ENCAPSIDATION OF RNA BY VSV N PROTEIN

ENCAPSIDATION OF RNA BY VSV N PROTEIN IN VITRO

By

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To my father and mother

with love and gratitude

Abstract

Sequences at the 5′ end of the nascent RNA are known to be important as signals for encapsidation of the genome of vesicular stomatitis virus. In order to define the specific sequences involved in this process and to develop an *in vitro* encapsidation system, *in vitro* transcription from SP64-based plasmids was used to synthesize RNA molecules corresponding to various portions of the viral 5′ plus strand sequence. Some of these RNAs were tested for their ability to bind the capsid N protein *in vitro*. N protein in this assay was provided either from VSV mRNA programmed reticulocyte lysates or from infected cell extracts or, in collaboration with Dr. Sue Moyer (Gainesville, Florida), purified from viral nucleocapsids.

This thesis describes the construction of the SP64-based plasmids and the use of their RNA transcription products in the experiments described above.

I also constructed a series of plasmids that could direct the synthesis of RNA molecules which have many of the features of VSV defective particle genomes. Two of the constructs generate a defective-like RNA carrying a reporter gene capable of expressing the bacterial lac Z protein. These RNAs have the potential, after *in vitro* encapsidation and transfection into mammalian cells, of producing readily detectable helper-dependent virions.

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Table of Contents

Results

Figures

Tables

List of Abbreviations

A	adenine
ATP	adenosine triphosphate
C	cytidine
Ci	curie
CsCl	cesium chloride
CTP	cytosine triphosphate
DEAE	dithylaminoethyl
DNA	deoxynucleic acid
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
EDTA	ethylenediaminetetracetic acid
EGTA	ethylene glycol bis(B-aminoethyl ether)-N,N,N',N'tetraacetate
G	guanine
GTP	guanosine triphophate
IPTG	isopropyl-beta-D-galactopyranoside
KCl	potassium chloride
L	litre
kDa	kilodalton
Lac Z	the B-galatosidase gene of E. coli
mg	milligram
ml	millilitre
Mg	magnesium ions
nm	nanometre
mM	millimolar
MW	molecular weight
N protein	nucleocapsid protein
NaI	sodium iodide
32P	a beta particle emitting isotope of phosphorus
RNA	ribonucleic acid
RNP	ribonucleoprotein; viral RNA coated with N protein
rpm	revolutions per minute
35S	a beta particle emitting isotope of sulphur
SDS	sodium doedocyl sulphate
T	thymidine
TCA	tricholoroacetic acid
TEMED	N,N,N',N'-tetramethylethlenediamine
Tris	(Tris[hydroxymethy]aminomethane)
U	uracil
u	micro
ug	microgram

ul	microlitre
UTP	uridine triphosphate
VSV	vesicular stomatitis virus
X-gal	5-bromo-4-chloro-3-indolyl-beta-D-galactoside

Introduction

The Genome and Proteins of VSV

Vesicular stomatitis virus (VSV), the prototype Rhabdovirus, is an enveloped, negative sense RNA virus. The virus consists of a helical ribonucleoprotein core and the spiked, bullet shaped envelope (reviewed by Wagner, 1984, 1987; Emerson, 1984). All five viral proteins are present in the assembled virus. In the nucleocapsid core the 11 kb genomic RNA (Schubert et al., 1984) is tightly complexed with 1258 N (49 kDa) protein molecules (Thomas et al., 1985), and less tightly associated (Emerson and Wagner, 1972) with 466 NS (29 kDa) and 50 L (241 kDa) protein molecules. The nucleocapsid is the template for transcription and replication, whereas the polymerase function (first described by Baltimore et al., 1970) is provided by NS and L protein. The matrix protein M (25 kDa), is required for the maturation (Knipe et al., 1977a; Weiss and Bennett, 1980), and packaging (Newcomb et al., 1982; De et al., 1982), of the virus. The M protein (25 kDa) contributes to the stability of the virus by serving as the bridge between the nucleocapsid and the

glycoprotein G (69 kDa) studded envelope (Zakowski and Wagner, 1980; reviewed by Wagner 1985, and 1987). The glycoprotein is the major antigenic determinant of the virus (Kelly et al., 1972) and mediates the absorption and penetration of the virus into host cells (Kelly et al, 1972; White et al., 1983; see reviews by Emerson, 1985; Wagner, 1984, 1988). The virus contains 1826 and 1205 molecules of M and G respectively (Thomas et al., 1985). The glycoprotein G is acquired, along with the membrane, during the budding process (Knipe et al., 1977a, 1977b).

The purified virus can be solubilized and fractionated to yield a single fraction of nucleocapsid with associated L and NS, with or without M. The enzymatic component (NS and L proteins) can be removed from the N-RNA complex by treatment with high salt (Emerson and Wagner, 1972).

The Viral Life Cycle

Primary Transcription

The first template mediated event in the viral life cycle is transcription of the nucleocapsid by its associated transcriptase. Transcription mapping by UV irradiation (Abraham et al., 1976; Ball et al., 1976), EM studies (Herman et al., 1978), and studies with in vitro transcription systems (reviewed by Banerjee, 1987; Emerson, 1987) have

permitted the partial dissection of the events involved in the process. Initiation of transcription occurs after the NS moiety of the transcriptase specifically binds to the RNP at a 15 base region located 16 nucleotides from the 3′ end of the genome (Keene et al., 1981). The L protein, the catalytic subunit of the transcriptase, subsequently binds the nucleocapsid (Mellon and Emerson, 1978; Emerson, 1982). The transcriptase complex then proceeds to sequentially synthesize (Abraham et al., 1976; Iverson and Rose, 1982), in diminishing quantities (Villareal et al., 1973), a 47 bp leader, followed by capped and polyadenylated N, NS, M, G, and L mRNAs (Banerjee et al., 1985).

Gene order is used for the polar control of the timing and the level of gene expression. Sequential transcription of the genes from the 3′ end accounts for the order of appearance of the transcripts, and pausing and attenuation by the transcriptase at each intergenic junction accounts for the differential levels of mRNA synthesis. Quantitatively, transcription decreases by 29 to 33% across the N-NS, NS-M, and M-G gene junctions (Iverson and Rose, 1981), presumably because the polymerase dissociates from the template at each intergenic region 29 to 33% of the time and can only reload at the 3′ end of the template.

The nontranscribed junction between the leader and the adjacent N gene consists of three A residues in the Indiana serotype and of four A residues in the NJ serotype (Rowlands, 1978; Keene et al., 1981). The mechanism by which this junction effectively acts as a transcription terminator and attenuator is unknown.

Whereas the leader-N gene junction is unique in the virus, the remaining junctions are identical in the NJ serotype, and almost identical in the Indiana serotype. The sequence at these junctions is 5′ CUGUUAGUUUUUUUCAUA 3′ (Rose, 1980). The mechanism by which this sequence directs transcription initiation, termination, and polyadenylation by the transcriptase is not known. The polymerase is postulated to "chatter" at the stretch of seven U residues and thereby synthesize the approximately one hundred and forty base long poly A tail of the transcripts (Banerjee et al.,1974). The 5′ AG 3′ dinucleotide does not appear in the mature transcript (Banerjee, 1987). The postulated transcription initiation sequence, 5′ CUGUU 3′, is absent at the end of the L gene; only the postulated termination and polyadenylation sequences are present. Thus the last 60 bases of the genome are not transcribed during primary transcription.

The mode of transcription of the nucleocapsid has not been established. The transcription process is most likely discontinuous (ie. the transcriptase terminates the

transcription of a particular gene, then polyadenylates the transcript, and reinitiates at the next gene (Banerjee et al., 1977). However, a continuous mode of transcription (ie. the enzyme transcribes polycistronic RNAs which are subsequently cleaved and processed to yield the mature mRNAs) (Colonno and Banerjee, 1976) cannot be ruled out. Low levels of polycistronic transcripts with intervening poly A tracts (Herman et al., 1978) resulting from readthrough at gene junctions, are detected in vitro. Readthrough transcripts are also found in infected cells (Chinchar et al., 1982; Schubert and Lazzarini, 1981). These include RNAs in which the leader transcript is attached 5′ to the N mRNA (Herman and Lazzarini, 1981) or trailer sequences are linked 3′ to the L mRNA. Readthrough occurs at all gene junctions but a single base change in the canonical junction sequence may account for the elevated frequency of readthrough at the NS-M gene junction of the Indiana serotype (Masters and Samuel, 1984).

Readthrough transcripts may result from the failure of the polymerase to obey "stop and restart" signals at the gene junctions, the failure of the putative cleavage mechanism, or from an inappropriate replication attempt by the polymerase (Banerjee, 1987).

Secondary Transcription

Secondary transcription occurs after viral replication has amplified the infecting templates. Positive sense RNPs, nonexistent during primary transcription, become available for transcriptase binding. Transcription of positive sense nucleocapsids is initiated by NS binding to the promoter element at position 17 to 37 from the 3′ terminus (Keene et al., 1981). Synthesis of the negative sense leader transcript (Leppert et al., 1979; Leppert and Kolakofsky, 1980) terminates at an attenuation signal located between positions 46 to 60 from the 3′ terminus of the positive sense RNP (see Nichol and Holland, 1987). The absense of downstream reinitiation signals ensures the dissociation of the transcriptase from the template.

Replication

During transcription the polymerase complex obeys the genomic signal sequences for initiation, termination, and polyadenylation of messages but successful replication requires the suppression of these signals (except the 3′ initiation signal) and the concomitant association of N protein with the newly synthesized, nascent RNA (Blumberg et al., 1981). The first step in the replication cycle of VSV

is the copying of the infecting negative sense RNPs into positive sense RNPs. These in turn serve as templates for the synthesis of negative sense RNP. The conservation of the initiation signal at both termini is refelected by the 80% homology between the 3′ termini of the genome and antigenome (Nichol and Holland, 1987).

The key requirement for replication is continuous protein synthesis; replication is arrested by protein synthesis inhibitors like cycloheximide (Pearlman and Huang, 1973; Wertz and Levine, 1973). Since neither positive sense nor negative sense genome RNA is found in infected cells, the association of N protein with the nascent RNA was proposed to lead to RNP formation (Blumberg et al., 1981).

Encapsidation and Replication

Blumberg (Blumberg et al., 1981) proposed that replication was necessarily coupled to encapsidation because the association of N protein with nascent leader sequences activates the replication mode of the polymerase. In this model, encapsidation is nucleated by specific N protein binding to the first 14 bases of the nascent leader sequence (this sequence is almost identical in both leaders) and further assembly of N protein to the growing RNA is mediated by the cooperative binding of N (Blumberg and Kolakofsky,

1983). Binding is thought to initiate on the nascent leader before the nucleation site is obscured by secondary structure of the lengthening RNA.

The highly self-aggregating nature of N protein (Sprague et al., 1983) satisfies the proposed requirement for cooperative assembly following the initial nucleation. The observation that the in vitro replication of the virus is unaffected by high levels of RNAse is also compatible with this model. The direct evidence for the nucleation site in the leader is that 1) all negative and positive sense leader is found bound to N protein late in infection (Blumberg and Kolakofsky, 1981) and, 2) studies using purified N protein show high affinity binding to the first 14 bases of positive and negative sense leader (Blumberg and Kolakofsky 1983).

According to Blumberg's model, a 47 base leader is produced during the transcription mode when, following initiation, the transcriptase encounters an attenuation signal located approximately 50 bases downstream from the 3' end of either the positive or negative sense genome. However, this attenuation signal and any other cis-acting signal on the template are ignored by the enzyme as long as nascent RNA is being encapsidated. Thus N protein binding to the nascent RNA serves the dual function of encapsidating the RNA and acting as an anti-terminator.

In this model N autoregulates its own synthesis by dictating the timing of the transcription-replication switch. Furthermore this model suggests a mechanism by which the virus can avoid the wasteful synthesis of functionless full length RNA: replication may initiate only when N protein levels are sufficient for the generation of full length RNPs.

Replication Systems: The Role of N:NS Complexes

In vitro studies have been used to analyze the roles and requirements of viral proteins in the replication process. Peluso and Moyer (1983,1984) developed an efficient cell-free replication system which consisted of the supernatant fraction of permeabilized VSV infected BHK cells. The cell extract contains sufficiently high levels of viral proteins to support the replication of input template viral RNPs; six to twelve percent of *de novo* initiated RNA made was full length RNP.

When the soluble protein extract was depleted of N protein by immunoprecipitation, the extract failed to support replication (Peluso and Moyer, 1988). Fractionation of the soluble proteins in the extract confirmed that N proteins existed in two forms; 25% of the N molecules were present as high molecular weight aggregates (MW > 500,000 kDa), and 75% of the N molecules, associated with NS protein, were present as low molecular weight complexes (Peluso and Moyer, 1988).

The low molecular weight fraction of free N protein and N-NS complexes supported replication, whereas the high molecular weight aggregates of N protein did not.

Other experiments confirmed the existence of stable N-NS complexes with a 1:1 molar ratio of N and NS (previously detected by Bell et al., 1984) and it was demonstrated that only the N-NS complexes supported replication in vitro and hence served as substrates for binding to nascent replicative RNA. The NS moiety dissociates from N following binding of N to the RNA. The role of NS protein was suggested to be one of keeping N protein in a soluble, replication competent state by preventing N-aggregate formation. This model was subsequently refined by Peluso and Moyer (1988) and by Wertz and coworkers.

Wertz and coworkers (Davis and Wertz, 1983; Patton et al., 1983; Wertz, 1983) developed a highly purified coupled transcription-translation system to study viral replication in vitro. In their system, viral proteins were provided by VSV mRNA programmed reticulocyte lysates, while enzymatically active defective RNPs served as substrates for replication (reviewed by Wertz et al., 1987). To ensure that the input mRNA was the only source of mRNA in the system, the RNP of the defective particle MST was used. The defective genome of MST contains only the 5′ 25% of the viral genome, and consequently, does not direct the synthesis of mRNA.

The switch from transcription, in which only naked leader was synthesized, to low levels of genome replication was triggered by the addition of N mRNA (Wertz, 1983). Over the range tested, there was a linear correlation between the amount of N mRNA added and the level of genome replication. Presynthesized N protein was inefficient at supporting replication. Later work with the system suggested a critical role for NS in determining the ability of N to support replication: the N/NS ratio governed the level of replication (Patton et al., 1984). Low levels of N protein alone support some replication. At higher levels, N protein aggregated and did not support replication unless NS protein was available to maintain N protein in a soluble, replication competent state. The optimal level of replication was obtained at a 2:1 molar ratio of N to NS. When the molar amount of NS exceeded that of N, replication was inhibited; an NS/N ratio of 2:5 produced 50% inhibition of RNA replication, and an NS/N ratio of 2:1 resulted in complete inhibition.

During an infection the level of NS protein may determine the balance between transcription and replication by controlling the level of N protein available for replication. The replication competent pool of N protein consists of free N protein and N:NS complexes. However, as NS protein levels rise, N:NS complexes act as a sink for free

N protein, making N less available for replication. At high levels of NS protein, NS competes with the nascent RNA for N protein binding (Patton et al., 1984). Davis et al. (1986) investigated the association of cotranslated N and NS proteins. The proteins formed multiple complexes, the stoicheometries of which depended on the concentration of NS relative to N protein.

Later work in Banerjee's lab (Masters and Banerjee, 1988a,b) defined the precise nature of the N:NS complexes that formed when (and as Masters and Banerjee showed, only when) both proteins were cotranslated. Nondenaturating electrophoresis following cotranslation of in vitro synthesized N and NS mRNA revealed the existence of six distinct N:NS complexes (Masters and Banerjee, 1988a). Complex formation required cotranslation because newly made N protein, in the absence of NS protein, immediately and irreversibly complexed with small species of RNA present in the reticulocyte lysate (Masters and Banerjee, 1988b). The binding of RNA to the N protein prevented N:NS complex formation (Masters and Banerjee, 1988b).

The Role of L and NS Proteins in Replication

Early investigations into the role of the viral proteins in replication and transcription revealed that an L-NS complex of unknown stoicheometry acts as the polymerase in the presence of N-coated genomes (Emerson and Yu, 1975). The NS moiety is an integral part of the polymerase complex, but it is likely the noncatalytic subunit and L is the catalytic subunit that mediates the enzymatic steps of transcription and replication (see reviews by Banerjee, 1987a and 1987; Emerson, 1987). However, L cannot perform any of its catalytic functions in the absence of NS. Even L binding to the RNP template is dependent on NS; only after NS binds to the promoter region at the 3' end of the genome can L enter the RNP (Mellon and Emerson, 1978). However, since NS protein is required in stoichiometric amounts for optimal RNA synthesis (De and Banerjee, 1985), template related functions other than promoter recognition and facilitation of L binding have been proposed (Emerson and Schubert, 1988; Hudson et al., 1986). Some of the hypothesized functions of NS protein are 1) packaging of L by serving as a bridge between the RNP and L protein (Mellon and Emerson, 1978), since NS has defined binding domains to both L and N proteins (Emerson and Schubert, 1988), 2) facilitating the elongation function of L protein by uncoiling the helical nucleocapsid (De and

Banerjee, 1985), 3) making template RNA accessible to the L protein by temporarily shifting N protein from the template during elongation (Hudson et al., 1986) and, 3) activating L protein catalytic functions by binding to it (Hudson et al., 1986).

The NS protein is phosphorylated, both by cellular kinases and by L protein at 21 possible serine and threonine residues, but constitutively at 5 sites at position 49 to 64 in the protein (Bell and Prevec, 1985; Hudson et al., 1986; Banerjee, 1987). The phosphorylation state of each molecule may dictate the particular role it plays in the viral life cycle (the most highly phosphorylated form of NS is the transcription competent species) (reviewed by Banerjee, 1987).

The Leader Gene and Transcript

The leader RNA sequence is remarkably multifunctional, with domains that have been implicated in the nucleation of encapsidation (Blumberg et al., 1983), initiation of transcription and replication (Mellon and Emerson, 1978; Emerson, 1982; Keene et al., 1981), host cell shut off (reviewed by Wagner, 1984 and 1987), and host protein La binding (Kurilla and Keene, 1983). Both a positive sense and a negative sense leader are found in infected cells (Blumberg and Kolakofsky, 1981). The 5′ ends of these leaders are

homologous: 14 of the first 17 bases are identical (Nichol and Holland, 1987). This sequence is also extensively conserved across the different serotypes (Nichol and Holland, 1987).

Host Cell Shutoff

VSV infection rapidly shuts off cellular DNA, RNA, and protein synthesis (reviewed by Wagner, 1984 and 1987). The leader sequence, which was shown to transiently localize to the nucleus during infection (Kurilla and Keene, 1982), has been convincingly implicated in the inhibition of initiation by cellular RNA pol I, II, and III directed transcription (Weck and Wager, 1978 and 1979; McGowan et al., 1982; Grinnell and Wagner, 1984 and 1985) and of cellular (McGowan and Wagner, 1981) and viral DNA replication (Remenick and McGowan, 1986; Remenick et al., 1988).

The ability of VSV to inhibit cellular RNA synthesis was shown to depend on primary transcription of the genome (Weck et al., 1979; Wu et al., 1980) and UV inactivation (Weck et al., 1979) studies suggested only synthesis of plus sense leader was required to effect these inhibitions. Kinetic studies demonstrated a direct correlation between the level of plus sense leader levels and the degree of cellular transcription inhibition (Grinnell and Wagner, 1983). In

vitro transcription (Grinnell and Wagner, 1985) and replication (Remenick et al., 1988) systems of adenovirus were extensively inhibited by the addition of leader, its cDNA homologue, or the ribonuclease TI fragment of leader that contained the AU rich sequence (at position 18 to 24) (Grinnell and Wagner, 1984).

Using oligonucleotides corresponding to various regions of the leader, Grinell and Wagner (1985) confirmed that the AUUAUUA palindrome was required for in vitro transcription inhibition. The highest inhibition was obtained with the oligonucleotide spanning bases 16 to 35 of the leader. The AU rich leader sequence bound to a 65 K cellular protein in the transcription extract. Addition of a fraction containing the 65K protein reversed the inhibition of initiation by pol II and pol III.

Both UV inactivation and kinetic studies also implicate the leader in cellular and viral (adenovirus and SV40) DNA synthesis inhibition. Using an adenovirus in vitro replication system, Remenick et al. (1988) showed that the plus sense leader transcript and its cDNA equivalent bound to and inhibited both the adenovirus polymerase and the Hela cell DNA polymerase alpha-primase. This would imply that these polymerases are the in vivo targets of cellular and adenovirus DNA synthesis inhibition.

Defective Interfering Particles

Defective interfering (DI) particles are subgenomic virus particles generated by deletion of trans sequences in wild-type virus genomes. The deficiency of coding sequences in DI genomes means they can not replicate independently; they require trans complementation from a helper (a coinfecting homologous parental virus) for replication. The phenomena of viral interference, extensively studied using the VSV system (reviewed by Perrault, 1981; Holland, 1987), refers to the preferential replication of the defective at the expense of the helper virus in a doubly infected cell.

Though primary transcription is unaffected, replication of the helper is stongly inhibited, and consequently secondary transcription is repressed. The low amount of viral protein produced by the doubly infected cell is almost exclusively used for the replication of the DI genomes and for their maturation into DI particles (Perrault, 1981). Effective competition for the replication proteins by the DI particle and its quicker replication due to its smaller size were proposed to result in the preferentially amplification of the DI particle and the virtual arrest of helper virus replication (Huang and Baltimore, 1970). In fact, single cells capable of yielding 5,000 infectious viruses consistently yielded no infectious virus when

coinfected with VSV and a single DI particle of a particular type (Sekellick and Marcus 1980). However, the extent of interference varies appreciably, depending on DI particle type, relative times of entry into the cell of the helper and the DI particle, cell type, and virus serotype (Holland, 1987).

The minimum sequence requirements of a defective virus are the cis-acting replication and encapsidation sequences present on the termini of the virus. But there may also be a minimum length requirement for packaging, because defective viruses consisting of less than 10% of the genome (shorter than about 1.2 kb) are not detected (Re and Kingsbury, 1988). Rather than possessing a 3′ and 5′ leader sequence, most DI particles retain only the 5′ leader sequence, replacing the 3′ leader sequence with the exact complement of the 5′ leader, the so-called copy-back 3′ terminus. This may account for the ability of defective viruses to outcompete wild type virus for polymerase binding <u>in vitro</u> (Re and Kingsbury, 1988; Giachetti and Holland, 1989).

The SP6 Transcription System

To study encapsidation *in vitro*, we required a source of viral leader RNA and a source of viral proteins. Viral RNA was synthesized by *in vitro* transcription of plasmids bearing VSV sequences placed downstream of the SP6 bacteriophage promoter. The cloning vector used was the plasmid pSP64 (Melton *et al.*, 1984), in which a polylinker sequence is placed downstream of the SP6 promoter sequence. Purified SP6 polymerase specifically initiates transcription at the promoter (Butler and Chamberlain, 1983) and runoff transcripts are synthesized from linear templates. The SP6 phage promoter sequence present in the plasmid includes the sequence 5′ AATA 3′ downstream of the G residue where transcription initiation occurs. Melton and colleagues (Melton *et al.*, 1984) showed that deletion of bases upstream of the initiating G residue results in markedly decreased transcription at the promoter.

