STRUCTURAL AND FUNCTIONAL ANALYSES OF VESICULAR STOMATITIS VIRUS P GENE PRODUCTS

by

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Abstract

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The growth of vesicular stomatitis virus requires two distinct RNA synthetic events: transcription of messenger RNA molecules, and replication of the viral genome RNA. This thesis reports the use of a panel of monoclonal antibodies directed against the viral phosphoprotein P in an attempt to assess the role of this protein in RNA synthesis. Using extracts derived from virus-infected cells, I show that several anti-P monoclonal antibodies have a specific inhibitory effect on genome RNA replication with minimal effects on mRNA transcription. I also show that the P protein to which one of these antibodies (6D11) is directed is not complexed with the N protein and that the amount of soluble P protein that binds to the 6D11 antibody in immunoprecipitation reactions can be increased by treating extracts with alkaline phosphatase. In addition, phosphatase treatment of infected cell extracts results in an increased level of genome RNA replication. These results suggest that a soluble sub-species of the P protein that functions in genome RNA replication exists in infected cells, and that this species of the P protein is not required for transcription. This thesis also reports the identification of two small basic proteins encoded by the P gene in an alternate reading frame. These proteins, which have not previously been shown to exist in VSV-Indiana, are called C and C' and are shown to be present in infected cells and to be associated with ribonucleoprotein complexes.

Acknowledgments

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LITERATURE REVIEW

Introduction

Vesicular stomatitis virus is a rhabdovirus that contains a cell-derived lipid envelope and a non-segmented, negative sense, single stranded RNA genome. The ease with which the virus can be grown to high titers in cell culture and purified has led to extensive research and the establishment of VSV as a prototype for other negative sense RNA viruses. During the past few decades, a great deal of information has been revealed about the virus, its gene products and its method of replication but some basic questions remain to be answered. In particular, the precise identity and functions of all of the proteins required for optimal RNA synthesis and the regulation of the switch between mRNA and genomic RNA synthesis remain areas of intense investigation.

Virus Structure

The genetic information of the Indiana serotype of VSV is contained in a single strand of RNA that has been entirely cloned into cDNA and sequenced. The genome is 11,161 nucleotides long (Rose and Schubert, 1987), is not infectious as unencapsidated RNA (Huang and Wagner, 1966), and is complementary to mRNA (negative sense). The virus carries out two separate types of RNA synthesis in infected cells: the transcription of the template to produce the leader and mRNAs; and the synthesis of full-length positive and negative sense copies of the genome in what is called replicative RNA synthesis (Emerson, 1987; Wertz, 1987). There are five major viral proteins synthesized by the virus in infected cells and all are present in the virus particle (Wagner, 1975).

In addition, three small non-structural proteins have been described that are present at low levels only in infected cells (Herman, 1986; Spiropoulou and Nichol, 1993). The viral envelope is composed of a lipid bilayer acquired by budding through the cellular plasma membrane and the viral glycoprotein (G) is found embedded in this envelope (Cartwright *et al.*, 1972). The matrix protein (M) lines the inner surface of the envelope and maintains contact with the ribonucleoprotein (RNP) complex (Dubovi and Wagner, 1977; Zakowski and Wagner, 1980). The viral RNP is infectious only at very high doses (10^5 above that of intact virus) and contains the remaining three viral proteins (Szilagyi and Uryvayev, 1973; Emerson and Yu, 1975). Approximately 1250 copies of the nucleoprotein (N) can be seen tightly complexed with the genomic RNA under high resolution scanning transmission electron microscopy (STEM: Thomas *et al.*, 1985) and the resultant nucleocapsid structure is highly resistant to nucleases (Emerson and Wagner, 1973). Also in the RNP is the transcriptase complex comprised of the large polymerase protein (L) and the phosphoprotein (P, formerly NS), which are estimated to be present at 50 and 466 copies respectively by STEM (Thomas *et al.*, 1985).

VSV RNA Synthesis

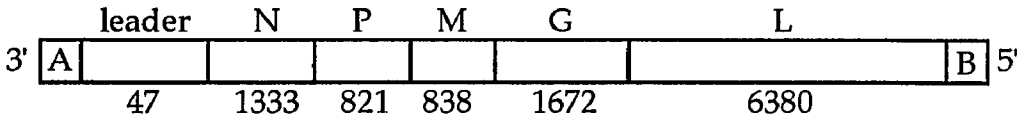
Transcription. Since VSV has a negative sense RNA genome which cannot itself function as mRNA, it requires an RNA-dependent RNA polymerase to carry out transcription of the genome to produce virus mRNA. This polymerase is found packaged into the virus particle and, upon infection of a susceptible cell, it commences the synthesis of mRNA. VSV was the first negative sense virus shown to have its own RNA polymerase (Baltimore *et al.* 1970) and the transcription of mRNA by VSV has been extensively studied.

Transcription of the VSV genome by the viral transcriptase results in the synthesis of six discrete RNA products. These are the uncapped, untranslated 47 nucleotide long leader RNA and five capped, polyadenylated mRNAs corresponding to the five VSV genes (figure 1; Banerjee and Barik, 1992). The leader gene begins at the exact 3' end of the genome and there are intergenic sequences consisting of three bases at the leader-N gene junction and two bases between each of the other genes (Rose and Schubert, 1987). The 5' end of the viral genome contains 59 non-transcribed bases after the L gene and, in all, 99.4% of the genomic RNA of VSV is transcribed into complementary RNA during transcription (Emerson, 1987). None of these RNA products are normally found encapsidated by N protein although the leader RNAs have been found to be encapsidated very late in infection (Blumberg and Kolakofsky, 1981). As shown in figure 1, the gene order is 3'-leader-N-P-M-G-L-5' and UV-inactivation studies showed that transcription proceeds in a polar, sequential manner from the 3' to the 5' end (Abraham and Banerjee, 1976; Ball and White, 1976; Iverson and Rose, 1981). The mRNAs of different genes are produced in different amounts (Villareal *et al.*, 1976) corresponding to the gene order due to the attenuation of transcription at the intergenic regions of the template (Iverson and Rose, 1981). This partial termination of transcription results in a 29-33% decrease in transcription from one gene to the one 5' to it. Thus the N protein is produced in greater amounts while the L protein is produced in the lowest amount. The transcriptase of VSV has an estimated error rate of one out of every one thousand bases transcribed (Pringle, 1982) and, since no correction mechanisms exist, this results in a high mutation rate (Schubert *et al.*, 1984; Steinhauer *et al.*, 1989).

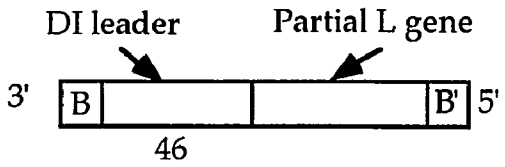
The availability of well defined *in vitro* systems for the study of VSV transcription has greatly aided the study of the protein requirements and mechanisms of mRNA synthesis. Early research demonstrated that disrupted

Figure 1. Schematic representations of the VSV wild type and MS-T defective-interfering particle genomes. The gene order is indicated above each gene while the length of each transcript is shown below the genome. The letters at the ends of the genomes represent the 3' and 5' end sequences. Both genomes are shown in the negative sense.

Wild-type VSV genome



DI genome



virions and isolated RNP complexes from purified virus were able to transcribe viral mRNAs when ribonucleoside triphosphates were provided in appropriate buffer conditions (Baltimore *et al.*, 1970; Bishop and Roy, 1972). High salt treatment can dissociate the RNP into soluble L/P and N-RNA fractions which can then be reassociated to reconstitute the transcriptase activity (Emerson and Wagner, 1972; Emerson and Yu, 1975; Naito and Ishihama, 1976). Early studies utilizing these systems showed that only the three proteins of the RNP complex are required for mRNA synthesis.

There have been several models of VSV transcription developed but most current data support the stop-start model of transcription. In this model, the transcriptase initiates only at the 3' end of the genome, transcribes the RNA, then terminates and reinitiates at the next downstream gene (Banerjee and Barik, 1992). Methylation and capping of mRNA species are thought to occur during the elongation step (Chanda and Banerjee, 1981) while polyadenylation of mRNA occurs just before termination. As mentioned above, the single polymerase entry site and the attenuation that occurs at the intergenic junctions are consistent with the sequential, attenuated transcription that is observed during infection. The numerous transcriptases packaged into virus particles are able to resume transcription after uncoating of the virus (Emerson and Wagner, 1972; Chanda and Banerjee, 1981), resulting in simultaneous transcription of several mRNAs.

Genome replication. The mechanism of replication of the genome RNA is less well understood than that of transcription. The RNA products of replication are full-length copies of the genome that are neither methylated nor polyadenylated. These RNAs must be encapsidated by the N protein in order to function as templates themselves. Protein synthesis is required for replication to begin but the precise viral and possibly cellular proteins required for optimal

replication have not been definitively established (Perlman and Huang, 1973; Wertz and Levine, 1973; Wertz, 1987).

In contrast to the ease in which transcription may be reconstituted *in vitro*, efforts to develop a system for assaying genomic RNA replication using pure components have not been successful. Several systems utilizing infected cell extracts (Condra and Lazzarini, 1980; Hill and Summers, 1982; Peluso and Moyer, 1983) or coupled translation/replication systems (Patton *et al.*, 1983) have been used but the heterogeneous nature of the components of these systems has made attempts to assay the exact mechanism of replication and the function of specific viral proteins or cellular cofactors difficult. In our laboratory, we employ the method of Peluso and Moyer (1983) in which infected cells are permeabilized with lysolecithin and cytoplasmic extracts are prepared. This system supports transcription, replication, and encapsidation and thus allows the study of all of these processes. RNA synthesis may be assayed directly in the infected cell extracts or they can be fractionated into soluble protein and nucleocapsid fractions and then combined to reconstitute RNA synthesis.

The use of defective interfering (DI) particles has made the study of genome replication much easier. DI particles contain only part of the genomic RNA and thus depend on a non-defective "helper" virus in order to replicate (Blumberg and Kolakofsky, 1983). The replication of DI particles takes place at the expense of the replication of the complete genomes and in this way DI particles interfere with the normal infection. The DI particles of VSV are smaller than wild type virus and can be purified and analyzed from a mixed infection. DI particles are especially useful in studying the replication of genomic RNA since an *in vitro* system containing only DI RNP complexes cannot express any viral proteins. Therefore, any replication which occurs is the result of the proteins which are added to the system. The Mudd-Summers DI-T particle (MS-

T) which contains a 3' end complementary to the 5' end of the wild-type VSV genome, the end of the L gene, and the 5' terminus of the genome RNA (figure 1) is widely used in studies of VSV replication (Peluso and Moyer, 1984, 1988; Peluso, 1988).

Since protein synthesis is required for replication and all replication products must be encapsidated by N, many groups postulated that a continuous supply of N protein is needed (Kingsbury, 1974; Leppert *et al.*, 1979; Simonsen *et al.*, 1979a; Blumberg *et al.*, 1981; Arnheiter *et al.*, 1985). Studies correlating the level of N protein in cells with the type of RNA synthesis occurring led to a model of replication in which the nucleocapsid protein is primarily responsible for the regulation of RNA synthesis (Blumberg *et al.*, 1981). According to this model, if no N protein is available for binding to a nucleocapsid assembly sequence within the nascent leader RNA, the transcriptase complex would terminate after transcribing the leader RNA and then commence mRNA synthesis. If there is a sufficient pool of N protein available, it would bind to the leader RNA and promote read-through of the termination signal and start the encapsidation of the nucleocapsid structure. This model has been supported by findings from several groups (De *et al.*, 1982; Hill and Summers, 1982) but recent studies have made it clear that the regulation of RNA synthesis may involve other proteins . In particular, studies of the role of phosphorylation, which is discussed later in this chapter, have led to revisions in the model of how RNA synthesis is regulated in VSV infected cells.

A system in which synthetic viral genome RNA produced from plasmids *in vitro* could be encapsidated into RNPs to form a functional template would be very useful in investigating the mechanism of VSV RNA synthesis. Such a system would allow the manipulation of the genome using the techniques of molecular biology and developing such a system has been a major focus in VSV

research. Early successes were achieved by using vectors expressing the three viral RNP proteins (L, P, and N) inside cells to promote replication and encapsidation of defective interfering particle genomes (Pattnaik and Wertz, 1990). A subsequent study in which all five viral genes were expressed showed that production of viruses could be supported in this system (Pattnaik and Wertz, 1991). More recently, several groups have reported successes in achieving replication of synthetic VSV RNAs introduced by transfection of plasmids into cells that are expressing the viral N, P, and L genes (Stillman *et al.*, 1995; Lawson *et al.*, 1995). In all of these systems, the cells are first infected with a recombinant vaccinia virus which expresses the T7 polymerase. Then, plasmids carrying genes for VSV proteins under the control of the T7 promoter are transfected into cells along with a separate plasmid encoding a synthetic VSV genome, also under T7 polymerase control. The T7 polymerase transcribes an RNA molecule which resembles the authentic VSV genome and also mRNAs which are translated to give rise to VSV proteins. If the N, P, and L proteins are coexpressed in the same cell, encapsidation and replication of the synthetic genome takes place. If the VSV M and G proteins are also expressed, the synthetic genomes, as RNPs, can be packaged into virus particles which are capable of infecting other cells (Pattnaik *et al.*, 1992). This strategy has culminated in the recent report of the production of a recombinant VSV, containing a full-length viral genome, entirely from cloned DNA (Lawson *et al.*, 1995). Although these systems are extremely inefficient at encapsidating synthetic genomes, once the initial encapsidation takes place the synthetic viral RNP can be transcribed, replicated, and packaged into virus particles. Once a synthetic virus is made, the infection of cells with the vaccinia virus is no longer necessary and the study of VSV processes can take place in a more isolated, natural situation. The ability to introduce specific

mutations into the genes of recombinant VSV viruses provides an unprecedented opportunity to study the life cycle of this virus.

The Role of Viral Proteins in VSV RNA Synthesis

The L protein. The large polymerase (L) protein of VSV has a molecular weight of 241 kd, has been entirely cloned, sequenced and expressed in bacteria (Schubert *et al.*, 1985) and is believed to carry out most of the enzymatic functions of the polymerase complex. The polyadenylation and mRNA methyl transferase activities have been mapped to the L protein by indirect evidence from *in vitro* reconstitution experiments and studies with temperature-sensitive mutants (Hutchinson *et al.*, 1974; Hunt *et al.*, 1984; Hercyk *et al.*, 1988) and host-range mutants (Horikami and Moyer, 1982). Several groups have shown a kinase activity to be associated with the L protein (Sanchez *et al.*, 1985; Hammond *et al.*, 1992a, 1992b) but more recent data indicates that this activity does not actually reside on the L protein (Massey *et al.*, 1990; Gao and Lenard, 1995). The L protein alone cannot bind to nucleocapsids and depends on the P protein to direct it to the template (Mellon and Emerson, 1978). Additional evidence that the L protein is the polymerase came from De and Banerjee (1985) who showed that the requirement for L in a reconstitution assay was catalytic and increasing amounts of L protein added in this system made no difference once the template was saturated. Approximately 50 molecules of L are present in the nucleocapsid structure (Thomas *et al.*, 1985) and immunogold labelling and electron microscopy indicate that these proteins are uniformly distributed along the template (Harmon *et al.*, 1985). The L protein may be disassociated from the nucleocapsid template in high salt conditions and has been shown to readily dissociate and reassociate with added template in *in vitro* assays (De and

Banerjee, 1984; Giachetti and Holland, 1989; Helfman and Perrault, 1989). High levels of L protein expressed *in vitro* leads to an arrest of transcription (Meier *et al.*, 1987) probably due to a relative lack of the P protein to complete the transcriptase complex.

The N protein. The nucleocapsid protein is a 49 kd protein that is required for transcription and replication and is a major component of the ribonucleoprotein complex (Simonsen *et al.*, 1979b). The N protein tightly encapsidates the viral RNA and in this way keeps the template in an extended conformation that is conducive to transcription. The N protein may also interact with the P protein to package the polymerase complex on the template. The requirement for N protein is absolute since unencapsidated RNA has never been shown to be functional as a template for the viral transcriptase. Encapsidation also prevents the association of the complementary positive and negative sense RNA strands that would probably block RNA synthesis. Evidence for the function of the N protein in replication has also come from the study of the polR mutants. These mutants exhibit an altered utilization of ATP (Helfman and Perrault, 1988) and the polymerase of these mutants is able to read through the termination sites on the genome as the result of an altered N protein (Perrault *et al.*, 1983; Perrault and McLear, 1984). As mentioned above, the level of N protein, complexed as N:P, is believed to have a role in regulating the balance between transcription and replication by controlling the encapsidation of the nascent RNA (Peluso and Moyer, 1984, 1988; Peluso, 1988).

The P protein. The 29 kd phosphoprotein P of VSV is the third known component of the ribonucleoprotein complex. P is required for both types of RNA synthesis but has not been shown to have an enzymatic function in these

processes. Early studies showed that the P protein recognizes the initiation sequence for transcription at nucleotides 16 through 30 in the leader gene and therefore acts as an initiation factor for the L protein (Keene *et al.*, 1981). The L protein cannot bind to the N-RNA template without the P protein and P is thought to direct the association of L with the template (Mellon and Emerson, 1978; Thornton *et al.*, 1984). Examination of the nucleotide sequence of the P proteins of the New Jersey and Indiana serotypes of VSV have revealed that the P genes are not highly conserved at the nucleotide (41%) or the amino acid (32%) levels (Gill and Banerjee, 1985). However, there is a significant similarity in the structure of the P proteins with regard to hydrophobic regions, potential phosphorylation sites , and the C-terminus. These data and studies utilizing deletion mutants of the P protein in *in vitro* transcription systems have identified several important domains within the P protein. The N-terminal one third of the protein (domain I) contains many hydrophilic residues and potential phosphorylation sites (Bell and Previc, 1985; Hsu and Kingsbury, 1985). This domain is also referred to as the "acid blob" in reference to the many acidic residues and the similarity to several transcription factors (Sigler, 1988). Emerson and Schubert (1987) used deletion mutants of P to show that this domain contains the L-binding activity. However, domain I is not essential for transcriptional activity in cell free systems and can be replaced by the acidic domain of tubulin (Chattopadhyay and Banerjee, 1988). In addition, a fusion protein consisting of the full-length β tubulin protein fused to the C-terminal 20% of the P protein (domains II and III) is functional in transcription (Chattopadhyay and Banerjee, 1988). The function of domain I can also be provided in *trans* by tubulin (Chattopadhyay and Banerjee, 1987b) along with a protein carrying only domains II and III. Thus the acidic domain of tubulin is similar enough to that of P protein to be a functional substitute, even though it differs greatly in both

sequence and size. Domain II is a 34 amino acid region from residue 213-247 near the C-terminus that is required for transcriptional activity (Gill *et al.*, 1986). Domain II is proposed to bind to the N-RNA template (Emerson and Schubert, 1987; Paul *et al.*, 1988). The third domain is the highly conserved 21 amino acids at the carboxy terminus of the P protein. This basic domain was shown by deletion mapping be dispensable for transcription but also involved in the binding to the N-RNA template (Chattopadhyay and Banerjee, 1987a). Interestingly, a synthetic peptide corresponding to domain III inhibits transcription when added to an *in vitro* system (Yamashita and Kawai, 1990).

A function of the P protein in replication was suggested by Peluso and Moyer (1984, 1988). Several complexes of the N and P proteins were found in infected cells and it was shown that the 1:1 complex between these two proteins was crucial for the ability of soluble protein to support replication by isolated RNP templates (Peluso and Moyer, 1984, 1988; Peluso, 1988). The P protein which binds to N functions as a chaperone to prevent free N protein from self-assembling into inactive complexes. The N:P complexes bind to newly synthesized replication products during nucleocapsid assembly. In this process, the complex is dissociated and the P protein is released into the cytoplasm while the N protein becomes bound to the nucleocapsids (Peluso and Moyer, 1988). Without P present, the N protein tends to aggregate into large multimeric complexes which are significantly less active in supporting genome replication (Sprague *et al.*, 1983). A second known function of the P protein in replication is as part of the replicase complex along with the L protein. It has not been conclusively determined whether the P protein of the polymerase complex which carries out replication differs from that which carries out transcription but there are many different forms of P present in infected cells which may function in different ways.

The Role of Phosphorylation in Regulating VSV RNA Synthesis

The phosphorylation of proteins is a common post-translational modification that has been implicated in the regulation of many cellular functions (Nishizuka, 1986). There are many examples of viral proteins which are phosphorylated including the P and M proteins of VSV. Amino acid sequence analysis of the P protein shows that there are 33 potential phosphorylation sites at serine and threonine residues and phosphopeptide mapping experiments have shown that at least 21 of these sites can indeed be found phosphorylated in infected cells (Hsu *et al.*, 1982). The P protein found in viruses contains an average of 4.88 phosphates (Gao and Lenard, 1995). No phosphorylation of tyrosine residues has ever been detected in the VSV P proteins.

Early studies showed that two forms of the P protein could be separated by either SDS-PAGE (Clinton *et al.*, 1978) or DEAE column chromatography (Kingsford and Emerson, 1980). These two isolated pools of P molecules showed a different overall phosphorylation content but both were heterogeneous. Clinton *et al.* (1978) reported that the less highly phosphorylated P molecules (P1) were preferentially associated with RNPs from virions and infected cells but Hsu and Kingsbury (1980) reported the opposite. Clinton *et al.* (1979) showed that the P protein could be acted upon by cellular phosphatases and virion associated kinase activities. These two activities were able to interconvert the two forms of P that were separated by SDS-PAGE (Clinton *et al.*, 1979). Several groups (Kingsford and Emerson, 1980; Masters and Banerjee, 1986) examined the activity of the two classes of P in an *in vitro* transcription system and found that only the more highly phosphorylated class (P2) was active in supporting transcription.

However, when both classes of P were included in the reactions, the level of transcription increased, indicating that P1 and P2 may have separate functions but may be interconvertible and act synergistically in transcription (Kingsford and Emerson, 1980).

