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MUTATIONAL ANALYSIS OF THE NS-1 PROTEIN OF THE MVM PARVOVIRUS

BY
MARIO H. SKIADOPOULOS

SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

AT

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Clinical	0622
Developmental	0620
Experimental	0623
Industrial	0624
Personality	0625
Physiological	0989
Psychobiology	0349
Psychometrics	0632
Social	0451

TABLE OF CONTENTS

CHAPTER 2 MATERIALS AND METHODS

page number

page number

List of Figures

Figure page number

List of Tables

ABSTRACT

The major non-structural gene (NS-1) of the minute virus of mice (MVM) parvovirus encodes a multifunctional protein that is absolutely required for replication of the viral genome and for transcriptional regulation of the two MVM promoters. A shuttle vector encoding the NS-1 protein was constructed and a replication complementation assay was developed, to study the effects of a series of insertion and point mutations in the NS-1 coding sequence, and the localization of activities required for viral DNA replication and transcriptional activation. Mutant NS-1 genes were transiently expressed in transfected COS-7 cells by using an SV40 promoter driven NS-1 expression vector. The ability of the mutant proteins to complement a replication defective NS-1 mutant of the infectious MVM plasmid pMM984 and to activate transcription from the MVM capsid gene promoter in chloramphenicol acetyl transferase (CAT) expression assays was determined.

Six independent insertions ranging in size from 2 to 12 amino acids inhibited the DNA replication activity of NS-1 between 20 and at least 100 -fold. There was no apparent correlation between the extent of inhibition of parvoviral DNA replication and the location of the mutations. The transcriptional activation function of NS-1 was inhibited between 1.5 and at least 20-fold and was therefore overall relatively less sensitive to mutagenesis than was its DNA replication function. An exception to this was a 5 amino acid insertion between Tyr543 and Gln544 that abolished transactivation as well as the ability of NS-1 to complement viral DNA replication. Two point mutations Ser249 to Ala and Lys250 to Gln and a one amino acid insertion between Asp606 and Leu607 had no effect on viral DNA replication and transactivation activities.

NS-1 has previously been shown to be covalently linked to the 5' ends of single stranded and replicative forms of the viral DNA. The linkage is stable under alkaline conditions and is therefore presumed to occur though a tyrosine residue. Tyrosine to phenylalanine mutations were constructed at the 11 conserved tyrosines of MVM NS-1. The ability of the mutant NS-1 proteins to complement the replication defective target plasmid in transient replication assays and to stimulate transcription from the capsid gene promoter of MVM in CAT assays was determined. F47, F227, and F543 replicated viral DNA and transactivated the capsid gene promoter at approximately wild type levels. Mutagenesis of Tyr6 to Phe and a double mutation Tyr550 to Phe and Ser445 to Thr exhibited increased levels of viral replication activity, while a Tyr to Phe conversion at position 209 replicated viral DNA at approximately 50% of wild type. F6 and F209 were wild type for transactivation, while F550/T445 transactivated the capsid gene promoter at approximately 50% of WT NS-1. Three mutations in the N-terminal portion of NS-1 (F188, F197 and F210) blocked the replication activity. F188 and F197 were defective for the transactivation function and F210 was wild type. Mutagenesis of Tyr310 and Tyr422 inhibited the replication activity but not transactivation. These mutations begin to define domains that are required for replication and suggest that the transactivation function may require more than one functional domain.

List of Abbreviations

A	adenine
a.a.	amino acid(s)
AAV	adeno-associated virus
AC	acetylated
ATP	adenosine 5'-triphosphate
bp	base pair(s)
BSA	bovine serum albumin
C	cytosine
CAM	chloramphenicol
CAT	chloramphenicol acetyl transferase
cc	cubic centimeter
cpm	counts per minute
CTP	cytidine 5'-triphosphate
^{o}C	degrees celsius
dATP	deoxyadenosine 5'-triphosphate
dCTP	deoxycytidine 5' -triphosphate
dGTP	deoxyguanosine 5' -triphosphate
dTTP	deoxythymidine 5' -triphosphate
dNTP	deoxynucleoside 5' -triphosphate
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ddNTP	dideoxynucleoside 5' -triphosphate
DMSO	dimethyl sulfoxide
ds	double stranded
EDTA	ethylenediaminetetra-acetic acid disodium salt
G	guanine
hr(s)	hour(s)
IPTG	isopropyl β-D-thiogalactopyranoside
kb	kilobase
KDa	kilodaltons
KOAc	potassium acetate
mA	milliamps

mg	milligrams
ml	milliliters
MVM	minute virus of mice
min	minutes
ng	nanograms
nt	nucleotide
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
rpm	revolutions per minute
RNA	ribonucleic acid
sec	seconds
ss	single stranded
SDS	sodium dodecyl sulphate
SSC	standard saline citrate
T	thymine
TEMED	N,N,N'N' - Tetramethylethylenediamine
µg	microgram
µl	microliter
u	units
UTP	uridine 5' -triphosphate
wt	wild type
X-gal	5-Bromo-chloro-3-indolyl-β -D-galactoside

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CHAPTER 1. INTRODUCTION

1.1 Discovery and classification of minute virus of mice

The minute virus of mice (MVM) was first isolated as a contaminant in a mouse adenovirus stock by Crawford in 1966. Subsequent characterization has placed this virus in the *Parvoviridae* family, a large family of small DNA viruses that infect a wide variety of vertebrate and invertebrate hosts. Electron microscopic studies have revealed that MVM, like all other parvoviruses, is an icosahedral, non-enveloped virus approximately 22 nm in diameter. The virus particles are highly stable and can be stored for years at 4 ^{o}C and still retain their infectivity. These viruses are also stable at pH ranges between 3 and 9 and are resistant to heating at 56 ^{o}C for up to one hour (reviewed by Tattersall and Cotmore, 1988). The genome of MVM consists of a single strand of negative sense DNA, approximately 5149 nucleotides in length, and like all parvoviruses contains terminal hairpin structures (reviewed by Astell, 1990). The nonstructural (NS-1) protein of MVM is covalently attached to the 5' terminus of the viral genome in infectious viral particles and it has been suggested that it protrudes from the capsid structure (Cotmore and Tattersall, 1989; Faust et al., 1989).

Parvoviridae are divided into three genera (Table 1) based on genome organization, helper function dependency and host specificity (Siegl et al., 1985). The autonomous Parvoviruses infect a wide range of mammalian and avian hosts. These viruses package predominantly the minus (non-coding) strand of the viral genome, with the exception of the human B19 parvovirus and the LuIII parvovirus, which package equal amounts of plus (coding) and minus strands (Bates et al., 1984; Siegl et al., 1985). The Dependoviruses, which package equal amounts of negative and positive sense DNA, are so named because of their requirement for adenovirus or herpesvirus coinfection for their replication (Berns and Hauswirth, 1984). Adeno-associated viruses can also replicate *in vitro* , if the cells are

Table 1. The *Parvoviridae* Family

Genus	**Virus** (host)
Parvovirus	
	MVM (mouse)
	H-1 (rat)
	FPV (feline)
	CPV (canine)
	PPV (porcine)
	ADV (mink)
	LuIII (rodent ?)
	B19 (human)
	BPV (bovine)
	LPV (lapine)
Dependovirus	
	AAV-1 (primate)
	AAV-2 (human)
	AAV-3 (human)
	AAV-4 (primate)
	AAAV (avian)
	AAV-X7 (bovine)
	CAAV (canine)
Densovirus	
	Galleria DNV (greater wax moth)
	Junonia DNV (Trinidadean buckeye caterpillar)
	Bombyx DNV (silkworm)
	Aedes DNV (mosquito)

pretreated with UV light or other DNA damaging agents (Yakobson et al., 1987; Yakobson et al., 1989). In the absence of a helper function the human dependovirus, adeno-associated virus (AAV), has the unique ability among mammalian DNA viruses to integrate into the host cell genome at a specific site on chromosome 19 (Kotin et al., 1990; Kotin et al., 1992). The third genus, Densoviruses, is less well characterized. These viruses package equal amounts of plus and minus sense DNA, do not require a helper function, and exclusively infect arthropods and arthropod cell lines (Siegl et al., 1985).

1.2 Parvovirus S-phase requirement

Parvoviruses have strict requirements concerning the states of proliferation and differentiation of potential host cell populations both *in vivo* and *in vitro*. It has been demonstrated that parvoviruses require actively dividing cells, that is, cells cycling through S-phase, for a productive infection to occur (Wolter et al., 1980). This requirement often manifests itself as a tropism for fetal and neonatal tissues. As each set of cell populations in the developing organism enters a relatively high rate of mitosis or a certain stage of differentiation, the virus selectively attacks those tissues. Parvovirus infection can have a wide range of effects, depending on the species of virus and the host. In the adult organism, most cell populations are relatively mitotically quiescent, with the notable exception of hematopoetic and gut epithelial tissues, and therefore the effects of parvoviral infection are often less devastating. Canine and feline parvoviruses, however, attack the gut epithelial cells and often cause extreme pathological consequences. The related rodent parvoviruses have less devastating effects on adult animals, while infection of the fetus or neonate is often fatal. When specific adult cell populations are artificially induced to enter a high rate of mitosis, these cells are then targeted by the virus. Partial hepatectomy, carbon tetrachloride exposure, or infection with the *Cysticercus fasciolaris* parasite, all of which induce high mitotic activity in the liver, allow the rat H-1 parvovirus to productively infect adult liver cells (reviewed by Cotmore and Tattersall, 1987). The human autonomous parvovirus, B19, attacks erythroid precursors and causes erythema infectiosum (a rash common to children), aplastic crisis in patients with hemolytic anemia and is also believed to be a causative agent in some forms of transient acute arthritis (reviewed by Pattison, 1990). Adeno-associated virus is not known to cause any pathological effects in humans.

In addition to a requirement for S-phase transition, parvoviruses require certain factors that are developmentally regulated. The MVMp (prototype strain) and MVMi (immunosuppressive strain) variants, which have over 97 % sequence similarity, are model examples for this requirement. The MVMp strain successfully infects certain mouse fibroblast cell lines, while it is unable to infect a transformed mouse lymphocyte cell line. The lymphotropic variant (MVMi) productively infects only T-lymphocyte mouse cell lines (Tattersall and Bratton, 1983). The block in infection is not due to a block in virus entry into the cell, but is due to an intracellular block in some other early stage of infection (Spalholz and Tattersall, 1983). Site-directed mutagenesis of two amino acids in the viral capsid protein VP-2 confers fibrotropism to the MVMi strain (Ball-Goodrich and Tattersall, 1992). The host cell specificity of feline and canine parvoviruses, which are also highly similar in their genomic sequence, is thought to be determined by amino acid substitutions in a similar region of their capsid proteins (Parrish, 1991).

The transformed state of the cell also affects the ability of the parvovirus to successfully infect that cell. PVR cells, a Balb/c 3T3 mouse cell variant, are normally refractory to MVM infection. If they are transformed by SV40, however, they are fully susceptible to MVM infection. If the PVR cells are only coinfected with MVM and SV40, MVM is unable to replicate, suggesting that the MVM requirement is not necessarily for an SV40 viral protein, but possibly for an SV40 activated cellular protein (Mousset and Rommelaere, 1982). Cellular transformation by specific oncogenes or chemical carcinogens results in an increased susceptibility to productive parvovirus infection (Chen et al., 1986; Mousset et al., 1986; Cornelis et al., 1988a; Van Hille et al., 1989; Salome et al., 1990).

1.3 Cytotoxicity

The isolation of various species of parvoviruses from tumors or as contaminants in tumor virus stocks, suggested at first that these viruses had oncogenic potential. It is now known that the converse is true. Because of their high replication rate, tumor cells provide an ideal environment for parvoviral replication. In fact, it has been demonstrated that parvovirus infection actually leads to a decrease in the incidence of spontaneous tumor formation. In one study, with a large population size, hamsters that had survived H-1 parvovirus infection as neonates exhibited a five-fold lower incidence of spontaneous

tumor formation as compared to their uninfected siblings (Toolan, 1967). Severely infected hamsters that exhibited craniofacial lesions had a twenty-fold lower incidence of tumor formation. This study suggested that H-1 infection somehow had a preventive effect on tumorigenesis. In another study, H-1 infection in nude mice significantly inhibited subsequent tumor formation of implanted tumor cells (Dupressoir et al., 1989).

Serological studies of human populations show that over 80% of the general population has previously been infected with AAV, while groups that had cervical or prostate carcinomas showed only a 14% incidence of previous AAV infection (Mayor et al., 1976; reviewed by Rommelaere and Tattersall, 1988). These studies also indicated that parvovirus infection may interfere with tumor formation. Although the mechanism of action is not known, it may simply be a result of parvoviral affinity for the transformed cell. The production of virus and subsequent lysis of the cell, however, is not necessary for this cytotoxic activity (Guetta et al., 1990; Cornelis et al., 1988b).

Expression of the viral structural proteins is not cytotoxic to cells (Pintel et al., 1984). The inability to obtain stably transformed cells expressing the nonstructural proteins, however, has implicated these proteins in the cytotoxic effect (Rhode, 1987; Ozawa et al., 1988). Cells with stably integrated copies of the MVM genome that are induced to produce the NS proteins show a cytotoxic effect (Caillet-Fauquet et al., 1990). In cotransfection experiments, the nonstructural proteins of MVM, H-1, or AAV have a cytotoxic activity towards transformation to neomycin resistance *in vitro* (Brandenburger et al., 1990; Labow et al., 1987; Ozawa et al., 1988; Rhode, 1987). The AAV nonstructural proteins inhibit bovine papillomavirus mediated transformation (Hermonat, 1989), as well as SV40 T antigen and E1A/*ras* mediated transformation (Khleif et al., 1991). The mechanism by which the nonstructural proteins achieve this cytotoxic action is not known, but it has been suggested that it may be due to transcriptional regulation of cellular promoters by these proteins (Li and Rhode, 1990).

The parvovirus nonstructural gene product is a transactivator. Initial attempts to construct cell lines that constitutively expressed the H-1 NS-1 gene by cotransfecting a selectable marker and plasmids expressing the NS-1 gene under the control of the nonstructural gene promoter failed to yield any stable clones (Rhode and Richard, 1987). A marked reduction in clones expressing neomycin resistance was noted and attributed to the

cytotoxic activity of NS-1 (Rhode, 1987). When a construct containing a capsid gene promoter-inducible NS-1 gene and chloramphenicol acetyl transferase (CAT) gene was transfected into permissive cells, along with a plasmid containing the neomycin resistance gene , G418-resistant clones were obtained. Low level expression of NS-1 and CAT was obtained by treating these cells with 5 mM sodium butyrate or superinfecting with H-1, CPV or MVM. Prolonged attempts at induction, however, resulted in a steady decrease of CAT activity (Rhode, 1987).

1.4 MVM genome organization

1.4.1 Coding strategy

Due to the relatively small size of their genomes, parvoviruses depend to a great extent on host cell functions for their replication. As a result, the evolution of parvoviruses has been closely associated with that of their hosts (Bando et al., 1987). Parvoviruses have developed different strategies, including alternative splicing, use of overlapping transcription units, and proteolytic cleavage of translation products, to maximize the coding potential of their small genomes. The MVM genome, like that of almost all other members of the *Parvoviridae* , is divided by convention into left and right halves which code for the nonstructural (NS) and structural (VP) proteins, respectively (Fig. 1). The only known exception to this organization is the *Junonia coenia* densovirus, which utilizes the complementary strand to produce the nonstructural proteins (Dumas et al., 1992).

1.4.2 Hairpin termini

Many parvovirus genomes have been cloned into prokaryotic vectors and their sequences have been determined. Like other such clones, the MVMp clone (pMM984) is infectious when transfected into permissive cell lines (Merchlinsky et al., 1983; Merchlinsky, 1984). Upon transfection, the MVM genome is excised, replicated and infectious virus is produced. Much of the work that has been done in determining key steps in the replication mechanism of parvoviruses has been done with this plasmid. Sequence analysis has revealed that the ends of all parvovirus genomes contain palindromic sequences that can form imperfect hairpin structures (Astell, 1990). The AAV

Figure 1 Coding organization of the MVM genome.

The three major mRNA transcripts (R1, R2, R3) and open reading frames (F1, F2, F3) are shown. The open reading frames corresponding to the major forms of each protein are also shown. (From Cotmore and Tattersall, 1987)

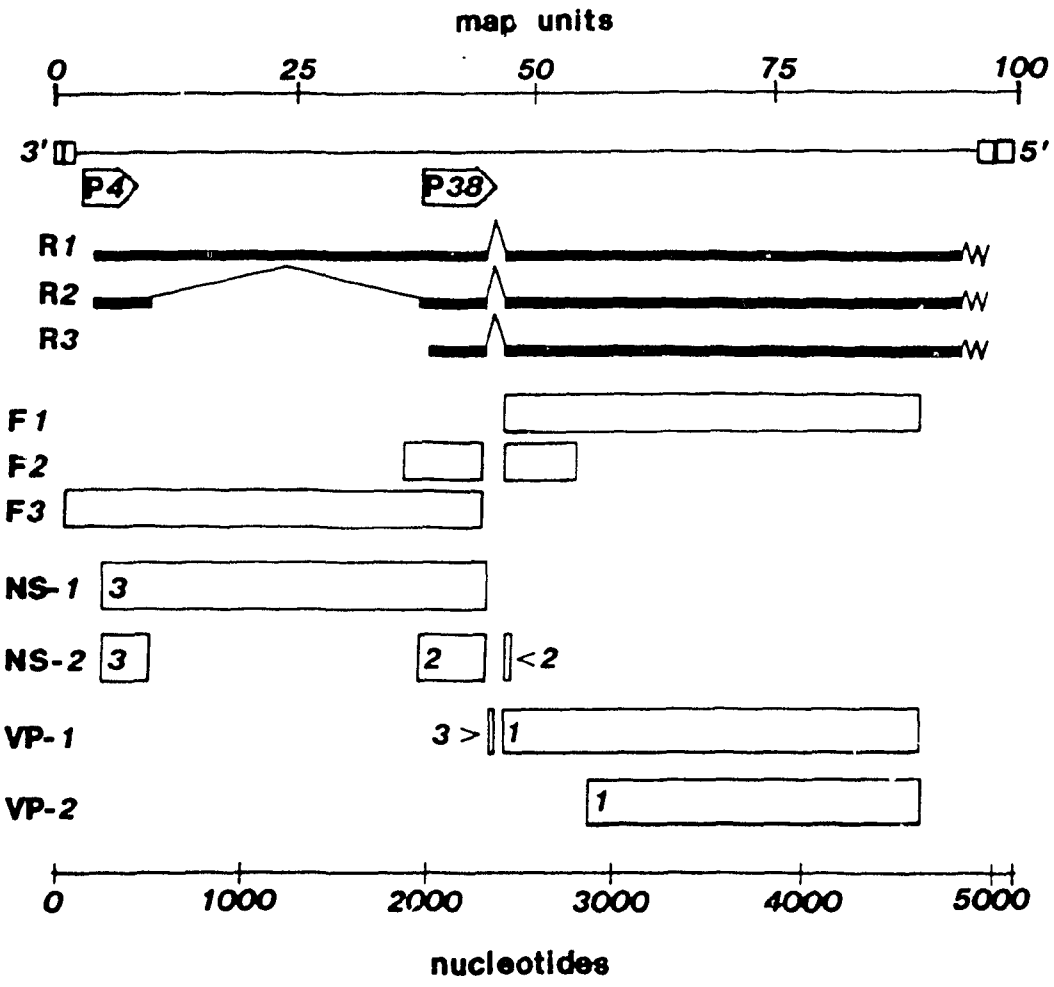


Figure 1 Coding organization of the MVM genome

dependovirus hairpins are 145 nt long, direct terminal repeats which contain palindromes. The sequences at the 3' and 5' ends are the inverted complement of each other, so that the single-stranded DNA is capable of forming a circle with a panhandle structure.