Materials and Methods

Plasmid Preparation

Large scale preparations of plasmids were prepared from 500 ml overnight cultures. These were started, in the case of Col E1 plasmids, by inoculating 1 ml of overnight culture into 2 litre flasks containing 500 ml of LB with 50 ug/ml Ampicillin. For M13 plasmids, a 500 ml log phase culture of JM 107 was infected with 1 ml of phage stock and grown for six to eight hours. The bacterial pellets were collected by spinning the cultures in 250 ml Nalgene polypropylene centrifuge bottles at 6000 rpm for 10 minutes in the Sorvall JA10 rotor. The pellets were resuspended in 40 ml of lysozyme solution (50 mM glucose [British Drug House (BDH)], 25 mM Tris-HCl [BDH], 10 mM EDTA [BDH], 5 mg/ml lysozyme [Sigma]) and left on ice for 30 minutes. Eighty ml of alkaline SDS (0.2 N NaOH [BDH], 1% SDS [Bio-Rad]) was added, the solution was gently mixed by inverting several times, and the mixture was placed on ice for 10 minutes, or until the solution had cleared. Forty ml of 5 M potassium acetate (pH 4.8)(BDH) was added next, the solution was gently mixed and placed on ice for one hour. The sample was filtered

through cheese cloth and divided into 40 ml Sorvall tubes and centrifuged at 15,000 rpm in a Sorvall GS 20 rotor for 30 minutes. The supernantants were collected into 250 ml Nalgene polypropylene centrifuge bottles, and 0.6 volumes of isporopanol was added. The mixture was shaken and left for half an hour at room temperature. The nucleic acid pellet was collected by spinning at 8,000 rpm in the GS 10 rotor of the Sorvall. The pellet was resuspended to a final volume of 5 ml in 0.1 X SSC (15 mM NaCl [BDH], 1.5 mM sodium citrate [BDH], pH 7.0), 2 ml of TE (50 mM Tris-HCl pH 8.0, 10 mM EDTA, pH 8.0 [both from BDH]), and 8.4 grams of CsCl (Terochem or Pharmacia) were added. After the CsCl was dissolved by shaking vigorously, the solution was evenly distributed to 2 VTi65 tubes (Beckman), 200 ul of 6 mg/ml ethidium bromide (Boehringer Mannheim) was added to each tube, and the tubes were filled to the top with light parafin oil (Fischer Scientific Company). The tubes were sealed, spun at 55,000 rpm overnight in Beckman VTi65.1 rotor at 15 degrees Celcius, and the DNA bands were collected by piercing the side of the tube with a needle. The ethidium bromide was removed by multiple extractions with isoamyl alcohol (Caledon Laboratories) and the DNA was precipitated by diluting the solution with three volumes of water, adding 2 volumes of cold ethanol, and spinning in 40 ml Sorvall tubes at 15,000 rpm for 30 minutes in a GS 20 rotor. The DNA pellet was washed twice

with 70 % cold ethanol, dried in the warm room for thirty minutes, and resuspended in a final volume of 1 ml. The DNA was then extracted twice with phenol, twice with chloroform, and then reprecipitated.

Preparation of Vector DNA

The vector DNA (previously purified by banding on CsCl gradients) was usually prepared by digestion with the appropriate restriction enzyme (about 5 units of the enzyme per microgram of DNA), followed by removal of the enzyme by phenol extraction, ethanol precipitation, and resuspension of the DNA in water at a concentration of about 100 ug/ml. Alternatively, the vector DNA was purified by extraction from an agarose or acrylamide gel.

Usually, the DNA was treated with calf intestinal phosphatase (Boehringer Mannheim) prior to its purification. Once digestion was completed, as determined by gel analysis, the DNA solution was made alkaline by the addition of 0.1 volume of 1M Tris (pH 8.5). Several units of the phosphatase were next added, and the mixture was incubated for 30 minutes.

Preparation of Insert DNA

Many methods for the purification of DNA molecules from acrylamide and agarose gels are available. To purify DNA fragments from acrylamide gels, the Maxam-Gilbert technique (Maxam and Gilbert, 1977) was always used. For isolation of DNA fragments from agarose the "GeneClean" (TM) (US Biochemical Corporation [USBC]) kit was used.

Purification of DNA with "GENECLEAN"(TM)

The digested DNA was fractionated by agarose gel electrophoresis, stained, and the desired DNA band was identified. The band was excised with a scalpel and treated according to the protocol provided with the kit from USBC. The agarose containing the DNA fragment was placed in an Eppendorf tube and was dissolved in a saturated solution of sodium iodide (NaI). Next, specially prepared glass beads were added. The liberated DNA bound to these glass beads at high ionic strength. After removal of the sodium iodide solution, the beads were washed with a high salt ethanol solution, and the bound DNA was released by the resuspension and incubation of the beads in water.

Purification of DNA from Acrylamide Gels

This technique, devised by Maxam and Gilbert (1977), was used for the purification of DNA fragments shorter than 1000 base pairs. The digested DNA was separated by (5%) polyacrylamide gel electrophoresis, stained with ethidium bromide, visualized by UV fluorescence, and the desired band was identified. The band was then excised using a scalpel and placed in a 1.5 ml Eppendorf tube. The acrylamide slice was then crushed with a pestle, or by repeated passage through a needle with a small bore syringe attached. One volume of elution buffer was then added (0.5 M ammonium acetate, 1 mM EDTA [pH 8.0]). The tube was then incubated overnight on a rotating wheel at 37 degrees Celcius. The supernatant was then collected by centrifuging the sample at 10,000 g for 10 minutes. An additional 0.5 volume of elution buffer was added to the acrylamide pellet, the tube was briefly vortexed, and recentrifuged. The supernantant was collected and added to the first supernantant. Any remaining fragments of acrylamide in the combined supernatants were removed by passing the supernatant over a small glass wool column. The DNA in the supernatant was then precipitated with ethanol and resuspended in water.

Ligations

Ligation conditions varied according to the type of ligation being performed; blunt end religation, sticky end religation, blunt end bimolecular ligation, sticky end bimolecular ligation, or ligation of linkers. For religations, 10 ng of DNA was used in a final volume of 20 microlitres, with one unit of T4 DNA ligase for the sticky end ligations, and two units of T4 DNA ligase for the blunt end ligations. For blunt end bimolecular ligations 100 ng of dephosphorylated vector DNA and 20 to 100 ng (calculated to give at least a 3 to 1 molar ratio) of insert were used in a final volume of 20 microlitres, with two units of T4 DNA ligase. For sticky end bimolecular ligations about 20 ng of dephosphorylated vector DNA was used with 10 to 50 ng of insert DNA (calculated to give a 2 to 1 molar ratio of insert to vector) in a final volume of 10 microlitres, with one unit of T4 DNA ligase. For ligation of linkers into linearized, blunt ended vectors, 20 ng of vector DNA and a 100 fold excess of linker were used (this usually corresponded to about one to two nanograms of linker) in a final volume of 10 microlitres, with one unit of T4 DNA ligase.

The ligations were performed in the following buffer (1X): 50 mM Tris HCl (pH 7.6), 10 mM magnesium chloride, 10 mM DTT, 1 mM ATP.

Preparation of Media and Plates for Bacterial Work

Bacteria were grown in sterile LB media (made by combining 10 g Bacto-tryptone [Difco], 5 g Bacto-yeast extract [Difco], and 10 g NaCl [BDH] in a final volume of 1 L of water and autoclaving the mixture), supplemented, when appropriate, with Ampicillin (final concentration 50 ug/ml). Transformed bacteria were plated on LB agar Ampicillin plates. The LB agar with Ampicillin was made by adding 15 g Bacto-agar (Difco) to 1 L LB media, autoclaving, cooling to 55 degrees Celcius, and adding Ampicillin to 50 ug/ml. Approximately 30 ml of the agar was added to each plate.

YT media, for growing JM 107 cells for M13 phage cloning, was made by combining 8 g Bacto-tryptone, 5 g Bacto-yeast extract, 5 g NaCl and water to make 1 L, and autoclaving the final solution. YT agar plates were made by adding 15 grams of Bacto-agar to 1 litre of YT medium, autoclaving, and pouring about 30 ml of the molten solution into petri dishes. Soft (top) agar for plating M13 phage was made by adding 6 g Bacto-agar to 1 litre of YT medium, and autoclaving. The molten solution was aliquoted into Gibco 100 ml bottles and melted by microwaving before use. For detection of clones with inserts during M13 cloning, IPTG (isopropyl-beta-D-thiogalactopyranoside), and X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactoside) were used.

A 100 mM solution of IPTG (BRL) was made by dissolving the powder in water to a final concentration of 23.8 mg/ml . A 2% solution of X-gal (BRL) was made by mixing 25 mg Xgal in 1.25 ml dimethylformamide (BDH).

Preparation of Competent E. coli and Transformation

Competent E. Coli, usually of the LE 392 or DH 1 strain for Col E1 plasmids, and JM 107 for M13 plasmids, were prepared as in Maniatis et al. (1982). Briefly, a 100 ml log phase cell culture (O.D. 550 nm = 0.5) was chilled on ice for ten minutes, and then the cells were harvested by centrifugation at 4000g for 5 minutes at 4 degrees Celcius. The cells were resuspended in 50 ml of an ice cold, sterile solution of 50 mM calcium chloride and 10 mM Tris HCl (pH 8.0). The cells were incubated on ice for 15 minutes, harvested as before, and resuspended in 7 ml of the same solution. These cells were usually used immediately.

Transformation of the competent LE 392 or DH1 cells by ligation mixtures containing Col E1 based plasmids was performed as follows. The ligation mixture was diluted five fold, added to 200 microlitres of competent cells, and the final mix incubated on ice for thirty minutes. The cells were placed in a 42 degrees Celcius water bath for two minutes, and immediately transferred to ice. One ml of LB broth was added

to the tube, which was incubated at 37 degrees Celcius for one hour. The cells were then plated onto Ampicillin plates (for ampicillin selection) or onto minimal media plates (see Maniatis et al., 1982) that contained X-gal (when selecting for Lac Z expression of DH I cells after transformation with p45 derivatives containing the Lac operator inserts-see Bautista and Graham, 1989). Usually plates had various volumes of the cell suspension added, ranging from 50 microlitres to 250 microlitres. The plates were left at room temperature until the liquid had been absorbed and were then incubated overnight at 37 degrees Celcius, at which time Ampicillin resistant colonies were picked for amplification and analysis of their plasmid content. When Lac Z selection was used to identify colonies bearing Lac operator inserts, blue colonies appeared after a 48 hour incubation.

Transformation of competent JM 107 cells by ligation mixtures containing M13 mp19 or mp18 vectors was more involved. The procedure was obtained from the BRL manual for M13 sequencing. After their transformation, varying amounts of cells were added to 3 ml molten soft agar (kept at 45 degrees Celcius) to which 100 microlitres of "lawn cells" (at log phase), 50 microlitres of 2% X-gal, and 10 microlitres of 100 mM IPTG had been added. This mixture was quickly vortexed and plated onto prewarmed YT plates. Turbid plaques

were detectable within eight hours. Only colourless "plaques" were picked for amplification and analysis of their DNA content.

Analysis of Transformants

Screening transformants to identify plasmids bearing the desired insert in the proper orientation was performed primarily by restriction enzyme analysis of "miniprep DNA". Candidates thus tentatively identified were usually subsequently confirmed by sequencing. For Col E1 plasmids the following procedure (modified by Dody Bautista from the procedure devised by Birnboim and Doly, 1979) was used to prepare DNA. A colony was picked from the plate, inoculated into 2 mls of LB with 50 ug/ml Ampicillin, and incubated a minimum of four hours (or a maximum of 16 hours) with shaking at 37 degrees Celcius. The cells were harvested by pouring approximately 1.5 ml of the culture into an Eppendorf tube and centrifuging in a microfuge for 15 seconds. The supernatant was removed by aspiration and the cells were immediately placed on ice. The remaining culture (about 0.5 ml) was frozen after the addition of an equal volume of 30% glycerol.

When analyzing M13 transformants, turbid plaques were picked, inoculated into 2.5 ml of YT media, and incubated with shaking for four hours at 37 degrees Celcius. Three hundred

ul of log phase JM107 cells was then added, and the tubes were left to shake at 37 degrees Celcius for another four hours. The cells were harvested as in the case of Col E1 plasmids, but the supernatant, which served as a stock for the clone, (because it contained high titer of infectious phage) was saved and stored at 4 degrees Celcius. The cells were resuspended by vortexing with one hundred microlitres of a lysozyme solution (25 mM Tris HCl [pH 8.0], 40 mM glucose, 10 mM EDTA [disodium form], and freshly added lysozyme [Sigma] to 10 mg/ml). After a 15 to 20 minute incubation on ice in this solution, two hundred microlitres of alkali-SDS (0.2 M NaOH, 1% SDS) was added. The contents of the tube were gently mixed by inverting several times and then incubated on ice for 5 minutes, or until the mixture became clear. The mixture was neutralized by the addition of 150 microlitres of 3 M sodium acetate (pH 4.5). The tube was quickly vortexed (1 to 2 seconds) immediately after the addition of the sodium acetate and kept on ice for one hour. The tube was centrifuged for 5 to 10 minutes and the clear supernatant was transferred into a clean tube containing 1 ml of cold (-20 degrees Celcius) ethanol. After vortexing to mix the contents, the tube was immediately centrifuged for 5 minutes. The supernatant was aspirated, the nucleic acid pellet was resuspended in 100 ul TE buffer (10 mM Tris HCl [pH 8.0]), and reprecipitated by the addition of 200 ul of cold ethanol, vortexing, and

centrifuging for 5 minutes. After aspiration of the supernatant, the pellet was washed by filling the tube with cold (-20 degrees Celcius) 70% ethanol, centrifuging for 60 seconds, and aspirating the supernatant. The plasmid DNA pellet was dried by incubating the tube in the warm room for 30 to 60 minutes. The pellet was resuspended in 100 ul of TE buffer.

This procedure usually yielded a minimum of 5 ug of relatively pure Col E1 plasmid DNA, with minimal tRNA, chromosomal DNA, or protein contamination. The DNA was suitable for restriction enzyme analysis, sequencing, and, even cloning. For restriction enzyme analysis, 1 ul was usually sufficient. For double stranded DNA sequencing, 10 ul were used.

The M13 plasmid DNA isolated by this procedure was only used for restriction enzyme analysis. Sequencing and site directed mutagenesis was performed using single strand M13 DNA. The DNA was purified from extracellular phage, which were harvested by polyethelyne precipitation from the media of infected cells (according to the protocol provided by BRL).

Agarose And Acrylamide Gel Analysis of DNA

Agarose gels, typically 1.2%, were cast and run in TAE buffer (40 mM Tris-Acetate, 2 mM EDTA). The stock of TAE used in the lab is 50X and is made by combining 242 g Tris base, 57.1 ml glacial Acetic Acid, 100 ml 0.5 M EDTA (pH 8.0), and adding water to a final volume of 1L.

Nondenaturing polyacrylamide gels used to analyze DNA fragments less than a thousand bases long were 5% acrylamide-bisacrylamide (30:1), cast and run in TBE buffer(0.089 M Tris-borate, 0.089 M boric acid, 2 mM EDTA). A 10X stock was made by combining 54 g Tris base, 27.5 g boric acid, 20 ml 0.5 M EDTA (pH 8.0), and water to make 500 ml. This same buffer was used in sequencing gels.

Extraction of DNA and RNA with Phenol

Extractions with phenol were used to eliminate protein contaminants from nucleic acids. The phenol used was redistilled from crystalline phenol (Fisher Scientific) at 60 degrees Celcius to remove potentially nucleic acid damaging contaminants. The phenol was first buffered with Tris-HCl (pH 8.0), then made 0.2% with 2-mercaptoethanol, and an equal volume of water-saturated chloroform-isoamyl alcohol (24:1 v/v) was added. Extractions with phenol were actually

performed with phenol:chloroform:isoamyl (1:1:0.04 v/v). Extractions with chloroform, performed subsequent to phenol extractions, remove any remaining phenol from the nucleic acid solution.

Preparation of Single Stranded DNA Sequencing Template

A 50 ml log phase culture of JM 107 was infected with 200 ul of phage stock. After six hours the cells were pelleted by centrifugation at 4000 g and the supernatant, containing the phage was collected. To 43 ml of the supernatant 7 ml of a solution of 20% polyethylene glycol (PEG 6000), 2.5 M NaCl was added. The mixture was shaken and incubated on ice for 30 minutes. The solution was divided between two 40 ml Sorval tubes and centrifuged for 30 minutes at 15,000 rpm at 4 degrees Celcius. The supernatant was discarded and any remaining supernatant on the walls of the tube was removed by wiping with a Kimwipe. The phage pellet was resuspended in 1 ml of TE and aliquoted into two Eppendorf tubes. Two extractions with phenol-chloroform, followed by two extractions with chloroform were performed. The single stranded DNA of the phage was precipitated with ethanol, washed with 70% ethanol, and dried for 30 minutes in a warm room. The pellet was resuspended in 100 ul of water and 1 ul was electrophoresed on a 1% agarose gel to check the quality

of the DNA. Twenty microlitres of the solution were diluted to 1 ml with water and a UV spectrum analysis was performed to determine the purity and concentration of the DNA. If the DNA appeared intact on the gel and if its UV spectrum confirmed its purity, then it was used for sequencing or site directed mutagenesis.

DNA Sequencing

Single strand M13 DNA sequencing and double stranded plasmid sequencing were performed according to established protocols. M13 sequencing was performed according to the BRL Manual entitled M13 Cloning/"Dideoxy" Sequencing Manual, using chemicals and enzymes provided by a kit (BRL). Double strand plasmid sequencing was performed either using the Sanger (Sanger, 1977) technique on linearized plasmids or by using the commercially available "Sequenace" (TM) kit (USBC) and protocol on supercoiled DNA. Depending on the availability of radioactive nucleotides, either internal labelling (with alpha 35S dATP) of the synthesized strands was used, or sequencing with 32P end labelled primer was performed. Excellent results were obtained by using "miniprep DNA" for double-stranded sequencing using the Sequenace system.

Double Strand Sequencing with End Labelled Primer

Ten ul of "miniprep DNA" was digested in a final volume of 15 ul. The primer was "kinased" in a reaction mixture that consisted of the following (the reaction was scaled down when necessary): up to 60 ng of primer, 5 ul 10X kinase buffer (same as ligase buffer, except there is no ATP included), 130 uCi of gamma-32P-ATP (NEN, 3000 Ci per millimole), 20 units T4 DNA kinase (BRL). The mixture was incubated for 45 minutes at 37 degrees Celcius. The template was denatured and hybridized to the primer by combining in a 1.5 ml Eppendorf 8 ul of DNA from the digestion, 5 ng of labelled primer, incubating the tube in boiling water for 5 minutes and placing it on ice for 30 minutes. To the hybridization mixture were added 1 ul of 0.1 M DTT (BMC), 1 unit Klenow fragment (Pharmacia), and 1 ul of 0.1 mM dATP (the deoxy-dideoxy mixes supplied by Pharmacia are intended for sequencing with an internal label, and hence they are difficient in dATP). The mixture was vortexed and quickly spun in a microfuge. Three microlitres of this mixture were aliquoted into 4 tubes separately labelled as A, T, C, or G containing two microlitres of the appropriate deoxy-dideoxy mix. Reactions were initiated by mixing and incubating at room temperature.

The protocol of the Sequenace kit (USBC) is a modification of the standard Sanger method (Sanger et al., 1977). Double-stranded plasmid DNA (we used" miniprep DNA" prepared as outlined above) is denatured by treatment with alkali and the enzyme used for sequencing is the modified T4 DNA polymerase. The manual provided by the United States Biotechnology Corporation gives detailed instructions of the use of the kit.

Processing of sequencing reactions and of primer extension reactions included the termination of the reactions by the addition of a formamide solution (supplied with the kits and consisting of 95% formamide, 0.1% xylene cyanol and bromophenol blue as tracking dyes --the formamide serves to arrest the reactions and facilitates the denaturation of the nucleic acids) to a final concentration of 50%, the denaturation of the contents by heating to 90 degrees Celcius for 2 minutes, and finally the electrophoresis of the reactions on denaturing acrylamide gels.

Denaturing (8 M urea [Bio-Rad]) sequencing gels of 6% or 8% polyacrylamide (1 in 20 bis-acrylamide to acrylamide ratio) were used. The buffer used was Tris-borate (1 M Tris [Bio-Rad], 1 M boric acid [Caledon Laboratories], 1 mM EDTA [BDH]. Following the preparation of the acrylamide solution (for 8% gels, it was made by combining 7.6 g acrylamide, 400 mg N, N'-methylene-bis-acrylamide, 10 ml 10X Tris-borate

buffer, and water to 100 ml) it was filtered through a 0.45 um filter (Nalgene). Just before pouring the acrylamide solution, 1 ml of 10% ammonium persulphate and 50 ul of Temed (N,N,N',N'-tetramethylethylenediamine (Bio-Rad) were added.

The glass plates to be used for sequencing gels were matched, washed with a 1% solution of SDS (Bio-Rad), rinsed with water, washed with ethanol, and dried. The long plate was treated with 20 ml of "repelsilane" (a 5% solution of dichlorodimethylsilane [Kodak] in 1,1,1-trichloroethane [Caledon Laboratories]) in two separate applications of 10 ml each. The solution was poured onto the plate and spread evenly over the entire area of the plate with Kimwipes. After 5 minutes, the solution was dry and the plate was polished with Kimwipes. After the second application of the solution, the plate was washed with ethanol, polished, and left to air dry. The short plate, to which the acrylamide gel was to bind, was treated in the same manner with 20 ml of "bindsilane", a solution consisting of 0.5% 3-(Trimethoxysilyl)propylmethacrylate (Aldrich Chemical Company), and 0.3% Acetic Acid (American Hospital Supply Canada Inc.) in 95% ethanol.

Once the 0.04 cm thick gel was formed by pouring the acrylamide solution into the assembled plates, it was allowed to polymerize for 1 hour. The comb was removed, the wells were washed, and the gel was prerun for one to two hours.

Before the samples were loaded, the wells were thoroughly washed out to remove the urea. Typically, the gels were run until the bromophenol blue reached the bottom. The plates were then disassembled and the gel, attached to the short plate, was then soaked in a 10% solution of acetic acid for 30 minutes, washed with water, and dried a minimum of 4 hours at 37 degrees Celcius. Kodak X-OMAT AR or X-OMAT RP film was placed directly on the gel, a glass plate was clamped on top to ensure good contact between the film and the gel.

Bal 31 Nuclease Digestion

Bal 31 nuclease is a double stranded DNA exonuclease that progressively and bidirectionally removes nucleotides from both strands of linear DNA. Because degradation occurs only at the ends of the DNA molecule, the enzyme can be used for the controlled deletion of sequences from the ends of the DNA molecule to be cloned (see Berger et al., 1987) The rate of exonuclease digestion is highly sequence dependent, and can be modulated by salt concentration, temperature, and amount of enzyme added to the reaction.

The rate of digestion of linearized plasmids was determined separately for each batch of DNA. Temperature, salt concentrations, and the DNA to enzyme ratio were manipulated to set the desired rate of digestion. The rate

of digestion under a given set of reaction conditions was determined by removing aliquots of the reaction at various times, stopping the reaction by adding EGTA [BDH] to 20 mM final concentration (the enzyme is calcium dependent), and analyzing the products by 5% acrylamide gel electrophoresis. The conditions of the pilot reaction yielding the desired rate were reproduced and usually aliquots were removed at three time points. The DNA was extracted with phenol, and precipitated with ethanol to remove the enzyme. The DNA was then treated with the Klenow polymerase in the presence of the four deoxynucleotides to make the ends blunt. The deleted plasmids were ligated in the presence or absence of linkers, transformed, and colonies were analyzed, initially by double enzyme digestion and polyacrylamide gel electrophoresis. Likely candidates were then sequenced.

A typical Bal 31 nuclease reaction combined the following in a 1.5 ml Eppendorf tube: 2 to 10 ug linear DNA, 5 ul 10X Bal 31 buffer (200 mM Tris-HCl pH 8.1, 125 mM magnesium chloride, 3 to 6 M NaCl, 10 mM EDTA), 1 to 4 units Bal 31 (BRL) per ug DNA, water to 100 ul. The tube was incubated at 20 degrees Celcius or 30 degrees Celcius.

T4 DNA Polymerase Deletion Mutagenesis of p011

T4 DNA polymerase (Promega Biotechnology Corporation) has a 5′ to 3′ polymerase function and a powerful 3′ to 5′ exonuclease that is functional in the absence of all four nucleotides. In the presence of one nucleotide the exonuclease activity will proceed until that particular nucleotide is reached, and then the enzyme "cycles", as the nucleotide is removed by the exonuclease activity and is promptly replaced by the polymerase activity.