Two main kinase activities which phosphorylate the P protein have been identified in VSV infected cells. One kinase activity, which is the cellular enzyme casein kinase-II (CK-II), phosphorylates sites in the N-terminal half of the P protein and can be inhibited by addition of heparin (Barik and Banerjee, 1992a,1992b). Unphosphorylated recombinant P protein expressed in bacteria has no activity in transcriptional assays (Barik and Banerjee, 1992a, 1992b) but can be activated by treatment of purified protein by CK-II. The requirement for casein kinase-II activity in VSV infections is indicated by a study that shows that VSV does not replicate well in cells deficient in this enzyme (Sleat *et al.*, 1992). Gao and Lenard (1995) recently determined that phosphorylation of P protein by CK-II at either of two sites in the amino terminal half of the protein was sufficient for activity. Substitution of the charged amino acid aspartate at either or both of the sites (serine 60 and threonine 62) was able to activate the protein to equal levels as phosphorylation. Thus, it is the net negative charge of the amino acid group, whether intrinsic to the amino acid residue or imparted by phosphorylation, that is important for activation. Of course, phosphorylation offers the possibility of regulating the activity of P so that it could be turned on or off during the life cycle of the virus. The idea that the negative charge of domain I of the P protein is responsible for regulating its activity is supported by data from Takacs *et al.* (1991). This group was investigating whether domain I of VSV-New Jersey P protein could activate transcription when fused to the DNA binding domain of the yeast transactivator GAL4. The wild type domain I of P was unable to activate transcription but when the net charge was reduced by

replacing basic amino acids with negatively charged ones, transactivation occurred.

The second kinase activity in VSV infected cells is found tightly associated with the L protein. This activity phosphorylates residues in the C-terminal half of the protein and can be inhibited by the ATP affinity analogue 5'-*p*-fluorosulfonylbenzoyl adenosine (FSBA) and the protease inhibitor Nα-p-tosyl-l-lysine chloro-methyl ketone (TLCK; Beckes and Perrault, 1992). The addition of protamine also inhibits the L associated kinase activity while it stimulates the CK II activity (Barik and Banerjee, 1992a,b). The relevance of phosphorylation by the L associated kinase has recently come into question. Although some reports maintain that phosphorylation of P by this activity is necessary for transcriptional activation (Barik and Banerjee, 1992a), recent reports indicate that this phosphorylation may not be necessary for transcription (Beckes and Perrault, 1992; Gao and Lenard, 1995). The L associated kinase activity can be removed from the L protein by gel filtration with no effect on transcriptional activity of the CK II phosphorylated P protein in reconstituted assays. Of course, since only transcription was examined in these studies, it is possible that the phosphorylation by this kinase is important for some other function of the P protein.

The role of phosphorylation in the replication of genomic RNA has not received as much study as that of transcription because of the difficulties of directly studying replication. It has long been recognized that synthesis of RNA by VSV involves a specific requirement for ATP at levels above that of the other nucleotides (Testa and Banerjee, 1979) but whether this requirement reflects a need for phosphorylation or some other process is not known (Beckes *et al.*, 1987). More recently, it has been shown that phosphorylation regulates genome RNA synthesis as well as transcription. When the serine/ threonine phosphatase

inhibitor okadaic acid is used to treat cells infected with VSV, genome replication is specifically inhibited (Chang *et al.*, 1994). Treating cells with okadaic acid results in the accumulation of highly phosphorylated P proteins; and, while no direct connection between the two observations has been demonstrated, it is suggested that highly phosphorylated P protein is functional in transcription while underphosphorylated P protein functions in replication. Hyperphosphorylation of the P protein would therefore result in loss of replicative activity without affecting transcriptional activity.

Alternate Coding Strategies of VSV

It has been known for many years that negative strand RNA viruses use a variety of mechanisms to increase the coding potential of their genomes. Two types of strategies can be employed to produce more than one protein from a single gene. The mRNAs transcribed from the gene can be spliced or modified by RNA editing to give rise to multiple mRNAs. Also, a single mRNA may be multicistronic and give rise to more than one protein through alternate translation of overlapping reading frames. The paramyxoviruses have been particularly well studied with respect to their use of overlapping reading frames in their P genes. Most members of this family contain additional reading frames in their P genes which give rise to additional protein products (Curran *et al.*, 1991). The P gene of Sendai virus expresses up to 6 proteins from additional reading frames and an additional two proteins from edited mRNAs. The functions of most of the P gene products, other than P itself, are unknown although it is thought that some of them function in RNA synthesis (Curran *et al.*, 1991; 1992).

Until recently, it has been thought that the expression of proteins from the P genes of rhabdoviruses like VSV, although close relatives of the paramyxoviruses, was straightforward and produced a single product, the phosphoprotein P. However, recent reports have contradicted this view. Analysis of the P gene of VSV shows that there are multiple potential open reading frames (ORFs) in both the New Jersey and Indiana serotypes (figure 2). The large ORF which spans almost the entire gene and the small ORF which is at the beginning of the gene are conserved between various isolates of these two serotypes (Bilsel *et al.*, 1990). Despite the knowledge that the P gene of VSV has the potential to encode proteins in addition to P itself, only two reports have appeared regarding additional protein products of these ORFs.

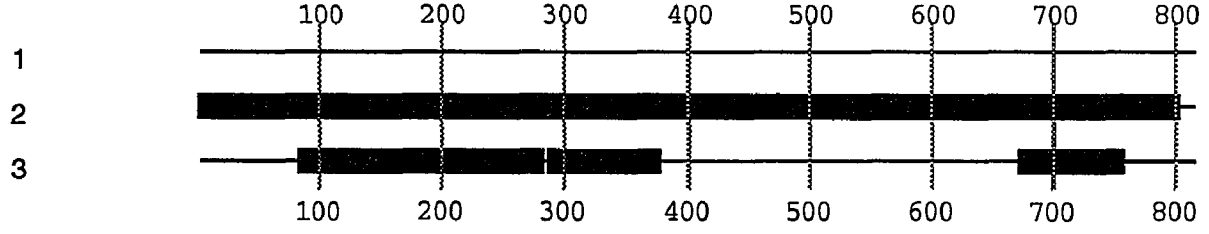
Herman (1986) reported the isolation of a second protein from *in vitro* translation of P gene mRNAs from VSV-Indiana. Hybrid arrest experiments proved that the second protein was encoded by the carboxy terminal one third of the P gene and was the result of an internal initiation event at a downstream AUG in the same ORF as the P protein. Translation from the internal start site resulted in the synthesis of a small protein which is identical to the carboxy terminal 61 amino acids of the P protein. This second protein, called 7k due to its mobility on SDS-PAGE gels, was also found in cytoplasmic extracts of VSV-infected cells and thus was not an artifact of the *in vitro* translation system. No further data has ever been reported about this protein and, indeed, it seems that this initial report has been disregarded in most reviews of VSV gene products.

Since the sequence encoding domain I of the P protein can be functionally replaced with tubulin, it is not expected that this region would be conserved during virus evolution. In fact, sequence comparisons among eighteen isolates of the New Jersey serotype of VSV reveals that this region is more conserved than either domain II or III (Bilsel *et al.*, 1990). In particular, the variability in the third

Figure 2. Open reading frame analysis of the VSV-Indiana and VSV-New Jersey P genes. ORF analysis was performed using MacVector™ (Eastman Kodak). The reading frame is shown to the left of each map and ORFs are denoted by black rectangles.

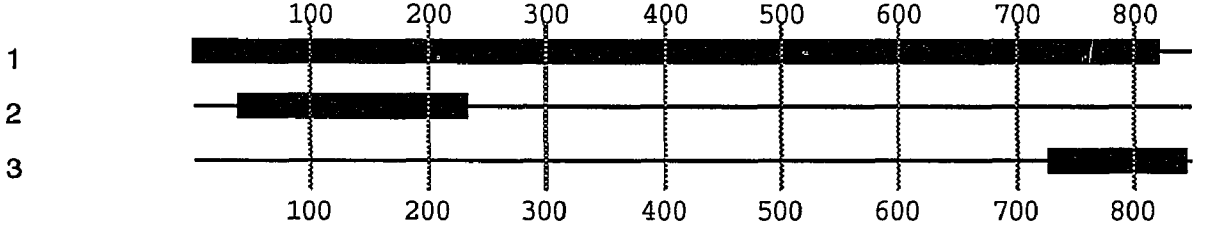
VSV-Indiana

Start/Stop Method: AA span >= 25
Genetic Code: universal



VSV-New Jersey

Start/Stop Method: AA span >= 25
Genetic Code: universal



base position is significantly reduced between bases 50 and 250 of the region encoding the P protein. This area coincides with the second ORF of the P gene (figure 2; Spiropoulou and Nichol, 1993). The third base position of the P protein reading frame corresponds to the second position of ORF2. These observations led to a search for a protein which could be encoded by this ORF. A polyclonal antisera preparation was obtained after immunization of a rabbit with a peptide representing a putative immunogenic region of the protein encoded by the second ORF. The antisera identified two proteins, named C' and C after the anlaogous proteins in paramyxoviruses, which were found by immunoprecipitations of the cytoplasm of infected cells. These proteins were determined to be C-terminally nested, highly basic proteins produced by alternate initiation of translation at two AUG codons within ORF2 of the P mRNA. The smaller protein C was found in higher levels than the C' protein, perhaps due to the optimal context of the sequences surrounding the initiation codon (Kozak, 1989; Spiropoulou and Nichol, 1993). The larger protein C' is identical to C except that it has 12 additional N-terminal amino acids . Both proteins are present in the cytoplasm of infected cells but are not detectable in purified virus. No direct evidence for the function of the C proteins of VSV has yet been presented. Since these proteins have not been found in virus particles, they could not have a function in at least the earliest stages of viral transcription.

MATERIALS AND METHODS

Cells ,Viruses, and Antibodies

BHK cells. The baby hamster kidney (BHK) cell line was used for all cell culture in this study. The cells were grown in complete medium (CM) containing minimal essential media (MEM, Gibco, Grand Island, New York) supplemented with 10% fetal calf serum, 4 mM L-glutamine, 1 mM pyruvate, 22 mM glucose, 100 units/ml penicillin and 100 µg/ml streptomycin. After the cell cultures grew to confluency, the cells were detached from the tissue culture flasks using a trypsin-EDTA solution. The cells were pelletted, suspended in fresh medium, and reseeded into new tissue culture plates or flasks. All cell cultures were incubated at 37 °C in a humidified chamber containing 5% CO_2.

Viruses. Stock preparations of the heat resistant (HR) strain of VSV-Indiana and the Mudd-Summers DI-T (MS-T) defective interfering particles were prepared in cell culture (Peluso and Moyer, 1983).

Antibodies. The polyclonal antibody against VSV-Indiana was purchased from Lee Biomolecular Research Inc. (San Diego, California). The panel of 13 monoclonal antibodies against the viral P protein were kindly provided by Dr. Susan Emerson (NIAID, Bethesda, Maryland) while the anti-N antibody was a gift from Dr. Douglas Lyles.

Infection of Cells

Subconfluent monolayers of BHK cells (60-80% confluent) in tissue culture plates were infected with virus at a multiplicity of infection (MOI) of 10 with or without a stock suspension of the MS-T DI particle (5 µl per 10^7 cells; Peluso and Moyer, 1983). The virus was adsorbed to the cells for one hour at 37 °C and plaquing media (PM: MEM supplemented with 4 mM L-glutamine, 1 mM pyruvate, 22 mM glucose, 100 units/ml penicillin, 100 µg/ml streptomycin, 25 mM Hepes pH 7.4, and 10 mM TES pH 7.4) was added. The infected cells were incubated in a humidified CO_2 incubator at 37 °C until radiolabelling or harvesting for cell extracts.

Isotopic Labelling of VSV Proteins.

Viral specific proteins were labelled using ^{35}S-methionine. Virus-infected cell cultures were incubated in methionine-free MEM at two hours post infection (p.i.). After 30 minutes, fresh methionine-free medium (Gibco) containing L-^{35}S-methionine (200 µci/ml, NEN-Dupont) was added and the cultures were incubated for 90 minutes. After labelling, the cells were placed on ice, washed with ice-cold buffer A (0.15 M sucrose; 30 mM Hepes, pH to 7.4 with KOH; 33 mM NH_4Cl; 7 mM KCl; 4.5 mM Mg acetate), and permeabilized for one minute in L-a-lysophosphatidylcholine, palmitoyl (lysolecithin, 125 µg/ml, Sigma). The cells were then rinsed with cold buffer A and scraped into a small volume of scraping salts (100 mM Hepes, pH 8.25; 100 mM NH_4Cl; 7 mM KCl; 4.5 mM $MgCl_2$; 1 mM dithiothreitol). The cells were disrupted by pipetting 10 times and the cell nuclei and debris were removed by centrifugation at 4000 x g for five minutes. The supernatant was then centrifuged at 50,000 rpm for 75 minutes in a

Beckman SW55 rotor. The supernatant (soluble protein) was removed and used for immunoprecipitations.

Viral phosphoproteins were labelled using radiolabelled phosphoric acid. The cell cultures were incubated overnight in phosphate-free medium (Gibco) prior to labelling. These cells were infected with virus and labelled with phosphate-free media containing radiolabelled phosphoric acid ($H_3{}^{32}PO_4$, NEN-Dupont) at a concentration of 400 µci/ml from two to four hours after infection.. Soluble protein extracts of the cells were prepared as described above.

Alternatively, when analysis of protein-protein interactions was not needed, the labelled cells were lysed directly in RIPA buffer containing: 10 mM Tris-HCl, ph 7.4; 150 mM NaCl; 1% deoxycholate; 1% Triton X-100; and 0.1% sodium dodecyl sulfate. The DNA in the lysate was sheared by passage several times through a small gauge needle and the lysate was clarified by centrifuging at 30,000 rpm for 45 minutes in a Beckman SW-55 rotor. The supernatant was then stored at -85 °C until use in immunoprecipitations.

Immunoprecipitation of Radiolabelled Viral Proteins

Viral proteins were isolated by immunoprecipitation with both polyclonal and monoclonal antisera. For immunoprecipitation under non-denaturing conditions, a solution of NET (0.15 M NaCl, 5 mM EDTA, 50 mM Tris pH 7.4) with 1% NP40 was added to samples of radiolabelled soluble proteins to a final concentration of 0.5% NP40. The appropriate antibodies were added to each sample, the samples were mixed and left overnight on ice. The next day, 30 µl of a 50% suspension of streptococcal protein G sepharose (Sigma) was added to each sample and each tube was rotated at 4 °C for 30 minutes. The sepharose beads, with the immune complexes, were then pelletted at 9000 x g for two

minutes and the supernatants were discarded. The beads were washed five times in ice-cold NET/0.5% NP40, or in RIPA for the RIPA lysates. After the final wash, the beads were resuspended in 40 µl of SDS-PAGE lysis buffer (4% SDS, 40% glycerol, 3% dithiothreitol, 100 mM Tris pH 6.8) and boiled for three minutes to release the viral proteins. The samples were then analysed by SDS-PAGE.

In vitro Translated Proteins

The ^{35}S-labelled proteins from in vitro translation of mRNAs carrying the P gene or the C'/C gene in rabbit reticulocyte lysates were kindly provided by Martin Lock.

Electrophoretic Analysis of Radiolabelled Viral Proteins

Gel electrophoresis. Radiolabelled viral proteins were resolved by a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) system described by Beckes and Perrault (1991). Briefly, the separating gels were composed of 10% acrylamide (33.5%:0.3%, acrylamide:bis), 0.375 M Tris pH 9.1, 0.1% SDS, 0.075% ammonium persulfate (APS), and 0.05% TEMED. The stacking gels were 3.9% acrylamide (30%:0.44%, acrylamide:bis), 0.125 M Tris pH 6.8, 0.1% SDS, 0.03% APS, 0.1% TEMED. The gels were usually electrophoresed overnight at 40 volts.

Analysis of low molecular weight proteins. Low molecular weight proteins were analyzed using tricine-SDS-PAGE since this method provides superior resolution of small proteins. The separating gels were composed of 16.5%

acrylamide and were run essentially as described by Schagger and Jagow (1987). Alternatively, 18% glycine-SDS-PAGE gels were used and run according to protocols established by Laemmli (1970).

Isoelectric focusing Radiolabelled viral proteins were also analysed by isoelectric focusing in a mini-tube gel apparatus (BioRad Min-PROTEAN II, Richmond, California). The immunoprecipitation of viral proteins was performed as described above except that, after the final wash of the protein G-sepharose beads, the immune complexes were released by resuspending the beads in a small volume (15 µl) of IEF sample buffer (9.5 M urea; 2% Triton X-100; 5% ß-mercaptoethanol; 1.5% Pharmalyte pH 4-6, Pharmacia, Uppsala, Sweden; 0.5% Pharmalyte pH 6-8) and incubating at room temperature for 15 minutes . The sepharose beads were spun down at high speed in a microfuge and the supernatant was loaded onto a 0.75 mm x 5 cm tube gel containing 9.2 M urea, 4% acrylamide (acrylamide:bis, 30%:0.4%), 20% Triton X-100, 1.5% Pharmalyte pH 4-6 (Pharmacia, Uppsala, Sweden), 0.5% Pharmalyte pH 6-8, 0.01% ammonium persulfate, and 0.1% TEMED. The samples were electrofocused for 4 hours at 750 volts using an upper chamber buffer of 20 mM NaOH and a lower chamber buffer of 10 mM H_3PO_4. After electrofocusing, the tube gels were removed from the capillary tubes and boiled for three minutes in equilibration buffer (0.0625 M Tris HCl, pH 6.8; 2.3% SDS; 5% ß-mercaptoethanol, 10% glycerol, 0.00125% bromophenol blue). The tube gels were then placed horizontally into wells in separate 10% SDS-PAGE gels and electrophoresed as described above. The gels were processed and autoradiographed as described below.

Peptide mapping by limited proteolysis. Structural analyses of viral proteins was accomplished using a peptide mapping procedure similar to that of Cleveland *et al.* (1977). Radiolabelled VSV proteins obtained by immunoprecipitation were resolved by SDS-PAGE and detected by autoradiography. The gel pieces containing the viral proteins of interest were excised and placed into tubes containing an enzyme solution with 2 μg of endoproteinase glu-C (Sigma Chemical) per ml and 10% glycerol. The gel pieces were left to digest overnight at room temperature and then loaded into the wells of a 17.5% acrylamide, 0.45% bis-acrylamide gel along with the enzyme solution. A tracking dye solution containing 0.1% bromophenol blue (Bio Rad, Richmond, California) was also added to each well and the gel was electrophoresed to completion at 100 volts constant voltage. The gels were processed as described above and the peptide products were visualized by autoradiography.

Processing and fluorography of SDS-PAGE gels. After electrophoresis was complete, the separating gel was removed from the gel apparatus and placed in a staining solution (0.1% coomassie brilliant blue in 50% methanol, 7% acetic acid) for one hour. The gel was then destained for one hour in a 20% methanol, 7% acetic acid solution. After destaining, gels containing ^{35}S were impregnated with Amplify (Amersham corp.), placed on filter paper, dried and exposed to Kodak X-AR film at -85 °C.

Enzymatic Dephosphorylation

The radiolabelled soluble protein fraction prepared as described above was treated with bacterial alkaline phosphatase (BAP) from E. coli (Worthington, Freehold, New Jersey or US Biochemical, Cleveland, Ohio). The

dephosphorylations were done in a buffer containing 100 mM Hepes, pH 8.25; 100 mM NH_4Cl; 7 mM KCl; 10 mM $MgCl_2$; and 1 mM dithiothreitol. The BAP was added to each sample and the tubes were incubated at 30 °C for one hour. Following BAP treatment, the samples were immunoprecipitated as described above. For the BAP treatment of infected cell extracts prior to RNA synthesis, the enzyme was added directly to these samples and they were incubated for 60 min at 30 °C. Following dephosphorylation, the BAP was inactivated by addition of $NaPO_4$ (Garen and Levinthal, 1960; Fernley and Walker, 1969), fresh NTPs were added, and RNA synthesis was assayed as below.

In vitro RNA Synthesis

For the analysis of viral RNA synthesis, subconfluent monolayers of cells were infected with virus as described above. At four hours p.i., the cells were placed on ice, washed with ice-cold buffer A (0.15 M sucrose; 30 mM Hepes, pH to 7.4 with KOH; 33 mM NH_4Cl; 7 mM KCl; 4.5 mM Mg acetate), and permeabilized for one minute in L-α-lysophosphatidylcholine, palmitoyl (lysolecithin, 125 µg/ml, Sigma). The cells were then rinsed with cold buffer A and scraped into a small volume of reaction mix (100 mM Hepes, pH 8.25; 100 mM NH_4Cl; 7 mM KCl; 4 mM $MgCl_2$; 1 mM dithiothreitol; 1 mM ATP; 1 mM CTP; 1 mM GTP; 0.1 mM UTP). The cells were disrupted by pipetting 10 times and the cell nuclei and debris were removed by centrifuging at 4000 x g for five minutes. Each reaction contained 5 X 10^6 cells (one half plate) in a volume of 200 µl. The cell extracts were then assayed directly for RNA synthesis by adding 20 µci of ^{32}P-UTP and incubating at 30 °C for 1 hour. Alternatively, the cell extract was fractionated by placing it onto a discontinuous glycerol gradient consisting of 3-4 ml of 30% glycerol (0.01 mM ATP, 0.01 mM $MgCl_2$) and a cushion of 50 µl

of 99% glycerol (0.01 mM ATP, 0.01 mM $MgCl_2$) in a 5 ml tube and centrifuging at 50,000 rpm for 75 minutes in a Beckman SW55 rotor at 4 °C. After centrifugation, the soluble protein fraction was collected from the top of the gradient, the 30% glycerol fraction was discarded, and the viral RNPs were collected by suspending the top of the 99% glycerol cushion in reaction mix. For reconstitution, the viral soluble protein fraction and the RNPs were combined along with 20 µci of ^{32}P-UTP and incubated at 30 °C for one hour. For the experiments determining the effects of antibodies on reconstituted RNA synthesis, immunoprecipitations of the soluble protein pool were performed prior to reconstitution. Antibodies were added to 20 µl of protein G sepharose beads (50% in PBS). The samples were rotated for 120 minutes at 4 °C then the antibody-protein G sepharose complexes were washed five times in 1 ml reaction mix. The antibody coated beads were then added to the soluble protein fractions and incubated for 1 hour while rotating at 4 °C. The complexes were removed by centrifugation at 10,000 x g for 1 minute and the antibody-depleted supernatants were removed and reconstituted with viral RNPs. ^{32}P-UTP was then added and the reactions were incubated as above. The resultant RNAs were either extracted directly from the reactions or after treatment with micrococcal nuclease to digest unencapsidated RNA (Peluso and Moyer, 1988). The RNAs were then analyzed by acid-urea-agarose gel electrophoresis as described (Lehrach *et al.*, 1977).

Addition of Protease and Kinase Inhibitors to Infected Cell Extracts

For the experiments in which inhibitors, with or without BAP, were tested for their abilities to inhibit RNA synthesis, each inhibitor was added to the *in vitro* extracts, incubated one hour at 30 °C, and then the cell extracts were assayed directly for RNA synthesis by adding 20 µci of ^{32}P-UTP and incubating at 30 °C

for 1 hour. When BAP was also added, the enzyme was added to the samples along with the inhibitors and they were incubated for 60 min at 30 °C. The BAP was inactivated by addition of $NaPO_4$ and RNA synthesis was assayed.

