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Functional analysis of the roles of the N:P protein complex and the C proteins of vesicular stomatitis virus in the replication of the viral genome

Lock, Martin William, Ph.D.

City University of New York, 1995

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FUNCTIONAL ANALYSIS OF THE ROLES OF THE N:P PROTEIN COMPLEX AND THE C PROTEINS OF VESICULAR STOMATITIS VIRUS IN THE REPLICATION OF THE VIRAL GENOME.

by

MARTIN WILLIAM LOCK

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1995

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Date

Chair of examining committee

November 3, 1994
Date

Executive Officer

Irwin Gelman

Supervisory Committee

THE CITY UNIVERSITY OF NEW YORK

ABSTRACT

FUNCTIONAL ANALYSIS OF THE ROLES OF THE N:P PROTEIN COMPLEX AND THE C PROTEINS OF VESICULAR STOMATITIS VIRUS IN THE REPLICATION OF THE VIRAL GENOME

by
Martin William Lock

Advisor: Richard Peluso, PhD.

Molecular events which distinguish the replication of the negative sense RNA genome of vesicular stomatitis virus (VSV) from viral transcription have been addressed. In contrast to transcription which entails the discontinuous synthesis of monocistronic mRNA, replication involves the synthesis of a full length copy of the genome and concurrent encapsidation of the nascent RNA by the nucleocapsid protein. *In vitro* studies performed here indicate that the recognized encapsidation substrate, the nucleocapsid protein:phosphoprotein (N:P) complex, is not able to directly and independently encapsidate nascent genomic RNA sequences. Rather, results from an *in vivo* replication system suggest that the N:P protein complex interacts with the viral RNA polymerase during replicative RNA synthesis and that this interaction may facilitate the delivery of the N protein to the nascent genome. A direct enzymatic role of the P protein component of the encapsidation complex in the replicative RNA synthetic reaction is not supported by the data. The effect of the recently demonstrated C proteins of VSV on viral RNA synthesis has been assessed using *in vitro* transcription and replication systems. In these systems, the C proteins are shown to exert negative and positive regulation of transcription and replication respectively. These results imply that the C proteins are important factors in the switch from the transcriptive to the replicative mode of VSV RNA synthesis.

To my father, Terry who passed away during my first
year as an undergraduate at Warwick University.
I will never forget the tears of pride that streaked
your face when I visited you at your hospital bed with the news
that I had been accepted at Warwick.
To know that those tears are rolling once more
is reward enough for me.

Also, to my mother, Pamela, and my wife, Tracy
for their love, patience and support.

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Jim Richardson, for his friendship and amazing ability to listen to all my problems.

Tom Moran, the ace of the links, for his friendship and advice, both on and off the golf course.

To those who I am sure I have inadvertently missed, my apologies.

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INTRODUCTION.

Vesicular stomatitis virus (VSV) is the prototype member of the family Rhabdoviridae (Wildy, 1971). Members of this family are widely distributed in both the animal and plant kingdoms and are causative agents of many diseases of livestock, crops and wildlife. Vertebrate rhabdoviruses for the most part fall into two genera, the vessiculoviruses, to which VSV is assigned and the lyssaviruses whose most prominent member is rabies virus (Wildy, 1971). Two major serotypes of VSV are recognized, Indiana and New Jersey, each of which comprises several strains (Clewly et al., 1977; Reichman et al., 1978) Both serotypes are causative agents of vesicular stomatitis in cattle, a disease which although non-lethal, is clinically indistinguishable from the more deadly foot and mouth disease (Shope and Tesh, 1987). Along with symptoms of weight loss and decreased milk production, this difficulty in differential diagnosis means that VSV epizootics can have grave economic consequences. With the one notable exception of rabies virus, humans disease produced by rhabdoviruses is rare and limited to acute influenza-like symptoms. (Shope and Tesh, 1987). However, many features of rhabdovirus genome replication and expression are common to a number of important human pathogens including measles virus, mumps virus, respiratory syncytical virus and the human parainfluenza viruses. Over the years VSV has become established as a model system in which to study RNA synthetic processes integral to the life cycle of such viruses. The wealth of knowledge pertaining to this system means that relevant insights and advances concerning fundamental virus replication events, continue to emerge.

THE RHABDOVIRUS LIFE CYCLE.

The rhabdovirus cycle of infection is a fairly typical one and has been described in some detail elsewhere (Wagner, 1987 and references therein). The virus particle attaches to the cell by means of an interaction of the viral glycoprotein (Bishop et al., 1975) with an as yet poorly defined host cell receptor (Schlegel et al., 1983), an event which permits entry to the cell by way of receptor-mediated endocytosis (Matlin et al., 1982). In a process known as uncoating, the particle is then delivered to the endosome where the low pH appears to allow the viral envelope to fuse with the endosomal membrane and deliver the nucleocapsid to the cytoplasm (Marsh et al., 1983). Once uncoating has occurred, primary viral transcription ensues which results in the production of viral messenger RNA and the translation of this message by the host cell machinery into viral protein (Huang and Manders, 1972). Viral protein production is a prerequisite for genome replication, i.e., the copying of the viral genome into an antigenome from which progeny genomes are produced (Wertz and Levine, 1973; Perlman and Huang, 1973). Once this process is complete, secondary transcription from progeny templates occurs with a resulting cycling and amplification of the preceding RNA synthetic events and the start of an exponential phase of virus multiplication which ultimately results in the production of up to 1000 particles per cell (Wagner, 1987). During the exponential growth phase, newly produced nucleocapsids locate to the cell surface (to which the glycoprotein has been independently trafficked) and bud through the membrane, thus gaining an envelope complete with glycoproteins in readiness to infect the next host cell (Wagner et al., 1971; Lenard and Compans, 1974).

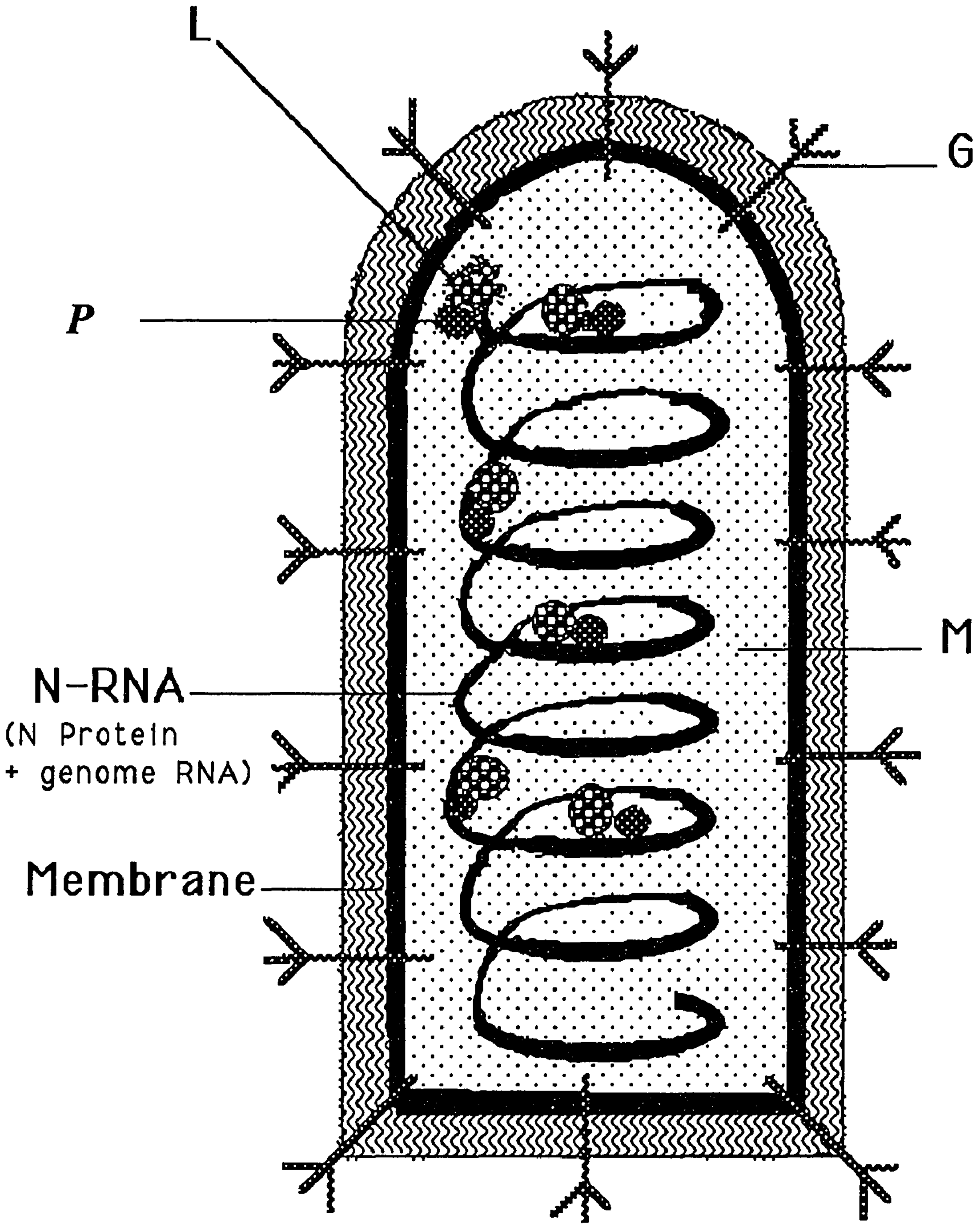
VIRION STRUCTURE AND VIRAL PROTEINS.

The classification of the family Rhabdoviridae was originally based upon the ubiquitous and distinctive rod or bullet shape morphology of the virus particle (rhabdos (Gk.) = rod). In negatively stained electron micrographs the virion can be seen to consist of a tightly coiled nucleocapsid surrounded by a lipoprotein bilayer with one flat and one curved end (Schematically represented in Figure 1). The lipid bilayer is derived by budding through the plasma membrane of the host cell (Lenard and Compans, 1974) and is associated with two viral proteins. The first is the externally oriented integral glycoprotein (G protein) which forms the spike-like projections which protrude from the lipid bilayer and is the major antigenic determinant of the virus (Cartwright et al., 1989; Kelly et al., 1972). The second is the matrix protein (M) which lines the inner surface of the viral envelope. This protein appears to bind to both the nucleocapsid and to the acidic phospholipid headgroups of the envelope and is thought to serve as the adhesive between the two (Wilson and Lenard, 1981; Zakowski et al., 1981)

The nucleocapsid consists of an RNA molecule and three virally encoded proteins: the nucleocapsid (N) protein (422 amino acids), the phosphoprotein (P) (265 amino acids) (previously termed by the misnomer "non-structural" (NS)) and the large (L) protein (2109 amino acids). These three proteins are involved in RNA synthesis in the infected cell and together with the RNA genome with which they are associated, are selectively packaged into the virus particle (Bishop and Roy, 1972).

Figure 1. The vesicular stomatitis virion

VSV is an enveloped virus which contains a helical, negative sense RNA genome tightly encapsidated in the nucleoprotein (N) . Two other proteins, the large (L) and the phosphoprotein (P) are associated with the N-RNA (N protein- RNA complex) The matrix protein (M) lines the inner surface of the membrane and provides a link between the nucleocapsid (N-RNA and associated L and P proteins) and the viral envelope. The glycoprotein is an integral membrane protein which forms the virion "spikes" and is the major antigenic determinant of the virus.

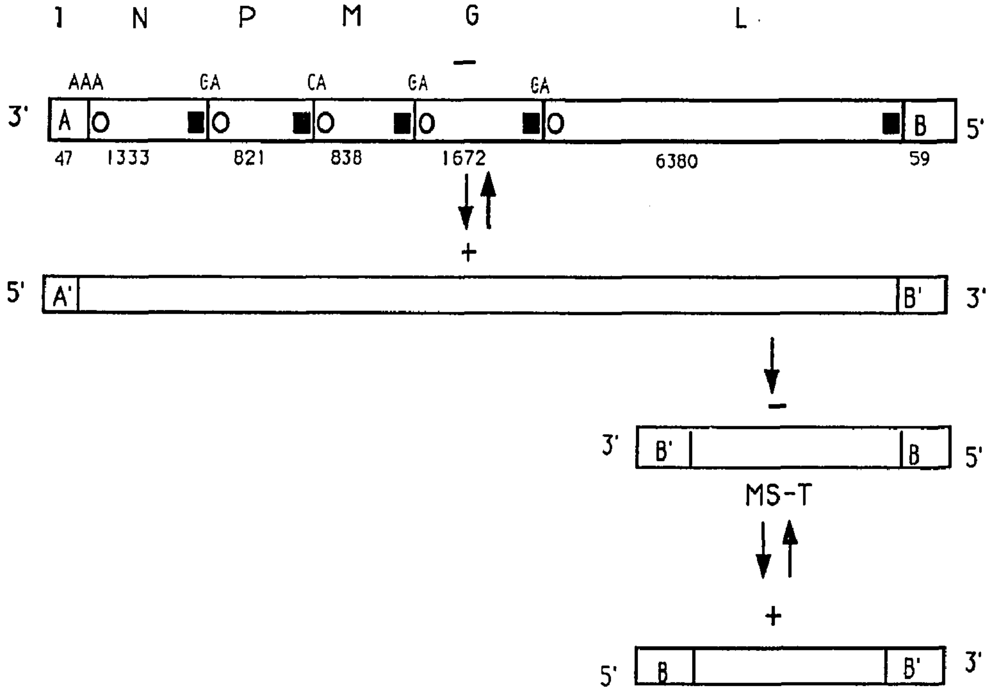

L
G
P
M
N-RNA
(N Protein
+ genome RNA)
Membrane

GENOME ORGANIZATION

The RNA genome of VSV is a linear single stranded molecule of negative sense (i.e., complementary to the viral message) and is 11,162 nucleotides in length (Schubert et al., 1984). The genome encodes six non-overlapping transcriptional products including the five messenger RNA's and a 47 nucleotide, positive sense leader RNA. (Banerjee 1987; Rose and Schubert, 1987) The order of the genes specifying these RNA products on the genome was first determined by the technique of UV transcriptional mapping (Ball and White, 1976; Abraham and Banerjee, 1976) This technique revealed that the genes were expressed in a sequential and polar manner in the order (3')-N-P-M-G-L-(5') and subsequent mapping of the leader RNA located the leader gene at the 3' terminus of the genomic RNA in a position preceding the other genes (Colonno and Banerjee, 1977) (Figure 2). The organization of the genome is extremely compact and all but 70 nucleotides are found in the leader and messenger RNA's. The non-transcribed nucleotides occur at the leader/N junction (3 or 4 nucleotides), the mRNA intergenic junctions (2 nucleotides per junction) and the 5' trailer region (59 nucleotides) (Rose and Schubert, 1987). Despite the compactness of this arrangement, coding capacity is expanded in only a limited way through the use of multiple reading frames and there is no apparent use of messenger RNA processing or editing. Hudson and colleagues (1986) were the first to recognize that the P protein mRNA of the Indiana serotype has the capacity to encode a small protein in a second open reading frame (ORF) located near the 5' terminus of the mRNA. Recently, two proteins originating from separate initiation codons, presumably by leaky ribosomal scanning or by independent initiation events, in the corresponding ORF of the New Jersey serotype have been demonstrated in the

Figure 2. Schematic representation of the VSV genome.

The genome of VSV is a non-segmented 11,162 base RNA molecule of negative sense (-). The five mRNA genes (3'-N, P, M, G, L-5') are indicated and are preceded by the 3' proximal leader gene (l). The length of each gene is indicated below the (-) sense genome and the intergenic nucleotides present in the Indiana serotype, above. A polyadenylation site (UACUUUUUUU) is present at the end of each mRNA gene (closed squares) and a repeated sequence (UUGUC), whose complement (AACAG) is present at the exact 5' terminus of each mRNA but not the leader RNA, is also represented (open circles). In contrast to transcription where monocistronic gene products are made, replication gives rise to the full length (+) complement of the genome, the antigenome which in turn is used to template the production of progeny genomes. The 2,208 base genome of the MS-T defective interfering (DI) particle is also shown and arises by a copy-back mechanism during the production of the standard (*wt)* genome. As a result this DI RNA molecule has complementary ends (B and B') which are separated by an incomplete stretch of the L gene. No messenger RNA products are produced from this genome but a short leader RNA is synthesized. In the presence of a helper virus, the DI genome will replicate to give high levels of both (+) and (-) complements.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B'
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3'
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B
5'
MS-T
+
5'
B
B'
3'

infected cell (Spiropolou and Nichol, 1993). These proteins, designated C and C' after similar proteins encoded from an overlapping reading frame of the P message of Sendai virus, are functionally uncharacterized in VSV and are of considerable interest. Additionally, a small protein of 7 kilodaltons (Kd) which derives from an internal translational initiation event at the extreme 3' end of the P mRNA has been demonstrated using an *in vitro* translation system (Herman, 1986). This protein is encoded in the same frame as the P protein and a product of similar size can be immunoprecipitated by anti-P antisera from the infected cell.

Other arrangements of the VSV genome have been recognized in defective interfering (DI) particles (Holland, 1987). The virion and nucleocapsid of DI particles are essentially identical in protein composition to the standard virus, but are physically truncated due to the fact that they contain partially deleted copies of the standard genome (Leamson et al., 1974). An example of a DI particle, the Mudd-Summers truncated particle (MS-T), is derived by an aberrant replication event called "copy-back" synthesis, which entails template switching by the polymerase from the antigenome to the nascent genome during replication. This process results in the production of a genomic RNA with complementary sequences at either end, representing the 3' trailer sequence of the genome and the 5' leader sequence of the antigenome respectively, linked by a portion of the L gene (Lazzarini et al., 1981; Figure 2). Therefore the MS-T genome lacks any functional genes and with the exception of a small leader transcript (Emerson et al., 1977), is transcriptionally inert. However, in the presence of a standard virus which supplies the defective functions *in trans*, the DI genome will replicate to high levels, a process which interferes with the replication of the helper virus (Holland, 1987).

Structurally, the VSV nucleocapsid is a left-handed helix of about 20nm in diameter (Nakai and Howatson, 1968) which may be loosely or more tightly coiled depending on the salt concentration of the environment (Heggeness et al., 1980). This flexibility of the helix is proposed to be functionally significant during transcription and replication although there is currently no data to support this theory. Dark field scanning electron microscope studies have revealed that the nucleocapsid consists of one RNA molecule and 1258, 466 and 50 copies of the N, P and L proteins respectively (Thomas et al., 1985). The N protein tightly binds to the RNA along the entire length of the genome, apparently via the sugar-phosphate backbone, and renders the RNA completely resistant to nuclease digestion (Soria et al., 1973; Emerson and Wagner, 1973; Keene et al., 1981). The L and P proteins are less tightly associated with the nucleocapsid structure and may be dissociated more readily under conditions of high ionic strength (Emerson and Yu, 1975).

In contrast to the positive strand RNA genomes of viruses such as poliovirus, the deproteinized genome of VSV is not infectious (Huang and Wagner, 1970). The explanation to this observation arises from an obligate requirement of negative strand viruses to copy their genomes into mRNA in order for their proteins to be expressed. The RNA-dependent RNA polymerase activity which accomplishes this task is not found in the uninfected cell, but rather is associated with the nucleocapsid (Baltimore et al., 1970). In addition, the template for this polymerase is a complex of the N protein and the genome RNA and not the RNA alone (Emerson and Wagner, 1972). Thus it is clear that the nucleocapsid structure is central to viral RNA synthetic events and indeed available evidence suggests that full length genomic and antigenomic RNA exists as a nucleocapsid throughout the infection cycle (Hill et al., 1979).

VSV TRANSCRIPTION

The detailed nature of the VSV transcriptase, first described by Baltimore and colleagues (1970), was to a great extent determined by the technique of dissociation of the virion and reconstitution of the resulting components *in vitro*. Separation of the M and G proteins from the nucleocapsid after detergent disruption of the envelope resulted in retention of transcriptional activity in the nucleocapsid fraction (Bishop and Roy, 1972). In an extension of this technique, Emerson and Yu (1975) demonstrated that separation of the G, M, L and P proteins from the N protein-RNA complex (N-RNA) completely abrogates the polymerase activity. However reconstitution of highly purified L and P proteins with the N-RNA template in the appropriate reaction mixture, reestablishes the ability to synthesize RNA while addition of either protein alone does not. This observation strongly points to the identity of the transcriptase and a subsequent demonstration that the L and the P proteins are able to form transcriptionally active complexes *in vitro* (Naito and Ishihama, 1976) provides confirmation that these two proteins together constitute the RNA-dependent RNA polymerase.

The large size of the L protein and the presence of conserved RNA polymerase motifs (Poch et al., 1989) within the L amino acid sequence indicate that this protein most likely contains the catalytic domain for RNA polymerization, although direct evidence for this assumption has not been forthcoming. Evidence does exist however for the role of the L protein in mediating auxiliary functions of the transcriptional process such as methyltransfer and polyadenylation activities (Hercyk et al, 1988; Hunt et al., 1984). In addition, the association of a kinase activity with the L protein which is able to phosphorylate the P protein has been well documented although there is some controversy as to whether this activity is intrinsic to the L protein itself or if it is present as the result of a tightly coupled host kinase (Sanchez et al.; 1985, Harmon et al.; 1983, Chattopadhyay and Banerjee; 1987, Massey et al., 1990).

Of all the viral proteins involved in RNA synthesis, perhaps the best characterized is the P protein. This protein appears to be devoid of catalytic activities and functions rather as an accessory protein to the L protein; however the importance of this small protein to RNA synthetic processes cannot be overstated. Reconstitutional studies have revealed that while the P protein is able to bind to the N-RNA template without other proteins present, the L protein is unable to bind in the absence of the P protein (Mellon and Emerson, 1987). Hence the P protein acts as a mediator allowing the L protein to access the template. Studies with recombinant P protein mutants expressed *in vitro* support this role since they have revealed two separate domains in the protein which bind to the N-RNA template (or the N protein in solution) and to the L protein respectively (Chattopadhyay and Banerjee, 1987; Emerson and Schubert, 1987).

The most striking aspects of the phosphoprotein are the highly phosphorylated state in which the protein exists and from which the name is derived and the overall acidity of the protein. Both of these features appear to be mainly attributable to the amino-terminal half of the protein (also termed domain I) with acidic residues constituting 32 out of the first 100 residues and with the majority of demonstrated phosphate additions occuring in this region (Bell and Prevac, 1985; Hsu and Kingsbury, 1985). This domain has been shown to interact with the L protein in solution (Emerson and Schubert, 1987) and due to the acidic nature it has been compared with the activation domain or "acid blob" of standard eukaryotic transcriptional activators (Chattopadhyay and Banerjee, 1988; Takacs et al., 1991). This analogy can be extended further since the domain responsible for binding to the N-RNA template, the extreme carboxy-terminal 21 amino acids of the P protein (domain III), is highly basic much like the DNA binding domain of transcription factors (Gill et al., 1986). Whereas the overall homology between the New Jersey and the Indiana P proteins is only 30 percent, a high degree of homology in domain III is exhibited. This conservation of sequence is consistent with the retention of a strict stereochemical conformation such as that required for sequence specific nucleic acid binding. Along these lines, Keene and colleagues (1981) have demonstrated that the P protein is able to bind to an A+U-rich sequence in the center of the leader gene, which resembles a Goldberg-Hogness transcriptional promoter site. However, this binding activity has not been mapped to any particular domain of the protein. From these detailed studies of the physical interactions of the P protein with the rest of the transcriptional apparatus it appears that this molecule mimics a basal transcription factor in precisely positions the polymerase complex (L+P) such that transcription initiates faithfully at a predetermined location, in this case at the 3' terminus of the genome and also seems to act as a transcriptional activator since it provides an acidic activation interface.

Phosphorylation of the P protein has long been appreciated to be functionally important in viral transcription, however it is only with recent advances that a clearer picture regarding this phenomenon has emerged. Analysis of the P protein in the infected cell and in virions has revealed that two distinct populations, low and high with regard to phosphorylation state, exist and that only the more phosphorylated species is transcriptionally active (Kingsford and Emerson, 1980). More recently, a different approach involving the expression the P protein in *E.coli* and the use of this purified product in reconstituted transcription reactions has been employed (Barik and Banerjee, 1991; Barik and Banerjee, 1992a, 1992b; Takacs et al., 1992). This form of P protein (P0) is unphosphorylated and transcriptionally inactive, but can be phosphorylated by a cellular enzyme, identified as casein kinase II, at two critical serine residues in domain I (residues 59 and 61) to a form called P1. The modified form is also inactive in reconstituted transcription reactions, but can be converted to a transcriptionally active form (P2) by the kinase activity associated with the L protein which has been reported previously to phosphorylate two serine residues (242 and 246) in domain II (amino acids 214-247) of the New Jersey P protein (Chattopadyhay and Banerjee, 1987). Therefore it is evident that a phosphorylation cascade, involving both host kinase and L protein-associated kinase activities, is required to produce a transcriptionally active P protein and that phosphorylation is an important means of transcriptional regulation. One additional, but no less interesting observation provided by this system is that the phosphates added by the L-associated kinase activity are rapidly turned over during transcription while the phosphates added in domain I remain stable. However, the functional significance of this observation and the manner in which the added phosphate groups are able to influence the activity of the polymerase remain unclear.