To delete the poly GC and AT tails at the ends of the VSV sequence of p011-19, we took advantage of the T4 DNA polymerase exonuclease function. The p011 fragment containing the 5′ terminal sequences of the VSV genome was cut out of the M13mp19 based plasmid p011-19 by Pst I digestion, isolated from a 5% polyacrylamide gel, and treated with the 5 units of enzyme per microgram of DNA for 30 seconds. This initial reaction was performed in the absence of any nucleotides so that the exonuclease activity could eliminate the nucleotides of the Pst I site. The reaction was performed in 50 ul final volume, with approximately 1 ug of purified fragment, 5 units of enzyme, and the manufacturer recommended buffer (67 mM Tris-HCl, pH 8.8, 16.6 mM ammonium sulphate, 7 mM magnesium chloride, 7 uM EDTA, 10 mM 2-mercaptoethanol [BDH]). Deoxythymidine triphosphate (dGTP) and deoxyguanosine

triphosphate (dGTP), to a final concentration of 0.5 mM were then added, and the reaction was incubated at 37 degrees Celcius for 15 more minutes. In the absence of deoxyadenosine triphosphate (dATP) and deoxycytosine triphosphate (dCTP), the exonuclease activity removed the poly C and poly A tails of the top strand. But because of the presence of dTTP and dGTP the polymerase function prevented the exonuclease activity from deleting any of the VSV sequences.

After this treatment the DNA was cloned into the filled in Xba I site of pUC 18. The Xba I site was regenerated if the insert terminated with a T residue. Thus transformants were screened by Xba I digestion; those that possessed an Xba I site were further analyzed by sequencing.

Site-Directed Mutagenesis

Site directed mutagenesis (Zoller and Smith, 1982) was used to produce the plasmid p45. The strategy was to eliminate non-VSV sequences that intervened between the SP6 promoter start site and the VSV sequence, including the initiating G residue of the promoter. The Sph I to Bam HI fragment of plasmid p60 (which contained the SP6 promoter linked to the 3′ genomic sequences of VSV, with intervening polylinker sequences and a tract of G residues) was cloned into the vector M13 mp19. The single strand DNA of the clone

was purified from the phage released into the supernatant of the culture and used in the mutagenesis protocol. The protocol (Zoller and Smith, 1982) involves 1) the annealing of a primer spanning the deletion site to the single stranded circular DNA, 2) the synthesis of a complementary strand by the Klenow fragment of E. coli DNA polymerase, 3) the ligation of the 3′ terminus of the newly synthesized strand to the 5′ terminus of the linker, 4) the transfection of the resulting double-stranded plasmid into competent JM 107 cells, 5) the screening of the recombinants, which in this case could be identified by a missing Hind III site, and 6) the sequencing of several of the Hind III negative recombinants to confirm the presence of the appropriate deletion. In our hands approximately one in forty of the transformants (turbid plaques) had the appropriate deletion.

In Vitro Protein Synthesis

In vitro synthesis of VSV proteins was performed using VSV mRNA programmed reticulocyte lysates. The reticulocyte lysates, buffers, and salts used in the translation system were obtained from BRL (Bethesda Research Laboratories) (catalogue number 8111 SA/SB). We provided 35S-labelled methionine (Amersham, 10 uCi/ul, 1000 Ci/mmole), and VSV mRNA was obtained by multiple extractions of the cytoplasmic

fraction of VSV-infected mouse L cells with phenol). A typical reaction contained the following ingredients: 1.3 ul potassium acetate (2 M, pH 7.2), 10 uCi 35S-labelled methionine, 3 ul reaction mixture (containing 240 mM Hepes buffer, an energy regenerating system consisting of phosphocreatine kinase and phosphocreatine, 500 uM each of 19 amino acids (minus methionine), 400 mM KCl), 10 ul of rabbit reticulocyte lysate (prepared by lysing reticulocytes of aneamic rabbits, and treated with micrococcal nuclease), approximately 0.5 ug to 1.0 ug hybrid-selected VSV mRNA, water to make a final volume of 30 ul. The contents were mixed gently and incubated at 30 degrees Celcius for 60 minutes. The translation system had an endogenous level of 1 mM magnesium chloride.

Determination of Isotope Incorporation in Translation Systems

After the 60 minute incubation, a 1 to 5 ul sample of the translation mixture was transferred to an 1.5 ml Eppendorf tube and treated with 100 ug/ml pancreatic RNAse (Sigma) for 15 minutes at 30 degrees Celcius. The aliquot was diluted to 50 ul with water, one hundred micrograms of carrier tRNA (Sigma) was added, and 1 ml of ice cold 10% tricholoroacetic acid (TCA, from J. T. Baker Chemical Company) was added. The sample was left on ice for 30 minutes. The precipitate was

collected by filtering through a glass fiber filter (BDH). The filter was washed ten times with 5 ml of ice-cold 10% TCA, followed by 5 ml of 95 % ethanol. The filter was placed into a scintillation vial and then dried in a 100 degree C oven for thirty minutes to one hour. Ten ml of a toluene-based scintillation fluid (made by combining 1 L of toluene (Fischer Scientific) and 5 grams of Permablend 11 [obtained from Packard Corporation] was transferred to the vial and the counts were determined in a Beckman scintillation counter.

Polyacrylamide Gel Analysis of In Vitro Synthesized Proteins

Proteins synthesized by the in vitro translation system were analyzed by disodium dodecyl sulphate polyacrylamide gel electrophoresis (Laemmli, 1970). The gel consisted of 12% acrylamide, 0.32% bisacrylamide (Bio-Rad), 0.1% SDS (Bio-Rad), 0.375 M Tris-HCl pH 8.8 (Bio-Rad), 0.1% ammonium persulphate (BDH), and 50 ul N,N,N',N'-tetramethylethylenediamine (TEMED, Bio-Rad). The gels were run at 5 V/cm for 12 hours with a reservoir buffer made of 25 mM Tris-HCl and 250 mM glycine (Bio-Rad), pH 8.3, and 0.1% SDS. The sample buffer consisted of 60 mM Tris-HCl (pH 6.8), 5% w/v SDS, 1% w/v DTT (Boehringer Mannheim), 20% glycerol (Fisher Scientific), 0.001% bromophenol blue (Sigma). Samples were processed by taking an aliquot of the translation

mix (no more than ten ul), adding twice the volume of sample buffer, and heating at 100 degrees Celcius for three minutes to denature the proteins. After the electrophoresis, the gel was dried onto Whatman filter paper and the proteins were identified by autoradiography (using Kodak X-Ray film in contact with the gel in a film cassette at -70 degrees Celcius).

In Vitro Transcription Reactions

The protocols for transcription were slightly modified from those provided by Promega Biotechnology Corporation, the supplier of SP6 polymerase. The transcription system consisted of 1X final concentration of the transcription buffer from a stock 5X transcription buffer (200 mM Tris-HCl, pH 7.5), 30 mM magnesium chloride, 10 mM spermidine, 50 mM NaCl), rNTPs at a final concentration of 0.5 mM from 10 mM stocks, 10 mM final concentration DTT from 100 mM stock, 1 unit/ul final concentration of placental ribonuclease inhibitor from a stock of 25 units/ul (Promega Biotechnology Corporation). For the synthesis of labelled RNA we used the following conditions: 1 ug linearized DNA (for p45 plasmid and its derivatives up to 5 ug were used), 2 ul 5X transcription buffer, 1 ul 100 mM DTT, 0.5 ul placental RNAse inhibitor, 2 ul 2.5 mM each of ATP, GTP, CTP, 2.4 ul 100 uM UTP (optional,

used early on in the work, but later it was found dispensible, since predominantly full length RNA could be obtained from Bam HI linearized plasmids in the absence of the cold UTP), 10 to 20 uCi alpha labelled 32P UTP (3000 Ci/mmole from NEN), 5 to 10 units of SP6 polymerase (when using p45 plasmids and derivatives the amount of polymerase was usually doubled). The reaction was incubated at 40 degrees Celcius for 60 minutes. An aliquot of the reactions was then combined with an equal volume of 95% formamide, 0.1 % bromophenol blue and 0.1% xylene cyanol , denatured by heating at 65 degrees Celcius for 5 minutes, and run on denaturing 6% or 8% acrylamide gels.

For high level synthesis of cold RNA (for analysis of RNA initiation by primer extension) the reaction was scaled up to 50 ul, all four cold ribonucleotides were included, and the amount of all constituents was increased fivefold.

Preparation of Transcripts for Mobility Shift Analysis

For mobility shift analysis, internally labelled or end-labelled transcripts were incubated with infected or uninfected cell extracts and subsequently run on mobility shift acrylamide gels. Transcripts that were to be kinased were synthesized in 20 ul volumes with 20 units of SP6 polymerase, 2 ug DNA (5 ug for p45), and 0.5 mM rNTPs, 1X SP6

buffer. After incubation at 40 degrees Celcius for one hour, the reaction was treated with DNAse, and the transcripts were dephosphorylated by treating with 10 units of calf intestinal phosphatase (BMC) in a 20 ul reaction buffered with 50 mM Tris-Cl, pH 8.0. The phosphatase was inactivated by multiple phenol-chloroform extractions in the presence of 0.1% SDS and 10 mM EGTA, and the RNA was purified by ethanol precipitation and/or passage through a G50 (Pharmacia) spun column. The RNA was then kinased in 10 ul reactions with 5 units of kinase, 10 to 20 uCi of gamma 32P ATP, and 1X kinase buffer.

When the RNA product was to be used for binding studies to viral proteins or for transcription initiation studies by primer extension, the samples were treated with DNAse (RNAse-free, 1 unit/ul, from Promega Biotechnology Corporation) at a concentration of 1 unit/ug DNA for 30 minutes at 37 degrees Celcius. The sample was then extracted twice with phenol, extracted twice with chloroform, and the RNA was recovered by precipitation with ethanol. Precipitations with ethanol were performed by adding one tenth volume of 3 M sodium acetate, adding 2.5 volume cold ethanol, mixing vigorously, incubating at -70 degrees Celcius for one hour, and spinning in a microfuge at 4 degrees Celcius for at least thirty minutes. The pellets were then resuspended in 10 ul of water.

Primer Extension Analysis of Transcripts

One half the amount of RNA obtained from a large scale transcription reaction was used (in the case of p45 the whole amount was used) to study initiation by primer extension. The primer SAM 79 (all primers used were synthesized by the Central Facility of the Molecular Biology Institute of McMaster) was kinased with 32P according to the same protocol outlined in the sequencing methods. Two nanograms were used to hybridize to the RNA in a final volume of 10 ul, in reverse transcriptase buffer (50 mM Tris-HCl pH 8.3, 100 mM KCl, 10 mM magnesium chloride, 10 mM DTT). Hybridization was achieved by heating the mixture to 65 degrees Celcius, and leaving it to slowly cool to 37 degrees Celcius. All four deoxynucleotides were then added to a final concentration of 0.5 mM, along with 1 ul placental RNAse inhibitor (25 units/ul stock), and 1 unit of AMV reverse transcriptase (BRL 1 unit/ul). The reaction was incubated at 37 degrees Celcius for one hour, extracted with phenol, precipitated with ethanol, and analyzed on a sequencing gel, with a DNA sequence as the marker.

Infected Cell Extracts

To study the association of viral proteins with in vitro synthesized RNA transcripts we used either VSV mRNA programmed reticulocyte lysates or infected cell extracts as the source of protein. We made the infected cell extracts according to a procedure originally devised by Miller (Miller et al., 1978) and later refined by Moyer (Peluso and Moyer, 1983). Like Moyer, we used monolayer cultures of baby hamster kidney cells and the HR11 strain of VSV (Indiana). The cells were grown in monolayers at 37 degrees Celcius in MEM F11 medium (Gibco) supplemented with 10% fetal calf serum (Gibco). Subconfluent (approximately ten million cells in a 100 mm dish) monolayers of BHK cells, split one in three the night before the experiment, were infected the next day with 20 to 50 plaque-forming units of VSV per cell. After 4.5 hours of infection, the cells were washed with PBS (Phosphate buffered salt solution), and all excess liquid was drained. The cells were treated with L-alpha-lysophosphatidylcholine, palmitoyl (lysolecithin) (Sigma), at 250 ug/ml for 1 minute at 4 degrees Celcius. The lysoclecithin solution (2.5 ml was used) was aspirated and the cells were washed with solution A. The solution consisted of 0.1 M HEPES adjusted to pH 7.4 with KOH, 0.2M ammonium chloride, 7 mM KCl, 4.5 mM magnesium acetate, 1 mM DTT. The cells from five dishes were then scraped into

600 ul of this solution and were disrupted by pipetting with a drawn out Pasteur pipette. The mixture was then centrifuged at 800g for 5 minutes to remove nuclei and cellular debris.

Encapsidation Attempts in Infected Cell Extracts

The cell-free supernatant fluid was incubated with heat denatured (heated for 5 minutes at 65 degrees Celcius, then chilled on ice) labelled RNA (about 100,000 counts) for one hour at 30 degrees Celcius. After this incubation, the extracts were treated with RNAse (either micrococcal nuclease [Pharmacia] or pancreatic RNAse [Sigma] at a final concentration of 100 ug/ml) for thirty minutes at 37 degrees Celcius. The samples were extracted twice with phenol in the presence of 0.1% SDS, 10 mM 2-mercaptoethanol, and 10 mM EGTA (if micrococcal nuclease had been used). After two chloroform extractions, the samples were made 300 mM in sodium acetate, and 10 ug of carrier tRNA was added. Any "protected" RNA was collected by precipitation with ethanol. After washing with cold 70% ethanol, the sample was dried, and redissolved in a minimal volume of water (about 5 to 10 ul). The samples were then analyzed by TCA precipitation and denaturing sequencing gels.

Encapsidation Attempts in Reticulocyte Lysates

Initially we attempted to synthesize RNA by SP6 polymerase in the reticulocyte lysates while VSV protein synthesis of the VSV mRNA-programmed lysates was in progress. Thus both nascent RNA and nascent VSV proteins would be available under such conditions. Later, we also tried to add presynthesized labelled RNA to pre-initiated lysates (30 minutes after VSV protein synthesis was initiated). In both cases after a further incubation of 60 minutes the samples were treated in the same way as described for the cell extracts (except that mobility shift analysis was not performed).

The coupled transcription-translation systems were performed in a final volume of 40 ul. The translation system was set up as suggested by the company literature (BRL), and protein synthesis was allowed to proceed for 15 minutes at 30 degrees Celcius, with no added magnesium, and no added potassium. At this point 15 units of SP6 polymerase, magnesium chloride to a final concentration of 3 mM, 5 ug linearized plasmid DNA, 20 uCi 32P UTP, and ATP, CTP, GTP to a final concentration of 0.5 mM were added. After a one hour incubation at 37 degrees Celcius, 35 ul of the reaction, to be treated with RNAse, was aliquoted to one tube, and the other 5 ul was aliquoted to another. The volume of each tube

was raised to 90 ul water, and 10 ul of 10 X micrococcal nuclease buffer (50 mM calcium chloride, 100 mM Tris-Glycine, pH 9.0) was added. Micrococcal nuclease (100 units) was added to the tube containing 35 ul of the original reaction. Both samples (+ and - RNAse) were incubated for one hour at 30 degrees Celcius, at which point EGTA was added to a final concentration of 10 mM. SDS was added to a final concentration of 0.1%, the samples were extracted twice with phenol-choloform, NaAc (pH 4.8) was added to a final concentration of 0.3 M, and the RNA was precipitated with ethanol. After two washes with 70% ethanol, the RNA was dried and redissolved in 5 ul of water. Before electrophoresis, 5 ul formamide was added, and the mixture was heated at 65 degrees Celcius for five minutes to denature the RNA. Five ul was applied to the sequencing gel.

Mobility Shift Analysis

An alternative method by which we tried to detect RNA was a modification of the method used for DNA-protein associations called mobility shift analysis (Singh et al., 1987). The technique relies on the retarded mobility of DNA-protein complexes or RNA-protein complexes during acrylamide gel electrophoresis relative to that of the unbound nucleic acid. In this procedure, an aliquot of the infected

cell extract incubated with labelled RNA and subsequently electrophoresed in a low ionic strength 4% polyacrylamide gel (acrylamide-bisacrylamide ratio 30:1). The buffer in the gel and the running buffer consisted of 1 mM EDTA, 3.3 mM sodium acetate, and 6.7 mM Tris-HCl, pH 7.5. Different values of pH of the buffer (pH 5.5, 6.5, 7.5) were tried.

Results

Overview of Experiments

The objective of these studies was twofold: 1. to prepare an *in vitro* system in which the sequence requirements of encapsidation might be defined in the absence of viral replication; 2. to construct a set of plasmids whose SP6 run off transcripts resemble the genomes of defective vesicular stomatitis virus.

The model system used to study encapsidation *in vitro* was the assembly of *in vitro* synthesized leader sequences by soluble N protein into RNase resistant nucleocapsids. The RNA was synthesized *in vitro* from pSP64-based plasmids, whereas viral proteins were obtained from VSV mRNA programmed reticulocyte lysates or from infected cell extracts. Several protocols were attempted : 1. cosynthesis of RNA and viral protein in VSV mRNA programmed reticulocyte lysates; 2. synthesis of RNA in infected cell extracts; 3. addition of presynthesized RNA to viral protein synthesizing lysates. Plasmids were made to analyze the sequence and length requirements, and directionality of encapsidation.

We initially constructed a pSP64 based plasmid with the 3′ genomic viral sequences under control of the SP6 promoter (p1). This plasmid was further modified so that transcription by the SP6 polymerase would produce a transcript containing only viral sequences (p45). Other plasmids that have deletions (p15) or additions (p60, psPL6) at the 5′ terminus of the VSV sequence were made in the process. The 5′ termini of the transcripts synthesized from these plasmids were analyzed by primer extension. Subsequently, deletion mutagenesis of p45 produced a set of nested deletions in the 3′ sequence of the leader transcript.

VSV mRNA programmed reticulocyte lysates and infected cell extracts were tested for their ability to support the encapsidation of input leader sequences. We also collaborated with Sue Moyer to test encapsidation by purified N protein.

In the second part of this project plasmids with sequences from both viral termini placed downstream of the SP6 promoter were made. These plasmids direct the synthesis of RNAs that are analagous to defective interfering genomes.

Construction of Plasmids with the 3′ Genomic Sequence

Construction of p1

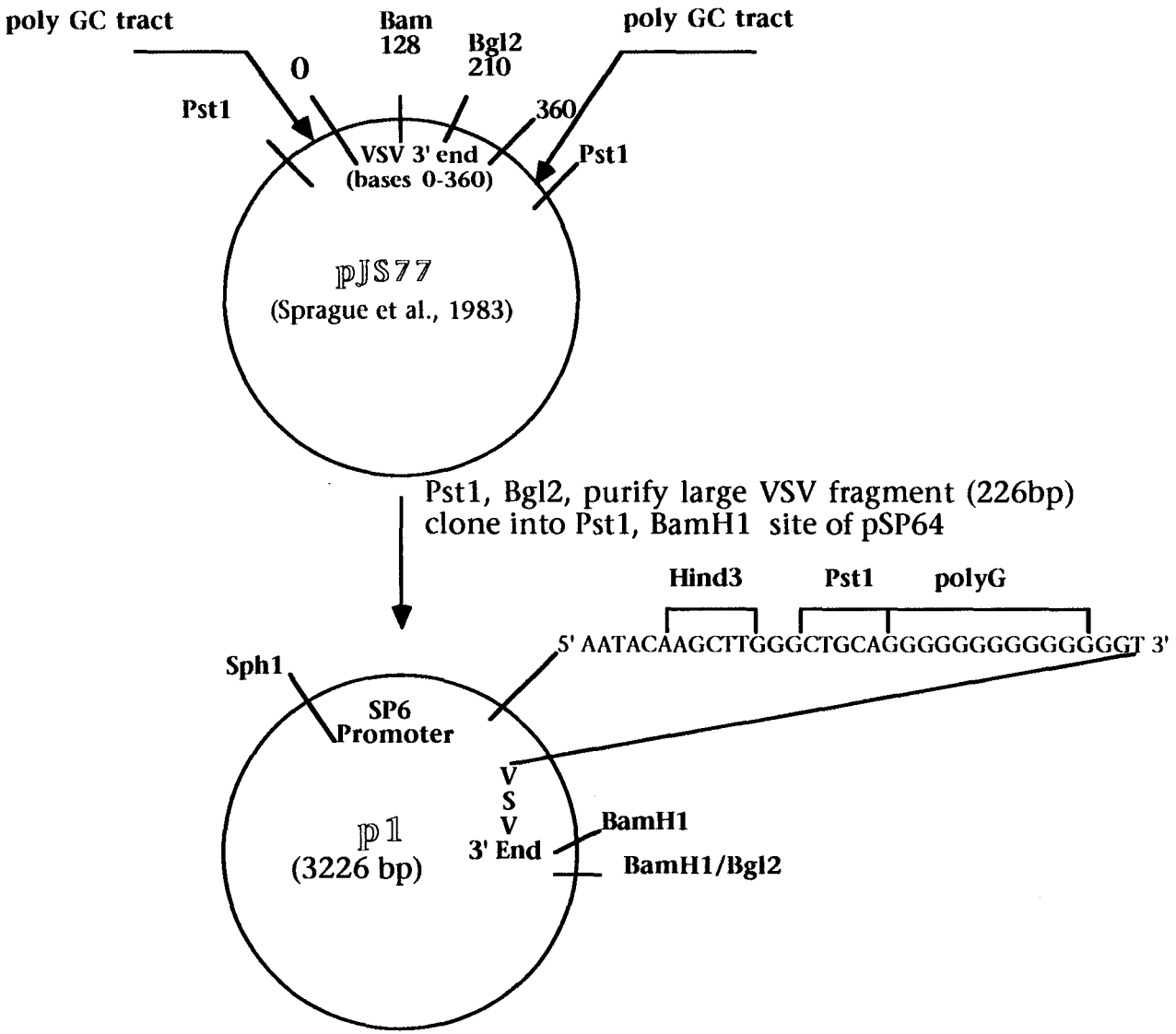
The construction of p1 is outlined in Figure 1. The plasmid PJS77 (Sprague et al., 1983), a gift from Dr. Lazarrini, contains a cDNA sequence corresponding to the 3′ end of VSV (approximately 270 bases) cloned into the Pst I site of pBR322. The Pst I to Bgl II fragment containing the the 210 3′ terminal sequences of the virus (the leader sequence and a portion of the 5′ terminus of the N gene) was inserted downstream of the SP6 promoter in the plasmid pSP64.

The plasmid, p1, contains the leader sequence and a portion of the N gene sequence positioned downstream of the SP6 promoter. However there are intervening sequences between the promoter and the leader: the polylinker sequence and a poly GC tract at the 5′ end of the leader (produced by the

Figure 1. Construction of p1

To assemble p1, the 226 base pair Pst I to Bgl II fragment of pJS77 (Sprague et al., 1983) was placed downstream of the SP6 promoter of pSP64 (Promega Biotec). The Pst I to Bgl II fragment, containing the leader gene and the 5′ terminus of the N gene, was purified from an agarose gel and ligated into the pSP64 vector that had been cut with Pst I and Bgl II. The sequences that intervened between the transcription start site of the SP6 promoter and the VSV sequence are indicated in the figure. These intervening sequences included part of the polylinker sequence and a poly GC tract.

Construction of p1



initial cDNA cloning of the viral sequences). DNA sequencing of p1 revealed that there are 35 nucleotides from the SP6 initiation site to the first nucleotide of the leader; 20 polylinker residues, 14 G residues, and one T residue.

Our strategy was to trim as much of the flanking 5′ sequences as possible without destroying the SP6 promoter. In the process, the plasmids p60, p15, and psPL6 were obtained. The plasmid p60 was further altered by site directed mutagenesis to create a plasmid, p45, in which the SP6 polymerase initiates at the first base (A) of the leader sequence.