RNA Analysis

For mRNA analysis, proteinase K (Sigma) was added to samples to a final concentration of 500 µg/ml along with SDS to a final concentration of 0.5% and the samples were incubated at 37 °C for 30 minutes. For genomic RNA analysis, $CaCl_2$ was added to a final concentration of 10 mM then 10 µg of staphylococcal micrococcal nuclease (MN, Pharmacia) was added to each sample and they were incubated at 37 °C for 30 minutes. After MN treatment, EGTA was added to a final concentration of 25 mM and the samples were treated with proteinase K as above. Following nuclease and/or proteinase treatment, the RNAs were isolated by phenol extraction and ethanol precipitation then analysed by electrophoresis on 1.5% acid-urea agarose gels (Lehrach *et al.*, 1977).

The GAL4 and VP16 Two Hybrid System

Construction of plasmids. The reporter plasmid (pUASGTATACAT), which contains five copies of the GAL4 17-mer binding site, the E1b TATA promoter, and the CAT gene, was provided by M. Ptashne and has been described previously (Martin *et al.*, 1990). The positive control was the pECEGAL4VP16 plasmid, containing the GAL4 and VP16 sequences together, was a gift of M. Green (University of Massachusetts, Wooster). pGAL4VSVC was created by restriction digest of the pGAL4MVN plasmid (a gift of Dr. Ron Harty; Appendix B) which contained the 441 bp region of GAL4 fused to the

measles virus N gene in the vector pECE, which contains: an SV40 early promoter, 3' poly(A) signal and poly(A) tract; a polylinker with sites for eight restriction enzymes with 6 bp recognition sequences; and 3' stop codons in all three reading frames prior to the polyadenylation signal. A 266 bp region of the P gene containing the C' gene was amplified by PCR using primers designed to create a SalI site immediately adjacent to the initiation codon for the C' ORF and a SacI site downstream of the termination codon. This fragment was amplified, purified and subcloned into pGAL4MVN plasmid which had been double digested with SalI and SacI to remove the measles virus sequences. The two fragments were ligated together and the resultant plasmid was transformed into bacteria, sequenced for accuracy, and purified for transfections.

The P gene of VSV was subcloned from a full length clone using PCR primers (Appendix A) to create a SalI site immediately upstream of the initiation codon and a SacI site downstream of the termination codon. PCR amplification and double digestion resulted in an 804 bp fragment which was subcloned into the pECE vector which had been double digested with SacI and BglII. The 236 bp fragment of VP16 was obtained by double digestion of the pVP16MVP plasmid (a gift of Dr. Ron Harty; Appendix B) with BglII and SacI. These three fragments were ligated together to form the pVP16VSVP plasmid.

Transfection of cells. BHK cells were grown as above in 60 mm dishes. For each transfection, 2.5 µg each of the test and reporter plasmids were mixed into 300 µl total OPTI-MEM I reduced serum medium (Gibco BRL). These samples were then mixed with 300 µl OPTI-MEM I containing 36 µl LipofectAMINE reagent (Gibco BRL) and incubated at room temperature for 30 minutes. 2.5 ml of OPTI-MEM I was added to each tube, mixed gently and placed dropwise onto cells which had been rinsed twice with 2 ml OPTI-MEM I.

The cells were incubated at 37 °C. After five hours, 3 ml of PM plus 20% fetal calf serum was added to each dish and they were incubated overnight at 37 °C. The following day, the medium was replaced with 5 ml CM plus 10% FCS and the cells were incubated an additional 24 hours. At 48 hours after transfection, the medium was aspirated and the cells were washed off the plate into 1.5 ml PBS and placed into sterile eppendorf tubes until CAT assays were performed.

CAT assay. The cells (in PBS) were centrifuged 3000 rpm for one minute. The supernatant was discarded and the cell pellet was resuspended in 100 µl 0.25M Tris-HCl, pH 7.5 (one dish of cells into 100 µl). The cells were disrupted by three courses of freezing in ethanol/dry ice for 5 minutes followed by 3 minutes in a 37 °C water bath. The cell debris was pelletted at 3000 rpm for one minute. The supernatant was transferred to a new tube and placed on ice. 50 µl of each supernatant was added to 100 µl of a solution containing 2 µl ^{14}C chloramphenicol, 20 µl acetyl-CoA (3.5 mg/ ml), 25 µl 1M Tris-HCl pH 7.5, and 53 µl distilled water. The positive control was 1 µl of commercial enzyme (+ 49 µl 0.25 M Tris-HCl pH 7.5) and the negative control was 50 µl 0.25 M Tris-HCl pH 7.5. The samples were incubated three hours at 37 °C. Following incubation, 1 ml ethyl acetate was added to each sample, the tubes were vortexed vigorously and then centrifuged at 3000 rpm for one minute. The top layer was collected into new tubes and dried down under vacuum. The dried samples were resuspended in 30 µl ethyl acetate and spotted onto thin layer chromatography plates and chromatographed to separate the acetylated forms of chloramphenicol (Gorman *et al.*, 1982). The finished plates were then exposed to X-ray film for detection.

Construction, Expression and Purification of GST-C'

Plasmid construction. The C' gene of VSV Indiana was amplified using the oligo primer JRC3 to create a BamHI site upstream of the C' coding region and JRC5 to create a EcoRI site downstream (Appendix A). The C' sequence was amplified and double digested with BamHI and EcoRI and cloned in frame into the multiple cloning site of the commercial vector pGEX-5X-1 (Pharmacia). This plasmid contains: a tac promoter, an internal lac I^q gene, and the coding sequence for the glutathione-S-transferase gene upstream of the multiple cloning site. The resultant plasmid from the ligation of C' into pGEX-5X-1 was pGEX-C'.

Expression and purification of GST-C'. The pGEX-C' plasmid was transformed into W31005 *E. coli* cells and grown to an optical density of 0.7-0.9, induced by addition of 1 mM IPTG, and incubated 5 more hours at 37 °C. The cells were harvested by pelletting at 4,000 rpm for 15 minutes, washed with PBS, and repelletted. The cells were resuspended in PBS, Triton X-100 was added to 1% and the cells were disrupted by sonicating. The cell debris was pelletted (10,000 X g for 5 minutes) and the supernatant was added to 1 ml glutathione-agarose (Sigma Chemical) and incubated at room temperature for 5 minutes. The beads were washed 5 times with PBS and the protein was eluted by incubating at room temperature in a solution containing 5 mM reduced glutathione, 50 mM Tris pH 8 for 2 minutes. The protein concentration of the eluate was determined and the purified protein was stored at -85 °C.

Construction, Expression and Purification of His-C' Protein

Construction of plasmids for his-C' expression. The histidine tagged C' protein was prepared using the QIAexpress protein expression and purification system (QIAGEN). The C' open reading frame was amplified by polymerase chain reaction (PCR) using primers which created a BamHI restriction site immediately upstream of the initiator AUG and a SacI site 63 bases downstream of the termination codon (Appendix A). This 265 bp PCR product was double digested with BamHI and SacI and gel purified. The vector pQE-30, which contains an optimized, regulable promoter/operator element consisting of the E. coli phage T5 promoter and two lac operator sequences, was also double digested and purified. The C' fragment was ligated into the vector to form the plasmid pQE-C'. Translation of the mRNA from this plasmid would produce a protein containing the C' protein sequence with eight additional amino acids at the N-terminus (six histidine residues plus the two residues encoded by the BamHI site).

Purification of his-C'. The his-C' protein was purified using the denaturing purification method described in the QIAexpressionist manual. The pQE-C' plasmid was transformed into JM109 E. coli cells. This strain of *E. coli* contains the lac I^q gene and produces excess lac repressor; making it a suitable strain for the inducible expression of proteins dependant on the addition of IPTG to inactivate the repressor. The cells containing the pQE-C' plasmid were grown in large scale cultures (1-2 liters) until the optical density was 0.7-0.9. The his-C' protein was found to be quite labile and to be present for only one hour after induction so the cells were grown for an additional four hours before inducing

expression of the protein by adding IPTG to 1mM. One hour after induction the cells were harvested and the protein was purified. Briefly, the cells were lysed in 6M guanidine HCl and the protein was purified using Nickel-agarose beads. The protein was eluted from the beads at low pH (4.9) and then refolded by dialysis against PBS using dialysis tubing with a MW cutoff of 500 daltons. The purified protein was kept at -85 °C until use.

STUDIES OF THE PHOSPHOPROTEIN

The systems in which the transcription and replication of VSV are studied contain many different components and the study of the function of individual viral proteins has proven difficult. Antibodies produced against VSV proteins have been used extensively to ascertain the role of the proteins in the transcriptase complex (Imblum and Wagner, 1975; Carroll and Wagner, 1978; Harmon and Summers, 1982). In particular, since there are many different forms of the P protein present in infected cells, the ability to identify and study subpopulations of this heterogeneous population would be useful. Dr Sue Emerson's lab prepared a panel of monoclonal antibodies to the VSV P protein with this goal in mind. These antibodies were utilized in preliminary studies of the function of the P protein in transcription using an *in vitro* system consisting of disrupted virus (Williams *et al.*, 1988). Our lab inherited this panel of anti-P antibodies and I started my work by using these antibodies to probe for functions of P in RNA synthesis using the previously described infected cell system. This system consists of cytoplasmic extracts of infected cells and it allows the analysis of viral transcription, replication and encapsidation of genomic RNA.

Effect of Antibodies to VSV Proteins on *in vitro* RNA Synthesis

The work of Williams *et al.* (1988a, 1988b) suggested that certain monoclonal anti-P antibodies bind to different functional domains within the P protein present in the virus particle. In order to determine the effects of a panel of monoclonal anti-P antibodies on viral transcription and genome RNA replication in infected cell extracts, each antibody was added to a separate *in vitro* RNA synthesis reaction. Extracts of VSV-infected BHK cells were prepared,

incubated with 2 μg of the indicated monoclonal antibody on ice and then ^{32}P-UTP was added to label the products of RNA synthesis. The ^{32}P-labelled RNAs synthesized in the presence of the antibodies are shown in figure 3. The bottom panel depicts RNA transcripts produced in the reactions and the top panel shows the nuclease-resistant 42S VSV genomic RNA indicative of replication. An untreated reaction and an anti-N monoclonal antibody treated reaction were included as controls (lanes 1 and 2). The anti-N and the anti-P 2A2 antibodies blocked all RNA synthesis by the infected cell extracts (bottom and top panels, lanes 2 and 4). The 2A2 antibody has been shown to cross-react with *in vitro* synthesized N protein (Williams *et al.*, 1988b) so this antibody and the anti-N antibody probably inhibited RNA synthesis by binding to N protein on the nucleocapsid template. None of the antibodies which react exclusively with P protein were able to totally block transcription, even when higher concentrations of antibodies were used (data not shown, see figure 4). The effect of the antibodies on viral genome replication was determined by examining the nuclease resistant encapsidated RNAs (top panel, figure 3). Several of the anti-P antibodies (6D11, 4F11, 2F2 and 6H1) specifically inhibit genome RNA synthesis and nucleocapsid assembly (figure 3; lanes 3, 5, 7, 15). The two antibodies that bind the N protein, anti-N and 2A2, and blocked transcription also blocked replication.

In some experiments, some of the anti-P antibodies inhibited viral transcription to some degree. I investigated the specificity of this effect by adding higher concentrations of the 6D11 monoclonal antibody to *in vitro* RNA synthesis reactions (figure 4). Genome RNA synthesis was blocked by as low as 1 μg of the antibody (lane 2) while transcription was only slightly inhibited at very high levels of antibody. In no experiments were any of the anti-P antibodies (except 2A2, which cross-reacts with the N protein) able to totally inhibit

Figure 3. Effect of antibodies to VSV proteins on *in vitro* RNA synthesis. The ^{32}P-labelled RNAs synthesized in the presence of 2 μg of the indicated antibodies were isolated and electrophoresed on a 1.5% acid urea agarose gel. The top panel shows the RNAs present after digestion with micrococcal nuclease while the lower panel shows isolated total RNA. The positions of the viral mRNAs and 42S genomic RNAs are indicated on the left.

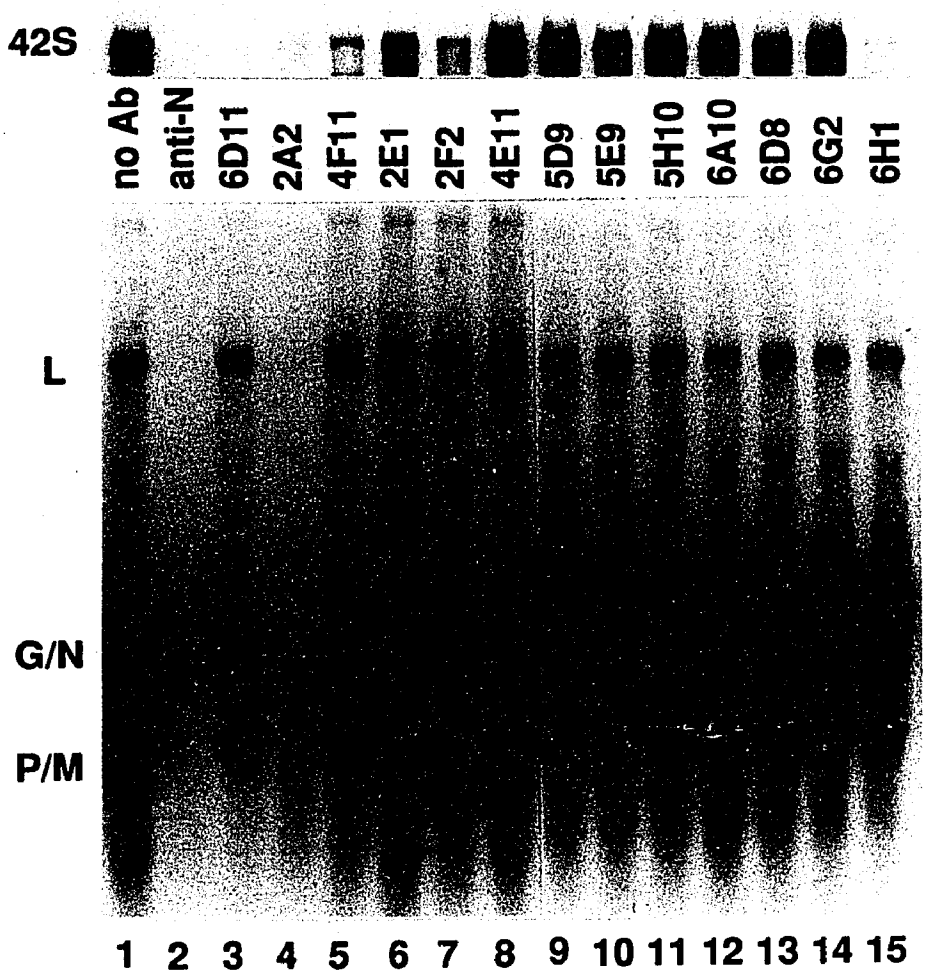
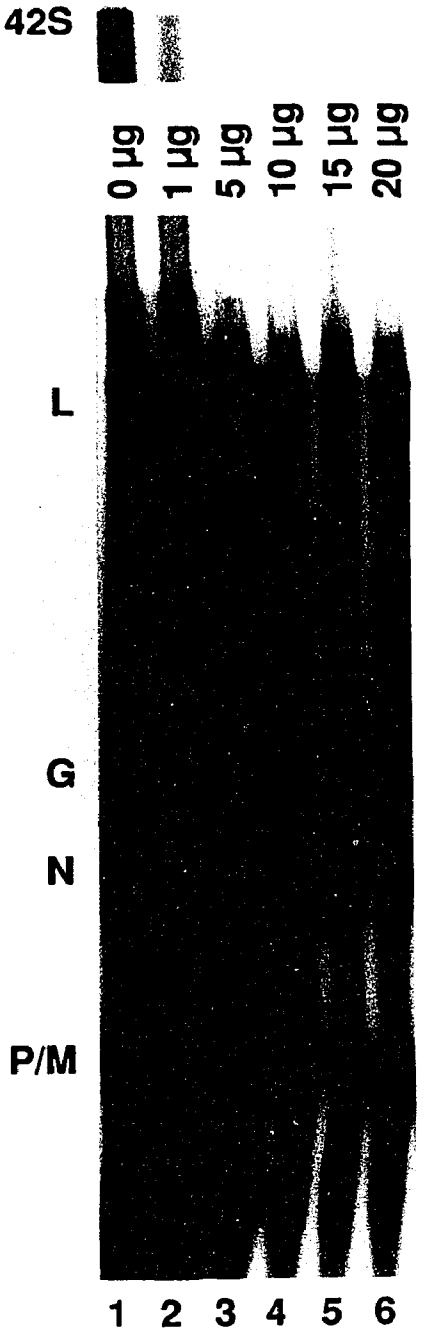

42S
no Ab
anti-N
6D11
2A2
4F11
2E1
2F2
4E11
5D9
5E9
5H10
6A10
6D8
6G2
6H1
L
G/N
P/M
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Figure 4. Effect of increasing amounts of 6D11 anti-P monoclonal antibody on *in vitro* RNA synthesis. Top and bottom panels are as in figure 1. The amounts of antibody used are shown above each lane. The positions of the viral mRNAs and 42S genomic RNAs are indicated on the left.


42S
0 µg
1 µg
5 µg
10 µg
15 µg
20 µg
L
G
N
P/M
1 2 3 4 5 6

transcription. In most of my following experiments, I utilized primarily the 6D11 antibody since we were able to obtain the hybridoma cell line which produces this antibody and it had a specific effect on replication of VSV genome RNA at low concentrations.

Ability of Soluble Proteins to Support Reconstituted Replication after Removal of Specific Proteins

Antibody to the P protein could affect genome RNA replication either by binding to P protein on the RNA template or by binding to a soluble form of the P protein that functions in genome RNA synthesis. To distinguish between these possibilities, I tested whether removal of antibody-bound proteins from the soluble protein fraction was able to affect RNA synthesis when added to the RNP templates. An infected cell extract was fractionated into a soluble protein fraction and a pellet fraction containing the RNP template. If these fractions are recombined, both transcription and genome RNA replication are reconstituted (Peluso and Moyer, 1983). The RNP fraction alone can carry out transcription of mRNAs, but the soluble protein fraction is needed for genome RNA replication to occur. Cells co-infected with wild type VSV (WT) plus the MS-T defective-interfering (DI) particle were used for these experiments in order to more easily assess genome RNA replication. The DI genome is smaller than WT and essentially only replicates, resulting in the synthesis of high levels of DI genomic RNA relative to WT. The antibodies were added to the soluble protein fraction of the extracts, allowed to react, and then antigen-antibody complexes were removed. The treated soluble protein was then reconstituted with the nucleocapsids in the pellet fraction, and RNA synthesis was analyzed (figures 5 and 6). Soluble protein treated using the monoclonal antibodies which were

shown to block replication in complete extracts no longer supported DI genome RNA replication (figure 5, lanes 3-6) while the soluble protein treated using normal rabbit serum was replication competent (lane 7). The soluble protein treated with the anti-N monoclonal antibody no longer supported genome RNA synthesis (lane 2), an expected result since the N protein is required for nucleocapsid assembly. When soluble protein was treated using any of the other anti-P monoclonals there was no effect on RNA synthesis (data not shown). The above data suggests that a soluble form of the P protein is required for genome RNA replication in these reactions and that the anti-P antibodies which block replication react with this P protein. Treatment of soluble protein with the antibodies had no effect on reconstituted transcription, as shown by figure 6, which shows the synthesis of a typical mRNA, the G mRNA band. This result is expected since the soluble protein fraction is not needed for this process.

Immunoprecipitation of VSV Proteins

Since several of the anti-P antibodies exerted specific effects in the *in vitro* assays, the proteins immunoprecipitated by these antibodies were examined. When the immunoprecipitates prepared under conditions which would permit N:P complex formation (no SDS) were analyzed by SDS-PAGE, several patterns emerged (Figure 7). The anti-P antibody 2A2 precipitated very little P protein but a large amount of the N protein. The antibody 4F11 precipitated both N and P while the 6D11 antibody brought down mostly P. There was no observable difference between the immunoprecipitates of the 6D11 and 2E1 antibodies except for a slight difference in the amount of P.

Figure 5 Effect of removal of specific proteins on the ability of soluble protein to support genome RNA synthesis in reactions reconstituted from fractionated WT and DI co-infected cell extracts. The ^{32}P-labelled, nuclease resistant RNAs synthesized from reactions combining RNPs with soluble protein treated using 2 μg of the indicated antibodies were isolated and electrophoresed on a 1.5% acid urea agarose gel. The bands shown correspond to the MS-T DI genome RNA.

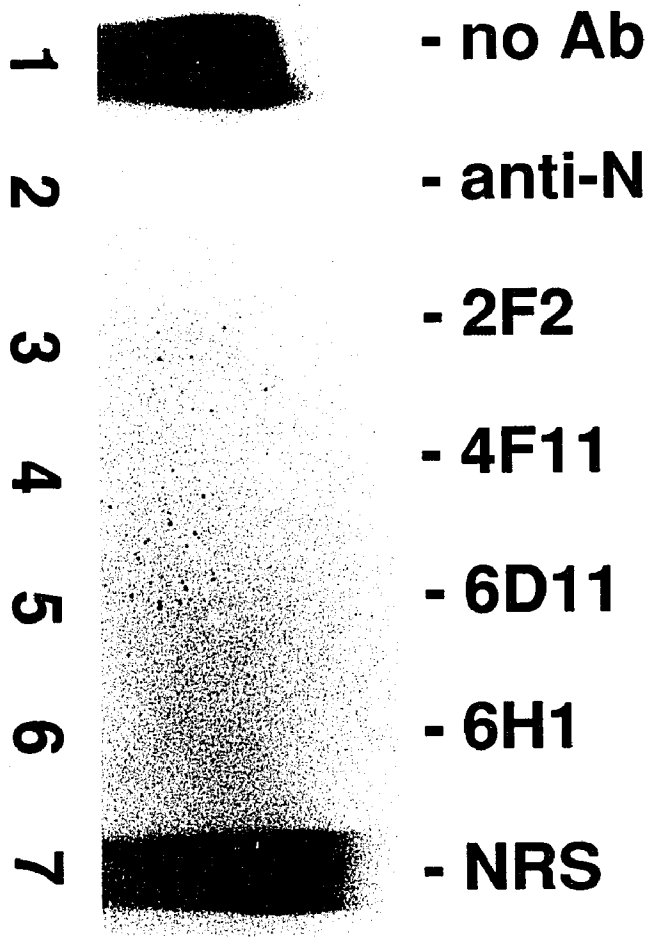

1
2
3
4
5
6
7
- no Ab
- anti-N
- 2F2
- 4F11
- 6D11
- 6H1
- NRS

Figure 6 Effect of removal of specific proteins on the ability of soluble protein to support RNA synthesis in reactions reconstituted from fractionated WT and DI co-infected cell extracts. The ^{32}P-labelled RNAs synthesized in reactions combining RNPs with soluble protein treated using 2 μg of the same antibodies as in figure 5 were isolated and electrophoresed on a 1.5% acid urea agarose gel. The band shown corresponds to the G mRNA.