Aside from the basic mechanics of transcription, the overall strategy of gene expression from the VSV genome is also of interest. Several models have been forwarded to explain how the individual monocistronic transcripts are made, but any plausible candidate must take into account the data generated from early UV transcriptional mapping studies. These experiments demonstrate that the order of gene expression is sequential in a 3' to 5' direction and that the accumulation of gene products occurs in a polar manner, i.e., the level of gene expression from successive genes is a function of the distance from the 3' terminus of the genome (Ball and White, 1976; Abraham and Banerjee, 1976). Perhaps the most credible model is that proposed by Emerson (1982) and has been dubbed the "stop-start" model. This version postulates a single polymerase entry site (or promoter) at the 3' end of the genome where transcription is initiated. At intergenic junctions the polymerase can either terminate, a characterized event known as attenuation (Iverson and Rose, 1981), fall off the template and relocalize to the 3' promoter or continue on and reinitiate at the start of the next gene. This mode of operation ensures sequential transcription of the genes since an internal cistron may only be transcribed in the event that the 3' proximal gene was first transcribed. The low level of 5' distal gene products, a result of polarity of gene expression, can be explained by the model since this feature would reflect the frequency with which the polymerase is able to reach the 5' portion of the genome without disengaging from the template. An elegant series of reconstitution experiments have been performed which quite convincingly support this model (Emerson 1982). An alternative version of the stop-start model whereby the polymerase binds and initiates at internal as well as at the 3' entry site has also been proposed (Testa et al., 1980). Although some evidence has been provided in support of this model (Thornton et al., 1984), it is much harder to reconcile internal entry of the polymerase with the UV mapping data.

GENOME REPLICATION.

The replication of the VSV genome in the infected cell may be compared to viral transcription only from the point of view that RNA synthesis is involved, otherwise the two processes are quite distinct. Genome replication entails the copying of the full length (-) strand RNA genome into a (+) strand antigenome, which in turn serves as the template for progeny (-) genomes. In contrast to viral transcripts which are unencapsidated, the newly synthesized genomic RNAs are exclusively found in the form of nucleocapsids, never as naked RNA (Hill et al., 1979) and are neither capped nor polyadenylated (Wagner, 1987). Furthermore, genome replication is characterized by a continuous requirement for protein synthesis, a demand which is not applicable to transcription (Wertz and Levine, 1973; Perlman and Huang, 1973). Although it is generally accepted that the subunits of the transcriptase, the P and the L proteins are also involved in genomic RNA synthesis , it is clearly evident that the polymerase is behaving differently in the two processes since by definition of the replication process, the hallmarks of the transcriptase, namely polyadenylation, capping and transcriptional attenuation at gene junctions, must be absent. Substantiated explanations for these differences are still notably lacking, however the development of *in vitro* replication systems over the past decade, has resulted in major contributions to our understanding of VSV genome replication.

The discovery of leader RNA nucleocapsid structures of both (+) and (-) sense in the infected cell which are complementary to the exact 3' termini of the viral genome and anti-genome respectively, led to the proposal of a model to account for the requirement for protein synthesis during replication and to explain how the switch between transcription and replication might occur (Leppert et al., 1979; Blumberg et al., 1981; Blumberg and Kolakofsky, 1981). In this model a critical concentration of the N protein is required to initiate the concurrent assembly of the nascent leader RNA into a nucleocapsid structure, an event which facilitates the antitermination of the leader-N gene junction and allows the polymerase to read through into the N gene. Evidence in support of this model has been obtained: first, a solubilized form of the N protein is found to selectively encapsidate leader RNA *in vitro*, a process which initiates within the highly conserved first 14 nucleotides of the 5' end of the (+) leader RNA (Blumberg et al., 1983). This region contains a repeating pattern of adenine residues at every third base and has been proposed to represent a nucleation sequence which dictates the start of the encapsidation process. Second, a reticulocyte lysate-based replication system which depends upon expression of hybrid selected viral mRNAs, has demonstrated that a low level of *de novo* synthesized N protein will support the replication of an added nucleocapsid with an associated polymerase (Patton et al., 1984). Although the N protein encapsidation model represented a large intellectual step forward at the time, a considerable caveat is present due to the artificial nature of the systems used to establish it. When expressed in cells from a eukaryotic expression vector or purified from nucleocapsids, the N-protein is extremely insoluble and this is largely a result of an innate ability of this protein to self-assemble (Sprague et al., 1983; Blumberg et al., 1983). In fact the leader encapsidation system described relies upon the solubilization of the N protein with guanidium hydrochloride and maintenance of the soluble state with high salt concentrations and low temperature (Blumberg et al., 1983). Similarly, in the reticulocyte system of Wertz and co-workers (Davis and Wertz, 1982, Patton et al., 1984), any aging of the N protein or synthesis at higher concentrations such that aggregates have the chance to form, results in an inability to support replication.

A more accurate picture of the replication process has been obtained by closely examining the state of the N protein in the infected cell. An *in vitro* system of genome replication has been developed in which infected cell extracts can be separated into soluble protein and nucleocapsid fractions (Peluso and Moyer, 1983). Neither of the two fractions are independently active in supporting genome replication but when reconstituted together *in vitro,* genomic RNA is replicated into a nuclease resistant form. Analysis of the soluble protein fraction revealed that the N protein was present in at least three forms, identified by their sedimentation rates on glycerol gradients (Peluso and Moyer, 1984; Peluso, 1988). Monoclonal antibodies were used to show that the slowest sedimenting form, which is the first to appear in infected cells, is an equimolar complex of the N and the P proteins. The ability to support RNA genome replication is exclusively associated with this complex while the faster sedimenting forms representing higher molecular weight aggregates of the N protein and which contain little or no P protein, are not active in supporting genome replication (Peluso and Moyer, 1983; Peluso and Moyer, 1984; Peluso, 1988; Peluso and Moyer, 1988). These studies demonstrate that in infected cell extracts the substrate for genome replication is a 1:1 complex of the N and the P proteins and not the N protein alone. This assertion is supported by the finding *in vivo* that interference with the formation of the complex by reducing the pH of the culture medium is accompanied by an inhibition of genome replication (LaFerla and Peluso, 1989). It is proposed that the function of the P protein in the N:P complex is to keep the N protein in a soluble, replication-competent state (Arnheiter et al.,1985; Hudson et al., 1986; Peluso, 1988) and that dissociation of this complex during replication leads to the formation of nucleocapsids, or at later times in infection, to the appearance of high molecular weight N protein aggregates (Peluso, 1988) The evidence from the infected cell indicates that the continuous protein requirement which defines the process of genome replication reflects the maintenance of a threshold level of the N:P complex and not of the N protein alone. However the hypothesis that encapsidation of the leader RNA drives the antitermination of the leader-N junction may still be valid although it is technically difficult to provide direct evidence that this is indeed the case.

Other aspects of the transcription to replication switch, such as the nature of the transcriptional termination signal and whether *cis* sequences and/or *trans*-acting factors are involved remain unresolved, although the recent development of a system which is able to package and replicate a recombinant genome *in vivo* may be useful in this regard (Pattnaik et al., 1992). The involvement of phosphorylation in regulating replication has received some attention largely because of the prominent role that this form of protein modification plays in transcription. Studies which include a β-γ imido analog of ATP, an unsuitable phosphate donor for kinase reactions, in an *in vitro* transcription system indicate that even in the absence of the N:P complex, a full length, unencapsidated (+) strand copy of the genome can be produced providing transcriptional initiation is first allowed to occur (Testa et al., 1980). This finding indicates that an inhibition of kinase activity may promote replication, although in this report no sequence analysis of intergenic junctions was performed to indicate whether the RNA obtained was an authentic replication product or a transcriptional artifact. A different approach has been reported which utilizes a phosphatase inhibitor, okadoic acid, in infected cells (Chang et al., 1994). This agent appears to have little effect on primary transcription but is inhibitory to the production of genome length RNA and in addition promotes the accumulation of a hyperphosphorylated form of the P protein. Taken together these two studies suggest that an underphosphorylated form of the P protein may play an important, but as yet undefined role in replication.

A new class of VSV proteins, known as the C proteins, have recently been described (Spiropolou and Nichol, 1993) (see "Genome Organization" above for more details) and to date remain functionally uncharacterized. Indirect evidence from temperature-sensitive (*ts*) mutants suggests that these proteins may play a direct role in replication. Mutations in the New Jersey P gene in a region which affects both the P and the C protein coding information, produce a replication-defective phenotype at the non-permissive temperature. However, mutations in the P gene which affect transcription but not replication at the higher temperature, invariably fall outside of the C coding region (Rae and Elliot, 1986). The C proteins are highly conserved between strains of a particular VSV serotype which is indicative of the retention of some important function. This function appears to be unrelated to transcription in the New Jersey serotype since the C proteins cannot be found in the New Jersey virion (Blissel et al., 1990; Spiropolou and Nichol, 1993).

THESIS OBJECTIVES

Despite the considerable progress described above in the area of VSV genome replication, a comprehensive understanding of this process has not been achieved and indeed the current indications are that the final picture is likely to be far more complex than was originally envisaged. The research presented here was initiated with the goal of investigating mechanisms and components of the replicative machinery in further detail and has concentrated mainly in two areas.

The role of the N:P complex as the substrate of the encapsidation reaction during genome replication and of the P protein in preventing the self-aggregation of the N protein has been well established (Peluso and Moyer, 1984; Peluso and Moyer, 1988; Peluso, 1988). In addition, the formation of this complex also functions to prevent the N protein from binding non-specifically to RNA (Masters and Banerjee, 1988). During the initial stages of this thesis project, the nature of the interaction of the N:P complex with the replicating nucleocapsid and the role of the complexed P protein in this interaction were largely undetermined. More specifically, no explanation had been advanced to account for the mechanism of localization of the N:P complex to the nascent genome or the observed release of the P protein (Peluso and Moyer, 1988) and the concomitant assembly of the N protein onto the newly synthesized RNA. As a first step in addressing these unknowns, two hypothetical models were conceived which appeared to be reasonable candidates for this process. The first suggests that complex formation between the N and the P proteins may result in the formation of a highly specific RNA binding site, which would bind to the nucleation sequence of the leader RNA but not to other RNAs [an idea also proposed by Masters and Banerjee (1988)], and that this binding event could sterically trigger the release of the P protein. The second alternative, largely based upon the ability of the P protein to bind to the L protein (Emerson and Schubert, 1987; Chatopadhyay and Banerjee, 1987), proposes that one function of the complexed P protein may be to "dock" the N protein to the RNA polymerase. In this version, the function of the P protein is to position the N protein in the locality of the emerging 5' terminal RNA,while the release of the P protein and subsequent binding of the N protein to the RNA would be concurrent events in the replication process. Experiments have been designed to test certain aspects of these models and although we cannot categorically eliminate or prove either alternative, the data obtained argue in favor of the second model over the first. Since the P protein is an integral component of the RNA-dependent RNA polymerase of VSV, additional studies to determine if the P protein in the N:P complex is involved in RNA synthesis have been performed. The results of these experiments indicate that the complexed form of the P protein is not required in an enzymatic sense for RNA synthetic events during replication and that the polymerase packaged in the DI particle is sufficient for this function.

The second area of focus of this thesis project concerns the role of the newly discovered C proteins in both viral transcription and genome replication. As discussed earlier (Genome Replication), the properties of P protein *ts* mutants suggest that the C proteins may be important factors in genome replication. Using *in vitro* systems which rely upon recombinant viral protein expression, I have shown that the C proteins can act as positive and negative regulators of replication and transcription respectively. The significance of these findings with regard to the transition from transcription to replication is discussed.

MATERIALS AND METHODS

SOLUTIONS

Buffer A. 0.15M sucrose, 33mM NH_4Cl, 7mM KCl, 4.5mM MgOAc and 30 mM HEPES adjusted to pH 7.4.

Complete medium. Minimal Eagles medium (MEM), pH 7.4, 10% heat inactivated fetal calf serum (FCS), 22mM glucose, 4mM glutamine, 1mM pyruvate, 100 U/ml penicillin and 100μg/ml streptomycin.

HN buffer. 10mM HEPES pH 8.0, 10mM NH_4Cl.

Infection medium. Complete medium minus FCS, supplemented with 25mM HEPES pH 7.4 and 10mM TES pH 7.4.

NET buffer. 0.15M NaCl, 5mM EDTA, and 50 mM Tris-HCl, pH 7.4.

5X NENS. 0.5M NaCl, 0.25M NaOAc pH 5.1, 0.05M EDTA and 2.5% SDS.

Phosphate buffered saline (PBS). 2.7mM KCl, 5mM KH_2PO_4, 0.15M NaCl and 8mM Na_2HPO_4, pH 7.2

Protein loading buffer. 4% SDS, 40% glycerol, 3% DTT and 62.5mM Tris pH 6.6.

Replication buffer. 100mM NH_4Cl, 100mM HEPES pH 8.0, 7mM KCl and 4mM MgOAc.

T7 RNA polymerase buffer. 40mM Tris-HCl pH 8.0, 20mM $MgCl_2$.

VIRUS STOCKS.

The HR strain of VSV (Indiana serotype) (Prevac and Kang, 1970) and the MS-T DI particle (Leamson and Reichmann, 1974) were grown and purified as described by Moyer and Gatchell (1979). Vaccinia virus stock containing the recombinant T7 RNA polymerase (vaccinia-T7) was derived from the original virus, vTF7-3 described by Fuerst *et al.* (1986). The Ogden strain of the New Jersey serotype of VSV was used (Gill and Banerjee, 1985).

CELL LINES

Baby hamster kidney fibroblasts (BHK) were used to propagate both the HR VSV and the MS-T DI particle. CV-1 monkey kidney cells were used to grow and plaque vaccinia-T7 virus.

ANTIBODIES.

Anti-N monoclonal antibody (Mab), 21F9 (LeFrancois and Lyles, 1982) and anti-P MAb, 6D11 (Williams et al., 1988) were kind gifts of Dr. D Lyles and Dr. S Emerson respectively. The anti-L rabbit antiserum (Schubert et al., 1984) raised against an amino-terminal peptide of the L protein was kindly provided by Dr. M. Schubert.

CLONING PLASMIDS.

pUC19 (Yanisch-Perron et al., 1985) was used for routine cloning and sequencing. pRc/CMV (Invitrogen Corp.) was used as the basis for expression vectors and contains the T7 RNA polymerase promoter upstream of the polylinker cloning site. pCITE-1 (Novagen) was the source of the CITE sequence and was used to construct pSVC-1, an intermediate cloning vehicle containing CITE, kindly provided by J. Chen.

CLONING PROCEDURES.

a) Reverse transcription (RT) and polymerase chain reaction (PCR).

Total infected cell RNA from VSV infected cells treated at 1 hour post-infection (p.i.) with actinomycin D (2μg/ml) was isolated by scraping cells directly into the medium, pelleting and washing with PBS. The pellet was resuspended in HN buffer, incubated on ice for 10 minutes after which Nonidet-P40 (NP-40) was added to 0.5%. Incubation was continued for a further 10 minutes and was followed by centrifugation for 5 minutes at 1000 xG to remove nuclei and cell debris, and digestion with proteinase K. Extracted, precipitated RNA at 0.1 mg/ml was reversed transcribed according to the method of Krug and Berger (1981) using AMV reverse transcriptase (Molecular Genetic Resources Inc.) and the negative sense oligonucleotide of the relevant PCR primer pair. The cDNA product was then subjected to 30 cycles of amplification (PCR) using Vent DNA polymerase (New England Biolabs) according to the manufacturers instructions.

b) Cloning and sequencing.

Standard procedures were used to clone PCR products into plasmid DNA (Sambrook et al., 1989), using convenient restriction sites built into the cloning primers. Clones were sequenced using the sequenase kit (United States Biochemical Corp.) according to manufacturers instructions.

c) Site directed mutagenesis.

The PCR-mediated overlap extension method of site directed mutagenesis (Ho et al. 1989) was employed for the introduction of most mutations. Essentially, two Vent polymerase PCR half reactions, each using an outside cloning primer and an internal primer containing the desired mutations, were performed and the products were purified on agarose gels. Since the internal primers were designed to be partially complementary, combination of the two half reaction products in a second PCR reaction resulted in the formation of an annealed overlap extendable in both directions to produce a full length product containing the directed mutations. Inclusion of the outside primers in this reaction results in the amplification of the full length product which may be cloned and sequenced to confirm the mutations.

CONSTRUCTS.

(PCR primers used in cloning and mutagenesis are listed in Appendix A).

The + leader/N and - leader/L sequences were amplified using Lead 5-1/ L-1 and Lead 3-1/ N16 PCR primer pairs respectively. Lead 5-1 and Lead 3-1 both contain truncated T7 RNA polymerase promoters such that the first base produced in a transcription reaction will be of VSV origin and orientated so that the sense of the + leader and - leader is preserved. The PCR sequences were cloned directionally into the pUC19 Xba1 and Kpn1 sites and linearization of the plasmids for run-off transcription was achieved using the EcoRV site in the L-1 and N16 primers. pCMC-P and pCMC-N were amplified using NS3/ NS10 and N12/ N19 primer pairs respectively and were cloned directly after the 3' terminus of the CITE sequence present in pSVC-1, using the restriction sites incorporated in the primers. The CITE-N fragment was excised and cloned directionally into the Xba 1 and Not1 site of the pRC/CMV polylinker region while the CITE-P fragment which contained internal Xba 1 sites, was excised with Not1 and Sma1 and cloned into the Not1 and Apa1 site [blunted by the exonuclease action of T4 DNA polymerase (New England Biolabs)]. This procedure resulted in the elimination of the Rc/CMV Apa1 site. The L gene was excised from pSV-VSL-1 with Xho1 and cloned into pUC19 in two halves separated at the unique BamH1 site in the L gene. The two fragments were reassembled in Rc/CMV using the HindIII and Sph1 sites and the final construct was examined extensively by restriction analysis for the correct arrangement. pCMC-P3S was derived directly by taking advantage of the unique Sty1 site in domain II of the P gene and replacing the 3' terminus of this gene with a PCR product amplified using the NS6/ NS10 primer pair.

With the exception of the pCMC-NJC construct, the ORF2 (C protein) expression vectors were all engineered using site-directed mutagenesis of the plasmid pCMC-P. In all cases, the outside primers employed were NS16 and NS17 which facilitated cloning of the full length mutated fragment into the Apa1 (CITE sequence) and Xba1 (P gene, position 689) sites. This procedure required that the internal Xba1 site at position 58 in the P gene be protected by the action of Dam methylase (New England Biolabs). The internal primer pairs used for the generation of each construct were pCMC-C'/C : NS12B and NS14 and pCMC-C : NS13B and NS15 (which result in the substitution of the CITE AUG with the C' and C AUGs; these constructs will express the C' and C proteins together or the C protein alone respectively but neither will express the P protein), pCMC-ΔC'/C : NS19 and NS20 (which result in the introduction of stop codons after both the C' and C AUGs; this construct will express the P protein but not the C proteins) and pCMC-GCG : NS22 and NS23 (which result in the replacement of the P AUG with the codon GCG; this construct will express the C proteins at wild-type levels but not the P protein). The New Jersey ORF2 was cloned directly into pSVC-1 from infected cells using an RT-PCR procedure and the primer pair NS25 and NS26. The CITE-NJC fragment was subcloned into pCMC-P using Apa1 and Xba1 replacing the corresponding CITE-P sequence. The resulting construct pCMC-NJC, will express the New Jersey C proteins but neither the New Jersey nor the Indiana P protein.

VIRUS INFECTIONS AND PLAQUING.

Wild-type VSV: 60mm or 100mm petri dishes containing ~5x 10^6 and ~1x 10^7 BHK cells in the form of a subconfluent monolayer were infected at a multiplicity of infection (MOI) of 10 plaque forming units (pfu) per cell in 0.5 and 1.0ml of infection medium respectively and incubated at 37^0C with periodic tilting. At 1 hour post-infection (p.i.) 2 or 4ml of infection medium were added and incubation was continued to 4 hours p.i. when extracts were made. For large scale preparations, the MOI was reduced to 0.1 pfu per cell and the infection was allowed to proceed overnight.

DI particles: Infections were performed using a similar protocol as used for the standard virus, except that an empirically determined amount of DI stock (giving the highest level of replication) was added along with the *wt* virus.

Plaque assays : 10 fold dilutions of HR- VSV stock were made in infection medium and 0.5 ml of each dilution was added to confluent BHK monolayers in 60mm petri dishes and washed once with infection medium. Plates were incubated for one hour at 37^0C and 5ml of agarose overlay (0.45 % agarose (FMC) in infection medium) was added and allowed to cool. Plates were inverted and incubated at 37^0C for 2-3 days. Vaccinia-T7 was plaqued by an identical method except that CV-1 cells were used.

IN VITRO TRANSCRIPTION AND TRANSLATION.

100-150 units of T7 RNA polymerase (United States Biochemical Corp.) were combined with 10mM DTT, 1 unit /μl RNasin (Promega Corp.), 0.5mM of each ribonucleotide, 100μg /ml acetylated BSA and 3-5 μg of DNA template in 100μl of 1X T7 RNA polymerase buffer. Reactions were incubated for 45 minutes at 37^0C and RQ1 DNase (Promega Corp.) was added to degrade the template. RNA was extracted with phenol/chloroform (2:1) and ethanol precipitated. Radiolabeled transcripts were produced in similar reactions except that cold UTP was present at only 12 μM and α^{32}P-UTP (NEN) was added to 2.5 mCi /ml. The ethanol precipitation step in this case was repeated twice from 1M ammonium acetate and 3 volumes of ethanol, to remove unincorporated nucleotides.*In vitro* translation was performed by the method of Pelham and Jackson (1976). 17 μl of nuclease treated rabbit reticulocyte lysate (Promega Corp.) was combined with 1μg of programming RNA, 40 units of RNasin, 20nM amino acid mixture minus methionine and 0.8 mCi /ml ^{35}S methionine (NEN) in a final reaction volume of 50 μl. Reactions were incubated at 30^0C for 60 minutes and the products were analyzed on the low molecular weight system of Schagger and Jagow (1987).

METABOLIC LABELING OF BHK MONOLAYERS AND CELL EXTRACT PRODUCTION.

VSV infected or transfected BHK cell monolayers were washed with infection medium, depleted of methionine by incubation for 20 minutes in methionine-free infection medium and then labeled by the direct addition of 100 μCi of ^{35}S methionine at 3-4 hours p.i. for VSV infected cells and 22-25 hours post vaccinia infection for transfected cells.

Transfected cells were lysed directly in 1ml NET buffer containing 0.1% Nonidet-P40 (NP40) and processed for immunoprecipitation. Metabolically labeled infected cells (5 x 100mm dishes) were lysed with lysolecithin (α–lysophosphatidylcholine palmitoyl; Sigma Chemical Co.) according to the method of Peluso and Moyer (1983). Briefly, monolayers were washed once in Buffer A and 125μg /ml lysolecithin in the same buffer was added for 60 seconds. The solution was aspirated, the monolayers were allowed to drain completely and were then scraped into a small volume of replication buffer (see Solutions). The permeabilized cells were pipetted 15 times and centrifuged at 800 xG to remove nuclei and cell debris. The resulting extracts were then pelleted through a 30% glycerol cushion containing 1mM ATP, 1mM MgOAc and 1mM HEPES pH 8.0 for 75 minutes at 4^0C. The resulting supernatant (designated "soluble protein") was loaded to a 12 ml 5-20% glycerol gradient made in replication buffer and was centrifuged in a SW41 rotor (Beckman) at 33,000 rpm for 22 hours. The gradients were fractionated into 1ml aliquots which were stored at -85^0C until use.

IMMUNOPRECIPITATIONS.

Transfected cell extracts were centrifuged for 30 minutes at 100K xG, brought to 1ml with NET/ 0.1% NP40 and pre-cleared overnight by the addition of 30μl of a 1:1 slurry of protein G-conjugated sepharose (Boeringer Manheim) and 1μg of non-specific rabbit or mouse IgG. 100μl of infected cell glycerol gradient fractions were brought to 1ml with NET/ 0.1% NP-40 directly. 1μg of anti-N or anti-P monoclonal antibodies, or 2μg of anti-VSV antiserum were added and the tubes were rotated end over end for either 6-8 hours or overnight. Immune complexes were collected by rotating the tubes for one hour at 4^0C in the presence of 30μl of the protein G-sepharose slurry. The beads were then washed 5 times with NET /0.1% NP40 before resuspension in protein loading buffer and analysis by 11% sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE). Resolved proteins were visualized by treating with Enhance (NEN) followed by autoradiography.

MICROCOCCAL NUCLEASE PROTECTION ASSAYS.