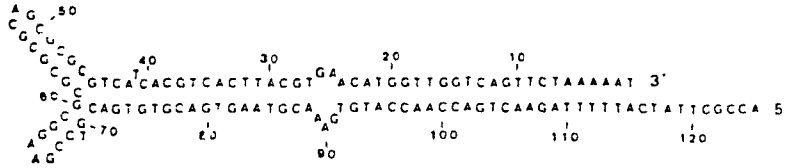
Autonomous parvoviruses, with the exception of the human parvovirus B19 which contains hairpins similar to AAV, contain different sequences at their respective 3' and 5' ends. The 5' end hairpin of MVM is 206 nt long and contains internal palindromes which allow the formation of a cruciform structure. Like the dependovirus hairpins, this hairpin is found in two sequence orientations, termed "flip" and "flop", which differ in the region that is not perfectly base paired, and are the inverted complement of each other (Fig. 2) (Astell et al., 1983). The 5' end of the MVM viral genome is approximately 18 nt shorter than the double-stranded or replicative (RF) form (Astell et al., 1983). This may be due to a purification artifact which removes the terminal protein along with these nucleotides (Cotmore and Tattersall, 1989; Faust et al., 1989). The MVM 3' hairpin is 115 or 116 nucleotides long and can form a Y-shaped structure (Fig. 2). This palindromic sequence is also imperfect, however, only one sequence is found in viral DNA (Astell et al., 1985). While the primary sequences may vary among species, the secondary structures, including an unpaired bubble region and potential cruciform structures, are conserved. These structures are crucial for replication of the genome, since mutations that can affect the secondary structure prove to be lethal (Lefebvre et al., 1984; Merchlinsky et al., 1983; Salvino et al., 1991).

It was recently proposed that parvoviruses arose from a common ancestor, which may itself have evolved from a cellular transposon (Fisher and Mayor, 1991). This protoparvovirus is hypothesized to have been defective for replication, and like modern-day dependoviruses it contained identical hairpin terminal ends. AAV is therefore thought to more closely resemble the ancestral state, and the human autonomous parvovirus B19, which has some structural features similar to dependoviruses, is believed to resemble an intermediate stage between the autonomous and dependovirus parvoviruses (Fisher and Mayor, 1991).

Figure 2 Structure of the MVM hairpin terminal ends.

Potential secondary structures of the A.) 3' end hairpin and B.) 5' hairpin.

C.) The two sequence orientations found at the 5' terminal hairpin. (From Astell, 1990)



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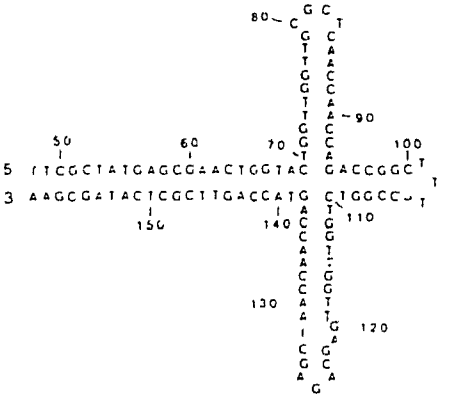

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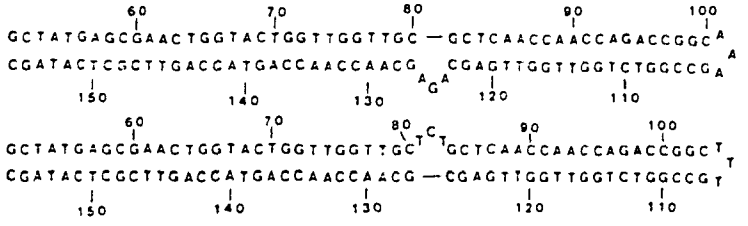

C

Figure 2 Structure of the MVM hairpin terminal ends

1.4.3 MVM proteins

The MVM genome codes for two sets of proteins: the nonstructural (NS) and the capsid proteins (VP) (Fig. 1). While both sets of proteins are required for some aspect of viral DNA replication, only the NS proteins are absolutely required. The VP-1 protein (83 kDa) is required for the production of infectious particles (Tullis et al., 1993). Besides formation of the capsid structure, mutagenesis studies reveal that the VP-2 protein (64 kDa) is also required for single-stranded viral DNA accumulation and encapsidation, while the VP-1 protein is dispensable for these functions (Merchlinsky, 1984; Tullis et al., 1993). In infectious capsids, the VP-2 protein is found in a 5:1 ratio with respect to VP-1. A third structural protein, VP-3 (63 kDa), whose function is unknown, is found in purified capsids containing genome-length DNA, and is a cleavage product of VP-2 (Tattersall et al., 1976; Tattersall et al., 1977). Several types of viral particles have been detected in preparations from infected cells. These particles are separated on the basis of differential buoyant densities in CsCl gradients. Full infectious particles contain full-length single-stranded genome, empty particles contain only the VP-1 and VP-2 proteins and defective particles contain deleted forms of the DNA genome (Tattersall et al., 1976; Faust and Ward, 1979).

The NS proteins are more intimately involved in viral DNA replication. The NS-1 protein (83 kDa) is the major non-structural protein. It is a multifunctional nuclear phosphoprotein, and is absolutely required for replication of viral DNA and transcriptional regulation of the two MVM promoters. In addition to these functions, NS-1 has been shown to have a cytotoxic effect when overexpressed in certain cell lines (van Hille et al., 1989; Caillet-Fauquet et al., 1990). The AAV nonstructural proteins (rep proteins) have been purified from infected cells (Im and Muzyczka, 1990; Im and Muzyczka, 1992), and the MVM nonstructural proteins have been overexpressed with baculovirus (Wilson et al., 1991) and vaccinia (Nuesch et al., 1992) expression vectors. As described below (section 1.7), these purified proteins have exhibited helicase, ATPase and site specific endonuclease activities.

NS-2 (25 kDa) is a predominantly cytoplasmic phosphoprotein (Clemens et al., 1990; Cotmore and Tattersall., 1990), and is required for DNA replication and cytotoxicity (Brandenburger et al., 1990) in a host-specific manner; that is, mutations in the NS-2 gene

affect the ability of the virus to replicate in some mouse cell lines, while replication in primate cells is not affected (Naeger et al., 1990; Li and Rhode, 1991; Cater and Pintel, 1992). NS-2 is also required for productive infection in newborn mice (Brownstein et al., 1992). Although its precise role is still unknown, it was recently shown that mutations in NS-2 affect the efficiency of translation of viral transcripts in mouse cells (Naeger et al., 1993).

1.4.4 MVM transcription units

Most parvoviruses have two promoters that regulate transcription of the nonstructural and capsid protein genes, respectively (reviewed by Cotmore, 1990). The nonstructural gene promoter is located at the left end of the genome (Fig. 1; map units 4-6), and the capsid gene promoter is located near the middle of the genome (map units 38-40) (Lebovitz and Roeder, 1986; Pintel et al., 1983). The AAV parvovirus has an additional promoter at map unit 19 which drives the production of additional species of Rep proteins (Mendelson et al., 1986). The human autonomous parvovirus B19 lacks a functional capsid gene promoter. Transcription of the capsid genes in this virus is driven from the 3' hairpin proximal promoter (p6), which also drives expression of the nonstructural genes (Liu et al., 1991a; 1991b). The MVM promoters contain a TATA box (TATATAA), an Sp1 transcription factor binding GC box (TGGGCGTGGT) and a CCAAT box (CCAATCT in p38 only) upstream of the site of transcription initiation (Pintel et al., 1983; Gavin and Ward, 1990; Ahn et al., 1992). A transactivation responsive element (tar) located approximately 130 nt upstream of the site of mRNA transcription initiation has also been reported to be required for full transactivation of the capsid gene promoter in the H-1 parvovirus (Rhode and Richard, 1987; Gu et al., 1992), but not for the MVM p38 promoter (Ahn et al., 1992).

All MVM RNA transcripts are copied from the viral DNA strand of double-stranded DNA intermediates. Three major species of viral encoded, spliced and polyadenylated RNA transcripts have been detected in MVM infected cell lines (Pintel et al., 1983). These transcripts are termed R1, R2 and R3 (Fig. 1). Although several potential polyadenylation signals are present, the same polyadenylation signal, located near nucleotide 4885, is used by all the transcripts (Clemens and Pintel, 1987). All three species of transcripts also have a similar intron sequence between map units 46 and 48

removed (Morgan and Ward, 1986). Each major RNA species has major and minor forms of alternatively spliced RNA's (Clemens et al., 1990; Jongeneel et al., 1986)

The promoter at map unit 4 (p4) drives the transcription of the R1 and R2 transcripts (Cotmore et al., 1983; Pintel et al., 1983; Cotmore and Tattersall., 1986b). These transcripts are formed from a common precursor (Pintel et al., 1983). Translation of R1 results in the production of NS-1 which is translated from one continuous open reading frame (Cotmore et al., 1983). R2 is translated into NS-2, which shares a common N-terminus (84 a.a.) with NS-1, but a different carboxyl terminal half, due to the removal of a large intron between map units 10 and 39 in R2 and a change in the reading frame (Cotmore and Tattersall., 1986b). The removal of additional alternative introns near the extreme 3' end of the R2 transcript (map units 44-46) results in three isoforms of NS-2 being produced which vary in the C-terminal 6 to 15 amino acids (Clemens et al., 1990; Cotmore and Tattersall, 1990). Regulation of splicing of the R1 and R2 transcripts and the relative protein stabilities of their products determine the abundance of the NS proteins (Cotmore and Tattersall, 1990).

The most abundant transcript, R3, is transcribed from the promoter located at map units 38-39. Two capsid proteins are produced from two alternatively spliced versions of this transcript (Labieniec-Pintel and Pintel, 1986). VP-1 results from translation initiating in a small open reading frame that is not present in the R3 transcript that encodes VP-2 (Paradiso et al., 1984). Translation of VP-2 begins at another AUG that is farther downstream. VP-2 therefore shares a common sequence with VP1 (Tattersall et al., 1977).

In an MVM infection there is a temporal phasing of transcription and translation. The R1 and R2 transcripts are detected within two hours of infection of highly synchronized cells, and the NS proteins are detected before any R3 transcripts are transcribed. The R3 transcripts, as well as capsid protein production, reach a high level of accumulation late in infection (Clemens and Pintel, 1988; Schoborg and Pintel, 1991).

1.5 MVM infection

Because parvoviruses are unable to induce the host cell to enter S-phase, like many other DNA viruses can (Tattersall, 1972), MVM infection of tissue culture cells leads to a

population of cells in various stages of viral replication. In order to study the time course of events associated with viral DNA replication, cells must be induced to enter S-phase in a synchronous manner. This is achieved *in vitro* by starving cells of isoleucine and by adding a DNA polymerase α inhibitor (aphidicolin) into the culture medium (Cotmore and Tattersall, 1987; Clemens and Pintel, 1988). Removal of these blocks leads to a synchronous entry of the culture into S-phase.

The initial steps involved in parvoviral entry and uncoating remain for the most part unknown. CPV uptake requires a low pH mediated step which suggests that this virus enters by endocytosis (Basak and Turner, 1992). While CPV enters the cell via small uncoated vesicles, MVM attaches to neuraminidase-sensitive cell surface receptors and enters the cell via coated vesicles (Linser et al., 1977). The virus particle finds its way to nucleus by an, as yet, unknown mechanism, where the DNA is extruded from the capsid.

In synchronized cells, viral NS and VP proteins can be detected within two hours of the cell entering S-phase, and reach a peak six hours after release of the aphidicolin block (Cotmore and Tattersall, 1987). Amplification of single stranded viral DNA to covalently closed duplex DNA occurs between 6 and 16 hours post-release (Ward and Dadachnji, 1978; Tattersall and Cotmore, 1990). By 16 hours post-infection most of the duplex DNA contains nicks (Ward and Dadachnji, 1978). Viral DNA isolated late in infection (38 hours) is found to occur as single-stranded, duplex, dimeric and tetrameric duplex forms. Cellular DNA replication is inhibited as parvoviral DNA replication reaches a peak (Tattersall and Cotmore, 1990). Single stranded DNA is packaged and virus is released by lysis of the cell (Siegl, 1984), although there have been reports of non-lytic productive infections (Salome et al., 1989; Guetta et al., 1990).

1.6 Steps in viral DNA replication

Several replication models which account for structural features of the viral genome and for certain replication intermediate molecules detected in infected cells have been proposed (Tattersall and Cotmore, 1990). The currently accepted model is a modification of the rolling circle replication model originally proposed by Cavalier-Smith (1974). In the modified rolling hairpin model (Fig. 3), it is postulated that the 3' end of the viral strand is used by a host cell polymerase(s) as a primer to synthesize the complementary DNA strand.

This generates a monomer duplex replicative form (RF) intermediate, which is covalently closed at the left end. It is postulated that a site-specific nickase creates a nick approximately 18 nucleotides inboard of the original 5' hairpin. At this point, the new 3' hydroxyl group is used to prime strand displacement synthesis creating dimer RF and then tetramer RF intermediates, which are then subject to nicking in several areas as indicated in Fig. 3. Isolated dimer RF DNA has been shown to collapse to unit length single strands and monomer RF molecules when electrophoresed under denaturing conditions (Ward and Dadachanji, 1978).

As shown in Fig. 3, the sequence that joins the monomer duplex molecules (dimer bridge) corresponds to the 3' terminal hairpin sequence. It is proposed that a site-specific nickase acts specifically on the parental strand, to resolve the 3' end termini. This step and the existence of these intermediates accounts for the fact that the 3' hairpin in progeny single-stranded viral DNA is found only in one configuration. A single orientation at the 3' end can only occur if the putative topoisomerase-like enzyme nicks the dimer bridge, exclusively in the parental strand. This model also takes into account the finding that the 5' hairpin sequence is found in one of two configurations, flip or flop (Fig. 3). These differ slightly in primary sequence in the areas of the hairpin where nucleotides are not perfectly paired. The flip and flop structures predicted to be formed by the hairpin transfer model have been detected in DNA isolated from cells transfected with clones containing a single sequence orientation (Merchlinsky et al., 1983; Tattersall and Cotmore, 1980).

All parvoviruses produce defective interfering particles at a high frequency, during each replication cycle (Carter, 1984). These particles contain varying amounts of viral DNA, which contains internal deletions of up to 90 % of the genome (Faust and Ward, 1979). The *cis*-acting sequences, which encompass the hairpin termini and are required for replication, make up most of the genome of the defective particles. Nonhomologous recombination between short repetitive sequences found near the termini may be the mechanism by which these deletions occur (Hogan and Faust, 1986). The sequence C T $^{A}/_{T}$ T T $^{C}/_{T}$ is found at or near the deletion junctions of these molecules. The site-specific nicking enzyme that resolves the hairpin termini is believed to be involved in the

Figure 3 Model for MVM DNA replication.

Modified rolling hairpin model for MVM DNA replication. ABa, 3' hairpin sequence; EFGf, 5' end hairpin sequence. T, site specific nickase; x, putative nickase site; V^{par}, parental viral DNA strand; V^{prog}, progeny viral DNA. V, virion strand; C, complementary strand. (from Berns, 1990)

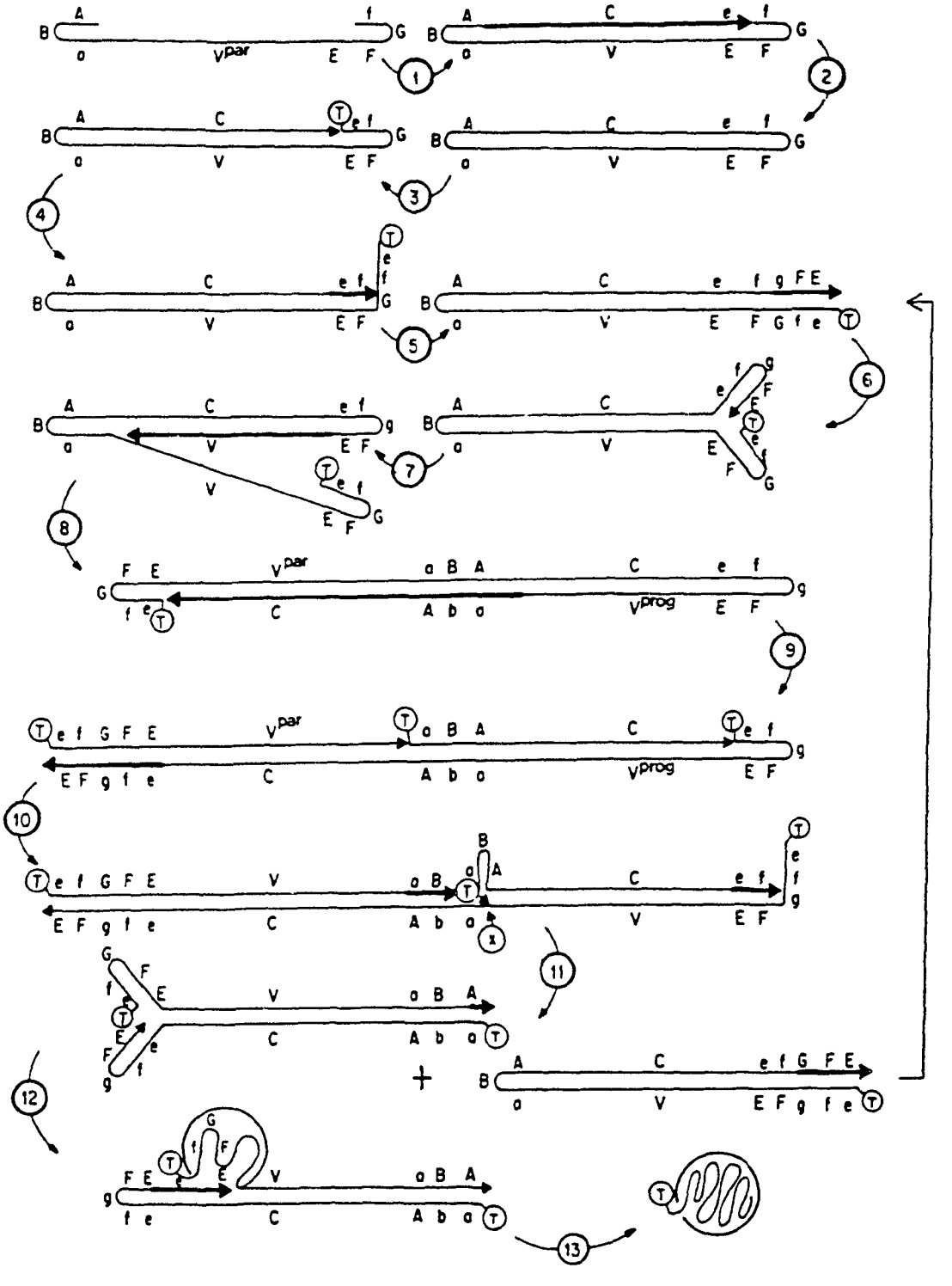

Figure 3 Model for MVM DNA replication

process that creates these deletions (reviewed by Faust and Hogan, 1990), since this consensus sequence is also found at the putative nicking sites of the 5' and 3' hairpins (Hogan and Faust, 1986).