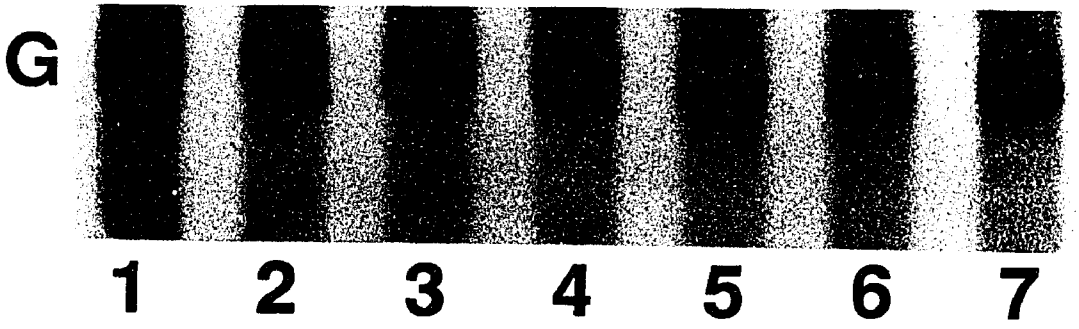
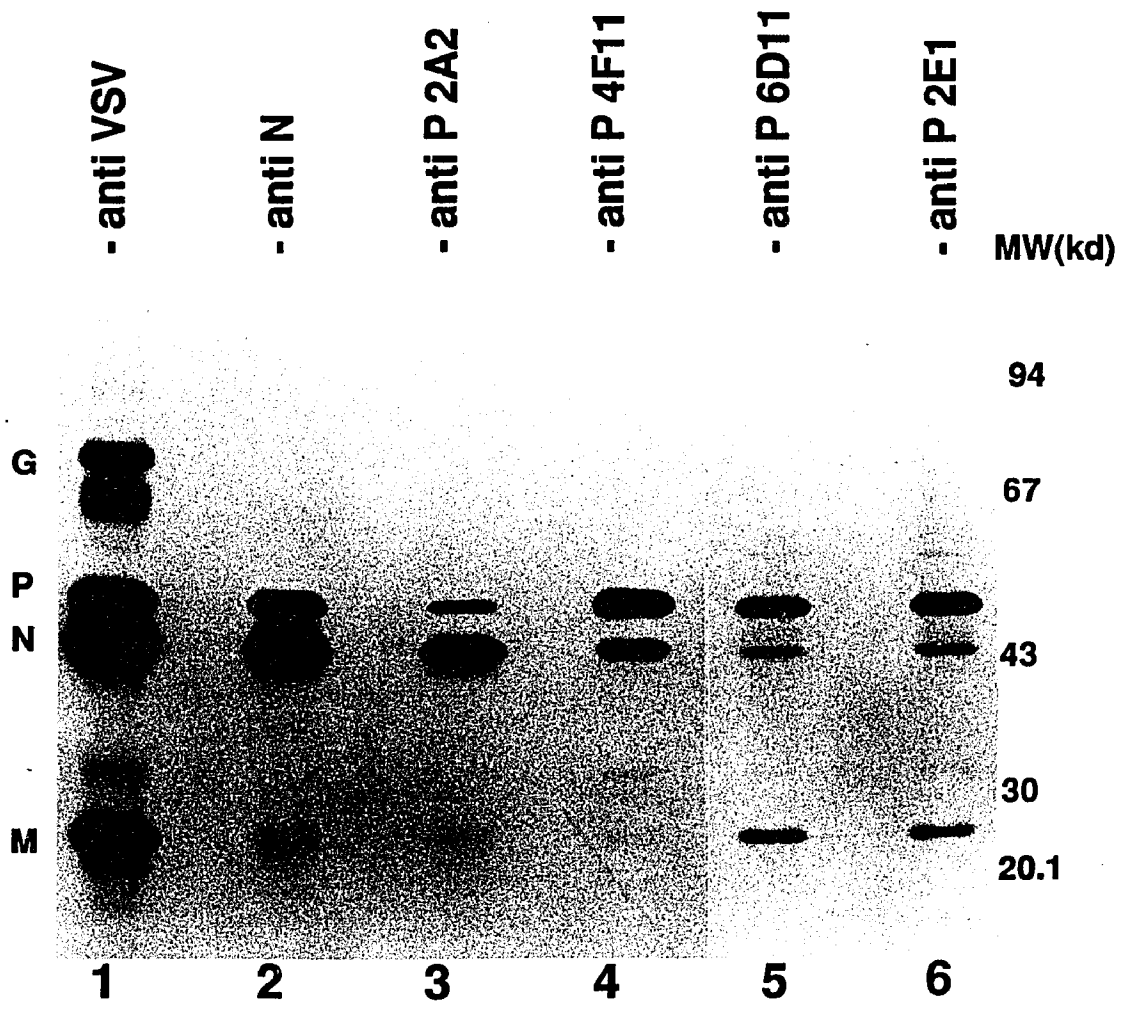

G
1
2
3
4
5
6
7

Figure 7 Immunoprecipitation of ^{35}S-labelled VSV-infected cell soluble proteins. Soluble proteins (non--denaturing) were prepared from ^{35}S-labelled infected cells and immunoprecipitated with 1 μg of the indicated antibodies. The immunoprecipitates were analyzed by polyacrylamide gel electrophoresis on an 10% gel as described. The positions of the viral G, P, N, and M proteins are indicated on the left while the migration of standard proteins is indicated on the right.


- anti VSV
- anti N
- anti P 2A2
- anti P 4F11
- anti P 6D11
- anti P 2E1
MW(kd)
94
67
43
30
20.1
G
P
N
M
1
2
3
4
5
6

Effect of Removal of N-P Complexes on Immunoprecipitation by Anti-P Antibodies

Several studies have shown that a 1:1 complex between the N and P proteins in the soluble protein fraction of infected cells is required for genome RNA replication and encapsidation (Peluso and Moyer, 1984, 1988; LaFerla and Peluso, 1989). It was possible that the 6D11 anti-P antibody was interacting with soluble P protein that was bound to N protein. Such an association would explain the ability of this antibody to specifically block genome RNA replication. Some of my data argued against this possibility. When immunoprecipitations were done under conditions which allowed the detection of N:P complexes, the 6D11 antibody was one of the least effective antibodies in precipitating these complexes (figures 7,8,9; lanes 6D11). It should be noted that the mature N protein is predicted to contain 13 methionine residues while the P protein contains 3. Thus, the intensity of the band corresponding to the N protein in these autoradiographs does not accurately reflect the molar ratios of these two proteins in immunoprecipitations. Additionally, one of the other anti-P antibodies (4F11) precipitated four fold more of N:P complexes than 6D11 (Williams *et al.*, 1988a and data not shown) and yet was not more effective at blocking replication. In repeated experiments, the 6D11 antibody has been shown to be the most effective at blocking replication. This evidence, although indirect, suggests that it is not the interaction of antibody with N:P complexes that results in the block of genome RNA replication by the 6D11 monoclonal antibody.

To further investigate this possibility, I tested the monoclonal anti-P antibody 6D11 for its ability to precipitate the P protein from soluble protein that had been previously depleted of N:P protein complexes. Soluble protein (^{35}S-

labelled) from VSV-infected cells was depleted of the N:P protein complex by repeated immunoprecipitation with an anti-N monoclonal antibody and then the complex-depleted soluble protein that remained was reacted with the monoclonal anti-P antibody 6D11 (Figure 8). As controls, the protein samples were also precipitated with a polyclonal rabbit anti-VSV serum and an anti-N monoclonal antibody. This procedure was effective in removing the N protein and the N:P protein complex (compare lanes 1 to 2, and lanes 3 to 4), yet the amount of P protein precipitated by the 6D11 antibody was not affected (lanes 5 and 6). Lanes 1, 3, and 5 were analyzed prior to N-depletion, and lanes 2, 4, and 6, after. This result indicated that the P protein to which the 6D11 monoclonal antibody was directed was not the form of P that is complexed to the N protein.

Since the conclusion that the 6D11 antibody affects genome replication by binding to proteins other than the N:P complex was central to my hypothesis for a third function of P protein, I checked this result in another way. Soluble protein which had been treated by immunoprecipitation with an amount of 6D11 sufficient to totally block reconstituted genome replication (2 μg per 5 X 10^6 cells, see figure 4) was subsequently immunoprecipitated with both the 6D11 and anti-N antibodies to check the levels of 6D11-reactive P protein and N:P complexes respectively. The amounts of antibodies used in the secondary immunoprecipitations were sufficient to precipitate all of the reactive proteins in each sample. As a control, an identical sample of soluble protein was immunoprecipitated first with normal rabbit serum (NRS) then with the 6D11 and anti-N antibodies. The results show that treatment of soluble protein by the 6D11 antibody has no effect on the amount of N:P complexes precipitated by the anti-N antibody (figure 9; compare lanes 1 and 2). The antibody treatment does remove a significant portion of the 6D11-reactive P protein in these samples (compare lanes 3 and 4). The 6D11 antibody does precipitate some L protein

Figure 8. Immunoprecipitation of ^{35}S-labelled, VSV-infected cell soluble proteins which have been depleted of N:P complex. Soluble proteins were prepared from ^{35}S-labelled infected cells and divided into two samples (10^6 cells each). One sample was depleted of N-P complexes by immunoprecipitation four times with 5μg each of anti-N antibody (depleted) while the other was immunoprecipitated in parallel using normal rabbit serum (normal). These two samples were each divided three ways and further immunoprecipitated using 1 μg of the indicated antibodies. The immunoprecipitates were analyzed by polyacrylamide gel electrophoresis on a 10% gel as described in the methods section. The positions of the viral P and N proteins are indicated.

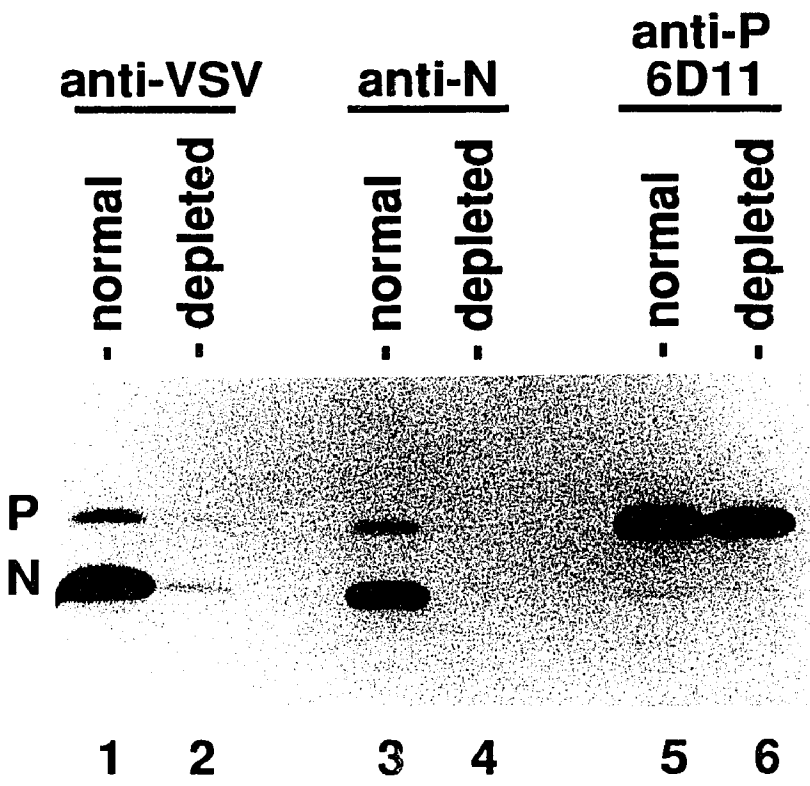
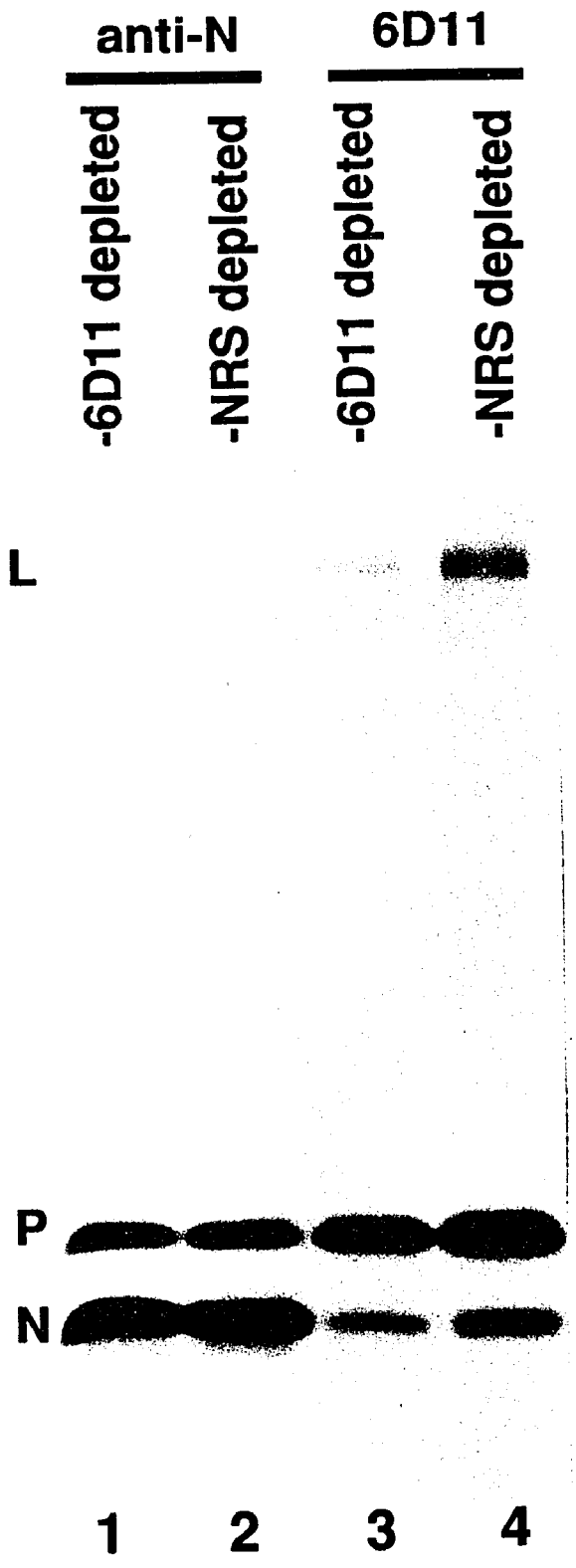

anti-VSV
anti-N
anti-P 6D11
- normal
- depleted
- normal
- depleted
- normal
- depleted
P
N
1
2
3
4
5
6

Figure 9. Immunoprecipitation of ^{35}S-labelled, VSV-infected cell soluble proteins which have been treated by prior immunoprecipitation. Soluble proteins were prepared from ^{35}S-labelled infected cells and divided into two samples (10^6 cells each). One sample was treated by immunoprecipitation with 0.4 μg of anti-P 6D11 antibody (4 μg per 100 mm dish of cells; 6D11-depleted) while the other was immunoprecipitated in parallel using normal rabbit serum (NRS-depleted). These two samples were each divided two ways and immunoprecipitated using 2 μg of the antibody indicated at the top of each pair of lanes. The immunoprecipitates were analyzed by polyacrylamide gel electrophoresis on a 10% gel as described. The positions of the viral P, L and N proteins are indicated on the left.


anti-N
6D11
-6D11 depleted
-NRS depleted
-6D11 depleted
-NRS depleted
L
P
N
1
2
3
4

under the non-denaturing conditions used in this experiment and it is not known whether this amount of L is functionally significant. The above experiments demonstrated the existence in VSV-infected cells of a soluble form of the P protein that is not complexed to the N protein, and that this form of the P protein is required for genome RNA replication and nucleocapsid assembly but not for transcription of viral mRNA.

Immunoaffinity Purification of the P Protein Bound by the 6D11 Antibody

The P protein bound by the 6D11 antibody is required for replication. I wanted to see if I could purify a this form of P protein for use in functional assays. I prepared an immunoaffinity column using the 6D11 antibody and used it to purify P proteins from both virus and infected cells. Neither of these purified components had any activity when added back to N-RNA templates along with L protein purified from either infected cells or virus. Addition of the purified 6D11-P to infected cell extracts also had no effect on RNA synthesis. It is possible that the proteins purified in this manner were denatured during purification and had lost any activity.

Isoelectric Focusing of Radiolabelled VSV Proteins

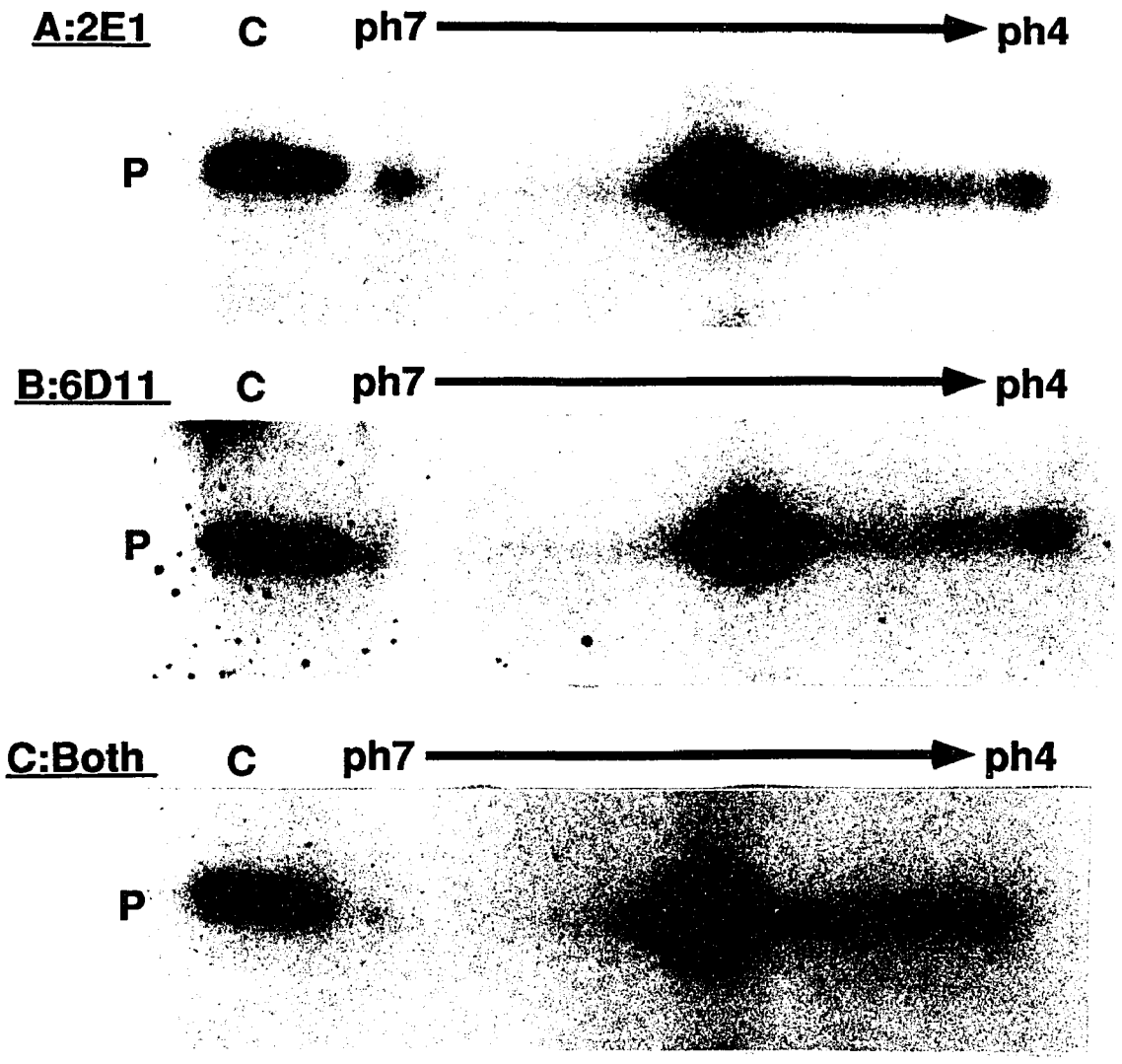
The 6D11 and 2E1 antibodies have different effects on RNA synthesis but the immunoprecipitates look identical by SDS-PAGE. Since no unique aspect of the 6D11-reactive P proteins was detected by one-dimensional electrophoresis, I examined the profile of the proteins precipitated by these two antibodies using two-dimensional electrophoresis. These experiments utilized isoelectric focusing in one dimension followed by SDS-PAGE. A difference in the phosphate content

of the immunoprecipitated P proteins would be expected to result in different mobilities in electrofocusing gels due to differences in isoelectric points. ^{32}P-labelled soluble proteins were analyzed by this technique. When the autoradiographs of the two-dimensional profiles were compared, no difference was seen between the 6D11 and 2E1 antibodies (figure 10). When the immunoprecipitates were combined before analysis, the proteins comigrated (figure 10, panel C). Thus, it appears that there are no detectable differences between the isoelectric points. It is still possible that differences exist in the specific residues which are phosphorylated within these two proteins that do not change the net charge or isoelectric point.