Fractions containing the N:P complex, as judged by co-precipitation of the P protein in anti-N immunoprecipitates were pooled and concentrated using Microcon 10 concentrators (Amicon). 30,000 Cerenkov counts of the radiolabeled transcripts were heated to 67^0C for 10 minutes and then quenched in a dry ice-ethanol bath to minimize secondary structure. The fraction concentrates and transcripts were combined, 1 unit /μl RNasin was added, the volume brought to 50 μl with scraping buffer and the reaction allowed to proceed for 60 minutes at 30^0C. For nascent protection assays, pooled gradient fractions containing the N:P complex were dialyzed overnight against T7 RNA polymerase buffer prior to concentration, the concentrate was added to T7 RNA polymerase reactions similar to those described for the production of radiolabeled transcripts and the reaction was incubated at 37^0C for 60 minutes. Both nascent and run-off reactions were divided into two equal aliquots, one of which was treated with micrococcal nuclease (MN; Pharmacia) while the other was left untreated. MN treatment was performed by the addition of $CaCl_2$ to 1mM and MN to 0.4 mg /ml followed by incubation at 37^0C for 30 minutes. The digestion was stopped by the addition of EGTA to 10mM after which all aliquots were treated with proteinase K (Gibco-BRL) in the presence of 1X NENS, phenol /chloroform extracted and analyzed on 5% polyacrylamide gels containing 7M urea. The gels were dried and products were visualized by autoradiography.

EXPRESSION OF PROTEINS *IN VIVO* USING THE VACCINIA-T7 SYSTEM.

BHK monolayers in 60mm dishes and at 50-60% confluence were infected with the vaccinia -T7 virus at an MOI of 1 pfu per cell in complete medium and were incubated at 37^{0}C for 1 hour with periodic tilting. During this time, plasmid DNA was prepared for transfection using lipofectamine reagent (Gibco-BRL) according to the manufacturers instructions (3μl of lipofectamine was used per μg of transfected DNA). DNA-lipid complexes were allowed to form for 20 minutes in Opti-MEM (Gibco-BRL), the monolayers were washed twice with Opti-MEM, DNA complexes were added dropwise to the monolayers and the plates were incubated at 37^{0}C. At 5 hours post-transfection, an equal volume of infection medium containing 20% FCS, was added and incubation was continued overnight.

DI PARTICLE *IN VIVO* REPLICATION SYSTEM.

DI particles were concentrated from culture medium and then purified by two sequential centrifugation steps on 5-15% sucrose gradients at 25,000 rpm for 60 minutes in an SW41 rotor (Beckman). Under these conditions the helper virus pellets while the DI particle forms a band one third of the way down the tube. Recovered DI particles were estimated for protein content using a micro-assay kit (Biorad) and 1.8μg was used to superinfect transfected monolayers 24 hours post-vaccinia infection. One hour after superinfection 30μCi ^{3}H-uridine (NEN) in 2.0ml of Opti-MEM was added and incubation was continued for a further 4 hours. Cells were scraped into 1ml of PBS, pelleted, resuspended in 300μl of HN buffer and lysed by 10 strokes of a Dounce homogenizer. Nuclei and cell debris were pelleted at 800 xG for 5 minutes and the supernatant was treated with MN as described for the MN protection experiments above. Extracted, precipitated RNAs were run overnight at 5 volts /cm and 4^0C on 1.5% agarose, 6M urea gels (Lehrach et al., 1977) The gels were then processed for fluorography as described by Bonner and Laskey (1974).

PREPARATION OF N-RNA TEMPLATES.

Sucrose gradient purified DI particle and wild-type VSV N-RNA were prepared according to the method of Helfman and Perrault (1989). Briefly, virions were disrupted with 0.1% Triton-N101 in the presence of 1M LiCl and nucleocapsids were pelleted through glycerol cushions. The disruption was then repeated and the nucleocapsids were centrifuged to equilibrium on 20-40% CsCl gradients. The N-RNA band recovered, was concentrated by centrifugation and resubjected to the disruption and CsCl gradient protocols. The final preparation was assayed for protein content and 1μg was analyzed by 11% SDS-PAGE and silver-staining (Biorad).

IN VITRO TRANSCRIPTION AND REPLICATION REACTIONS.

Extracts of transfected cells were made by lysolecithin permeabilization (see Metabolic labeling and cell extract preparation above), scraped into 60μl of replication salts, pipetted 15 times and centrifuged at 800 xG for 5 minutes to pellet nuclei and cell debris. The supernatant was divided into four equal aliquots and frozen at -85^0C. 15μl (transcription reactions) or 30μl (replication reactions) of extract representing ~1.25 $x10^6$

and ~5x 10^6 cell equivalents respectively, were treated with 50 units of DNase (RNase-free) (Boeringer Manheim) and 0.5μg of actinomycin D at 30^0C for 30 minutes and then reconstituted with 1μg of N-RNA, judged to be free of associated P and L proteins. 1 unit /μl RNasin, 1mM ATP, GTP, CTP and 50μM UTP were added in a final volume of 50μl and the entire mixture transferred to a tube containing 20μCi of dried α^{32}P-UTP. The reactions were allowed to proceed for 2 hours at 30^0C after which time replication reactions were treated with MN followed by proteinase K (see MN protections assays above) and transcription reactions were treated with proteinase K alone. All RNAs were phenol /chloroform extracted, precipitated twice from 1M ammonium acetate and 2.5 volumes of ethanol, resuspended in 50% formamide, boiled for 2 minutes and electrophoresed overnight on 1.5% agarose /6M urea gels. RNA products were visualized by autoradiography of the dried gel.

WESTERN TRANSFERS

Proteins resolved on 11% SDS-PAGE gels were transferred to Zetabind membranes (Cuno) by the quantitative electroblotting procedure described by Peluso and Rosenberg (1987). Blots were blocked with 5% non-fat milk (Carnation) in PBS / 0.1 % Tween-20 and probed with anti-VSV (1/500), anti-P (1/200) or anti-L (1/300) antibodies. Blots were directly developed from this point, either with ^{125}I-conjugated protein G (NEN) and autoradiogrphy, or with the Renaissance chemiluminescence reagent (NEN) using anti-rabbit or anti-mouse peroxidase-conjugated goat IgG, according to the manufacturers instructions.

RESULTS

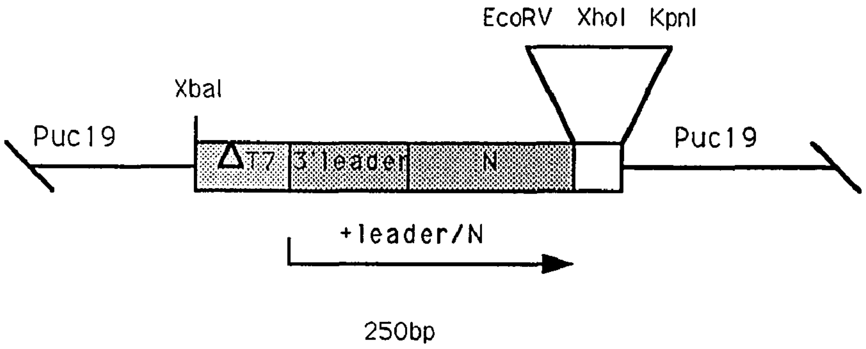
I: THE N:P PROTEIN COMPLEX AND GENOME REPLICATION.

1) Is the N:P protein complex able to protect synthetic RNA species representing the 5' ends of the VSV genome and antigenome?

The innate ability of solubilized N protein to bind to and encapsidate free leader RNA and synthetic RNA genome substitutes to a nuclease resistant form has been demonstrated (Blumberg et al., 1983, Moyer et al., 1991). However, in the infected cell, the N protein is only competent for replication in the form of the N:P protein complex and hence RNA binding is most likely to be mediated in some way by this complex. To test whether the complex is able to bind to and encapsidate genomic RNA directly, constructs were produced which contain the 5' ends of the antigenome and genome respectively downstream of a truncated T7 RNA polymerase promoter (+ leader/N and - leader/L, Figure 3). Linearization of these constructs at the EcoRV site and transcription *in vitro* gives rise to RNAs of 250 bases in length which represent the exact sequence of the 5' termini of the antigenome and genome and include the putative encapsidation sequence and the leader /N (+ leader) and the leader /L (- leader) gene junctions. The N:P complex was separated from other forms of the N protein by centrifugation of VSV-infected cell extracts through glycerol gradients, a process which was monitored by the immunnoprecipitation of gradient fractions with an anti-N monoclonal antibody. Those fractions containing the complex were identified by assessing co-precipitation of the P protein (Figure 4) and were incubated with radiolabeled run-off transcripts at 30^{0}C, an

Figure 3. Templates for the production of synthetic VSV genome sequences.

The constructs shown schematically here were produced by amplification of infected cell RNA using RT-PCR reactions to give products representative of the 3' termini of the genome and antigenome respectively, which were subsequently cloned into pUC19. The 5' primer used in these amplification reactions, contained a truncated T7 RNA polymerase promoter orientated such that the first base produced in a T7 RNA polymerase reaction would be the first base of either the + leader RNA (top construct) or the - leader RNA (bottom construct), produced from the genome and anti-genome respectively. Linearization at the EcoRV site followed by run-off transcription gives rise to 250 base RNA's which contain either the + or - sense leader sequence, leader-N or leader-L gene junctions and portions of the N or the L genes (+ leader/N and - leader/L respectively).


EcoRV
XhoI
KpnI
XbaI
Puc19
ΔT7
3'leader
N
Puc19
+leader/N
250bp

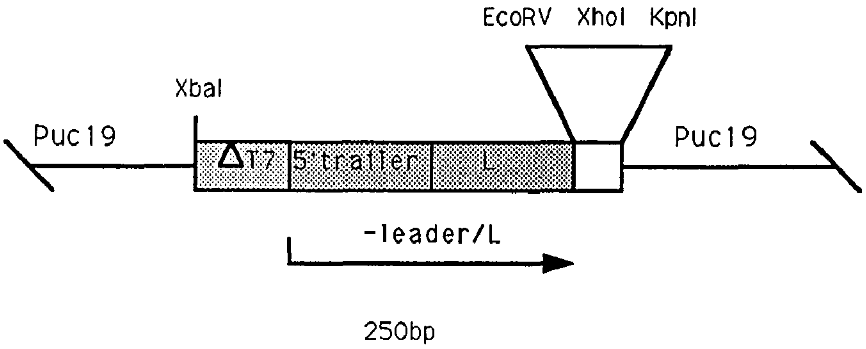
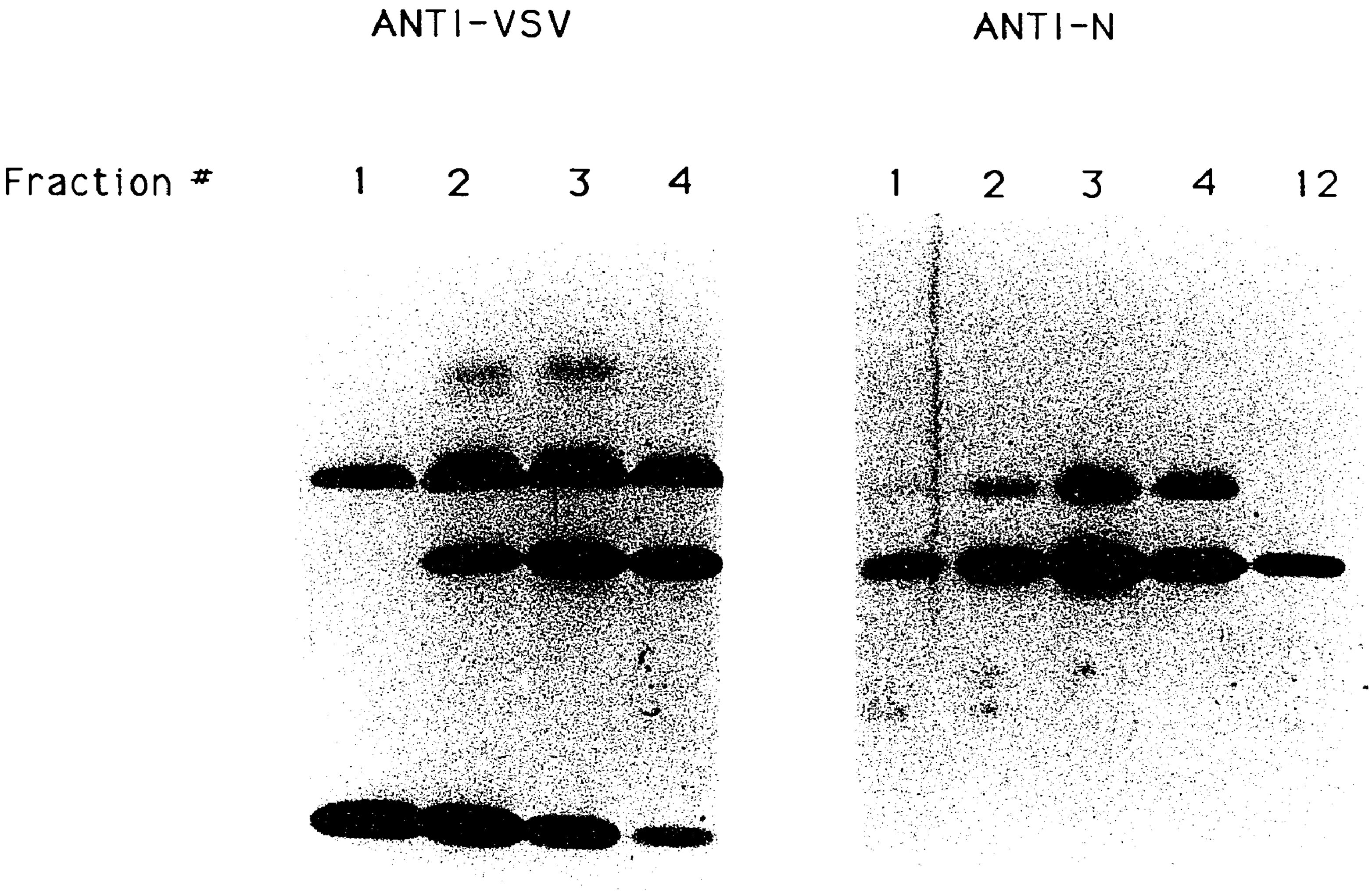

EcoRV
XhoI
KpnI
XbaI
Puc19
ΔT7
5'trailer
L
Puc19
-leader/L
250bp

Figure 4. Isolation of the N:P complex from infected cells.

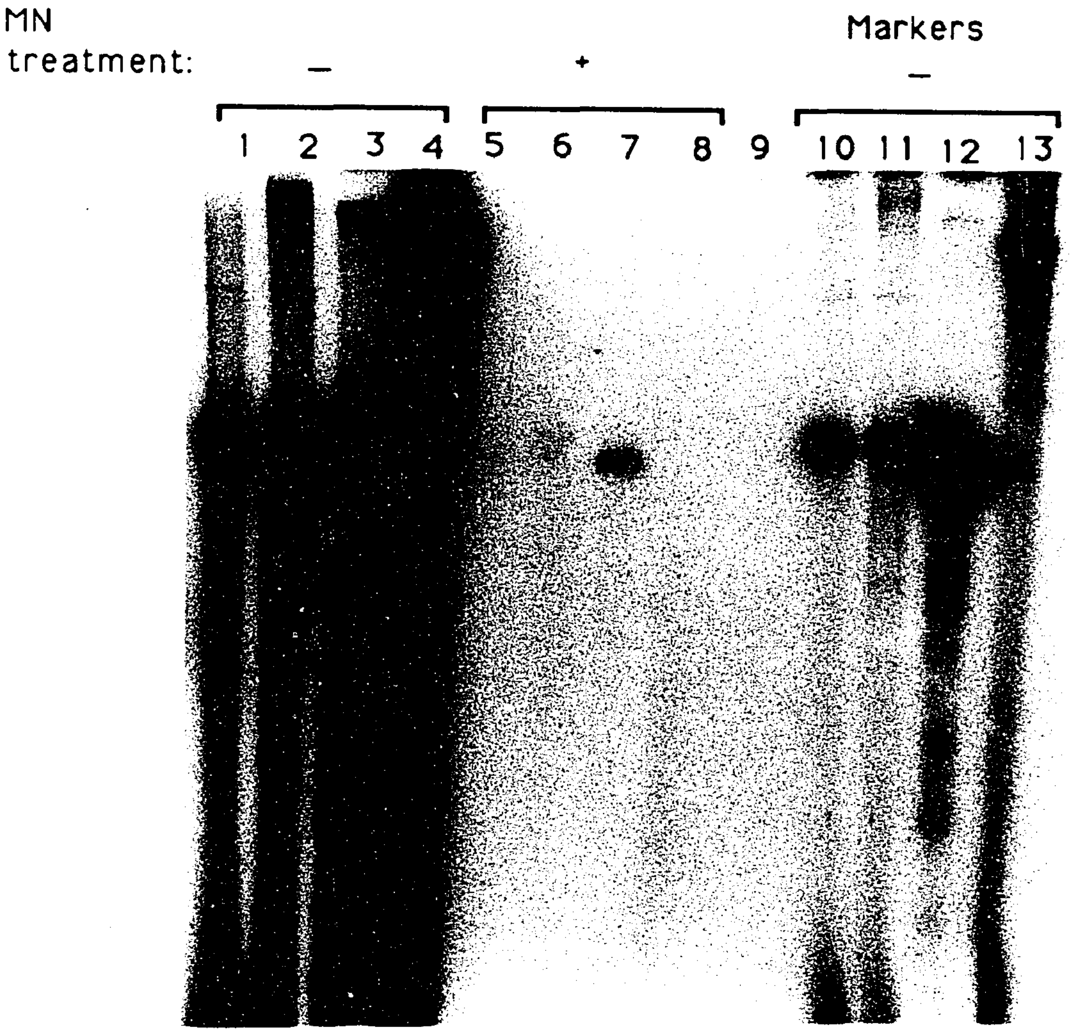
Cells labeled with ^{35}S methionine and infected with VSV were permeabilized with lysolecithin and cell extracts were loaded onto glycerol gradients for centrifugation. The gradients were fractionated and analyzed for total viral protein content (left figure) with anti- VSV antiserum and for complex formation with anti-N monoclonal antibody (right figure). In the anti-N immunoprecipitates, the N protein (lower band) and the P protein (upper band) can be seen. In the anti-VSV immunoprecipitates, the N and P proteins co-migrate with the same proteins in the anti-N immunoprecipitates and in addition, the M protein (very bottom band) and the G protein (band above the P protein) may also be seen. Fractions containing the complex were indicated by the co-precipitation of the P protein in anti-N precipitates.


ANTI-VSV
ANTI-N
Fraction #
1
2
3
4
1
2
3
4
12

optimal temperature for replication, to allow binding and encapsidation of the RNA. The transcripts were then assayed for resistance to micrococcal nuclease. The results of this type of " run-off protection" experiment are shown in figure 5. The + leader/N, - leader/L RNAs and a control RNA of similar length transcribed from the plasmid pGEM, were all relatively stable for the duration of the incubation period [compare lanes 1, 2, and 3 with the marker lanes (not incubated with the complex)]. However, incubation of the RNA with the N:P complex did not render the + leader/N and - leader/L RNAs resistant to MN treatment (lanes 5 and 6). Some apparent protection of the pGEM RNA is observed (lane 7), however, this RNA is synthesized at a far higher level than the other two since unlike the other plasmids, the T7 promoter in pGEM is not truncated. Thus the RNA band seen in lane 7 is more likely to result from an overloading of this reaction with RNA rather than a non-specific protection effect. The VSV-CAT RNA in lanes 4, 8 and 13 is derived from a construct containing both ends of the VSV genome linked by an inserted chloramphenicol acetyl transferase (CAT) gene and is also unprotected by the N:P complex from the action of the nuclease (lane 8). The design of the encapsidation templates for the foregoing experiment was influenced mainly by a desire to reproduce the exact 5' ends of the genome and antigenome such that artificially added bases at these locations could not influence binding of the complex. In addition, the leader/N and leader/L junctions were also included since we were interested to know whether these junctions would present a barrier to the encapsidation process, if indeed it did occur. However, the possibility existed that despite efforts to denature the RNA prior to incubation with the N:P complex, the length of these transcripts might permit formation

Figure 5. Microccocal nuclease protection of synthetic RNA transcripts.

Glycerol gradient isolated N:P protein complexes were incubated with various radiolabeled T7 RNA polymerase transcripts (indicated to the right of the figure), divided into two equal aliquots and either treated (+MN) or left untreated (-MN) with microccocal nuclease. Subsequently the RNA was extracted, separated on 5% polyacrylamide gels containing 7M urea and visualized by autoradiography. The 250 base + leader/N and - leader/L RNAs have been described in the legend to Figure 3 and the pGEM RNA is transcribed from the pGEM plasmid (Promega) linearized at the Ban 1 site and is of similar size to the + and - leader transcripts. The VSV-CAT RNA contains both terminal regions of the VSV genome separated by an inserted CAT gene. The marker lanes represent radiolabeled transcripts which were not incubated with gradient fractions or treated with MN and were run directly on the gel.

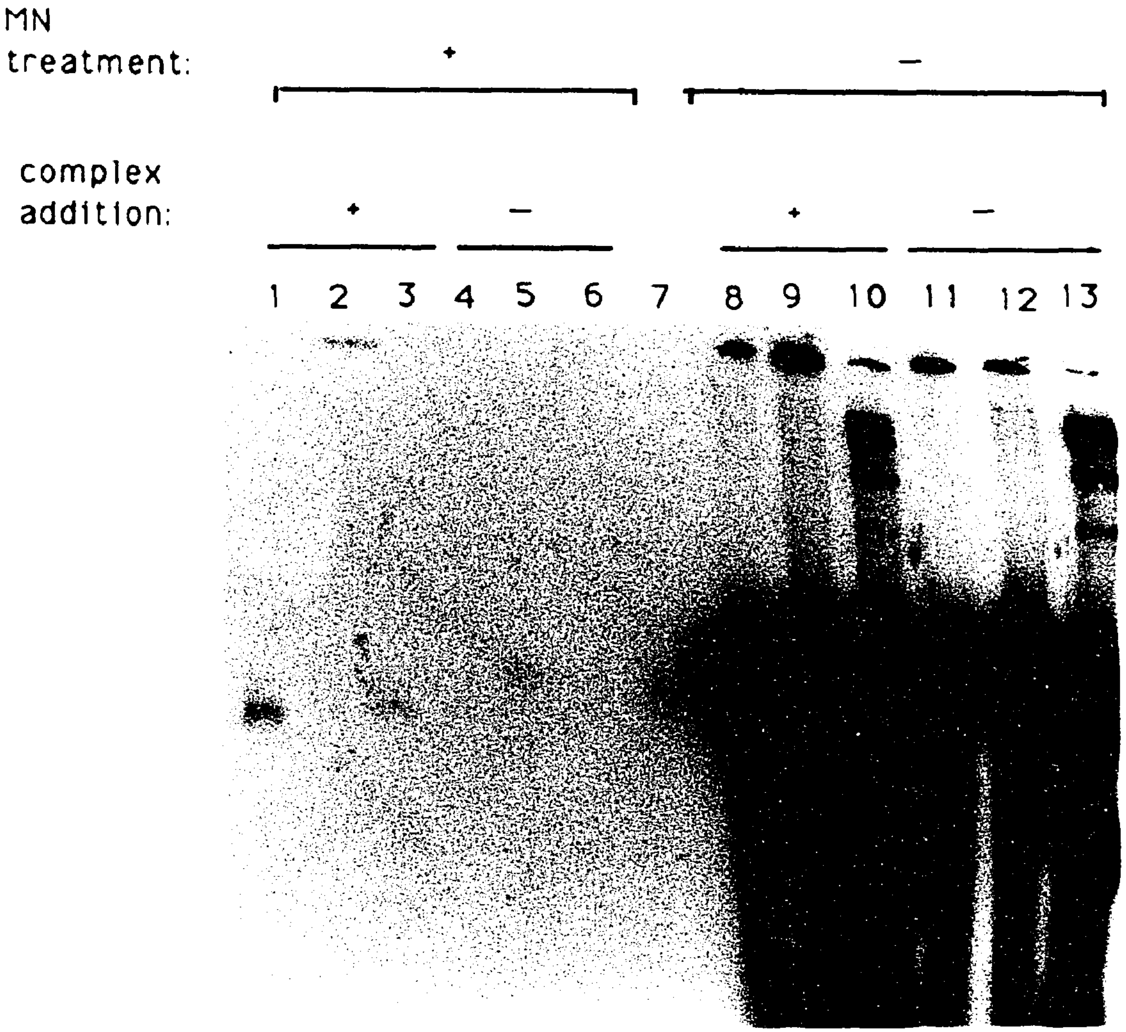


LANE#	RNA
1	+ LEADER/N
2	- LEADER/L
3	pGEM
4	VSVCAT
5	+ LEADER/N
6	- LEADER/L
7	pGEM
8	VSVCAT
9	—
10	+ LEADER/N
11	- LEADER/L
12	pGEM
13	VSVCAT

of secondary structure and occlude the putative nucleation sequences at the 5' terminus of the RNA. To address this problem, I adapted a technique which has been successful for the encapsidation of synthetic influenza genomic segments (Luytjes et al., 1989). Gradient fractions containing the N:P complex were first dialyzed against a buffer suitable for the activity of the T7 RNA polymerase. The enzyme, α^{32}P-UTP and linearized plasmid were added and the polymerization reaction was allowed to proceed. In this way, I hoped to continuously provide nascent genomic ends to the N:P complex such that we could mimic the natural situation in the infected cell more closely. The results of these experiments (figure 6) show that high levels of RNA are synthesized in both the presence and the absence of the complex (lanes 8-13), however, only a very low level of the + leader /N RNA (lane 1) and none of the - leader/L RNA (lane 2) were resistant to MN. While the low level of protection of the + leader/N RNA observed may be the result of a specific encapsidation event, it is also possible that saturating amounts of RNA produced in the reaction or non-specific RNA binding, as suggested by a similar level of protection of the pGEM RNA (lane 3), could be responsible for this effect. Considered together, the protection experiments indicate that interaction of the N:P complex with encapsidation substrates is at best very inefficient.