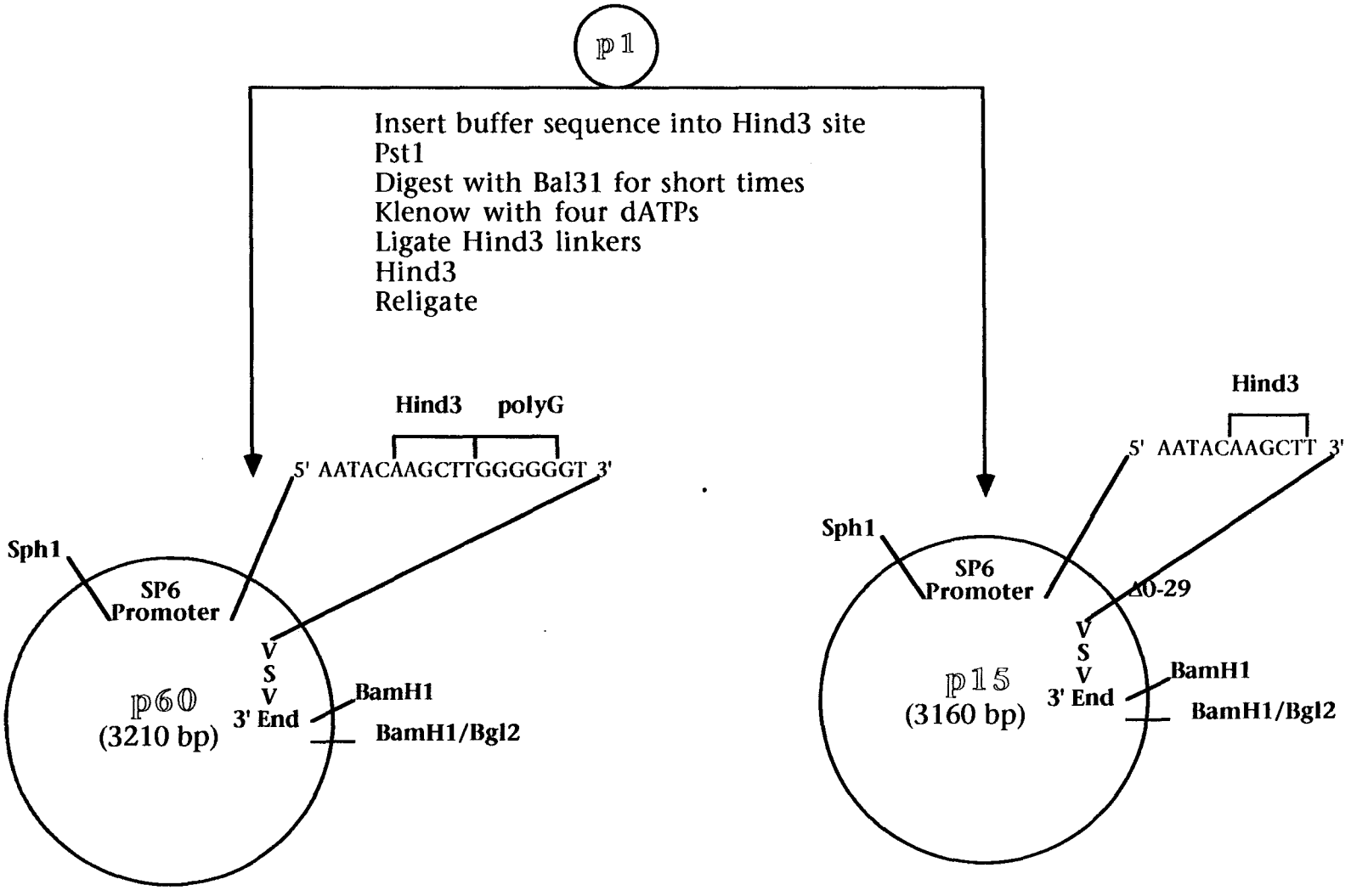
Construction of p60, p15, psPL6, psPL8, psPL18

The construction of p60 and p15 is outlined in Figure 2. Exonuclease treatment from the Pst I site was used to eliminate the poly GC tract in p1. However, to protect the promoter we ligated in a 700 bp pBR322 Hind III fragment into the Hind III site of p1. The plasmid thus produced, p3, was subsequently linearized at the Pst I site and treated with Bal 31 or T4 DNA polymerase in the absence of deoxynucleotides. Following exonuclease treatment, the ends of the plasmid were made flush by treating with the Klenow fragment in the

Figure 2. Construction of p60 and p15

The plasmids p60 and p15 were produced from p1 by exonuclease digestion from the Pst I site. To protect the SP6 promoter during the exonuclease treatment we first inserted a buffer sequence into the Hind III site of p1. The resultant construct was linearized with Pst I, and digested with Bal 31 for short times. After treatment with the Klenow polymerase to repair the ends of the plasmid, the plasmid was religated in the presence of Hind III linkers. The sequence intervening between the initiating G residue of the promoter and the VSV sequence in the plasmids is indicated in the figure. The viral sequence of p60 is intact, whereas the viral sequence of p15 lacks the first 29 bases from the 5′ terminus of the leader gene.

Construction of p60 and p15



presence of all four nucleotides, and religation was performed in the presence of Hind III linkers. After this ligation step, the mixture was treated with Hind III and religated. The final ligation mix was treated with Pst I to eliminate background.

Transformants were initially screened for the presence of a Hind III site. Further screening involved the estimation of the length of the Hind III to Bam HI fragment by acrylamide gel electrophoresis. Two plasmids were deemed potentially useful; p60 because it apparently deleted about 12 bases from the GC tract, and p15 because it had a deletion of about 30 bases of the leader.

As presented in Figure 3, the plasmid p60 was further trimmed by exonuclease treatment from the almost symmetrically placed Hind III site. Size analysis of the Sph I to Bam HI fragment on polyacrylamide gels was used to identify potentially useful transformants. Four plasmids with Sph I-Bam H I fragments of about 500 bases were sequenced. Only one, psPL6 (see sequence gel in Figure 4), was transcriptionally active. The others (psPL8, psPL18, psPL8) had sequence alterations in the SP6 promoter sequence that inactivated the promoter.

Figure 3. Construction of psPL6, psPL8, psPL18, and p45

The plasmid psPL6 was created from p60 by exonuclease treatment from the Hind III site. The plasmid p60 was linearized with Hind III, and treated with Bal 31 exonuclease for short periods of time. After treatment with the Klenow polymerase to repair the ends, the plasmid was religated. Three interesting deletions were identified by sequencing: psPL6, psPL8, and psPL18.

The plasmid p45 was made by site directed mutagenesis of the small (582 bp) Sph I to Bam HI fragment of p60 in M13mp19. After the desired deletion was confirmed by sequencing, the Hind III to Bam HI fragment was cloned into the Hind III and Bam HI sites of the vector pUC8.

Construction of psPL6 and p45

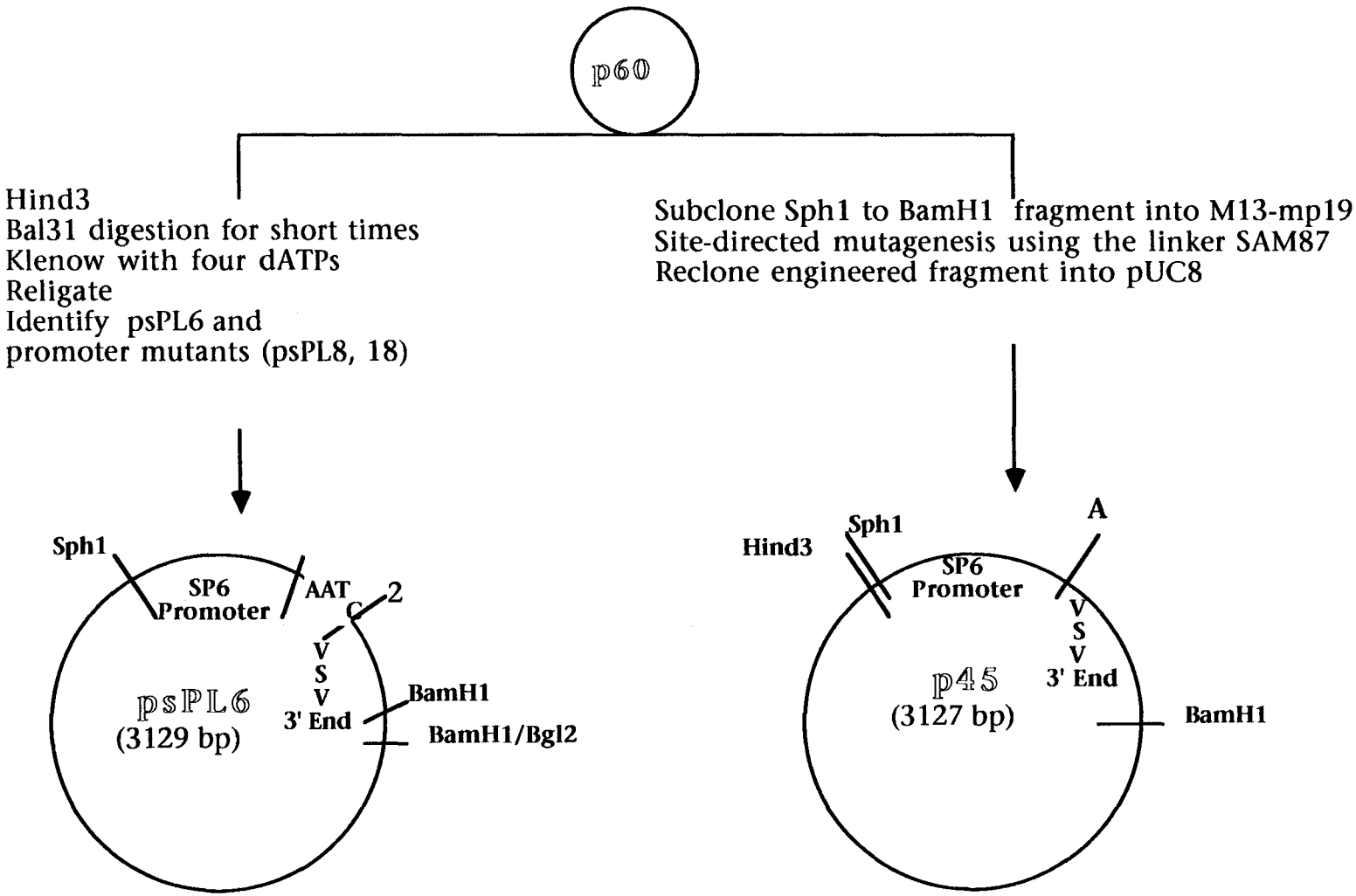
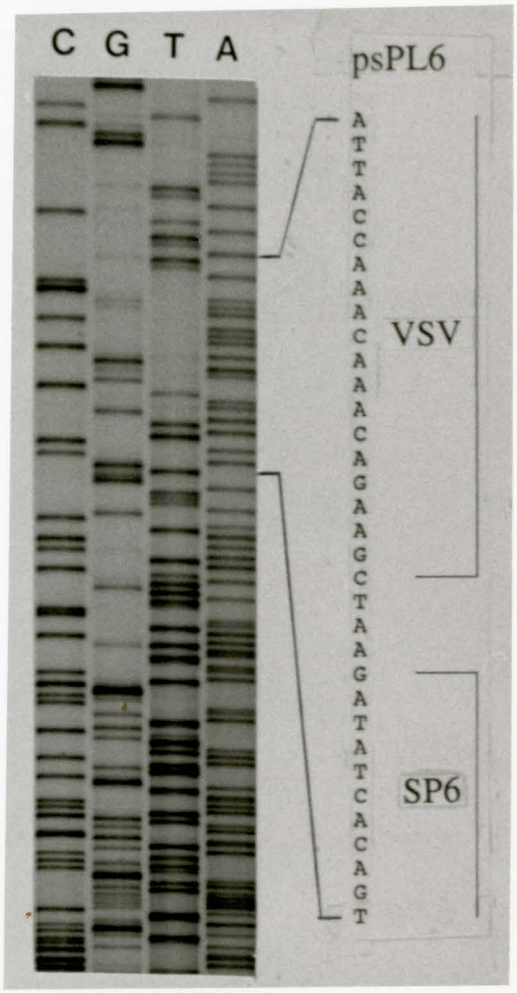


Figure 4. Sequence of psPL6

The sequence lanes are indicated on the figure. Sequencing was performed on double stranded DNA with the end labeled primer AB79. This primer is 17 bases long and hybridizes to the VSV sequence at position 79 to position 53, counting from the 3 ' terminus of the virus.


C G T A
psPL6
ATTACCAAACAAACAGAAGCTAAGATATCACAGT
VSV
SP6

Construction of p45

The plasmid p45 makes possible the *in vitro* synthesis of an RNA molecule identical to the viral positive sense leader transcript. The residues between base 0 of the SP6 promoter and base 1 of the VSV sequence in p60 were deleted by site directed oligonucleotide mutagenesis (Zoller and Smith, 1982). The oligonucleotide used, 5′ GGT GAC ACT ATA ACG AAG ACA AAC 3′, had 12 nucleotides of the SP6 promoter and 12 nucleotides of the VSV leader sequence.

The sequence at the junction of the SP6 promoter and the VSV sequence of p45 is presented in Figure 5. The sequences present downstream of the SP6 promoter in the plasmids p1, p60, p15, psPL6, psPL18, and p45 are listed in Table 1.

Figure 5. The SP6 Promoter-Leader Junction of p45

The sequence of the SP6 promoter-leader junction was obtained using a primer that hybridzes to bases 63 to 79 of the 5′ terminus viral sequence. The sequence of the junction, starting with base 20 in the viral sequence, is:

```
3′ ATTACCAAACAACCAGAAGCA  ATATCACAGTGGATT ... 5′
        base 1 of VSV |  SP6 promoter
```

The orientation of the sequence is the inverse of that on the gel because the complement of the sequence lanes is actually labelled on the gel.

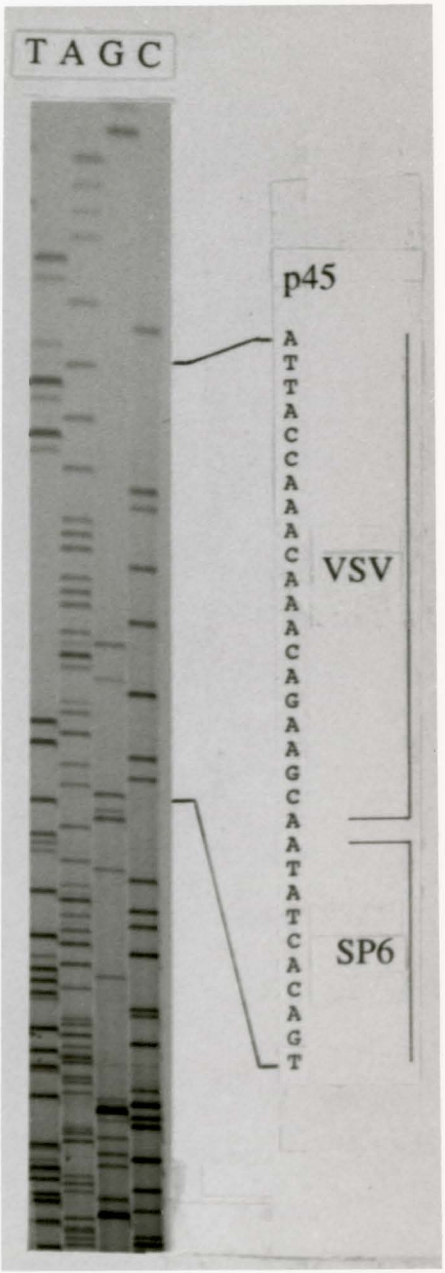

T A G C
p45
ATTACCAAACAAACAGAAGCAATATCACAGT
VSV
SP6

Table Legend

The designation SP6 refers to the portion of the promoter sequence upstream of the G residue at which transcription is initiatited. The VSV sequence is underlined. Deletions in the VSV sequence are described beneath the sequence. The SP6 promoter sequences, intervening sequences, and VSV sequences are separated by a double space.

Table 1. DNA Sequence of p1, p60, p15, psPL6, p45

p1

5′ SP6 AATACAAGCTTGGGCTGCAGGGGGGGGGGGGGT

ACGAAGACAAACAAACCA...3′

p60

5′ SP6 AATACAAGCTTGGGGGGT ACGAAGACAAACAAACCA ... 3′

p15

5′ SP6 AATACAAGCTT AAAAGGCTCAGGAGAAA ... 3′
VSV starting at base 29 of the leader

psPL6

5′ SP6 AAT CGAAGACAAACAAACCA ... 3′
VSV starting at base 2 of the leader

(see Figure 1)

psPL8

5′ ...TTTAGGTGACACTA CAACATCATA ... 3′
SP6 promoter minus VSV starting at base 115
5′ TAG 3′at -1 to +1

psPL18

5′ ...TTTAGGTGACACTA GCAAGCTTGCGGAT ACGAAGACAAACAAACCA ..3′
SP6 promoter minus
5′ TAG 3′at -1 to +1

p45

5′ ...TTTAGGTGACACTATA ACGAAGACAAACAAACCA ... 3′
SP6 promoter minus G (+1)

Transcription of the Plasmids

The time course of transcription from psPL6 and p45 is presented in Table 2. Table 2 shows that the replacement of the G residue at position +1 of the SP6 promoter by an A residue in p45 disrupts promoter function.

The dependence of transcription on template concentration was also investigated. Table 3 presents the effect of plasmid concentration on level of transcription of psPL6, and Table 4 presents the effect of plasmid concentration on the transcription of p45.
Transcription of linearized p45 increased with increasing plasmid concentrations (up to 0.5ug/ul), whereas no increase in transcription was observed for linearized psPL6 at template concentrations greater than 0.1ug/ul. High plasmid concentrations were subsequently used to increase the yield of p45 transcripts.

Table 2. Time Course of Transcription in SP6 Buffer

Time (min)	CPM (psPL6)	CPM (p45)
0	2,200	1,900
30	50,000	7,600
60	68,500	9,300
90	75,900	9,200

Reactions were performed using 0.5 micrograms of each plasmid linearized with Bam HI. The reactions were in 1X SP6 buffer in a 10 microlitre volume, with 5 units of SP6 polymerase, and 20 microcuries of tritiated UTP. After incubation at 40 degree Celcius for the specified time, the TCA precipate was collected by filtration, and the radioactivity was determined in toluene based scintillation fluid. The above results represent the average of duplicate samples, processed in parallel.

Table 3. Effect of Plasmid Concentration on the Transcription of Bam HI Linearized and Supercoiled psPL6

Concentration (ug/10ul)	CPM (Bam HI)	CPM (uncut)
0.00	2,000	3,000
0.10	32,900	86,300
0.25	49,700	120,800
0.50	167,500	426,200
1.00	210,000	997,400
2.00	200,900	908,600

Reactions were performed in 1XSP6 buffer, with 5 units SP6 polymerase, and 20 microcuries of tritiated UTP. The above figures represent the average of duplicate samples, processed in parallel.

Table 4. Effect of Plasmid Concentration on the Transcription of Bam HI Cut and Supercoiled p45

Concentration (ug/10ul)	CPM (Bam HI)	CPM (uncut)
0.00	1,200	1,800
0.50	3,100	37,700
1.0	12,300	99,400
2.0	18,600	219,300
3.0	30,200	200,600
4.0	38,000	155,300
5.0	42,000	130,000

Reactions were performed in 1X transcription buffer, with 5 units of SP6 polymerase, and 20 microcuries of tritiated UTP. After incubation for one hour at 40 degrees Celius, the radioactivity of the TCA precipitates was determined. The average of duplicate samples, processed in parallel was calculated.

Transcript Mapping

Primer extension of the transcripts was used to determine the start site of transcription on the parent plasmids and to quantify initiation on linear and supercoiled plasmids. Figure 6 shows primer extension analysis on transcripts from linearized and supercoiled psPL6 (lanes 1 and 2) and p60 (lane 7 and 8).

The lanes containing cDNAs of transcripts from supercoiled plasmids (1 and 8) differ from lanes with cDNAs of run off transcripts only in band intensities. The sites of transcription initiation were not affected by supercoiling, though, as indicated by band densities on the gel, initiation occurs more frequently on supercoiled plasmids. Two initiation sites were observed for psPL6 and p60 . Initiation occurs at the G residue at position +1 in the SP6 promoter and at the A residue at position 0 of the promoter. For psPL6 the G residue is the preferred site of initiation, whereas for p60 initiation occurs more frequently at the A residue.

Figure 6. Transcript Mapping of psPL6 and p60

Lane 1. cDNA of transcripts from supercoiled psPL6

Lane 2. cDNA of transcripts from Bam HI cut psPL6

Lane 3. cDNA of transcripts from Bam HI p60

Lane 4. C lane of sequence of p1

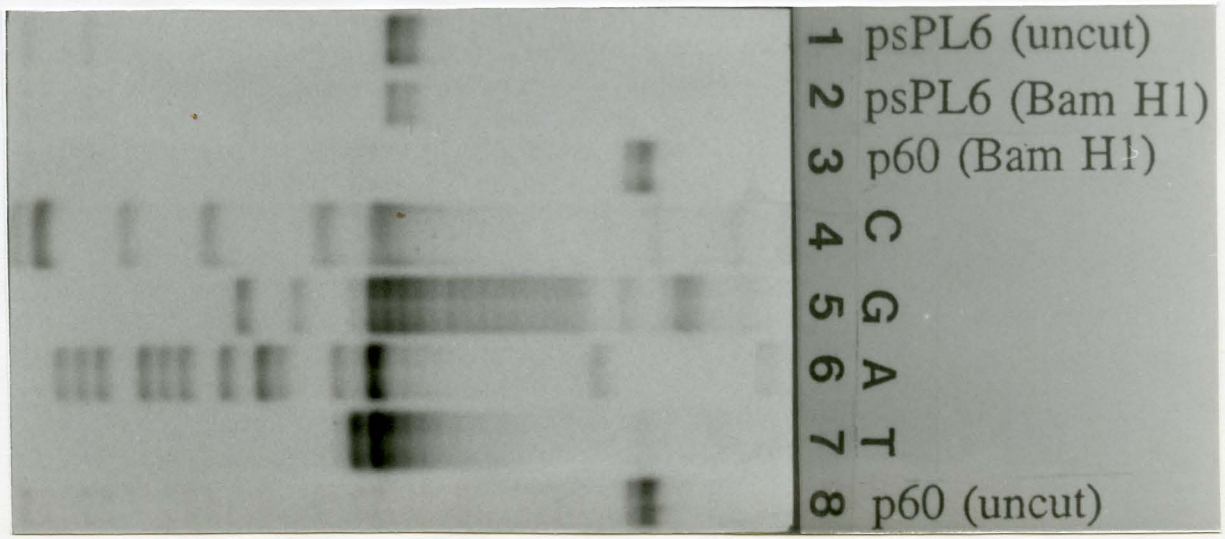
Lane 5. G lane of sequence of p1

Lane 6. A lane of sequence of p1

Lane 7. T lane of sequence of p1

Lane 8. cDNA of transcripts from supercoiled cut p60

The procedures used are described in the Materials and Methods section. Briefly, transcripts were synthesized, the DNA was removed, the RNA was purified, and cDNA synthesis was primed with the primer SAM 79. Only one third of the RNA synthesized from the supercoiled plasmids was used for cDNA synthesis, whereas all the RNA synthesized from the linearized plasmids was used.


1 psPL6 (uncut)
2 psPL6 (Bam H1)
3 p60 (Bam H1)
4 C
5 G
6 A
7 T
8 p60 (uncut)

Primer extension was also performed on transcripts from p45. Figure 7 (lane 1) shows that there are three sites of transcription initation on p45. The most frequently chosen site is the first A residue of the VSV sequence, which replaced the G present at position +1 of the intact SP6 promoter. Initiation was also observed, albeit at a lesser extent, at the adjacent upstream A residue at position 0 of the promoter. The least frequently used site of initiation is the G residue at the third position of the VSV sequence.

Transcription in Reticulocyte Lysates and Cell Extracts

Transcription in Reticulocyte Lysates

Wertz (Wertz, 1984) showed that reticulocyte lysates programmed with VSV mRNA were a good source of soluble N protein for the replication and encapsidation of VSV genomes. We wanted to test for the encapsidation of transcripts synthesized by the SP6 polymerase in reticulocyte lysates programmed with VSV mRNA. The effect of the components of the translation system on the SP6 polymerase was examined.

