native protein itself could not elicit (Green *et al.*, 1982). Therefore, it appears that the number of antigenic determinants of intact proteins does not set a limit on the

number of protein fragments which carry immunogenic determinants.

i. Choice of Appropriate Amino Acid Sequence

There are a number of factors to consider when selecting a region of a protein which is likely to be immunogenic. There are no generalities that apply but some guidelines have been suggested by other researchers (reviewed in Lerner, 1984; Walter, 1986). The first point to consider is that the selected sequence be located on the protein surface which makes contact with the aqueous environment, whereas hydrophobic residues often make up the internal part of a protein. In order to facilitate the search for polar regions, several computer hydropathy plot programs have been developed which are based on a polar scale for the 20 amino acids (Hopp and Woods, 1981; Kyte and Doolittle, 1982; Rose and Roy, 1980).

As there are generally many regions of a protein that are hydrophilic, the secondary structure may also be considered. Computer programs are also available for secondary structure predictions. Residues such as asparagine, aspartic acid, proline and glycine often occupy turns and protrusions from

the main body of the protein (Chou and Fasman, 1978). However, there is no evidence that secondary structure considerations are useful in limiting the number of choices that are presented by hydropathy plots. In fact, Kabsch and Sander (1984) found that identical pentapeptides can have completely different structures in different proteins.

It has been found that peptides corresponding to the amino or carboxyl termini are useful in the production of antibodies as these regions may be conformationally less restrictive than other parts of the molecule and, therefore, might be recognized by a larger fraction of the antibodies to the peptides (Walter *et al.*, 1980). Some researchers have found that antibodies directed against the terminal peptides are superior to those directed against internal regions (Friedrich *et al.*, 1986), while others have found little or no difference (Green *et al.*, 1982; Lerner *et al.*, 1981). Tainer *et al.* (1984) reported that antibodies to internal regions are highly reactive against the native protein if they are derived from regions of high flexibility (high B-values). Again, computer programs are available for predicting flexible segments of the primary amino acid sequence.

A final point to consider is the length of the peptide. Although peptides as short as six amino acids have sometimes been found to be satisfactory, especially if they correspond to termini, peptides of no less than ten residues should be used for coupling (Harlow and Lane, 1988). The best strategy, if finances permit, is to make several small peptides, 10-15 amino acids, to several different regions of the protein.

ii. Applications of Resulting Antipeptide Antibodies

Antipeptide antibodies have been extremely useful in detecting proteins predicted from nucleic acid sequences. The largest amount of work has been carried out in viral systems, especially for the detection of the products of oncogenes and proto-oncogenes including: the Maloney murine sarcoma virus (Papkoff *et al.*, 1981); Maloney C murine leukemia virus (Sutcliffe *et al.*, 1980); Simian virus 40 (Walter *et al*, 1980); polyoma (Walter *et al.*, 1981); Simian Sarcoma virus (Robbins *et al.*, 1982); adenovirus (Green *et al.*, 1983).

In addition to detecting putative gene products, antipeptide antibodies are used to: i) distinguish between similar proteins, such as ras p21 and the cellular p21 with a single amino acid change (Clark *et al.*, 1985); ii) demonstrate

protein similarity as in the sis oncogene protein and platelet derived growth factor (Doolittle *et al.*, 1983; Waterfield *et al.*, 1983); iii) determine subcellular localization of proteins; iv) study proteolytic processing; v) study protein function; vi) study exon usage during gene expression; vii) study frameshift mutations; viii) purify proteins by immunoaffinity techniques; and ix) make potential vaccines (reviewed in Lerner, 1982; 1984; Walter, 1986).

4. Research Objectives and Rationale

The research work presented in this thesis involves the identification and characterization of the MV V protein. The MV V protein is derived from an edited MV P gene and consequently has the same amino terminal region but a different carboxyl terminal region than the P protein. Evidence exists for a V protein-specific mRNA transcript but no protein corresponding to this message has yet been identified in vivo (Cattaneo *et al.*, 1989).

The present project has identified the MV V protein by the use of antisera directed against several synthetic peptides corresponding to relatively hydrophilic domains, as predicted from the amino acid sequence of the V protein. Peptide

sequences were chosen in the amino terminus, thus common to P and V proteins, and in the carboxyl terminus, thus unique to V. The antisera were used in immunoprecipitation experiments to isolate the MV V protein from MV-infected Vero cells.

To confirm the specificity of the peptide antisera, mRNAs corresponding to the P and V proteins were synthesized *in vitro* using the SP6 promoter system. The *in vitro* synthesized P and V transcripts were translated in an *in vitro* cell-free rabbit reticulocyte system and used in immunoprecipitation experiments.

MATERIALS AND METHODS

1. Cells and Viruses

Vero cells (obtained from American Type Tissue Collection [ATTC]) were grown in Dulbecco's modified Eagle's medium (DMEM) (Flow Laboratories) supplemented with 10% fetal bovine serum (FBS), 25 ug/ml amphotericin B, and 50 ug/ml gentamicin. The Edmonston strain of MV (obtained from ATTC) was propagated by infecting Vero cells at a multiplicity of infection (MOI) of 0.0001 plaque forming units (PFU) per cell and harvesting supernatants after 72-96 hours of incubation at 37° when approximately 85-90% of the cells were fused. MV stocks were titered by plaque assay on Vero cells. Only those with a titer 10^8 PFU/ml were used.

2. Measles Virus Plaque Assay

Monolayers of Vero cells were grown to confluence in 60 mm diameter plastic petri dishes in a 37° incubator supplemented with 5% CO_2. The dishes were inoculated with 0.5 ml aliquots of serial ten-fold dilutions (in DMEM). After a one hour adsorption period at 37° the cell monolayers were washed and then overlaid with 5 ml of DMEM containing 5% [V/V] FBS and

0.9% [W/V] agarose. Three days after inoculation a second layer of agar containing 0.0025% [W/V] neutral red was added and plaques were counted after 5 hours.

3. Preparation of Antisera

Synthesis of five peptides that corresponded to various regions of the MV V protein was commissioned from Immuno-Dynamics Inc. (California, U.S.A.). The peptides were coupled to carrier proteins using m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS). A terminal cysteine residue was added to each peptide for coupling. Peptides were coupled to keyhole lympet hemocyanin (KLH) and bovine serum albumin (BSA) separately. Uncoupled peptide was also supplied.

Duplicate New Zealand White rabbits were initially injected with peptide-KLH conjugate in Freund's complete adjuvant (1:1). An emulsion was made by passing the liquid mixture through two syringes. Approximately 0.2 mg of peptide conjugate was injected subcutaneously at each of five sites along the back. The rabbits were subsequently boosted four times, at 4-6 week intervals, in a similar manner with peptide-conjugate in Freund's incomplete adjuvant (1:1). All antisera were tested for their ability to react with the

peptides used in immunizations by enzyme-linked immunosorbant assays (ELISA) according to the protocol described by Harlow and Lane (1988). Pre-immune sera was used as a negative control.

4. DNA Purification

a) Plasmid DNA Purification

To obtain small quantities of plasmid DNA (1-10 µg) a quick plasmid preparation (miniprep) was done according to the method described by Holmes and Quigley (1981). This DNA is useful for rapidly screening bacterial clones for size and orientation of plasmid inserts but there are too many impurities for other techniques such as in vitro transcription. Large quantities (500-2000 µg) of purified plasmid DNA (maxipreps) were obtained using a cesium-chloride gradient technique (Maniatis *et al.*, 1982).

b) Purification of Plasmid Insert DNA

Maxiprep plasmid DNA was digested with the appropriate restriction enzymes in order to isolate insert DNA. The sample was subsequently run on a 1% agarose electrophoresis gel according to standard procedures. The desired DNA fragment was detected by ethidium bromide staining and the

band was excised from the gel. DNA was eluted from the gel using the Geneclean® Kit according to the directions of the manufacturer (Bio 101).

5. DNA Modification

a) Restriction Endonucleases

All restriction enzymes were used according to the specifications of the manufacturer (Pharmacia).

b) DNA ligation

Ligation of insert DNA was performed using 5 units of the T4 DNA ligase (Pharmacia) in 20 µl of ligase reaction buffer (70 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 1 mM ATP, 15 mM dithiothreit [DTT]) and 50-500 ng of DNA. Ligation reactions were incubated overnight at 15° (sticky ends) or 22° (blunt ends) and then heated to 65° for 10 minutes to inactivate the enzyme.

c) Generation of Blunt-ended DNA

DNA digested with restriction enzymes yielding 3′ recessed ends were back-filled using 1 unit of the klenow fragment of Eschericia coli DNA polymerase I (Pharmacia) in 25 µl of nick translation buffer (50 mM Tris-HCl pH 7.2, 0.1 mM DTT, 10 mM $MgSO_4$, 50 ug/ml BSA, 2 mM each of all four deoxyribo-

nucleotides) with 1-10 ug of DNA. Reactions were incubated for 30 min at room temperature. The reaction was heat inactivated by incubating tubes at 75° for 15 min followed by ethanol precipitation.