Figure 6. Micrococcal nuclease protection of nascently produced synthetic RNA.

Glycerol gradient fractions containing the N:P complex were dialyzed against T7 RNA polymerase buffer and were included in T7 RNA polymerase reactions containing linearized template DNA and α^{32}P-UTP. Control reactions were also performed which did not contain the N:P complex and all reactions were split into equal aliquots and were either treated (+MN) or left untreated (-MN) with micrococcal nuclease. The RNA was extracted, run on 5% polyacrylamide/7M urea gels and visualized by autoradiography. The RNA's produced in each reaction are noted to the right of the figure.



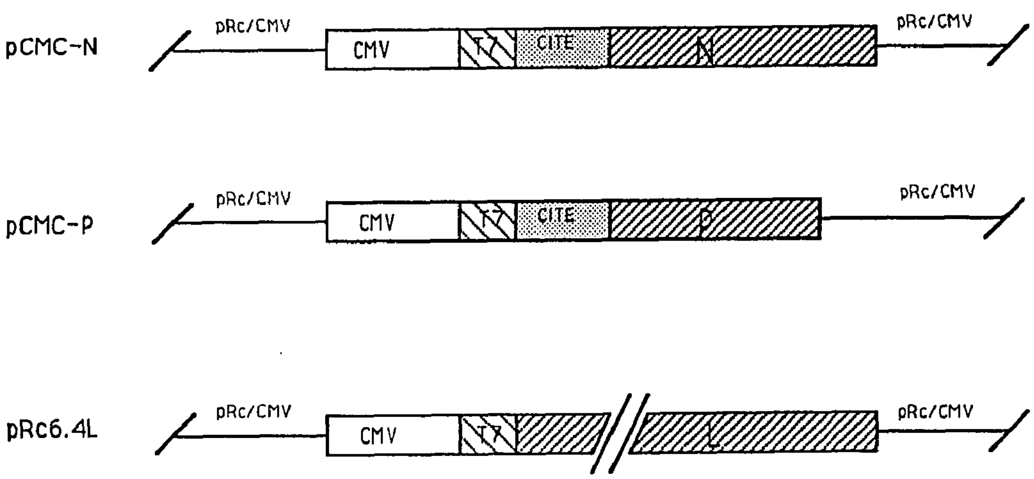
LANE*	RNA
1	+LEADER/N
2	-LEADER/L
3	pGEM
4	+LEADER/N
5	-LEADER/L
6	pGEM
7	—
8	+LEADER/N
9	-LEADER/L
10	pGEM
11	+LEADER/N
12	-LEADER/L
13	pGEM

2) *Establishment of an in vivo replication system*

The results of the *in vitro* protection experiments suggested that the N:P complex alone does not function to directly encapsidate free viral RNA sequences and that perhaps the encapsidation reaction may only be efficient in the context of the viral polymerase and template. In this light, it was felt that further analysis of the role of the N:P complex in replication could only be undertaken if a system was developed in which the viral nucleocapsid was responsible for RNA synthetic events and where components of the N:P complex could be manipulated at will. Such a system has recently been developed (Pattnaik and Wertz, 1990) and relies upon the high level expression of the VSV N, P and L proteins from plasmids containing the T7 RNA polymerase promoter by means of a recombinant vaccinia virus expressing the T7 RNA polymerase gene (vaccinia-T7). The co-expression of all three VSV proteins was reported to support the replication of a VSV DI genome which lacks the capacity to encode proteins of its own. In order to reproduce this system in our laboratory, plasmids containing the N, P and L genes under the control of the T7 promoter were constructed as detailed in Materials and Methods (Figure 7). A cap-independent translational enhancer (CITE) sequence from the encephalomyocarditis virus was included in these constructs, directly preceding the initiator AUG, since this sequence has been shown to result in increased levels of target protein synthesis in the vaccinia T7 system (Elroy-Stein et al., 1989). Infection of BHK cells with the recombinant vaccinia virus followed by transfection with these constructs, resulted in levels of expression at 24 hours post-transfection which approached those seen in VSV-infected cells at peak times of replication (Figure 8). Transfected cells expressing such

Figure 7. VSV protein expression vectors.

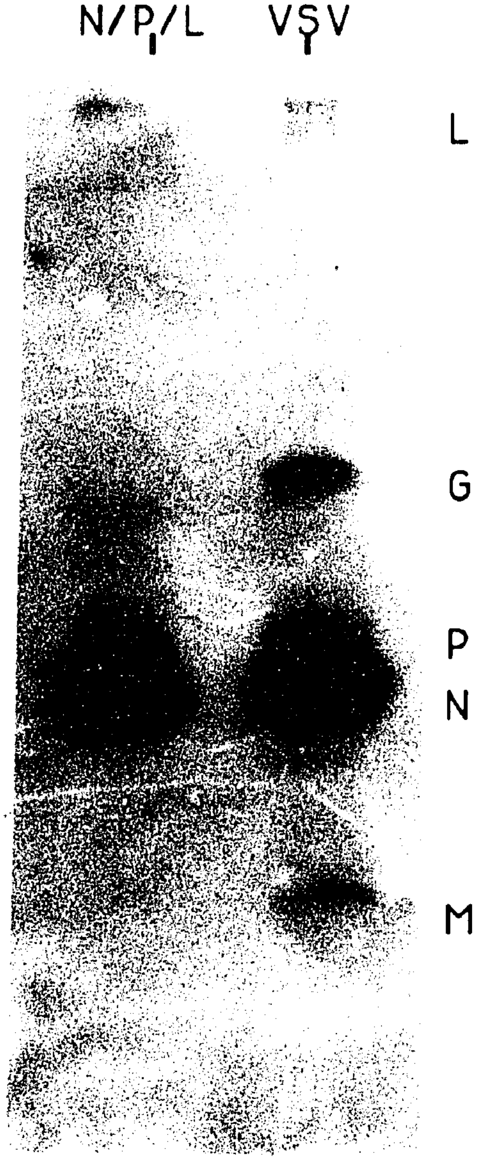
The plasmid pRc/CMV was used as a basis to construct eukaryotic expression vectors containing the VSV N, P and L genes. pRc/CMV contains both the cytomegalovirus (CMV) and the T7 RNA polymerase (T7) promoters in addition to a polylinker cloning region directly following the T7 promoter. The N and P genes were first cloned into an intermediate vector containing the cap-independent translational enhancer (CITE) of encephalomyocarditis virus (EMC) and the CITE-N and CITE-P fragments were introduced into pRc/CMV, while the L gene was cloned directly into the polylinker region of pRc/CMV. The resulting constructs are suitable vectors for use with the vaccinia-T7 expression system described in the text (Results section I.2).


pCMC-N
pRc/CMV
CMV
T7
CITE
N
pRc/CMV
pCMC-P
pRc/CMV
CMV
T7
CITE
P
pRc/CMV
pRc6.4L
pRc/CMV
CMV
T7
L
pRc/CMV

levels of recombinant viral protein were super-infected with DI particles which had been purified away from wild-type virus by successive separations on sucrose gradients. Replication of the DI particle genome RNA was followed by labeling the cells with ^{3}H-uridine and resolving micrococcal nuclease protected products purified from extracts of these cells, on acid-urea-agarose gels. Figure 9 shows clearly that the recombinant N, P and L proteins will support replication of the 19S DI RNA (lane 3) to a level which is close to that produced by a standard co-infection of the *wt* virus and the defective particle (lane 1). While the purified DI particle genome does not replicate in the absence of artificially expressed proteins (lane 4), the unpurified supernatant from a co-infection with the DI particle and the wild-type virus has no such requirement for genome replication because of the presence of contaminating *wt* helper virus in this stock (lane 2). This result demonstrates the necessity to carefully purify the DI stock from the helper virus so that complete dependence upon recombinant protein expression can be established. The absence of genome replication observed when purified DI particle stock is used to infect vaccinia-T7 infected cells (lane 4), indicates that the DI preparation employed in this experiment and for subsequent studies is free of *wt* VSV. Therefore the level of replication seen in lane 3 is supported solely by protein expresssion from recombinant vectors.

Figure 8. High level expression of VSV proteins in the vaccinia-T7 expression system.

BHK Cell monolayers were infected at an MOI of 1 pfu per cell with a recombinant vaccinia virus containing the gene for the T7 RNA polymerase and then transfected with 3μg of each of the constructs shown in figure 7, which contain the L, P, and N gene downstream of the T7 RNA polymerase promoter. To assess expression from this promoter *in vivo,* lysolecithin permeabilized extracts were made at 24 hours after infection and an aliquot of the extract was run alongside an equivalent portion of VSV infected cell extract, on an 11% SDS-polyacrylamide gel. The proteins were transferred to Zetabind membranes and the blot was probed with an anti-VSV antiserum and ^{125}I protein G and then visualized by autoradiography.


N/P/L
VSV
L
G
P
N
M

3) *Formation of a triple complex between recombinant N, P and L proteins in transfected cells.*

The fact that the N, P and L proteins expressed from recombinant plasmids in the vaccinia-T7 virus infected cell will support DI genome replication, indicates that the properties of these proteins with regard to genomic replication are preserved. Therefore efficient complex formation between the N and the P proteins would be expected. Co-immunoprecipitation experiments (Figure 10) using monoclonal antibodies specific for the P protein (lane 3) or the N protein (lane 5) show that in both cases large amounts of either protein co-precipitate with the antibody to the other protein, and this confirms appropriate complex formation (the specificity of the monoclonal antibodies used is shown in figure 11, lanes 2, 4, 7 and 9). More interestingly, when all three recombinant nucleocapsid proteins were expressed in the same cell and extracts were precipitated with anti-N monoclonal antibody, it was observed that some portion of the N protein was in a triple complex with the P and L proteins (Figure 10, lane 1), a result which is not due to a cross reaction of the antibody with either the P or the L proteins (Figure 11, lanes 6 and 7). A similar triple complex, where the N and the L proteins co-precipitated with the P protein was obtained when the anti-P monoclonal antibody was used (data not shown). While we have not characterized this complex further, the fact that it exists demonstrates that the N:P complex may be able to interact with the L protein alone , or more importantly with the viral polymerase (L+P).

Figure 9. Support of DI particle genome replication by recombinant VSV proteins *in vivo*

N, P and L proteins expressed with the vaccinia-T7 system in BHK cells, were super-infected with 1.8 μg of sucrose gradient purified MST DI particles (Purified DI) and incubated for 5 hours in the presence of ^{3}H uridine. Cell extracts were treated with MN and the RNA extracted and run on 1.5% agarose gels containing 6M urea. The 19S DI RNA was visualized by fluorography and can be seen to be present as a doublet, corresponding to the genome (top band) and the antigenome (bottom band) respectively. Additions (+) and omissions (-) of the various components are indicated at the top of the figure (Vac-T7: vaccinia -T7, N/P/L: co-transfected pCMC-N (6 μg), pCMC-P(4 μg) and pRc6.4L(2 μg), DI: unpurified DI particle stock, wtVSV: wild-type VSV).

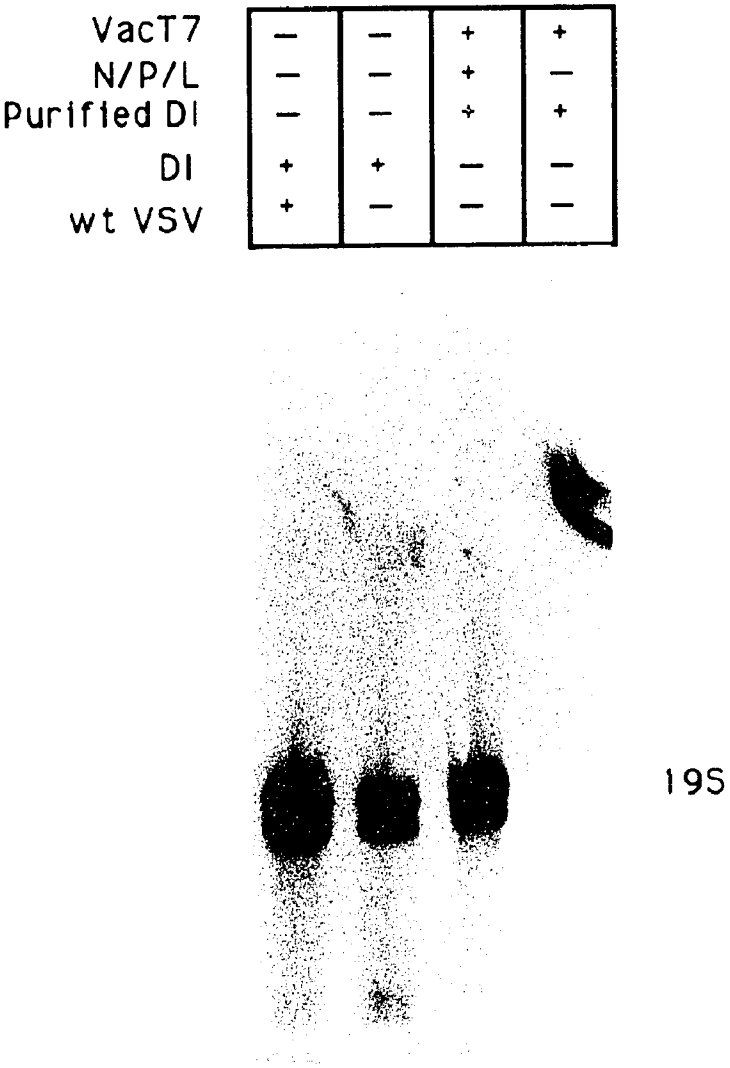
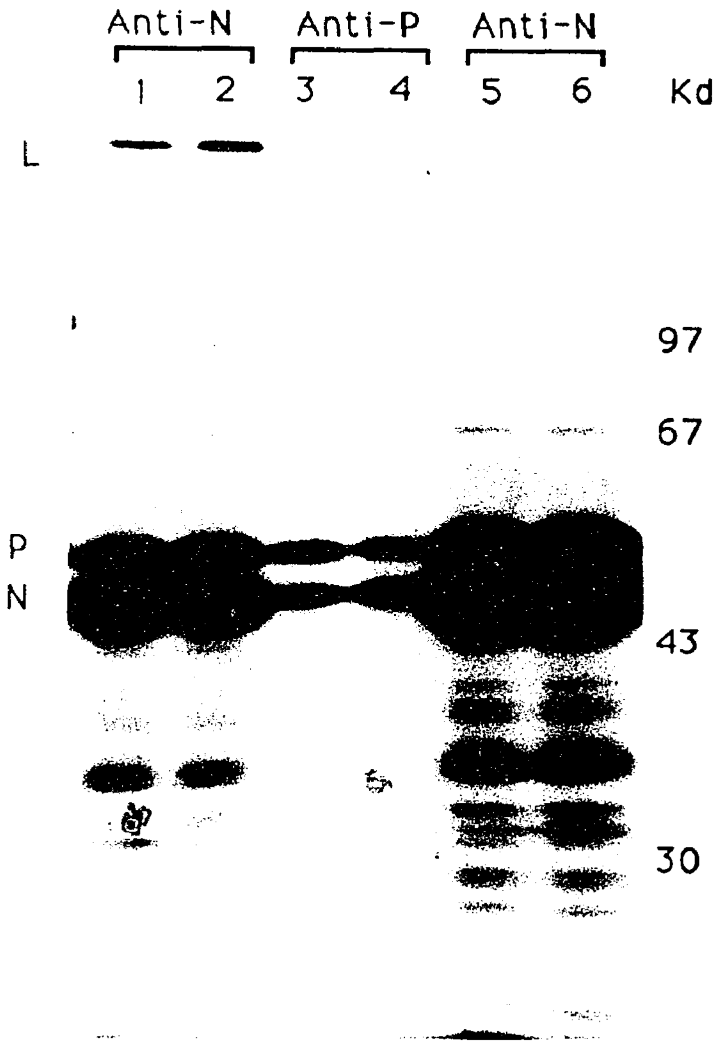

VacT7 — — + +
N/P/L — — + —
Purified DI — — + +
DI + + — —
wt VSV + — — —
19S

Figure 10. Complex formation between VSV proteins expressed by the vaccinia T7 system in BHK cells.

The L, N, P and P3S proteins were expressed in BHK cells using the vaccinia-T7 system and were metabolically labeled from 22-25 hours p.i.. Extracts were immunoprecipitated with monoclonal antibodies to the N and P proteins. 2, 4 and 6 micrograms of the pRC6.4L, pCMC-P or p CMC-P3S and pCMC-N constructs were transfected respectively and the combination transfected is indicated to the right of the figure. The P3S construct contains a mutant P protein with introduced amino acid substitutions as indicated in figure 13. The immunoprecipitates were run on 11% SDS-polyacrylamide gels and the precipitated proteins were visualized by fluorography. The antibody used in each lane is indicated at the top of the figure.



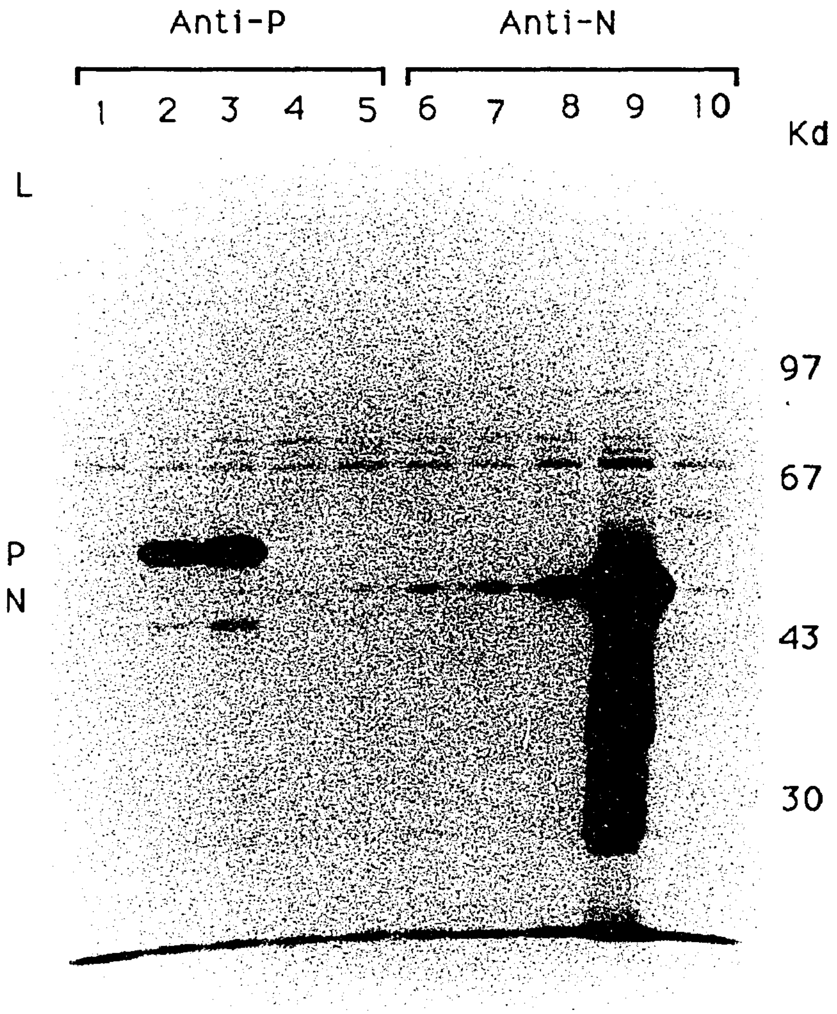
Lane#	Plasmid Transfected
1	L/N/P
2	L/N/P3S
3	N/P
4	N/P3S
5	N/P
6	N/P3S

4) The N and P proteins supplied in trans and the virion associated polymerase are sufficient to support the replication of the DI particle in vivo.

The initial report of the vaccinia-T7 based system for the support of DI genome replication *in vivo* stipulated that all three nucleocapsid proteins must be expressed in order for replication to occur (Pattnaik and Wertz, 1990). However, a repeat of this experiment with my system yielded quite different results (Figure 12). In agreement with the previous report, expression of N, P or L proteins individually (Figure 12, lanes 1, 2 and 4) would not support DI genome replication, but when these proteins were expressed together, replication resulted (lane 5). However, if the L protein was omitted and just the N and P proteins were expressed, a similar level of genome replication to the level obtained when all three proteins were provided was observed (lane 8). This result indicates that the polymerase packaged within the DI particle is active during replication since this is the only source of the L protein and thus the catalytic domain for viral RNA synthesis, in the transfected cells. The N:P protein complex is the substrate for encapsidation of genomic RNA *in vivo* and the previous results (section I.1) show that this complex does not act independently in encapsidating RNA. Therefore the results of this experiment strongly suggest that the N:P protein complex formed here is interacting directly with the nucleocapsid associated polymerase to replicate the DI genome.

Figure 11. Specificity of the Anti-P and Anti-N monoclonal antibodies.

Immunoprecipitations of the L, P, P3S and N proteins expressed individually in the vaccinia-T7 system are shown. Immunoprecipitates brought down with the identical monoclonal antibodies to those used for the experiment in figure 10, were visualized as before. The antibodies used in each lane are indicated at the top of the figure and the construct transfected to the right.

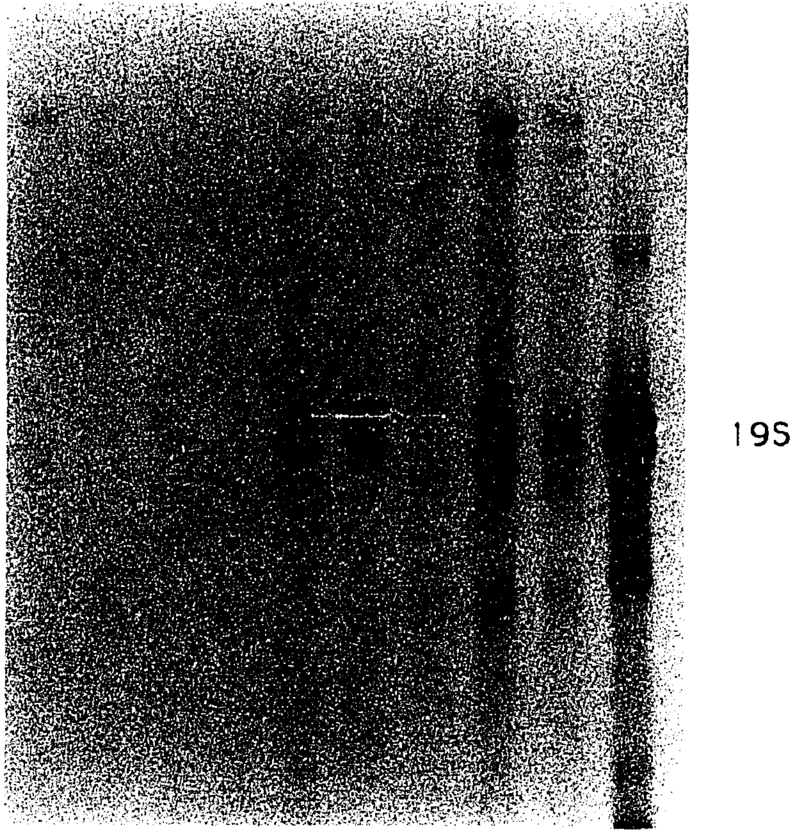


Lane#	Plasmid Transfected
1	L
2	P
3	P3S
4	N
5	NONE
6	L
7	P
8	P3S
9	N
10	NONE

Figure 12. Minimum requirements for the replication of the DI genome *in vivo.*

Protein requirements for the support of DI particle genome replication were addressed using the vaccinia-T7 based DI replication system previously established. Vaccinia-T7 infected BHK cells were transfected with 2, 4 and 6 μg of L, P or P3S and N constructs, in various combinations as indicated at the top of the figure (addition:(+), omission(-)) and 19S RNA products were labeled with ^{3}H uridine and processed as described. (Materials and Methods; Figure 9 legend). Purified RNAs were resolved in agarose-urea gels and were visualized by fluorography. Support of the DI plasmid by wild-type VSV is shown.