Figure 7. Transcript Mapping of p45

Lane 1. cDNA of transcript from Bam HI cut p45

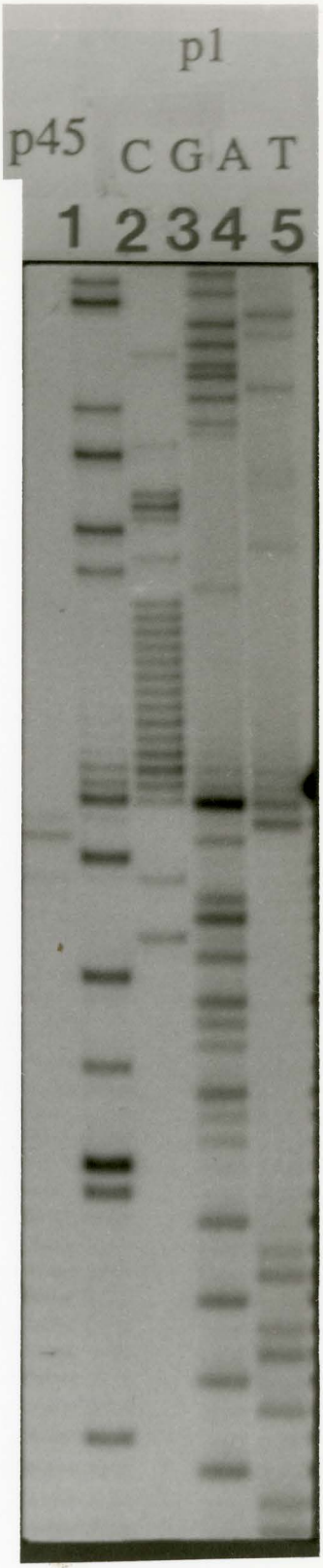
Lane 2. T lane of p1 sequence

Lane 3. A lane of p1 sequence

Lane 4. G lane of p1 sequence

Lane 5. C lane of p1 sequence

Transcript mapping and sequencing using the primer SAM 79 were performed as described in the Materials and Methods section.


p1
p45
C G A T
1 2 3 4 5

The effect of magnesium ion concentration on transcription is displayed in Table 5 and its effect on the translation of VSV mRNA is displayed in Table 6. Transcription and translation are differentially affected by magnesium ion concentration. Whereas transcription efficiency was optimal at a concentration of 6 mM magnesium, translation efficiency was optimal with no added magnesium (1 mM final concentration).

The effect of magnesium ion concentration on the translation of Indiana mRNA in reticulocyte lysates was confirmed by SDS polyacrylamide gel electrophoresis (Laemmli, 1970). Figure 8 shows that raising the concentration of magnesium ions in the lysates by 2 mM (lane 4) significantly reduced translation of all VSV mRNAs.

The endogenous concentration of potassium chloride in the translation system is 125 mM. Table 7 examines the effect of potassium chloride levels on the SP6 polymerase. At a concentration of 100 mM, potassium chloride strongly inhibited the transcription of p45, both supercoiled and linear. However, little effect was observed on the

Table 5. Effect of Magnesium Chloride Concentration on the Transcription of Bam HI linearized p60

Magnesium (in mM)	CPM
0	1,600
3	11,200
4	15,800
6	27,400

Approximately 0.1 ug of linearized p60 was used in the standard transcription assay using tritiated GTP. One half the reaction was precipitated with TCA and counted with toluene. The counts represent an average of duplicate samples, processed in parallel. The magnesium ion concentrations represent the final concentration in the test tube.

Table 6. Effect of Magnesium Ion Levels on the Translation of VSV mRNA

Magnesium (in mM)	CPM (35S)
0	53,900
1	39,300
2	17,800
4	6,400
Background	4,000

The reactions were performed in a final volume of 10 microlitres using standard conditions. Methionine was not included in the mix and 1 microlitre was used for TCA precipitation. DTT at 10mM reduced translation to background levels, as did 100 mM ammonium chloride (data not shown). The magnesium ion concentrations refer to added magnesium. The endogenous level of magnesium is 1 mM. The sample used to determine the background had no added VSV mRNA. Samples were processed and counted in duplicate to obtain an average count.

Figure 8. Effect of Magnesium Chloride Concentration on the Translation of VSV (Indiana) mRNAs by Reticulocyte Lysates

Lane 1. Translation of Indiana mRNA, no exogenous magnesium

Lane 2. Translation of Piry mRNA, no exogenous magnesium

Lane 3. Translation of Indiana mRNA, 1 mM exogenous magnesium

Lane 4. Translation of Indiana mRNA, 2 mM exogenous magnesium

Translations were performed as in the Materials and Methods. Approximately the same amount of VSV RNA was used in each reaction. To one half the reaction (15 ul) an equal volume of sample buffer was added and the mixture was incubated in boiling water for 5 minutes. The mixture was loaded on a 12% SDS polyacrylamide gel and run at 5 V/cm until the bromophenol dye reached the bottom.


1 Indiana
2 Piry
3 Indiana + 1 mM Mg
4 Indiana + 2 mM Mg

Table 7. Effect of KCl on Transcription of Supercoiled and Linear Plasmids

Supercoiled Plasmid (1.0 ug)	CPM (0 mM KCl)	CPM (100 mM KCl)
p1	838,000	739,000
p45	127,000	6,000
Bam HI Cut Plasmid (2.0 ug)		
p1	200,000	156,000
p45	22,000	4,000

Reactions were performed in 1X transcription buffer, with five units of SP6 buffer, and 10 uCi of 32P GTP (alpha labelled). The results represent the average of duplicate samples, which were processed in parallel.

transcription of plasmids with a complete SP6 promoter sequence. Initiation at the SP6 promoter of p45 was extremely sensitive to disruption, both by potassium ions and by ammonium ions, as will be shown later.

Since the reticulocyte lysate translation system consists of two components, the reticulocyte buffer, and the reticulocyte lysate, we determined the effect of each on transcription by SP6 polymerase. The results are presented in Table 8.

Both the reticulocyte lysate and the "reticulocyte buffer" strongly inhibited the SP6 polymerase, even when supplemented with magnesium. Thus a coupled transcription-translation system in reticulocyte lysates was not feasible. But since infected cell extracts also support viral replication (Peluso and Moyer, 1983), we next determined if transcription by the SP6 polymerase could take place in infected cell extracts.

Table 8. Transcription of Supercoiled p45 in the Reticulocyte Lysate

Conditions	CPM
in 1X transcription buffer	973,600
in 1X retic lysate alone	2,300
in 1X retic buffer, 1X retic lysate	1,400
in translation system (TS), + 2 mM exogenous Mg	6,000
in TS + 5 mM exogenous Mg	32,300

TS represents the complete translation system. Reactions were performed in a final volume of 10 microlitres, 10 units enzyme, 20 microcuries of alpha labelled (32)P UTP. The whole reaction was precipitated with TCA after the 60 minute incubation period.

Transcription in Cell Extracts

Transcription by SP6 polymerase in cell extracts was very inefficient, yielding very low incorporation of radioactivity into RNA species of the correct size. Table 9 indicate that this was partially due to ammonium chloride inhibition of the polymerase. The inhibition of transcription of the plasmid p45 by ammonium chloride was more pronounced and occurred at much lower concentrations than did the inhibition of transcription of plasmids with a normal SP6 promoter.

But even cell extract prepared without ammonium chloride inhibited transcription by the SP6 polymerase (Sue Moyer, personal communication). Since transcription by SP6 polymerase in the extract was so inefficient we abandoned this system and chose to use presynthesized VSV leader sequences in cell extracts.

Table 9. Effect of Ammonium Chloride Concentration on the Transcription of Sph I Linearized Plasmids

Ammonium Conc. (mM)	CPM (p45)	CPM (p60)
0	28,700	810,800
10	15,900	750,900
20	12,300	691,000
30	11,500	634,300
40	10,800	570,000
50	8,300	528,400
100	8,100	181,600
Background	7,900	

Reactions were performed with 2 ug DNA 15 units SP6, 20 uCi 3H GTP, in a final volume of 10 ul. The whole reaction was precipitated with TCA and the radioactivity of the precipitates was counted by toluene based scintillation. The background counts were determined on a sample to which no DNA was added.

Deletions of p45

As part of the the mutational analysis of the leader encapsidation signal a set of nested deletions was created in p45, starting from the Bam HI at the 3' end of the VSV sequence. RNAs identical (with the exception of several extra bases coming from the linearized restriction enzyme site) to the leader or representing 3' deleted leader were used for encapsidation studies.

The manipulations used to created nested deletions in p45 from the Bam HI site are presented in Figure 9. After linearization with Bam HI the plasmid was treated with Bal 31 for various lengths of time. The amount of plasmid, the number of units of Bal 31, and the time of incubation were varied to obtain deletions varying from about thirty bases to one hundred and fifty. After the exonuclease treatment the plasmids were incubated with the Klenow polymerase and four dNTPs and ligated in the presence of Bam HI linkers. Transformants were screened for the presence of a Bam HI site. Approximately one out of ten transformants had a Bam HI site and these were further analyzed on polyacrylamide gels to assess the extent of the deletion. Deletions within the

Figure 9. Construction of p45 Deletions

To generate a series of nested deletions spanning the VSV sequence of p45, Bal 31 deletion mutagenesis from the Bam HI site of p45 was used. The plasmids that were used in the construction of the small defective encoding plasmids are indicated in the figure.

Construction of p45 deletions

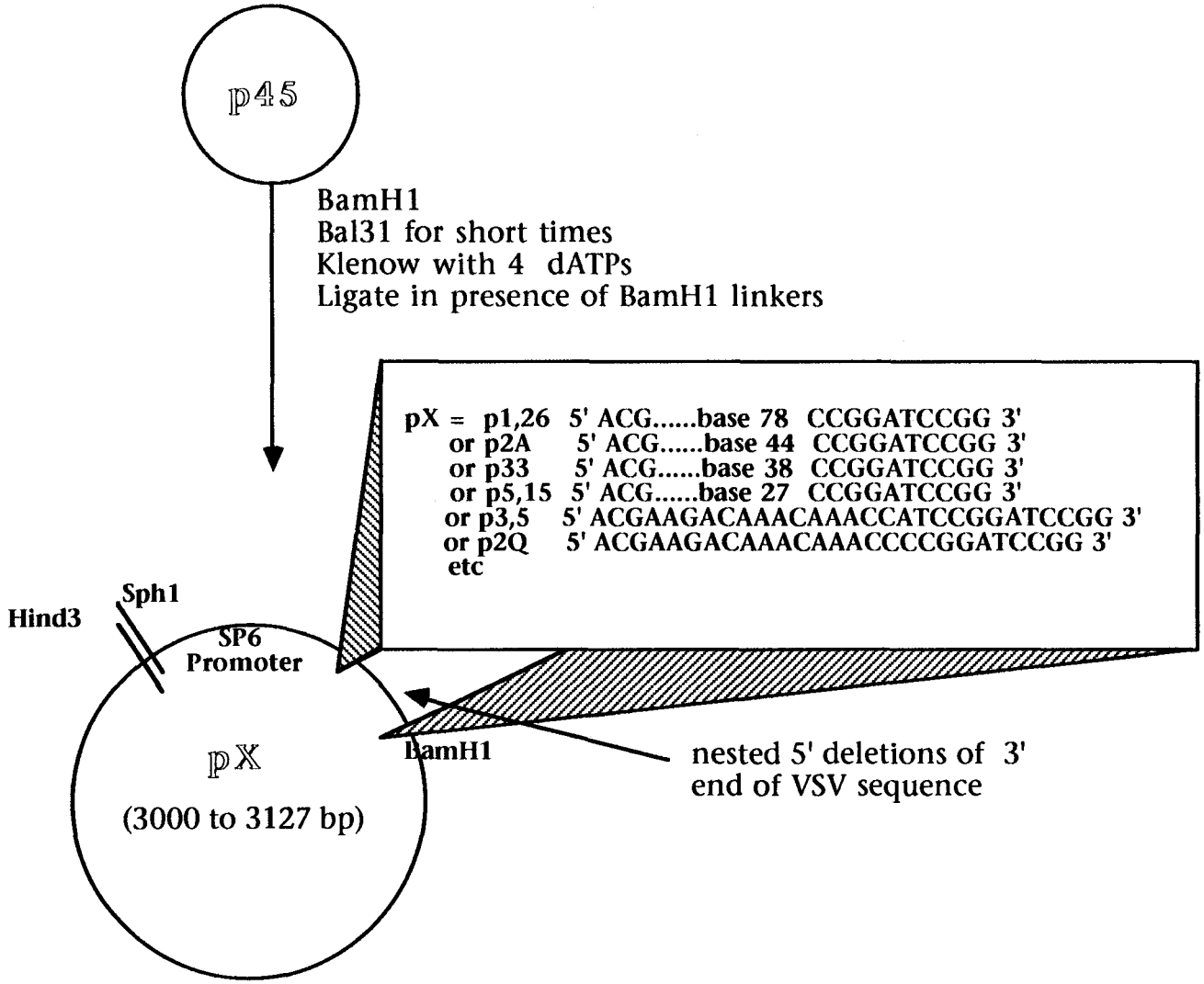


Table 10. Deletions of p45

Plasmid	Length of VSV Sequence
5,38	---------- 61 (S/L)
5,17	---------- 40 (S/L)
5,19	---------- 46 (S/L)
5,6	---------- 99 (S/L)
5,22	---------- 121(S/L)
2	---------- 19 (L)
3	---------- 112(S/L)
72	---------- 59 (S/L)
59	---------- 17 (S/L)
26	too short to bind primer
35	---------- 76 (S/L)
1,26	---------- 78 (S/L)
1,A	just like p45
2Q	---------- 17 (S/L)
9	---------- 16 (L)
3,5	---------- 19 (S/L)
68A	---------- 17 (S/L)
60	too short to bind primer
31	---------- 17 (L)
46	---------- 38 (S/L)
5,32	---------- 45 (S/L)

39	----------	118(S/L)	only length analysis gave good resolution (+/-4)
69	----------	30 (L)	band was very faint
5,15	----------	27 (S/L)	
5,34			too short to bind primer
6	----------	19 (L)	
5,30	----------	119(L)	only approximate
5,41	----------	80 (L)	only approximate
27	----------	41 (L)	
5,24	----------	104 (L)	only approximate
70	----------	15 to 17	
33	----------	38 (S/L)	
49	----------	41 (L)	
28	----------	107(L)	
19	----------	17?(L)	
41	----------	<17 (L)	
62	----------	<17 (L)	
5	----------	<17 (L)	
68 B	----------	60 (L)	only approximate
5,32	----------	45 (S/L)	
71	----------	<17 (L)	
2A	----------	44 (S/L)	
5,42	----------	41 (S/L)	
5,23	----------	74 (S/L)	followed by 5′ CCGGATCGCCGGATCCGG 3′

30	----------	66 (S)	
32	----------	74 (S/L)	followed by 2 Bam HI sites, confirmed by 2 sequences and 1 chain extension

S indicates that the plasmid was sequenced and L indicates that length analysis was performed.

appropriate range were sequenced and analysed by primer extension of the linearized plasmids. The primer used (5′ ACG AAG ACC ACA AAA CCA 3′) hybridizes to the first 17 bases downstream of the SP6 promoter of the VSV insert. The plasmids that were analyzed are listed in Table 10.

Encapsidation Assays

Two procedures were used to promote association between the proteins and RNA: 1. the addition of RNA to preformed proteins in the reticulocyte system, 2. the addition of RNA to infected cell extracts. Nucleoprotein complexes were assayed by RNase resistance or mobility shift analysis.

Encapsidation Assay in Reticulocyte Lysates

Labelled leader RNA was purified, heat denatured, and added to VSV reticulocyte lysates in which protein synthesis had proceeded for 30 minutes. Samples were incubated for another hour in the presence of the leader RNA. The samples were divided in two and one aliquot was treated with RNase, processed, and precipitated with TCA or electrophoresed on a sequencing gel.

In the experiment presented in Figure 10, 10,000 counts of input transcript were used. After RNase treatment, residual full length transcripts from Bam HI linearized p45 (lane 1), psPL6 (lane 3), p15 (lane 5), and p60 (lane 7) were evident. However, some protection of p1 (lane 9) or psPL6 (lane 11) transcripts incubated with globin mRNA programmed lysate was also observed. Furthermore, scintillation counting of the TCA precipitates from the samples were indistinguishable from background. Similar experiments were performed using 100,000 counts of transcripts, with similar results (data not shown).

Encapsidation Assay in Infected Cell Extracts

Transcripts made from p1, p60, psPL6, and p45 were not detectably encapsidated in infected cell extracts (data not shown). We postulated that the longer transcripts used in these experiments were not efficiently encapsidated because RNA secondary structure impeded encapsidation, possibly by obscuring the required sequences. As described above, we produced a set of nested deletions in p45 from the Bam HI site into the leader sequence and attempted to use transcripts from

Figure 10. Incubation of Leader Sequences with VSV mRNA Programmed Translation System

Lane 1. p45 transcript incubated in VSV mRNA programmed translation system, +RNase

Lane 2. p45 transcript incubated in VSV mRNA programmed translation system, -RNase

Lane 3. psPL6 transcript incubated in VSV mRNA programmed translation system, +RNase

Lane 4. psPL6 transcript incubated in VSV mRNA programmed translation system, -RNase

Lane 5. p15 transcript incubated in VSV mRNA programmed translation system, +RNase

Lane 6. p15 transcript incubated in VSV mRNA programmed translation system, -RNase

Lane 7. p60 transcript incubated in VSV mRNA programmed translation system, +RNase

Lane 8. p60 transcript incubated in VSV mRNA programmed translation system, -RNase

Lane 9. p1 transcript incubated in globin mRNA programmed translation system, +RNase

Lane 10. p1 transcript incubated in globin mRNA programmed translation system, -RNase

Lane 11. psPL6 transcript incubated in globin mRNA programmed translation system, +RNase

Lane 12. psPL6 transcript incubated in globin mRNA programmed translation system, -RNase

Ten thousand counts of labelled transcripts (synthesized by SP6 polymerase from Bam HI cut plasmids) were heat denatured and added to a VSV mRNA or globin (for lanes 11 - 14) programmed translation system. The samples were then subdivided into two equal portions, only one of which was treated with RNase. After multiple extractions with phenol-chloroform and percipitation with ethanol, one fifth of the non-RNase treated samples and the whole reaction from the RNase treated samples was applied to the gel.

VSV mRNA
globin mRNA
p45 + RNAse
p45 RNAse
psPL6 + RNAse
psPL6 RNAse
p15 + RNAse
p15 RNAse
p60 + RNAse
p60 RNAse
p1 + RNAse
p1 RNAse
psPL6 + RNAse
psPL6 - RNAse
1 2 3 4 5 6 7 8 9 10 11 12

some of these plasmids (2Q, 3,5, 2A, 33, 1,26) as substrates for encapsidation in the infected cell extracts. However, encapsidation of the short transcripts was not detected (data not shown).

Mobility Shift Analysis

We tried to adapt mobility shift analysis to detect the specific binding of N protein to the in vitro synthesized RNA transcripts. However, preliminary experiments revealed that the mobility of the transcript was retarded in the gels both after incubation with infected and incubation with uninfected cell extracts. Apparently, the leader transcripts were binding to factors present in non-infected cell extracts.

To further investigate this phenomenon, we performed mobility shift analysis on short and long transcripts. Labelled transcripts from Bam HI cut p1, p60, psPL6, p45, p2Q, p3,5, p5,15, p33, and p2A were incubated with uninfected cell extracts and analyzed by mobility shift analysis. They all formed complexes having the same retarded mobility though only about one percent of the total input radioactivity was retarded. As seen in Figure 11, transcripts from psPL6

Figure 11. Mobility Shift Analysis of Transcripts from psPL6, p60, and p1

Lane 1. p1 transcript (150 bases)

Lane 2. psPL6 transcript incubated in cell extract

Lane 3. psPL6 transcript incubated with BSA

Lane 4. p60 transcript incubated in cell extract

Lane 5. p60 transcript incubated in BSA

Lane 6. p1 transcript incubated in cell extract

The transcripts were synthesized from Bam HI linearized plasmids and were internally labelled with alpha 32P UTP. Fifty thousand counts of the transcripts (only 10,000 counts of p2A) were incubated with 30 ul of cell extract for one hour, and electrophoresed in mobility shift polyacrylamide gels, pH 7.5. An equal number of counts of each transcript was applied to the gel in the control lanes. These transcripts were not incubated with cell extract prior to electrophoresis.

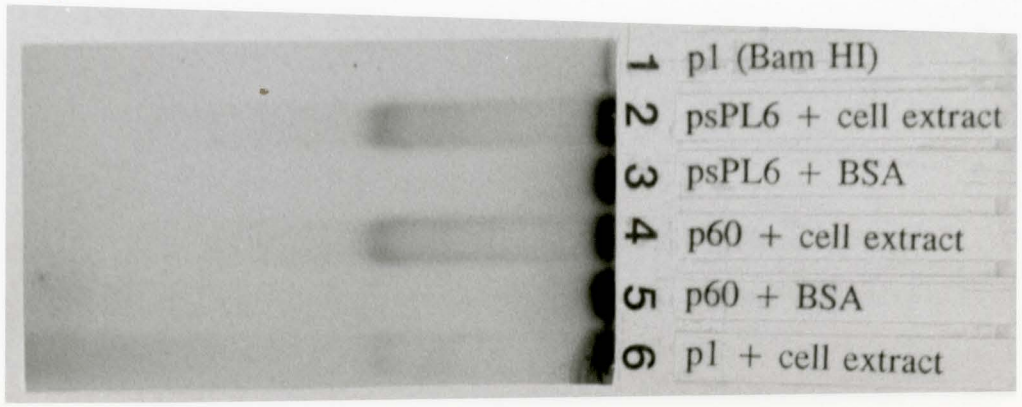

1 p1 (Bam HI)
2 psPL6 + cell extract
3 psPL6 + BSA
4 p60 + cell extract
5 p60 + BSA
6 p1 + cell extract

Figure 12. Mobility Shift Analysis of
Transcripts from p45 and p3,5

Lane 1. p60 Hind III transcript (3.3 Kb)

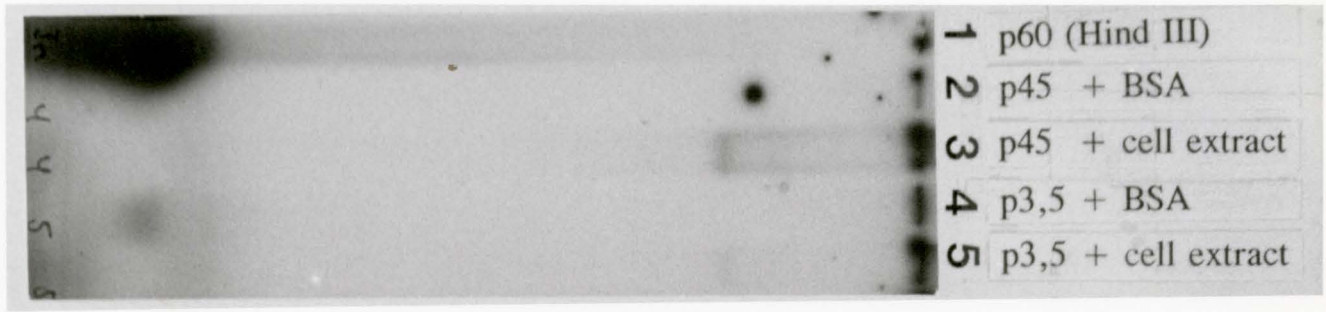
Lane 2. p45 transcript

Lane 3. p45 transcript incubated in cell extract

Lane 4. p3,5 transcript

Lane 5. p3,5 transcript incubated in cell extract

The transcripts were synthesized from BamHI linearized plasmids and were internally labelled with alpha 32P. Fifty thousand counts of the the p45 transcript and 5,000 of p3,5) were incubated with 30 ul of uninfected cell extract. The same amount of transcript was run in the control lanes. These transcripts were not incubated with cell extract prior to electrophoresis.


1 p60 (Hind III)
2 p45 + BSA
3 p45 + cell extract
4 p3,5 + BSA
5 p3,5 + cell extract

(lane 2), p60 (lane 4), and p1 (lane 6) exhibited retarded mobility. Figure 12 reveals that transcripts from p45 (lane 3), and p3,5 (lane 5) were also retarded in their mobility. Figure 13 (pH 5.5, pH 6.5, pH 7.5) presents the results of mobility shift assays with p3,5 transcript and several single stranded 17 base long DNA primers. These primers have very different sequences and were used as size markers. They may form complexes with single strand DNA binding proteins present in the cell extract.