d) Dephosphorylation

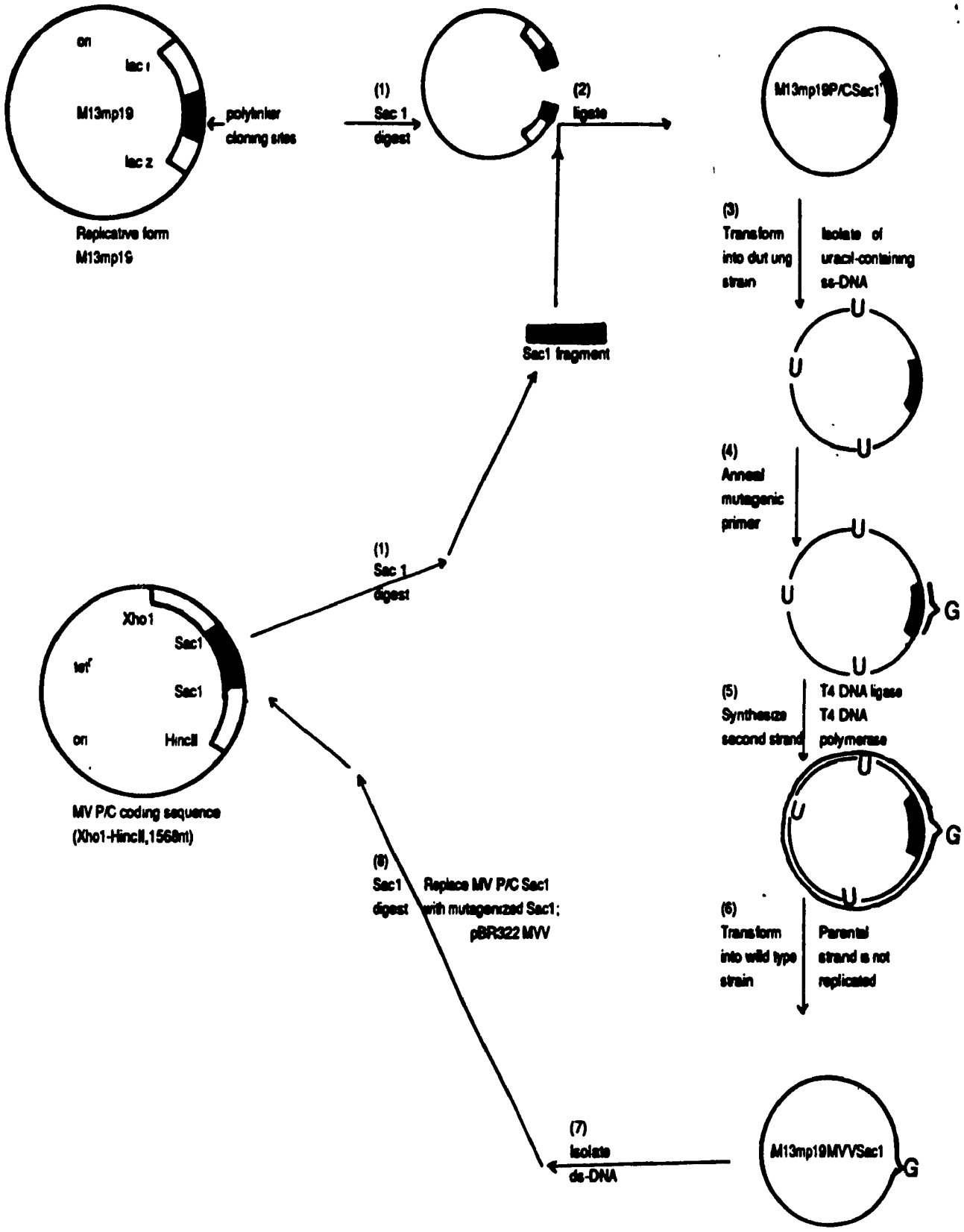
DNA was dephosphorylated using bacterial alkaline phosphatase (BAP) (Bethesda Research Laboratories) in BAP buffer (50 mM Tris-HCl pH 8.1, 10 mM $MgCl_2$). Reactions were incubated at 65° for 90 minutes followed by phenol/chloroform extraction and ethanol precipitation.

6. Construction of Plasmids

a) Mutagenesis of P/C Gene

The MV V transcript differs from the MV P transcript by the addition of a G nucleotide at position 755. To construct a V gene the following strategy was employed (Fig. 4). The cDNA of the MV P/C coding region had previously been cloned into the Pst 1 site of pBR322 (Alkhatib *et al.*, 1988). The coding region is 1565 nucleotides and is flanked by a 5′ Xho I site and a 3′ Hinc II site. Digesting of this plasmid with the restriction endonuclease Sac I released a fragment of 765 nucleotides that contains the G insertion site.

Figure 4. Schematic representation of mutagenesis of MV P/C gene and cloning of pBR322MVV.


ori
lac I
M13mp19
lac z
polylinker cloning sites
Replicative form M13mp19
(1) Sac 1 digest
(2) ligate
M13mp19P/CSac1
(3) Transform into dut ung strain
Isolate of uracil-containing ss-DNA
U
U
U
Sac1 fragment
(4) Anneal mutagenic primer
U
U
U
G
(1) Sac 1 digest
Xho1
Sac1
ter
Sac1
ori
HincII
MV P/C coding sequence (Xho1-HincII,1568nt)
(5) Synthesize second strand
T4 DNA ligase T4 DNA polymerase
U
U
U
G
(8) Sac1 digest
Replace MV P/C Sac1 with mutagenized Sac1; pBR322 MVV
(6) Transform into wild type strain
Parental strand is not replicated
(7) Isolate ds-DNA
M13mp19MVVSac1
G

This fragment was ligated to the dephosphorylated Sac I of the replicative form (RF) (ie. double-stranded) of the M13mp19 bacteriophage vector (Bio-Rad) to generate M13MVP/C Sac I. Mutagenesis, the addition of the G nucleotide, was performed as instructed by the manufacturer. Briefly, the M13mp19P/C Sac I was transformed into a dut/ung double mutant bacterium. When DNA is synthesized in a dut, ung mutant bacterium, the nascent DNA carries a number of uracils in thymine positions as the result of the dut mutation which inactivates the enzyme dUTPase and results in high intracellular levels of dUTP. The ung mutation inactivates the enzyme dUTPase and results in high intracellular levels of dUTP. The ung mutation inactivates uracil N-glycosylase which allows the incorporated uracil to remain in the DNA. This uracil-containing strand is used as template for the in vitro synthesis of a complementary strand primed by an oligonucleotide containing the desired mutation. In this case the oligonucleotide 5′ dCCATTAAAAAGGGGCACAGAG 3′ (Regional DNA Synthesis Laboratory, University of Calgary) was used, where the underlined G is the added nucleotide. The second strand was synthesized with T4 DNA polymerase and T4 DNA ligase and the double-stranded DNA were used to transform a bacterial strain with proficient uracil N-glycosylase. The uracil-containing strand is thus inactivated with high efficiency, leaving the non-uracil

containing survivor (strand with desired mutation) to replicate (Kunkel, 1985; Kunkel *et al.*, 1987). The resulting clones were sequenced using the Sequenase™ Kit (United States Biochemical Corp) according to the protocol described by the manufacturer. The primer used for sequencing was 5′ dCTTTCCGAAGCTTGG 3′ (Regional DNA Synthesis Laboratory, University of Calgary), which is 72 base pairs upstream of the G insertion site. A M13mp19MVP/C Sac I clone containing the desired mutation (M13mp19MVV Sac I) was digested with the restriction enzyme Sac I and the Sac I fragment was ligated back into the dephosphorylated Sac I site of the pBR322MVP/C vector to complete the V coding sequence, pBR322MVV.

b) In Vitro Transcription Vectors

Cloned cDNA corresponding to the complete coding region of the P and V genes were separately inserted into pAM vectors (Amersham) to allow in vitro transcription of these genes. The pAM18 and pAM19 vectors contain both the SP6 and T7 RNA polymerase promoters, a polylinker with multiple unique cloning sites, and the ampicillin resistance gene (refer to Fig. 5). The pBR322MVP/C (Alkhatib *et al.*, 1988) and pBR322 MVV (described in section a) plasmids were digested with Xho I and Hinc II to release the P and V coding sequence respectively and gel purified. The fragments were treated

with the E. coli Klenow fragment of DNA polymerase I and inserted into the Hinc II site of a dephosphorylated pAM vector by blunt end ligation. The P coding region was inserted into pAMmp18 and the V coding region into pAMmp19. The only difference between these two vectors is the orientation of the polylinker, and they were used according to availability.

c) Bacterial Transformation and Screening of Recombinant Clones

The E. coli strain DH5 was transformed with the plasmids pBR322MVV, pAM18MVP, and pAMMVV, in order to be able to amplify the plasmids with ease by mini- or maxi-preps. Briefly, 100 ng of plasmid DNA in 100 µl of ice cold transformation buffer (10 mM Tris-HCl pH 8.1, 10 mM $CaCl_2$, and 10 mM $MgCl_2$) was mixed with 200 µl of competent DH5 cells and left on ice for 30 minutes. The cells were then heat shocked for 90 sec in a 37° waterbath. One ml of Luria broth (LB) (1% [W/V] tryptone, 0.5% [W/V] yeast extract, 1% [W/V] NaCl) was added to the cells and incubated for 1 hr at 37°. Bacterial suspensions containing the pAM plasmids were plated onto ampicillin-containing LB agar plates (LB broth containing 1.5% [W/V] agar and 200 µg/ml ampicillin). Those containing the pBR322MVV plasmid were plated onto tetracyclin-containing LB

agar plates (20 µg/ml tetracycline). Plates were incubated at 37° for 12-14 hr.

Drug resistance alone was not enough to select for bacterial colonies containing plasmids with insert DNA. The pBR322 plasmids in the tetracycline resistant colonies were amplified my miniprep and analyzed for size and orientation of the insert by restriction endonuclease analysis. The ampicillin resistant colonies containing pAM plasmids were first rapidly screened by "colony cracking". Briefly, bacteria from individual colonies were transferred, using sterile toothpicks, into 50 µl of cracking buffer (50 mM Tris pH 7.5, 20 mM ethylene-diaminetetra-acetic acid [EDTA], 1% [W/V] sodium dodecyl sulphate [SDS], 14% [W/V] sucrose, and 0.1% [W/V] bromophenol blue), vortexed and left at room temperature for 15 min. Samples were centrifuged in a microfuge (Fisher) and the supernatant was analyzed on a 0.8% agarose gel. Colonies containing plasmids with insert were amplified by miniprep and analyzed for size and orientation of insert by restriction endonuclease analysis.

As the insert DNA is bordered by the SP6 and T7 promoters, the plasmids were sequenced using Sequenase™ with the SP6 and T7 primers (New England Nuclear) to ensure that the entire coding

sequence of the P, or V, gene was inserted. In addition, the colonies were sequenced to ensure whether or not there was an additional G nucleotide.

Bacterial transformations were also performed at two stages of the in vitro mutagenesis procedure: i) when initially cloning the DNA fragment to be mutagenized into M13 phages ii) after synthesis of the mutagenized strand on uracil-containing template. The bacterial transformation is essentially the same except that E. coli strain MV1190 was used. Also, 10-100 ul of transformed cells were added to 0.3 ml of an MV1190 overnight culture and 2.5 ml of molten top agar (0.7% Bacto-Agar [W/V]) and poured onto H agar plates (1% Bacto-tryptone [W/V], 0.5% NaCl [W/V], 1.5% Bacto-Agar). Plates were incubated at 37° for 12-16 hr.

The cloning site in the M13mp19 vector is within a segment of the ß-galactosidase gene and plaques were screened for inserts by a color assay. This was done by including 50 ul of 2% 5-bromo-4-chloro-3-indolyl-ß-D-galactosidase (X-gal) (dissolved in dimethyl formamide) and 20 ul of 100 mM isopropyl-1-thio-ß-D-galactosidase (IPTG) in the top agar. Plaques without inserts are blue; those with inserts are clear. RF M13mp19 were amplified by miniprep and analyzed for size and

orientation of insert by restriction endonuclease assay. The M13mp19MVV plasmids were screened by sequencing as described earlier.