In addition to the terminal hairpin sequences other *cis*-acting sequences required for replication have been identified. The H-1, FPV, CPV and MVMp parvoviruses contain multiple 55-65 nt long repetitive A/T rich elements inboard of the 5' end hairpin (Tattersall and Cotmore, 1990). MVMp contains two tandemly arranged 65 nt repeats, while the MVMi variant has only one. Deletion of one of the MVMp repeats in pMM984 caused the viral genome to replicate at only 10 % of wild type levels in mouse A9 cells, while replication in the African green monkey cell line COS-7 was reduced to less than 1 % of wild type (Salvino et al., 1991).These results indicate that this element acts in *cis* to regulate replication, although its precise role in this process remains unknown. Tam and Astell (1993) also described *cis*-acting sequences inboard of the 5' end terminal hairpin that are required for replication.

1.7 Role of the nonstructural proteins in viral DNA replication

1.7.1 NS-1 is required for replication

Transfection of the MVM clone, pMM984, into permissive cells results in excision and replication of the MVM genome. An insertion mutation in the middle of the NS-1 coding sequence in pMM984, which disrupts the reading frame, caused this plasmid to become fully defective for both excision and replication (Merchlinsky et al., 1983; Merchlinsky, 1984). Subsequent studies have confirmed the essential role of the nonstructural proteins of MVM and other parvoviruses in viral DNA replication. Single amino acid insertions at a number of sites in the NS-1 coding sequence affected the ability of pMM984 to produce plaques on mouse A9 cell monolayers (Tullis et al., 1988). Some of these mutants had a temperature-sensitive phenotype for plaque formation and were therefore useful in demonstrating that NS-1 is indeed required for MVM replication. The NS-1 protein was shown to be required for excision, as well as replication (Rhode, 1989).

The AAV nonstructural gene codes for four proteins that are collectively called rep proteins. These proteins are all encoded from the same open reading frame. The AAV p5

promoter drives transcription of the rep78 and rep68 proteins. The transcript that encodes rep68 undergoes a splicing event that removes sequences present near the C-terminal end of rep78. The p19 promoter drives transcription for the rep40 and rep52 proteins. The transcript that encodes the rep40 protein undergoes a similar splicing event as the rep68 RNA transcript (Becerra et al., 1988; Carter et al., 1990). The 78 kDa and 68 kDa proteins are absolutely required for replication of the viral genome (Hermonalt et al., 1984; Tratschin et al., 1984). The rep52 and rep40 proteins are required for infectious virus production (Chejanovsky and Carter, 1989). Purified rep78 and 68 bind to the viral hairpin terminal ends and cut at a putative nickase site (Im and Muzyczka, 1989; 1990). Im and Muzyczka (1990, 1992) have also shown that these proteins contain a helicase activity that depends on the binding or hydrolysis of ATP. This helicase activity is thought to unwind the hairpin terminal end during replication.

1.7.2 *In vitro* concatemer resolution

The requirement for the nonstructural gene in a key step in the replication of parvovirus DNA was recently confirmed *in vitro*. The AAV rep78 and 68 proteins were purified from AAV infected cells. They were shown to bind the AAV terminal repeat only when it was in a hairpin conformation, and were able to protect it from DNase digestion (Im and Muzyczka, 1989). Extracts from adenovirus and AAV coinfected cells contained an activity that resolved a covalently closed AAV DNA substrate by nicking the parental strand at a site near the end of the hairpin, the terminal resolution site (TRS), and synthesized new DNA to extend the hairpin (Snyder et al., 1990a). Purified AAV 68 kDa protein was found to contain a magnesium ion and ATP-dependent, strand and site-specific endonuclease activity and a helicase activity; furthermore, this protein was found to be covalently attached to the 5' end of the DNA at the site of nicking in similar *in vitro* reactions (Im and Muzyczka , 1990). This attachment was through a tyrosine residue on rep68 and the 5' phosphate of a thymidine residue (Snyder et al., 1990b). In a normal AAV infection a terminal protein attached to the viral DNA has not been detected (Snyder et al., 1990b). The *in vitro* resolution reaction was also insensitive to topoisomerase I and II inhibitors and the RNA polymerase II inhibitor, α amanitin, but was inhibited by high concentrations of p-n-butylphenyl dGTP and p-n-butylanilino dATP, suggesting that the cellular polymerase δ was responsible for leading-strand synthesis at the resolution site (Snyder et al., 1990b).

Viral DNA fragments isolated from MVM infected cells and consisting of MVM dimer bridges of left end:left end and tetramer right end:right end bridge fusions were cloned into *E.coli* plasmids. Mouse A9 cells were transfected with these plasmids and were then superinfected with wild-type MVM. The concatemers were resolved and the plasmids replicated as linear molecules with MVM termini (Cotmore and Tattersall, 1992). The 3' end bridge plasmid resolved and replicated, yielding three distinct forms: a resolved and replicated covalently closed turnaround form representing the major product of the reaction, a resolved and replicated extended form containing the NS-1 protein attached to the 5' ends of the molecule, and a small amount of a newly synthesized palindromic duplex that was still unresolved. The right end:right end plasmid resolved to predominantly one form, a linear molecule with the NS-1 protein attached at the extended 5' ends. The existence of turnaround forms on the replicated 3' end fusion molecules provided further evidence for a nicking and ligation step, as was suggested previously (Cotmore et al., 1989). These results showed that the 3' and 5' ends are processed differently, and that the structures produced *in vitro* are similar to intermediates that have been detected in MVM infected cells (Cotmore et al., 1989; Ward and Dadachanji, 1978).

NS-1 protein produced by recombinant vaccinia virus in conjunction with HeLa cell extracts was capable of resolving and replicating the 5' end fusion sequence *in vitro* , yielding a linearized plasmid with extended termini at each end and a covalently attached NS-1 protein on the 5' ends (Nuesch et al., 1992). Similar assays using the 3' end junction plasmid showed that NS-1 resolved these sequences *in vitro* asymmetrically (Cotmore et al., 1993). The major product of this reaction was a linearized plasmid containing an extended hairpin at one end with a covalently attached NS-1 protein on the 5' overhang, and a covalently closed turnaround form at the other end of the linearized plasmid. However, although only one strand was nicked in this reaction it is not the viral strand sequence as predicted by the current model. What is remarkable about the role of NS-1 in the resolution reactions is that the primary sequences at the resolution sites and the putative secondary structures adopted by the 3' and 5' palindromes are quite different, yet NS-1 is able to catalyze both sets of reactions. It has been suggested that the reactions mediated by NS-1 are somewhat analogous to the role of the A protein of ϕX174 in replicating the phage genome through a rolling circle replication mechanism (Astell et al., 1985; Cotmore et al., 1993). The A protein has been shown to be a topoisomerase that

mediates nicking and rejoining of phage DNA through two closely situated tyrosine residues (van Mansfeld et al., 1986).

1.7.3 Nucleotide-binding domain

In most parvoviruses a stretch of approximately 200 amino acids, comprising the central region of the NS-1 proteins, is highly conserved (Astell et al., 1987). Sequence comparisons have revealed that the parvoviral NS-1 and rep proteins contain a highly conserved A-type nucleotide binding motif ($^{G}/_{A}$ X_4 G K $^{S}/_{T}$) that has been identified in other ATP-binding proteins such as SV40 large T-antigen and the E1 proteins of papillomaviruses (Anton and Lane, 1986; Astell et al., 1987). This consensus is found in the middle of the highly conserved region of NS-1. Mutagenesis of the putative active site lysine (Lys405) to serine in the H-1 parvovirus NS-1 protein completely disrupted its DNA replication, transactivation and cytotoxicity activities (Li and Rhode, 1990). Mutagenesis of the analogous lysine residue in the AAV rep proteins resulted in a dominant negative DNA-replication phenotype (Chejanovsky and Carter, 1990). Using vaccinia-produced MVM NS-1 protein, it was demonstrated that conversion of the putative active site lysine to arginine or methionine abolished transactivation of the p38 promoter and concatemer resolution, while translocation to the nucleus was unaffected (Nuesch et al., 1992). MVM NS-1 protein produced in insect cells, using a baculovirus expression vector, exhibited ATPase and ATP-dependent helicase activities (Wilson et al., 1991).

1.7.4 NS-1 nuclear localization signals

Wild type NS-1 is predominantly localized to the nucleus (Cotmore and Tattersall, 1986a). A putative nuclear translocation signal was described for NS-1 by Nuesch and Tattersall (1992). A deletion of this motif, encompassed by residues 198-234, in recombinant vaccinia virus produced MVM NS-1, resulted in cytoplasmic compartmentalization of NS-1. Mutagenesis of the putative nuclear localization signal KKK (a.a. 214-216) also resulted in the majority of the protein localizing in the cytoplasm. A C-terminal end deletion, however, did not affect the localization of NS-1 . When the C-terminal deleted NS-1 protein and the KKK substitution mutant were expressed together,

both proteins were localized to the nucleus. This suggested that the proteins may normally be found as multimers, and that the C-terminal deleted protein facilitated the nuclear localization of the KKK mutation. Another motif that may be involved in nuclear localization was described by Chen et al. (1986). The sequence GKRN (a.a. 390-393), found in the highly conserved region of NS-1, is similar to the nuclear localization signal used by the yeast mating type locus protein (Hall et al., 1984).

1.7.5 SV40 large T-antigen

In many respects the parvoviral rep proteins are similar to other viral replication proteins. The simian virus 40 (SV40) large T-antigen is a multifunctional protein required by the virus for replication of its DNA, and for regulation of the viral early and late promoter. The large T-antigen suppresses transcription of the early promoter gene, which is expressed constitutively in its absence. Late in infection, the large T-antigen transactivates capsid protein mRNA production from the SV40 late promoter. This protein also transactivates cellular genes that are required for viral DNA replication, binds the cell growth factor p53 and the retinoblastoma suppresser protein, and also causes the immortalization of primary cells. It has also been shown that the large T-antigen binds the DNA polymerase α-primase complex, which in conjunction with DNA polymerase δ, PCNA, and topoisomerase I and II is responsible for replicating the viral genome by semidiscontinuous DNA synthesis (reviewed by Eckhart, 1990).

The large T-antigen plays a key role in the synthesis of viral DNA during the course of infection. The known activities include binding DNA at the site of initiation of viral DNA replication (Arthur et al., 1988), ATPase and DNA helicase activities that are required for viral DNA replication, and induction of ribosomal RNA synthesis and DNA synthesis (Gaudray et al., 1980). Extensive site-directed mutagenesis of this protein has revealed much of its domain structure (Shenk et al., 1976; Clarke et al., 1983; Fanning, 1992). The activities described above are localized to specific regions on the polypeptide and can often function as independent polypeptide units (Wun-Kim and Simmons, 1990). T-antigen is a heavily phosphorylated nuclear protein. Phosphorylation at serine or threonine residues by cellular kinases appears to play a critical role in creating T-antigen molecules that are active or inactive for replication functions, depending on the states of phosphorylation. Mutagenesis studies and *in vitro* studies, using purified large T-antigen

and cellular phosphatases and kinases, have revealed that dephosphorylation of four serine residues results in an activation of the large T-antigen to function in DNA binding and replication (Scheidtmann et al., 1991). Phosphorylation of a single threonine residue is also required for DNA replication. The state of phosphorylation, which directly affects the progression of the infection, is modulated by interactions between the T-antigen and the cellular proteins (reviewed by Prives, 1990).

1.8 Role of the nonstructural proteins in transcriptional regulation

1.8.1 NS-1 *trans*-activates the capsid gene promoter

The parvovirus nonstructural gene has been shown to activate transcription from the capsid gene promoter in *trans* (Rhode, 1985; Rhode and Richards, 1987; Doerig et al., 1988). By creating a C-terminal truncation mutation of NS-2, Doerig et al. (1988) showed that the NS-1 protein is the only viral encoded protein required for transactivation of the MVMi p38 promoter in HeLa cells or in mouse EL-4 cells in CAT assays. It was demonstrated that the transactivation was brought about by increasing mRNA production from the capsid gene promoter. Relatively few mutations that affect transactivation have been described. As discussed above, mutagenesis of the nucleotide binding motif has been shown to affect the transactivation function. Also, deletion of residues near a.a. 357-546, 546-610 and truncation mutations deleting the C-terminal half of NS-1 abolished the transactivation function (Rhode and Richard, 1987).

The uninduced p4 promoter is constitutively strong and the p38 promoter is weak (Ahn et al., 1989). Deletion mutations show that the GC box and the TATA box are the only *cis* -acting elements required for transcription from the p4 and p38 promoters (Ahn et al., 1989; Gavin and Ward, 1990; Ahn et al., 1992). It is possible that NS-1 interacts with cellular transcription factors such as Sp1 and TFIID, which are known to bind with the GC and TATA boxes (Ahn et al., 1992). Deletion of the CCAAT box sequence located in the p38 promoter affects transcription *in vivo* but not *in vitro* (Gavin and Ward, 1990). The mechanism of NS-1 mediated transactivation of the MVM promoters is not known. Krauskopf et al. (1990) suggested that NS-1 binds to a *cis*-acting element 282 to 647 base pairs downstream of the site of transcription initiation in the p38 promoter *in vitro*, and that this binding may be through a cellular factor. However, the significance of this *cis*-acting

element *in vivo* is not clear. The transactivation of the p4 promoter by NS-1 is less well defined, but it has been shown that the terminal repeats and replication of the genome are required for full stimulation of this promoter (Hanson and Rhode, 1991; Ahn et al., 1989).

NS-1 was shown to inhibit transactivation of heterologous promoters including the SV40 early promoter, the Rous sarcoma virus (RSV) long terminal repeats (LTR), the human immunodeficiency virus (HIV) LTR, the Ha-*ras* and the H-1 parvovirus p4 promoters (Rhode and Richard, 1987). In a recent study using site-directed mutagenesis, Legendre and Rommelaere (1992) determined that a C-terminal and N-terminal region of the NS-1 protein are required for full heterologous promoter repression and cytotoxicity.

1.8.2 The rep78 and rep68 proteins regulate transcription

The AAV rep78 and rep68 proteins regulate transcription of the AAV promoters. Mutations in these proteins disrupt their ability to activate the capsid gene promoter. In the absence of helper virus coinfection, these proteins repress transactivation (Tratschin et al., 1986; Labow et al., 1986). Mutational analysis of these proteins has begun to uncover their domain structure. Insertion mutations have revealed regions that are required for transactivation as well as suppression of E1A/*ras* mediated transformation, and that these activities are genetically separable (Yang et al., 1992). In that study, the N-terminal region of rep78 was required for transformation repression, and the C-terminal end was dispensable for transactivation. In another study, McCarty et al. (1992), used deletions and point mutations to uncover domains that were required for hairpin binding, transactivation, replication and concatemer resolution. Mutations that affected transactivation and replication did not affect binding to the hairpin termini. However, no distinct transactivation or DNA replication domain has been identified, as has been done for other viral transactivating proteins including the well characterized adenovirus E1A protein.

1.8.3 E1A as a model viral transactivator protein

While the SV40 large T-antigen is the paradigm viral DNA replication protein, the adenovirus E1A proteins have been studied extensively in order to dissect the processes involved in transcriptional regulation. E1A plays a central role in adenovirus replication by activating or repressing viral and cellular promoters. Like the SV40 large T-antigen, E1A

is involved in immortalization of primary cells, and binds the retinoblastoma suppresser protein, as well as a host of other cellular proteins. Adenovirus infection has been shown to stimulate production of topoisomerase I in HeLa cells, and it has been proposed that this induction may be due to E1A activation (Chow et al., 1985). E1A transactivates the transcription of endogenous cellular genes such as the 70 kDa heat shock protein (Nevins, 1982; Wu et al., 1986), the β -tubulin gene (Stein and Ziff, 1984), and transiently introduced cellular genes such as the rabbit and human β-globin genes (Green et al., 1983; Svenson and Akusjarvi, 1984). E1A represses transcription from enhancers such as the SV40, polyoma virus and the Ad 5 E2A enhancers (Zajchowsi et al., 1985; Velcich and Ziff, 1985; Velcich et al., 1986). It is also able to induce cellular DNA synthesis in serum-starved cells, stimulates proliferation of cells by induction of growth factors, and induces undifferentiated F9 teratocarcinoma cells to undergo differentiation (Lillie et al., 1987; Montano and Lane, 1987; Thangue and Rigby, 1987).

Sequence analysis of the promoters that E1A acts on shows that they do not have significant homology (Zajchowski et al., 1985) and E1A has not been shown to bind any promoter sequences (Feldman et al., 1982). Rather, E1A acts with cellular factors to transactivate genes. An E1A-inducible factor (E2F) present in HeLa cells and in undifferentiated F9 cells is involved in promoter binding and activation (Kovesdi et al., 1986). This factor or its affinity for the promoter is greatly increased by adenovirus infection.

Comparison of the amino acid sequences of human adenovirus serotypes 5, 7, 12 and simian adenovirus type 7 has revealed considerable conservation in three regions of the 289 a.a. E1A protein, as well as a possible C-terminal rapid nuclear localization signal (KRPR) (Kimmelman et al, 1985). Studies have shown that certain E1A functions are closely associated with these regions. Site-directed mutagenesis of the E1A proteins has defined one of these regions as the transactivation domain (Jelsma et al., 1988; Schneider et al., 1987; Lillie et al., 1986). Microinjection of a 49 a.a. peptide encompassing this domain into HeLa cells along with an E1A-responsive promoter resulted in transactivation of the promoter (Lillie et al., 1987). This domain has been further dissected and shown to function as a general regulator of transcription. Fusion of the E1A transactivation domain to the yeast GAL4 DNA-binding domain, resulted in E1A activation of promoters containing GAL4 DNA binding sites (Martin et al., 1990). The E1A transactivation region

is grouped with several other "acidic" activating proteins including GAL4 and VP16 (Mitchell and Tijian, 1989). These proteins are believed to activate transcription through a highly acidic region that may form an α -helix in the polypeptide, and which interacts with cellular transcription factors. The importance of an acidic and α -helical region in GAL4, however, has been recently challenged (Van Hoy et al., 1993; Leuther et al., 1993).

1.9 Purpose of this study

Site-directed mutagenesis of multifunctional proteins has yielded important information about their domain structure and about how such proteins interact with cellular factors in DNA replication or transcriptional regulation events. In order to study the biochemical properties of the NS-1 protein, expression vectors that produce the MVM NS-1 polypeptide were constructed. A replication complementation assay was developed, and mutations were introduced into the coding sequence of NS-1 to study their ability to complement a replication defective MVM clone. The effect of these mutations on the ability of NS-1 to transactivate the MVM p38 promoter in CAT expression assays is also described. This collection of mutations begins to define domains required for the replication and transactivation activities of NS-1.