The best resolution of the retarded p3,5 RNA complexes was achieved at pH 6.5 (Figure 13, lanes 1 and 6). At this pH, three species of different mobilities can be clearly identified. However, electrophoresis at pH 7.5 produced the least diffuse bands with retarded mobility. At pH 5.5 binding of the primers to protein was eliminated, whereas some binding of p3,5 transcript to two or three species of protein was still detectable.

Figure 13. Mobility Shift Assay of the p3,5 Transcript

Lane 1. p3,5 transcript incubated with uninfected cell extract

Lane 2. AB51 primer incubated with uninfected cell extract

Lane 3. AB71 primer incubated with uninfected cell extract

Lane 4. AB72 primer incubated with uninfected cell extract

Lane 5. p3,5 transcript

Lane 6. p3,5 transcript incubated with infected cell extract

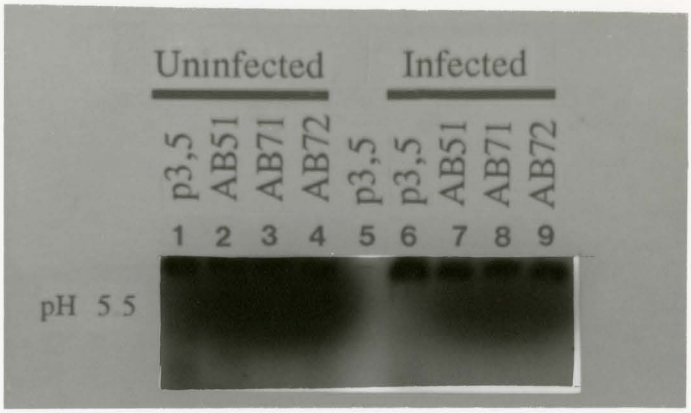
Lane 7. AB51 primer incubated with infected cell extract

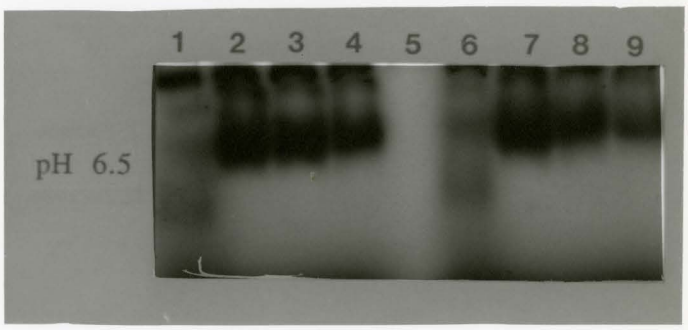
Lane 8. AB71 primer incubated with infected cell extract

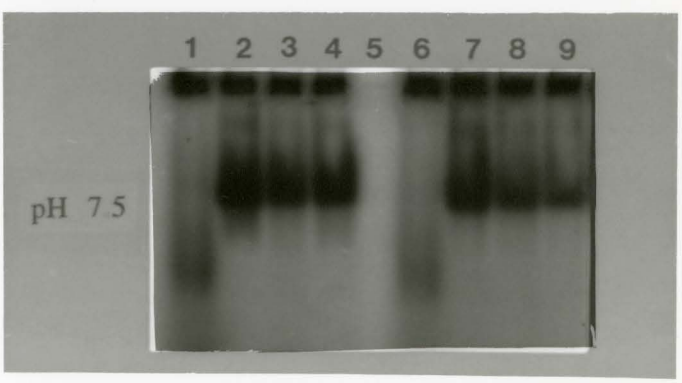
Lane 9. AB72 primer incubated with infected cell extract

The p3,5 transcript, internally labelled with alpha 32P CTP, was synthesized from Bam HI cut p3,5. The primers used were kinased with gamma 32P ATP. The labelled transcript and primers were incubated for one hour in cell extracts and the whole reaction was loaded

onto mobility shift polyacrylamide gels. The gels and the running buffer were at pH 5.5, 6.5, and 7.5.


Uninfected
Infected
p3,5
AB51
AB71
AB72
p3,5
p3,5
AB51
AB71
AB72
1 2 3 4 5 6 7 8 9
pH 5.5


1 2 3 4 5 6 7 8 9
pH 6.5


1 2 3 4 5 6 7 8 9
pH 7.5

Collaboration with Sue Moyer

Our failure to detect protection of the both long and short transcripts in the cell extract led us to collaborate with Sue Moyer (University of Florida). She also failed to obtain encapsidation of leader transcripts in infected cell extracts. High levels of specific encapsidation were eventually achieved by using purified N protein, prepared by a modification of the method first described by Blumberg (Blumberg et al., 1984).

Initially, Moyer found that purified N protein nonspecifically protected any RNAs shorter than one hundred bases from RNase. She subsequently found that the addition of uninfected cell extract along with the N protein confers specificity to the encapsidation reaction. In the presence of cell extract only 10% of added non-VSV RNA was protected from RNase, whereas more than 60% of added VSV RNA was protected. The observation that in a mixture of VSV RNA and a heterologous sequence, only VSV RNA was protected further confirmed the specificity of the encapsidation reaction in the presence of cell extract. The poly A tails of mRNA in the cell extract are probably responsible for the inhibition of nonspecific binding by N protein because cell extracts could be replaced by poly A oligonucleotides. Using our constructs she obtained high levels of specific encapsidation of

transcripts shorter than 80 bases long. But transcripts with only 10 residues of the 5′ terminus of the plus sense leader sequence were also encapsidated. Transcripts less than 80 bases long from psPL6, p1, p60, and p45 were fully encapsidated, in spite of their divergent 5′ sequences. She showed that encapsidation can proceed bidirectionally and is not inhibited by heterologous sequences upstream of the encapsidation signal.

The problem of encapsidating the long defective-like RNAs was circumvented by synthesizing a shorter defective like RNA by the procedure of Milligan et al. (1987). The transcript is 78 bases long with 26 bases from the 5′ end of the VSV genome and 51 bases from the 3′ end of the virus. The encapsidated transcript serves as an effective template for transcription or replication by purified VSV polymerase. The switch from transcription, which yields the positive sense leader transcript, to replication, was triggered by the addition of purified N protein.

While Sue Moyer was testing for encapsidation of the transcripts produced by our plasmids we continued to construct plasmids. Of particular interest to us was the possibility that after encapsidation defective like transcripts from some of the plasmids could serve as substrates for in vitro replication.

Construction of "Control" Plasmids

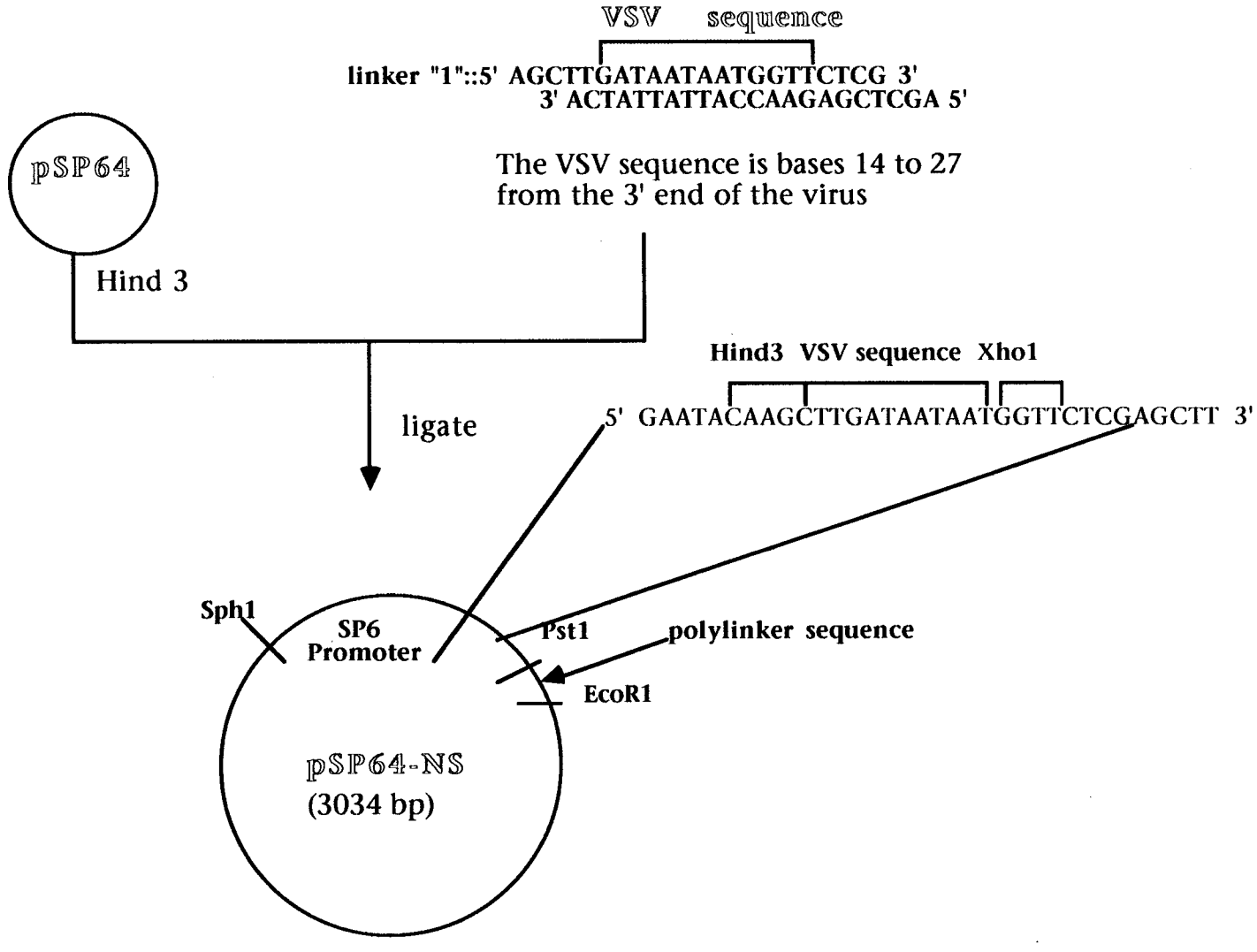
In the course of the in vitro encapsidation experiments conducted by Sue Moyer using leader sequence transcripts and purified N protein, the need arose for a negative control (non VSV) short transcript. The transcript from this construct was needed to test the binding specificity of the N protein preparation, which exhibited very high affinity for all transcripts that were initially tested, even the transcript from p15 (in which about 30 bases of the 5′ terminus of the VSV leader sequence are deleted). For the construction of the control plasmid (pSP64-NS), presented in Figure 14, we inserted a short DNA sequence downstream of the SP6 promoter into the Hind III site of the vector pSP64. A 27 base pair oligonucleotide with Hind III overhangs and an asymmetrically placed Xho I site was made and inserted at the Hind III site of pSP64. The sequence of the oligomer is:

```
5′  AGCTCGAGAACCATTATTATCA     3′
3′       GCTCTTGGTAATAATAGTTCGA 5′
```

Figure 14. Construction of pSP64-NS

To construct pSP64-NS, we inserted a double strand linker (linker 1), which had Hind III overhangs and an asymmetrically place Xho I site, into the Hind III site of pSP64. The appropriate plasmid, in which the orientation of the linker placed the Xho I site furthest downstream of the SP6 promoter, was identified by sequencing.

Construction of pSP64-NS



In the above orientation the top strand of the insert from base 9 to base 22 corresponds to the VSV plus sense leader sequence from base 14 to base 27 (5'AACCAUUAUUAUCA 3'). However, the desired plasmid, identified by sequencing, was one in which the sequence was inserted in the opposite orientation downstream of the SP6 promoter. Linearization of the plasmid with Xho I, followed by transcription by the SP6 polymerase yields a 26 base long transcript with the following sequence:

5' GAAUACAAGCUUGAUAAUAAUGGUUC 3'
| VSV |

This sequence corresponds, 12 bases downstream of its 5' terminus, to the VSV minus sense genomic sequence from bases 14 to 27 (counting from the 3' terminus of the virus). This transcript was expected to serve as a negative control for encapsidation experiments because it did not possess the putatitive encapsidation signal presumably present only at the 5' terminus of the plus sense leader sequence. Interestingly, this transcript contains part of the putative NS binding domain, as identified by Keene (Keene et al., 1981). Thus after encapsidation with N protein, it should serve as a substrate for NS binding.

Moyer's encapsidation experiments suggested the need for another set of plasmids that might facilitate the identification of the minimum encapsidation signal. The difficulty encountered was that the short leader transcripts, synthesized by SP6 polymerase from p2Q, p3,5, and p33 were not efficiently internally labelled. Furthermore, the potentially low stability of small ribonucleoprotein particle may impede their formation and/or detection. The solution was to extend the short transcripts of p2Q, p3,5, and p33 by the addition of non-leader sequences to their 3' termini. This was achieved by inserting a short sequence with an asymmetrically placed restriction enzyme site into the Bam HI site of the p45 derived plasmids. The procedure is presented in Figure 15.

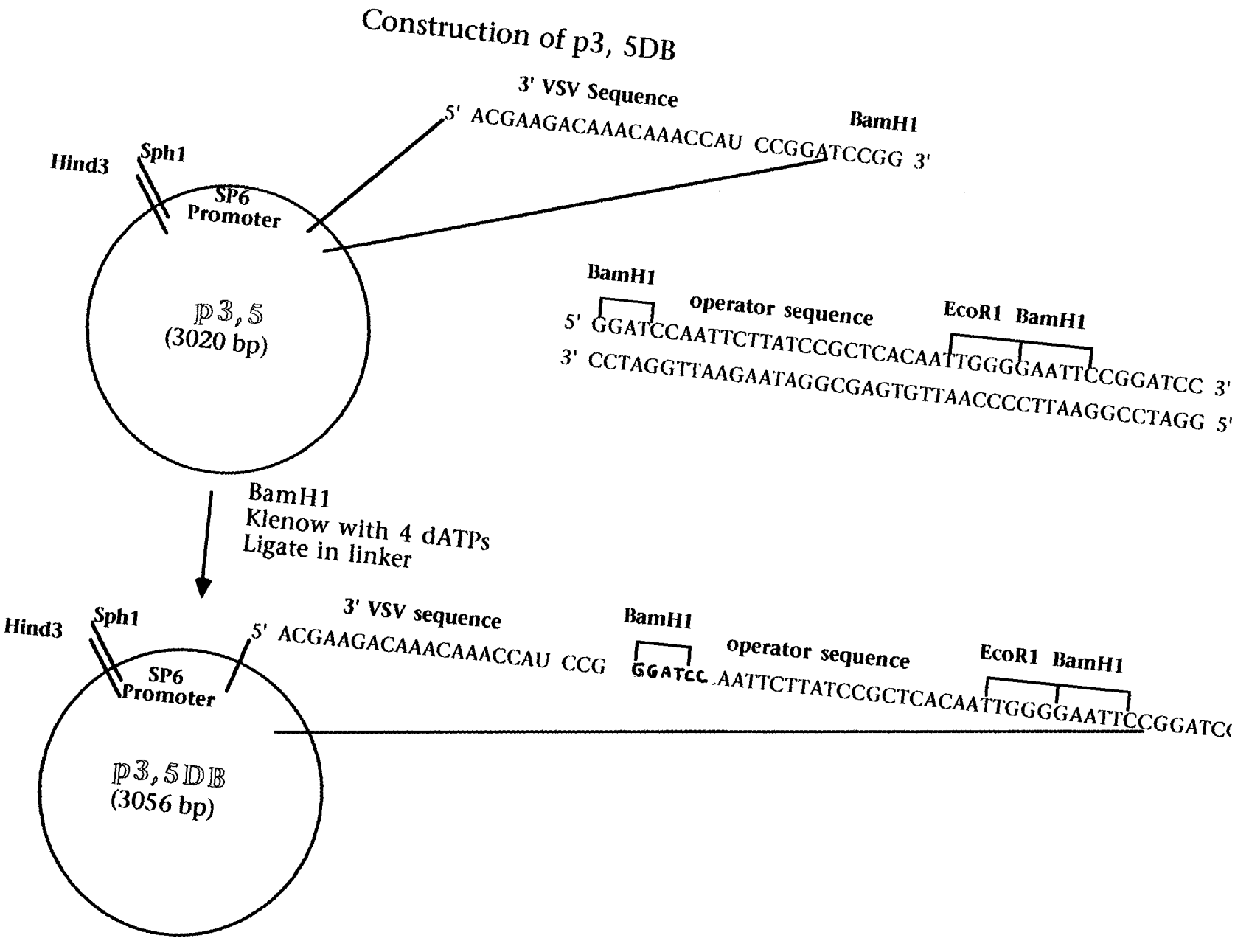
The insert sequence used, a double-stranded DNA oligomer with the sequence:

```
5' GGATCCAATTCTTATCCGCTCACAATTGGGGAATTCGGATCC 3'
   Bam HI|        Lac operator          | Bam HI
```

was a gift from Dody Bautista (Bautista and Graham, 1989). Since this sequence contains the operator element of the Lac Z operon, clones bearing the insert are identifiable as blue colonies on X-gal plates, if the host bacteria carry the intact Lac Z gene. The oligomer is flanked by Bam HI sites, but also has an asymmetric Eco RI site.

Figure 15. Construction of p3,5DB

The plasmid p3,5DB was constructed by inserting a linker (provided by Dody Bautista) into the flushed Bam HI site of p3,5. The operator sequence of the Lac-Z operon is flanked by a Bam HI site and an asymmetrically placed Eco RI site. The orientation of the inserted linker which places the Eco RI site furthest from the promoter sequence was identified by sequencing. Transcription of the Eco RI cut construct yields a transcript with the 5′ 19 bases of the plus sense leader transcript at its 5′ terminus and the operator sequence at its 3′ terminus.


Construction of p3, 5DB
3' VSV Sequence
5' ACGAAGACAAACAAACCAU CCGGATCCGG 3'
BamH1
Hind3
Sph1
SP6
Promoter
ℙ3,5
(3020 bp)
BamH1
operator sequence
EcoR1 BamH1
5' GGATCCAATTCTTATCCGCTCACAATTGGGGAATTCCGGATCC 3'
3' CCTAGGTTAAGAATAGGCGAGTGTTAACCCCTTAAGGCCTAGG 5'
BamH1
Klenow with 4 dATPs
Ligate in linker
Hind3
Sph1
3' VSV sequence
5' ACGAAGACAAACAAACCAU CCG
BamH1
GGATCC
operator sequence
EcoR1 BamH1
AATTCTTATCCGCTCACAATTGGGGAATTCCGGATC
SP6
Promoter
ℙ3,5𝔻𝔹
(3056 bp)

The sequence was inserted into the mung bean nuclease treated Bam HI site of the plasmids. Sequencing was used to identify two recombinants in which the operator was cloned in the two possible orientations in the plasmid p3,5. The Eco RI run off transcript of the useful orientation, in which the first 5′ 19 bases of the plus sense leader sequence are followed by 35 heterologous bases, is given below:

5′ACGAAGACAAACAAACCAU CCG GGATCCAATTGTTATCCGCTCACAATTGGGGA
VSV sequence (p3,5) BamHI lac operator

Other plasmids were constructed (see Figures 20 and 21--their construction will be presented in another section) which when appropriately (Xba I) linearized and transcribed (by SP6 polymerase) yield RNA transcripts analogous to short defective genomes. Linearization with an alternate restriction enzyme (Bgl II), allows the generation of transcripts in which the leader sequences of p3,5, p45, p33, p1,26, and p2Q at the 5′ terminus are adjoined to a short tract (54 bases long) of non-leader sequences at the 3′ terminus. The non-leader sequences at the 3′ terminus of the transcripts come from the carboxy terminus of the L gene sequence.

The 5′ Genomic Terminus of VSV

The plasmid p011-2 (Yang and Lazarrini, 1983), contains the 400 5′ terminal sequences of the virus, inserted into the unique Pst I site of the of pBR322. The viral sequences are flanked on both sides by an AT tail and GC tail both of undetermined length, generated during the cloning of the sequence from the viral RNA. To sequence the insert and to determine the length of the tails the VSV insert of p011-2 was cloned into the single strand phage M13 (see Figure 16). The Pst I insert of the plasmid was purified from an 5% polyacrylamide gel by the Maxam-Gilbert technique (Maxam and Gilbert, 1977) and ligated into a Pst I linearized M13 mp19 vector. Recombinant plaques were screened for the presence of the Pst I fragment. The insert, including the poly A and poly C tracts, was sequenced with the modified T7 DNA polymerase (Tabor and Richardson, 1987) using an internal primer (5′ CCAAACTTTAAGTATCA 3′) that hybridizes to the VSV sequence (from base 82 to base 66, according to Yang and Lazzarini's numbering). The Klenow fragment and reverse transcriptase had both failed to traverse the homopolymer tracts on single or double stranded templates.

Engineering the 5′ Viral Terminus

The sequences from the 5′ end of the virus were needed to complete the construction of a plasmid that directs the transcription (by SP6 polymerase) of an RNA species analogous to that of a defective viral genome. The 5′ terminus of the defective genome was provided by p1,26 and the 3′ terminus was provided by p011-2. To eliminate nonviral sequences from the 3′ terminus of the transcript, the deletion of the poly AT and poly GC tracts and the insertion of a unique Xba I site at the 3′ end of the viral sequence in p011-2 was undertaken. The Xba I site was chosen for two reasons: 1. it was not present within the viral sequence, the Lac Z sequence or SP6 promoter sequence; 2. cleavage by the Xba I enzyme generates a 3′ terminal T residue, which is the last nucleotide in the virus complementary sequence. Thus Xba I run off transcripts of the final defective coding construct have only VSV sequences at the 3′ terminus of the RNA.

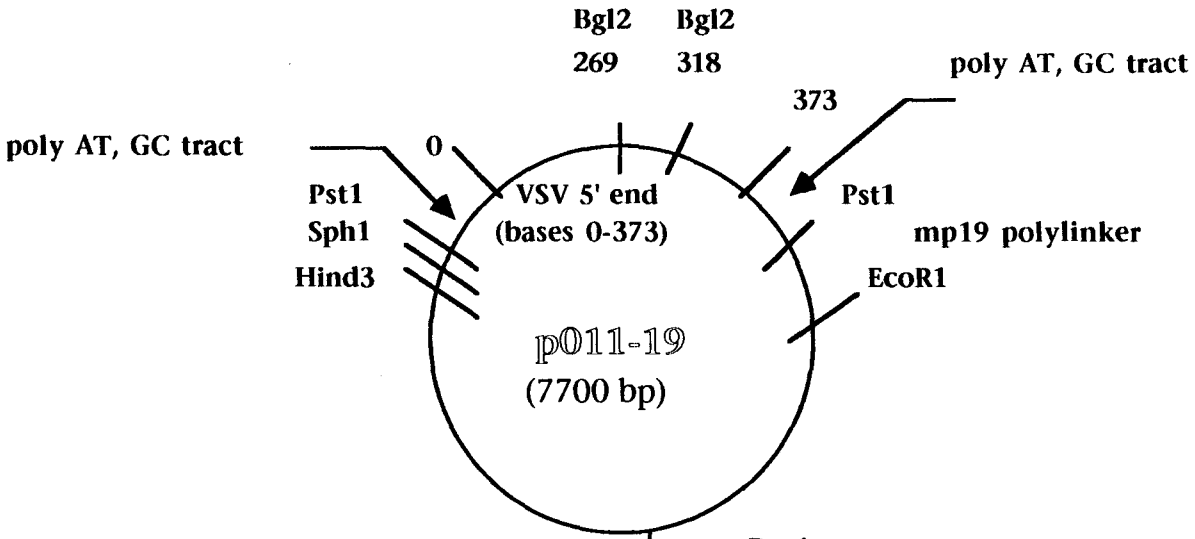
A modification of the T4 DNA polymerase deletion mutagenesis procedure was used to delete the non-VSV sequences from the p011-2 insert and to introduce the Xba I site at its terminus. As illustrated in Figure 16, the Pst I fragment of p011-19 was purified from a polyacrylamide gel, treated with

Figure 16. Construction of p44pU19

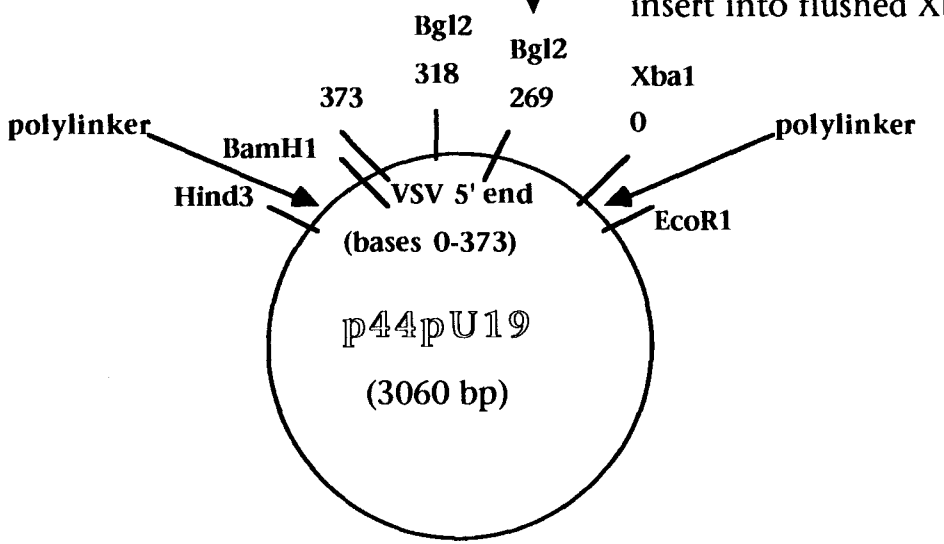
The plasmid p44pU19 has the first 373 bases (numbering according to Yang and Lazarrini, 1983) of the 5′ genomic sequence of VSV. The first base of the VSV sequence constitutes the 5′ A residue of an Xba I site. To construct this plasmid, we first cloned the p011-2 (Yang and Lazarrini, 1983) viral sequence, including flanking AT and GC tails, into the Pst I site of the vector M13mp19. The viral sequence was then excised from pM13mp19-011, purified from an polyacrylamide gel, treated with T4 DNA polymerase with dGTP and dTTP, and cloned into the flushed Xba I site of pUC19. The desired plasmid was identified by sequencing.