7. In Vitro Transcription

Purified pAM18MVP and pAM19MVV, with the desired DNA insert in the SP6 orientation, were used for in vitro transcription (Fig. 5). One µg of linearized plasmid was incubated for 1 hr at 37° with rNTPs (400 µM ATP, CTP, UTP and $M^7G(5')ppp(5')G$, 40 µM GTP), 30 units RNase inhibitor (Pharmacia), 50 units SP6 polymerase (Pharmacia) in transcription buffer (40 mM Tris-HCl (pH 7.9), 6 mM $MgCl_2$, 10 mM DTT, and 4 mM spermidine). The RNA produced was extracted once with buffered phenol and twice with chloroform. RNA was precipitated with 35 mM ammonium acetate, ethanol (70% V/V) and 20 µg of glycogen. The RNA was resuspended in water and precipitated in the same manner. RNA was resuspended in water and used for in vitro translation.

8. In Vitro Translation

The RNA was translated in nuclease treated rabbit reticulocyte lysate according to the recommendations of the manufacturer (Promega Corp.). Briefly, RNA was incubated with rabbit

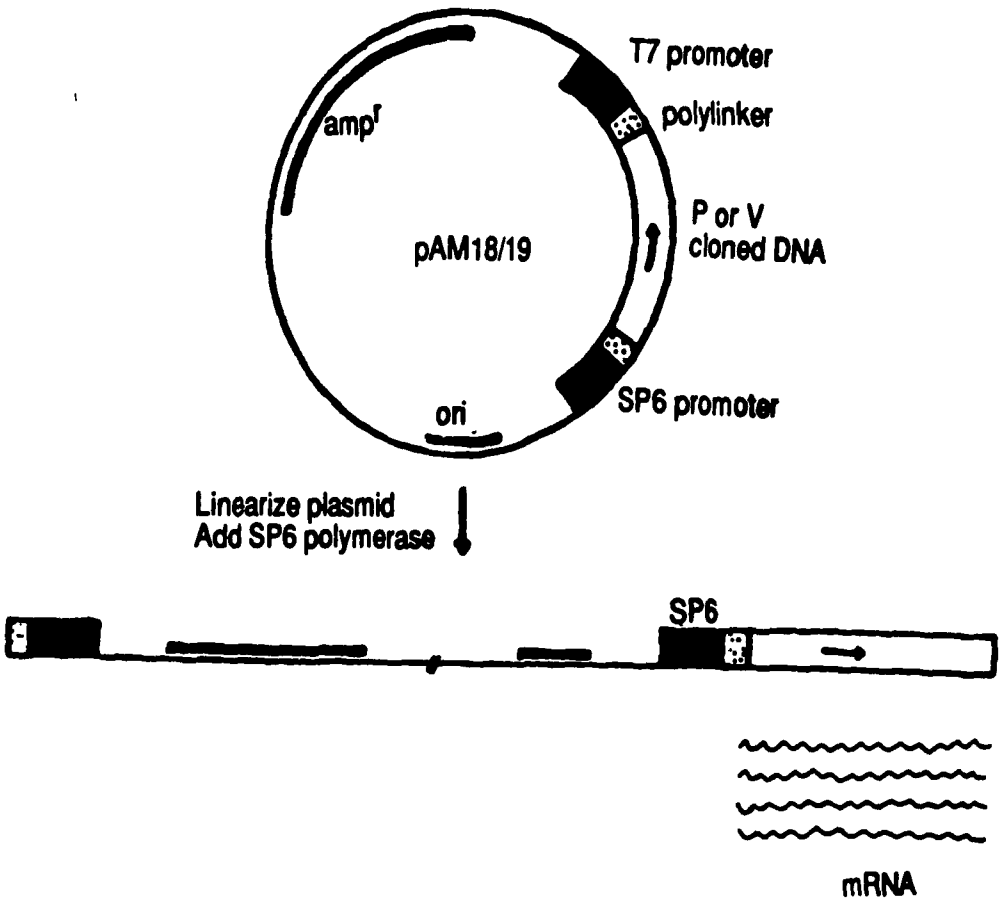
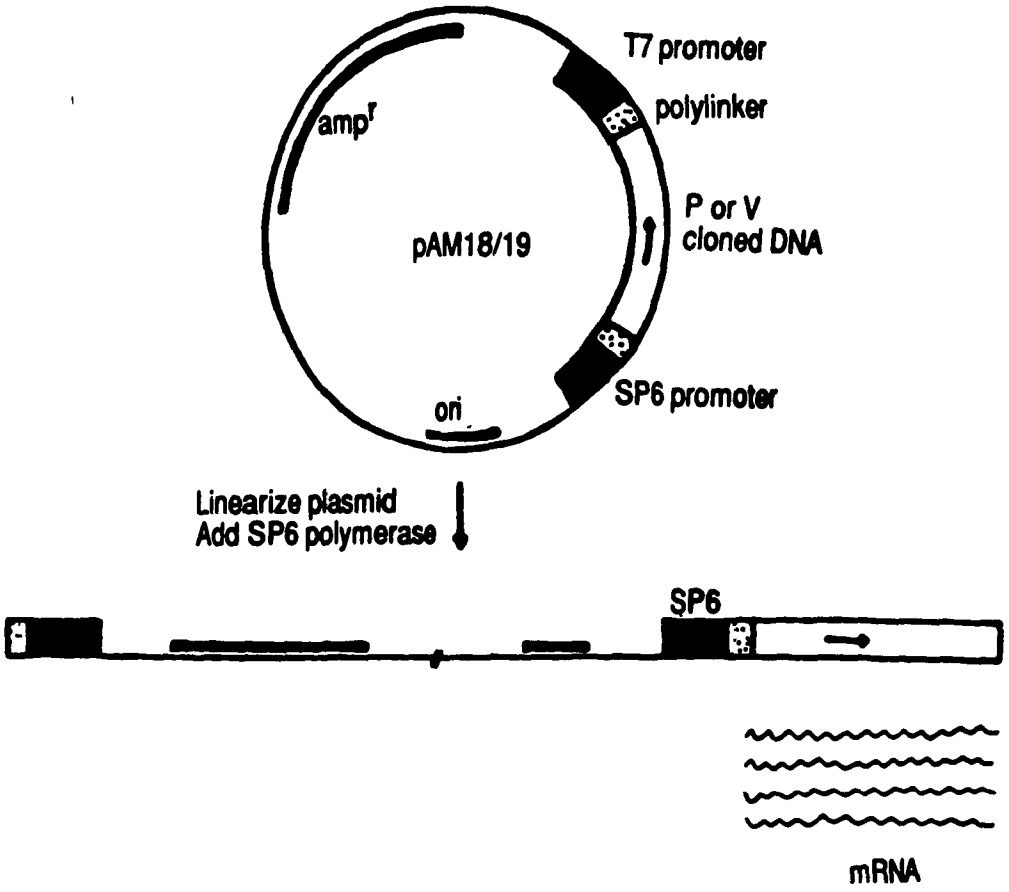

T7 promoter
polylinker
amp^r
P or V
cloned DNA
pAM18/19
ori
SP6 promoter
Linearize plasmid
Add SP6 polymerase
SP6
mRNA

Figure 5. Schematic representation of preparation of substrate RNA using recombinant vector pAM18/19 (Amersham).


T7 promoter
polylinker
amp^r
P or V
cloned DNA
pAM18/19
ori
SP6 promoter
Linearize plasmid
Add SP6 polymerase
SP6
mRNA

reticulocyte lysate, amino acid mixture minus methionine, RNase inhibitor, and [^{35}S] methionine (0.8 mCi/ml) (Amersham) for 1 hr at 30°. Proteins were also labeled with [^{35}S] cysteine (0.8 mCi/ml) (Amersham) using amino acid mixture minus cysteine. The protein products produced were mixed with protein lysis buffer (60 mM Tris pH 6.8, 40% [V/V] glycerol, 4% [W/V] SDS, 3% [W/V] DTT and 0.002% [W/V] bromphenol blue) and analyzed on a discontinuous SDS-polyacrylamide gel according to the method described by Laemmli (1970).

9. In Vivo Labeling of Infected Cell Proteins

Confluent layers of Vero cells were infected with MV at MOI of 10 PFU/cell. The cells were incubated for 90 min and then the medium was replaced with DMEM. After 16 hr, the medium was removed and replaced, sequentially, with methionine-free minimal essential medium (MEM) (Flow) without radiolabel for 30 min, methionine-free MEM containing 100 uCi [^{35}S] methionine (Amersham Corp.) for one hour, and then complete DMEM without radiolabel for 30 minutes. For cysteine or phosphate labeling a similar procedure was used except that cysteine-free media (Select-Amine, Bio-Rad) or phosphate free media (Flow) was used in place of methionine free MEM and [^{35}S] cysteine or [^{32}P] orthophosphate (Amersham Corp.) was used for radiolabeling.

Cells were harvested in radioimmuno-precipitation assay (RIPA) buffer (150 mM NaCl, 1% [W/V] deoxycholic acid, 1% [V/V] triton X-100, 0.1% [W/V] SDS, 10 mM Tris-HCL pH 7.4, 1 mM phenylmethylsulfonyl fluoride, 1 mM methionine and 20 µg/ml aprotinin) with 0.1% BSA using a rubber policeman. The cells were centrifuged in a microfuge for 10 minutes and the supernatant used for immunoprecipitations

10. Immunoprecipitations

In vivo and in vitro synthesized MV proteins were incubated in RIPA buffer containing 0.1% BSA and the appropriate antiserum for 2 hr at 4°. Pre-swollen protein-A Sepharose beads (Pharmacia) were added to each assay and incubated for 1 hr at 4°, during which time the tubes were vortexed every 10 min to keep the beads in suspension. Immune complexes were washed three times with RIPA buffer and 0.1% BSA and twice with RIPA buffer without BSA. A 35 µl aliquot of protein lysis buffer was added to the beads. The sample were incubated at 37° for 10 min, heated to 95° for 3 min, and analyzed on discontinuous SDS polyacrylamide gels.