	1	2	3	4	5	6	7	8	9	10
N	+	—	—	—	+	+	+	+	+	—
P	—	+	—	—	+	—	+	+	—	—
P3S	—	—	+	—	—	+	—	—	+	—
L	—	—	—	+	+	+	+	—	—	—
Purified DI	+	+	+	+	+	+	—	+	+	+
wt VSV	—	—	—	—	—	—	—	—	—	+



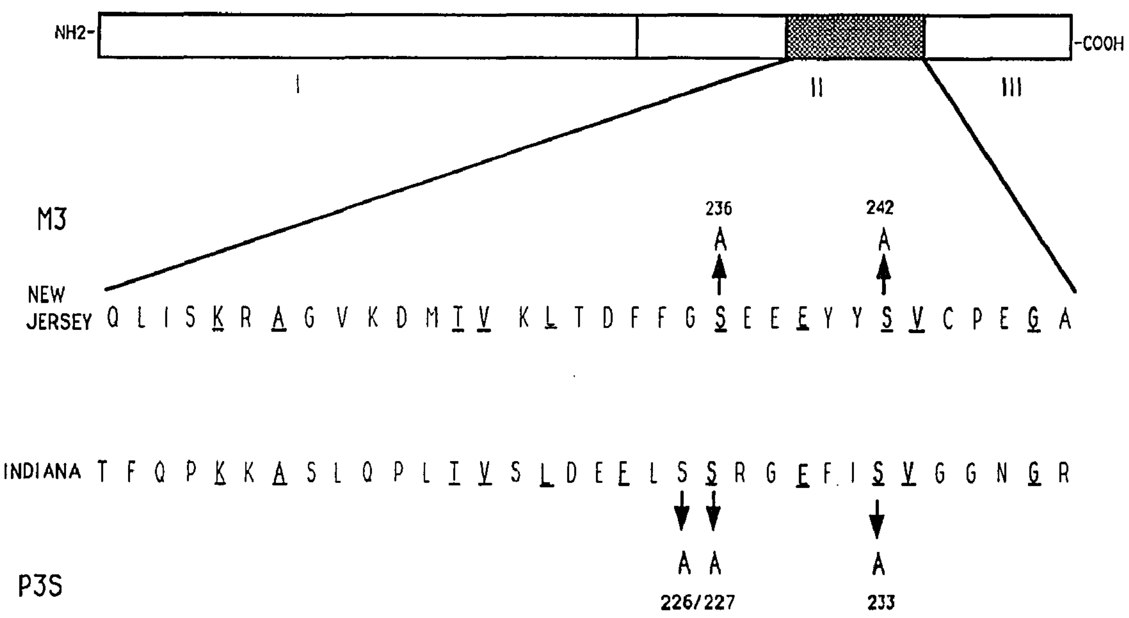
5. *A P protein mutant which is defective in RNA synthesis will support the replication of the DI genome in vivo when supplied in trans with the N protein.*

If the N: P protein complex is indeed interacting with the viral RNA polymerase during replication, the distinct possibility arises that the P protein component of this complex plays an auxiliary role in the process of RNA synthesis. In order to test this hypothesis a mutant P protein which is defective in RNA synthesis, yet retains the ability to complex to the N protein, was required. A candidate which potentially possesses both of these properties has been engineered from the P protein of the New Jersey serotype of VSV and has been called M3 (Chattopadhyay and Banerjee, 1987; Figure 13). This mutant contains serine to alanine mutations at two conserved positions in domain II of the P protein, which effectively eliminates the ability of this mutant to act as a substrate for the L kinase and interferes with the capacity to effectively bind to the N-RNA template. The overall effect of the introduced mutations is to severely handicap transcriptional activity when the mutated P protein is reconstituted with the L protein and the N-RNA template *in vitro*. In order to produce a similar mutant P protein of the Indiana serotype, the conserved amino acid residues in domain II were substituted with alanine by introducing mutations at the DNA level into the construct pCMC-P to give the plasmid pCMC-P3S. When this plasmid was expressed *in vivo,* a polypeptide with the same mobility and which could be immunoprecipitated with the same monoclonal antibody as the *wt* protein was produced (Figure 11, lanes 2 and 3). Co-expression of the mutant with the N protein alone or with both the N protein and the L protein resulted in the formation

Figure 13. P protein domain II mutants.

A schematic division of the P protein into three domains is shown and the amino acid sequence of domain II is indicated below the diagram. Serine to alanine amino acid substitutions introduced into domain II of the New Jersey serotype P protein to produce the M3 mutant, are indicated and the numbers above the substitutions refer to their position in the P protein sequence. Completely conserved amino acid residues between the New Jersey and Indiana sequences in domain II are underlined. Substitutions introduced into the New Jersey serotype were duplicated by PCR directed mutagenesis at the conserved serine residues in the Indiana P protein sequence (positions 226, 227 and 233, shown below the New Jersey sequence) to produce the mutant P3S.

P PROTEIN DOMAIN II



PROPERTIES OF M3

(Chattopadhyay and Banerjee, Cell 49, 1987)

1) Reduces transcription levels to 10% of the wildtype P protein.

2) N-RNA template binding levels are only 10% of the wildtype level

3) M3 is not a substrate for the L kinase.

of two-component or three-component complexes respectively which exhibited identical protein ratios to those displayed by the *wt* protein (Figure 10, lanes 2, 4 and 6).

Since the P3S mutant was behaving identically in terms of complex formation to the *wt* protein, it was important to establish that P3S possessed the same transcriptional phenotype as the M3 protein. This was achieved by taking advantage of an *in vitro* extension of the vaccinia-T7 system developed by Canter *et al.* (1993) which depends upon the production of cell extracts containing recombinant L and P proteins. These extracts were capable of directing transcription from an N-RNA template stripped of all associated viral polymerase. In this *in vitro* system, protein synthesis from the wt N-RNA template is not significant and transcription is totally dependent upon recombinant protein expression. Incubation of cell extracts containing wild-type P and L proteins expressed from the plasmids pCMC-P and pRC6.4L, with stripped N-RNA templates and the appropriate reaction mix, resulted in the production of viral transcripts of the same size and at the same level as those produced by infected cell extracts (Figure 14, compare lane 5 and lane 1). This result indicates that the proteins produced *in vivo* are functional in the *in vitro* assay. However, incubation of the N-RNA template with cell extracts containing recombinant vaccinia virus but no expressed VSV proteins, did not result in viral transcription as expected (Figure 14, lane 7). When extracts were used which contained the L protein and the P3S mutant, an expected lower level of transcriptional activity was obtained (Figure 14, lane 6). The low transcriptional activity observed in the P3S reaction may be due to the presence of trace amounts of wild-type P protein left bound to the N-RNA template since it is notoriously difficult to remove the P protein completely from nucleocapsids (Keene et al., 1981). In support of this possibility, it should be noted that extracts which contained the L protein alone but not those containing just the P or P3S proteins, also produced a similar low level of transcription (Figure 14, lanes 2, 3 and 4). Therefore it appears that the Indiana mutant P3S shares the RNA synthesis defect of the original New Jersey mutant, but retains the ability to bind to the N protein. Use of the P3S mutant in the *in vivo* replication system which employs proteins expressed from recombinant plasmid vectors to support DI particle genome replication, is shown in Figure 12. In this experiment, P3S was expressed with both the L and the N proteins or with just the N protein (lanes 6 and 9) and the cells were then super-infected with DI particle. Quite clearly, the level of genome replication supported in both cases approaches that achieved with the wild-type P, N and L proteins (lane 5). Since the demonstrated defect of the P3S mutant in RNA synthesis does not interfere with DI genome replication, the idea that the polymerase packaged in the DI particle is sufficient for replication (Section 1.4) is re-enforced. Secondly, the P3S:N complex is clearly functional for replication despite the defective nature of the P3S protein and therefore the enzymatic involvement of the P component of the N:P complex in replicative RNA synthesis, is not required.

The results of this section show that the N:P protein complex does not independently encapsidate VSV genomic RNA, that a complex between the N, P and the L proteins can form *in vivo* and that the N:P complex supplied *in trans* to a DI particle containing the RNA polymerase is sufficient to support genome replication *in vivo.* With regard to the latter point, it is shown that the polymerase packaged in the DI particle will supply the necessary functions for RNA synthesis during replication and that there is no mandatory

Figure 14. The P3S mutant is defective in RNA synthesis.

Extracts made from cells transfected with various combinations of the L, P and P3S constructs, as indicated at the top of the figure (transfection/addition:(+), omission(-)), were reconstituted with N-RNA from the wild-type virus in a transcription mixture containing α^{32}P-UTP. The reaction products were extracted, separated in a 1.5% agarose-urea gel and visualized by autoradiography. Products of an *in vitro* reaction using endogenous proteins and N-RNA templates of VSV infected cells (lane 1),.and a control lane where vaccinia-T7 infected cell extract was incubated with *wt* N-RNA (Lane 7), were also included. All lanes shown originate from the same autoradiograph.

	1	2	3	4	5	6	7
VacT7	-	+	+	+	+	+	+
VSV	+	-	-	-	-	-	-
L	-	+	-	-	+	+	-
P	-	-	+	-	+	-	-
P3S	-	-	-	+	-	+	-
wt N-RNA	-	+	+	+	+	+	+

enzymatic requirement for the P protein component of the N:P complex in replicative RNA synthesis.

Taken as a whole, these results suggest that an interaction between the N:P complex and the N-RNA template bound polymerase can and most likely does occur *in vivo* and that this interaction is important in mediating encapsidation and hence replication of DI genome RNA.

II. ROLE OF THE C PROTEINS IN VSV TRANSCRIPTION AND REPLICATION.

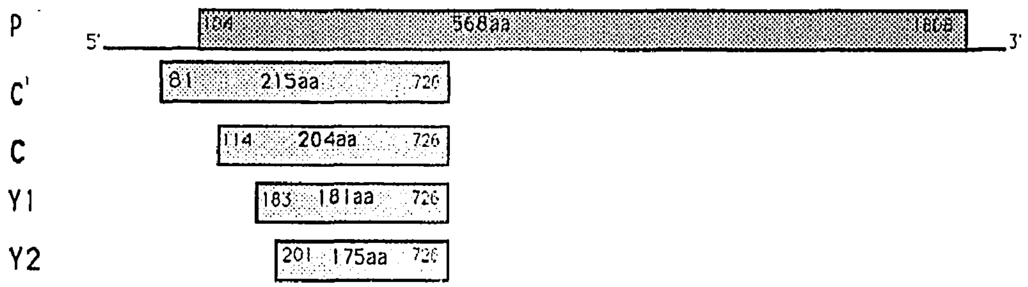
1. Expression of the C proteins in vitro and in vivo from a second open reading frame of the cloned P gene.

The C' and C proteins of VSV are encoded as a nested set in a second open reading frame (ORF) of the P gene (figure 15). The initial demonstration that these proteins are synthesized in the infected cell was made using the New Jersey serotype (Spiropolou and Nichol, 1993) and since that time, no functional characterization in either serotype has been reported. With the aim of using cell extracts containing proteins expressed from the C ORF in *in vitro* transcription and replication systems, I have cloned various combinations of the C genes and derivatives of the P gene into the same expression vector used to set up the vaccinia-T7 system (Figure 16). Using PCR mediated site-directed mutagenesis, the initiation codons (AUGs) of both the C' and C proteins were substituted for the initiation codon at the 3' terminus of the CITE sequence (pCMC-C'/C and pCMC-C respectively) such that T7 RNA polymerase transcripts of these genes would direct the expression of the C' and C proteins but would not encode the P protein (see Materials and Methods). Conversely, a construct (pCMC-ΔC'/C) was engineered which included stop codons in the second open reading frame shortly after both the C' and C protein AUGs, such that synthesis of the P protein would not be affected while expression of the C proteins would be eliminated. In order to assess appropriate expression from these plasmids, the DNA's were linearized at positions following the C stop codon (in pCMC-C'/C and pCMC-C) or the P stop codon (in pCMC-ΔC'/C), uncapped run-off T7 RNA polymerase transcripts were prepared and were subsequently

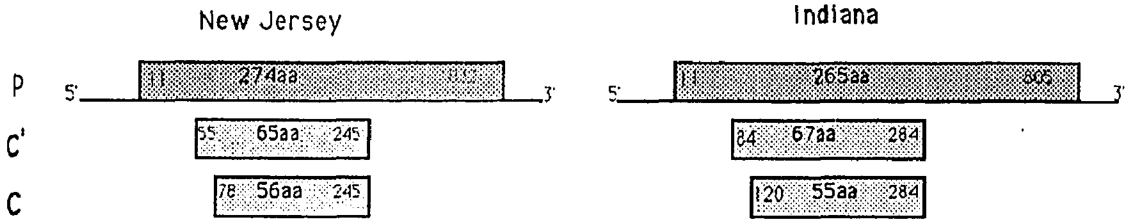
Figure 15. Comparison of the second open reading frame of the P gene of Sendai virus and vesicular stomatitis virus.

The P (dark stipple) and C (light stipple) open reading frames (ORFs) in the P gene of both Sendai virus (top) and vesicular stomatitis virus (New Jersey and Indiana serotypes) (bottom) are shown. In each case the C ORF (ORF2) is +1 in relation to the P gene and the numbers at the end of each box represent the distance of the start and terminus of each coding region from the 5' terminus of the P gene. The ORF2 gene products are all 3' co-terminal, however, the different size indicated (e.g. 215aa: 215 amino acid residues) occurs as a result of utilization of different initiation codons. The calculated molecular weights (Mr) and the pI in the tables at the bottom of the figure refer to properties of the C proteins of the two serotypes of VSV.

SENDAI VIRUS



VESICULAR STOMATITIS VIRUS

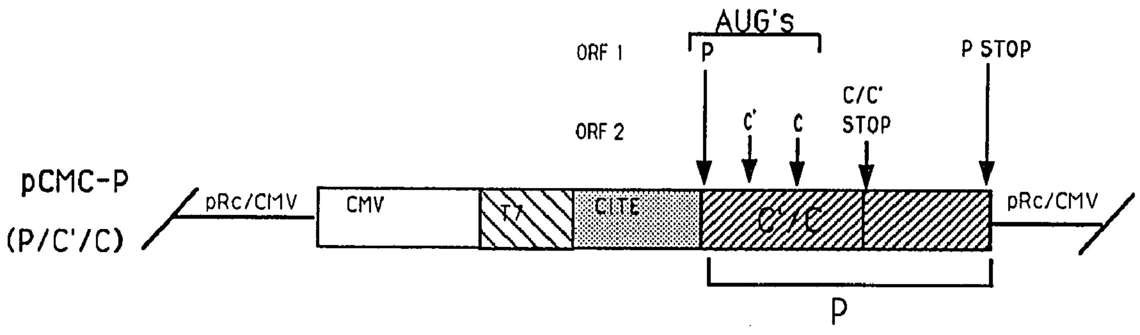


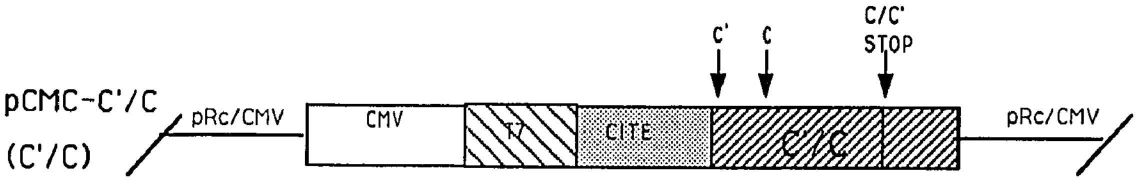
	C'	C
Mr	7930	6600
pI	12.26	12.09

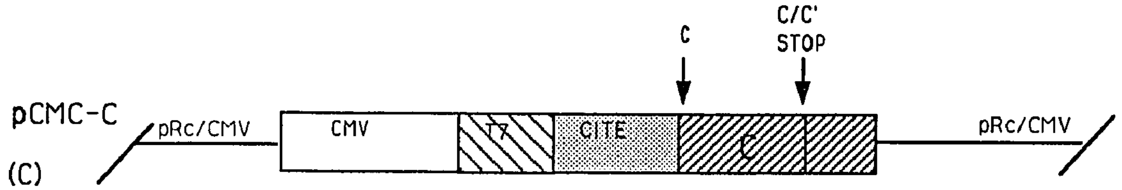
	C'	C
Mr	7960	6500
pI	11.84	11.92

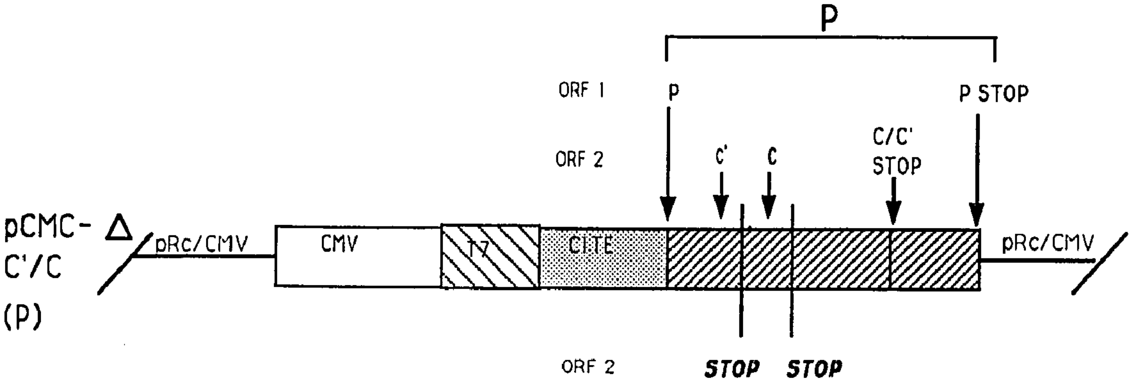
Figure 16. C gene expression vectors.

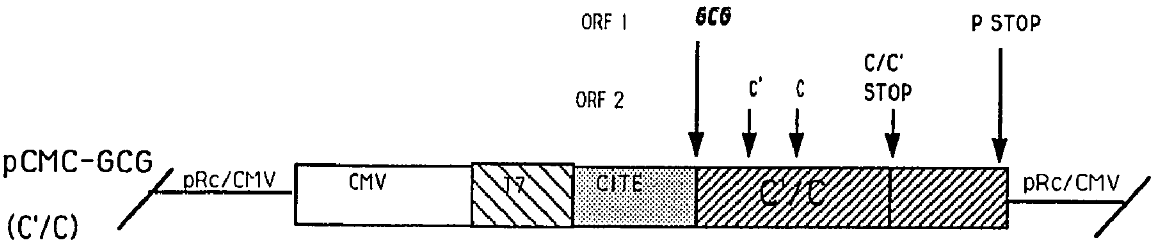
The wild-type P proteins expression vector pCMC-P described earlier, (legend to Figure 7) and various derivatives are shown. pCMC-P has the potential to express the P protein from an open reading frame (ORF) which has been designated ORF1 and in addition, the C and C' proteins in ORF2. Substituting the P protein AUG with each of the C protein initiation codons (AUG's) and deletion of the intervening sequences, results in the production of pCMC-C'/C and p CMC-C which lose the potential to express the P protein but encode the C' and C proteins together (pCMC-C'/C), or the C protein alone (pCMC-C). pCMC-ΔC'/C is produced by the introduction of stop codons directly after each C protein AUG in ORF2 of pCMC-P, such that P protein expression from ORF1 is not affected, while C protein expression is eliminated. Replacement of the P protein AUG with a non-initiator codon GCG, results in the production of a derivative of pCMC-P, designated pCMC-GCG. This construct retains the capacity of the P construct to express C proteins but is unable to express the P protein. The C coding region of the New Jersey P gene was cloned via a intermediate plasmid containing the CITE sequence, into the pCMC-P vector with the elimination of most of the Indiana P gene and the resultant clone pCMC-NJC, has the potential to express both New Jersey C proteins.

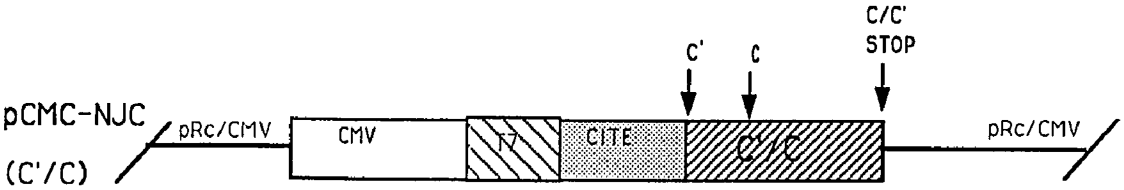

AUG's
ORF 1
P
P STOP
ORF 2
C'
C
C/C' STOP
pCMC-P
(P/C'/C)
pRc/CMV
CMV
T7
CITE
C'/C
pRc/CMV
P


C'
C
C/C' STOP
pCMC-C'/C
(C'/C)
pRc/CMV
CMV
T7
CITE
C'/C
pRc/CMV


C
C/C' STOP
pCMC-C
(C)
pRc/CMV
CMV
T7
CITE
C
pRc/CMV


P
ORF 1
p
P STOP
ORF 2
C'
C
C/C' STOP
pCMC-Δ C'/C
(P)
pRc/CMV
CMV
T7
CITE
pRc/CMV
ORF 2
STOP
STOP


ORF 1
GCG
P STOP
ORF 2
C'
C
C/C' STOP
pCMC-GCG
(C'/C)
pRc/CMV
CMV
T7
CITE
C'/C
pRc/CMV

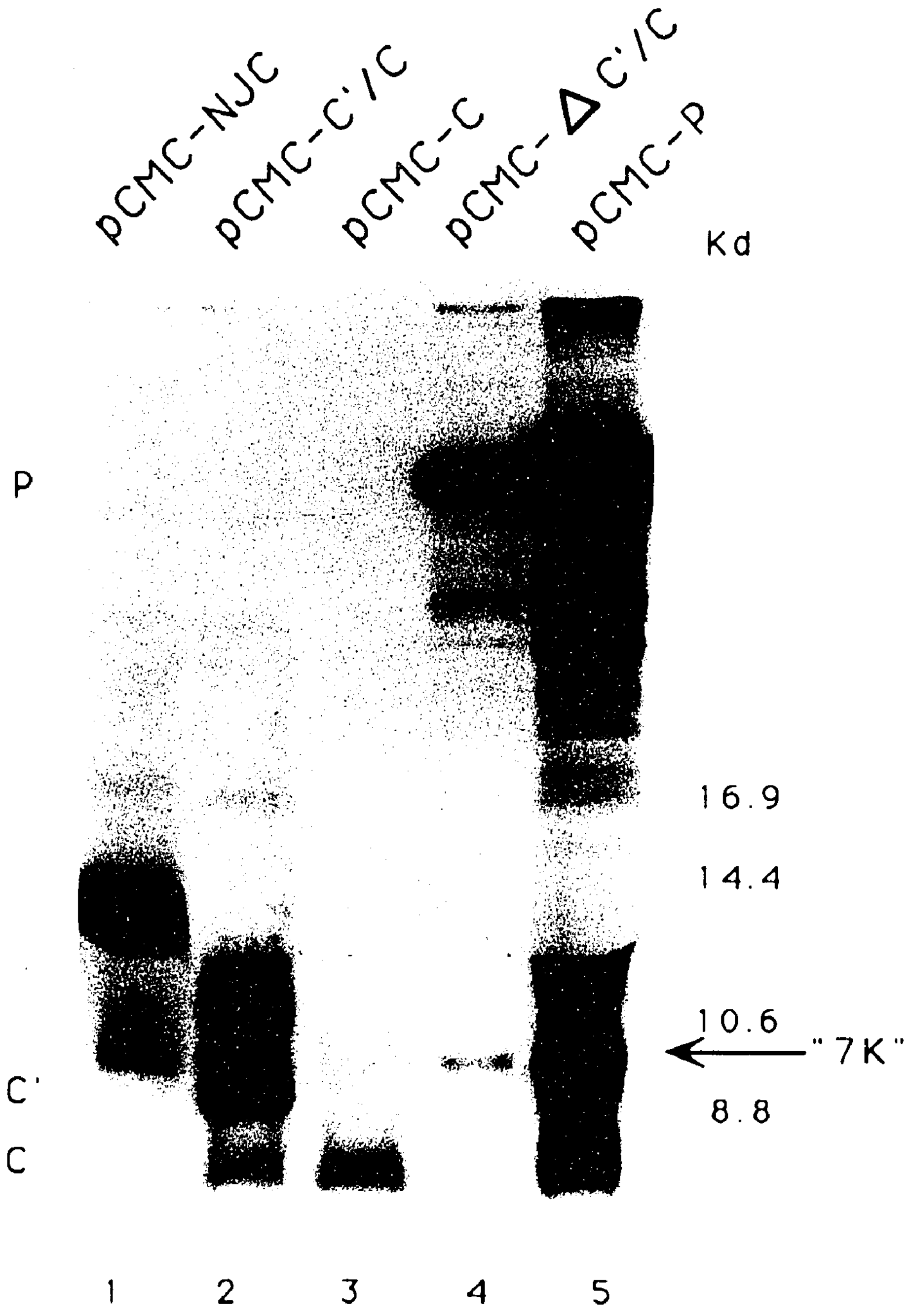

C'
C
C/C' STOP
pCMC-NJC
(C'/C)
pRc/CMV
CMV
T7
CITE
C'/C
pRc/CMV

translated in rabbit reticulocyte lysates (Figures 17 and 18). The products of expression of the C'/C and C constructs in this system (Figure 17, lanes 2 and 3; Figure 18, lanes 11 and 12) co-migrate with the products expressed from the *wt* P gene construct, presumably by internal initiation or by leaky ribosomal scanning (Figure 17, lane 5; Figure 18, lane 13). Expression of the ΔC'/C construct *in vitro* results in the production of the P but not the C proteins as expected (Figure 17, lane 4; Figure 18, lane 9). In addition, a product migrating just above the C' protein which is similarly present when the *wt* P gene is expressed (Figure 17, lane 5; Figure 18, lane 13). Truncation of the P gene at the 3' terminus results in the disappearance of this product (data not shown) which indicates that it represents the previously characterized "7K" protein produced by an independant ribosomal initiation at the 3' terminus of the P gene (Herman, 1986). The bands migrating between the C and P proteins (Figure 17, lanes 4 and 5; Figure 18, lanes 11 and 13) are artefacts of the *in vitro* translation system and are thought to derive from internal initiations in the P mRNA.