The Effect of Alkaline Phosphatase Treatment on Immunoprecipitation of Infected Cell Proteins

The P protein exists in infected cells as a heterogeneous pool of differentially phosphorylated molecules (Hsu and Kingsbury, 1982). Since phosphorylation of P has been shown to regulate the function of the protein in RNA synthesis and I had shown that the P protein which bound to the 6D11 antibody is functionally different from other P protein, I wanted to know if there was a discernible structural difference in the phosphorylation pattern of the 6D11-reactive P. Other studies have shown that phosphorylated P protein can be dephosphorylated by bacterial alkaline phosphatase (BAP; Kingsbury *et al.*, 1981; Hsu *et al.*, 1982) so I decided to use this enzyme to try and identify a structural difference that distinguishes the P protein recognized by the 6D11 antibody from other forms. If the 6D11 antibody recognizes an epitope that is conformationally dependent on phosphorylation, then dephosphorylation of the P protein would affect the binding of the antibody. I used BAP to dephosphorylate ^{35}S-labelled

Figure 10. Analysis of immunoprecipitates by two dimensional electrophoresis. ^{32}P-labelled infected cell soluble proteins were immunoprecipitated with the 2E1 and 6D11 anti-P antibodies. The immunoprecipitates were subjected to electrofocusing in tube gels as described. After electrofocusing, the tube gels were placed in a well of a 10% SDS-PAGE gel and electrophoresed . The pH gradient obtained in the electrofocusing gels is indicated at the top of each panel while the C lane contains a sample of the original immunoprecipitate which was not electrofocused. Panel A contains the two dimensional analysis of an immunoprecipitation by the 2E1 antibody. Panel B similarly contains proteins immunoprecipitated using the 6D11 antibody. Panel C contains the analysis of a mixture of precipitates using both of the above antibodies. The position of the P protein in each panel is indicated on the left.


A:2E1
C
ph7
ph4
P
B:6D11
C
ph7
ph4
P
C:Both
C
ph7
ph4
P

soluble protein and then performed immunoprecipitations as before.

Treatment of infected cell soluble protein with BAP (Figure 11; lanes 4-6) resulted in an increase in the amount of the P protein that is immunoprecipitated by the 6D11 antibody (compare lanes 3 and 6). In contrast, the amount of P protein reactive with the 2E1 anti-P protein monoclonal antibody, which has no effect on genome RNA replication (see figure 3), decreased (lanes 2 & 5). The amount of protein precipitated by the polyclonal anti-VSV serum remains unchanged by BAP treatment (lanes 1 and 4). The above results were confirmed in several subsequent experiments, including an experiment using increasing amounts of BAP (Figure 12). The total amounts of N and P proteins precipitated by anti-VSV and anti-N antibodies did not change with enzyme treatment (first 8 lanes), but the amount of 6D11-reactive P protein increased as the amount of enzyme increases (lanes 9-12). Quantitation of the respective bands of this gel by scintillation counting of excised gel pieces revealed a 45% increase in the P protein precipitated by the 6D11 antibody after BAP treatment. The above experiments indicate that the epitope recognized by this antibody is available for binding when a potential phosphorylation site is not phosphorylated.

The Effect of Addition of Alkaline Phosphatase on *in vitro* RNA Synthesis by Infected Cell Extracts

Since I could increase the amount of the P protein that is reactive with the 6D11 antibody by treatment with BAP, and since this form of the protein is needed for genome RNA replication, I tested the effect of BAP treatment of cell-free extracts on genome RNA replication. Our experiments involved adding BAP to cell extracts made from WT and DI co-infected cells and incubating for one hour. The BAP was then inhibited by adding inorganic phosphate, fresh

Figure 11. Immunoprecipitation of untreated and BAP treated soluble protein by antibodies to VSV proteins. Lanes 1-3 were immunoprecipitations of untreated ^{35}S-labelled soluble protein while lanes 4-6 were treated with BAP prior to immunoprecipitation with 1 μg of the antibodies indicated above each lane. The positions of the viral P and N proteins are indicated.

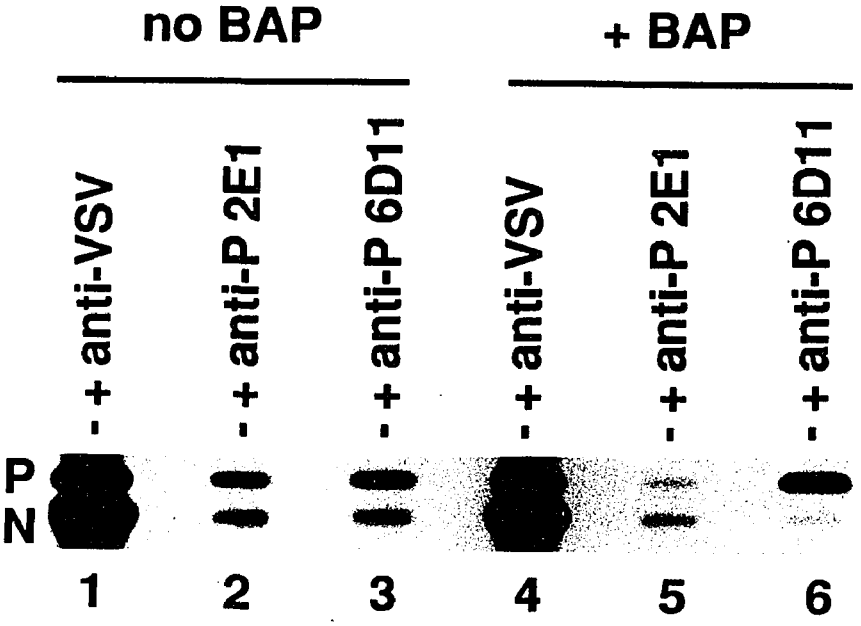
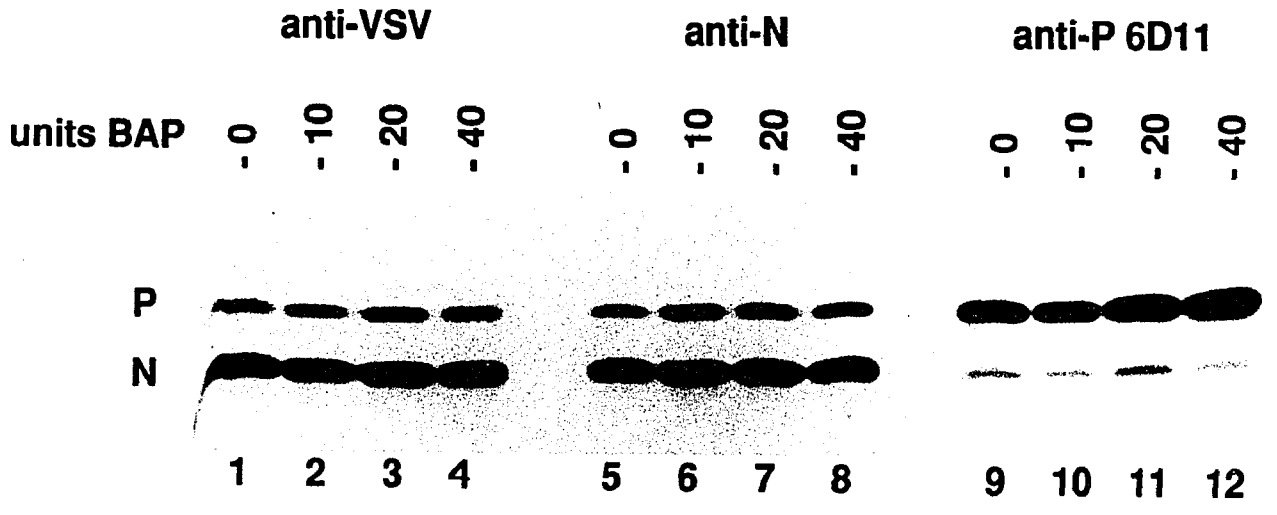

no BAP
+ BAP
- + anti-VSV
- + anti-P 2E1
- + anti-P 6D11
- + anti-VSV
- + anti-P 2E1
- + anti-P 6D11
P
N
1
2
3
4
5
6

Figure 12. Immunoprecipitation of soluble proteins treated with varying amounts of BAP. Notation and immunoprecipitations are similar to those in figure 11 and the amount of BAP used is indicated above each lane. The antibody used for immunoprecipitation is indicated above each quartet of lanes.


anti-VSV
anti-N
anti-P 6D11
units BAP
0
10
20
40
0
10
20
40
0
10
20
40
P
N
1
2
3
4
5
6
7
8
9
10
11
12

NTPs were added, and the extracts were incubated at 30 °C to allow RNA increased production of nuclease resistant genome RNA (figure 13). This stimulation of RNA replication was dose-dependent with the most genome RNA made at the highest level of added BAP. In addition, there was a concomitant decrease in the level of transcription in these reactions (figure 14). Treatment of soluble protein alone with alkaline phosphatase resulted in a similar, although less clear, stimulation of genome replication (figure 15). Although the amount of genome replication varies in the experiment shown, there is a clear stimulation of replication at the highest level of BAP used (lane 6). The conclusion that BAP is having an effect on genome replication by affecting some soluble molecule is supported by the finding that treatment of isolated nucleocapsids alone had no effect on genome replication when reconstituted with untreated soluble proteins (data not shown).

Effect of Protease and Kinase Inhibitors on RNA Synthesis in Infected Cell Extracts

Several groups working on the importance of phosphorylation to the function of the P protein in viral transcription have used inhibitors of specific kinases to study this process (Barik and Banerjee, 1992; Beckes and Perrault, 1992). There are at least two specific kinase activities which phosphorylate the P protein in infected cells. One of the kinase activities may be casein kinase II (CK II) and phosphorylates sites in domain I of the P protein while the other kinase activity is tightly associated with the L protein and phosphorylates the molecule at sites in the C-terminal half of the protein. Heparin has been used to inhibit the CK-II like activity (Barik and Banerjee, 1992) and protamine (Barik and Banerjee, 1992), the adenine analog FSBA (Beckes and Perrault, 1992), and the protease

Figure 13. Effect of BAP on synthesis of nuclease resistant RNA by VSV-infected cell extracts. VSV wild type and DI co-infected cell extracts (5 X 10^6 cells/lane) were treated with BAP (lane 1, 0 units; lane 2, 0.03 units; lane 3, 0.15 units; lane 4, 0.3 units; lane 5, 0.6 units; lane 6, 1.2 units) for 60 minutes at 30 °C, and then the ^{32}P-labelled, nuclease resistant RNAs were isolated and analyzed as described in the methods. The top band is the minus sense DI genome RNA, and the bottom, the plus sense.

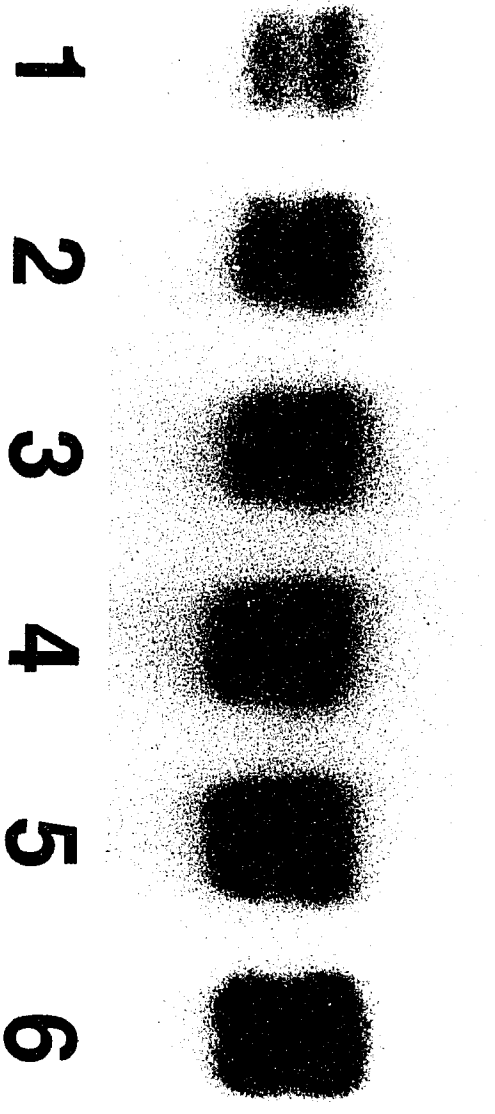

1
2
3
4
5
6

Figure 14. Effect of BAP treatment of infected cell extracts on synthesis of total RNA. VSV wild type and DI co-infected cell extracts (5 X 10^6 cells/lane) were treated with BAP (lane 1, 0 units; lane 2, 0.03 units; lane 3, 0.15 units; lane 4, 0.3 units; lane 5, 0.6 units; lane 6, 1.2 units) for 60 minutes at 30 °C, and then the ^{32}P-labelled RNAs were isolated and analyzed as described in the methods. The positions of the viral mRNAs are indicated on the left.

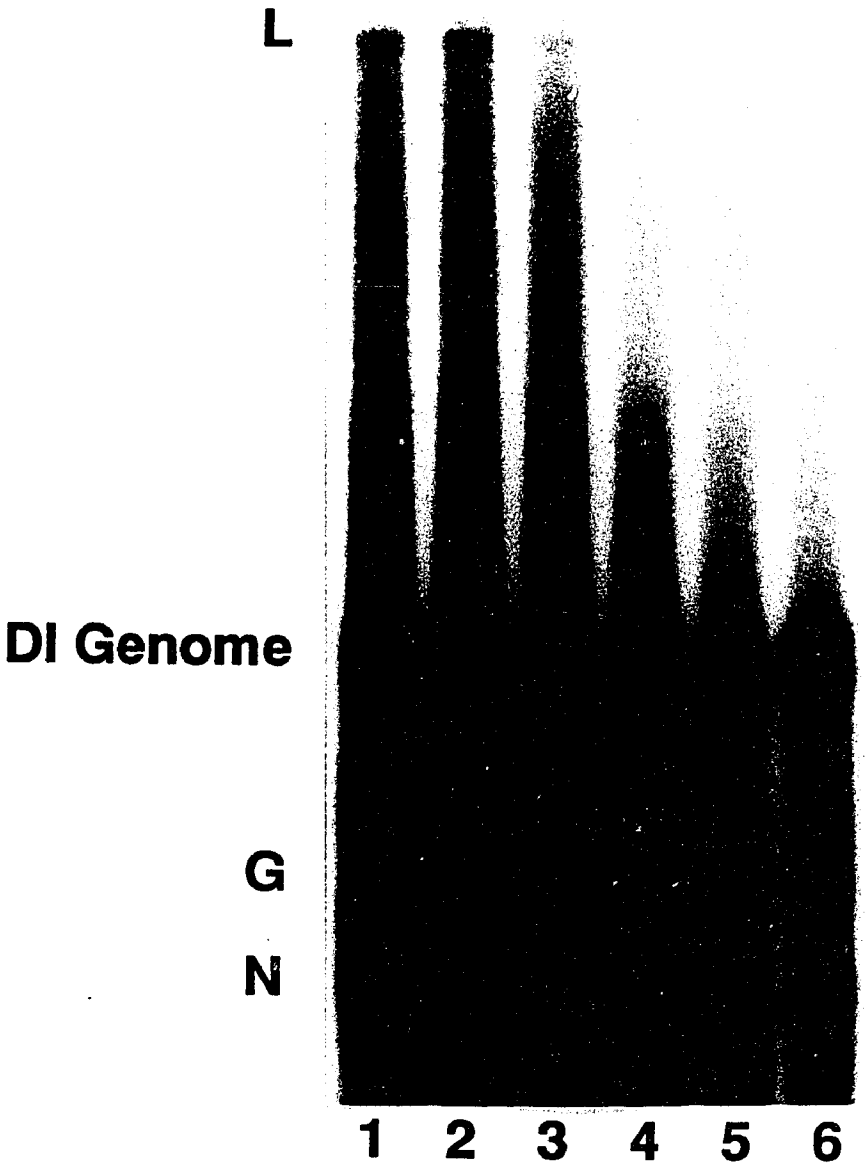
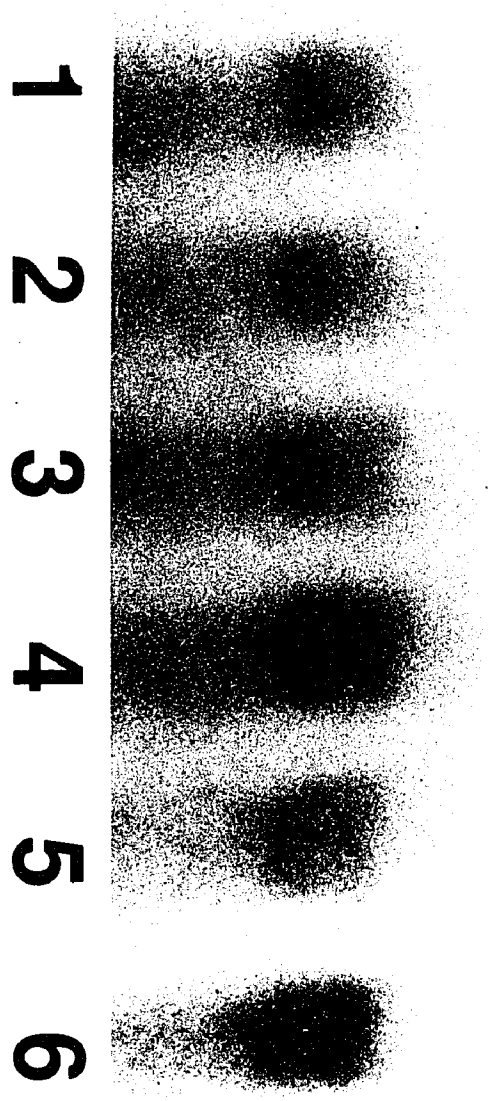

L
DI Genome
G
N
1 2 3 4 5 6

Figure 15. Effect of BAP treatment of soluble protein on synthesis of nuclease resistant RNA by reconstituted VSV-infected cell extracts. VSV wild type and DI co-infected cell extracts (5 X 10^6 cells/lane) were fractionated into soluble protein and RNP fractions and the soluble protein alone was treated with BAP (lane 1, 0 units; lane 2, 0.03 units; lane 3, 0.15 units; lane 4, 0.3 units; lane 5, 0.6 units; lane 6, 1.2 units) for 60 minutes at 30 °C. The ^{32}P-labelled, nuclease resistant RNAs were then isolated and analyzed. The top band is the minus sense DI genome RNA, and the bottom, the plus sense.


1
2
3
4
5
6

inhibitor TLCK (Beckes and Perrault, 1992) have been shown to inhibit the L associated kinase activity. I wanted to see if, by adding one of these reagents, I could alter the balance of phosphorylated molecules in VSV-infected cells and thus affect the balance of RNA synthesis between transcription and replication.

When I tested the effects of these inhibitors on *in vitro* RNA synthesis, the following results were obtained. The addition of FSBA seemed to increase RNA synthesis non-specifically in infected cell extracts (figures 16 and 17; lanes 2 and 3). The addition of protamine and heparin to infected cell extracts had no obvious effects except for a slight stimulation of RNA synthesis at low levels of protamine while the addition of TLCK inhibited all RNA synthesis (figures 16 and 17).

Since the proteins present in the infected cell extracts are presumably already phosphorylated, the addition of kinase inhibitors might have had no effect because further phosphorylation is unnecessary. Therefore, I tested the effect of adding kinase inhibitors after the extracts had been treated with alkaline phosphatase. The addition of kinase inhibitors to dephosphorylated extracts would presumably inhibit the rephosphorylation of proteins. The addition of FSBA did inhibit the replication of genome RNA (figure 18) but there was also a decrease in mRNA synthesis (figure 19). Thus, the use of phosphatase inhibitors was not helpful in determining the role of phosphorylation in *in vitro* RNA synthesis by VSV.

Figure 16. Effect of addition of protease and kinase inhibitors on the synthesis of RNAs by VSV-infected cell extracts. VSV wild type infected cell extracts (5 X 10^6 cells/lane) were treated with the amounts of the reagents indicated above each lane for 60 minutes at 30 °C. Then, ^{32}P-UTP was added and the reactions were incubated an additional 60 minutes at 30 °C. The ^{32}P-labelled RNAs were isolated and analyzed as described. The positions of the viral mRNAs are indicated on the left.

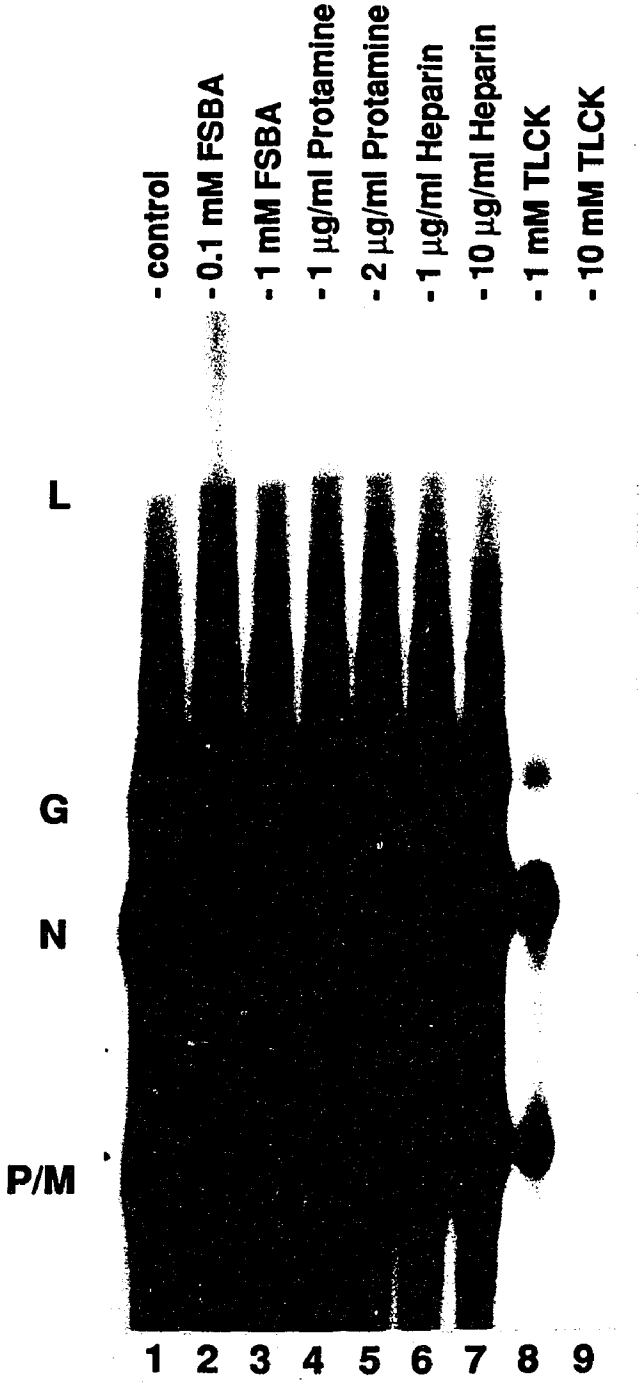

- control
- 0.1 mM FSBA
- 1 mM FSBA
- 1 µg/ml Protamine
- 2 µg/ml Protamine
- 1 µg/ml Heparin
- 10 µg/ml Heparin
- 1 mM TLCK
- 10 mM TLCK
L
G
N
P/M
1 2 3 4 5 6 7 8 9

Figure 17. Effect of addition of protease and kinase inhibitors on the synthesis of nuclease resistant RNAs by VSV-infected cell extracts. VSV wild type infected cell extracts (5 X 10^6 cells/lane) were treated with the amounts of the reagents indicated above each lane for 60 minutes at 30 °C. Then, ^{32}P-UTP was added and the reactions were incubated an additional 60 minutes at 30 °C. The ^{32}P-labelled nuclease resistant RNAs were isolated and analyzed as described. The position of the viral 42S genomic RNA is indicated on the left.