11. Indirect Immunofluorescence

Vero cells were grown to 40% confluence on microscope cover slips before infection with MV. Uninfected Vero cells were used as a control. After 16 hr, the cells were washed with phosphate-buffered saline (PBS) and then fixed by treatment with 2.5% paraformaldehyde for 20 min. Cells were permeabilized by treating them with 0.5% Triton in PBS for 10 min. Following three washes with PBS the cells were treated with normal goat serum (Cedarlane) for 30 min and then primary antibody, PV1, PV2, or V2, for 1 hr. A control was done whereby there was no primary antibody added, for both uninfected and infected cells. The cells were washed three times with PBS and then treated with goat-anti-rabbit-fluorescein-isothiocyanate (FITC) (Cedarlane) for 30 min. Cells were again washed with PBS and then cover slips were inverted on a drop of glycerol containing citofluor™ (Amersham) on glass slides for photography.

12. Computer Assisted Analysis

The DNA and protein sequence analysis programs of Pustell (International Biotechnologies, Inc.) version 2.02, were used on an IBM-PC computer.

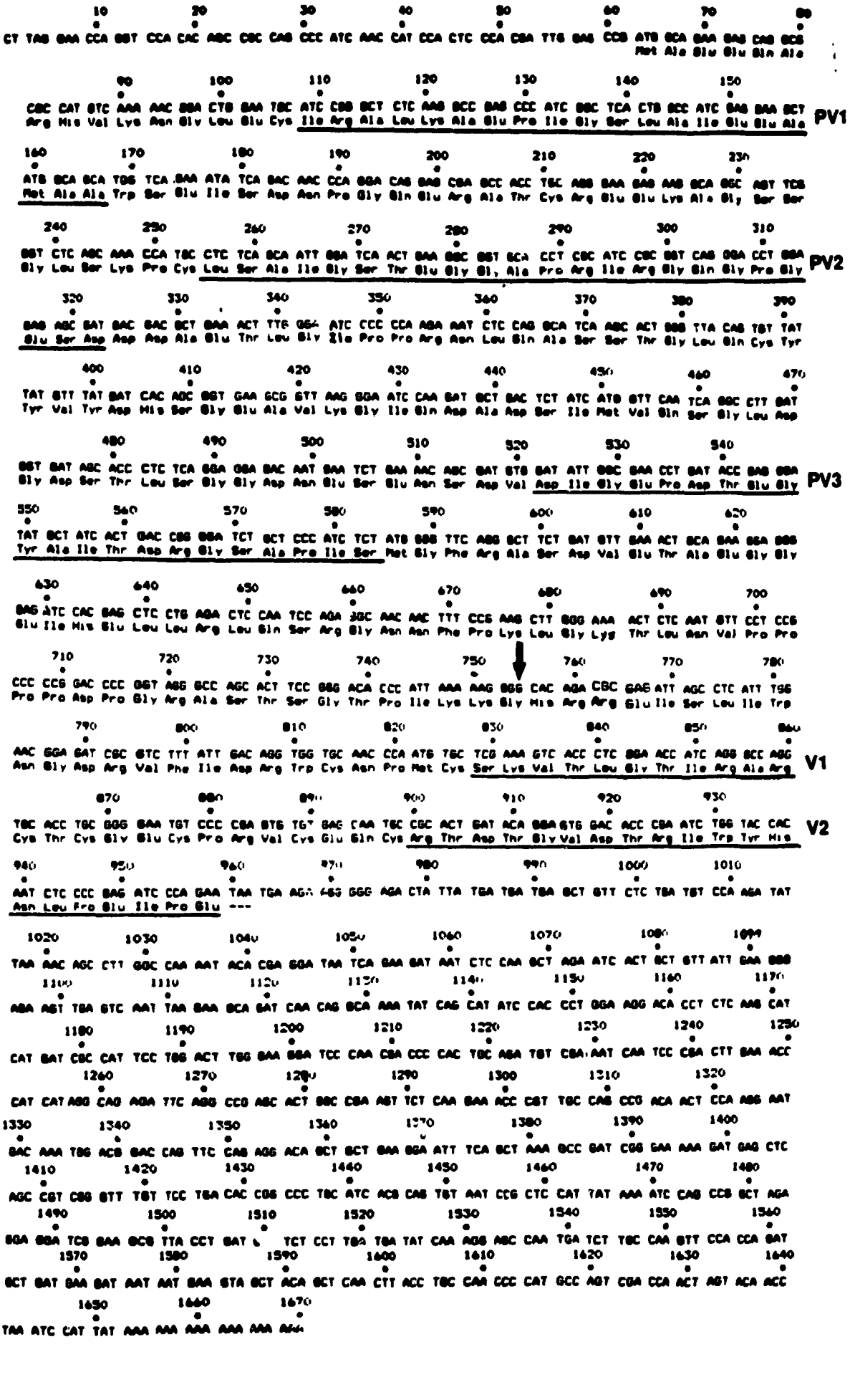
RESULTS

1. Preparation of Antibodies Directed Against Synthetic Peptides

The MV V protein has been predicted to be encoded from the virus P gene (Cattaneo *et al.*, 1989). A V-specific mRNA is thought to be transcribed from the P gene by means of an "editing" mechanism whereby an extra nontemplated G residue is inserted at position 755, thereby changing the protein-coding reading frame from that of P to that of V (Fig. 6). P and V would be amino coterminal but distinct at their carboxy termini. Although such an edited transcript of the P gene has now been identified in MV-infected cells by cDNA cloning and in vitro translation, no corresponding protein has yet been found in vivo (Cattaneo *et al.*, 1989).

The use of antibodies directed against synthetic peptides has been successful in identifying previously unknown gene products (Lerner *et al.*, 1981; Lerner, 1984; Walter, 1986). In order to examine whether the V protein was expressed in MV-infected cells, five peptide sequences were chosen from the predicted amino acid sequence of the V protein for the production of anitpeptide-antibodies (Fig. 6). Peptide

Figure 6. The MV V protein mRNA nucleotide and amino acid sequences. The site of nontemplated G insertion is designated by an arrowhead at position 755 but the actual insertion could take place one or two positions before. The sequences of the 5 peptides chosen for antibody production are underlined (PV1, PV2, PV3, V1, and V2).

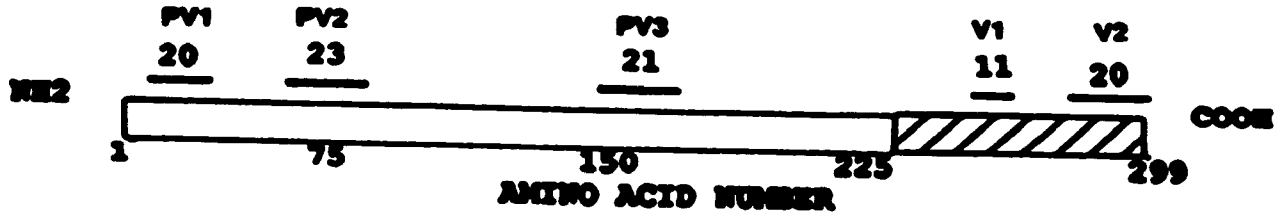

PV1
PV2
PV3
V1
V2

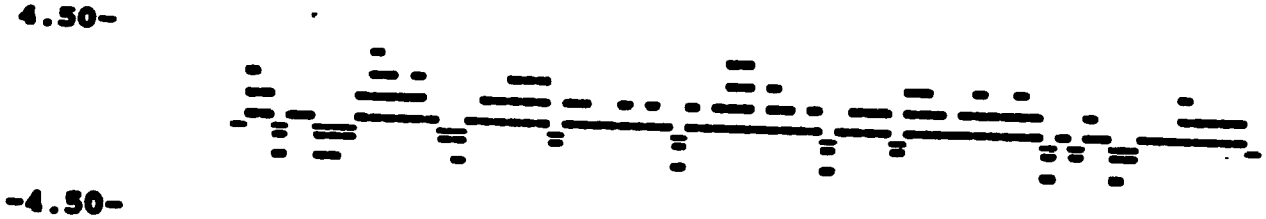
sequences were chosen to maximize their hydrophilicity, while also optimizing location and secondary structure predictions (Fig. 7). The peptides ranged in length from 11 to 23 amino acids. The first three peptides, PV1, PV2, and PV3, map to the amino terminal end of the V protein in the region where it is amino coterminal with P and thus antibodies directed against these peptides should the able, possibly, to recognize both P and V. The remaining two peptides, V1 and V2, are located in the unique V protein carboxyl terminus and antibodies directed against these two peptides should be able to recognize the V protein only.

The peptides were conjugated to KLH and each used to immunize two New Zealand White rabbits. Resulting antibody titers were determined by ELISA using the pre-immune serum as a negative control. After four immunizations, all antisera, except that directed against V1, had titers of $\geq$ 1/100,00. The V1 antiserum had a titer of 1/50,000. Nevertheless, the VI antibody titer would normally be considered adequate for use in immunoprecipitation and/or immunofluorescence. Pre-immune sera (Pre) from all 10 rabbits were pooled and then used as negative controls. Additionally, peptide antiserum PC20 (generously provided by Dr. C.D. Richardson) directed against a carboxyl-terminal region of P (Richardson *et al.*, 1985) not

Figure 7. Relative location and length of peptide sequences used for antiserum production. The open box indicates the P/V amino coterminal region. The hatched box represents the carboxyl region unique to V. A hydropathy plot is placed below (IBI Pustell Sequence Analysis Programs Version 2.02). Regions above the baseline are hydrophilic while regions below the baseline are hydrophobic.

PEPTIDE LOCATIONS





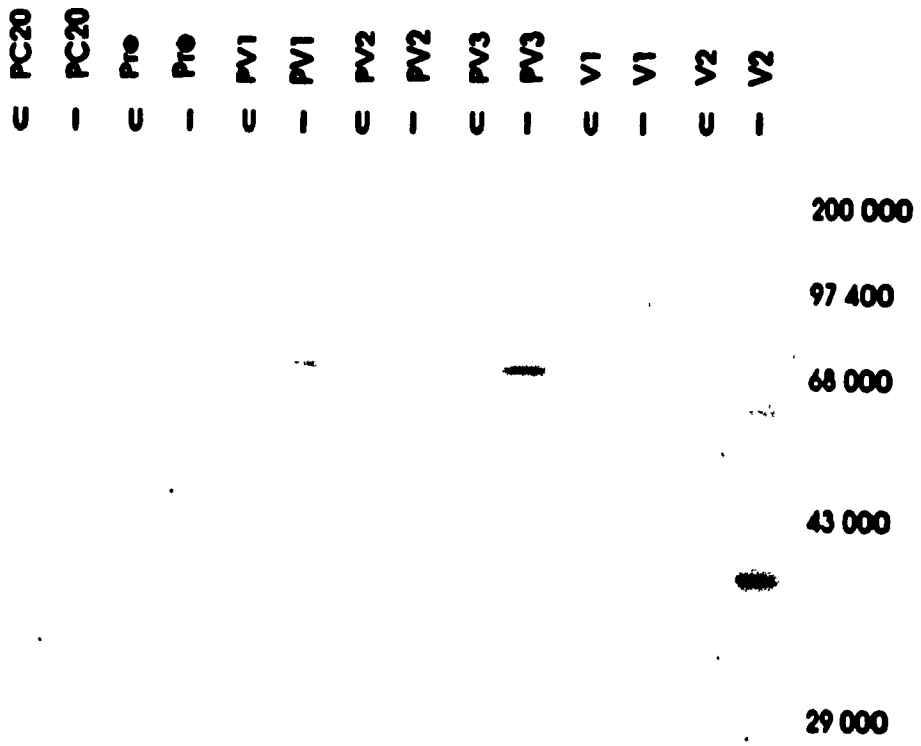
HYDROPATHY PLOT

expressed in the V protein was also used as a control.