The object of cloning the C genes was to express them in the vaccinia -T7 system and to use cell extracts containing the expressed C proteins in transcription and replication reactions *in vitro*. Although it was reasonable to expect appropriate expression in the vaccinia-T7 system since with the exception of the inserted gene, the constructs to be used were identical to those employed earlier (Results, Section I.2), it was desirable to demonstrate the presence of the C proteins in transfected cell extracts. However, a major hindrance in this regard was the extreme difficulty experienced in our laboratory in raising an antibody to the Indiana C proteins. To overcome this lack of a

Figure 17. Expression of recombinant C proteins *in vitro.*

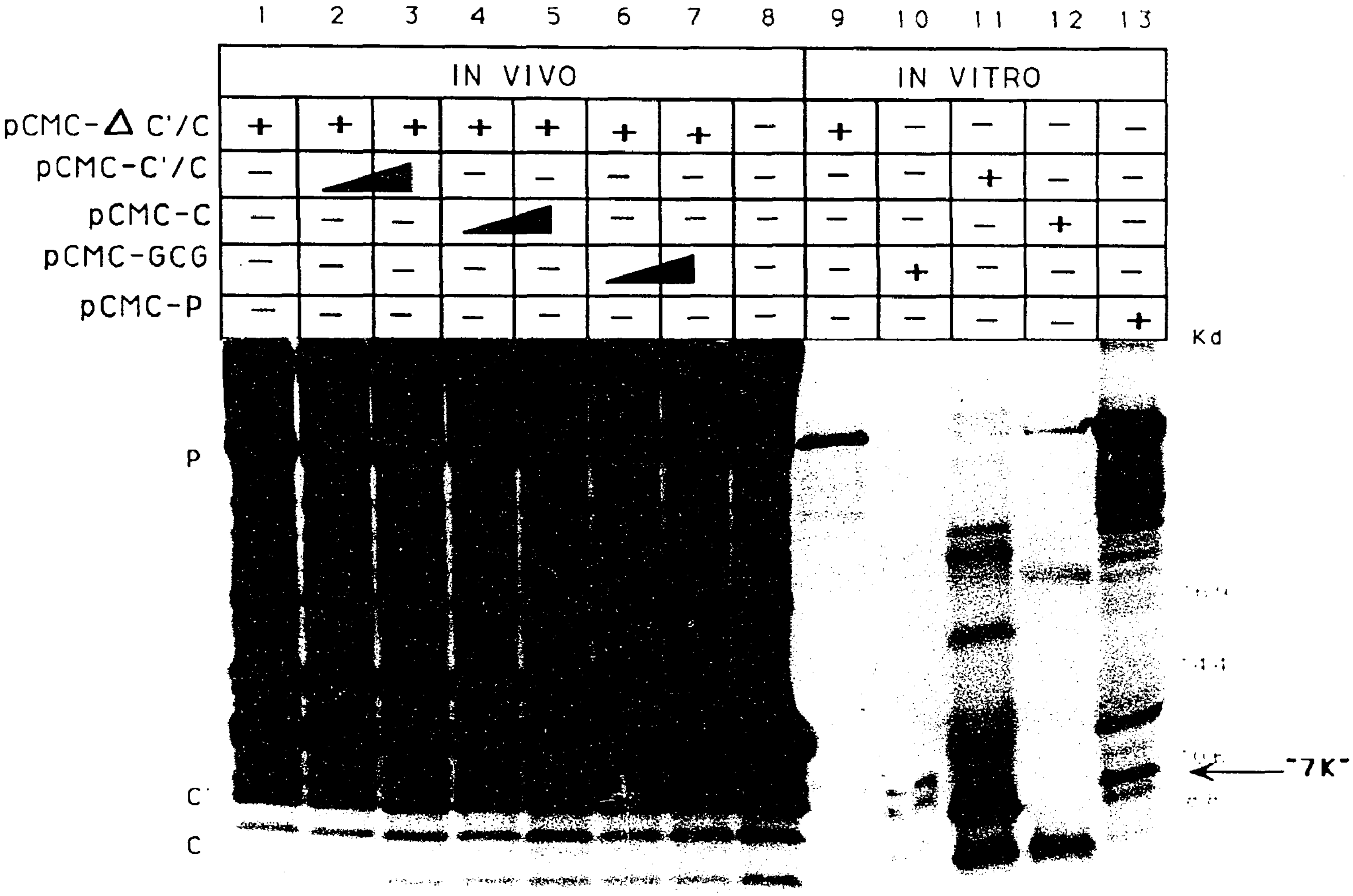
Expression products from vectors described in the legend to figure 9 were obtained by T7 RNA polymerase transcription followed by translation in rabbit reticulocyte lysates in the presence of ^{35}S methionine. Products were separated on the low molecular weight gel system described by Schagger and Jagow (1987) and visualized by fluorography. The DNA templates for the transcription reaction are shown at the top of the figure and Indiana gene products are indicated to the left. The 7K product described by Herman (1986), is seen clearly in lane 4 and is indicated to the right. (This product is not seen in lanes 2 and 3 because of the absence of the 3' terminus of the P gene in these constructs). The position of molecular weight markers is indicated at the right.


pCMC-NJC
pCMC-C'/C
pCMC-C
pCMC-ΔC'/C
pCMC-P
Kd
P
16.9
14.4
10.6
"7K"
8.8
C'
C
1
2
3
4
5

specific antibody, an attempt was made to metabolically label the expressed C proteins and to visualize the products against the vaccinia-T7 infected cell background in polyacrylamide gels (Figure 18). When either the C'/C or the C plasmids were transfected, a low-level product could be seen (lanes 3 and 5) which co-migrated with the *in vitro* translated C protein (lane 12), but which was absent from either the mock-transfected vaccinia-T7 cell extract (lane 8) or the ΔC'/C transfected extract (lane 1). The low level of expression of the recombinant C protein obtained *in vivo* compared to the co-expressed P protein cannot be explained at present, however it is possibly due to an instability of the C protein *in vivo.* Visualization of the C' product in transfected cells by metabolic labeling was not possible (Figure 18, lane 2 and 3), probably as a result of a co-migrating host or vaccinia protein.

Figure 18. *In vivo* expression of recombinant C proteins.

C protein expression vectors were introduced into vaccinia-T7 infected cells, metabolically labeled with ^{35}S methionine and equal aliquots of the extracts were loaded onto a low molecular weight gel system (Schagger and Jagow, 1987) (lanes 1-8) alongside *in vitro* translation products, (lanes 9-13) and the products were visualized by autoradiography. The location of proteins expressed from the CMC vectors is indicated on the left of the figure, while the position of the 7K protein and molecular weight markers is shown on the right. The use of (+) or omission (-) of various vectors is shown at the top of the figure and transfection of increasing amounts of the C protein expression vectors is indicated by the filled triangles. The amount of each plasmid transfected were: pCMC-ΔC'/C : 3μg; pCMC-C'/C : 1 and 3 μg, pCMC-C : 1 and 3 μg, pCMC-GCG : 1 and 3 μg.


1 2 3 4 5 6 7 8 9 10 11 12 13
IN VIVO
IN VITRO
pCMC-Δ C'/C + + + + + + + − + − − − −
pCMC-C'/C − − − − − − − − + − −
pCMC-C − − − − − − − − − + −
pCMC-GCG − − − − − − − + − − −
pCMC-P − − − − − − − − − − − − +
Kd
P
C'
C
"7K"

2. *Effect of the C proteins on viral transcription in vitro.*

The evidence from studies with *ts* mutants of the P protein and the fact that the C proteins cannot be detected in the New Jersey serotype virion which is transcriptionally active (Spiropolou and Nichol,1993), suggests that these proteins play no critical role in viral transcription but rather are involved in some aspect of genome replication (see Introduction:Genome Replication). The effect of the C proteins on viral transcription has been directly tested *in vitro* by reconstituting transcription using an N-RNA template stripped of all associated polymerase and extracts containing various combinations of recombinant P, L and C proteins. Cells were transfected with the plasmids pRc6.4L, encoding the L protein and pCMC-P which directs the synthesis of the P protein and both C proteins at wild-type levels. An extract of these cells was made and combined with N-RNA template in a transcription reaction (Figure 19). The level of transcription obtained with this extract was lower than, but approached that produced by VSV-infected cell extracts. Replacing the *wt* P construct with pCMC-ΔC'/C which is unable to make either C protein, results in a slight, but detectable increase in the level of transcription (Figure 19, lane 3) A much more obvious change however, is seen when increasing amounts of the C'/C plasmid (encoding C' and C) are co-transfected with pRC6.4L (encoding L) and pCMC-ΔC'/C (encoding P) (Figure 19, lanes 4 and 5). Here an inhibition in transcription is observed when both the C' and C proteins are expressed (lane 5) but not when the C protein in the absence of the C' protein is expressed (lanes 6 and 7). There was some concern that this decrease in transcription may reflect a lower expression level of either the P or the L protein rather than an effect of the added C proteins. To address this

Figure 19. Effect of the C proteins on reconstituted viral transcription *in vitro*. I.

Extracts of cells transfected with the plasmids indicated to the top left of the figure were incubated with 1μg of *wt* N-RNA template and the ^{32}P labeled products were extracted and resolved on 1.5% agarose urea gels are shown. The (+) and (-) symbols at the top of the figure represent the transfection/addition or omission of various components of the reaction respectively and the expected proteins expression from each construct is shown at the top right. The filled triangles represent increasing amounts of DNA transfected. The amounts of the relevant constructs transfected were pRc6.4L : 3μg, pCMC-P : 1.5μg; pCMCΔC'/C : 1.5μg; pCMC-C'/C : 1 and 3μg; pCMC-C : 1 and 3 μg. The identity of the transcripts produced, as verified by the co-migration of transcripts synthesized in an endogenous VSV reaction, is indicated to the left. A control reaction with non-transfected extracts is also shown (lane 8).

	1	2	3	4	5	6	7	8	Proteins expressed
VacT7	-	+	+	+	+	+	+	+	
VSV	+	-	-	-	-	-	-	-	
pRc6.4L	-	+	+	+	+	+	+	-	(L)
pCMC-P	-	+	-	-	-	-	-	-	(P/C'/C)
pCMC-△C'/C	-	-	+	+	+	+	+	-	(P)
pCMC-C'/C	-	-	-	◢	◢	-	-	-	(C'/C)
pCmC-C	-	-	-	-	-	◢	◢	-	(C)
wt N-RNA	-	+	+	+	+	+	+	+	

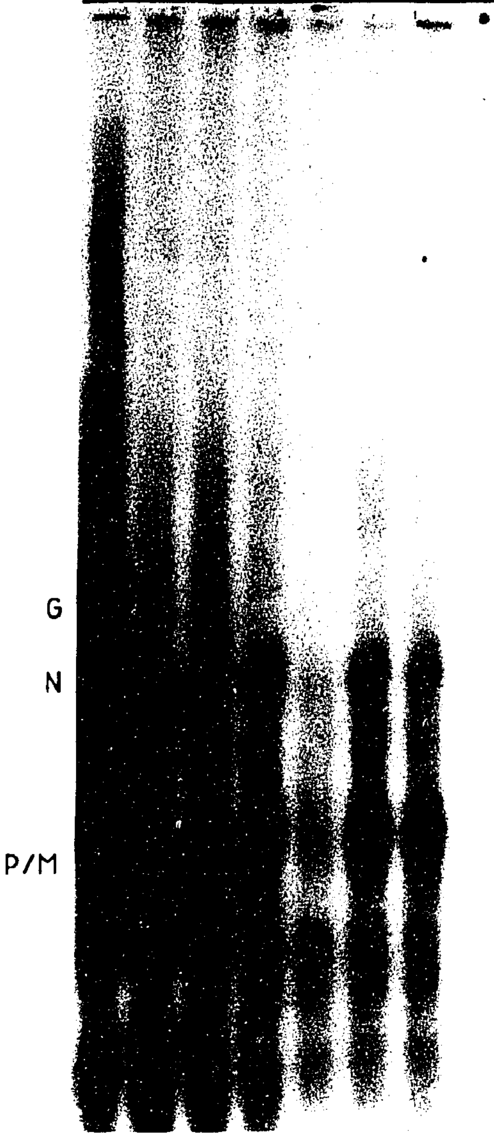
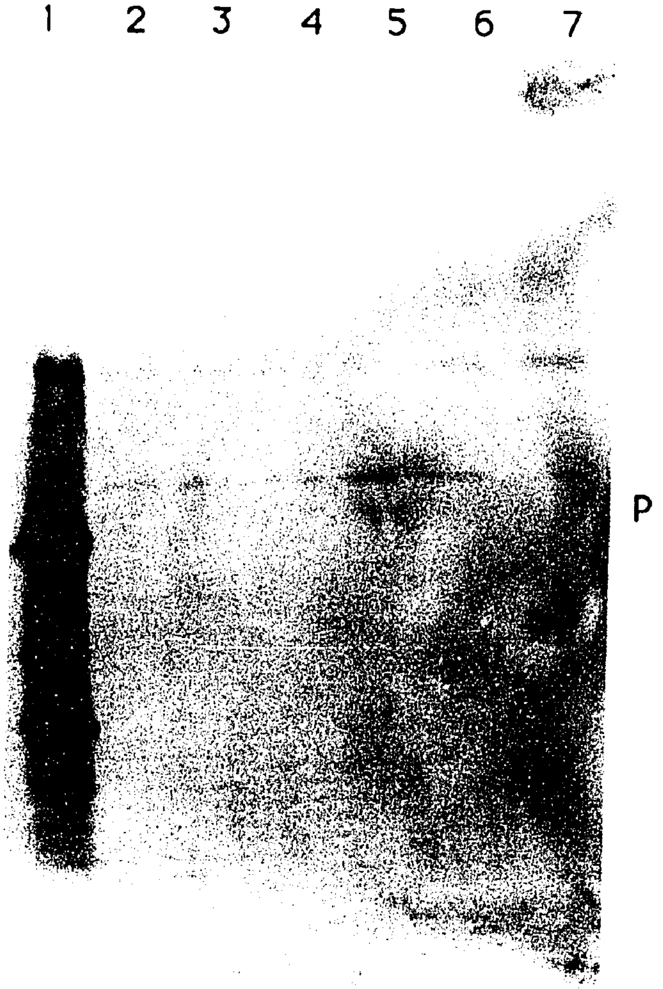


Figure 20. Western blot of extracts used to support reconstituted viral transcription *in vitro.*

Equal aliquots of the extracts used for the experiment shown in Figure 19 were electrophoresed on 11% SDS-polyacrylamide gels and were transferred to Zetabind membranes. The blot was probed with an anti-VSV antiserum and developed with a peroxidase conjugated goat anti-rabbit secondary antibody and the Renaissance (NEN) chemiluminescence substrate. The extracts run in lanes 1-7 here are identical to those used in the reactions shown in lanes 1-7 of Figure 19. The position of the P protein is indicated to the right of the figure.

Anti-VSV



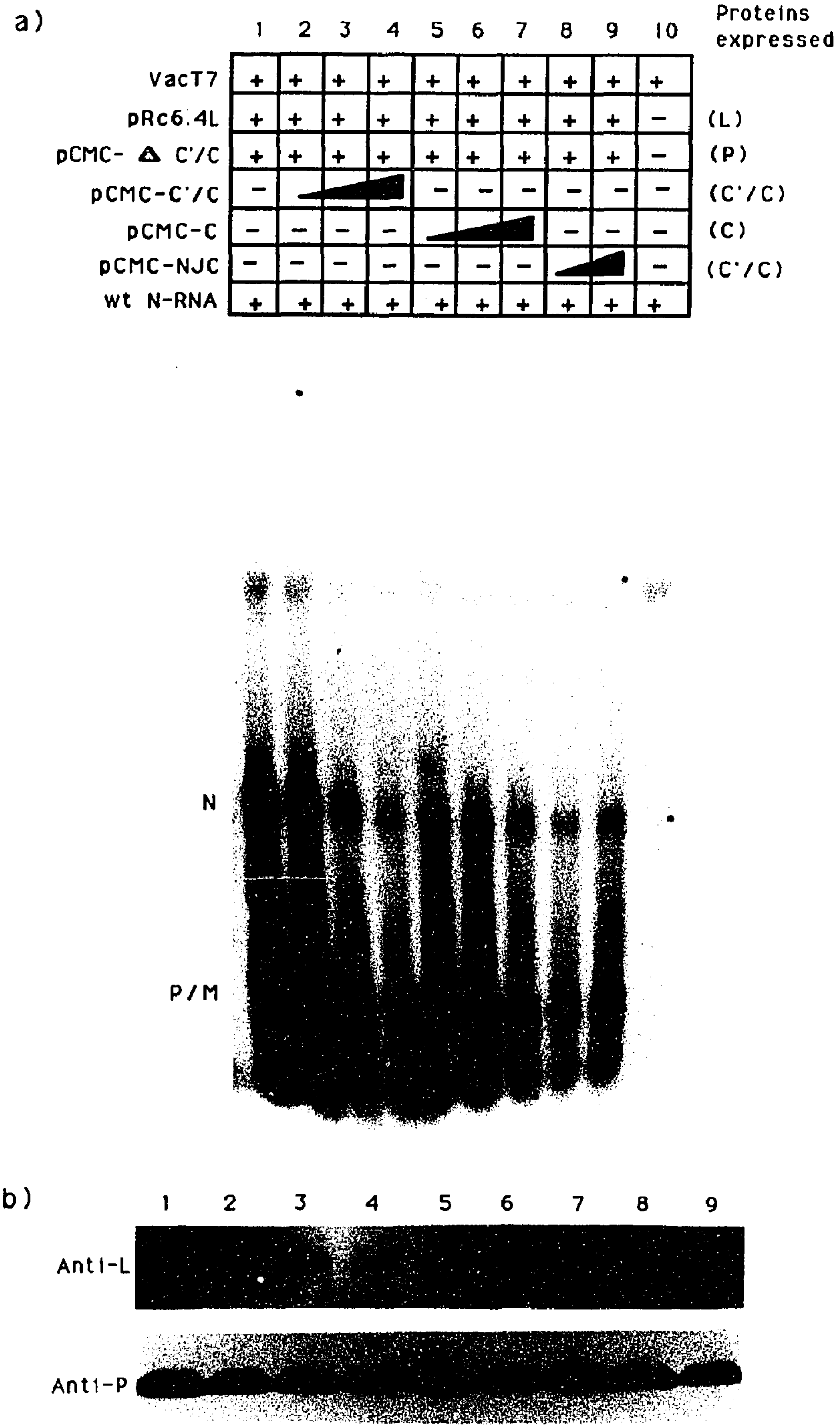
possibility, a western blot of the extracts used in Figure 19 was probed with a polyclonal anti-VSV antiserum in order to assess protein expression levels. This antiserum has a low titer against the P and the L proteins and consequently the L protein proved to be below the level of detection in all lanes; however the P protein could just be detected at similar levels in each reaction. (Figure 20, lanes 2-7).

Nevertheless, it was felt that both P and L levels needed to be monitored to be sure that the effect observed was dependent upon the transfection of the C'/C construct. A separate but similar experiment was performed and the results are shown in Figure 21. An identical effect of the C'/C construct on levels of transcription *in vitro* was demonstrated (Figure 21). Lanes 1-4 of Figure 21 show that transfecting increasing amounts of the C'/C construct results in a coordinate inhibition of the ability of these extracts to support mRNA synthesis from the N-RNA template. This result, along with those of the experiment shown in Figure 19 (lanes 3-5), indicates that the two C proteins together are able to negatively regulate viral transcription. Expression of increasing amounts of the C protein in the absence of the C' protein (Figure 21, lanes 5-7) does not produce the clear titration of transcriptional activity observed when both proteins are expressed together. However, an overall lower level of transcription is apparent at all levels of C protein expression (Figure 21, lanes 5-7). This more uniform effect of the C protein on transcription cannot be consistently reproduced, both in the experiments shown here (compare Figure 19, lanes 6 and 7 with Figure 21, lanes 5-7) and in other experiments (not shown) and may be non-specific. A western blot (Figure 21-b) of the extracts used for this experiment was probed with a monoclonal antibody specific for the P protein and also with a rabbit polyclonal antibody which recognizes the L protein.

Figure 21. Effect of the C protein on reconstituted viral transcription *in vitro*. II.

a) Transcriptional products are shown which were produced in reactions similar to those described in the legend to Figure 19 and were visualized in an identical manner. The plasmids transfected are indicated at the top left and the various components transfected/added (+) or omitted (-) are shown in the table at the top of the figure. Increasing amounts of DNA transfected are indicated by filled triangles and the amounts of each construct transfected is as follows: pRc6.4L : 2μg; pCMC-ΔC'/C : 4μg; pCMC-C'/C : 2, 4 and 8μg; pCMC-C : 2, 4 and 8μg; pCMC-NJC : 4 and 8μg. The expected protein products from each construct is indicated at the top right of the table.

b) A Western blot of equal aliquots of the extracts used in A above, (lanes 1-9) is shown. The blot was first probed with anti–L antiserum and developed with peroxidase conjugated goat anti-rabbit secondary antibody and Renaissance (NEN) chemiluminescence reagent. The blot was then stripped of antibody, reprobed with anti-P monoclonal antibody and developed as before with the exception that a peroxidase-conjugated goat anti-mouse secondary antibody was used.


a)
1 2 3 4 5 6 7 8 9 10
Proteins expressed
VacT7 + + + + + + + + + +
pRc6.4L + + + + + + + + + − (L)
pCMC-Δ C'/C + + + + + + + + + − (P)
pCMC-C'/C − − − − − − − (C'/C)
pCMC-C − − − − − − − (C)
pCMC-NJC − − − − − − − − (C'/C)
wt N-RNA + + + + + + + + + +
N
P/M
b)
1 2 3 4 5 6 7 8 9
Anti-L
Anti-P

The higher affinity of these antibodies allowed for a much closer examination of recombinant protein expression levels. For reasons which remain unknown, the L protein was expressed at higher levels in the extracts probed in lane 1 of Figure 21-B whereas L protein expression in the other extracts was lower but consistently uniform (Figure 21-b, lanes 2-9). The level of the P protein appears to be similar in all the extracts (Figure 21-b, lanes 1-9) although close examination of the original X-ray film shows a slightly lower amount of the P protein in the extract transfected with the highest amount of the C'/C plasmid (Figure 21-b, lane 4). The effect of the C'/C construct on the level of the P protein in the absence of any great change in the level of the other recombinant proteins produced, is reproducible (See Figure 25-b, lane 6) and may be significant in mediating the effect of the C proteins on transcription. The observation in Figures 19 and 21 that the C protein alone cannot reproduce the effect that the C' and C proteins mediate together, suggests that this effect is specific and perhaps a property of the unique amino-terminal region of the C' protein which distinguishes these otherwise identical proteins (figure 15). To further demonstrate such specificity, the region of the New Jersey P protein message encoding the two C proteins was cloned using an RT-PCR procedure (Materials and Methods; Figure 16). The New Jersey C proteins are similar to those of Indiana serotype since they are small and highly basic, however, they share no sequence homology at either the DNA or the protein level. *In vitro* transcription and translation of the New Jersey C (NJC) construct gave rise to a product which migrated in polyacrylamide gels just above the position to which the Indiana C' protein migrates (Figure 17, lanes 1 and 2), but did not result in a product migrating in the molecular weight range of the Indiana C protein. It appears then, that at least the New Jersey C' protein and possibly both C proteins are produced *in vitro.* Co-transfection of the plasmid encoding the New Jersey C proteins with the L and ΔC'/C constructs gave rise to extracts which did not exhibit a drastic inhibition in transcriptional levels with increasing amounts of pCMC-NJC DNA (Figure 21, lanes 8 and 9) as was typically demonstrated with the Indiana C'/C construct. In comparison with reactions containing the ΔC'/C, L and C constructs (lanes 5-7) there is a uniform depression in transcription at both high and low levels of New Jersey C protein expression which once again may represent a non-specific effect.

To summarize, the evidence accumulated in the *in vitro* transcription system shows that the VSV C proteins together but not the C protein alone, function as negative regulators of transcription.

3. Establishment of an in vitro genome replication system using proteins expressed from cloned genes.

The results of transcriptional studies with the C proteins described above were intriguing since a specific negative effect on transcription might be indicative of a switch of the RNA polymerase to a replicative mode; therefore, a system in which to test the effects of the C proteins on genome replication became highly desirable. The presence of the C proteins in the virion of the New Jersey serotype of VSV (Spiropolou and Nichol, 1993) could not be demonstrated, however the C proteins of Sendai virus do appear to be represented in the virion at very low levels, perhaps as a result of an interaction of the C

Figure 22. Optimization of recombinant protein expression for the support of DI particle replication *in vivo*.