CHAPTER 2. MATERIALS AND METHODS

2.1 Bacterial strains

Strains were maintained as 15% glycerol stocks at -25 ^{o}C and were grown in LB (1 % Bacto-tryptone; 0.5 % Bacto-yeast extract; 171 mM NaCl) broth at 37 ^{o}C with shaking. *Escherichia coli* strains used in this study included:

BW 58 (gift of G. Johnston) dam3 dam6.
CJ236 (Kunkel et al., 1987) dut1 ung1 thi1 relA1
DH5α (BRL) n^- F^- endA1 hsdR17 supE44 thi1 recA1 gyrA96 relA1 ul69
DH5α F' (BRL) F'Φ80DLacZΔM15Δ(LacZYA-ArgF), recA1
JC8111 (Boissy and Astell, 1985) recBC sbcB recF
HMS174 (gift of F.W. Studier) F^- recA r^-_{K12} m^+_{K12}1Rif^R
BL21 (gift of F.W. Studier) F^- ompT $r^-_B m^-_B$
N4830-1 (Pharmacia) F- su- his- ilv- galKΔ8 Δ(chlD-pgl) l, ΔBam N+.cI_{857} ΔH1
SURE (Stratagene) uvrC unuC sbcC recB recJ Δ(hsdR mcrA mcrB mrr)

2.2 Tissue culture cell lines and infections

Murine A9 ouab^{r}11(Littlefield, 1964) and COS-7 cells (ATCC CRL 1651) were grown in monolayer in Dulbecco's modified minimal essential medium (GIBCO) supplemented with gentamycin (50 ug/ml) and 10% fetal bovine serum (GIBCO), at 37 ^{o}C in a humidified CO_2 incubator. Ehrlich ascites mouse tumor cells (van Venrooij et al., 1979) were grown in suspension in Joklik's minimal essential medium supplemented with 5% fetal bovine serum. For infections COS-7, A9 and EA cells were incubated with infective MVMp inoculum (10 PFU/cell) for 1 hr and were harvested 24 hr postinfection, as described previously (Faust and Gloor, 1984; Faust et al., 1989).

2.3 Virus strains

The minute virus of mice prototype strain (MVMp) was used in infections. High titer inoculum was obtained by infecting EA cells in suspension at a low multiplicity (1PFU/5 cells) for 72 - 96 hr.

2.4 Transformation of *E. coli* strains

Bacteria were made competent for the uptake of DNA by treatment with calcium chloride. One ml of overnight culture was added to 100 ml LB and was incubated at 37 ^{o}C with shaking until OD_{600} = 0.4 - 0.6 . Cells were harvested by centrifugation in an SS-34 rotor at 5,000 rpm for 10 min at 4 ^{o}C. The cells were resuspended in 20 ml cold 5 mM Tris (pH 7.6); 75 mM $CaCl_2$ and were incubated on ice for 30 min. The bacteria were pelleted as described above and were resuspended in 5 ml cold 5 mM Tris (pH 7.6); 75mM $CaCl_2$. Cells treated in this manner were used within 24 hr or were stored as 20% glycerol stocks at -80 ^{o}C.

Competent bacteria (100 ul) were incubated with 1-10 ul DNA solution for 30 min, on ice. The cells were then heat shocked at 42 ^{o}C for 45 sec. For transfections with M13 clones, aliquots (5-60 µl) were plated onto LB plates with 100 µl of DH5α F' (OD_{600} = 0.8) and 3 ml plating agar (0.8 % agar). For transformation with ampicillin resistance encoding plasmids, the cells were placed on ice for 2 min after which 900 ul SOB was added. The bacteria were then incubated at 37 ^{o}C for 60 min with shaking. Aliquots (50-200 µl) were plated onto LB plates (100 ug carbenicillin/ml) and were then incubated at 37 ^{o}C overnight.

2.5 Purification and manipulation of DNA

2.5.1 Plasmid DNA purification

2.5.1.1 Small scale plasmid DNA preparation

Plasmid and RF M13 DNA was isolated by a modification of the method of Birnboim and Doly (1979). Single colonies were picked from LB plates (100µg/ml

carbenicillin) and inoculated into 2 ml LB broth with ampicillin (50 µg/ml) and were incubated at 37 °C with shaking for up to 16 h . An overnight DH5α F' culture (1.5 ml) was centrifuged in an Eppendorf centrifuge for 5 min at 4 °C. All of the supernatant was removed and the bacterial pellet was resuspended in lysis buffer with 5 mg/ml hen egg white lysozyme. The cells were incubated on ice for 10 min and 200 ul 0.2 M NaOH; 1 % SDS were added and incubation was for an additional 10 min on ice. One hundred and fifty ul 3 M KOAc (pH 5.0) were then added and the lysate was incubated on ice for an additional 10 min. Cell debris was removed by centrifugation in an Eppendorf centrifuge for 20 min at 4 °C. The supernatant was transferred to a new tube and the DNA was precipitated by the addition of 1 ml 95% ethanol and incubation at -20 °C for 10 min. The DNA was pelleted by centrifugation in an Eppendorf centrifuge for 10 min at 4 °C. The precipitate was resuspended in 200 µl0.15 M NaCl and reprecipitated with the addition of two volumes of 95% ethanol at -80 °C. The DNA was pelleted as described above and washed with 70% ethanol, air dried and finally resuspended in 50-100 µlH_2O.

2.5.1.2 Large scale plasmid DNA preparation

To 500 ml sterile LB broth (50 µg/ml ampicillin), 1 ml overnight culture carrying a plasmid encoding ampicillin resistance was added and the culture was incubated at 37 °C with shaking. Plasmid DNA was amplified by the addition of chloramphemicol (15µg/ml) to the culture at an OD_{600} = 0.5 - 0.7. The cells were then incubated at 37 °C overnight with shaking. The bacteria were harvested by centrifugation in JA-14 rotor at 9000 rpm for 5 min at 4 °C. The supernatant was discarded and the bacterial pellet was resuspended in 8 ml lysis buffer (50 mM glucose; 25 mM Tris pH 8.0; 10 mM EDTA). Lysis buffer (2 ml) with hen egg white lysozyme (25 mg/ml) were then added, and the cells were incubated on ice for 10 min. Ten ml 0.2 M NaOH; 1% SDS were added and the cells were incubated for an additional 10 min or until the lysate had cleared, after which 7.5 ml 3 M KOAc (pH 5.0) were added and the lysate was incubated on ice for 10-15 min. High molecular weight DNA and cellular debris were sedimented by centrifugation in an SS-34 rotor at 14,000 rpm for 25 min. The supernatant was transfered to a new tube and approximately 11 ml isopropyl alcohol were added and incubated at room temperature for 10 min. Plasmid DNA was recovered by centrifugation in an SS-34 rotor at 10,000 rpm for 10 min at room temperature.

The pellet was resuspended in 2 ml 10 mM Tris (pH 7.6); 15 mM NaCl and 60 ul RNase A (10mg/ml) were added and incubated for 30 min at 37 °C. Proteinase K (60 ul) (20mg/ml) were added and the DNA was incubated for 0.5 -1 hr at 37 °C. Two grams CsCl were added to the DNA solution and the volume was brought up to 6ml with a solution of 0.9 g/ml CsCl. Ethidium bromide solution (10mg/ml) was added to a final concentration of 60 ug/ml and the solution was placed in a Beckman 13 X 51mm quick seal ultracentrifuge tube. The DNA was centrifuged in a Beckman VTi 65.2 rotor at 55,000 rpm for 18-22 hr at 15 °C. The plasmid DNA bands were visualized with low intensity long wave ultraviolet light and were recovered from the gradient with a 16 gauge hypodermic needle and a 3 cc syringe. The volume was brought up to 4 ml with H_2O and the ethidium bromide was removed by 4-5 extractions with equal volumes of water saturated n-butanol. The DNA was precipitated by the addition of 10 ml absolute ethanol at -20 °C overnight, and was pelleted by centrifugation in an IEC centrifuge at 3000 rpm for 20 min at 4 °C. The DNA was resuspended in 500 ul 0.15 M NaCl and 1ml 95% ethanol was added and incubated at -80 °C for 5 min. The DNA was pelleted by centrifugation in an Eppendorf centrifuge for 10 min at 4 °C. The pellet was resuspended in 500 ul 0.15M NaCl and was ethanol precipitated again. The precipitate was washed with 70% ethanol and air-dried. Plasmid DNA was resuspended in 500-1000 µl H_20. The DNA concentration at 260 nm was determined by scanning the optical density of a 1:50 dilution from 210 nm to 310 nm with a Beckman DU-64 spectrophotometer.

2.5.2 M13 DNA purification

2.5.2.1 Small scale RF DNA preparation

LB broth (3 ml) was inoculated with 40 ul of an overnight DH5α F' culture, and a single M13 plaque. The culture was incubated for seven hrs with shaking at 37 °C. The bacteria (1.5 ml) were harvested and RF DNA was isolated using the procedure for small scale isolation of plasmid DNA.

2.5.2.2 Large scale RF DNA preparation

LB broth (500 ml) was inoculated with 500 ul of an overnight culture of DH5α F' and 50 ul phage supernatant. The culture was incubated at 37 °C with shaking for up to 7 hrs. Bacteria were pelleted by centrifugation as described above. The cells were

resuspended in two ml sucrose buffer (25 % sucrose; 50 mM Tris, pH 8.0; 10 mM EDTA). Lysozyme solution (1 ml of 50 mM Tris, pH 7.5; 5mg/ml hen egg white lysozyme) was then added and the cells were incubated on ice for 5 min . Two ml RF TE buffer (0.25 M Tris. pH 7.8; 50 mM EDTA) were added and the lysate was incubated on ice with 250 ul RNase A (10mg/ml) for 5 min. Triton-X 100 (2.5 ml) was added and the cells incubated on ice for 10 min. High molecular weight DNA and cellular debris were pelleted by centrifugation in an SS-34 rotor at 12,000 rpm for 20 min at 4 ^{o}C. The supernatant was transfered to a 10ml graduated cylinder, to which 8 g of CsCl was added and the volume brought up to 10 ml with RF TE buffer. The sample was transfered to a 16 X 76 mm Beckman Quick-Seal centrifuge tube and 40 ul ethidium bromide (10mg/ml) were added. The tube was heat sealed and centrifuge at 55,000 rpm for 24 hrs at 15 ^{o}C.

2.5.2.3 ss M13 DNA purification

Single-stranded M13 DNA was isolated from infected *E. coli* medium using the method described by Sambrook et al. (1989). Cleared supernatant was incubated with one-eighth volume 20 % PEG 8000; 2.5 M NaCl for at least one hour on ice. M13 phage were pelleted by centrifugation in an Eppendorf microfuge or in an SS-34 rotor at 14,000 rpm for 15 min at 4 ^{o}C. The phage pellet was phenol extracted twice, phenol:chloroform:isoamyl (50:48:2) extracted twice, chloroform:isoamyl (24:1) extracted once and then ethanol precipitated twice. The single-stranded DNA was resuspended in H_2O. Optical density was determined by scanning at 260 nm.

2.6 Gel Electrophoresis

2.6.1 Agarose gel electrophoresis

Loading buffer (5 X: 0.2 M sucrose; 0.4 M urea; 0.1% (w/v) bromophenol blue; 10 mM EDTA) was added to the DNA solution. Agarose gel electrophoresis was carried out in 0.8-1.0 % agarose gels, at 30-100 v for 1-16 hr, in 1X TBE (0.089 M Tris-HCl; 0.0 89M boric acid; 0.002M EDTA) and approximately 1ug/ml ethidium bromide. For purification of small DNA fragments (< 700 bp), SeaPlaque agarose was used. For purification of DNA fragments using the Geneclean kit, gels were made and

electrophoresis was carried out in 1X TAE buffer (40 mM Tris acetate, 0.001 mM EDTA, pH 8.0).

2.6.2 Dideoxy chain termination sequencing of ds and ss DNA

Single stranded and double-stranded DNA sequencing was carried out using the Sequenase Kit (USB) reagents and protocol. Briefly, double stranded plasmid DNA was first denatured with NaOH and was ethanol precipitated as described in the Sequenase kit instructions. The primer (20 ng) was annealed to the DNA (1μg single stranded; 5μg double-stranded) by incubating at 70 °C for 2 min and cooling slowly to room temperature. The annealed DNA was then incubated in the presence of DTT, labeling mix, [α -^{35}S] - dATP, and T7 DNA polymerase for 5 min at room temperature. Aliquots of this reaction were added to each ddNTP mix and incubation was continued for an additional 5 min at 37 °C. The reaction was stopped with the addition of stop buffer. The samples were denatured at 75 °C for 2 min and 2μl aliquots were loaded onto a 0.4 mm thick, 6.5 % polyacrylamide; 7 M urea polyacrylamide gel and electrophoresed at 50 mA and at 50 °C for 1-4 hr. The gel was then fixed in 10% glacial acetic acid; 15% methanol for 15 min, and was dried onto 3MM chromatography paper (Whatman) under vacuum at 80 °C for 1 hr.. Kodak XAR 5 X-ray film was used for autoradiography.

2.6.3 SDS-PAGE (discontinuous SDS-polyacrylamide gel electrophoresis)

Protein extracts were subjected to discontinuous SDS-polyacrylamide gel electrophoresis according to the method of Laemmli (1970). Gels were typically 0.5 to 1 mm in thickness. The stacking gel contained 0.125 M Tris-Cl pH 6.8; 0.1%w/v SDS; 5.25% w/v acrylamide (30:1 bis), 0.08 % ammonium persulfate, 5 mM TEMED. The separating gel contained 0.37 M Tris.Cl pH 8.8, 0.1% w/v SDS, 6.5% acrylamide (30:1 bis), 2 mM EDTA, 0.08 % ammonium persulfate, 5 mM TEMED. Samples were made up in 1X sample buffer (0.25 M Tris.Cl pH 6.8, 4% w/v SDS, 1.4 M 2-mercaptoethanol, 10% glycerol, 0.0002% bromophenol blue). Samples were placed in a boiling water bath for two minutes and then chilled on ice prior to loading. The gel was run in 1X running buffer (50 mM Tris-glycine, pH 8.2; 0.1% SDS (w/v) electrophoresis was at 70 v for 2 hr.

2.6.4 Coomassie staining of polyacrylamide gels

Immediately after electrophoresis, SDS-PAGE gels were placed in a solution of 0.2% Coomassie Blue; 10% glacial acetic acid; 45% methanol, for 30 to 60 min with shaking. Excess stain was removed by soaking the gel in 10% glacial acetic acid, 45% methanol with shaking.

2.7 Enzyme reactions

2.7.1 DNA restriction endonuclease reactions

DNA restriction enzyme digestions were carried out in the buffer recommended by the manufacturer. Typically, for screening of plasmid clones 200 ng DNA was digested with 5 U restriction enzyme at 37 ^{o}C for 2 hr. For the isolation of specific DNA fragments 10-60 ug DNA were digested with up to 100 U restriction enzyme for 2-3 hr at 37 ^{o}C.

2.7.2 DNA ligations.

DNA fragments were ligated using T4 DNA ligase (BRL) in ligation buffer provided by the manufacturer. Typical ligations reactions contained 10-30 ng vector DNA and 60-120 ng insert DNA. Ligations were carried out at 15 ^{o}C for ends containing 5' overhangs, or at room temperature for blunt ended DNA, for 4 to 16 hr.

2.7.3 Blunt ending of 5' overhangs

5' overhangs generated by restriction enzyme endonucleases were filled-in using the Klenow fragment of DNA polymerase (4-7 U) and 0.25 mM dNTP's at 37 ^{o}C for 15 min in the same buffer used for the restriction enzyme digestion.

2.7.4 5' end DNA phosphorylation

5' ends of double stranded or oligonucleotide DNA were phosphorylated with T4 polynucleotide kinase in 1 X kinase buffer (50 mM Tris, pH 7.6; 10 mM $MgCl_2$; 5 mM

DTT; 0.1 mM spermidine; 0.1 mM EDTA) at 37 °C for 1 hr. The kinase was then inactivated by heating at 65 °C for 10 min.

2.7.5 5' end dephosphorylation

5' phosphate groups were removed by incubation of the DNA with 8 U calf intestine alkaline phosphatase (CIP) in 1X CIP buffer (20 mM Tris pH 8.0; 1 mM $MgCl_2$; 0.05 mg/ml BSA) for 30 min at 37 °C for DNA ends containing 5' overhangs. An additional 8 U of CIP was added and the DNA was incubated at 37 °C for another 30 min. DNA with blunt ends was incubated for 15 min at 37 °C and 15 min at 56 °C, after each addition of CIP enzyme. The CIP was inactivated by heating at 80 °C for 10 min. The DNA was phenol:chloroform:isoamyl alcohol (50: 48: 2) extracted once, chloroform:isoamyl alcohol (24:1) extracted once and ethanol precipitated.

2.8 Construction of vectors

2.8.1 Isolation of DNA restriction fragments

DNA fragments were isolated using the Geneclean kit (BIO 101) and protocol. Briefly, the DNA was electrophoresed overnight in a 0.8-1% agarose; 1X TAE gel in 1X TAE buffer (with approximately 1µg/ml ethidium bromide). The DNA was visualized with a long wave UV light source and excised from the gel with a scalpel blade. The agarose containing the DNA fragments was chopped into small pieces and dissolved in 2.5 volumes sodium iodide at 50 °C for 5 min. Glassmilk (5-20 µl glass beads) were added and incubated for 5-15 min on ice. The Glassmilk was then pelleted by centrifugation in an Eppendorf centrifuge for 30 sec, and washed 3 times with 400 µl NEW wash buffer . The DNA was released from the Glassmilk by incubation with an equal volume of H_2O at 50 °C for 3 min. DNA purified in this manner was quantified by agarose gel electrophoresis and comparison with DNA markers of known concentration. Plamids used in this study are summarized in Table 2.

Table 2: Plasmids and M13 clones

Vector	Source	Description
M13mp18	(Pharmacia)	bacteriophage M13 cloning vector
pSVL	(Pharmacia)	T-antigen inducible expression cloning vector
pCAT-Basic	(Promega)	CAT expression vector lacking a promoter
pRIT2T	(Pharmacia)	inducible protein A fusion protein expression vector
pSVNS1-1	(see section 2.8.7)	T-antigen inducible NS-1 expression vector
pSVNS1-2	(see section 2.8.8)	T-antigen inducible NS-1 expression vector
pP39CAT	(see section 2.8.6)	capsid gene promoter driven CAT expression vector
M13NS1-4	(see section 2.8.5)	M13/NS-1 vector
pETNS-1	(see section 2.8.9)	T7 promoter driven NS-1 expression vector
pVLNS-1	(see section 2.8.3)	baculovirus NS-1 expression vector
pP10NS-1	(see section 2.8.10)	baculovirus NS-1 expression vector (X-gal selection)
pPA/NS-1	(see section 2.8. 4)	protein A/NS-1 fusion expression vector
pMM984	(gift of P. Tattersall)	infectious MVM clone
ins 20B	(gift of R. Salvino)	mutant NS-1 MVM clone
pVL941	(gift of M. Summers)	baculovirus cloning vector
pJVP10	(gift of C. Richardson)	baculovirus cloning vector with X-gal selection
pET-11d	(gift of F.W. Studier)	T7 promoter driven expression vector
pSP64-5	(gift of R. Salvino)	SP6 promoter driven *in vitro* MVM RNA transcription vector

2.8.2 Construction of NS-1 cassette

The MVM genome was excised from pMM984 as a *Bam*HI fragment and was purified by agarose gel electrophoresis using the Geneclean kit (Bio 101). The DNA was digested first with *Hga* I (New England Biolabs) and then with *Aha* II (New England Biolabs), and the resulting fragments were blunt ended with the Klenow fragment of DNA polymerase I and 0.25 mM dNTP's. The 2050 bp fragment containing the NS-1 coding sequence (MVM nt 241-2291) was purified by agarose gel electrophoresis and *Bam*HI linkers (5'-CGGATCCG-3') were added to the ends with T4 DNA ligase (Pharmacia). Excess linkers were digested with *Bam*HI for 3 hr. The DNA was then purified by TAE agarose gel electrophoresis as described above.