2. Detection of V Protein in Infected Cells Using Immunoprecipitation

To determine whether the MV V protein was present in vivo, [^{35}S] methionine-labeled proteins from MV-infected Vero cells were immunoprecipitated with the different antisera and then analyzed by SDS-PAGE (Fig. 8). The MV P protein has an M_r of approximately 72 kDa (Norrby and Oxman, 1990) and the V protein has been predicted to have an M_r of approximately 40 kDa (Cattaneo *et al.*, 1989). No proteins were detected when uninfected cell lysates were immunoprecipitated using any of the peptide antisera or when infected cell lysates were immunoprecipitated with the pre-immune serum. However, two polypeptides (with M_r respectively, of 70 kDa and 40 kDa) were detected in MV infected cell lysates in different patterns of the different peptide antisera and are likely to be the P and V proteins. Evidence that supports this is that the 70 kDa polypeptide migrated at the same position as P immunoprecipitated by the PC20 antiserum (lane 2) and that it was recognized by the antisera specific to both P and V: PV1, PV2, and PV3, but not by the antisera specific to V only: V1 or V2. Furthermore, the 40 kDa polypeptide was recognized by

Figure 8. Immunoprecipitation of [^{35}S] methionine-labeled polypeptides from uninfected Vero cells as well as from MV-infected Vero cells using peptide antisera PC20, PV1, PV2, PV3, V1, and V2. I = infected. U = unifected. Pre = preimmune serum. Samples were analyzed on a 10% SDS-PAGE gel. Positions of molecular weight markers (lane not shown) are indicated on the right.


PC20 U
PC20 I
Pre U
Pre I
PV1 U
PV1 I
PV2 U
PV2 I
PV3 U
PV3 I
V1 U
V1 I
V2 U
V2 I
200 000
97 400
68 000
43 000
29 000

the V specific antiserum V2 as well as by the antisera specific to both P and V: PV1 and PV3, but not by the P only-specific antiserum PC20.

The V1 antipeptide antiserum failed to immunoprecipitate V. However, the V1 peptide was predicted to be somewhat hydrophobic. There are only 62 amino acids after the G insertion site in the unique V region and the V1 sequence seemed, after V2, to be the most favourable for antibody recognition based on secondary structure predictions. The PV2 antiserum was able to immunoprecipitate the 70 kDa polypeptide but not the 40 kDa polypeptide, although the sequence of the peptide was derived from a portion of the P/V amino coterminal region. This may indicate that the two proteins are folded differently at or near this region such that the PV2 sequence is not exposed at the surface of the V protein but is exposed at the surface of the P protein.

3. Immunoprecipitation of Differentially Radiolabeled Proteins from Measles Virus Infected Cells

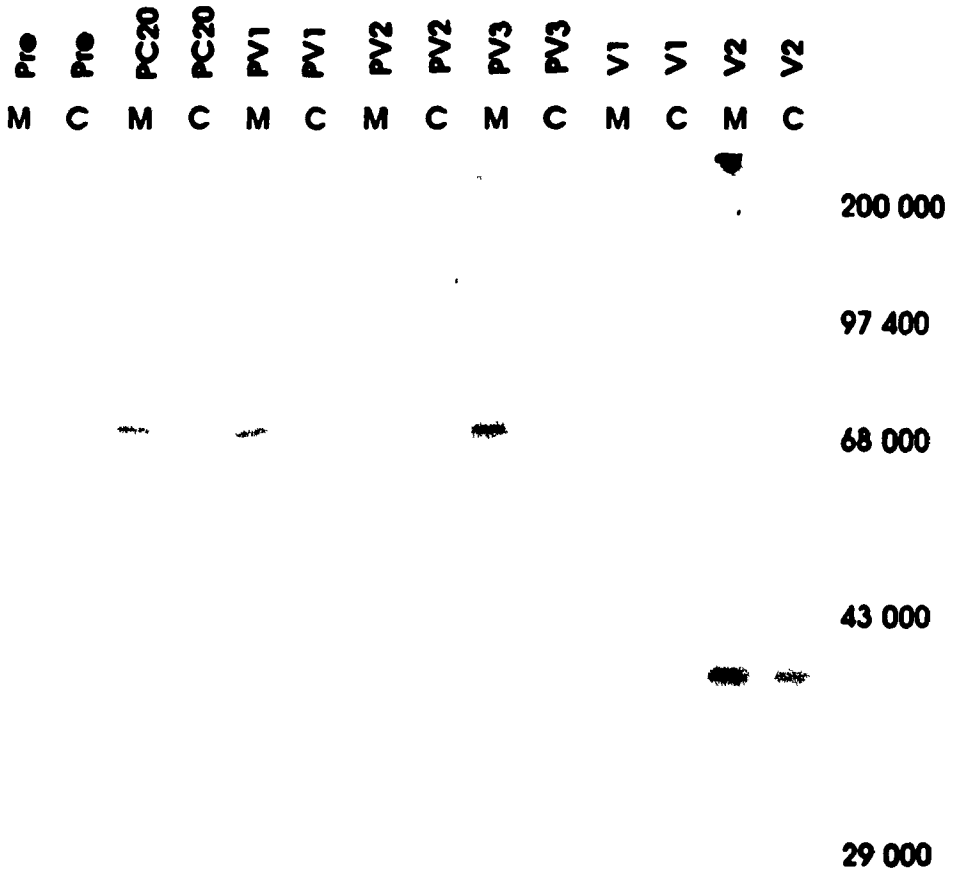
In order to provide evidence that the 70 kDa and 40 kDa polypeptides are identical to, respectively, the P and V proteins, MV-infected cells were separately radiolabeled with

either [^{35}S] methionine or [^{35}S] cysteine, immunoprecipitated with the different antisera, and analyzed by SDS-PAGE (Fig. 9). The MV P protein contains 11 methionine and 6 cysteine residues whereas the V protein should contain 5 methionine and 11 cysteine residues. It can bee seen in the control lanes using PC20 antiserum, that the efficiency of cysteine-labeling was not as efficient as that of methionine-labeling, since the cysteine band was less intense than the methionine band. This could be due to a number of factors including cellular amino acid pool size. Similar problems have been described by others (Paterson and Lamb, 1990; Thomas *et al.*, 1988). The 70 kDa polypeptide was readily labeled with methionine but not with cysteine whereas the 40 kDa polypeptide was readily labeled with either methionine or cysteine.

4. Synthesis of V Protein During MV Infection

To determine the time course of V protein synthesis in vivo, cells were labeled with [^{35}S] methionine at different times following MV infection. Labeled proteins were immunoprecipitated with either PV1 or V2 antisera and analyzed by SDS-PAGE. For both the P and V proteins maximal synthesis

Figure 9. Differential radiolabeling of the P and V proteins. MV-infected Vero cells were radiolabeled with either [^{35}S] methionine or [^{35}S] cysteine, immunoprecipitated with the anitsera indicated, and analyzed by SDS-PAGE on a 10% polyacrylamide gel. M = methionine label C = cysteine label. Positions of molecular weight markers (lane not shown) are indicated on the right.


Pre
Pre
PC20
PC20
PV1
PV1
PV2
PV2
PV3
PV3
V1
V1
V2
V2
M C M C M C M C M C M C M C
200 000
97 400
68 000
43 000
29 000

occurred at 16 hours after infection at which time approximately 80-90% of the cells were fused (Fig. 10). Non-immunoprecipitated MV-infected cell lysates are shown for comparison (Fig. 11).

5. Analysis of Proteins Synthesized In Vitro

To further confirm that the peptide antisera were specific for the P and V proteins, they were tested for reactivity with in vitro synthesized P and V proteins. To obtain a copy of the edited V protein mRNA a fragment of the cDNA copy of the P gene containing the G insertion site was mutated by oligonucleotide sited directed mutagenesis in the vector M13mp19. Resultant clones were sequenced to confirm the presence of an extra G nucleotide (Fig. 12). Two plasmids, pAM18MVP and pAM19MVV, were then constructed capable of producing SP6 RNAs specific to the V and P proteins, respectively, by in vitro transcription. Rabbit reticulocyte lysate was subsequently used for in vitro translation reactions. The resulting polypeptide products were immunoprecipitated and analyzed by SDS-PAGE.

Proteins of the approximate molecular weight of P and V were immunoprecipitated by the appropriate peptide antisera and

Figure 10. Time course of synthesis of V protein during MV infection. Vero cell proteins were labeled with [^{35}S] methionine at different times following MV infection, immunoprecipitated with PV1 or V2 antisera, and analyzed using SDS-PAGE on a 10% polyacrylamide gel. The cells were radiolabeled at the indicated times (h) after infection. Un= uninfected. Positions of molecular weight markers (lane not shown) are indicated on the right.