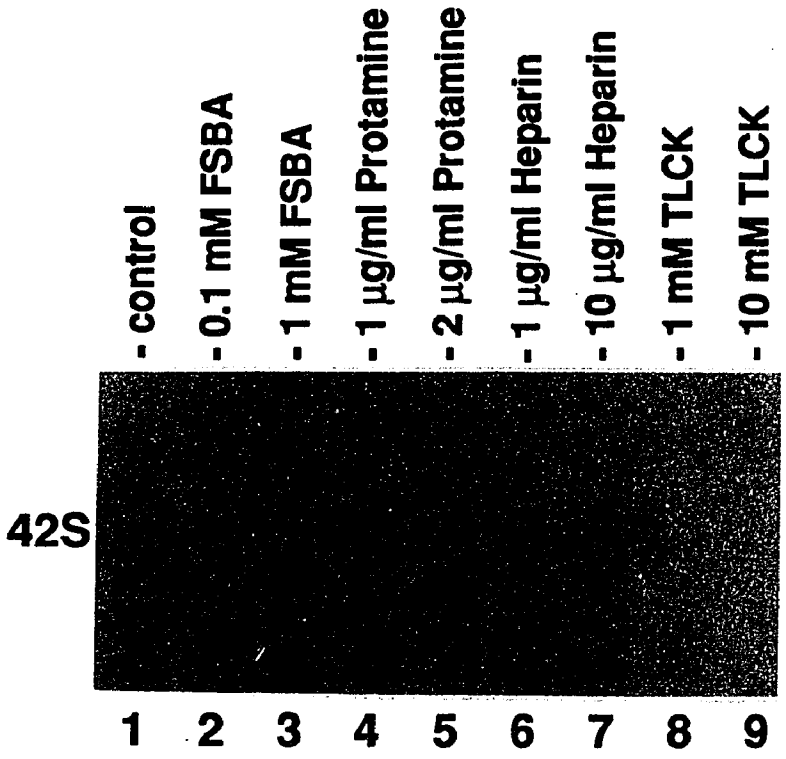
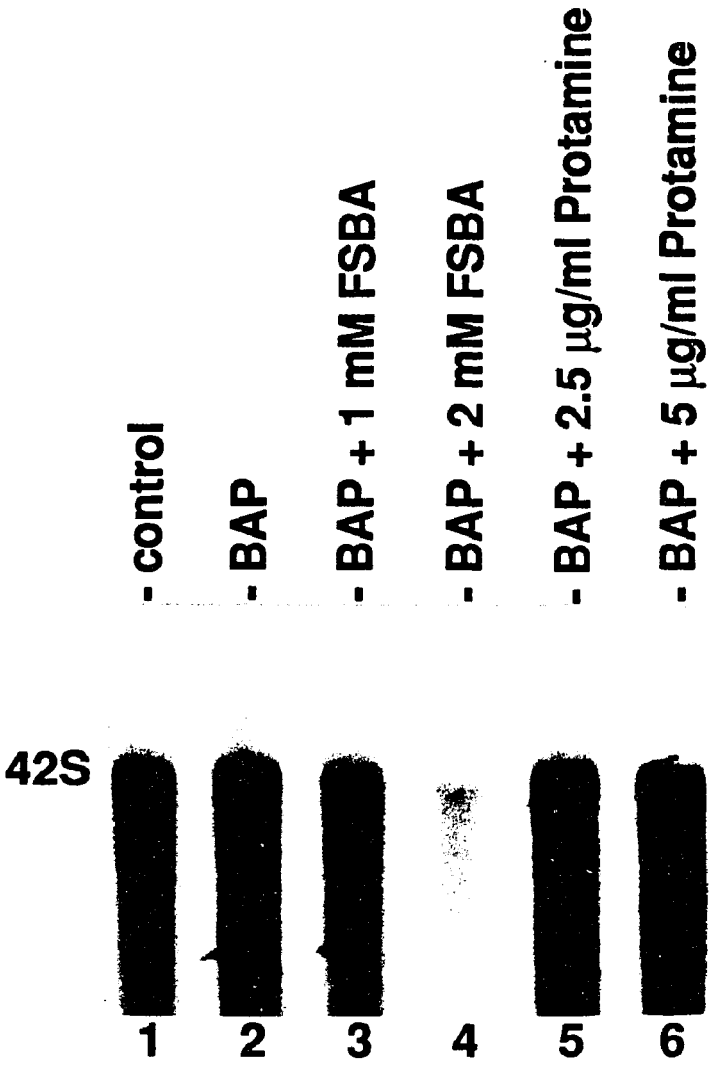

- control
- 0.1 mM FSBA
- 1 mM FSBA
- 1 µg/ml Protamine
- 2 µg/ml Protamine
- 1 µg/ml Heparin
- 10 µg/ml Heparin
- 1 mM TLCK
- 10 mM TLCK
42S
1 2 3 4 5 6 7 8 9

Figure 18. Effect of addition of protease and kinase inhibitors along with BAP on the synthesis of RNAs by VSV-infected cell extracts. VSV wild type infected cell extracts (5 X 10^6 cells/lane) were treated with the amounts of the reagents indicated above each lane (BAP treatment was 1.2 units) for 60 minutes at 30 °C. Then, the BAP was inhibited, ^{32}P-UTP and fresh NTPs were added and the reactions were incubated an additional 60 minutes at 30 °C. The ^{32}P-labelled RNAs were isolated and analyzed as described. The positions of the viral mRNAs are indicated on the left.


- control
- BAP
- BAP + 1 mM FSBA
- BAP + 2 mM FSBA
- BAP + 2.5 µg/ml Protamine
- BAP + 5 µg/ml Protamine
42S
L
G
N
P/M
1
2
3
4
5
6

Figure 19. Effect of addition of protease and kinase inhibitors along with BAP on the synthesis of nuclease resistant RNAs by VSV-infected cell extracts. VSV wild type infected cell extracts (5 X 10^6 cells/lane) were treated with the amounts of the reagents indicated above each lane (BAP treatment was 1.2 units) for 60 minutes at 30 °C. Then, the BAP was inhibited, ^{32}P-UTP and fresh NTPs were added and the reactions were incubated an additional 60 minutes at 30 °C. The ^{32}P-labelled nuclease resistant RNAs were isolated and analyzed as described. The position of the viral 42S genomic RNA is indicated on the left.


- control
- BAP
- BAP + 1 mM FSBA
- BAP + 2 mM FSBA
- BAP + 2.5 µg/ml Protamine
- BAP + 5 µg/ml Protamine
42S
1
2
3
4
5
6

STUDIES OF THE SMALL GENE PRODUCTS OF THE VSV P GENE

The P gene of VSV has the potential to encode several small proteins in addition to P itself. Herman (1986) showed that the P gene of VSV encoded an additional small protein in the same reading frame as P. This protein was named 7k due to its migration on SDS-PAGE gels. In analogy to the designations used for the Sendai virus P gene, this protein may be called X. It is a small, basic protein of 61 amino acids in length produced by initiation of translation at an internal AUG located at position 623 of the P gene. No other studies have been done on the X (7k) protein of VSV.

More recently, Spiropoulou and Nichol, (1993) reported the identification of two small, highly basic proteins encoded by the P gene of VSV New Jersey. Studies of the sequence conservation among different isolates of this serotype of VSV had indicated that an additional protein might be encoded by the first half of the P gene in a +1 reading frame relative to P. Antisera prepared against a peptide corresponding to 20 amino acids of the putative protein precipitated a protein of the appropriate size from infected cell extracts. Analysis of the sequence of the P genes from isolates of both serotypes of VSV reveals that, although the sequence of the second ORF is not conserved between the two serotypes, there is conservation within isolates of both serotypes of a open reading frame which could encode a small, highly basic protein.

Association of Small Viral Proteins with the P Protein in VSV-Infected Cells

Since the C proteins had been detected in cells infected with VSV-New Jersey, I conducted several experiments to try and find the C proteins in cells infected with VSV-Indiana. Since it had been hypothesized that the C proteins

may be involved in RNA synthetic events, and since no antibody was yet available for the Indiana C proteins, I decided to see if I could detect the association of the C proteins with the P protein by coprecipitation using anti-P antibodies. As a preliminary experiment, I simply performed non-denaturing immunoprecipitations of ^{35}S-labelled infected cell soluble protein with several anti-P antibodies and analyzed the precipitates on gels optimized for resolution of low molecular weight proteins. When this was done, I detected a small protein of approximately 9 kd which was immunoprecipitated by the 6D11 antibody (figure 20). This protein was immunoprecipitated exclusively by the 6D11 antibody and not by any of the other anti-P antibodies or the anti-N antibody. This protein was not detected by western blot of immunoprecipitates by the 6D11 antibody (data not shown), so it appeared that it was co-precipitating with the P protein brought down by 6D11 and not directly binding to the antibody.

In order to determine the origin of the small protein and since *in vitro* translation of mRNA had been used by Herman (1986) to detect the X protein of VSV, I next performed immunoprecipitations of ^{35}S-labelled proteins from *in vitro* translation of the P gene message in a rabbit reticulocyte lysate. The P gene message was used because I thought that the P protein was binding to the small protein seen in infected cell soluble protein. Immunoprecipitation of these samples also revealed a small protein in the 6D11 precipitate only (figure 21). This was interesting because the 6D11 antibody was shown to bind to a soluble protein which was necessary for replication and I thought that it was possible that the association of the small protein with 6D11 bound P protein might have been partly or wholly responsible for the effects of this antibody on replication of genome RNA.

Figure 20. Analysis of small proteins coprecipitating with antibodies to VSV proteins. Soluble proteins (non--denaturing) were prepared from ^{35}S-labelled infected cells and immunoprecipitated with 1 μg of the indicated antibodies (lanes 1-6). The immunoprecipitates were analyzed by 16.5% acrylamide tricine SDS-PAGE. Lane 7 shows ^{35}S-labelled proteins from *in vitro* translation of mRNA containing the C'/C ORF. The migration of low molecular weight standard proteins is indicated to the right of the figure.

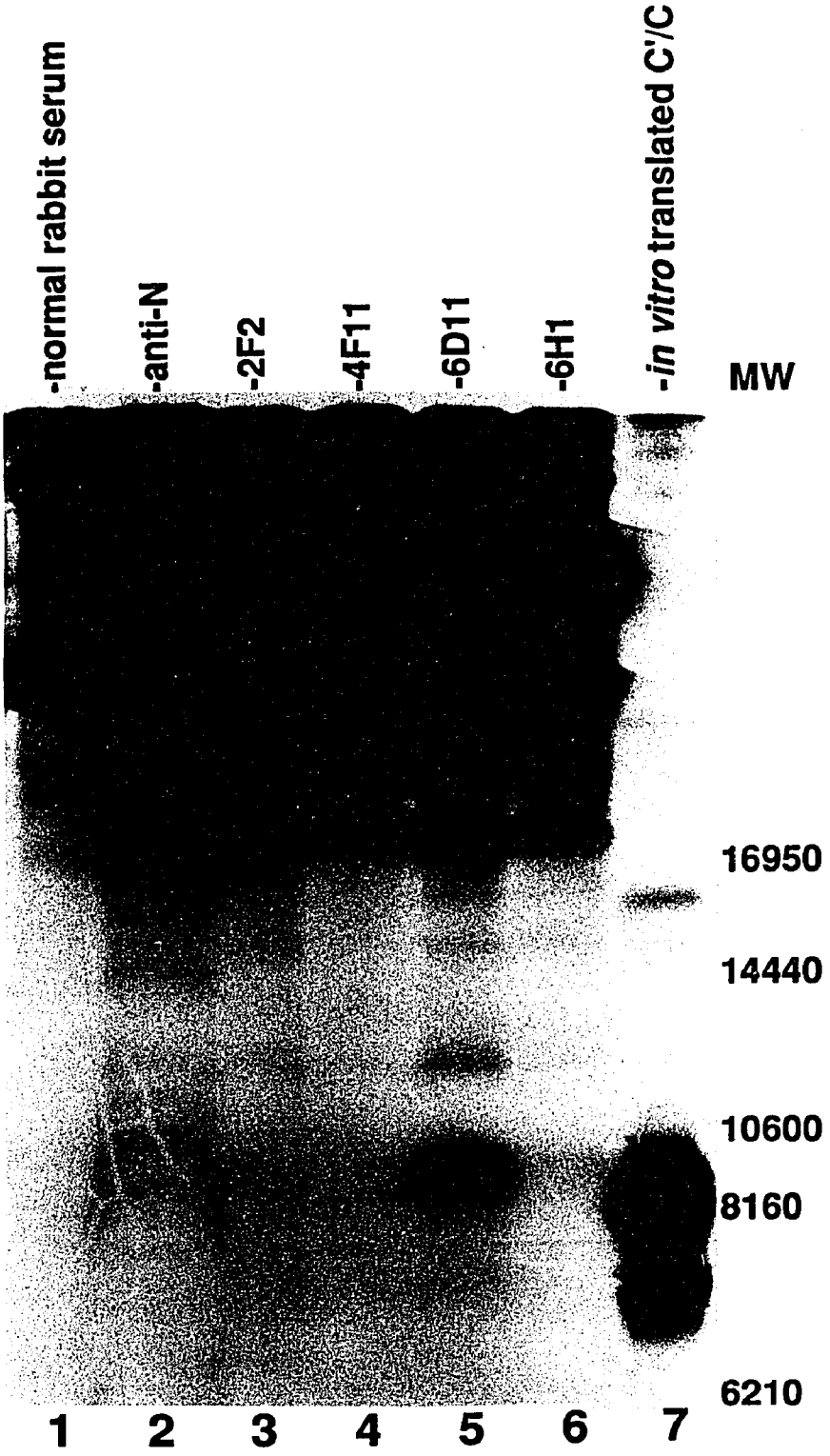
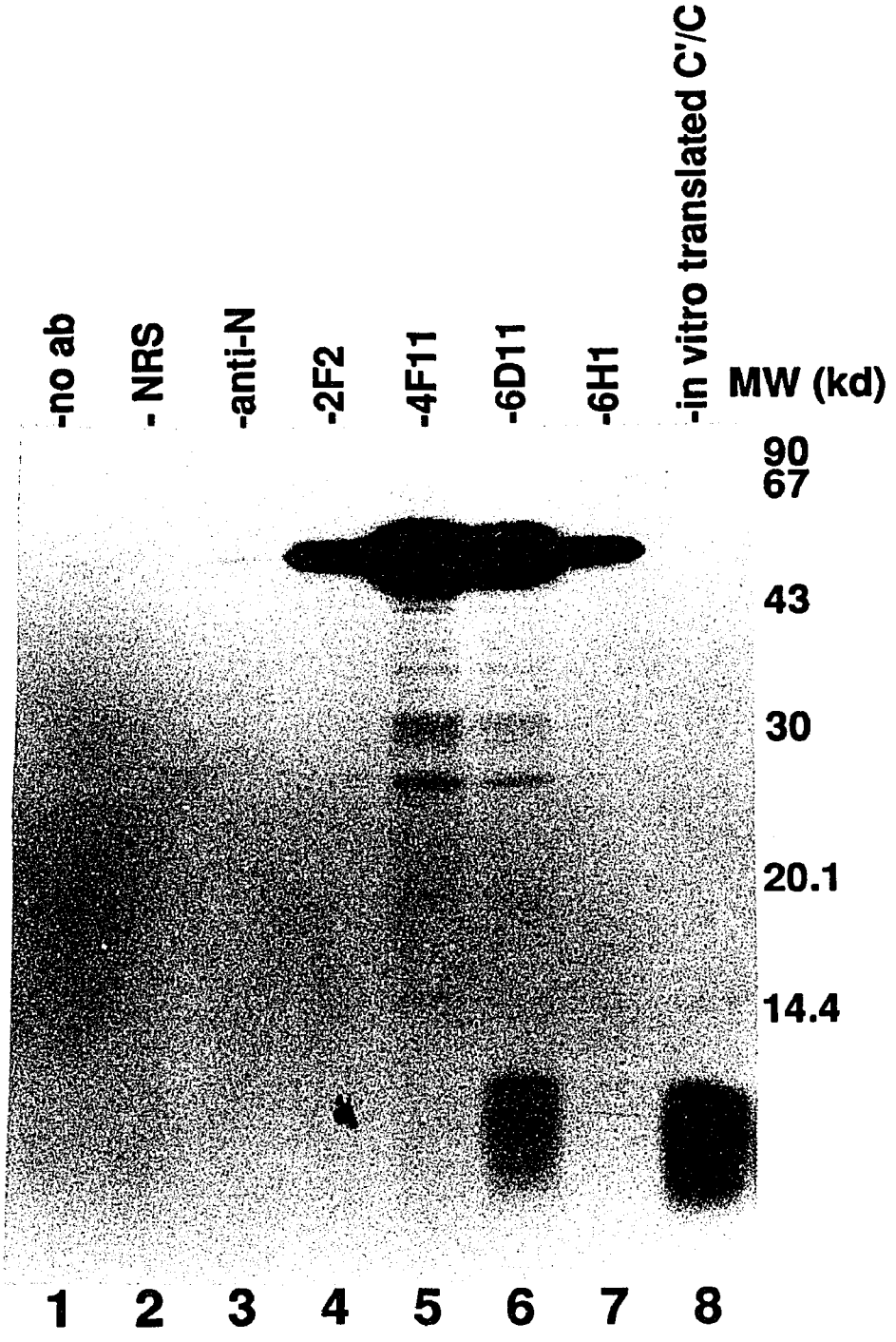

-normal rabbit serum
-anti-N
-2F2
-4F11
-6D11
-6H1
-in vitro translated C'/C
MW
16950
14440
10600
8160
6210
1
2
3
4
5
6
7

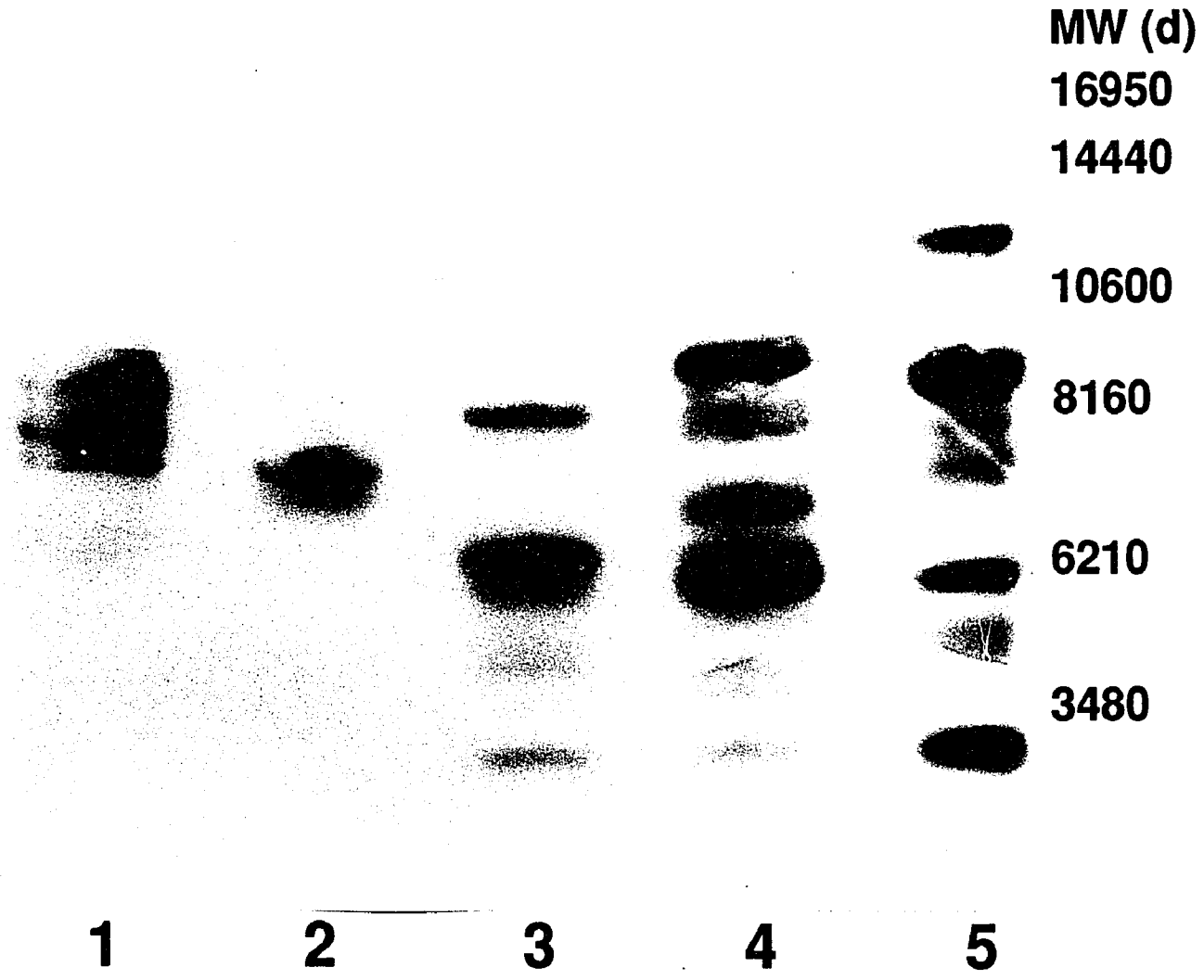
Figure 21. Coprecipitation of small proteins in immunoprecipitations of *in vitro* translation products of the P gene message. ^{35}S-labelled proteins from *in vitro* translation of mRNA containing the P gene message were immunoprecipitated with 1 μg of the indicated antibodies (lanes 1-7). The immunoprecipitates were analyzed by 18% acrylamide SDS-PAGE. Lane 8 shows ^{35}S-labelled proteins from *in vitro* translation of mRNA containing the C'/C ORF. The migration of low molecular weight standard proteins is indicated to the right of the figure.