2.8.3 Construction of pVLNS-1

The *Hga I/ Aha II* fragment with *Bam* HI linkers was cloned into the unique *Bam*HI site of pVL941 (gift of M. Summers) using T4 DNA ligase. DH5 α cells were transformed with the ligation products and a clone designated pVLNS-1 was isolated.

2.8.4 Construction of pSVNS1-1

The *Bam*HI fragment containing the NS-1 coding sequence from pVLNS-1 was excised by digestion with *Bam* HI in React -3 buffer (BRL). The resulting fragments were separated by TAE agarose gel electrophoresis and the NS-1 fragment was purified using the Geneclean kit, and was inserted into the unique *Bam*HI of pSVL (Pharmacia), an SV40 large T-antigen inducible, SV40 late promoter driven expression vector, using T4 DNA ligase to produce pSVNS1-1 (Fig. 4 A). A second construct (pSVRNS1-1) containing the NS-1 coding sequence in the opposite orientation to that of pSVNS1-1 was also produced by this procedure. Clones containing the insert in each orientation were grown in *E. coli* strain DH5α.

2.8.5 Construction of pSVNS1-2

This expression vector was created by inserting the blunt-ended *Hga* I fragment of pMM984 (MVM nt 241- 2487), which contains the entire coding sequence of the NS-1 and

NS-2 genes, into the *Sma* I site of pSVL. The initial clone (pSVRNS1-2) contained the insert in the opposite orientation such that the MVM genes could not be transcribed. The MVM sequence was excised from this clone by digestion with restriction enzymes *Bam* HI and *Xba* I, which cut at sites flanking the insert. The insert was blunt ended with the Klenow fragment of DNA polymerase, and was inserted into the *Sma* I site of pSVL in the proper orientation.

2.8.6 Construction of M13NS1-4

The 2058 bp *Bam*HI fragment containing the NS-1 coding sequence was purified from pSVNS1-1 by gel electrophoresis and was inserted into the unique *Bam*HI site of M13mp18 (Pharmacia). A clone (M13NS1-4; Fig. 4 B) containing the NS-1 insert with nt 241 of the NS-1 gene proximal to the universal priming site of M13 was isolated from *E. coli* strain DH5α F' and phage DNA was purified as described previously (Sambrook et al., 1989).

2.8.7 Construction of pP39CAT

A *Pst* I fragment of pSVNS1-1 (nt 1350 to 2129 of MVM DNA) was isolated by gel electrophoresis. This fragment, which contains the p38 promoter, was inserted into the unique *Pst* I site of pCAT (Pharmacia) with T4 DNA ligase. Clones containing the insert in the proper orientation (pP39CAT; Fig. 4 C) were grown in *E. coli* strain DH5α.

2.8.8 Construction of pPA/NS-1

The *Bam* HI fragment from pVLNS1 encoding the NS-1 protein was inserted into the unique *Bam* HI site of the protein A expression vector pRIT2T (Pharmacia) to create pPA/NS-1. This vector encodes an IgG binding portion of protein A as the N-terminal half of the fusion protein followed by the entire NS-1 protein sequence.

2.8.9 Construction of pP10NS1

The *Bam*HI fragment containing the NS-1 coding sequence was excised from pSVNS1-1 using *Bam*HI and the ends were rendered blunt with the Klenow fragment as

Figure 4 Plasmid map of: A. pSVNS1-1; B. M13NS1-4; C. pP39CAT

Constructs and unique restriction sites used for mutagenesis are shown. The positions of the SV40 late promoter and SV40 origin of replication relative to the NS-1 gene of MVM in pSVNS1-1 are indicated in A. pP39CAT contains the chloramphenicol acetyl transferase (CAT) gene downstream of the MVM p38 promoter located within a *Pst*I fragment of the MVM genome.

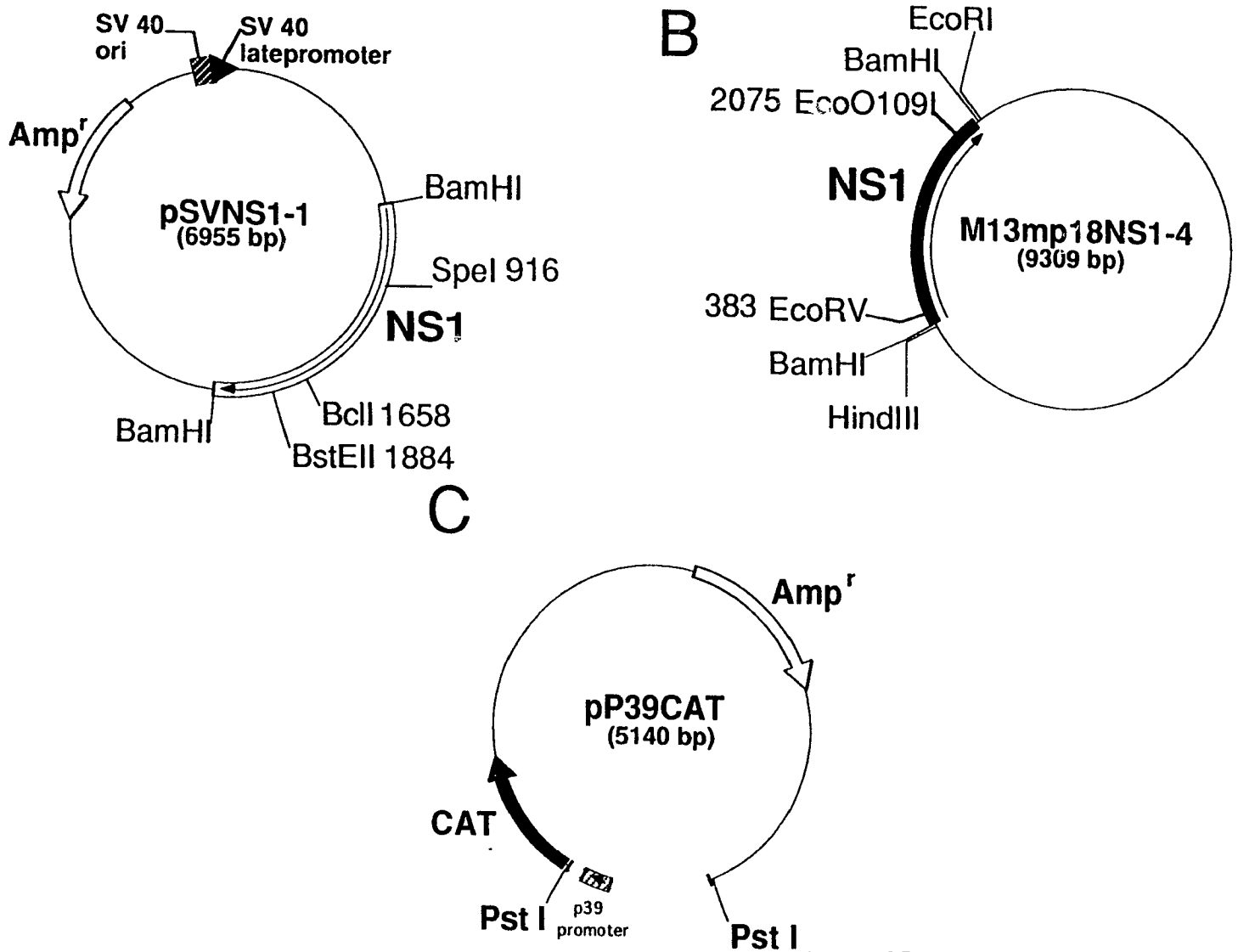

Figure 4 Plasmid map of : A. pSvNS1-1; M13NS1-4; C. pP39CAT

described previously. *Nhe* I DNA linkers (GCGCTAGCGC) were added onto the end with T4 DNA ligase. The resulting fragments were digested with *Nhe* I to remove excess linkers, and the fragments were purified by agarose gel electrophoresis using Geneclean as described previously. The purified fragment contained the NS-1 coding sequence with *Nhe* I linkers at either end. This fragment was cloned into pJVP10 (gift of C. Richardson), using T4 DNA ligase. *E. coli* strain DH5α was transformed with the resulting ligation products and colonies were picked and screened for plasmids containing the insert in the proper orientation. One such clone was designated pP10NS1.

2.8.10 Construction of pETNS1

The *Bam* HI fragment containing the NS-1 coding sequence was isolated from pSVNS1-1 as described above. The end were blunt ended with Klenow polymerase . *Nhe* I linkers were added using methods outlined above. The *Nhe* I cassette was inserted into the unique *Nhe* I site of the inducible expression vector pET11d (gift of F.W. Studier) using T4 DNA ligase.

2.9 Construction of mutations

2.9.1 Construction of linker insertions

Insertion mutations were created by inserting DNA linkers in restriction sites that were unique to NS-1 in M13NS1-4 or pSVNS1-1. Mutations made in M13NS1-4 were subcloned into pSVNS1-1. Mutations were distinguished from wild type by restriction enzyme analysis. Crude DNA extracts were prepared as described previously. In separate reactions, the DNA was cut with: a) the restriction enzyme used to initially cleave the wild type DNA, and b) the restriction enzyme specific for the new restriction site included in the inserted linker. Subsequently, clones were sequenced through the mutated region using the dideoxy-sequencing method. Clones that contained the proper mutation as determined by restriction enzyme analysis and DNA sequencing were subcloned into pSVNS1-1. The resulting clones were then analyzed for the presence of the proper restriction enzyme sites, as described previously.

2.9.1.1 Construction of *Eco*RV insertion mutations

ΔEVH and ΔEVM were constructed in M13NS1-4 by digesting the RF DNA with *Eco*RV and dephosphorylating the linearized DNA with CIP (Boehringer). *Mlu* I linkers (8bp; 5'- GACGCGTC -3') or *Hpa*I linkers (5'-GTTAAC-3'), obtained from Pharmacia, were added with T4 DNA ligase and excess linkers were removed by digestion with *Mlu* I or *Hpa* I, respectively. The DNA was purified by agarose gel electrophoresis. Each of the gel purified fragments were then self-ligated with T4 DNA ligase. *E. coli* strain DH5α F' was transformed with the resulting ligation products. Plaques were picked and screened for the absence of an *Eco* RV site and the presence of an *Mlu* I (ΔEVM) or *Hpa* I (ΔEVH) site. The NS-1 *Bam*HI fragment was then isolated from M13NS1-4 and inserted into the *Bam*HI site of pSVL. *E. coli* strain DH5α transformants were picked and screened for the presence of the insert in the proper orientation.

2.9.1.2 Construction of *Spe* I insertion mutations

ΔSIb, ΔSIM1, and ΔSIM4 were constructed in pSVNS1-1 by digestion of pSVNS1-1 with *Spe* I and blunt ending the resulting 5' overhangs with Klenow polymerase. pSV-ΔSIb was created by self-ligating the blunt ended DNA with T4 DNA ligase. pSV-ΔS1M1 was created by adding *Mlu* I linkers (8bp) as described above. pSV-ΔSIM4 was sequenced and found to contain an in tandem insertion of four *Mlu* I linkers.

2.9.1.3 Construction of *Bcl* I insertion mutations

ΔBIb, ΔBIM1, and ΔBIM4 were constructed by digesting pSVNS1-1 (grown in the dam$^-$ *E. coli* strain BW48) with *Bcl* I and blunt ending the 5' overhangs with Klenow polymerase. pSV-ΔBIb was made by self-ligating the blunt ended DNA. pSV-ΔBIM1 was made by inserting *Mlu* I linkers (8bp) onto the blunt ended DNA as described above. pSV-ΔBIM4 was sequenced and found to contain an in tandem insertion of four *Mlu* I linkers. These constructs were grown in *E. coli* strain BW48.

2.9.1.4 Construction of *Bst* EII insertion mutations

ΔBIIb and ΔBIIM1 were constructed by digesting pSVNS1-1 with *Bst* EII (New England Biolabs), and blunt ending the 5' overhangs with the Klenow fragment of DNA polymerase I. pSV-ΔBIIb was made by self-ligating the blunt ended DNA. pSV-ΔBIIM1 was made by adding *Mlu* I linkers (10bp; 5'-CGACGCGTCG-3') to the blunt ended DNA, as previously described.

2.9.1.5 Construction of *Eco* O109 I insertion mutation

pSV-ΔEOb was constructed by digesting M13NS1-4 with *Eco* O109 I (New England Biolabs) and blunt ending the 5' overhangs with the Klenow fragment of DNA polymerase I. The blunt ended DNA was then self-ligated. The NS-1 *Bam*HI fragment was gel purified and inserted into the *Bam*HI site of pSVL.

2.9.2 Oligonucleotide-directed mutagenesis

Mutagenic oligonucleotides were purchased from the Regional DNA Synthesis Lab of the University of Calgary. Point mutations were made using the method described by Kunkel et al. (1987). Each 5' end phosphorylated oligonucleotide was hybridized to single stranded M13NS1-4 uracil-containing DNA, prepared in *E. coli* strain CJ236, at 70 °C in 1.25X SSC for 5 min, and cooled slowly to room temperature. Double stranded RF DNA was synthesized in 1X polymerase buffer (20 mM Tris-HCl, pH 8.8; 2 mM DTT; 10 mM $MgCl_2$; 0.5mM dNTP's; 1 mM rATP) for 5 min on ice, followed by 10 min at room temperature and then 2 hr at 37 °C, with the modified form of T7 polymerase (New England Biolabs). The ends were ligated with T4 DNA ligase. The reaction products were used to transform *E. coli* strain DH5 α F'.

2.9.2.1 Construction of ΔA249 and ΔQ250

ΔA249 and ΔQ250 were created by Wey Leang Leong. The oligonucleotide 5' CCATCTAGTGGCCAAACTATAC-3' was used to make ΔA249, in which the serine codon AGC is changed to the alanine codon GCC. The oligonucleotide 5'-CGCCATCTGGTGAGCCAACTATAC-3' was used to make ΔQ250, in which the lysine

codon AAA is changed to a glutamine codon CAA. A second point mutation specified by this oligonucleotide changed A to G in the wobble base of a leucine codon (CTA) and provided a new *Hph* I restriction site. ΔA249 and ΔQ250 clones were isolated and screened by dot blot hybridization (Zoller and Smith, 1984) as described below and by restriction enzyme digestion, respectively. The mutations were confirmed by dideoxy-sequencing. The entire DNA segment from nucleotides 383-916 (*Eco*RV to *Spe* I) was sequenced and subcloned into pSVNS1-1. The clones were sequenced again through the mutated region in the pSVNS1-1 background.

2.9.2.2 Construction of tyrosine to phenylalanine mutations

Tyrosine to phenylalanine mutations were created in M13NS1-4 with oligonucleotides converting A to T in the third position of the tyrosine codon (Table 3). Mutations were initially screened with the dot blot hybridization technique. Potential clones were sequenced through the mutated region and then subcloned into a wild type pSVNS1-1 background, and sequenced again to ensure that no other mutations were present. Representative autoradiograms of dideoxysequencing of the equivalent of the MVM viral strand showing the A to T conversion for each mutation is presented in Fig. 5, 6 and 7.

2.9.3 Hybridization screening

2.9.3.1 Dot blot hybridization

Point mutations were initially screened by dot blot or plaque lift hybridization, using the mutagenic oligomer as a probe. For dot blot hybridization, M13 phage was prepared by infecting 2 ml cultures of DH5α F' with well separated, individual plaques and incubating at 37 ^{o}C with shaking for up to 7 hrs. Phage was precipitated from the cleared supernatant as described previously, and resuspended in ddH_2O. Aliquots of resuspended phage were spotted onto a nitrocellulose filter and allowed to air dry. The nitrocellulose was then baked at 80 ^{o}C for two hrs, and phage were screened for the mutation as described below.

Table 3: Oligonucleotides used for Tyr to Phe mutagenesis

Mutation	Oligonucleotide sequence
F6:	GGAAATGCTTTCTCTGATGAAG
F47:	GGAATAGTTTCAAAAAAGAGC
F188:	CTACTTACTTTTAAGCATAAGC
F197:	CCAAAAAAGACTTTACCAAGTGTG
F209:	CATGATTGCTTTCTATTTTTTAAC
F210:	CATGATTGCTTACTTTTTTTTAAC
F209/F210:	CATGATTGCTTTCTTTTTTTTAAC
F227:	GAGACGGCGGCTTTTTTCTTA*
F310:	GCCAGACAGTTTCATTGAAATGATG
F422:	CAATGTTGGTTGCTTTAATGCAGC
F543:	GAATGGTTTCCAATCTACCATGGC
F550:	GGCAAGCTTCTGTGCTAAAT
F550/T545:	CCATGGCAAGCTTCTGTGCTAAATG

* also contains an A to C conversion in the wobble base of the glycine codon (GGA) and removes an *Mnl* I restriction site

Figure 5 Autoradiogram of dideoxysequencing of Tyr to Phe mutations F6, F47, F188, F197.

Tyrosine to phenylalanine mutations in M13NS1-4 or in pSVNS1-1 were sequenced using the dideoxysequencing method as described. The mutation is shown at the top of each panel. Lanes containing each ddNTP are indicated at the bottom. The mutated nucleotide is indicated with an arrow.

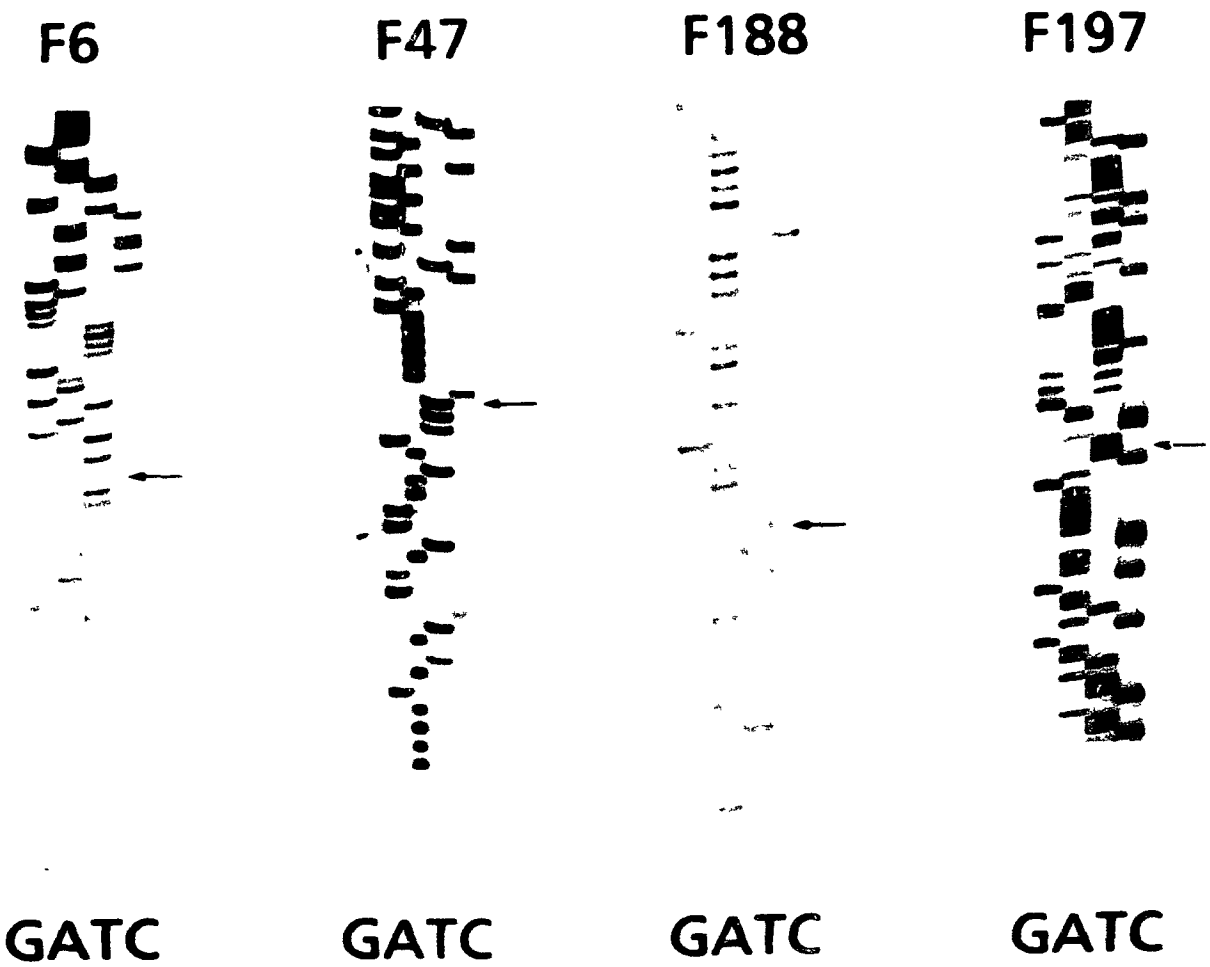

Figure 5 Autoradiogram of dideoxysequencing of Tyr to Phe mutations
F6, F47, F188, F197

Figure 6 Autoradiogram of dideoxysequencing of Tyr to Phe mutations F209, F210, F209/F210, F227.