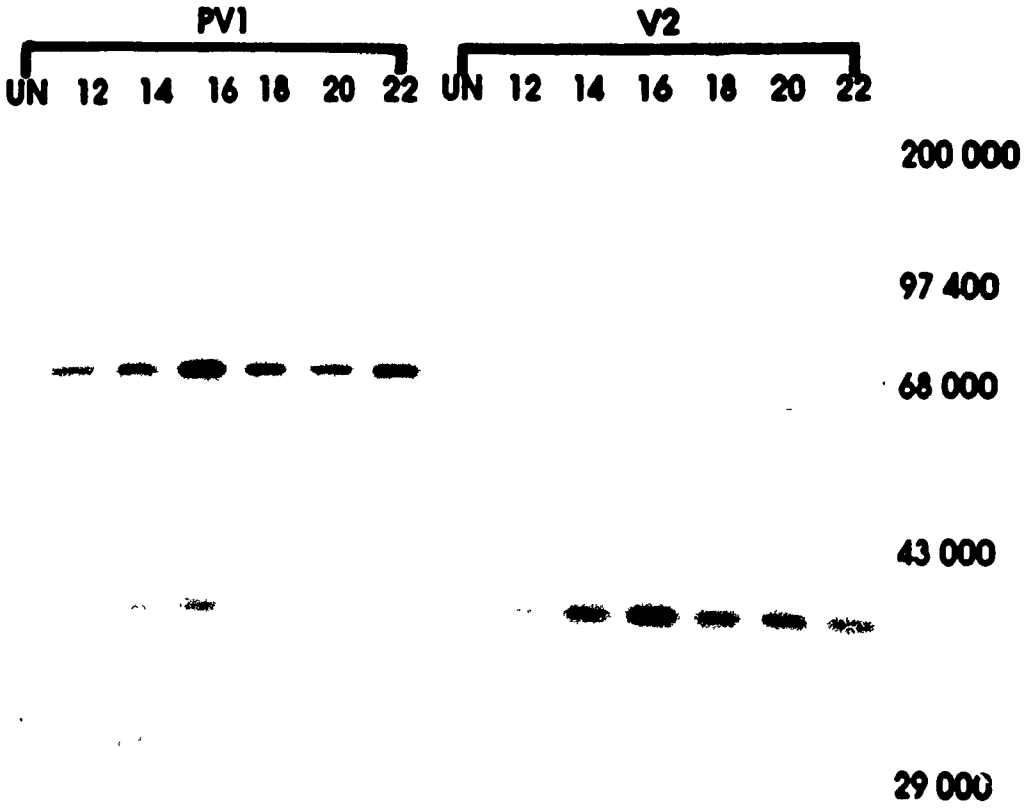
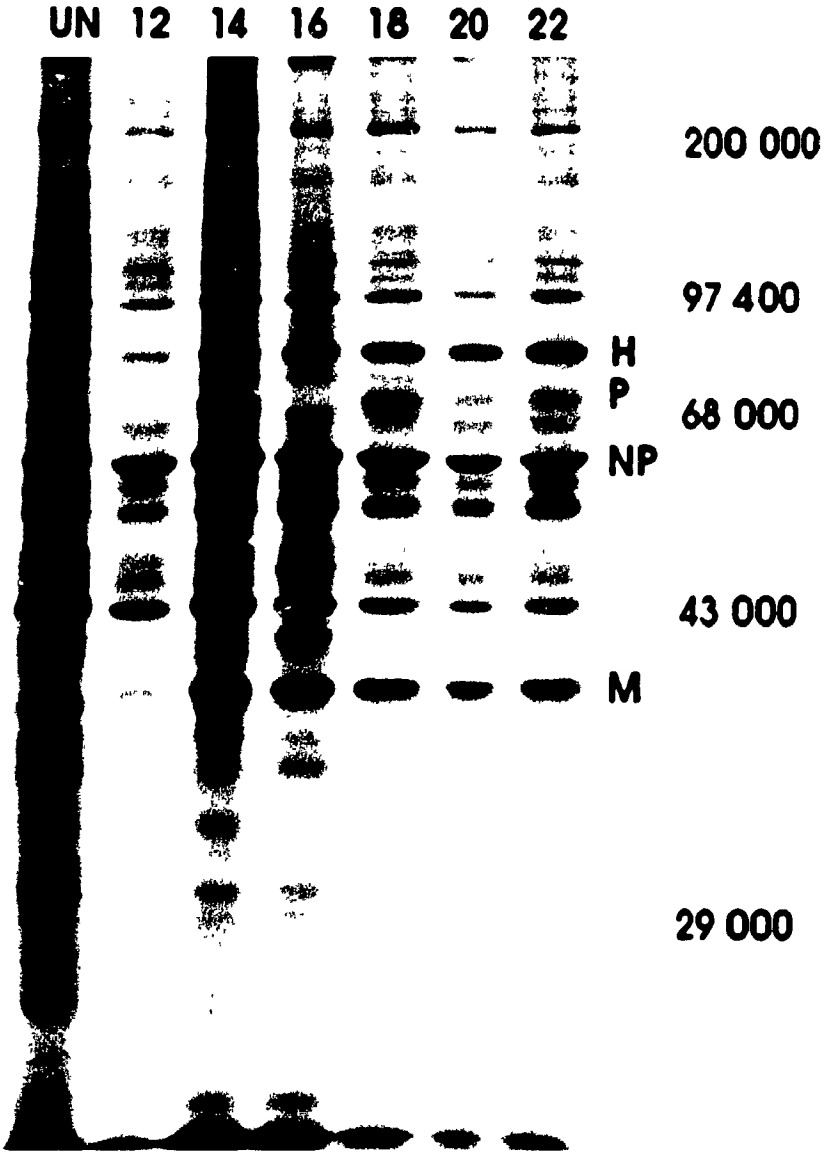

PV1
V2
UN 12 14 16 18 20 22 UN 12 14 16 18 20 22
200 000
97 400
68 000
43 000
29 000

Figure 11. Time course of synthesis of MV proteins during infection. Vero cells were radiolabeled with [^{35}S] methionine at different times after infection and analyzed by SDS-PAGE on a 10% polyacrylamide gel. The cells were radiolabeled at the indicated times (h) after infection. Un = uninfected. H = hemagglutinin, P = phosphoprotein, NP = nucleoprotein and M = matrix protein. Positions of molecular weight markers (lane not shown) are indicated on the right.


UN
12
14
16
18
20
22
200 000
97 400
H
P
68 000
NP
43 000
M
29 000

were not present in RNA-minus in vitro translation reactions (Fig. 13). For convenience, only the PV1 peptide antisera was chosen for P and V recognition, and V2 for V-only recognition. Proteins were also immunoprecipitated from [^{35}S] methionine labeled MV infected cell lysates, using the same peptide antisera, to compare migration of in vivo and in vitro synthesized proteins. The in vivo synthesized proteins migrated slightly slower than the corresponding in vitro proteins. This probably indicates that the P and V proteins undergo different post translational modification in vivo and in vitro.

The in vitro synthesized proteins were also differentially labeled with [^{35}S] methionine or [^{35}S] cysteine and analyzed by SDS-PAGE. Under these in vitro conditions, the P was preferentially labeled by methionine and the V by cysteine (Fig. 14) in a pattern entirely consistent with the different expected ratios of methionine to cysteine in these proteins. Since cell pool size, etc. is not a factor in labeling intensity in vitro, these results strongly confirm the identity of these proteins.

Figure 12. Nucleotide sequence of 4 different M13 clones after site-directed mutagenesis. The Sac I fragment of the P/C gene was inserted into M13mp19 and an extra G nucleotide was added at position 755 using a specific oligonucleotide. The first clone (left side) has the desired stretch of 4 G residues. The second clone has the sequence of GCGG at the insertion site likely indicating heterogeneity within the mutagenesis primer. The remaining two clones have only 3 G nucleotides at the insertion site and therefore represent wild type background.

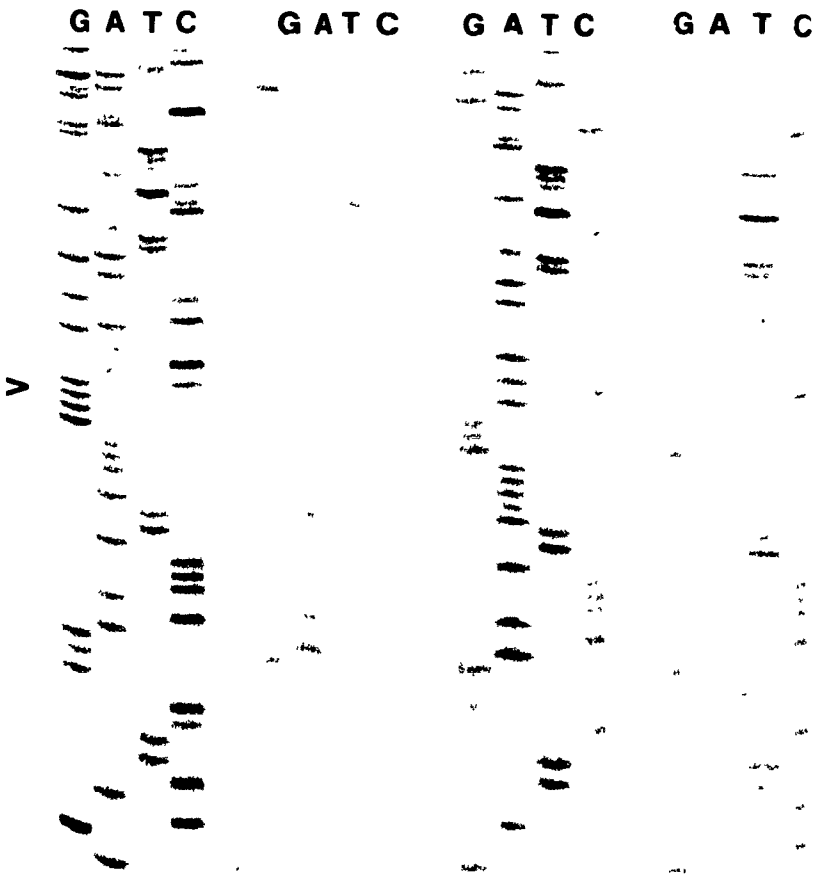

G A T C
G A T C
G A T C
G A T C

Figure 13. Immunoprecipitation of P and V proteins synthesized *in vitro* and *in vivo*. Using *in vitro* transcribed mRNAs, the P and V proteins were synthesized *in vitro* using rabbit reticulocyte lysates and [^{35}S] methionine as label. The *in vitro* translation products were immunoprecipitated with PV1 or V2 antisera. For comparison, [^{35}S] methionine-labeled polypeptides were also immunoprecipitated from MV-infected Vero cell lysates. Immunoprecipitated polypeptides were analyzed by SDS-PAGE on a 10% polyacrylamide gel. Positions of molecular weight markers (lane not shown) are indicated on the right.