Expression of the N, P and L proteins in the Vaccinia-T7 system was optimized for maximum support of purified DI particle genome replication, by varying the amount of plasmid transfected. The table at the top of the figure indicates the microgram amounts of the plasmids pRc6.4L (L); pCMC-P (P) and pCMC-N (N) transfected and the position of the ^{3}H urididine labeled19S RNA [(+) genome, (-) antigenome] produced is indicated on the left. The asterisks above the table indicate the amount of each plasmid (from left to right: L, P and N) judged to be optimal for the support DI replication. The last lane of the figure shows the level of support of the purified DI particle by wild-type VSV. All RNA products shown were treated with micrococcal nuclease prior to separation on the gel.

			*						*				*	
L	0.25	0.5	1.0	2.0	4.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	DI + wt
P	4.0	4.0	4.0	4.0	4.0	0.5	1.0	2.0	4.0	4.0	4.0	4.0	4.0	
N	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	1.0	2.0	4.0	8.0	

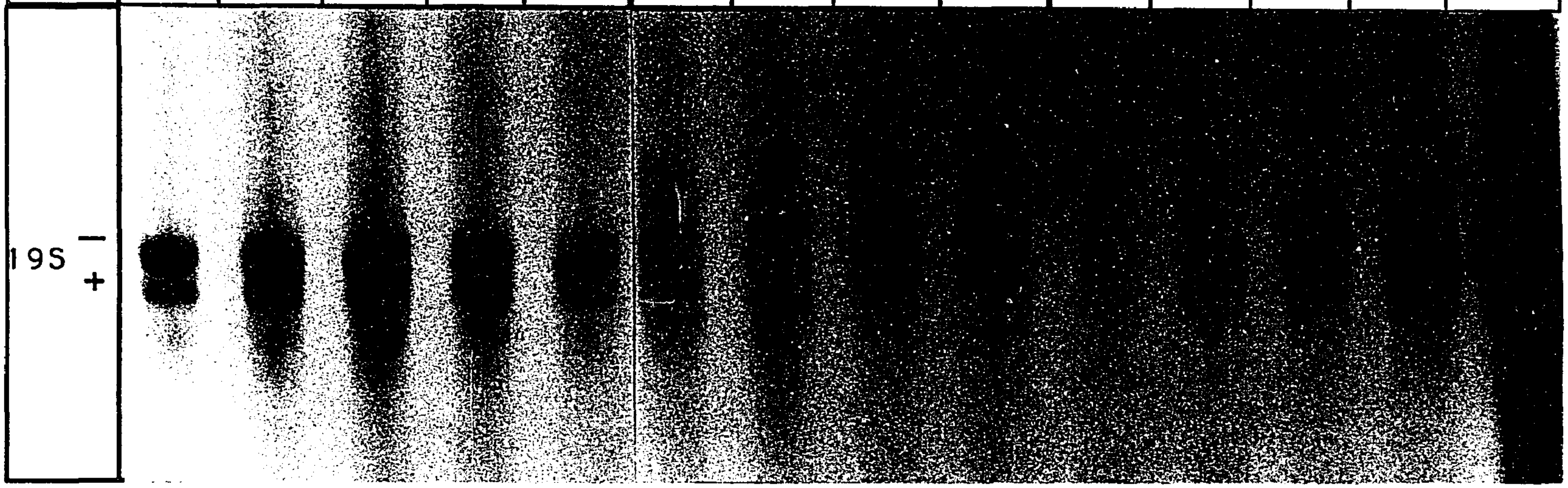
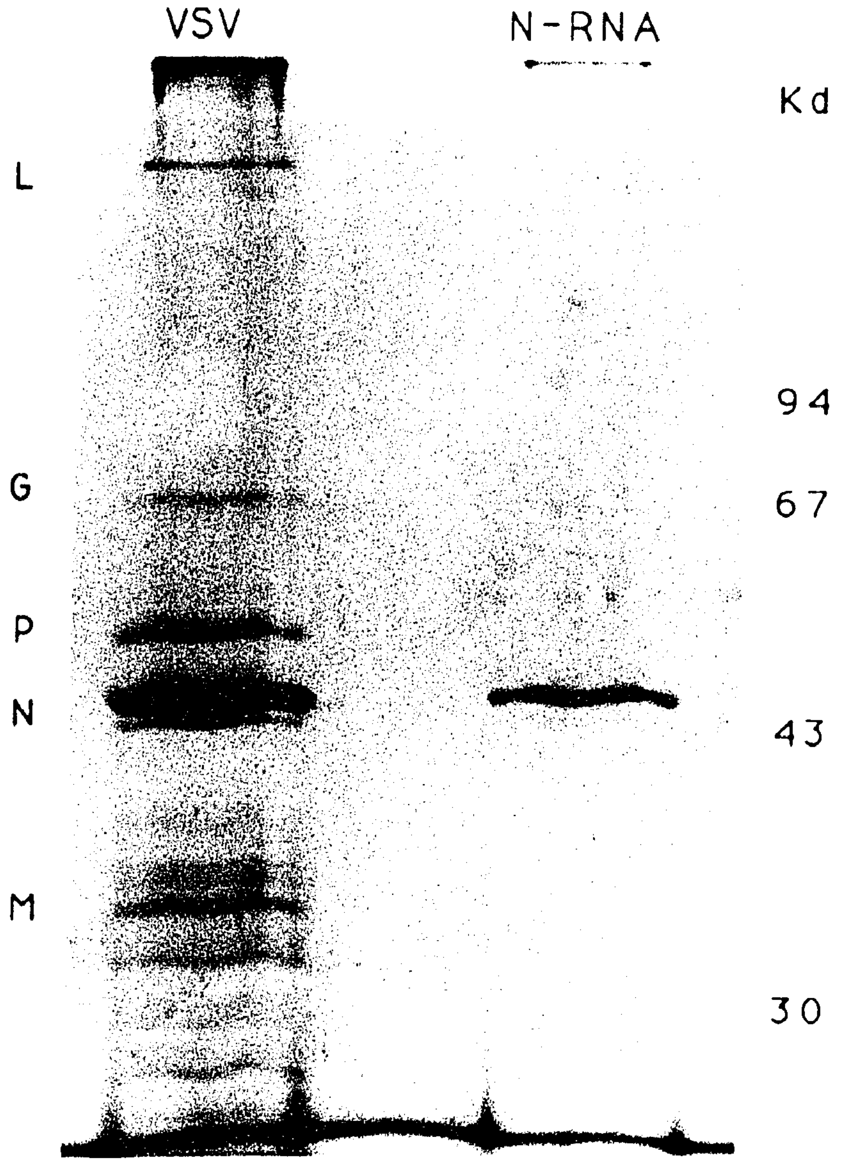


Figure 23. Preparation of DI N-RNA free of associated proteins.

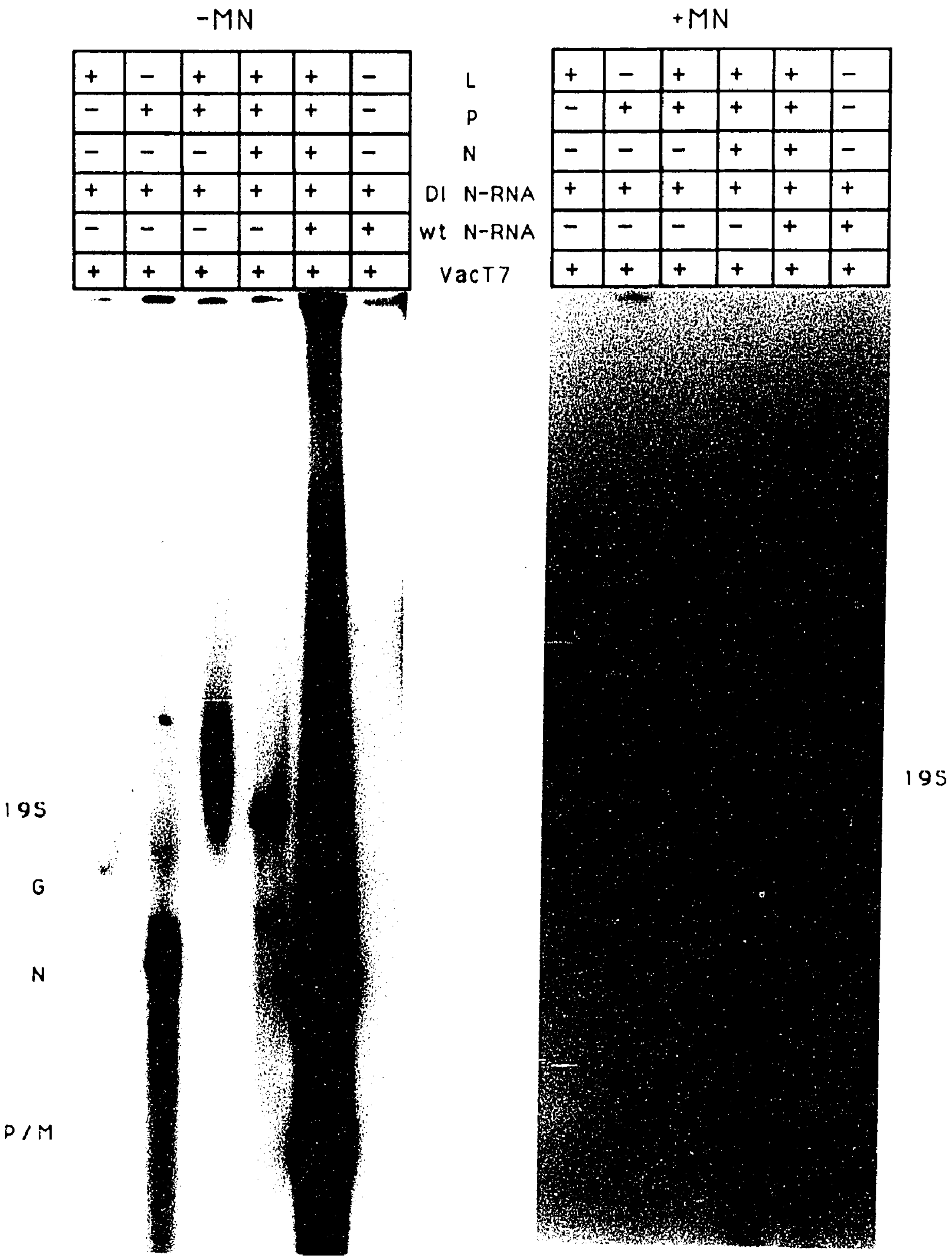
A comparison of the protein content of detergent solubilized virus (VSV lane) and DI particle N-RNA, purified by sequential centrifugation through Cesium Chloride gradients is shown. 2μg and 1μg of the virus and the N- RNA preparation respectively, were electrophoresed on an 11% SDS-polyacrylamide gel and the viral proteins indicated to the left, were visualized by silver staining. The position of molecular weight markers is shown to the right of the figure.


VSV
N-RNA
Kd
L
G
P
N
M
94
67
43
30

proteins with the P protein (Yamada et al., 1990; Curran et al., 1992). It was decided therefore, that the optimal system to study replication using the C protein expression vectors would involve nucleocapsids stripped of the associated polymerase to reduce the chance that endogenous C proteins would influence results. No previous reports of this type of system for VSV have been published, but the use of infected cell extracts, produced by the lysolecithin permeabilization technique for the replication of intracellular nucleocapsids, is well documented (Peluso and Moyer, 1983; 1984). Given the success of lysolecithin-permeabilized, transfected cell extracts for the support of *in vitro* transcription reactions (Canter et al., 1993; Curran et al, 1992; Results sections I.5, II.2), the adaptation of this system to *in vitro* N-RNA replication seemed feasible. It was necessary to first optimize the amount of each plasmid transfected in the *in vivo* replication system and to use these amounts in preparing extracts for the support of replication. Such an experiment is shown in Figure 22 and optimal microgram amounts of each plasmid are indicated (* above Lanes 3, 9 and 13). This experiment shows that efficient replication is dependent upon high levels of the P protein and especially of the N protein, but in agreement with previous reports (Meier et al., 1987, Pattnaik and Wertz, 1990) over-expression of the L protein, becomes inhibitory to genome replication. Minimization of the possibility of endogenous C proteins on the N-RNA template required successive bandings of the DI nucleocapsid in cesium chloride gradients and yielded a preparation which was free of detectable P or L proteins, as judged by silver staining (Figure 23). When this template was reconstituted with extracts of cells transfected with optimal amounts of the L, P and N expression vectors, 19S RNA of

Figure 24. Establishment of an *in vitro* system for DI genome replication using recombinant proteins expressed *in vivo.*

Extracts of cells expressing P, L and N proteins in various combinations were used to reconstitute DI genome replication *in vitro* from added DI N-RNA template. Equal aliquots of *in vitro* reactions treated (+MN) or untreated (-MN) with micrococcal nuclease are shown on the right and left respectively. The plasmids Rc6.4L (L), pCMC-P (P) and pCMC-N (N) transfected into vaccinia-T7 virus infected (vacT7) BHK cells and the types of N-RNA template added to the transfected cell extracts (DI and *wt* : 1µg of each) is shown between the two figures. The transfection of plasmids or addition of template N-RNA are indicated in the tables above the figures (+) and the position of the 19S DI RNA and viral transcripts are shown beside the figures. The reaction products were labeled with ^{32}P-UTP and each reaction was treated with micrococcal nuclease. The protected products were separated on an agarose-urea gel.



-MN

+	−	+	+	+	−	L
−	+	+	+	+	−	P
−	−	−	+	+	−	N
+	+	+	+	+	+	DI N-RNA
−	−	−	−	+	+	wt N-RNA
+	+	+	+	+	+	VacT7

+MN

+	−	+	+	+	−
−	+	+	+	+	−
−	−	−	+	+	−
+	+	+	+	+	+
−	−	−	−	+	+
+	+	+	+	+	+

predominantly (+)-sense (bottom band) but with some (-)-sense (top band) was produced (Figure 24 (-MN), lane 4). These products were demonstrated to be authentically replicated nucleocapsid structures since they were resistant to treatment with MN (Figure 24 (+MN), lane 4). Reconstitution of the template with extracts containing the L, N or P proteins alone did give rise to some extraneous RNA products ((-MN), lanes 1-3). However these RNAs did not represent replicated nucleocapsids since they exhibited no resistance to MN ((+MN), lanes 1-3) and their origin remains undetermined. An additional feature of the *in vitro* replication system is the ability to carry out independent transcription and replication reactions using the same cell extract. This property can be seen when both DI and *wt* N-RNA templates are added to extracts containing all three recombinant proteins (Figure 24, + and - MN, lane 5). High levels of transcripts are produced in this reaction (-MN, lane 5) which are completely sensitive to MN treatment (+MN, lane 5), whereas the level of DI in this reaction(obscured by the over-exposure; +MN, lane 5) is much lower than in reactions with DI N-RNA alone (-MN, lane 4) but can be seen clearly in the MN treated aliquot of this reaction (+MN, lane 5). The lower level of DI replication in this reaction is thought to be due to competition between templates for the recombinant polymerase.

4. Effect of the C proteins on DI genome replication in vitro.

The establishment of a system for genome replication *in vitro* dependent upon cloned gene products, meant that the C protein constructs used previously for transcription reactions, could be similarly employed in the *in vitro* replication system (Figure 25-a). Reconstitution of the DI N-RNA with extracts containing L, P and N proteins produced high levels of MN resistant 19S RNA (lane 3) while incubation of this template with extracts expressing either the L or the P proteins alone did not support replication (lanes 1 and 2). When the ΔC'/C construct, which is incapable of expressing C proteins, was substituted for the *wt* P gene and expressed with the other two plasmids, a large drop in replicated 19S RNA levels was observed (lane 4), a striking result which indicates that the C proteins are required for efficient genome replication. Moreover, the expression of the C'and C proteins in these extracts directed by the co-transfection of the C'/C expression vector, led to the gradual restoration (lanes 4 and 5) of the level of genome replication produced by cell extracts containing the wt P construct (lane 3). The enhancement of genome RNA synthesis mediated by the C proteins expressed together could not be reproduced by the plasmid which encodes the C protein but not the C' protein (lanes 7 and 8) and this observation correlates well with the lack of effect of the C construct on inhibition of viral transcription *in vitro* (Figures 19 and 21). With the exception of the extract transfected with the highest amount of the C'/C construct, the levels of L, P and N protein expression are fairly constant (Figure 25-b). Transfection of high amounts of the C'/C construct seems to lead to lower levels of P protein expression and perhaps to a slight decrease in the level of the L protein, while the level of the expressed N protein remains high. This effect on P protein expression levels has been noted earlier (Results section II.2) and may be important in the mechanism of the action of the C proteins. An expression vector which is derived from the *wt* P construct by replacing the P protein translational initiation codon (AUG) with a GCG codon has also been produced (pCMC-GCG, Figure 16). This construct should be unable to initiate

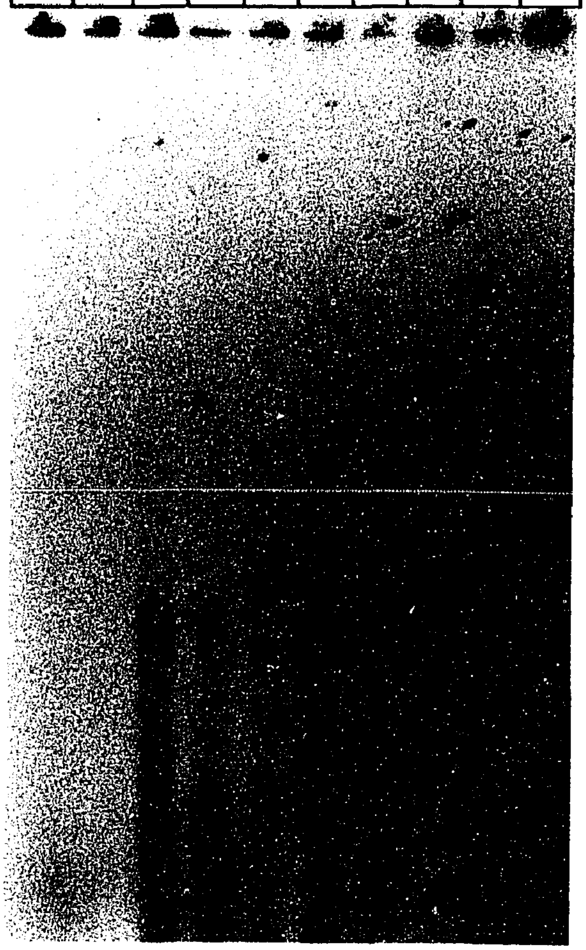
Figure 25. Effect of the C protein on reconstituted replication *in vitro.*

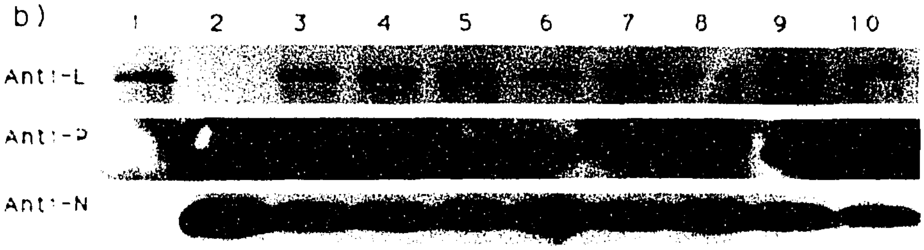
a) The established *in vitro* replication system (Results Section II; Figure 24) was used to assess the effect of the C proteins on replication *in vitro* and the results of such an experiment is shown. The presence of 1μg of DI N-RNA in the reaction and the combination of plasmids used to transfect cells is indicated in the table at the top of the figure (+) while the identity of the construct used is indicated to the left of the table. The amounts of DNA transfected were pRc6.4L : 1μg, pCMC-P : 4μg, pCMC-L: 1μg, pCMC-N : 6μg, pCMC-ΔC'/C : 4μg, pCMC-C'/C : 1 and 3μg, pCMC-C : 1 and 3μg, pCMC-GCG : 1 and 3μg. The transfection of increasing amounts of DNA is indicated by filled triangles. The position of the 19S DI RNA is indicated to the left of the figure and the expected protein expression from each construct appears to the right of the table. RNA products were labeled with ^{3}H-UTP and each reaction was treated with micrococcal nuclease. The protected products were separated on an agarose-urea gel.

b) A Western blot of equal aliquots of the extracts used to reconstitute replication in A, is shown, and lanes 1-10 in A and B, are equivalent. The blot was probed and developed as described in the legend to Figure 21, with the exception that an additional round of stripping and reprobing with anti-VSV antiserum was performed, in order to detect the N protein.

a)

	1	2	3	4	5	6	7	8	9	10	Proteins expressed
DI N-RNA	+	+	+	+	+	+	+	+	+	+	
pRc6.4L	+	–	+	+	+	+	+	+	+	+	(L)
pCMC-P	–	–	+	–	–	–	–	–	–	–	(P/C'/C)
pCMC-N	–	+	+	+	+	+	+	+	+	+	(N)
pCMC-ΔC'/C	–	–	–	+	+	+	+	+	+	+	(P)
pCMC-C'/C	–	–	–	–	◢		–	–	–	–	(C'/C)
pCMC-C	–	–	–	–	–	–	◢		–	–	(C)
pCMC-GCG	–	–	–	–	–	–	–	–	◢		(C'/C)
VacT7	+	+	+	+	+	+	+	+	+	+	





P protein synthesis, while synthesis of the C proteins by independent initiation events should be unaffected. Appropriate expression from this plasmid is demonstrated in figure 18 (lane 10) and when this construct was assayed in reconstituted replication reactions in the experiment described above (Figure 25-a, lanes 9 and 10), a slight increase in the level of 19S RNA (lane 9) over that produced in the absence of the C proteins (lane 4) was obtained. No product could be detected when a greater amount of the GCG construct was transfected (lane 10). Since genome replication is dependent on high levels of N protein expression, the absence of 19S RNA may be explained by the low amount of N protein detected in this extract (Figure 25-b, lane 10).

To conclude, the results obtained in the *in vitro* replication system indicate that the C' and C proteins together but not the C protein alone, are able to directly stimulate the process of DI genome replication. This positive effect of the co-expressed C' and C proteins on genome RNA replication is in direct contrast to the negative regulatory effect that these two proteins exert on viral transcription. The opposite effects exerted on transcription and replication suggest that the C proteins may play a critical role in the transition between transcriptive and replicative modes of RNA synthesis.

DISCUSSION

The process by which vesicular stomatitis virus is able to switch from one form of RNA synthesis (transcription) to another (replication) is a central feature of the viral life cycle and is probably the least understood. Although a model exists for this process (Leppert et al., 1979), it was proposed over a decade ago and therefore cannot accommodate the more recent data regarding VSV genome replication. A prominent advance over the last few years has been the identification of the N:P protein complex as the substrate for genome encapsidation, a finding which poses new questions concerning the mechanics of the replication process. The work described in the first section of this thesis has addressed the role of this complex during replication and provides indirect evidence for an interaction with the viral RNA polymerase.

The observation that formation of a complex between the N and the P proteins inhibits the non-specific binding of the N protein to RNA, led to the proposal that the two proteins could form a binding site with specificity for the genomic encapsidation sequence (Masters and Banerjee, 1988). A similar situation in a very different system, the U2 small nuclear ribonucleoprotein (snRNP), has been demonstrated (Scherly et al., 1989) and in this case, a recognized RNA binding motif is also the site of protein-protein interaction between U2A' and U2B", two cognate binding proteins, both of which are required to interact with the U2 snRNA. Therefore, it did not seem unreasonable that this type of arrangement could impart genomic RNA binding specificity to the N:P complex. However, the results of the MN protection experiments (Results section I.1) show that synthetic RNA transcripts representing the exact 5' termini of both the genome and antigenome could not be efficiently encapsidated by N:P complexes isolated from infected cells, irrespective of whether the RNAs were preformed or nascently transcribed by the T7 RNA polymerase. The competence of the glycerol gradient fractions used to act as substrates for encapsidation reactions is well established, since similar fractions have supported high levels of DI replication *in vitro* with the resultant production of MN resistant nucleocapsids (Peluso and Moyer, 1983; Peluso and Moyer, 1984; Peluso and Moyer, 1988; Peluso, 1988) and in addition, there is no detectable RNA already associated with the N:P complex isolated in this way (La Ferla, 1989). Thus, with the possible caveat that the T7 RNA polymerase poses an initial steric hindrance to the encapsidation reaction and that directly thereafter secondary structure forms to occlude the encapsidation sequence, it appears that the N:P complex shows little or no specificity for putative genomic encapsidation signals and is not able to independently encapsidate genomic RNA. The low level of protection of the +-leader/N RNA observed in the nascent protection experiment (Figure 6, lane 1) is not necessarily a specific effect, since a similar low level of protection of the pGEM RNA is also seen (Figure 6, lane 3) and both effects may be due to the over-saturation of the system with RNA. A specific binding activity for the N:P complex cannot be totally eliminated however, but the results obtained suggest that this activity is an unlikely driving force for the encapsidation reaction and for replication in general.