Tyrosine to phenylalanine mutations in M13NS1-4 or in pSVNS1-1 were sequenced using the dideoxysequencing method as described. The mutation is shown at the top of each panel. Lanes containing each ddNTP are indicated at the bottom. The mutated nucleotide is indicated with an arrow.

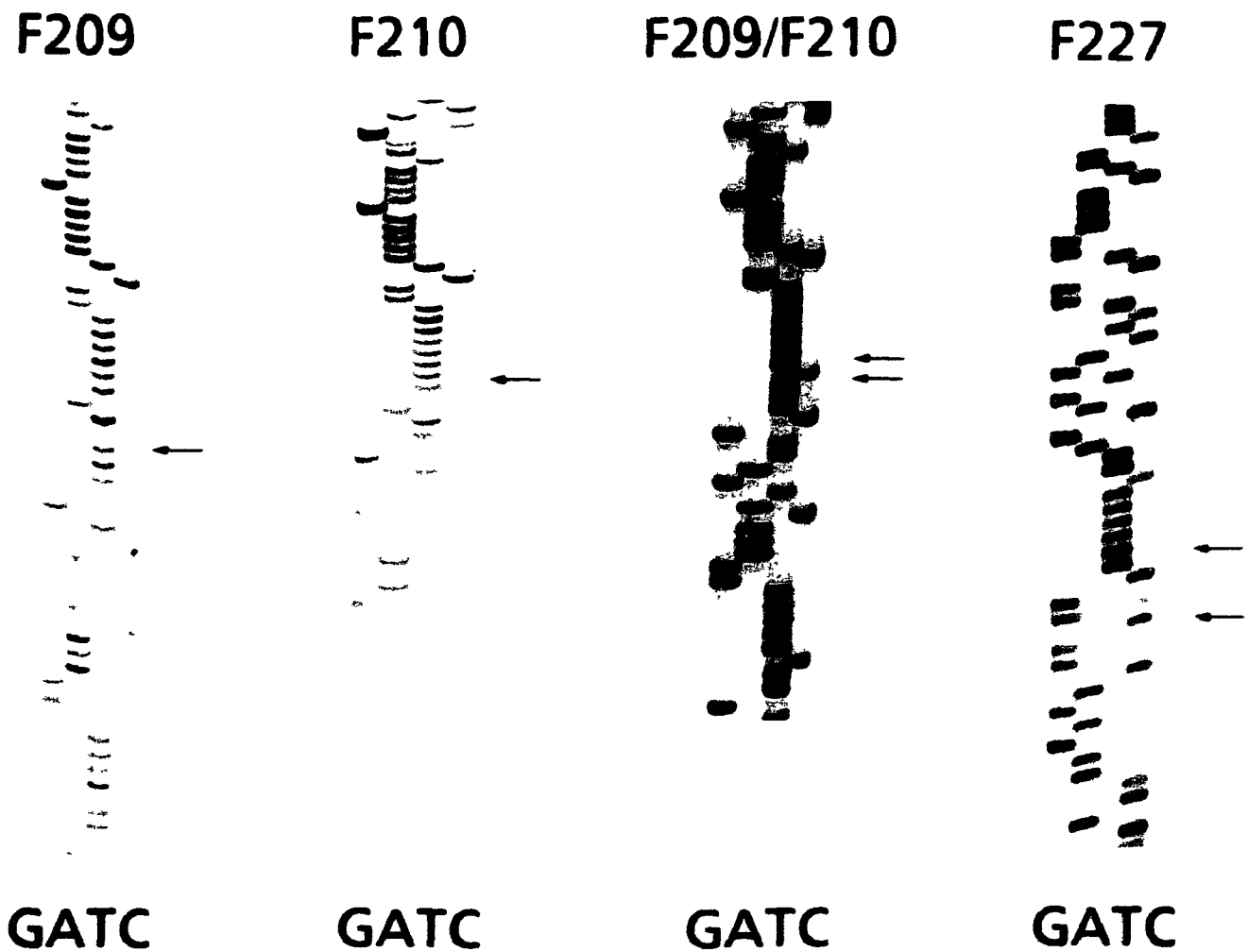

Figure 6 Autoradiogram of dideoxysequencing of Tyr tp Phe mutations
F209, F210, F209/F210, F227

Figure 7 Autoradiogram of dideoxysequencing of Tyr to Phe mutations F310, F422, F543, F550, F550/T545.

Tyrosine to phenylalanine mutations in M13NS1-4 or in pSVNS1-1 were sequenced using the dideoxysequencing method as described. The mutation is shown at the top of each panel. Lanes containing each ddNTP are indicated at the bottom. The mutated nucleotide is indicated with an arrow.

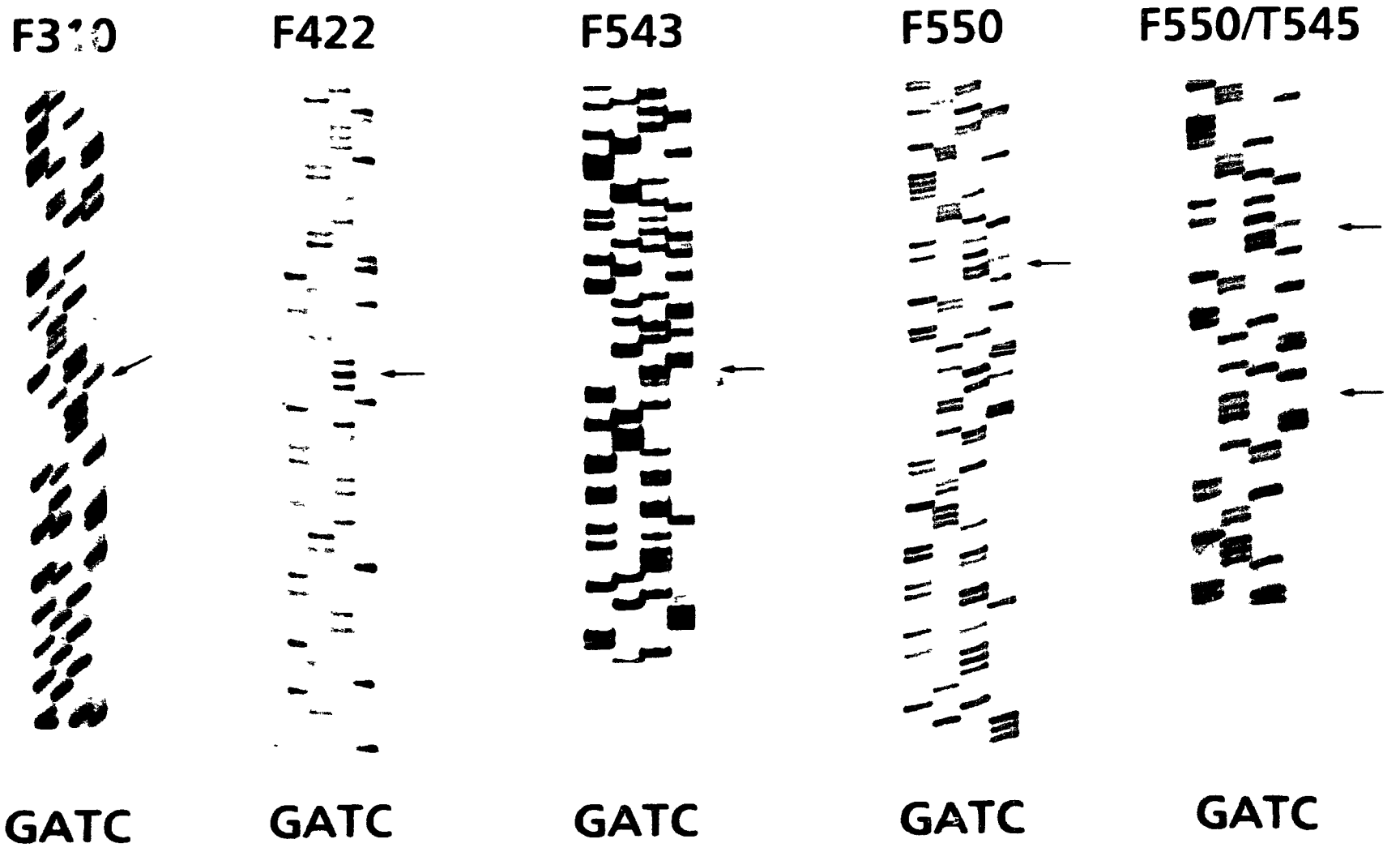

Figure 7 Autoradiogram of dideoxysequencing of Tyr to Phe mutations
F310, F422, F543, F550, F550/T545

2.9.3.2 Plaque lift hybridization

M13 plaques were formed on top agar as described previously, using M13NS1-1 that had been subjected to mutagenesis. A piece of nitrocellulose filter was placed directly on top of the agar containing the plaques for 1 min. The nitrocellulose filter was then removed and washed for several minutes in 6X SSC to remove any adherent agar and bacteria. The nitrocellulose was allowed to air dry and was baked at 80 ^{o}C for 2 hrs.

2.9.3.3 Hybridization screening

The nitrocellulose filter from a plaque lift or with dot blotted phage was prepared for hybridization by prehybridizing with 10 ml prehybridization solution (6X SSC; 10X Denhardt's solution; 1% SDS) in a sealed bag for one hr at 67 ^{o}C with shaking. The prehybridization solution was removed and the filter was washed three times with 40 ml 6X SSC at room temperature.

Fifty ml of hybridization solution (6X SSC; 10 X Denhardt's solution) were then added, followed by the γ-[^{32}P] -ATP 5' end labeled mutagenic oligonucleotide. The bag was sealed and hybridization was allowed to take place at room temperature for 1 hr, with occasional shaking. The nitrocellulose was washed three times in 50 ml 6X SSC at room temperature. The filter was then autoradiographed with Kodak XAR5 film for one hour at room temperature. The nitrocellulose was then washed at successively higher temperatures in 6X SSC, for three minutes with shaking, starting at 6 ^{o}C below the predicted temperature of dissociation of the oligonucleotide (T_D = 4[G+C] + 2[A+T]), until the signal from putative wild type clones could no longer be detected with a hand held Geiger-Muller counter. The filter was autoradiographed overnight with Kodak XAR5 film. Clones that gave a signal after washes at or above the predicted T_D were further screened by dideoxy sequencing (Fig. 5, 6, 7). The frequency of mutations detected by dot blot hybridization was typically between 25 and 80 %.

2.10 Assays

2.10.1 Transfection of DNA into mammalian cells

COS-7 and A9 cells were transfected by the calcium phosphate precipitate method (Graham et al., 1974; Graham et al., 1980) as described previously (Salvino et al., 1991). Cells were seeded at a density of 0.5 X 10^6 cells / 100mm ^{2} dish twenty four hours before transfection. Two hours before the addition of the DNA precipitate, the medium on each dish was replaced with five ml of fresh supplemented medium. The calcium phosphate precipitate was prepared by slowly adding 0.5 ml DNA solution (20 µg DNA; 50 mM $CaCl_2$) to 0.5 ml of 2X HBS buffer (1.64% (w/v) NaCl, 1.2% (w/v) Hepes and 0.04% (w/v) Na_2HPO_4 /pH 7.10) while bubbling air into the solution. The precipitate was then allowed to form for 15-25 minutes. One ml of precipitate in 1X HBS buffer was added dropwise to each plate. Following a 3-4 hour incubation period at 37°C the cells were glycerol-shocked with 2 ml 20% glycerol in 1X HBS buffer for one minute and were then washed with 4 ml 1X HBS buffer. Ten ml of fresh supplemented medium were added to each plate and the cells were incubated at 37°C in a humidified CO_2 incubator, for an additional 40-44 hours before harvesting

2.10.2 Viral DNA replication assay

Cells were transfected as described above. Forty eight hours after transfection, cells were washed with phosphate buffered saline (PBS), and pelleted in PBS. Viral DNA was isolated by a modification of the method of Hirt (Hirt, 1967; Salvino et al., 1991). Cell pellets were resuspended in 100 ul Hirt wash buffer (150 mM NaCl, 20 mM Tris-HCl, pH 7.5. 10 mM EDTA). A 5' [32P] ATP-labeled (40-60,000 cpm) 1.1 kb *Bam*HI /*Eco*RI DNA fragment of pMM984 (MVM 1-1084) was also added to monitor recovery of DNA. Hirt lysis buffer (400 µl of 1.25 M NaCl; 20 mM Tris, pH 7.6; 10 mM EDTA; 0.75% SDS; 100 µg/ml proteinase K) were then added to each sample. The lysates were incubated at 37 °C for 3-4 hr, followed by incubation on ice for 16 hr. Cell debris was pelleted by centrifugation in an Eppendorf centrifuge for 1hr at 4 °C. The supernatant was transfered to a new tube, and DNA was precipitated by the addition of 5 µl yeast tRNA (50 µg) and 1ml 95% ethanol. DNA was recovered by centrifugation and was resuspended in 200 ul TE containing 100 µg/ml RNAse A and incubated at 37 °C for 30 min. The

samples were phenol:chloroform extracted once, followed by one chloroform extraction. DNA was precipitated by the addition of 5 ul yeast tRNA (50 µg), 8 ul 5 M NaCl, and 400 µl 95% ethanol. The DNA was recovered by centrifugation and was washed once with 70% ethanol. The precipitate was air-dried and resuspended in 45 µl H_2O. For some experiments, viral DNA extracts were not phenol:chloroform extracted in order to increase the yield of viral DNA.

The amount of radioactivity recovered was determined by counting the entire sample in a Beckman liquid scintillation counter. Equal amounts of DNA, as determined by counting radioactivity, were digested with 5 U of *Dpn* I restriction endonuclease for three hours at 37 °C. The samples were then loaded onto a 0.8% agarose gel in 1X TBE and electrophoresed at 2.4 mA/cm for 14 hrs. The DNA was transfered to a nylon membrane (Magnagraph) by the method of Southern (1975). The membrane was baked at 80 °C for 1-2 hrs, followed by prehybridization in (50% deionized formamide, 5X Denhardt's solution [0.1% BSA, 0.1% Ficol, 0.1% polyvinyl pyrrolidone]; 5X SSC ; 0.1% SDS (w/v); 0.1% BSA (w/v); 20 mM sodium phosphate, pH 7.0; 200 µg/ml sheared denatured salmon sperm DNA; 200 µg/ml denatured yeast tRNA) at 50 °C for 2 hr. The membrane was probed with pSP64-5 (Salvino et al., 1991), a radiolabeled RNA Riboprobe complementary to the 5' end of the MVM genome (4177-5149), in hybridization buffer (50% deionized formamide, 5X Denhardt's solution [0.1% BSA, 0.1% Ficol, 0.1% polyvinyl pyrrolidone]; 5X SSC ; 0.1% SDS (w/v); 20 mM sodium phosphate, pH 7.0; 200 µg/ml sheared denatured salmon sperm DNA; 200 µg/ml denatured yeast tRNA , > 1 X 10^6 cpm probe/ml hybridization buffer) for 12 hrs at 50 °C. The membrane was then washed with two changes of 5X SSPE (0.75 M NaCl; 50 mM $NaH_2PO4{\cdot}2H_2O$; 0.5% (w/v) SDS; 5 mM EDTA at pH 7.4) for 30 min at room temperature, followed by a 30 min wash in two changes of 1X SSPE; 0.5% (w/v) SDS at 37 °C, and a 30 min wash in two changes of 0.1X SSPE; 1.0% (w/v) SDS at 65 °C. Kodak XAR-5 film and an intensifying screen were used for autoradiography.

2.10.3 Transient chloramphenicol acetyl transferase expression assays

Transient chloramphenicol acetyl transferase (CAT) expression assays were performed as previously described (Gorman et al., 1982), with some modifications. 100 mm^2 tissue culture plates were cotransfected with 10 ug pP39CAT and 10 ug

pSVNS1-1 as described above and harvested 40-44 hrs later. The cell were washed with PBS and scraped off the plates in 4 ml TEN (40 mM Tris pH 7.8, 1 mM EDTA, 150 mM NaCl) buffer. The cells were pelleted in a Damon IEC centrifuge at 3000 rpm for 5 min at 4 ^{o}C. The cell pellets were lysed in 400 µl 0.25 M Tris-HCl (pH 7.8) by several freeze-thaw cycles using a dry ice/ethanol bath and a 37 ^{o}C water bath. Cell debris was pelleted by centrifugation in a Damon IEC centrifuge at 3000 rpm for 20 min. at 4 ^{o}C. Deacetylases were inactivated by incubating the cell lysates at 60 ^{o}C for 10 minutes. The lysates were cleared by centrifugation in an Eppendorf centrifuge for 10 min at 4 ^{o}C.

One eighth of the lysate was then incubated in the presence of 1 mM acetyl CoA and 0.2 µCi [^{14}C] -labeled chloramphenicol (specific activity 54 mCi/mmole) at 37 ^{o}C for 20-30 min. The reaction products were separated on a thin layer silica gel G plate (Analtech) with chloroform:methanol (95:5) and visualized by autoradiography using Kodak XRP 5 X-ray film. Acetylation was quantified by scraping the acetylated and non-acetylated forms of chloramphenicol from the plate and determining the radioactivity.

2.10.4 Western blot analysis

Expression of NS-1 was determined using western blot analysis techniques essentially as described in Sambrook et al., 1989. COS-7 cells were transfected with a wild type or mutant pSVNS1-1 plasmid or infected with MVM_p. Infected cells were harvested after 24 hrs. Transfected cells were harvested after 48 hrs. Cells were harvested in TEN buffer (40 mM Tris pH 7.8; 1 mM EDTA; 150 mM NaCl) and pelleted by centrifugation. The cell pellet was resuspended in two volumes of TEN buffer. Cells were then lysed by four freeze-thaw cycles. Cell debris was removed by centrifugation in an Eppendorf centrifuge.