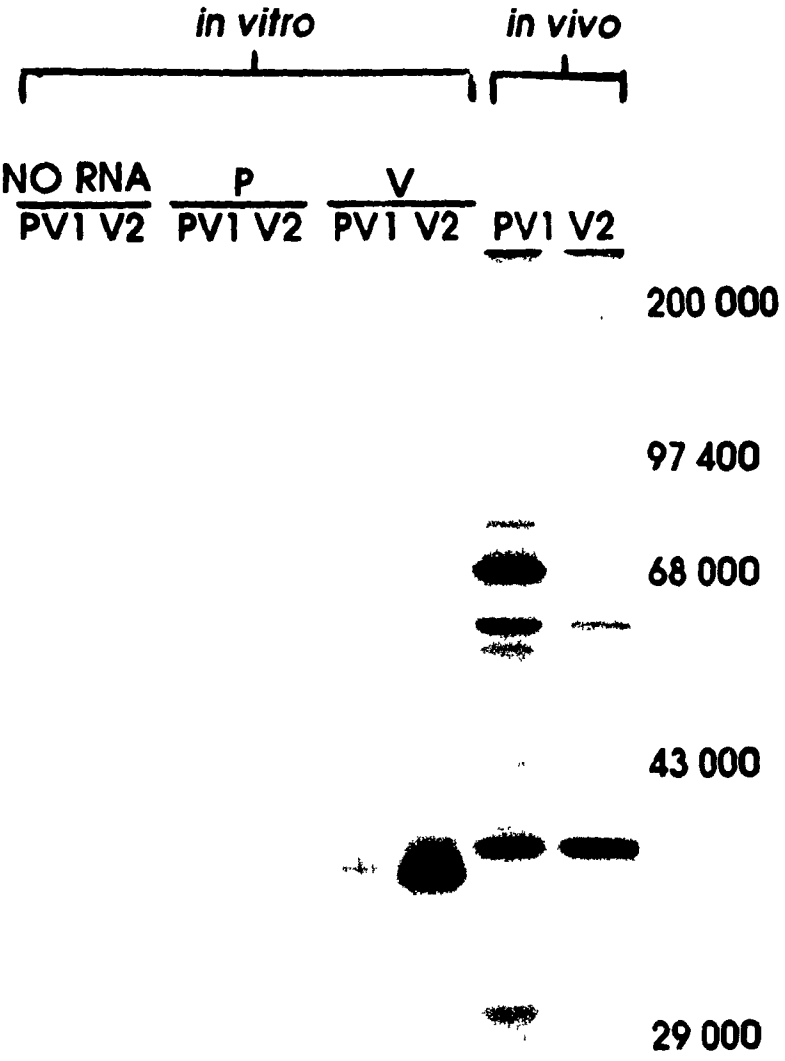
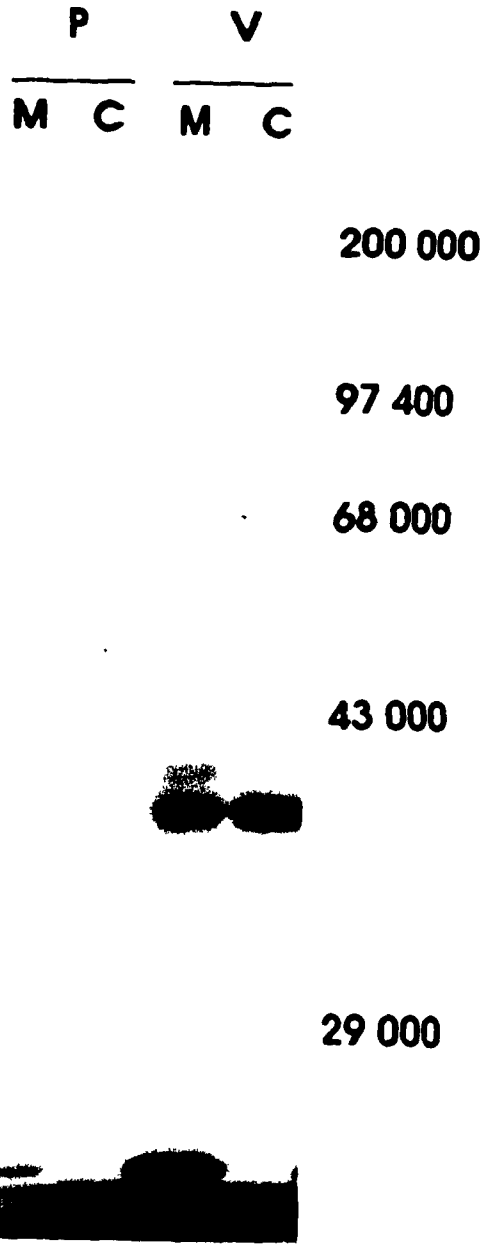

in vitro
in vivo
NO RNA
PV1 V2
P
PV1 V2
V
PV1 V2
PV1 V2
200 000
97 400
68 000
43 000
29 000

Figure 14. Differential radiolabeling of *in vitro* synthesized proteins. *In vitro* transcribed mRNAs corresponding to P and V were translated *in vitro* using rabbit reticulocyte lysates and labeled with either [^{35}S] methionine (M) or [^{35}S] cysteine (C). Translation products analyzed by SDS-PAGE on a 10% polyacrylamide gel. Positions of molecular weight markers (lane not shown) are indicated on the right.


P
V
M C M C
200 000
97 400
68 000
43 000
29 000

6. *Analysis of [^{32}P]-labeled P and V Proteins*

To determine whether the V protein is phosphorylated in vivo, MV-infected Vero cells were labeled with [^{32}P]-orthophosphate, immunoprecipitated, and analyzed by SDS-PAGE (Fig. 15). The P protein has shown to be highly phosphorylated (Graves, 1981) and our results confirm this (Fig. 15). Additionally, the results (Fig. 15) also found V to be highly phosphorylated.

7. *Localization of P and V Proteins in MV-Infected Cells*

In order to determine the cellular localization of the P and V proteins in MV-infected cells, indirect immunofluorescence (IF) analysis was performed. As the PV2 antisera only recognized P during immunoprecipitation experiments, it was hoped that it would similarly localize only P during IF analysis. The V2 antiserum was used for V localization, and the PV1 for combined P and V localization. Uninfected cells showed no fluorescence with any of the antisera (Fig. 16). The PV2 antiserum resulted in specific fluorescence in small discrete cytoplasmic inclusions. This is similar to that previously described (Bellini *et al.*, 1985) using a peptide antiserum against a carboxy-terminal region of P which is not present in V. Hence, the PV2 immunofluorescence does seem to

Figure 15. Analysis of [^{32}P]-labeled P and V proteins. Uninfected and MV-infected Vero cells were labeled with [^{32}P]-orthophosphate, immunoprecipitated with the antisera indicated, and analyzed by SDS-PAGE on a 10% polyacrylamide gel. U = Uninfected. I = Infected. Positions of molecular weight markers (lane not shown) are indicated on the right.

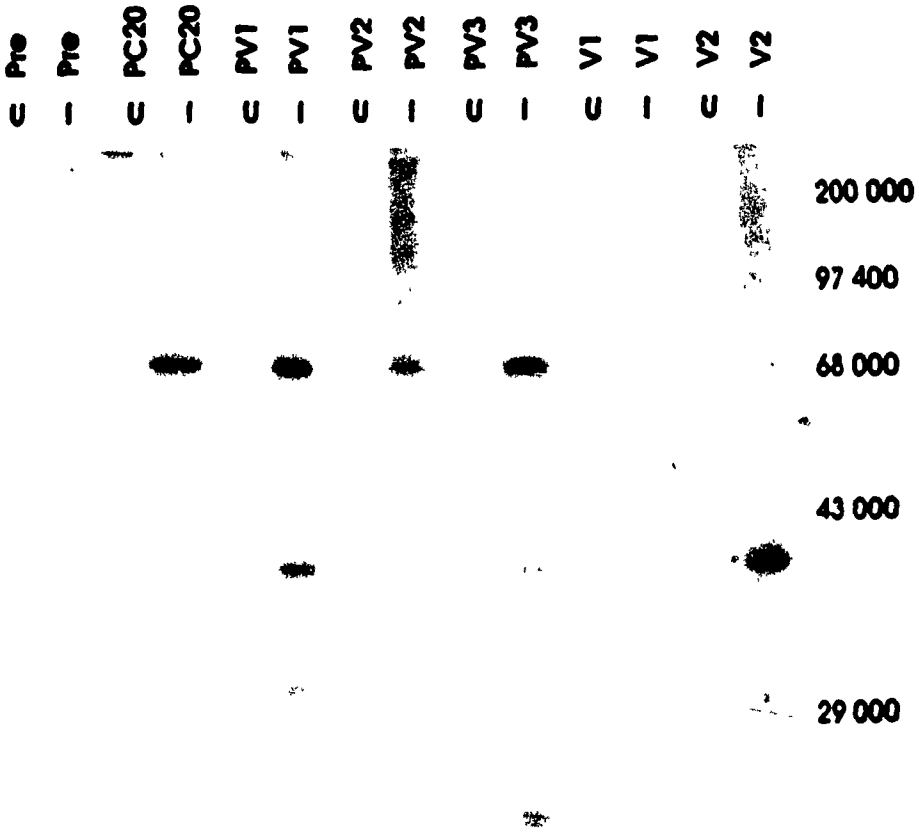
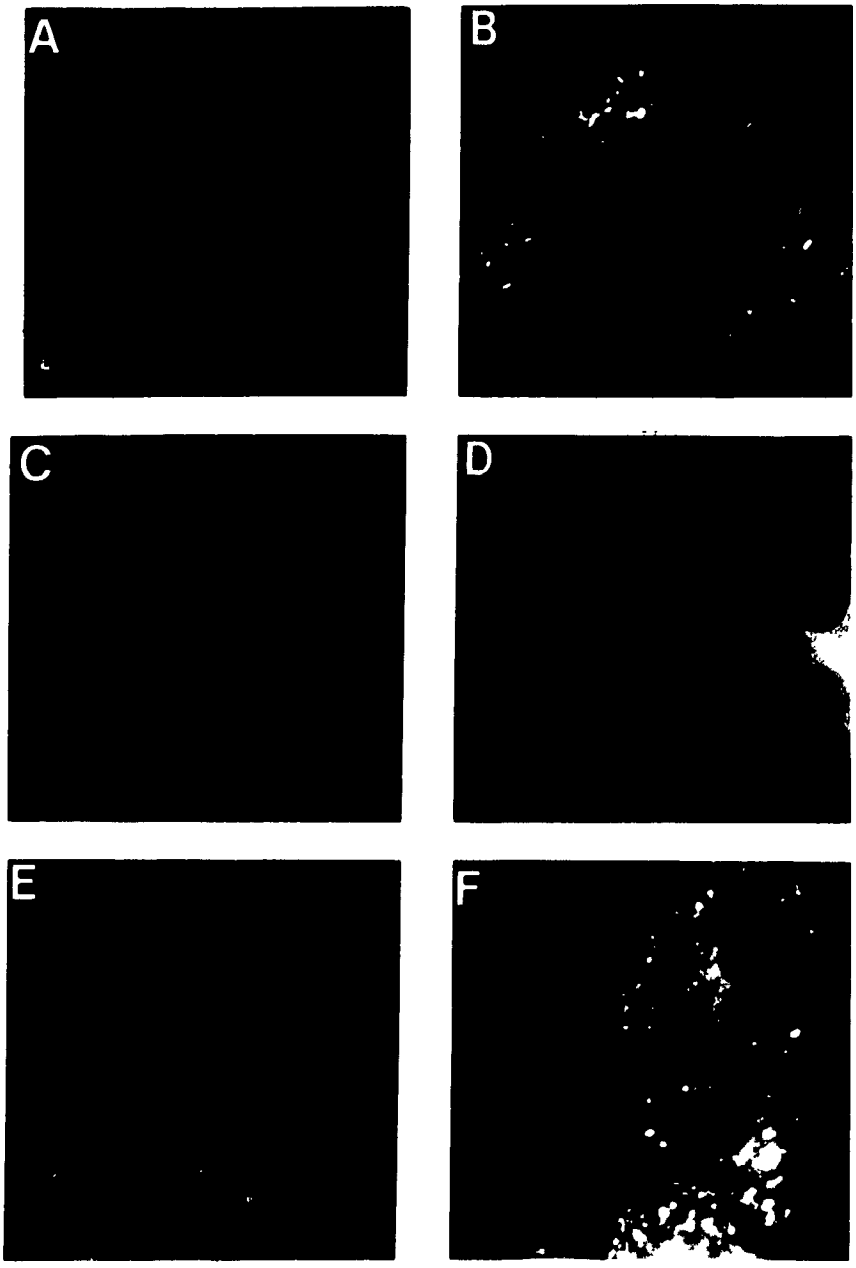