Construction of p44pU19

Insert the small Pst1 fragment of p011-2 (Yang and Lazzarini, 1983)
into the M13 vector mp19 to create p011-19



Pst1
isolate small fragment, T4 DNA pol. with dGTP, dTTP
insert into flushed Xba1 of pUC19



T4 DNA polymerase for a brief period in the presence of dGTP and dTTP and subsequently cloned into the filled-in Xba I site of pUC 18 (the site was made flush by filling in with the Klenow polymerase in the presence of all four deoxynucleotides). Transformants that regenerated the Xba I site and possessed the two internal Bgl II sites of p011 were most likely plasmids that had the properly trimmed VSV sequences from p011-2 correctly inserted. These transformants were sequenced to confirm the presence of the intact VSV sequence with the Xba I site properly placed.

DNA sequencing of the recombinants identified five relevant plasmids. Three of these were perfect deletions; ei. they completely eliminated the AT and CG tracts while leaving the VSV sequence intact. Two others had interesting terminal deletions in the VSV sequence. One of the plasmids (p44pU19) was used in the subsequent constructs.

Construction of Plamids With the VSV Termini Downstream of the SP6 Promoter

Peluso (1988) reported that naked RNA of a short defective of VSV can be encapsidated in vitro with N protein. In his system naked viral RNA was incubated in infected cell extracts. Our failure to detect significant encapsidation of plus sense leader sequences by N protein from infected cell extracts was in marked contrast to his findings. It was

possible that encapsidation in his system was facilitated by the presence of both viral ends on the RNA. To test this hypothesis and to lay the ground work of future studies of encapsidation and viral replication, we constructed plasmids (see Figure 18 and 19) that direct the synthesis of short RNAs that are analogous to plus sense viral defective genomes. The transcripts generated by these plasmids consist of the viral plus sense leader sequence at the 5′ terminus or 3′ deletions of this sequence, the viral minus sense leader sequence at the 3′ terminus, and intervening spacer. Nested 3′ deletions of the viral plus sense leader sequence (p45 deletion plasmids p33, p2A, p2Q, p3,5, p1,26, and p45) were used in the construction of the plasmids and these constitute the 5′ termini of the various transcripts that are synthesized from the plasmids. If encapsidated, the transcripts produced by the plasmids become in essence functional defective viral templates.

The plasmid constructs involved the assembly of different 5′ termini derived from the p45 deletion plasmids to the trimmed viral sequence of p44pU19. The Hind III to the Bam HI fragment from p1,26, p2Q, p3,5, p2A, p33, and p45 was inserted upstream of the 3′ terminal viral sequence of p44pU19. The plasmid p44pU19 was cut with both Bam HI and Hind III, the large fragment was gel purified and ligated with the small gel purified Hind III to Bam HI fragment of the p45

deletion. The constructs in this series (Figure 18), designated pXmVD (X= 2Q, 3,5, 2A, p1,26, p45) contain the complete viral sequence (373 bases) of p44pU19. Alternatively, the p45 deletion fragment was cloned into the large Hind III to Bgl II fragment of p44pU19. The resulting set of constructs, designated pXmmVD (X= 2Q, 3,5, 2A, p1,26, p45), contain 269 bases of the viral sequence of p44pU19 (see Figure 19). The plasmids were screened by cleavage with Hind III and Bam HI in the case of the longer ones, or with Hind III and Xba I in the case of the longer ones, and eventually sequenced to confirm their identity.

The plasmids pXmVD and pXmmVD were derived from the plasmid p1,26mVD. The plasmid, whose construction is outlined in Figure 17, was made using pVDZ-19 (see Figure 20).

Figure 17. Construction of p1,26mVD

The plasmid p1,26mVD has the SP6 promoter sequence and 3′ VSV end from p1,26 joined to the 5′ VSV end from p44pU19. Transcription of the Xba I cut plasmid yields a defective-like RNA transcript. The plasmid was constructed by inserting the Bam HI to Xba I fragment of p44pU19 into the Bam HI and Xba I sites of pVDZ-19 (see Figure 20). The Bam HI to Xba I fragment of p44pU19 represents the 5′ genomic VSV sequence (first 373 bases) and the large fragment of pVDZ-19 has the SP6 promoter and 3′ genomic viral sequences of p1,26. Both fragments were purified from a gel prior to ligation.

Construction of p1,26mVD

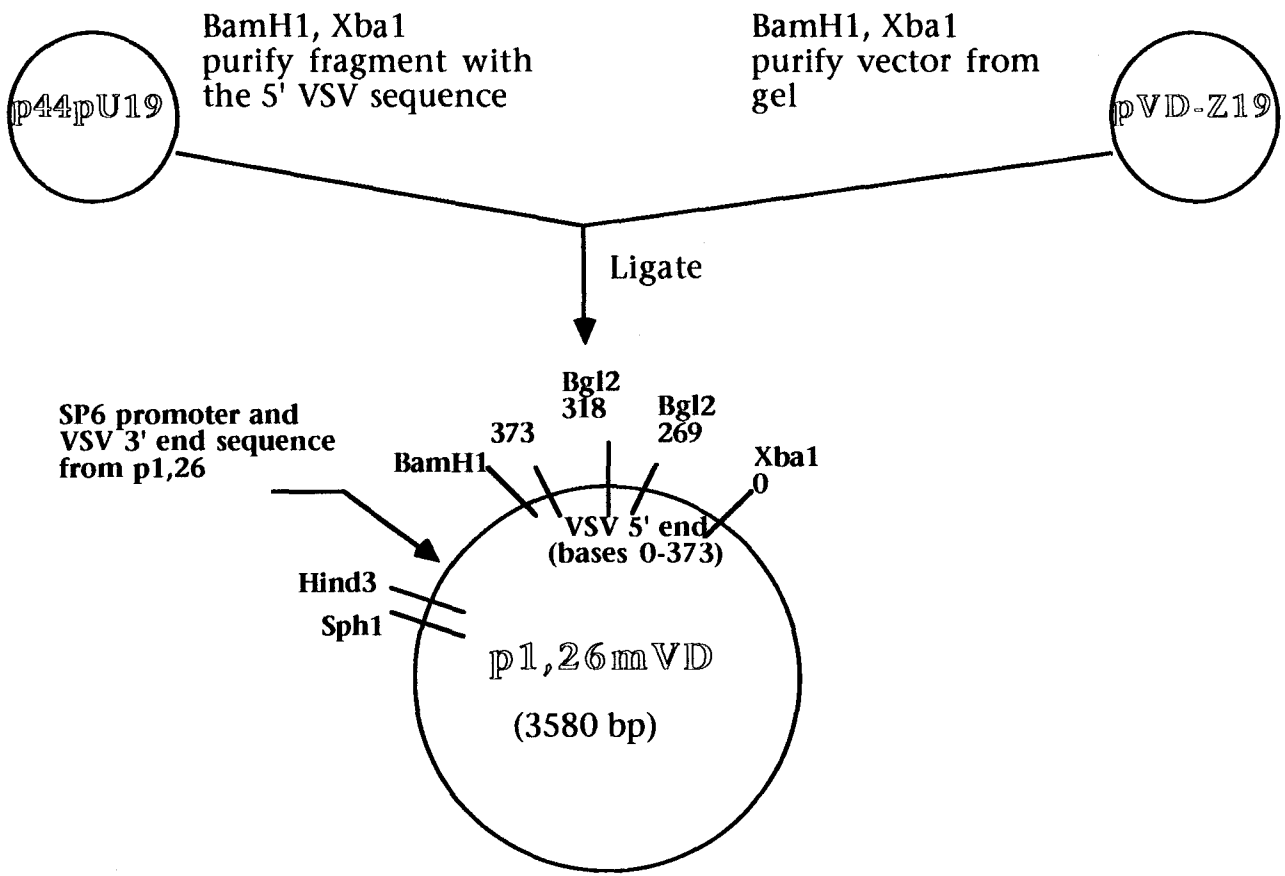


Figure 18. Construction of pXmVD Series

The set of plasmids designated pXmVD was constructed by inserting the small Bam HI to Hind III fragment of p2Q, p3,5, p33, p2A, and p45 into the Bam HI and Hind III sites of p1,26mVD. The plasmids were sequenced across the viral sequences using a primer that hybridizes to the SP6 promoter and a primer (PGA 1) that hybridizes to the viral sequence of p44pU19.

Construction of the pXmVD Series

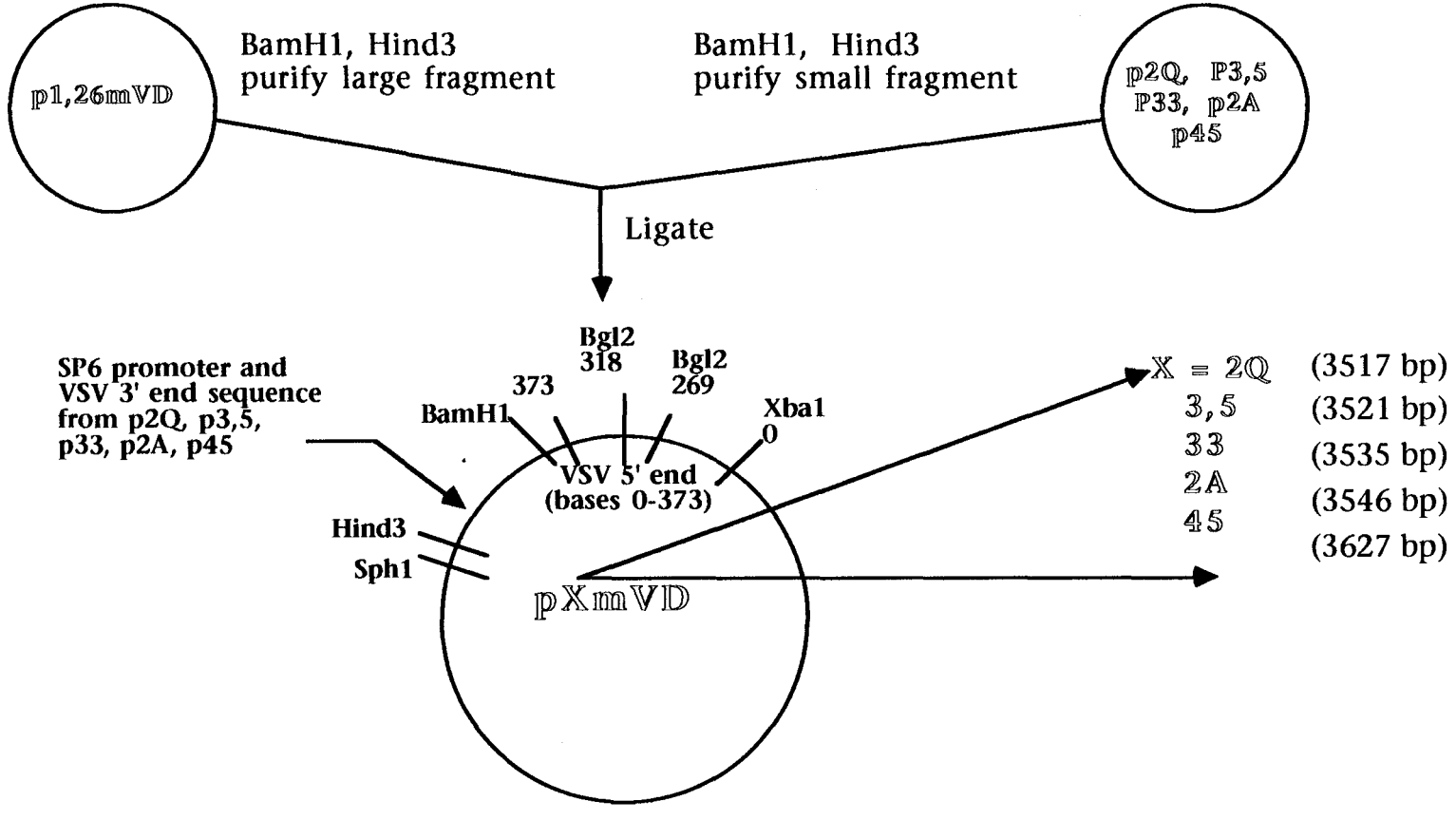
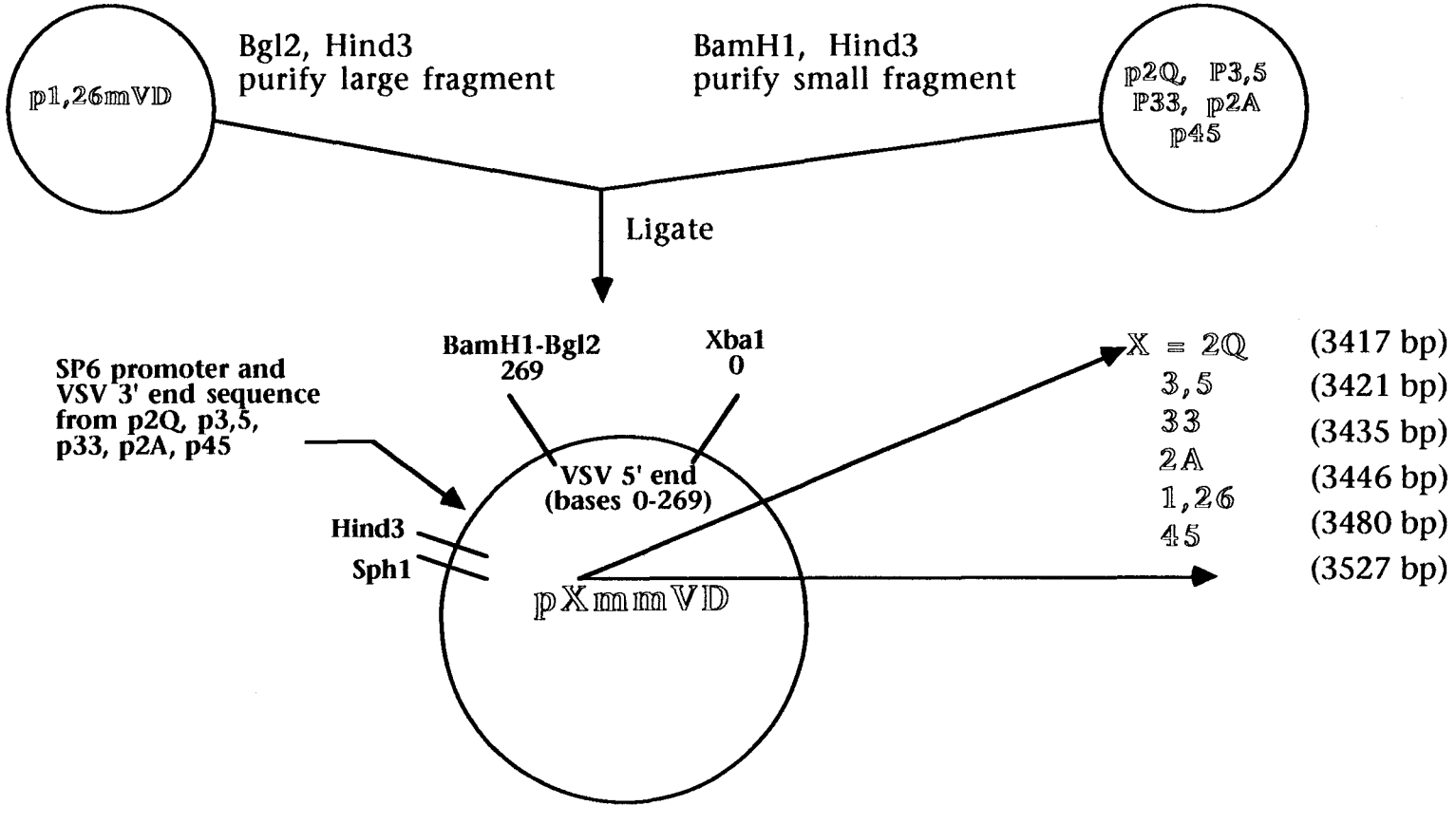


Figure 19: Construction of the pxmmVD Series

The set of plasmids designated pXmmVD was constructed by inserting the Bam HI to Hind III fragment of p2Q, p3,5, p33, p2A, and p45 into the Bgl II and Hind III sites of p1,26mVD. The constructs do not have a Bam HI or Bgl II site. The plasmids were sequenced across the viral sequences using a primer that hybridizes to the SP6 promoter and a primer (PGA 1) that hybridizes to the viral sequence of p44pU19.

Construction of the pXmmVD Series



Constructs Containing Lac Z Flanked by VSV Terminal Sequences

As a first step to the development of a VSV based helper-dependent vector system and in preparation for future studies of viral cis sequences in vivo, a defective VSV genome with a detectable marker was assembled in DNA form downstream of the SP6 promoter. Encapsidation of the transcript from the plasmid would yield a functional viral nucleocapsid. The production of packaged defective virus from the nucleocapsid requires the transfection of VSV infected cells with the nucleocapsid by the calcium phosphate precipitation method (Graham and Van Der Eb, 1973), or by DEAE dextra-mediated transfection (Rochovansky and Hirst, 1976).

The replication and transcription of the transfected defective nucleocapsid results in the amplification and expression of the marker gene. The Lac Z gene was chosen as the marker gene because its expression is readily detectable and quantifiable, even after cells cease to produce the protein. The cleavage of X-gal by B-galactosidase produces a persistent blue colour. Consequently, when X-gal is included in the tissue culture medium, the appearance of blue plaques signals the spread of the defective particles into the surrounding. Clearly, the phenomenon (blue plaques) is contingent on the successful execution both in vitro and in vivo of a series of (RNA) sequence-dependent events: the

encapsidation of the defective-like RNA, its replication and transcription in the cell, and expression of the marker gene. Inclusion of in the transcript the appropriate sequences was the first step in ensuring the successful performance of the defective.

The 5′ terminus of the defective genome was provided by the p45 derivative, plamid 1,26, in which the SP6 promoter directs transcription of the first 80 bases of the 3′ terminus of the virus. This plasmid contains the sequences for the leader gene, the attenuation signal, the N gene promoter, the N gene ribosome binding site, and the N gene initiation codon followed by 7 other codons (4 belonging to the N gene, and 3 being generated by the Bam HI site).

The B-galactosidase gene from the plasmid PMC1871 (Casabadan et al., 1983) was the source of the selectable marker. The gene is flanked by Bam HI sites in the plasmid and the joining of its 5′ terminus to the Bam HI site of plasmid 1,26 creates an in-phase fusion, in which the first 8 codons of the wild type gene are replaced by the 7 extra codons downstream of the N gene initiation codon. Expression of Lac Z is under control of the N gene promoter.

The 3′ terminus of the defective genome was obtained from p011-2, or p44pU19. Initially, a plasmid, pVDZ-19, was constructed (see Figure 20) in which the viral sequence of the p011-2 plasmid was not trimmed. The vector used, pTEQ-4

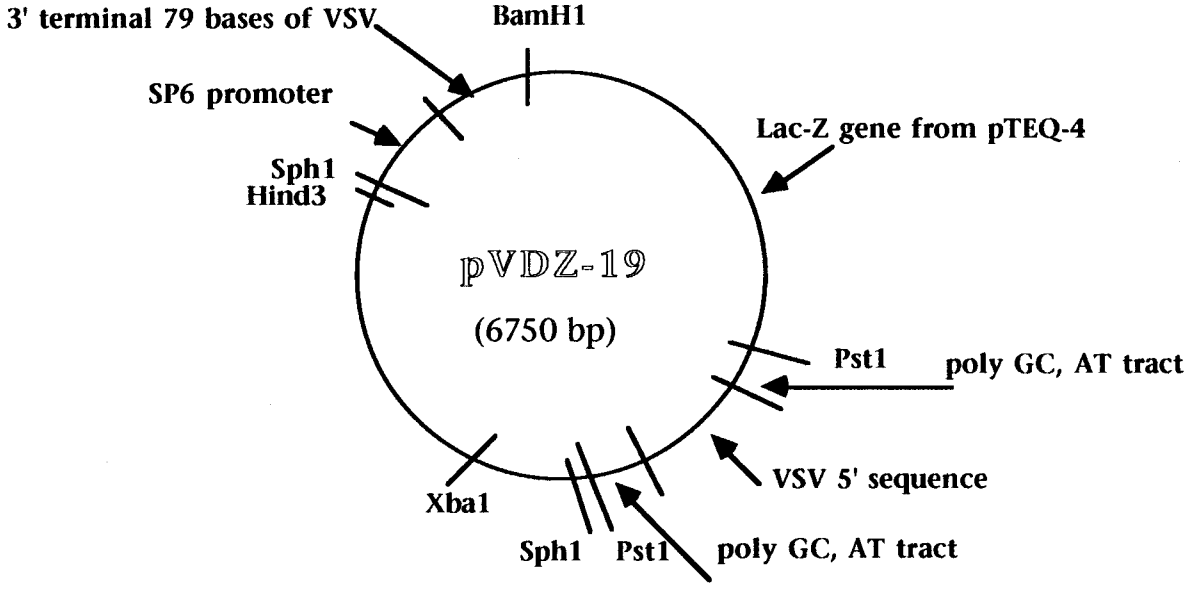
(Bautista and Graham, 1989) has a polylinker sequence upstream of the Bam HI site of the Lac-Z gene from PMC1871. The 3′ terminus of the gene possessed a unique Sma I site, a 1000 bases downstream of which was a unique Xba I site. The Hind III to Bgl II fragment of p011-19 containing the 5′ terminus of the virus was made blunt by treating with Klenow polymerase in the presence of four deoxynucleotides, purified from an agarose gel, and ligated into the dephosphorylated Sma I site of pTEQ-4. The transformants were screened for the presence of a Pst I site adjacent to the filled-in Hind III site of the VSV sequence insert. Sequencing of the plasmid (designated pTEQ-4-011) was performed with a primer (5′ TCC AAA CTT TAA GTA TG 3′) that corresponded to the viral sequence of p011-2 (position 83 to 67 according to Lazzarini's numbering). Sequencing was used to confirm the desired clone and to determine the length of the poly GC and poly AT tracts flanking the VSV sequences.