A contradictory view that complex formation does impart specificity and enables encapsidation has been expressed (Das and Banerjee, 1992), although a crucial difference in this report was the source of the nascent genomic RNA. Unlike the system I have established, employing an unrelated RNA polymerase, Das and Banerjee were able to take advantage of a transcribing VSV nucleocapsid to provide the nascent genomic RNA to reticulocyte translated N:P complex and produce RNase resistant genomes. Since nascent RNA was present in both cases, the major factor determining successful encapsidation between the two systems is likely to be the type of RNA polymerase or template used, which is suggestive of the requirement for a specific interaction of either one with the N:P complex.

The results obtained in the *in vivo* DI replication experiments are supportive of the idea that the N:P complex interacts with the VSV RNA polymerase to produce N protein encapsidated genomic RNA. A primary indication is that the presence of recombinant N and P proteins together with the DI nucleocapsid in transfected cells is sufficient to produce genome RNA replication (Figure 17) and in the absence of a direct interaction with nascent RNA, the N:P complex formed in these cells must fulfill its role as the encapsidation substrate by some other mechanism. The suggestion that the N:P complex and the RNA polymerase (L:P) can interact is supported by the formation of soluble triple complexes between the L, N and P proteins *in vivo* (Figure 10). These complexes may have assembled by a number of different routes and among them is the possibility that the N:P complex formed first and subsequently bound to the L protein, or to a soluble form of the RNA polymerase (L+P) to give an N:P/L:P "replicase" type structure. The triple complex observed would be less interesting if it represented the arrangement of the three proteins already known to exist in the nucleocapsid; however, given the property of the N protein to self assemble even in the absence of RNA, this kind of arrangement would probably occur mainly as a high molecular weight form and would not be present in the supernatant after centrifugation. Therefore, the complexes observed probably comprise mostly N:P heterodimers with smaller amounts of a non-nucleocapsid arrangement of the triple complex. The existence of an interaction between the N:P and the L:P complexes in cells expressing the N, P and L proteins would suggest that a similar interaction might occur when the DI particle containing the L:P polymerase is used to superinfect cells expressing the N and the P proteins. Three situations, involving interaction of the N:P complex and the incoming DI nucleocapsid can be envisaged. The first would require that the N:P complex bind directly to the N-RNA-associated DI polymerase (L+P). A second scenario in which the L protein would dissociate from the DI nucleocapsid and bind to the N:P complex to give an N:P/L arrangement is also feasible since the polymerase proteins are known to freely dissociate from the N-RNA template (Peluso and Moyer, 1984; Helfman and Perrault, 1989). Lastly, the N:P complex may bind directly to the N-RNA template and might only interact with the polymerase when it passes during RNA synthesis. The second alternative is effectively ruled out by the studies with the P3S mutant, which is defective in RNA synthesis yet supports DI replication *in vivo* when co-transfected with the N protein. If replication were dependent on the formation of an N:P/L arrangement, DI genome replication would not occur in cells expressing the N and P3S proteins since an N:P3S/L complex would not be capable of synthesizing RNA. The third possibility is also rendered unlikely by the P3S experiment, since the transcriptional defect of the original mutant of the New Jersey serotype (M3) was associated with a greatly reduced ability to bind to the N-RNA template (Chattopadhyay and Banerjee, 1987). More solid evidence in the form of direct binding studies is required to further confirm the interaction between the N:P encapsidation substrate and the template bound RNA polymerase, however, studies of this sort in our laboratory have been inconclusive due to the lack of an adequate assay system (data not shown) and perhaps further genetic studies aimed at more closely dissecting the interaction of the P and L proteins during transcription and replication, will be more informative.

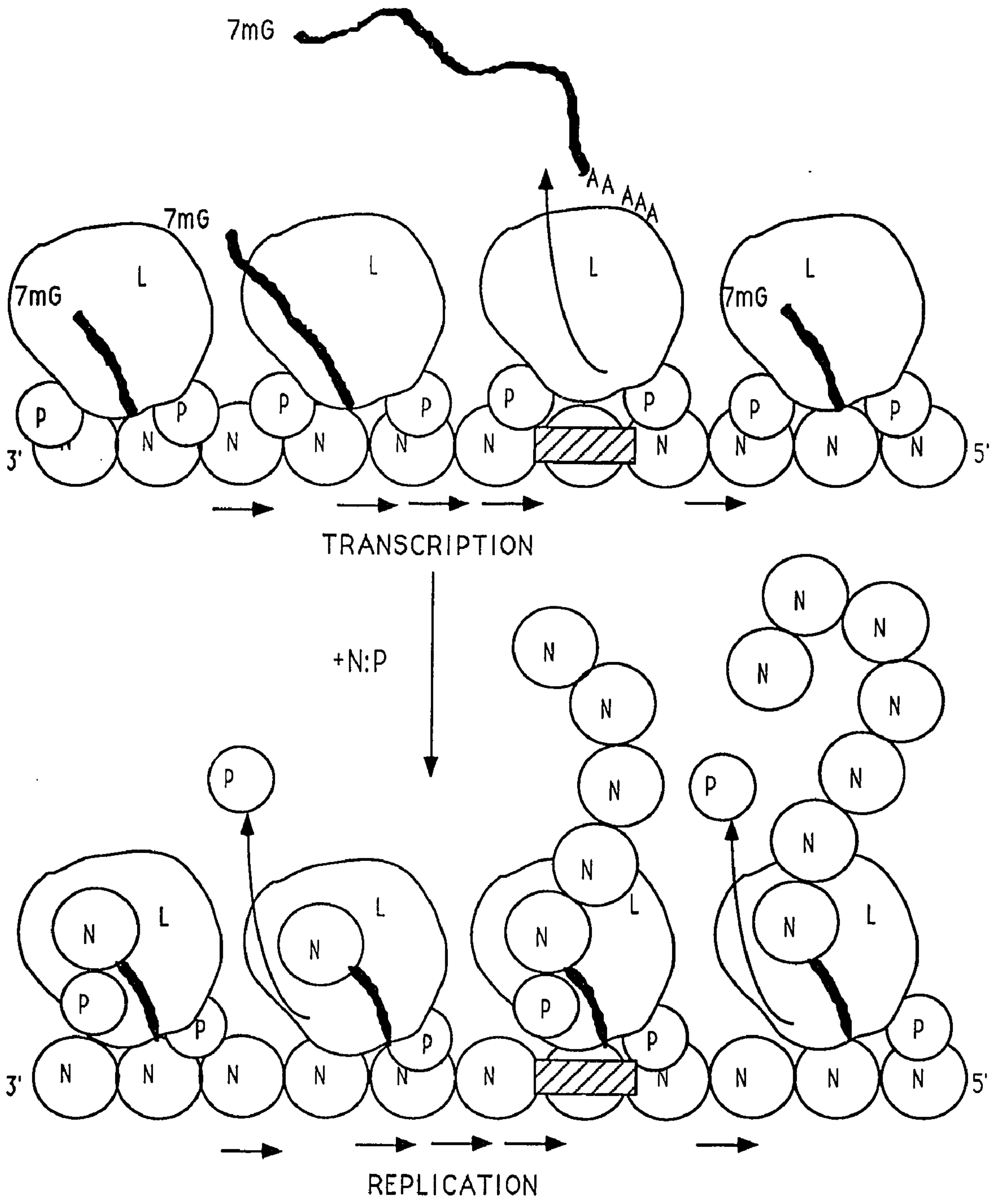
The contradiction in the minimum requirements of recombinant proteins for the support of DI genome replication, between the initial report (Pattnaik and Wertz, 1990) where all three nucleocapsid proteins were found to be necessary, and the results presented here (Results section I.2) are likely to be due to differing amounts of DI particles used to super-infect transfected cells. In the initial report, the concentration of DI particles was determined by hemagglutination of goose erythrocytes and particles were used at a multiplicity of one, while in the system I have established, the optimal amount of DI stock to produce maximal replication was determined empirically. Consequently far more than one particle per cell may have been present in my system, resulting in a much higher concentration of endogenous polymerase and thus eliminating the need for an exogenous supply of the L protein. While the overall requirement for all three nucleocapsid proteins remains the same between the two systems, the source of these proteins differs and note should be taken of parallel studies to the *in vivo* system reported here, that have been established *in vitro*. In these studies the N:P complex is formed independently in reticulocyte lysates and can complement functional nucleocapsids containing the RNA polymerase to produce authentic replication (Howard and Wertz, 1989; Das and Banerjee, 1992).

Theoretically, an interaction between the N:P complex and the RNA polymerase could entail an enzymatic involvement of the complexed P component in replicative RNA synthesis. In this case, a mutant such as the transcriptionally defective P3S mutant would be inhibitory to genome replication. Since the P3S/N complex can be demonstrated by co-immunoprecipitation (Figure 10) and is clearly able to support replication (Figure 12), there can be no enzymatic requirement for the P protein component of the N:P complex for RNA synthesis during replication, although a redundancy in this activity cannot be ruled out. A possible caveat in this experiment is the ability of *wt* P protein to dissociate from the template, which might lead to the formation of complexes with newly synthesized, unaggregated N protein, an event which would compromise the interpretation of the P3S results. However, a strong argument against this occurrence is the fact that the N protein expressed alone (Figure 12, lane 1) will not support replication; in this situation there is perhaps a greater likelihood that nucleocapsid-derived P protein could complex with nascently produced N protein since there is a lack of competition for binding with a vast excess of recombinant P protein.

A phenotype which has not been directly demonstrated for the P3S mutant, but which is implicated due to the transcriptional defect, is a property originally established for the New Jersey M3 mutant, namely the inability to act as a substrate for the L kinase (Chattopadhyay and Banerjee, 1987). If the surmised interaction of the N:P complex and RNA polymerase is correct, it is possible that the L-associated kinase is able to phosphorylate the complexed P component and that this event would constitute part of the replicase action, perhaps by causing the release of the P protein from the complex and allowing the N protein to bind to nascent genomic RNA. The ability of the complexed P3S protein to support replication argues against this mechanism however, since the conserved serine residues in this protein, a defined target of the L-associated kinase in the New Jersey serotype, have been replaced with alanine. Thus, from the current data, it seems likely that the role of the complexed P protein is simply to "dock" the complex to the RNA polymerase and position the N protein close to the emerging genomic RNA, such that concurrent encapsidation may occur in a coordinated manner. This model predicts that there must be two sites of P protein interaction on the L protein, although an alternative arrangement with the P protein in dimeric form is conceivable. Early studies with the polymerase complex (Naito and Ishihama, 1976) suggested that the polymerase was made up of equal molar amounts of the L and P proteins, however, this conclusion was based upon estimated molecular weights of the two proteins which made the L protein four times larger than the P protein. Calculated molecular weights determined from sequences which were not available at the time of the initial study, show that the L protein is approximately eight times larger than the P protein and therefore the complex may consist of two P proteins for every L protein (Rose and Schubert, 1987). A working model based upon this ratio is represented schematically in Figure 26.

Figure 26. Schematic model for the interaction of the N:P protein complex and the viral RNA polymerase during replication.

A working model for the interaction of the N:P protein complex and the viral RNA polymerase is shown. The viral RNA polymerase is represented with a P to L protein ratio of two to one. The N-RNA is shown as a linear arrangement of the N protein (N) and intergenic junctions are indicated by the boxed region on the N-RNA template. During transcription (top) the polymerase initiates RNA synthesis at the 3' terminus of a transcription unit (here, the 3' end of a structural gene), caps the 5' terminus of the nascent RNA and polymerizes until it reaches the poly-A site immediately preceding the gene junction. The polymerase pauses and produces a poly-adenylate sequence by reiterative synthesis on the stretch of uridine residues present within the poly-A site. The mRNA is released and the polymerase reinitiates at the start of the next gene. According to the model for replicative RNA synthesis (bottom), the polymerase initiates polymerization at the 3' terminus of the leader gene, the N:P protein complex binds to the polymerase via a P-L interaction and delivers the N protein to the nascent 5' terminus of the RNA. The P protein is released resulting in a free site ready for the binding of the next N:P complex. The nucleocapsid assembly process is thus directed by the N:P complex and together with anti-termination events at gene junctions,would result in the production of a full-length genome complement.


7mG
AAAAA
7mG
7mG
L
L
L
7mG
L
P
P
P
P
P
P
P
P
3'
N
N
N
N
N
N
N
N
N
N
5'
TRANSCRIPTION
+N:P
N
N
N
N
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N
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N
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P
P
P
P
P
P
3'
N
N
N
N
N
N
N
N
N
N
5'
REPLICATION

The demonstration of the New Jersey serotype C proteins in the infected cell (Spiropolou and Nichol, 1993) and the implication of their role in genome replication through analysis of P protein *ts* mutant phenotypes (Rae and Elliot, 1986) prompted the initiation of studies to determine the effect of these proteins on viral transcription and replication. The results presented here show that the C' and C proteins of the Indiana serotype of VSV expressed together, but not the C protein alone, can have opposite negative and positive regulatory effects on transcription and replication respectively, in *in vitro* systems (Figures 19, 21 and 25). The fact that the C protein alone does not mediate the effect seen on transcription and replication suggests that, either a combination of the two proteins is required or that the C' protein may be solely responsible for this phenomenon. Because the two genes are present as a 3' co-terminal nested set with separate initiation codons, the difference between the proteins resides in the amino terminal 12 residue peptide which is unique to the C' protein. It is possible then, that this sequence determines the regulatory activity of the protein. However this observation is somewhat surprising, since in terms of Kozak's rules for ribosomal scanning (Kozak, 1989), the C protein and not the C' protein would be expected to be the most abundant protein in the infected cell since the C initiation codon is in the strongest context. In the New Jersey serotype, the C protein is also in the strongest context and has been demonstrated by immunoprecipitation to be present in 7-fold excess over the C' protein in the infected cell (Spiropolou and Nichol, 1993). Analysis of the relative levels of the Indiana serotype C' and C proteins, expressed by a "natural" process from the GCG construct and from the *wt* P gene in the rabbit reticulocyte system (Figure 18, lanes 10 and 13), suggest that the two proteins are produced at similar levels. However, if ribosomal scanning is operative, it is not clear what effect the CITE sequence included in these constructs would have on secondary initiation events in the reticulocyte system and this uncertainty is compounded by the fact that the *in vitro* ratio of the two proteins is not necessarily duplicated *in vivo.* Placing the C' gene directly adjacent to the CITE sequence in the C'/C construct, effectively uncouples the wild-type ratio of these two proteins, since only the C' protein is targeted in this way for direct ribosomal entry while C protein expression still relies upon an independent initiation event. Whether the observed effects of the C'/C construct on transcription and replication is a direct result of uncoupling the ratio of the C proteins or alternatively, is due mainly to the over-expression of the C' protein, remains to be determined. In this regard, further experimentation with the GCG construct and derivatives of the C construct engineered to express only the C' protein should prove to be enlightening, although identification of the predominant species of the C proteins in the infected cell through production a of specific antiserum or monoclonal antibody is necessary to properly interpret the *in vitro* results. It should be noted that in contrast to the severe drop in transcription produced by the C'/C construct, elimination of "wild-type" levels of C protein expression in extracts transfected with the ΔC'/C construct expressing the P protein but no C proteins, led to only a slight increase in transcription (Figure 19, lanes 2 and 3). Therefore, it appears that over-expression from the C'/C vector greatly exaggerates the effect on transcription and may not be an exact representation of the situation in the infected cell, where more subtle regulation may be in operation.

In the *in vitro* replication system however, the ΔC'/C construct displays a more noticeable effect and leads to a significant drop in the level of the 19S DI genome produced (Figure 25), which may indicate a more important role of the C proteins in promoting replication than in the negative regulation of transcription. The specificity of the Indiana C proteins in inhibiting transcription from the Indiana serotype nucleocapsid is demonstrated by a lack of a similar inhibition produced by the New Jersey C protein plasmid (Figure 21). This specificity is tentatively suggestive of a requirement for an interaction between proteins of the same serotype, since cis-acting transcriptional signals are generally more conserved than protein sequences between Indiana and New Jersey serotypes. A consistent negative effect on the level of the P protein in the absence of a significant effect on other transfected proteins, in extracts transfected with the C'/C construct has been noted (Results, sections II.2 and II.4). It is tempting to speculate from this result that the C proteins are able to down-regulate either total levels of the P protein or a particular subset of the P protein involved in transcription. However, further investigation is required to establish whether the effect of the C proteins on P protein levels is significant with regard to the regulation of transcription and replication..

The C proteins of VSV are so designated after similar small, basic proteins translated by independent initiation events in a second ORF of the Sendai virus P gene. Since these viruses are functionally related in terms of transcription and replication, comparison of the two systems is useful, although findings do not necessarily translate. Functional analysis of the Sendai virus C proteins reveals that they are fairly potent inhibitors of viral transcription *in vitro* when expressed at wild-type levels (Curran et al., 1992). They have been shown to have little effect on genome replication (Curran et al., 1992) and do not seem to be absolutely required for this process (Curran et al., 1991). However, this latter report did not take into account the demonstrated low levels of the C proteins in the Sendai virion (Yamada et al., 1990), which may have facilitated the replication of the DI particle used. An interesting result from the *in vitro* transcription system is some indirect evidence that the C and P proteins may interact to produce transcriptional down-regulation (Curran et al., 1992). The C proteins of Sendai virus and VSV can therefore be considered to be functionally similar with regard to their ability to negatively regulate transcription, although in the Sendai system, this negative transcriptional regulation could not be demonstrated to be balanced by a positive effect on replication.

The results of the *in vitro* transcription and replication experiments reported here, suggest that the C proteins may play a critical role in the switch between these two RNA synthetic processes. The mechanism by which this is accomplished is not directly addressed by the current work, but several possibilities exist. The C proteins bear a striking resemblance to the bacteriophage anti-terminator (N) proteins which are also small and highly basic (Franklin, 1985). The N proteins are able to engage the bacterial RNA polymerase and modify it to a termination resistant form. Although the RNA polymerase in this case is DNA dependent, it appears that sequences in the transcribed RNA (*nut* sites) are able to form hairpins which are recognized by an amino terminal, arginine-rich motif of the N protein and that this event promotes the formation of a stable, termination resistant polymerase complex (Lazinski et al., 1989). Given the overall similarity between the VSV C and the bacteriophage N proteins, there is a possibility that a similar situation occurs at VSV gene junctions. Analysis of the C' specific amino-terminal peptide reveals that it is not arginine rich in either serotypes. However, the C proteins (or perhaps just the C' protein) may contain a different, serotype specific motif which enables them to interact with secondary structure formed in the nascent genome RNA and/or with the RNA polymerase. Sequence analysis of the 3' terminus of each of the VSV transcription units reveals a degree of inverted complementarity which invariably includes the polyadenylation site. Formation of secondary structure in RNA synthesized from this region would be interesting, since it might impede the progression of the polymerase and cause it to stutter on the stretch of uridines present at the polyadenylation site and thus produce a poly-A tail. Pausing of the polymerase at gene junctions has been demonstrated (Iverson and Rose, 1981) and the production of the poly-A sequence by reiterative synthesis is generally accepted (Rose and Schubert, 1987). In accordance with this model, the C proteins might overcome the physical barrier produced by secondary structure formation, promote read-through of the gene junction by the polymerase and thus facilitate a replicative mode of synthesis. The consequence of this anti-termination event would be the bypass of both poly-adenylate synthesis and polymerase reinitiation and therefore the elimination of mRNA production. Although this model accomodates the data concerning the positive and negative regulatory effects of the C proteins on replication and transcription respectively, it is highly dependent upon the ability of the inverted complementarity observed, to form secondary structure. In this regard, it should be noted that the putative secondary structure would be extremely A-U rich and may not be thermodynamically stable. However, a transient stem-loop structure may be sufficient to halt polymerase progression and produce reiterative synthesis. Whether the C proteins are acting as antiterminators or not, an interaction with the RNA polymerase may still result in the adoption of a replicative mode of RNA synthesis. In this respect, the transcriptional evidence from the Sendai virus system (Curran et al., 1992) that the C proteins are perhaps interacting with the P protein is interesting, although direct biochemical evidence for the interaction of these proteins in this system, was not obtained. The putative C-P interaction may involve the P component of the N:P protein complex and could be important for the functional status of this encapsidation substrate. At present however, there are no indications or evidence to suggest how this might occur. Finally, the C proteins may exert their effect by interacting with undefined host proteins critical for either transcription or replication. In addition to the difficulty that these type of interactions would create in explaining the specificity of the C proteins, the involvement of host proteins in the VSV life cycle is a poorly defined area and as a result, any role that they may play in mediating C protein induced regulation, remains highly speculative.

Appendix A

<u>Oligonucleotides*</u>

1) Lead 5-1 (-)**

Xba 1 Truncated T7 Promoter -Leader

5'-GGTCTAGATAATACGACTCACTATAACGAAGACCACAAAACC-3'

2) Lead 3-1 (+)

Xba 1 Truncated T7 Promoter +Leader

5'-GGCTCTAGATAATACGACTCACTATAACGAAGACAAACAAACC-3'

3) L-1 (+)

Kpn 1 EcoRV L

5'-GCCGGTACCTCGAGATATCATGTCGTACAGTGGA-3'

Xho 1

4) N12 (+)

Msc1 N

5'-GCCATGGCCATTACAGTCAAGAGAATCA-3'

5) N16 (-)**

Kpn 1 EcoRV N

5'-GCCGGTACCTCGAGATATCTTCCGGATTTGAGGCCT-3'

Xho 1

6) N19 (-)

Sal1 N

5'-CGGGTCGACCATATGCATAATAT-3'

*oligonucleotide sense indicated in brackets.

**(+) and (-) refers to the VSV sequences.

7) NS3 (+)

Msc1 P
5'-GCCATGGCCAATCTCACAAAAGTTCGT-3'

8) NS6 (+)

Sty1 ala ala ala
5'-TATCCTTGGATGAATTGTTCGCAGCTAGAGGAGAATTCATCGCTGTC

GGAGGTAACGGACGA-3'

9) NS10 (-)

Sal1 P
5'-CGGGTCGACCATAGTCTACAGAGAAT-3'

10) NS12B (-)

C' GTA CITE
5'-CGTTATGCTTCGATCT$\boxed{\text{CAT}}$ATTATCATCGTG-3'
ORF1
Stop

11) NS13B (-)

C GTA CITE
5'-CGTCCTCTTAGAACAACT$\boxed{\text{CAT}}$ATTATCATCGTG-3'
ORF1
Stop

12) NS14 (+)

CITE C'
5'-CACGATGATAAT$\boxed{\text{ATG}}$AGATCGAAGCATAAGC-3'
ORF1
Stop

13) NS15 (+)

CITE C
5'-CACGATGATAAT$\boxed{\text{ATG}}$AGTTGTTCTAAGAGGACG-3'
ORF1
Stop

14) NS16 (+)

Apa1
5'-AUGUGAGGGCCCGGAAAACC-3'

15) NS17 (-)

Xba1
5'-ATTCTCCTCTAGATGAGA-3'

16) NS19 (-)

5'-CAACTCATAATTGGACTTTTCAGCTCGTTGTGCTTCTATCTCATC-3'
ORF2
stop (C')

17) NS20 (+)

5'-GTCCAATTATGAGTTGTTCCAAGAGGACGGAGTAGAAGAG-3'
ORF2
stop (C)

18 NS22 (+)

CITE P
5'-CACGATGATAAT$\boxed{\text{GCG}}$GATAATCTC-3'

19) NS23 (-)

```
        P                CITE
   ___________    ___________
5'-GTGAGATTATC|CGC|ATTATATCATC-3'
```

20) NS25 (+)

```
            Msc1     NJC
           ______ _____________
5'-GCCACTTATGGCCATTTGGATTCTGCC-3'
```

21) NS26 (-)

```
          Xba1        NJC
        ______ ________________
5'-CCCCTTCTAGAGTCTATTCTTTCATCC-3'
                ___
                ORF1
                stop
```

Appendix B

Sequencing of the HR-VSV N and P genes.

The following HR-VSV nucleotide changes in the N and Pgenes from the published sequence of the Glasgow strain of VSV Indiana (DePolo et al., 1987) have been detected. The point mutations were confirmed by sequencing three separate clones from two RT-PCR reactions. The resulting amino acid changes are noted (three letter code).

A) N gene

HR position	Codon change	Amino acid change
348	GGC⇒GCC	Gly⇒Ala
397	AGA⇒AGC	Arg⇒Ser
477	AAG⇒AGG	Lys⇒Arg
952	GCT⇒GCA	Ala⇒Ala (silent)
1039	GCC⇒GCT	Ala⇒Ala (silent)

B) P gene

HR position	Codon change	Amino acid change
49	TCT⇒TCC	Ser⇒Ser (silent)
208	CCG⇒CGA	Pro⇒Pro (silent)
298	GCA⇒GCG	Ala⇒Ala (silent)
304	GAT⇒GAG	Asp⇒Glu
429	TCG⇒TTG	Ser⇒Leu
665	GTA⇒ATA	Val⇒Ile

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