C Pre
I Pre
C PC20
I PC20
C PV1
I PV1
C PV2
I PV2
C PV3
I PV3
C V1
I V1
C V2
I V2
200 000
97 400
68 000
43 000
29 000

Figure 16. Indirect immunofluorescence analysis of the MV P and V proteins in MV-infected Vero cells. Cells were fixed 16 hours post infection in 2.5% paraformaldehyde in PBS, permeabilized with 0.5% Triton in PBS and then incubated sequentially in goat normal serum, P and/or V-specific rabbit antiserum, goat-anti-rabbit-FITC, and citofluor™ in glycerol. Panels A, C, E, uninfected Vero cells treated with PV2, V2, and PV1, respectively. Panels B, D, F MV-infected Vero cells treated with PV2, V2, and PV1. respectively. Ultraviolet photomicrographs taken at 400 X magnification. Photographic exposure times for panels A, C, and E were adjusted to be the same as for B, D, and F.


A
B
C
D
E
F

represent P alone. The V2 antiserum resulted in a very different pattern: diffuse cytoplasmic fluorescence with a perinuclear accentuation. The PV1 antiserum (specific to both P and V) gave a result combining the patterns of PV2 and V2.

DISCUSSION

In the brief period since Thomas *et al.* (1988) found that an edited P gene-specific mRNA gave rise to the SV5 virus P protein, there have been a number of groups reporting RNA editing in transcripts of other paramyxovirus P genes. Along with the discovery of such RNA editing, came the identification of a previously unknown paramyxovirus-encoded protein, designated V. In cells infected by SV5, mumps, or PIV-2, the V protein is encoded by the native genomic sequence of the P gene while the synthesis of the P protein requires the addition of G residues at a specific site in the mRNA (Ohgimoto *et al.*, 1990; Paterson and Lamb, 1990; Southern *et al.*, 1990; Takeuchi *et al.*, 1990; Thomas *et al.*, 1988). On the other hand, in cells infected by MV or Sendai viruses the native genomic sequence is used to encode the P protein and the edited sequence, with a single nontemplated G residue inserted, gives rise to the V protein (Cattaneo, *et al.*, 1989; Vidal *et al.*, 1990). The V protein has been detected in virus-infected cells, for some of the paramyxoviruses, by the use of the antibodies derived in a number of ways. P-specific monoclonal antibodies were employed for detection of the V proteins of SV5 and PIV-2 viruses (Southern *et al.*, 1990; Thomas *et al.*, 1988), while antisera directed against

synthetic peptides were used for mumps virus (Takeuchi *et al.*, 1990). However, for MV and Sendai virus, the edited P mRNA has been detected in virus-infected cells, but there has not yet been any direct evidence that the V protein exists in vivo. For this purpose peptide-antisera were produced in the present study directed against the MV V protein. To maximize chances of success, five different peptide sequences were selected from the predicted amino acid sequence of the MV V protein. Three were from the P and V amino coterminal region, and thus were expected to be able to recognize both the P and V proteins. The remaining two peptide amino acid sequences were located after the G insertion site, and thus were expected to be specific only for the V protein. Polyclonal antisera were produced in rabbits and used to immunoprecipitate proteins from MV-infected Vero cells. Depending upon the specific antiserum used, one or two proteins were detected with molecular masses of 70 kDa and 40 kDa respectively (Fig. 8). These two proteins corresponded to the P and V proteins, respectively. It had previously been determined that the P protein migrates at 70 kDa (Graves *et al.*, 1978). Rima *et al.* (1981) obtained evidence for in vivo production of a MV P-related protein (40 kDa) using partial protease digestion to indicate that it was not simply a P breakdown product. They also reported that this protein

comigrated with the MV M protein. In the current study, the 40 kDa protein also comigrated with the M protein. This may explain why the V protein had thus far gone undetected. Previously no strong signal at 40 kDa has been found in MV-infected cells after immunoprecipitation with antibodies directed against P (Bellini *et al.*, 1985), possibly because the paramyxovirus P protein carboxyl terminus is more immunogenic than the rest of the protein (Vidal *et al.*, 1988).

Further evidence that the two proteins immunoprecipitated by our sera were P and V was provided by differential metabolic in vivo and in vitro labeling with [^{35}S] methionine or [^{35}S] cysteine. The P protein has almost twice as many methionine residues as cysteine residues while the reverse is found true for the V protein. Indeed, experiments proved that the 70 kDa protein was preferentially labeled with methionine whereas the 40 kDa protein was preferentially labeled with cysteine (Fig. 9).

Gel migration of the proteins immunoprecipitated from MV-infected cells was compared to those immunoprecipitated from in vitro translation reactions. The in vitro synthesized P and V proteins were found to migrate slightly faster than those from MV-infected cells (Fig. 13). This could indicate

that different post translational modification occur in vivo and in vitro. The P protein is known to be phosphorylated and, since the V protein shares 231 of its 299 amino acids with the P protein, the possibility of phosphorylation of the V protein was examined. Immunoprecipitation of [^{32}P] orthophosphate-labeled proteins from MV-infected Vero cells indicated that the V protein was indeed highly phosphorylated (Fig. 15). Differential phosphorylation in vitro and in vivo of the V protein may explain differences in gel migration. This does not exclude the possibility that other modifications may distinguish the V protein synthesized in vivo.

Paterson and Lamb (1990) have reported that an additional protein, I (M_r 19,000), was produced by an additional type of RNA editing of the mumps virus P gene. In this case, four G residues were added to the transcript instead of the two which gave rise to the P message. In addition, Takeuchi *et al.* (1990) have reported that a mumps protein of M_r 24 kDa was immunoprecipitated with peptide-antisera raised against the P/V coterminal region. They postulated that this 24 kDa protein could arise from an mRNA in which 4 G residues were added. No evidence that a similar protein was produced by MV was found in the current study. In some instances, lower molecular weight proteins were indeed immunoprecipitated from

MV-infected cells using the peptide antisera. However, these proteins did not appear to be P/V coterminal proteins as in some instances they were also immunoprecipitated using PC20 antiserum which is directed against a carboxyl terminal region of P (Fig. 9). The nature of the these proteins was not determined.

The MV V protein was found to be diffusely distributed throughout the cytoplasm, with perinuclear accentuation, in MV-infected Vero cells (Fig. 16). In contrast, P localizes in a distinctly different pattern, that of small bright cytoplasmic inclusions (Fig. 16). Previous studies have shown that the P protein colocalizes with cytoplasmic inclusions which also contain viral nucleocapsids (Hogan *et al.*, 1983; Nakai *et al.*, 1969). Therefore, if the P protein can be assumed to be a nucleocapsid marker, the present study indicates that most MV V protein is not associated with nucleocapsids. This is supported by the observation that V did not colocalize with P in MV-infected cells that were treated with an antisera able to recognize both P and V since a mixture of both fluorescent patterns was seen (Fig. 16). However, these data do not rule out the possibility that some small amount of V protein may be associated with nucleocapsids.

The function of the V protein is still unknown. It is likely that it has an important biological role as its sequence is highly conserved among the paramyxoviruses. Others have suggested that the V protein may be a factor involved in RNA transcription or replication (or both), since the cysteine-rich region bears resemblance to a zinc finger domain, a motif that has been identified in nucleic acid binding regulatory proteins (Cattaneo *et al.*, 1989; Thomas *et al.*, 1988). An attempt was made to determine if the MV V protein could bind zinc using the protocol described by Schiff *et al.* (1988). By using carbonic anhydrase, a protein known to bind zinc, it was determined that a minimum of 100 ng of protein was required for the assay. Unfortunately not enough V protein could be generated from MV infected cells or from in vitro translation. For this, and other functional assays, it will be necessary to produce large amounts of purified V protein. This can be achieved by expression of the protein in a high level expression vector such as adenovirus or baculovirus.

CONCLUSION

The work presented in this thesis was designed to characterize the MV V protein in MV infected cells. Antisera directed against synthetic peptides were produced against regions of the V protein. Using these antisera in immunoprecipitation and immunofluorescence experiments it was possible to determine that (1) V protein was present in MV-infected cells, (2) V migrates as a 40 kDa polypeptide in infected cells, but somewhat faster when synthesized in vitro, (3) V is phosphorylated (4) V has a diffuse cytoplasmic localization with perinuclear accentuation - a pattern quite distinct from that of P, and (5) most V protein exists independent of virus nucleocapsids.

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