Figure 20. Construction of pVDZ-19

The plasmid pVDZ-19 encodes a defective-like RNA with the Lac Z marker. However, the 3' terminal sequence of the transcript contains the 5' genomic VSV sequence flanked by non-VSV sequences. These sequences are complementary and can form a panhandle structure. Two steps were involved in the construction of the plasmid. Initially, the plasmid pTEQ-4-011 was constructed by inserting the large Bgl II to Hind III fragment of p011-19 into the Sma I site of pTEQ,4 (Bautista and Graham, 1989). Prior to ligation into pTEQ-4, the Bgl II to Hind III fragment of p011-19 was purified from an polyacrylamide gel, and flushed by treating with Klenow polymerase in the presence of all 4 dATPs. The small Hind III to Bam HI frament of p1,26, purified from an polyacrylamide gel, was then inserted into the Hind III and Bam HI sites of pTEQ-4-011. The final plasmid was designated pVDZ-19.

Construction of pVDZ-19

Isolate large Bgl2, Hind3 fragment of p011-19,
Flush with Klenow and 4 dATPs
Insert into Sma1 site of pTEQ-4 (see appendix).
Identify the recombinant (pTEQ-4-011)
Insert the small Hind3, BamH1 fragment of p1,26 into
the Hind , BamH1 sites of the pTEQ-4-011.



The plasmid pTEQ-4-011 was cut with Bam HI and Hind III, the large fragment was purified from a gel and ligated with the small Bam HI to Hind III fragment of the p45 derivative, p1,26 (also purified from a gel). The transformants were screened for the presence of the p1,26 insert by digestion with Bam HI and Hind III, which released the 430 bp insert. Sequencing of the final plasmid, pVDZ-19, further confirmed the sequence and the correct fusion of p1,26 with pTEQ-4-011.

Once assembled, the plasmid was sequenced at all its significant junctions. Using four different primers three isolates were sequenced at the SP6-1,26 junction, the 1,26-B-galactosidase junction, the B-galactosidase-011 junction, and the 011-pTEQ-4 junction.

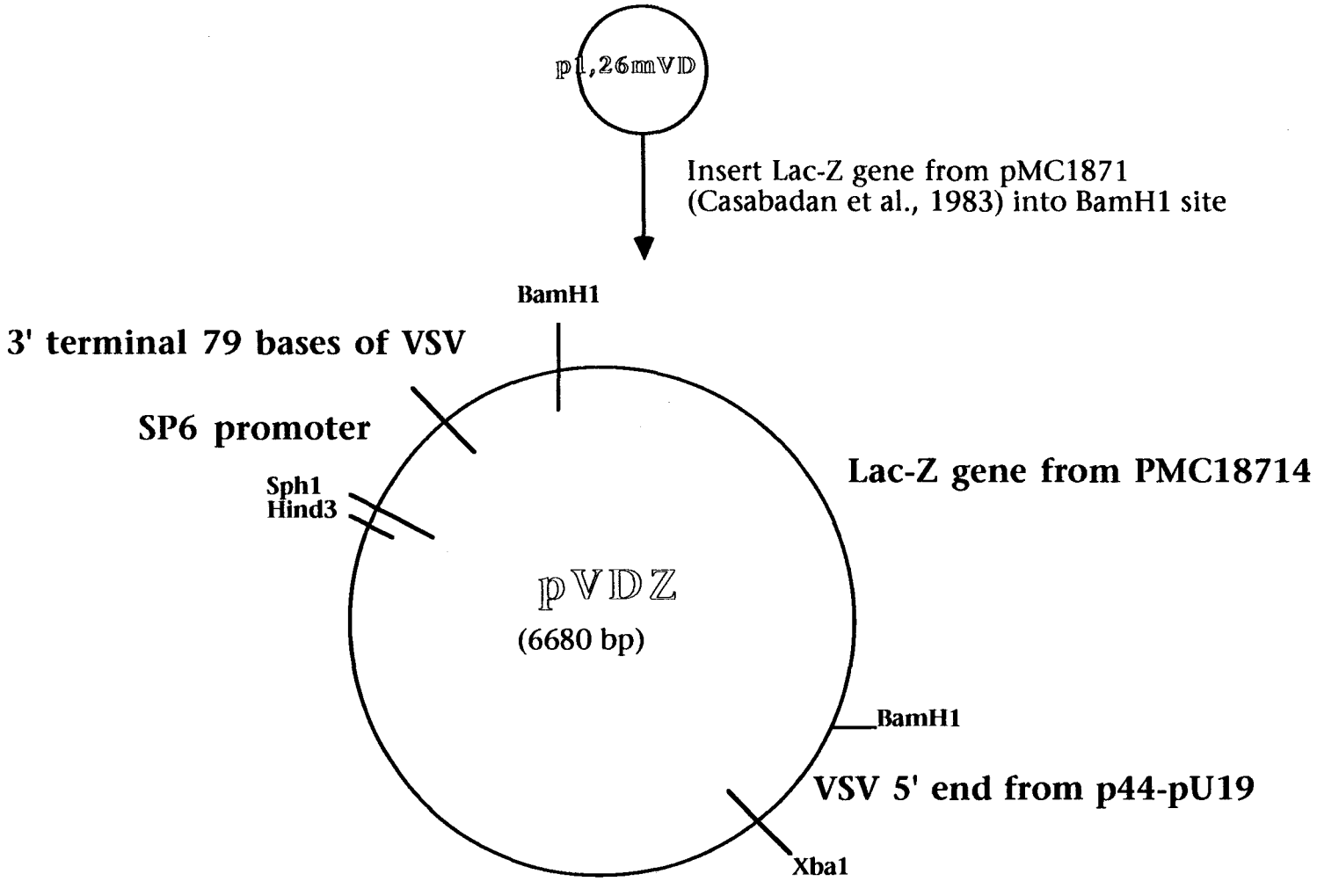
The sequence of the RNA produced by the vector is:
5′ VSV sequence of positive strand 5′ end to position 78, CCGGATCCC, B-gal sequence from codon 9 (valine) to nucleotide position 3,103 of the B-gal sequence of pMC1871, GGATCGATCCCCGATCT, the sequence from base 312 of the published p011-2 sequence to base 1, 22A, 12C CTGCAGGCATGCATGCT GGGAAGG
Pst I Sph I

The plasmid pVDZ can synthesize an Xba I run off transcript with completely viral termini. As outlined in Figure 21, the gel purified Bam HI flanked B-galactosidase

Figure 21. Construction of pVDZ

The plasmid pVDZ was made by inserting the Lac Z gene missing the promoter and the first 8 condons, from pMC1871 (Casabadan et al., 1983) into the Bam HI site of p1,26mVD. The Lac Z gene was excised from pMC1871 by Bam HI digestion and subsequently purified from a gel. It was then ligated into the Bam HI site of p1,26mVD and transformants were screened for the proper orientation of the Lac Z gene in relation to the N gene start codon. Sequencing was consequently used to confirm the construct and to check all significant junctions.

Construction of pVDZ



gene of PMC1871 was ligated into the Bam HI site of the plasmid. The clone was identified by Bam HI digestion, which released the large Lac Z insert. The orientation of the gene was determined by digestion of the asymmetrically placed Eco RI site and of the Xba I site at the 3′ viral terminus of the construct. The three significant junctures of the plasmid were sequenced; the 5′ terminus, the N promoter-Lac Z junction (Figure 22B), and the 3′ viral terminus (Figure 22A). The sequences obtained were as expected.

Figure 22: Sequences from pVDZ

Figure 22A. The 3′ Terminal Sequence

Figure 22B. The N Gene Promoter-Lac Z Junction

These figures represent the sequence analysis performed on pVDZ. Sequencing was performed on double stranded DNA using the Sequenace protocol. The sequence of the N gene promoter-Lac Z gene junction was obtained using a primer that hybridizes to the first 17 bases upstream of the transcription initiation site (AB51). The 3′ terminus viral sequence was obtained using a primer that hybridizes to bases 80 to 63 (PGA1).

Figure 22A. The 3′ Viral Sequence

The sequence of the 3′ viral terminus located at the 3′ (relative to the SP6 promoter) end of the construct, starting from base 20 (according to Yang and Lazarrini's numbering) of the 3′ viral sequence is:

```
5′ TCTGGTTTTGTGGTCTTCGTCTAGA  GACCT 3′
            VSV              |Xba I  plasmid sequence
```

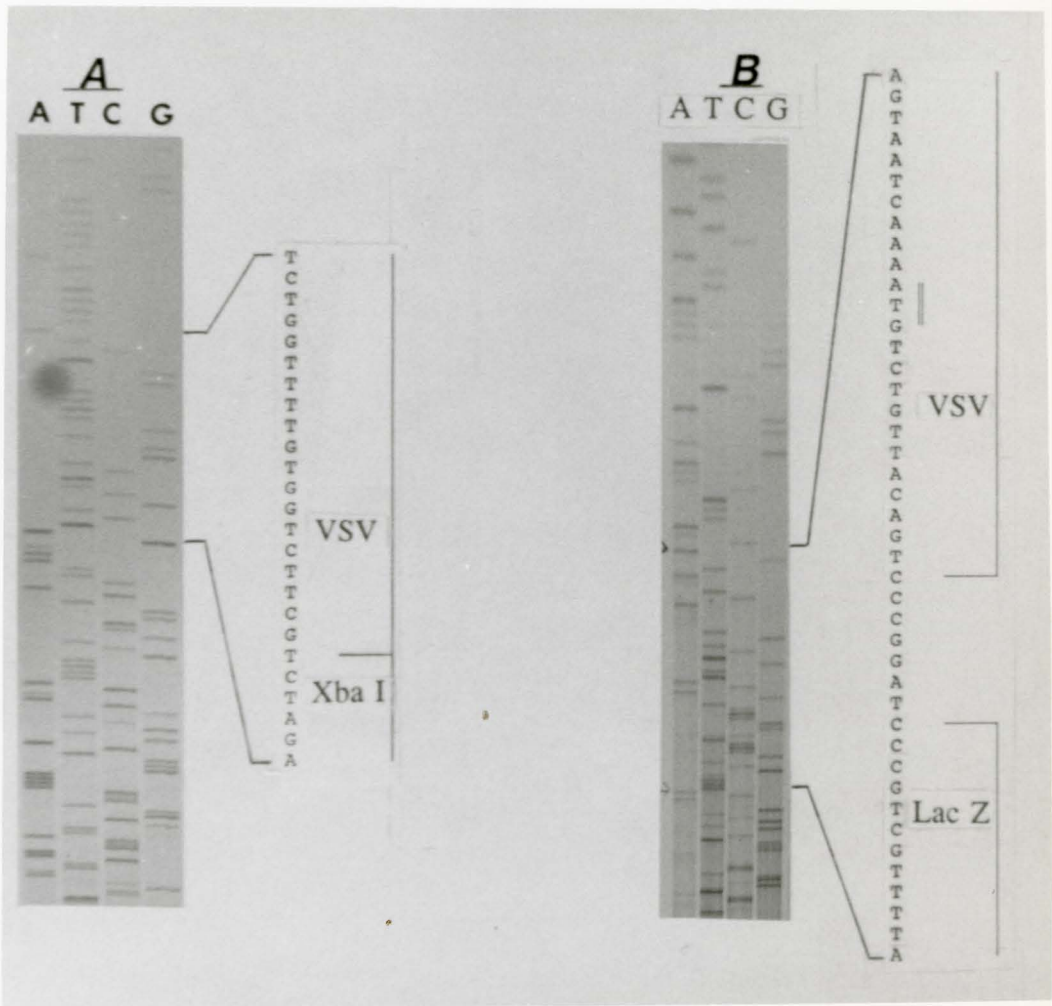
The transcript produced by pVDZ terminates with the first T residue of the Xba I site (indicated by a dashed line in the sequence), which is also the first T residue of the VSV sequence.

Figure 22B. The N Gene-Lac Z Junction

The sequence of the junction, starting ten bases upstream of the start codon of the N gene, is:

```
5'  AGTAATCAAA ATG TCT GTT ACA GTC CCG GAT CCC GTC GTT
                |                  |Bam HI|  |valine
          N start codon                       Lac Z
```

The N gene promoter preceeds the start codon (marked by a double line on the figure). The valine codon (GTC) indicated is the eighth codon of the wild-type Lac Z gene and the first triplet of the Lac Z gene in pMC1871.


A
A T C G
TCTGGTTTTGTGGTCTTCGTCTAGA
VSV
Xba I
B
A T C G
AGTAATCAAAATGTCTGTTACAGTCCCCGGATCCCGTCGTTTTA
VSV
Lac Z

Discussion

The discussion will consist of two parts; the first addresses the experimental data obtained, the second examines the potential application of plasmids we constructed during the course of our work. Our work consisted of: 1. the construction and analysis of plasmids in which the positive sense leader sequence was placed downstream of the SP6 promoter; 2. the use of transcripts from these plasmids to detect RNA-N protein association; 3. the construction of plasmids for collaboration with Sue Moyer; 4. the construction of plasmids for future work.

In the process of engineering plasmids in which SP6 polymerase synthesizes leader sequences with a minimal number of non-VSV 5′ residues, three mutants of SP6 promoter were obtained; two were transcription negative (psPL8, psPL18), and one (p45) was weakly transcription positive. The intact SP6 promoter sequence 5′ CTATAG 3′ spanning nucleotides -4 to +1 is replaced by 5′ CTAAGC 3′ in psPL18 and by 5′ CTACAA 3′ in psPL8. The mutation that inactivates the SP6 promoter of psPL18 is a deletion of the T residue at position -1. Studies with p45 showed that the G residue at position 1 of the SP6 promoter is also importat for promoter function.

Replacement of the G residue at position +1 of by an A residue (in p45) resulted in an inefficient promoter with an anomalous initiation pattern. The plasmid p45 directed initiation at the 5′ terminal A residue of the plus sense leader sequence, at the upstream A residue of the SP6 promoter, and unexpectedly at the G residue at position 3 of the viral sequence (see Figure 7, lane 1).

The analysis of initiation by the plasmids with an intact SP6 promoter (p60 and psPL6) revealed that the SP6 polymerase initiated transcription at the expected G residue and at the upstream A residue (Figure 6, lanes 1, 2, 3, and 8). Interestingly, the proportion of initiations at the G or A residue differed among the plasmids. For the plasmid psPL6 (lanes 1 and 2) initiation at the G residue was much more frequent than for p60 (lanes 3 and 8). These results suggest that sequences downstream of the SP6 promoter affect transcription initiation by the polymerase.

Transcription in cell extracts and in reticulocyte lysates was very inefficient, particularly for p45. Inhibition of transcription of p45 by the endogenous potassium chloride in the reticulocyte lysate and by the ammonium chloride in cell extracts made coupled systems unfeasible, particulary for p45 and its derivatives. But the p45 deletion derivatives are suitable for future studies on the sequence-dependence of leader-mediated inhibition of

eukaryotic transcription and replication in in vitro systems. Previous studies (Grinell et al., 1984) have used single stranded DNA sequences corresponding to various portions of the leader. These plasmids offer the alternative of using RNA corresponding to various sections of the leader in the in vitro inhibition studies. Furthermore, the transfection of these Bam HI linearized plasmids along with a plasmid encoding the SP6 polymerase should offer a method for studying the in vivo inhibition of cellular functions mediated by leader sequences. Such a procedure can identify which leader sequences, if any, are involved in cell killing.

Encapsidation

Soluble N protein for encapsidation is available from three sources: infected cell extracts, VSV mRNA programmed reticulocyte lysates, and preparations of purified N protein. Infected cell extracts and VSV mRNA programmed reticulocyte lysates were shown to support replication of input viral templates (Peluso and Moyer, 1983, 1984, 1988; Davis and Wertz, 1983; Patton et al., 1983; Wertz, 1983, 1987). The balance and levels of viral proteins in these systems reflect the intracellular situation more closely than preparations of purified N protein. Furthermore, the viral proteins in VSV programmed reticulocyte lysates and infected cell extracts are

in their native state, whereas purified N protein must be denatured to prevent its aggregation in the absence of NS protein (Sprague et al., 1984; Blumberg et al., 1983).

The failure to detect significant levels of encapsidation in VSV mRNA programmed reticulocyte lysates and in infected cell extracts, even of short leader sequences, is puzzling. Moyer (personal communication) also did not detect any encapsidation of leader sequences added to or synthesized in infected cell extracts. In the in vitro replication system devised by Wertz (Patton et al.), leader RNAs produced prior to replication were not encapsidated after replication (and encapsidation of nascent genomes) had been triggerred by high levels of N protein. Thus, in this system, nascent leader sequences in the replication complex effectively compete with free leader transcripts for N binding. Free leader sequences exhibit a low affinity for N protein; the requirement of high levels of N protein for their assembly is met late in infection, but not in the N mRNA programmed reticulocyte lysates.

The inability of the systems we used to promote encapsidation in the absence of replication may indicate that either the pool of N protein available for leader binding is the limiting factor, or that encapsidation may be necessarily coupled to replication of the virus.

One possibility is that encapsidation initiates with the interaction of N with the replication complex, which, properly defined, consists of the template, the associated polymerase complex, and the attached nascent RNA. Interaction with the replication complex may facilitate the displacement of the NS moiety from N:NS complexes. Alternatively, initiation of encapsidation may be a multistep event, in which sequence specific interaction with the nascent RNA is secondary to N interaction with the replication initiation complex. Thus sequence alone would be insufficient to initiate encapsidate, or it may initiate it inefficiently.

Data obtained by Dillon and Gupta (1988) suggest that the encapsidation of replicating genomes in infected cells may be mechanistically different from the encapsidation of leader RNA *in vivo*, or by pure N protein *in vitro*. They found that the earliest stable RNP complex formed during replication possesses the 5' terminal 65 nucleotides of genome RNA. The encapsidation of nascent genomes may thus require anti-termination. Furthermore, this complex and the leader RNP complexes differ significantly in times of appearance and protein content; leader nucleocapsids appear much later in infection and are not associated with NS protein.

We had hoped to avoid using purified N protein for the encapsidation experiments. Our analysis of Blumberg *et al.*'s (1983) control data suggested that, in their system, the

purified N protein exhibited promiscuous binding to RNA. This analysis was experimentally confirmed by Sue Moyer (personal communication), who found that purified N protein avidly binds any RNA, and has a very high affinity for poly A tracts. Fortunately, Moyer found that the nonspecific binding of RNA by the purified N protein was eliminated by the inclusion of uninfected cell extracts in the encapsidation reaction. Since NS protein curtails the nonspecific association of nascent N protein with small RNAs (Masters and Banerjee, 1988b), its ability to confer specificity to the encapsidation reaction is being explored.

In collaboration with Sue Moyer the encapsidation signal was narrowed to the first 5′ ten bases of the leader sequence. The signal is a true nucleation origin because heterologous sequences downstream and upstream of the site are effectively encapsidated. Since encapsidation is not sensitive to slight sequence alterations of the signal the more transcriptionally active psPL6 should have been used as the parent plasmid of all our constructs, rather than p45.

The major limitation to date has been the inability of the purified N protein preparation to encapsidate transcripts longer than one hundred nucleotides. Secondary structure in long transcripts may obscure the encapsidation signal of long

transcripts or may arrest encapsidation once it is initiated. The problem of secondary structure may be alleviated by synthesizing the transcript in the presence of N protein.

The small defective-like RNA used by Moyer contains all the necessary sequences for its encapsidation, transcription, and replication *in vitro* and *in vivo*. Consequently the viral cis sequences involved in transcription, replication, in the switch from viral transcription to replication, and in the inhibition of eukaryotic replication and transcription, are available for mutational analysis.

This system may elucidate the mechanism by which N protein triggers the switch from transcription to replication. The apparent coupling of encapsidation to replication *in vitro* and *in vivo* may be correlational and not causal as Blumberg suggests (Blumberg *et al.*, 1983); encapsidation may initiate with replication, and not necessarily initiate it. The availability of N protein may act by some unknown pathway to initiate replication; the interaction of soluble N or N:NS complexes with L may cause it to switch to the replicative mode. Also, a soluble pool of N protein may simultaneously, but by separate pathways, induce replication and encapsidation.

One way to distinguish between Blumberg's model (Blumberg *et al.*, 1983) and these alternative hypotheses is

to determine if replication can be uncoupled from encapsidation *in vitro*. This may be possible if particular mutations in 3′ terminus of the positive sense defective-like RNA do not affect polymerase binding but eliminate the encapsidation signal in the 5′ terminus of the negative sense molecule. Blumberg's model (Blumberg *et al.*, 1983) would be disproved if replication of the mutant genome proceeds without encapsidation of the nascent genome.

Mobility Shift Analysis

Binding of the trancript from p3,5 suggests that complex formation only requires the first 5′ 19 bases of the VSV sequence. Mobility shift analysis of transcripts from p3,5 at pH 6.5 (middle gel in Figure 15, lanes 1 and 6) showed three complexes with retarded mobility. This suggests the binding of three proteins to the leader sequence. But the specificity of the binding requires confirmation, perhaps by binding competition analysis with poly I-C. The transcript from p15 was intended as a negative control for protein binding because it lacks the 5′ terminal 29 bases of the leader sequence.

In Vitro Generated Defective Viruses

The inability to target mutations in negative sense RNA viruses has hindered the molecular analysis of their life cycle. Manipulation of RNA virus sequences has traditionally been limited to: a) the creation of random mutations by treatment of the virus with mutagens, and 2) the identification and isolation of naturally occurring mutants that contaminate the "wild-type" population. These techniques have been applied to study viral trans sequences because deleterious mutants can be propogated at permissive temperatures, or rescued by complementation. One key limitation is that mutations in trans sequences can only be detected when they affect the phenotype (thus neutral mutations are difficult to identify), and precisely locating the mutation/s is an arduous, if not impossible task.

Viral cis sequences are even much less amenable to dissection by traditional methodologies. Viruses bearing deleterious mutations in cis sequences cannot be propogated, and hence such mutations cannot be positively identified. Presently, cis sequences of negative sense RNA viruses are only "identifiable" by their conservation across serotypes.

We have devised a VSV-based vector system that makes possible the generation, propagation, and identification of lethal mutations in viral cis and trans sequences. Defective

viruses are generated by encapsidating transcripts made by the SP6 system and transfecting the nucleocapsids into VSV infected cells. Directed mutagenesis of VSV sequences in plasmids is used to create and propagate novel defective-like genomes. The independent mutational analysis of encapsidation and replication is possible in this system because encapsidation of the vector transcript proceeds in the 5′ to 3′ direction, whereas replication of the nucleocapsid proceeds from the 3′ to 5′ direction.

The Lac Z gene encoded by the defective genome of pVDZ facilitates the screening and identification of mutants. The expression of the Lac Z gene in the infected cells is dependent upon viral replication and transcription. Expression of the gene can monitered by plaque colour (blue when X-gal is present in the medium) and quantified by biochemical assays. Consequently, plaque colour provides a means to classify mutations in the defective as lethal (white plaques), deleterious (the more deleterious, the lighter the colour of the plaque), and neutral (blue plaque).

The large defective genome in pVDZ is intended for the <u>in vitro</u> analysis of mutants. The small defective genomes are ideal substrates for <u>in vitro</u> replication and transcription systems because their small size ensures efficient replication and transcription. They are also suited for studies on the phenomenon of viral interference <u>in vitro</u>

and in vivo. All the viral cis sequences are represented on the small defective encoding plasmids p1,26mVD and p45mVD. The remaining plasmids in the pXmVD and pXmmVD series are missing VSV cis sequences: the leader-N junction attenuation signal (p2QmVD, p3,5mVD, p33mVD), the N promoter and ribosome binding site (p2QmVD, p3,5mVD, p33mVD, p2AmVD), and the NS binding domain (p2QmVD, p3,5mVD).

The trans sequences of the virus can also be analyzed by the vector system. In fact, if encapsidation of the run-off transcript from a plasmid containing the entire VSV sequence can be accomplished, the entire viral genome becomes amenable to analysis by directed mutagenesis.

In vitro generated defective viruses may also serve as efficient vector systems for the expression of large quantities of proteins in mammalian cells. VSV infected cells almost exclusively express viral proteins because of the total host cell shut-off mediated by the virus. A VSV-based, helper-dependent vector system should permit the purification of large quantities of cloned protein, relatively free of cellular contaminants.

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