One fourth to one third of the lysate was electrophoresed on an SDS/7.5% polyacrylamide gel. The proteins were electroblotted onto a nitrocellulose filter (Micro Filtration Systems) in Western blot buffer (20 mM Tris-HCl, pH 8.5; 150 mM Glycine; 20 % methanol), at 70 v/ 0.2 A for 4 hr and the filter was blocked with 5% Carnation instant skim milk overnight in TS buffer (10 mM Tris pH 7.4, 150 mM NaCl). The blot was incubated with a 1:200 dilution of a polyclonal rabbit antiserum prepared against the C-terminal 16 amino acids of NS-1 in TS buffer with 5% Carnation instant skim milk. The

polyclonal rabbit antibodies used in this study were generated against the 16 C-terminal amino acids of NS-1 (GAQPLKKDFSQPLNLD) conjugated through a cysteine residue to keyhole limpet hemocyanin (Cambridge Biochemicals). Bound anti-NS-1 antibody was detected by incubating the immunoblot with a 1:500 dilution of an anti-rabbit IgG swine immunoglobulin peroxidase conjugate (DAKO Corp.) in TS with 0.1% Tween 20 and 0.5% bovine serum albumin. The immunoblots were developed in TS with 0.1% Tween 20, 0.03% 4-chloro-1-naphthol and 0.0002% H_2O_2.

2.11 Protein A/NS-1 fusion polypeptide expression

The *Bam*HI fragment containing the NS-1 coding sequence was placed adjacent to the sequence for the IgG binding region of the *staphylococcus aureus* protein A gene in pRIT2T. The resulting clone pPANS-1 is predicted to encode a 110 kDa fusion polypeptide consisting of an N-terminal IgG binding protein A domain fused to the MVM NS-1 protein. This vector was transfected into the *E. coli* strain N4830-1 which allows the heat inducible expression of a protein A /NS-1 fusion product.

2.11.1 Heat induction of expression

Three ml overnight culture containing pPANS-1 was grown in LB broth (50 ug ampicillin/ml) at 28 °C with shaking. One hundred and fifty ml LB broth (50 ug/ml ampicillin) were inoculated with 1.5 ml overnight culture and incubated at 30 °C with shaking until OD600 = 1.2 . The cells were heat shocked by the addition of LB broth (50 µg/ml ampicillin) which had been prewarmed to 54 °C. The cells were incubated at 42 °C with shaking for 90 min. Cells were harvested by centrifugation in a JA-14 rotor at 9000 rpm for 10 min at 4 °C. The cells were then used immediately or were stored at -80 °C.

2.11.2 Purification of fusion product

Cells were resuspended in 20 ml TST buffer (50 mM Tris -HCl, pH 7.6; 150 mM NaCl; 0.05% Tween 20) and were passed through a French press at a setting of 13,000 psi. Eighty ml of TST buffer were added to the lysate. Cell debris was removed by centrifugation in a Beckman SW28 swinging bucket rotor at 20,000 rpm for 30 min at 4 °C.

2.11.3 Affinity chromatography

An IgG Sepharose 6 Fast Flow (Pharmacia) column (5-10 ml) was prepared for chromatography by washing the gel beads with at least 5 bed volumes TST . The column was then equilibrated with 2-3 volumes of 0.1 M acetic acid (pH 3.4), followed by 2-3 volumes TST buffer. This process was repeated once and the lysate was then applied to the column. The column was washed with 140 ml TST followed with 30 ml 5 mM ammonium acetate (pH 5.0). The bound protein was eluted with 0.1 M acetic acid (pH 3.4). Three quarter ml fractions were collected and immediately neutralized with the addition of 500 ul 0.5 M potassium phosphate buffer (pH 12.4). Protein concentration was quantified by determining the OD at 280 nm.

CHAPTER 3 RESULTS

3.1 Introduction

The NS-1 gene of MVM is a multifunctional protein whose known activities include its requirement for replication of the MVM genome, transcriptional regulation of the two MVM promoters, hydrolysis of ATP, covalent binding of single-stranded and replicative forms of MVM DNA, concatemer resolution, and a cytotoxic activity towards neoplastic cells. The construction of inducible shuttle vectors expressing the NS-1 protein and their use in determining the effects of a series of insertion and point mutations on the DNA-replication and transactivation activities are described.

3.2 Isolation of NS-1 coding sequence and construction of NS-1 vectors

The NS-1 coding sequence was excised from the MVM genome and *Bam*HI linkers were added onto the blunted ends. The DNA was cloned into several prokaryotic and eukaryotic expression vectors. An SV40 promoter driven, large T-antigen inducible, expression vector (pSVNS1-1) was used to study the effects of mutations on the viral DNA replication and transactivation function of NS-1 (Skiadopoulos et al., 1992). A [^{32}P] CTP labeled RNA probe that is complementary to the 5' end of the viral DNA strand was used to detect MVM sequences. In extracts of MVM infected cells, this probe detects the single stranded viral genome, the monomer replicative form (RF) as well as other slower migrating intermediates (Fig. 8, lane 1). In extracts that were not phenol:chloroform extracted, additional slower migrating species of ss and RF DNA were also detected (Fig. 8, lane 2). As shown in Fig. 9, pSVNS1-1 complements the replication defective target plasmid ins 20B. Ins 20B contains an NS-1 frame shift mutation that should lead to the production of a severely truncated NS-1 polypeptide consisting of 276 N-terminal amino acids (Salvino et al., 1991). This plasmid is unable to replicate in transient DNA replication assays in A9 cells (Salvino, 1990) and in COS-7 cells (Salvino et al., 1991). The ins 20B

Figure 8 pSP64-5 Riboprobe hybridizes to MVM DNA from infected COS-7 cells.

COS-7 cells were infected with MVMp as described. Cells were harvested 24 hr post infection and Hirt extracts were prepared. The DNA was separated by agarose gel electrophoresis and transfered onto nitrocellulose by the method of Southern (1975). A [^{32}P] CTP labeled RNA probe complementary to the right end of the viral MVM strand was prepared from pSP64-5 and hybridized to the blot (see Materials and Methods for additional details). Lane 1 contains MVM DNA that was phenol:chloroform extracted before electrophoresis. Lane 2 contains DNA that was not phenol:chloroform extracted. RF, monomer RF MVM DNA; ss, ss MVM DNA.

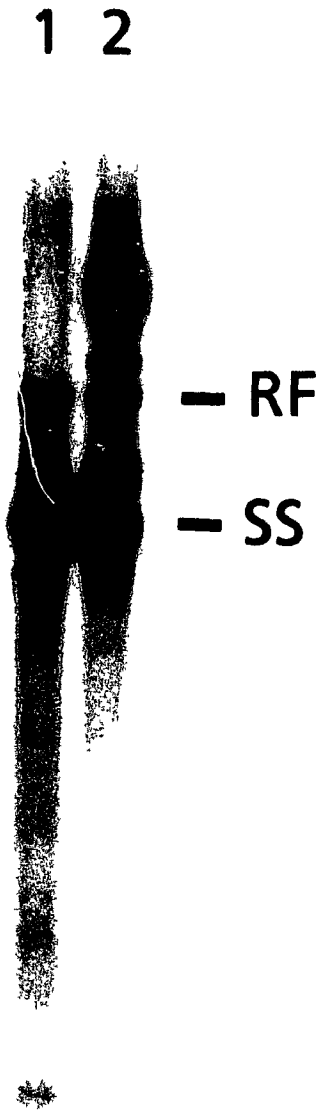

Figure 8 pSP64-5 riboprobe hybridizes to MVM DNA from infected COS-7 cells

Figure 9 Complementation of ins 20B replication by pSVNS1-1.

COS-7 cells were transfected with 20μg sheared calf thymus DNA (mock), 10μg ins 20B DNA + 10μg sheared calf thymus DNA (ins 20B) or 10μg ins 20B + 10ug pSVNS1-1. Hirt extracts were prepared and subjected to Southern blot analysis (See Methods). An undiluted aliquot (1:1) and dilutions of the extract from ins 20B and pSVNS1-1 cotransfection are indicated. ss, single stranded DNA ; RF, monomer replicative form DNA.

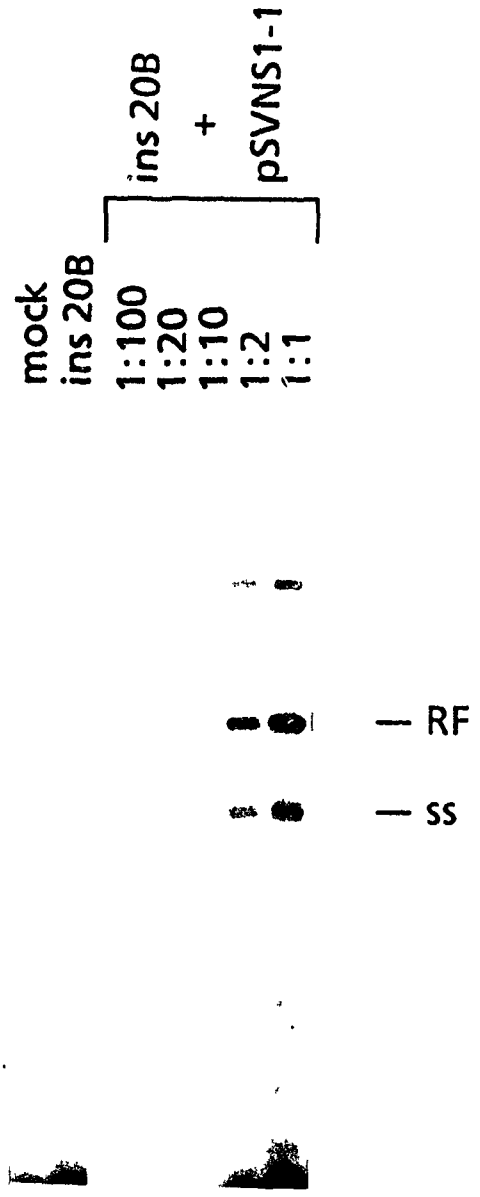

Figure 9 Complementation of ins 20B replication by pSVNS1-1.

reporter plasmid has a unique *Mlu* I site which was introduced along with the frame shift mutation to distinguish it from wild-type viral DNA which is not cleaved by *Mlu* I (Salvino et al., 1991) . MVM DNA cleaved with *Mlu* I migrates just above ss MVM DNA. In a pSVNS1-1/ins20B cotransfection of COS-7 cells, 1-5% of the replicated DNA was not cleaved with *Mlu* I (Fig. 10). Therefore, the majority of the replicated viral DNA scored in the viral DNA replication assay represents ins 20B, while there may be a low level of recombination that produces wild type viral DNA. In this study, DNA was not generally cleaved with *Mlu* I and therefore any resultant recombinants have been included. Double recombinants might be expected to arise at a low frequency of 1% or less. This theoretical level of wild type viral DNA arising by recombination between mutant NS-1 genes (in a pSVNS1-1/ins 20B cotransfection) would be barely detectable and within the range of experimental variation of the DNA replication assay. Therefore, the slight overestimate of DNA replication levels that might result from the inclusion of wild type recombinants in the assay of ins 20B replication would be insignificant and have virtually no effect on the results. Actually, many mutants of NS-1 that readily exhibited a rep$^-$ phenotype (< 1% replication) were obtained, demonstrating that wild type double recombinants are indeed rare (for example see Fig. 12 below).

Cotransfection of pSVNS1-1 along with ins 20B in transient replication assays allowed excision and replication of the ins 20B genome to occur in COS-7 cells as judged by the detection of viral DNA forms in Southern blots of extracts from transfected cells (Fig. 9), but not in A9 cells (data not shown). Replication of ins 20B in COS-7 cells in the presence of pSVNS1-1 produced single stranded DNA and a 5.1 kbp RF DNA (Fig. 9). The sensitivity of this assay is such that at least 5% of the pSVNS1-1 replication level could be detected routinely and for certain experiments the sensitivity was increased up to 5-fold by overexposure of the autoradiograms. Estimates of the replication activity of mutant NS-1 genes were made by comparison with standard dilutions of pSVNS1-1. Thus, pSVNS1-1 effectively complements the defect in ins 20B by providing a functional NS-1 protein in*trans*. The DNA sequence encoding the extreme carboxyl terminus of NS-2 is absent from pSVNS1-1. However, NS-2 is encoded by ins 20B, and it has been shown that NS-2 is not required for viral DNA replication in COS-1 cells (Naeger et al., 1990). This experimental system permitted the direct evaluation of the effect of mutations in the NS-1 gene on viral DNA replication.

Figure 10 Complementation of ins 20B replication by pSVNS1-1 and cleavage of ins 20B genome by *Mlu* I.

COS-7 cells were transfected with pSVNS1-1 (10μg) and ins 20B (20μg), and Hirt extracts were prepared. The DNA was digested with *Dpn* I only (—), or *Dpn* I and *Mlu* I (MluI) and subjected to Southern blot analysis as described. Dilutions of the DNA sample that was only treated with *Dpn* I (—) are shown (1:2; 1:10; 1:20; 1:100). RF, MVM monomer replicative form; ss, single stranded MVM DNA.

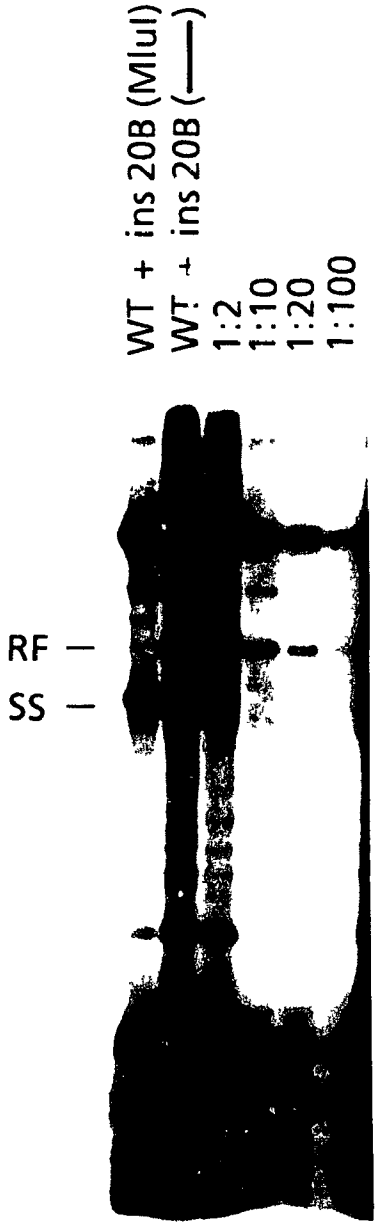

Figure 10 Complementation oif ins 20B replication by pSVNS1-1 and cleavage of ins 20B genome by Mlu I

3.3 Effect of frame-shift mutations in NS-1 on DNA replication

To determine if the rep function of NS-1 could be localized to a specific N-terminal domain of the protein, frame shift mutations were introduced into the NS-1 gene so as to produce a series of truncated NS-1 polypeptides that were missing increasing amounts of the carboxyl-terminus of the NS-1 protein (Fig. 11, Table 4). None of these mutants (ΔEVM, ΔSIb, ΔBIb, ΔBIIb) displayed any detectable DNA replication activity (Fig. 11) suggesting either that there is a single rep domain whose integrity is adversely affected by each of the mutations tested, that there are multiple rep domains, at least one of which is affected by each of the mutations tested, or that an essential rep domain lies within or is affected by the carboxyl-terminal truncation of 129 amino acids.

3.4 Effect of in-frame linker-insertion mutations in NS-1 on DNA replication

To determine whether a distinct rep domain could be identified within the NS-1 protein, insertion and point mutations were introduced throughout the NS-1 coding region of pSVNS1-1. The mutations were located between amino acids 41 and 607, inclusive (Table 4 and Fig. 12). The effect of each mutation on the ability of NS-1 to support the replication of a cotransfected ins 20B target plasmid was tested in an *in vivo* transient DNA replication assay in COS-7 cells. The results are summarized in Table 4.

Insertion mutations between Asp41 and Ile42 (ΔEVH), Thr219 and Ser220 (ΔSIM4), Asp467 and Gln468 (ΔBIM1, ΔBIM4), and Tyr543 and Gln544 (ΔBIIM1) each reduced the *in vivo* rep activity of the NS-1 protein between 20 and 100-fold relative to that observed for the wild type expression plasmid pSVNS1-1 (Fig. 12, Table 4) as measured by comparison to serial dilutions of viral DNA isolated from a pSVNS1-1 and ins 20B cotransfection. The insertion mutations that exerted these effects ranged in size from 2 amino acids at position 41-42 to 12 amino acids at positions 219-220 and 467-468. Interestingly, an insertion of 4 amino acids between Thr219 and Ser220 (ΔSIM1) did not affect the rep activity of NS-1 (Fig. 12). A single amino acid insertion between Asp606 and Leu607 (ΔEOb) also had no apparent effect on the rep activity of NS-1. Two point mutations converting Ser249 to Ala249 (ΔA249) and Lys250 to Gln250 (ΔQ250) were

Figure 11 Map of location of insertion and point mutations in NS-1.

The upper portion of the figure depicts the DNA sequence of the NS-1 gene with the locations (nt) of the unique restriction sites used for insertion mutagenesis. A diagram of the NS-1 protein showing the position of insertion (▽) and point mutations (⌇) is also presented. The diagram also depicts the NS-1 truncation and insertion mutants used in this study. The hatched boxes (▨) represent missense amino acid sequences arising from frame shift mutations in the NS-1 gene. The solid blocks (■) represent the locations and relative sizes of insertions in the wild type NS-1 polypeptide.