-no ab
- NRS
-anti-N
-2F2
-4F11
-6D11
-6H1
-in vitro translated C'/C
MW (kd)
90
67
43
30
20.1
14.4
1
2
3
4
5
6
7
8

Peptide Mapping of the Small Protein Precipitated by 6D11

In order to demonstrate whether the small protein I detected was actually the C protein, I performed peptide mapping analyses of the proteins from 6D11 immunoprecipitates along with authentic C' and C proteins from *in vitro* translations of the C ORF. I also analysed the P protein immunoprecipitated by the 6D11 antibody as a control. In the first dimension, the precipitates of the 6D11 antibody sometimes resolve into two small bands migrating at approximately 10kd and 9kd (data not shown). Both of these bands were analyzed in this experiment. The results (figure 22) show that the small proteins precipitated by the 6D11 antibody are not the C' or the C proteins. There are many more bands present than would be expected from digestion of the C proteins. The peptide mapping profiles of the two small proteins (lanes 3 and 4) share some similarities with the P protein (lane 5), such as the bands at 2400, 6200 and 8200 daltons and therefore I thought that these bands might represent the X protein of Herman (1986). The C' protein (lane 1) has two possible cleavage sites and was digested to give three bands: the top band corresponds to the uncut C' protein, the second band corresponds to the expected cleavage product from a single cut between residues 7 and 8, and the third band corresponds to the cleavage product from a cut between residues 20 and 21. The C protein (lane 2) has only one cleavage site and was digested into 2 bands corresponding to the uncut protein and the cleavage product from a single cut between residues 8 and 9. The origin of the sequence encoding the small proteins immunoprecipitated by the 6D11 antibody was confirmed by another member of the lab when immunoprecipitations were done on ^{35}S-labelled proteins from *in vitro* translations of an mRNA which contained the C'/C coding sequences but did not contain the downstream sequences which encode X. This experiment showed

Figure 22. Peptide mapping by limited proteolysis using the endoproteinase Glu-C. Selected proteins were removed from 16.5% acrylamide tricine SDS-PAGE gels containing immunoprecipitations of soluble cell proteins by the 6D11 antibody and proteins from *in vitro* translation of mRNA containing the C'/C ORF. These proteins were subjected to proteolysis as described in the methods and the digestion products were analysed on a 17.5% acrylamide, 0.45% bis-acrylamide SDS-PAGE gel. Lanes 1 and 2 represents the bands corresponding to *in vitro* translated C' and C proteins, respectively. Lanes 3 and 4 represent two bands taken from the 9 kd and 10 kd regions of a lane containing proteins precipitated by the 6D11 antibody from infected cells. Lane 5 contains the band corresponding to the P protein immunoprecipitated by 6D11 and analysed by 10% SDS-PAGE. The migration of low molecular weight standard proteins is indicated to the right of the figure.

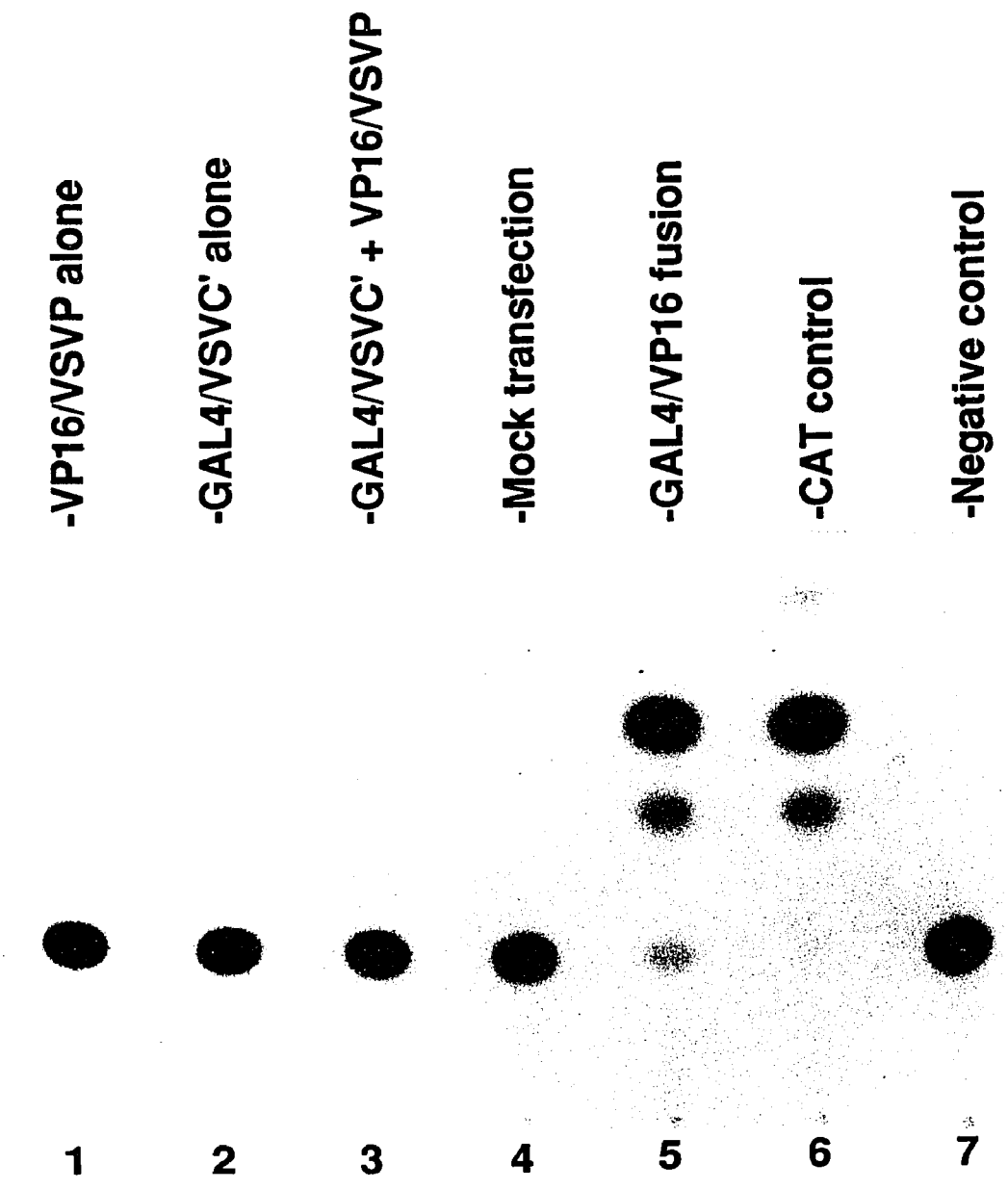

MW (d)
16950
14440
10600
8160
6210
3480
1
2
3
4
5

that the existence of the 9 kd protein in *in vitro* translations of P gene sequences depended on the expression of the sequences encoding the X protein (data not shown). The above result was disappointing in that the small proteins were not the C proteins but this is the only confirmation of the findings of Herman (1986) regarding the presence of the X protein in infected cells. I did not investigate the X protein further since I was concentrating my efforts on examining the C proteins but I believe this protein does merit further study since it is possible that this small protein has some function in infected cells

Test for the Interaction between C' and P Using the GAL4-VP16 Two Hybrid System

If the C proteins do function in VSV RNA synthesis, they may do so by binding to and interacting with other proteins which function in these processes. Since the P protein is known to function in RNA synthesis, I tested for an interaction between the C' protein and the P protein using a two hybrid system utilizing fusion proteins containing the GAL4 and VP16 protein sequences. In this study, the C' gene of VSV was fused to the coding sequences for the DNA binding motif of the GAL4 protein and the P gene of VSV was fused to the transactivation domain of VP16. Plasmids containing the fusion genes were transfected into cells and expressed, along with a reporter containing the chloramphenicol acetyl transferase gene downstream of five GAL4 binding domains. A typical experiment is shown in figure 23. These experiments failed to detect an interaction between the GAL4VSVC fusion protein and the VP16VSVP fusion protein (lane 3). Similar results were obtained when the corresponding fusion proteins GAL4VSVP and VP16VSVC were used (data not shown). Also, this system was unable to detect the interaction of GAL4VSVC

Figure 23. CAT assay of cells transfected with plasmids encoding fusion proteins containing GAL4 and VP16 sequences. The plasmids indicated above each lane were transfected along with the pUASGTATACAT reporter plasmid into BHK cells. 48 hours after transfection, the CAT activity in these cells was determined. The CAT and negative controls are described in the methods section.


-VP16/VSVP alone
-GAL4/VSVC' alone
-GAL4/VSVC' + VP16/VSVP
-Mock transfection
-GAL4/VP16 fusion
-CAT control
-Negative control
1
2
3
4
5
6
7

with VP16VSVC or GAL4VSVP with VP16VSVP (data not shown).

Immunoprecipitations of Infected Cell Proteins by Antibody to the C' Protein

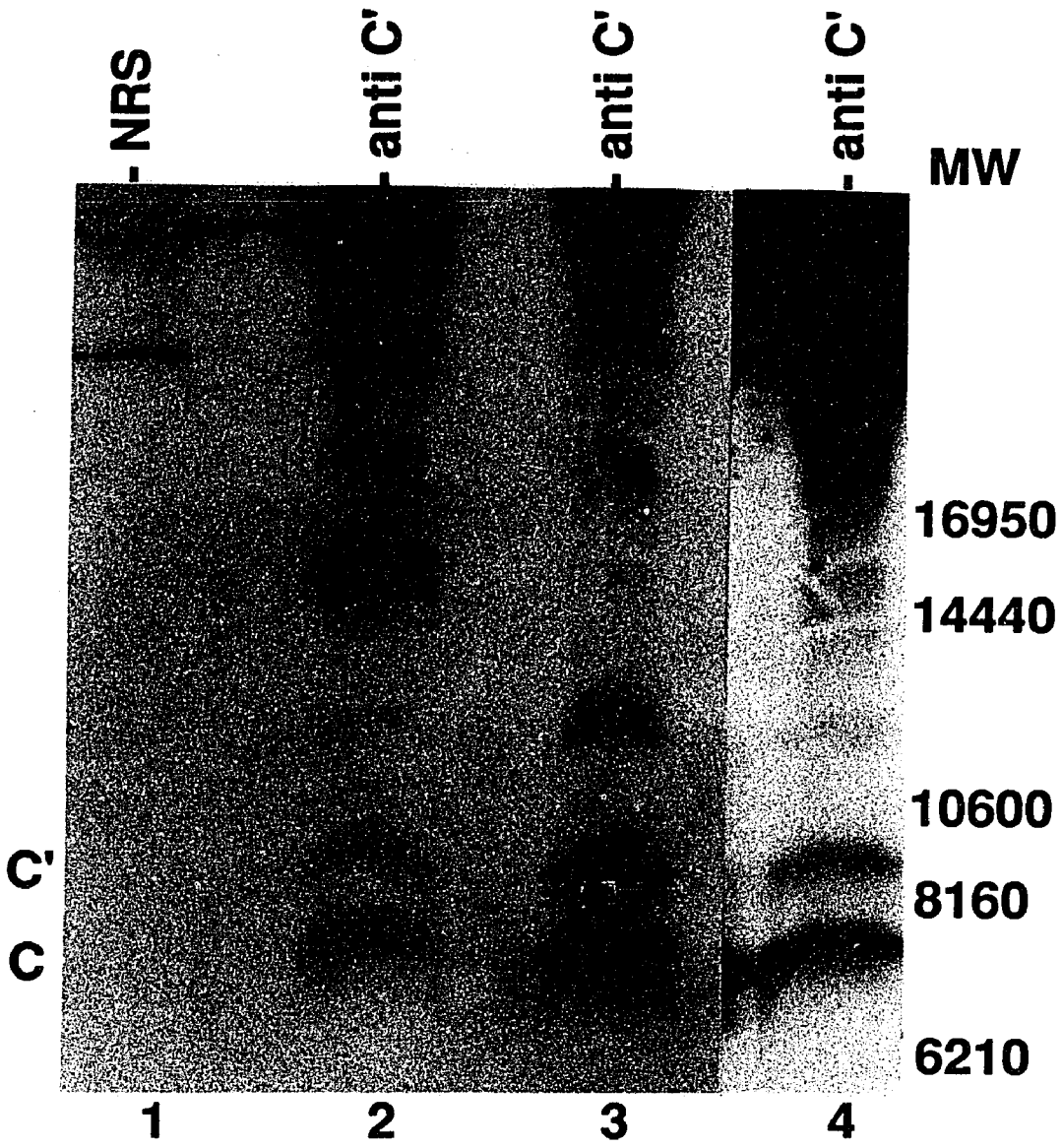
Antibodies to a synthetic peptide corresponding to 23 aa of C'. In order to determine if the C proteins were made during infections by VSV-Indiana, antisera was prepared against a 23 a.a. portion of the C proteins of this serotype (residues 13-35 of C', 1-23 of C), similar to that prepared by Spiropoulou and Nichol, (1993). The antisera prepared by immunization of a rabbit with this peptide was unable to precipitate any proteins from infected cells although it did react against the peptide used in immunization. It should be noted that the C proteins translated *in vitro* bind non-specifically to protein G agarose and thus are not suitable for testing of antisera. The problem with antibody prepared against the C peptide had been encountered before with peptides from Sendai virus C protein (Portner *et al.*, 1986). When a peptide corresponding to the unique N-terminal amino acids of the Sendai virus C protein was used to immunize a rabbit, the antisera raised failed to react with authentic C protein even though it had a high titer against the immunogen. The authors hypothesized that the peptide had assumed a non-native conformation and had immunogenic epitopes that were not present on the full-length molecule. This could be the problem with my peptide antisera also.

Antibodies to a glutathione-S-transferase-C' fusion protein. I next prepared a fusion protein consisting of the glutathione-S-transferase protein fused to the entire coding sequence for the C' protein. It was expected that expressing the full-length C' protein would enhance the chance that antibodies produced to the fusion protein would react with the C proteins in infected cells.

Fusion proteins containing the glutathione-S-transferase protein can be easily purified using commercially available glutathione-agarose resin. This protein was cloned and expressed in bacteria and purified by virtue of its association with glutathione-agarose beads. The purified protein was used to immunize a rabbit and antisera was obtained. The antisera prepared in this manner was found to react exclusively with the GST portion of the fusion protein and thus was not useful for our studies. Since the C' protein is small (8kd)relative to the GST protein (30 kd), I thought that the larger GST protein may have masked the immunogenicity of the C' protein. Therefore, a different strategy was called for in order to prepare antisera which would react with C proteins.

Antibodies to a histidine tagged C' protein. As a third attempt, the C' coding sequence was cloned into a QIAexpress vector in frame with 6 histidine residues. The histidine residues provide a means of purifying the fusion protein by virtue of its affinity for nickel containing resin . When this protein was expressed, purified, and used to immunize a rabbit, it elicited antibodies which were capable of precipitating proteins from infected cells. The antisera prepared in this manner was used to immunoprecipitate ^{35}S-labelled infected cell extracts. The anti-C' antisera was able to precipitate two small proteins with migrations on tricine-SDS polyacrylamide gels similar to that expected of the C proteins (figure 24) from RIPA lysates of infected cells (lane 2) as well as from the soluble protein fraction (lane 3) and purified nucleocapsids (lane 4). I was unable to detect the C proteins in purified virions either by immunoprecipitation of ^{35}S-labelled cell extracts, by western blotting of purified virus, or by westerm blotting of immunoprecipitates of virus proteins (data not shown). It is possible that the C proteins are present in very small quantities in virus particles and the use of more sensitive methods may resolve this question.

Figure 24. Immunoprecipitation of C proteins from infected cells using the anti-his-C' antisera. Samples containing ^{35}S-labelled proteins were immunoprecipitated with the antibodies indicated above each lane. The samples in lanes 1 and 2 are from RIPA lysates of infected cells. The sample in lane 3 is from soluble protein prepared from infected cells and the sample in lane 4 is from RNPs purified by density and velocity gradients. The positions of the C' and C proteins are shown on the left while the migration of low molecular weight standard proteins is indicated to the right of the figure.

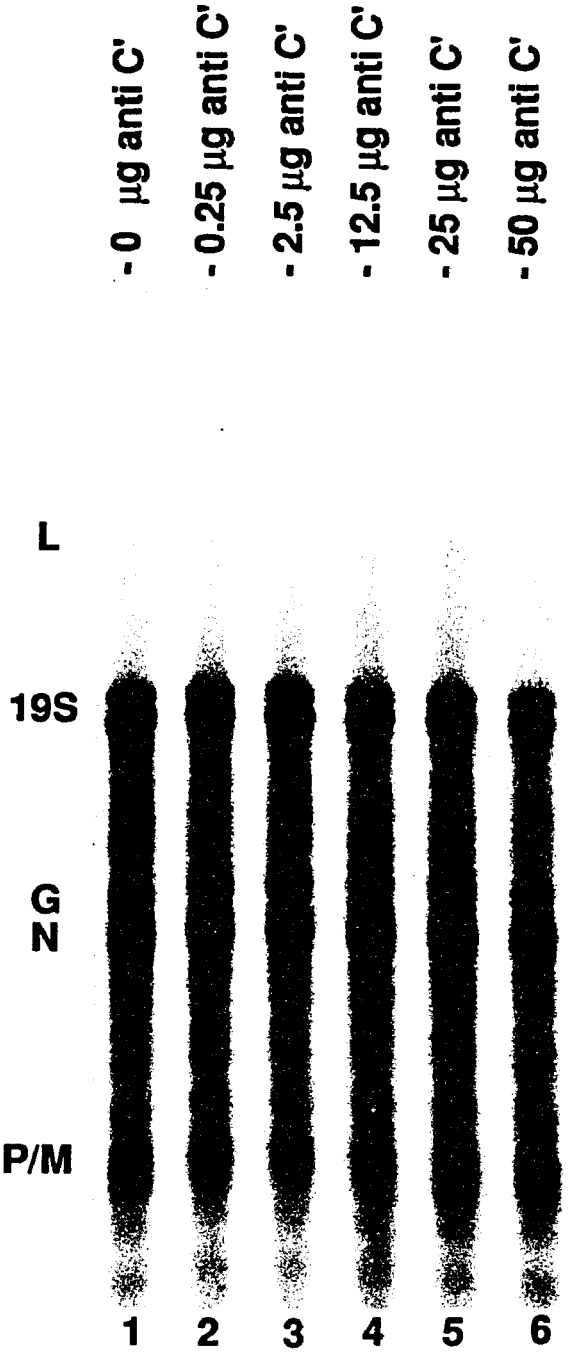

NRS
anti C'
anti C'
anti C'
MW
16950
14440
10600
8160
6210
C'
C
1
2
3
4

Effect of Anti-C Antisera on *in vitro* RNA Synthesis

In this study and others, antibodies to viral proteins have been used as probes for determining the function of specific proteins. Since the anti-C' antibody was shown to bind the C proteins present in infected cells, I tested whether addition of this antibody to infected cell extracts had any effects on VSV RNA synthesis. When RNA synthesis in the presence of anti-C' antibodies was examined, it was seen that the antibodies had no effect on *in vitro* RNA synthesis, even at high levels (figure 25). I also tested whether removal of soluble C proteins affected RNA synthesis using the same method used for treatment of soluble protein with the anti-P antibodies. The removal of soluble C proteins from the soluble protein fraction had no effect on reconstituted *in vitro* RNA synthesis (data not shown).

Three possibilities exist to explain the above results. First, the C proteins may not function during RNA synthesis at all or just under the *in vitro* conditions used in this study. This possibility can not be discounted but several lines of evidence (see Literature Review) indicate that this may not be true. Second, the C proteins functioning in infected cells may not be available for binding to the antibody. Since the C proteins are very small in size, they could easily be covered by the larger proteins present in ribonucleoprotein templates. Third, binding of the anti-C' antibody to C proteins may not impede their functions. The inability to block RNA synthesis by depleting soluble protein with anti-C' may be explained by the fact that the nucleocapsids present in the RNP fraction, which were shown to have C proteins associated, may be saturated with these components and the soluble C proteins may be in excess of what is required for RNA synthesis.

Figure 25. Effect of antibodies to his-C' protein on *in vitro* RNA synthesis by wild type and DI co-infected cell extracts. The ^{32}P-labelled RNAs synthesized in the presence of the indicated amounts of anti-his-C' antibody were isolated and electrophoresed on a 1.5% acid urea agarose gel. The positions of the viral mRNAs and the 19 S MS-T genomic RNA are indicated on the left.


- 0 µg anti C'
- 0.25 µg anti C'
- 2.5 µg anti C'
- 12.5 µg anti C'
- 25 µg anti C'
- 50 µg anti C'
L
19S
G
N
P/M
1
2
3
4
5
6

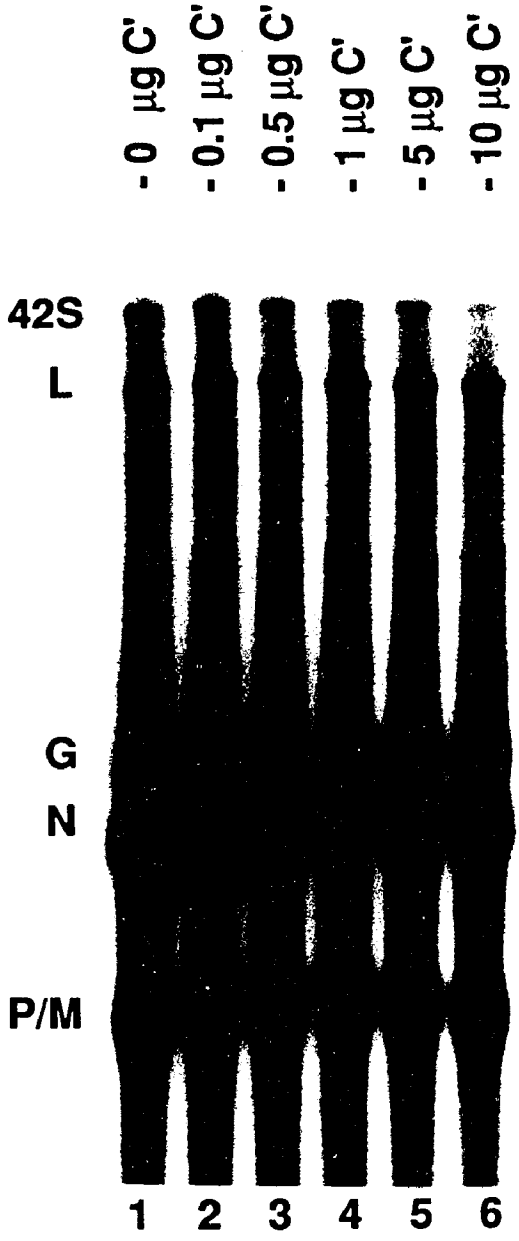
Effect of His-C' Protein on *in vitro* RNA Synthesis

Since I could obtain purified C' protein from the expression of the fusion construct in bacteria, and since this fusion protein was shown to have epitopes which were present on the proteins present in infected cells, I tested whether the addition of purified C' to infected cell extracts had any effect on *in vitro* synthesis of RNA. Addition of purified His-C' had no effect on *in vitro* viral RNA synthesis (figure 26), except at the highest level used (10 μg, lane 6). Again, it is possible that the components needed for genome RNA replication, which are present in the forms and quantities needed for RNA synthesis, are sufficient and excess C' protein may not have a chance to function. Also, in order for proper function, some proteins which interact with each other need to be coexpressed at the same time in the same cell (Horikami *et al.*, 1992). Thus, the preformed, purified his-C' protein may not be able to make up the functional form of a putative protein complex.

Phosphorylation of C' and X

Since the P protein is highly phosphorylated and the P and X proteins share sequences which contain potential phosphorylation sites, I performed immunoprecipitation of ^{32}P-labelled infected cell lysates with the 6D11 antibody. Since the C proteins of Sendai virus have been shown to be phosphorylated (Hendricks *et al.*, 1993), I also used the anti-C' antibody to try and determine whether the C proteins were also phosphorylated in infected cells. No bands corresponding to the small non-structural proteins of VSV were detected in the precipitates (data not shown).

Figure 26. Effect of addition of purified his-C' protein on *in vitro* RNA synthesis by VSV-infected cell extracts. The ^{32}P-labelled RNAs synthesized in the presence of the indicated amounts of his-C' were isolated and electrophoresed on a 1.5% acid urea agarose gel. The positions of the viral mRNAs and 42S genomic RNAs are indicated on the left.


- 0 µg C'
- 0.1 µg C'
- 0.5 µg C'
- 1 µg C'
- 5 µg C'
- 10 µg C'
42S
L
G
N
P/M
1
2
3
4
5
6

DISCUSSION

The P Protein

The phosphoprotein P of VSV is known to have multiple functions in infected cells. It is an essential subunit of the viral RNA polymerase and is thought to bind directly to the nucleocapsid template and facilitate the association of the catalytic subunit, the L protein, to the template (Mellon and Emerson, 1978; Thornton *et al.*, 1984). The P protein alone is not believed to have an enzymatic function during RNA synthesis. In addition, the P protein also functions during genome RNA synthesis by complexing with the N protein to form the functional substrate for the encapsidation of newly synthesized genome-length RNAs (Peluso and Moyer, 1984, 1988; LaFerla and Peluso, 1989). The P protein which binds to N functions as a chaperone to prevent free N protein from self-assembling into inactive complexes. The N:P complexes bind to newly synthesized replication products during nucleocapsid assembly. In this process, the complex is dissociated and the P protein is released into the cytoplasm while the N protein becomes bound to the nucleocapsid. The possibility that the P protein carries out additional functions during the viral life cycle is being actively investigated.

It has long been recognized that the P protein exists in infected cells and virions as a heterogeneous pool of differentially phosphorylated molecules. This observation, along with the early identification of different functional subsets of P protein that differred in their phosphate content, led to the hypothesis that the function of P is regulated by phosphorylation (Kingsford and Emerson, 1980; Masters and Banerjee, 1986). Recent studies have made it clear that phosphorylation of specific residues regulates the function of P in RNA

synthesis. The phosphorylation of two specific amino acids in the negatively charged amino terminal half of the P protein has been shown by several groups to be required for the activity of the P protein in transcription (Barik and Banerjee, 1992a,1992b; Gao and Lenard, 1995). Recently, it has been suggested that phosphorylation of P regulates its activity during replication as well (Chang *et al.*, 1994).

An interesting extension of the above work is the recent suggestion that phosphorylation of P may regulate complex formation by the P protein (Gao and Lenard, 1995). This group showed that the transcriptionally active form of the P protein is a multimer, probably a homotetramer. Phosphorylation of P protein by a casein-kinase II-like activity is required for the formation of this complex. Since the formation of a heterodimer complex between the N and P proteins is not believed to require phosphorylation, the phosphorylation and dephosphorylation of P may regulate the balance between the phosphorylated, transcriptionally active homotetramer and the N:P heterodimer which is required for replication.

I present evidence here that indicates that a soluble form of P protein performs an essential function during genome RNA replication that is unnecessary during viral transcription. This hypothesis is based on the fact that several anti-P monoclonal antibodies, when added to extracts of VSV-infected cells that support transcription and replication of the viral genome, specifically block genomic RNA synthesis and nucleocapsid assembly while having minimal effects on transcription. The anti-P antibody 6D11 was shown to inhibit replication by binding to P protein that was soluble and not complexed with N protein. This distinction is important since P complexed with N is necessary for the production of encapsidated, full-length genome RNA. In the reconstituted reactions discussed in this study, there is a functioning RNA polymerase that is

capable of producing transcription products. There are also N:P complexes present which should be able to encapsidate the products of replicative RNA synthesis. According to the most prevalent model of replication (Blumberg and Kolakofsky, 1981; Arnheiter *et al.*, 1985), these two components should be sufficient to produce full-length genomic RNAs from the RNPs present in infected cells. However, in soluble protein depleted of 6D11-reactive P protein there is a loss of the ability to support replication. At this time I are unable to determine the specific function of this soluble P protein during the replication process. The 6D11 bound P protein does associate in a soluble complex with some L protein (figure 9). The removal of this small amount of L-P complex has no effect on transcription but the loss of L protein could contribute to the inability of the depleted soluble protein to support replication, particularly if this L:P complex is responsible for some critical aspect of replicative RNA synthesis.

The present study is an extension of the previous work of Williams *et al.* (1988a, 1988b). Their work showed that different monoclonal anti-P antibodies could affect different steps of viral transcription when added to an *in vitro* system consisting of disrupted virions. In those studies, the 6D11 antibody was able to block the initiation of RNA synthesis by viral nucleocapsid templates while the 2A2 antibody, which reacts with N protein, blocked the elongation of initiated transcripts. It is possible that the soluble P protein bound by 6D11 is involved in the initiation of replication but not initiation of transcription. The experiments described in this paper can not discriminate between the inhibition of initiation of replication and inhibition of later steps in the replicative process. Since the disrupted virus is a simpler, less biochemically complex system, it is possible that the 6D11 antibody could bind to P protein which is not available for binding under the conditions present in infected cells.

Since the P protein which is bound by the 6D11 antibody is so crucial to replication, it would be useful to know what structural features distinguish this P protein from the rest of the P protein found in infected cells. The depletion of soluble protein by 6D11 antibody does not remove all of the P protein found in this fraction. At levels sufficient to totally block replication, the depletion protocol removes about one half of the 6D11 reactive P protein from a sample of the soluble protein pool (figure 9), and a much smaller percentage of the total soluble P protein in the cell. Thus the inhibition of replication is consistent with the hypothesis that decreasing the level of some critical component below a certain point results in the shutoff of replication. The only information that I determined about the unique nature of the 6D11-bound P protein is that the phosphorylated state of an unknown residue was shown to be different in this subset of P protein than that which binds to an antibody (2E1) which has no effect on RNA synthesis. The dephosphorylation of soluble protein by BAP results in more P protein being available for binding to the 6D11 antibody but less for binding to the 2E1 antibody. Williams *et al.* (1988a) mapped the binding sites of these antibodies to the same region, amino acids 177-227, of the P protein. They also showed that these two antibodies competed for binding to P protein in plate binding assays, indicating that they both bind to similar regions. Our findings indicate that the epitopes bound by these antibodies are changed by dephosphorylation. The 2E1 antibody binds to a P protein which is phosphorylated at an unspecified site while the 6D11 antibody binds to P which is not phosphorylated at some site. It is possible that these antibodies bind to epitopes which are affected by phosphorylation of the same amino acid, but this remains to be determined.

It is important to note that the amount of BAP used to demonstrate an increase in P protein binding by monoclonal antibody 6D11 is greater than the

amount required to stimulate genome replication. There may not be a direct correlation between the measured increase in 6D11 precipitable P protein and the increase in genome RNA replication. However, it is also possible that the enzyme treatment is in fact stimulating the level of genome RNA replication by its action on the P protein, and this is not being seen in the immunoprecipitation assays I employed, where subtle changes in protein levels would not be detected. If the dephosphorylated form of the P protein is required in catalytic rather than stoichiometric amounts, a small increase in the level of this protein could have a large functional effect.

Recently published results support the hypothesis that an incompletely phosphorylated form of the P protein is required for the production of full-length genome RNAs (Chang *et al.*, 1994). Addition of the phosphatase inhibitor okadaic acid to infected cell extracts results in the accumulation of highly phosphorylated P protein. Treatment of extracts with okadaic acid also results in the inhibition of genome RNA synthesis while transcription continues unaffected. Together with our finding that treatment of infected cell extracts with alkaline phosphatase increased genome replication, these data indicate that phosphorylation of P protein regulates its activity during genome replication as well as transcription, and that hypophosphorylation of P biases RNA synthesis toward genome RNA replication, and away from transcription.

The results presented in this thesis, along with the data cited above, indicate that the P protein is a multifunctional protein with at least three functions during viral RNA synthesis. One is to function as part of the viral transcriptase to produce leader and mRNAs. A second is as a complex with the N protein that serves as the substrate for encapsidation of nascent RNA into ribonucleoprotein templates. Third, soluble P protein must provide some unknown function during replication, possibly as part of a viral replicase. It is

this third function which is apparently inhibited by binding of the 6D11 antibody.

Since phosphorylation of the P protein is important in regulating the RNA synthetic activities of VSV, it would be useful to identify which amino acids and domains of the P protein are most important for replication. This could be accomplished using site-directed mutagenesis to produce specifically altered forms of P protein that could be tested for replicative activity. Recently developed systems which support RNA replication using L, P, and N proteins derived from transfected DNAs would be useful for this type of study (Pattnaik and Wertz, 1990; Pattnaik *et al.*, 1992; Lawson *et al.*, 1995; Stillman *et al.*, 1995). The information from such experiments would provide evidence as to which specific P molecules perform which functions and how the phosphorylation of specific sites regulates these activities.

The X and C Proteins

The results presented in this thesis indicate that VSV-Indiana expresses several proteins from the P gene in addition to P itself. The presence of the small X proteins in infected cells was confirmed and two proteins previously shown to exist in the New Jersey serotype of VSV, the C proteins, were found both soluble and associated with nucleocapsids in cells infected with VSV-Indiana. None of these small P gene products were found in purified virions. I was unable to detect the specific association of the C' protein with either the P protein, the C' protein itself, or any other viral or cellular proteins. I was also unable to detect the phosphorylation of any of these proteins in infected cells. No direct evidence is presented relating to the functions of these proteins during infection by the virus but no data rules out a function for these proteins in RNA synthesis.

It is not surprising that the P gene of VSV encodes multiple proteins since the analogous genes in the closely related paramyxovirus family are multicistronic. Also, the nature of the P protein allows a great deal of sequence variability, which would be suitable for the evolution of alternate reading frames. It has been recognized for a long time that the P protein ORF is not highly conserved between different isolates of both serotypes of VSV. It seems that, as long as certain structural features are conserved, the primary nucleotide and amino acid sequences can be significantly altered. This flexibility of sequence, plus the high error rate of the VSV polymerase, provides the optimal conditions for the evolution of alternate reading frames which could encode functional proteins while still maintaining the function of the P protein. If the proteins produced from such events proved to have a function useful to the virus and conferred a growth advantage, it would be expected that such a reading frame would be conserved.

A study of the sequence variation of the P gene of natural isolates of VSV led to the initial identification of the C proteins of VSV-New Jersey. Since the first half of the P protein sequence (Domain I) can be functionally replaced by that of tubulin in an *in vitro* transcription reaction (Chattopadhyay and Banerjee, 1988), it is not expected that the RNA sequence in this part of the gene would be conserved. In fact, contrary to the expectation, the sequence corresponding to domain I of the P protein is more conserved than that of the either domain II or III (Bilsel *et al.*, 1990). In particular, detailed analysis revealed that the third codon base position, the "wobble" base, was less variable between positions 50 and 250 of the P protein ORF. Since ORF analysis of the VSV genome predicts a second ORF in this region in the +2 reading frame (P is in the +1 frame, the 0 frame is extensively blocked; Herman, 1986), and since the third base position of P relates to the second base position in ORF 2a, the presence of a protein product was investigated. When antibody directed against part of the putative protein was made, it reacted with a protein of approximately the predicted size. This protein was shown to exist in the cytoplasm of infected cells and was not found in purified virus.

The P gene of VSV-Indiana differs from that of VSV-NJ in that it contains four open reading frames (figure 2). The data presented here indicate that two of these can be used to produce four protein products, two (P, and X) from the P ORF and two (C' and C) from the C ORF, by alternate initiation of translation in overlapping reading frames. By analogy with the names of the Sendai virus P gene products these proteins may be called: P, C', C, and X. The C proteins are produced from a reading frame which is +1 relative to that of the P protein. The C' protein reading frame starts at base 84 of the VSV-Indiana P gene and continues until base 284. Translation of this sequence results in a 67 amino acid protein which has a predicted molecular weight of 7936 daltons and an isoelectric

point of 11.37. The smaller C protein starts at base 120 of P and is coterminal with the C' ORF. Initiation of translation at this site results in the production of a 55 amino acid protein which has a predicted molecular weight of 6500 daltons and an isoelectric point of 11.92. The migration of proteins precipitated from infected cells by the antibody directed against the C' protein and the peptide mapping studies confirm that the proteins I show in figures 20 and 21 are indeed the VSV-Indiana equivalent of the C proteins described by Spiropoulou and Nichol (1993). The C proteins were shown in this work to be present in soluble form and associated with nucleocapsids in infected cells. They were not detected in virus particles by either immunoprecipitation with anti-C' antibody or western blot of virus proteins.

The X protein was initially identified by Herman (1986), who called it the 7k protein due to its migration in SDS-PAGE gels. In the present work the X protein, which is analogous to the 7k protein, was identified in imunoprecipitations with the 6D11 antibody. Cleveland peptide maps of the proteins indicated that this protein was not related to the C proteins but instead had sequences in common with the P protein. The identification of this protein as X was confirmed in our lab by the *in vitro* translation of mRNAs carrying the C' ORF with and without the X coding region downstream of the C'/C termination signal. Translation of these mRNAs showed that the coding sequence for the X protein was necessary to produce the protein which migrated in the region of 8-10 kd. The data presented in this study confirmed the findings of Herman (1986) that the X protein was present in the cytoplasm of infected cells and was not found associated with nucleocapsids. The X protein was also not detected in purified virus. Whether the X protein has a function in infected cells or is just the result of an aberrant , low frequency translational event remains to be determined.

It is surprising that the *in vitro* translations of the P mRNA done by Herman (1986) did not reveal the C proteins. Translation of the P gene message produces all of the small gene products of the P gene in addition to P. All of the small proteins are clearly detectable in *in vitro* translations and the use of specific antisera easily allows their detection. These proteins are present in much lower levels in infected cells but are still readily detectable by immunoprecipitation. It is possible that the SDS-PAGE system used in the study of Herman was not able to resolve the C proteins as distinct bands separate from the X protein.

The functions of these proteins in the viral life cycle remain unknown. None of these small proteins were detected in the virus by either immunoprecipitation or western blot. However, they are all present in very low levels in infected cells and it is possible that the methods used would not detect extremely low concentrations of the proteins. If they indeed are not structural components of the virus, this would indicate that they are not required for the initial events that occur when the virus enters a cell. Since purified viral RNPs are capable of transcription, these proteins would presumably have no critical roles in this process.

Analysis of the sequence data from temperature-sensitive mutants hints at a possible role for the C proteins in genome replication. A mutant with a defect in transcription has mutations in the P ORF but not in C while one with a defect in replication has mutations in the coding region of P as well as C (Rae and Elliot, 1986b ; Spiropoulou and Nichol, 1993). Of course, the defects can not be linked exclusively to one or the other proteins because of the shared sequence.

If the ORF containing the C proteins of Sendai virus is knocked out by mutation, *in vitro* replication of a DI genome is unaffected (Curran *et al.*, 1991). In transcription however, the C proteins of Sendai virus have been shown to be inhibitory (Curran *et al.*, 1992). The coexpression of C'/C with the P and L

proteins inhibited transcription of N-RNA template. It has been hypothesized that coexpression of the C proteins with the other polymerase proteins results in the formation of inactive complexes which are inactive in transcription but still active in replication. Whether this is the case with VSV is not known. I was unable to detect the specific association of C proteins with any other proteins by coprecipitation or between the C' and P proteins by using the two hybrid system. Although the experiments designed to investigate whether the C' protein bound specifically to any other proteins failed to show any interactions, the possibility remains that it does so in infected cells. The methods used here may not have the sensitivity to detect an interaction between C' and other proteins. Alternatively, the C' protein may not have been in its native form when expressed as fusion proteins with the GAL4 and VP16 proteins.

The VSV proteins are considerably smaller than those of Sendai virus, 67 aa for the C' protein as compared to 215 aa for that of Sendai C, but all of the C-type proteins have a basic nature. This is interesting because the P protein itself has both acidic and basic domains which react with other proteins and have functional activity on their own. It is possible that the basic C proteins could interact with the P proteins directly or with one of the other proteins by mimicking the basic region of P. Either of these possibilities could affect the function of the P protein and RNA synthesis. It is also possible that these small proteins do not provide a critical function under normal infection conditions but do confer a selective advantage in certain circumstances or in certain cell types, or during the infection of an animal host. The fact that these proteins have been so well conserved throughout the evolution of both serotypes of viruses, even in laboratory strains, suggests that they have an important function which confers a selective advantage.

The X protein is also basic in nature. The X protein of Sendai does not bind to nucleocapsids and its absence has no effect on genome replication (Curran *et al.*, 1992). The X protein of VSV contains both domains II and III of the P protein. These domains are believed to be involved in binding the N-RNA template. A truncated P protein similar to the X protein was shown to be capable of binding to N-RNA and was active in transcription when the negatively charged tubulin molecule was also added (Chattopadhyay and Banerjee, 1987b). This raises the possibility that the X protein could function in RNA synthesis. Since the domains carried by the X protein have been shown to be active in transcription in *trans*, they could be functioning in concert with the acidic domain of P proteins present in infected cells. Expression of the X proteins could also be envisioned to regulate RNA synthesis by competing for binding to N-RNA with full-length P protein.

The data presented above indicates that the P gene of VSV, like those of the closely related paramyxoviruses, encodes several small basic proteins in addition to P. These proteins do not seem to be present in the virus particle but all are found in the cytoplasm of infected cells. The C proteins in particular are interesting because they are found associated with nucleocapsids in infected cells. There is much more to be learned about all of the small proteins encoded by the P gene and the newly developed systems enabling the reverse genetic manipulation of protein coding sequences should provide opportunities to study the structure and functions of these proteins in more detail.

Appendix A: Oligonucleotides

Cloning of GAL4VSVC and VP16VSVP fusion proteins:

1) 5CSAL- upstream of the C' ORF, incorporating a SalI restriction site

C' ORF
5'-CCCGGGTCGACATGAGATCGAAGCAC-3'
SalI

2) 3CSAC- downstream of the C' ORF, incorporating a SacI restriction site

C' ORF
5'-CGCGCGAGCTCAGGCTGTTTC-3'
SacI

3) 5PSAL- upstream of the P ORF, incorporating a SalI restriction site

P ORF
5'-CCCGGGTCGACATGGATAATCTCACA-3'
SalI

4) 3PSAC- downstream of the P ORF, incorporating a SacI restriction site

P ORF
5'-CGCGCGAGCTCCATAGTCTACAGAGA-3'
SacI

Cloning of glutathione-S-transferase-C' fusion protein:

5) JRC3- upstream of the C' ORF, incorporating a BamHI restriction site

C' ORF
5'-CCCGGATCCCCATGAGATCGAAGC-3'
BamHI

6) JRC5- downstream of the C' ORF, incorporating a EcoRI restriction site

C' ORF
5'-GGGAATTCAAGGCCCCTGTAT-3'
EcoRI

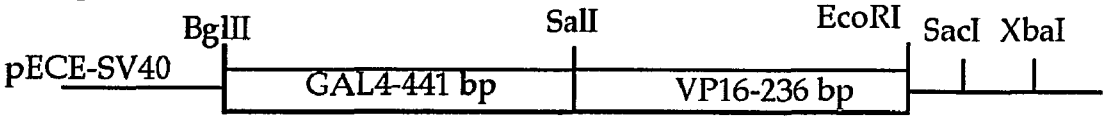
Cloning of the histidine-C' protein

6) 5CBAM- upstream of the C' ORF, incorporating a BamHI restriction site

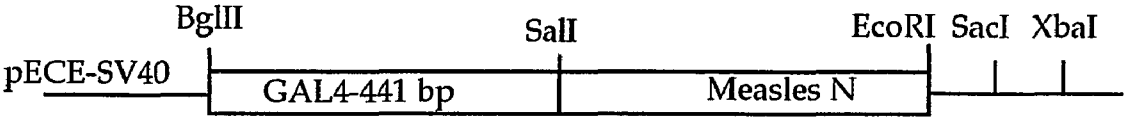
C' ORF
5'-CGCGGGATCCCATGAGATCGAAGCAC-3'
BamHI

Appendix B: Plasmids

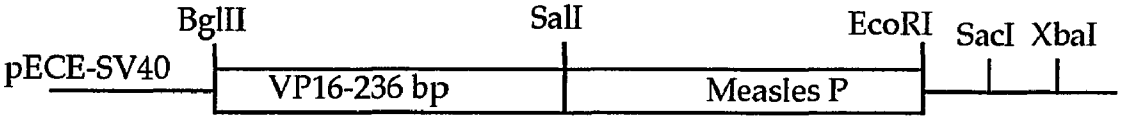
1) pECEGAL4VP16



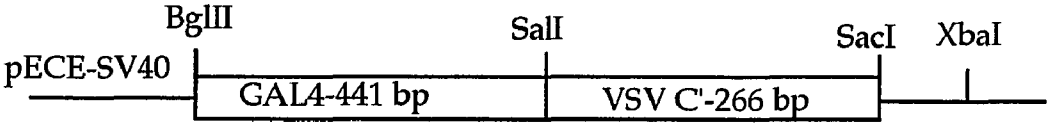
2) pGAL4MVN



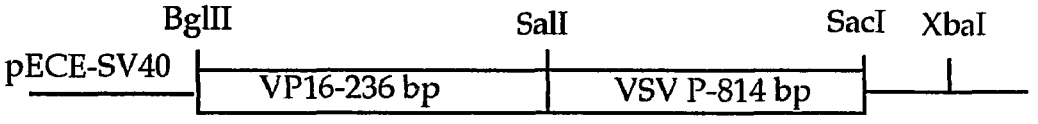
3) pVP16MVP



4) pGAL4VSVC



5) pVP16VSVP



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