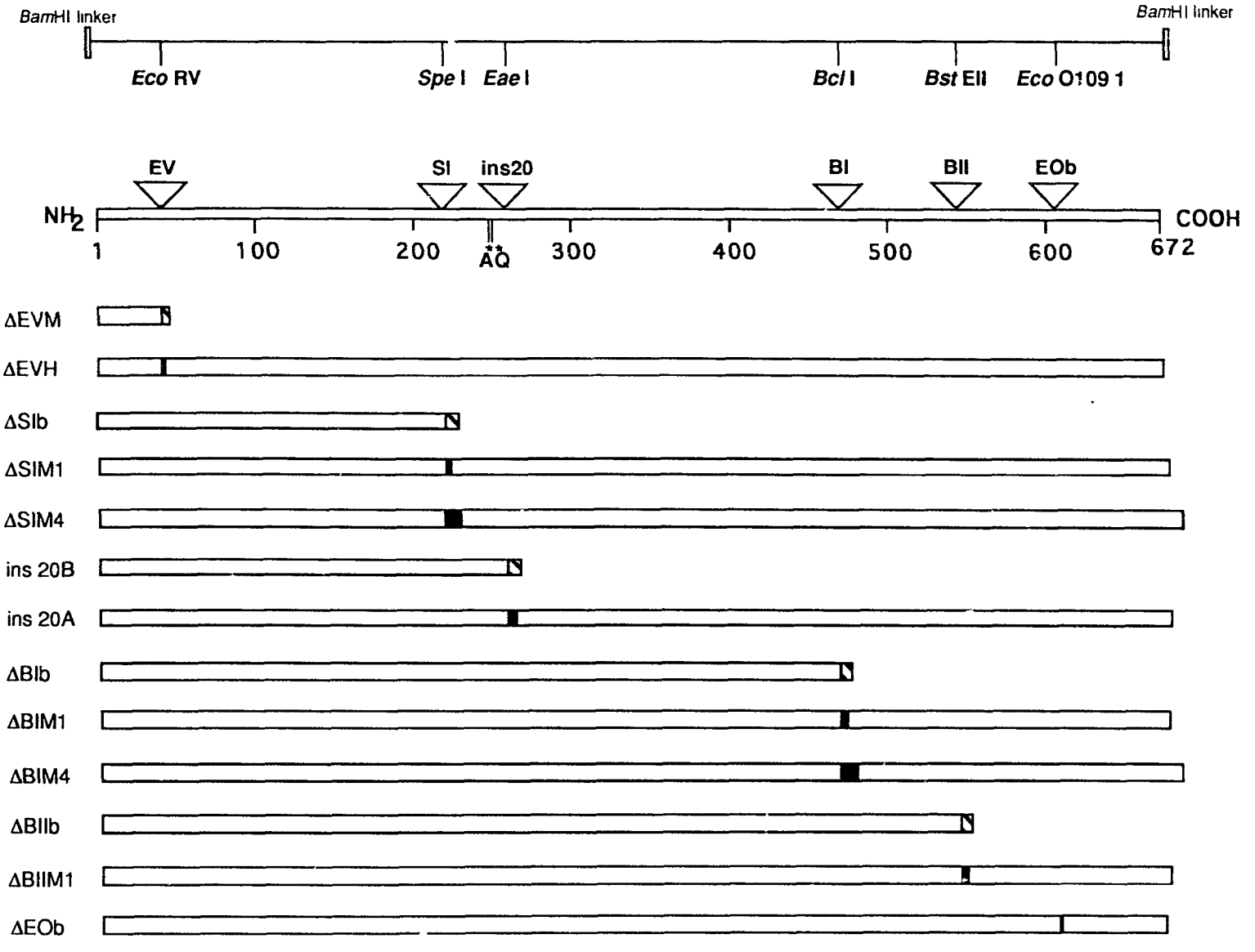

Figure 11 Map of location of insertion and point mutations in NS-1

Figure 12 Complementation of ins 20B replication by NS-1 insertion and point mutations.

Ins 20B (10μg) was cotransfected into COS-7 cells with 10 ug calf thymus DNA (CT), 10 μg pSVNS1-1(WT) or 10 μg each of the NS-1 mutants as indicated. Hirt extracts were prepared and analyzed as described in Material and Methods. Dilutions of DNA isolated from pSVNS1-1 and ins 20B cotransfections are indicated (1:10; 1:100). RF, MVM monomer replicative form; ss, single stranded MVM DNA.

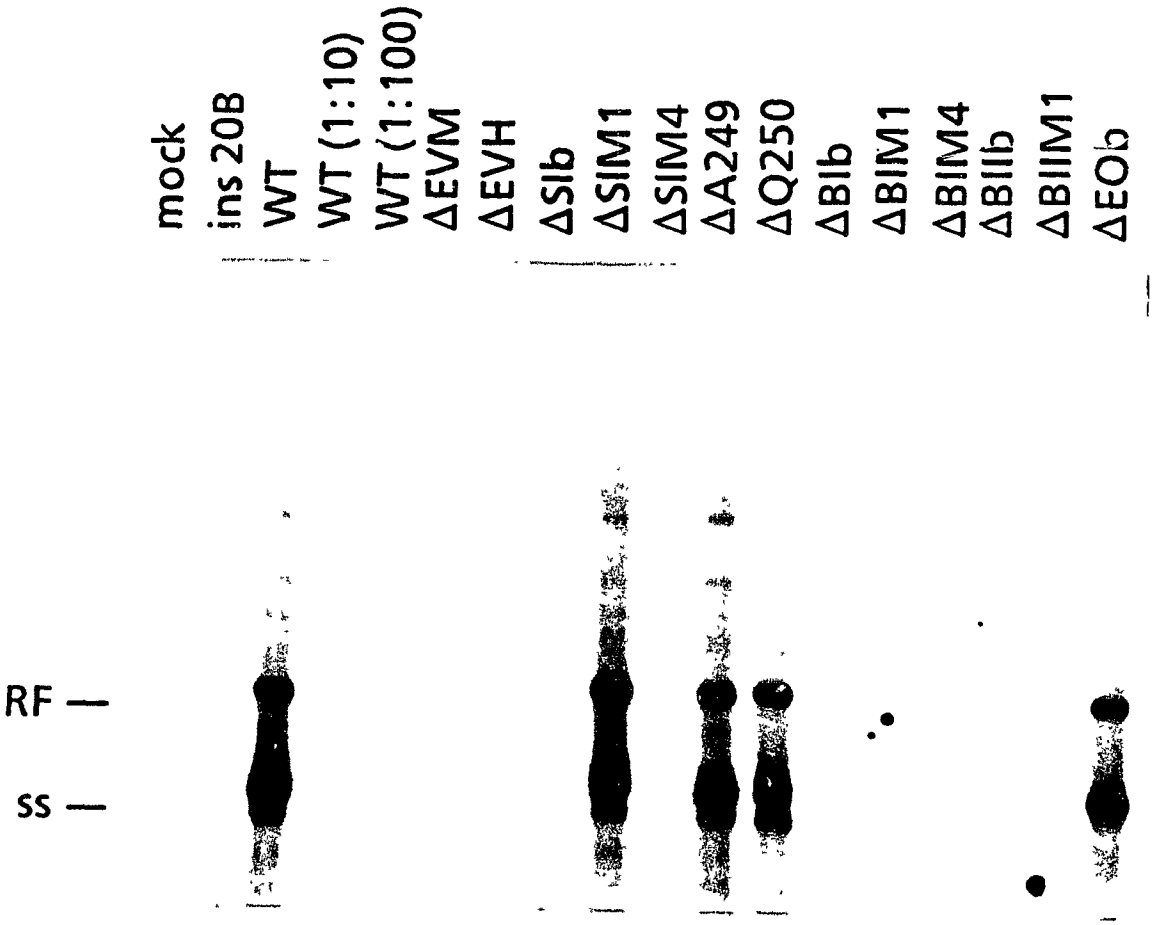

Figure 12 Complementaion of ins 20B replication by NS-1 insertion and point mutations

Table 4 Insertion and point mutations: effects on DNA replication and transactivation functions

CONSTRUCTS	INSERT	CODON POSITION	REP[a]	CAT[b]
pSVNS1-1	---	wild type	++	100
pSV-ΔEVM	frame shift (deletes 42-672)	41- 42	-	3.2 ± 0.9
pSV-ΔEVH	VAL-ASN	41- 42	≤1%	74.9 ± 6.0
pSV-ΔSIb	frame shift (deletes 220-672)	219-220	-	1.2 ± 0.1
pSV-ΔSIM1	ARG-THR-ARG-PRO	219-220	++	64.5 ± 2.8
pSV-ΔSIM4	ARG-THR-ARG-ARG-ARG-VAL-ASP-ALA-SER-THR-ARG-PRO	219-220	-	8.0 ± 0.7
pSV-ΔA249	SER to ALA	249	++	100.7± 2.0
pSV-ΔQ250	LYS to GLN	250	++	93.4± 1.3
ins 20B[c]	frame shift (deletes 259-672)	258-259	-	1.4 ± 0.6
pSV-ΔBIb	frame shift (deletes 468-672)	467-468	-	1.1 ± 0.1
pSV-ΔBIM1	ARG-ARG-VAL-ASP	467-468	-	19.1 ± 3.1
pSV-ΔBIM4	GLY-ARG-VAL-ASP-ALA-SER-THR-ARG-ARG-ARG-VAL-ASP	467-468	-	8.9 ± 1.3
pSV-ΔBIIb	frame shift (deletes 544-672)	543-544	-	2.0 ± 1.5
pSV-ΔBIIM1	ARG-ARG-VAL-GLY-TYR	543-544	-	1.3 ± 0.4
pSV-ΔEOb	ASP	606-607	++	103.1 ± 5.4

[a] rep activity of NS-1 as determined in in vivo transient replication assays. The lower limit of detection in this assay was between 1-5% of wild type. ΔEVH replicated at 1% of wild type in one of six experiments. [b] values are the result of at least three experiments and indicate the percent transactivation (± standard error) normalized to wild type NS-1 which is set at 100%. Results were compiled from experiments in which the acetylation levels were below 65%. [c] values are compared to pMM984.

also tested. These point mutations did not effect the ability of NS-1 to support viral DNA replication (Fig. 12).

In addition to these results it was also shown that none of the rep$^-$ mutants with 1, 2, 4 or 5 amino acid insertions exhibited temperature sensitivity when their rep activities were compared at 32 ^oC and 37 ^oC (data not shown). Thus, relatively minor perturbations of the primary structure at a variety of locations in the NS-1 protein severely affect rep activity.

3.5 Transcriptional activation of the p38 promoter by pSVNS1-1

NS-1 has previously been shown to greatly enhance transcription from the capsid gene promoter located at map units 38-39 (Doerig et al., 1988; Rhode III, 1987). The pSVNS1-1 construct and pMM984 were tested for their ability to transactivate the p38 promoter in COS-7 cells, in cotransfection assays with a p38 promoter driven CAT expression vector, pP39CAT (Fig. 4 C). In these assays the p38 promoter in pP39CAT is constitutively weak in COS-7 cells (Fig. 13), as it is in many other transformed cell lines (Doerig et al., 1988; Rhode III, 1987). With cotransfection of pSVNS1-1 or pMM984, the promoter was turned on to a high level of expression (Fig.13). The rate of the acetylation reaction was determined by assaying aliquots of cell extracts from a pSVNS1-1 and pP39CAT cotransfection (Fig. 14). Below approximately 70 % acetylation, the reaction was in the linear range.

3.6 Effect of in-frame insertions in NS-1 on transactivation of the p38 promoter

To further characterize the domain structure of NS-1, each of the NS-1 mutants was tested for its ability to stimulate transcription from the MVM p38 promoter in CAT assays. Insertions of between 1 and 12 amino acids between Asp41/ Ile42, Thr219/Ser220, Asp467/Gln468 and Asp606/Leu607 in NS-1 failed to completely block transactivation of the p38 promoter, although these mutations reduced transactivation between 1.5 and 10-fold compared to wild type (Figs. 15 A, Table 4). The point mutants ΔA249 and ΔQ250 were able to transactivate the p38 promoter at wild-type

Figure 13 Transactivation of pP39CAT by pSVNS1-1 and pMM984.

COS-7 cells were cotransfected with pP39CAT (10μg) and 10μg of sheared calf thymus DNA (—), pSVNS1-1 or pMM984 as indicated. Crude extracts were prepared and CAT enzyme was assayed as described in Materials and Methods. The percent acetylation is indicated at the bottom of the figure. C, chloramphenicol; AC, acetylated chloramphenicol.

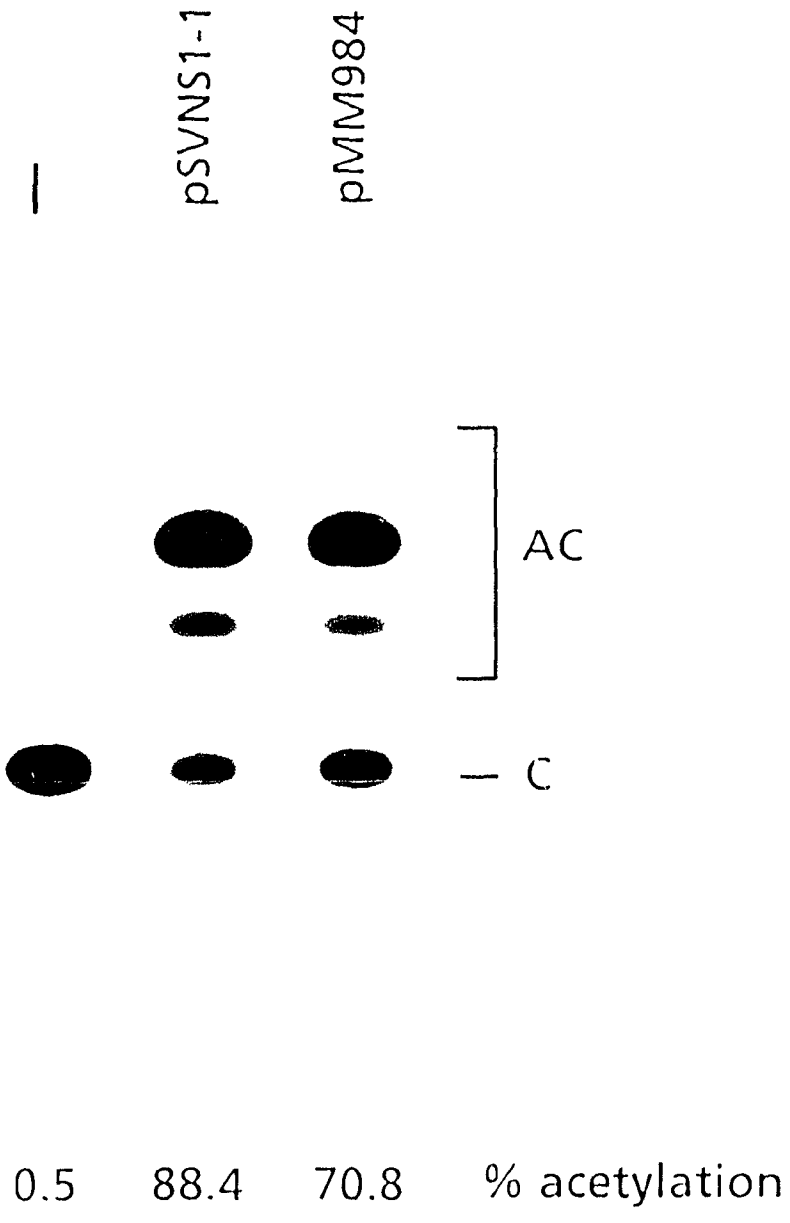

Figure 13 Transactivation of pP39CAT by pSVNS-1 and pMM984

Figure 14 Assay of chloramphenicol acetyl transferase activity in crude extracts of COS-7 cells.

COS-7 cells were cotransfected with pSVNS1-1 (10 μg) and pP39CAT (10 mg) as described. Crude CAT extracts were prepared and assayed for their ability to acetylate ^{14}C- labeled chloramphenicol. Aliquots were removed and the reaction was stopped at the indicated time points with the addition of ethyl acetate. The samples were prepared for chromatography, and conversion of ^{14}C labeled chloramphenicol to acetylated forms was determined as described in Materials and Methods.

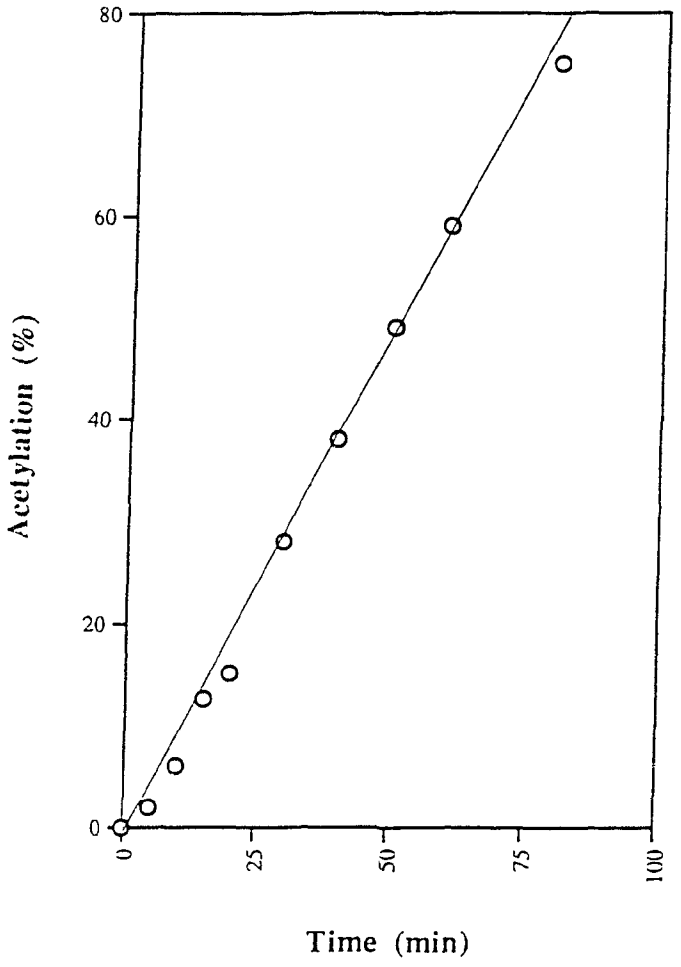

Figure 14 Assay of chloramphenicol acetyl transferase activity in crude extracts of COS-7 cells

levels (Fig. 15 B). In contrast, a 5 amino acid insertion between Tyr543 and Gln544 (ΔBIIM1) completely blocked the transactivation function of NS-1 (Fig. 15 A, Table 4).

The ability of the in-frame insertion mutants to transactivate the p38 promoter at 32 ^{o}C and 37 ^{o}C was also examined. The relative efficiency of transactivation at the two temperatures was the same for ΔEVH, ΔSIM4, ΔBIM4, ΔBIIM1 and ΔEOb. However, ΔBIM1 was 1.5 - 2.5 fold more efficient in transactivating the p38 promoter at 32 ^{o}C than at 37 ^{o}C in two out of three experiments (data not shown). Certain insertion mutations that reduced the rep activity of NS-1 between 20 -100 -fold had only a relatively small (1.5- 5 fold) inhibitory effect on transactivation (compare the effect of the ΔEVH, and ΔBIM1 mutations on replication and CAT activities in Table 4).

3.7 Effect of frame-shift mutations in NS-1 on transactivation of the p38 promoter

Each of the frame shift mutations listed in Table 4 completely abolished the transactivation activity of NS-1 toward the p38 promoter (Fig. 15). Since the smallest truncation created by these frame shift mutations was a carboxyl-terminal deletion of 129 amino acids (ΔBIIb), the amino terminal 543 amino acids, or a portion thereof, is apparently insufficient to maintain its transactivation activity.

3.8 BIIM1 does not display a dominant negative phenotype in transactivating p38

ΔBIIM1 was assayed for its ability to inhibit wild type NS-1 transactivation of p38 in transient expression assays by cotransfecting increasing amounts of ΔBIIM1 with pSVNS1-1 and pP39CAT. The results showed that at equal amounts of wild type and mutant NS-1 there is no significant decrease in the ability of wild type NS-1 to stimulate transcription from p38 (Fig. 16 A). However, at increasing concentrations of ΔBIIM1 there was a decrease in transcriptional activation as measured in the CAT assay (Fig.16 A).

As a control, ΔEVM, which codes for a severely truncated NS-1 polypeptide (Fig. 16 B), was tested for its ability to inhibit wild type transactivation of p38 in similar cotransfection assays. A parallel experiment using pSVRNS1-1 as the competing plasmid was also performed. pSVRNS1-1 contains the NS-1 coding sequence inserted into

Figure 15 Transactivation of pP39CAT by NS-1 insertion (A) and point mutations (B).

COS-7 cells were cotransfected with pP39CAT (10μg) and 10μg of sheared calf thymus DNA (—), pSVNS1-1 (10 μg) or pSVNS1-1 carrying (A) insertion mutations, and (B) point mutations. Samples were assayed for CAT enzyme activity as described in Materials and Methods. Percent acetylation is indicated. AC, acetylated chloramphenicol; C, chloramphenicol.

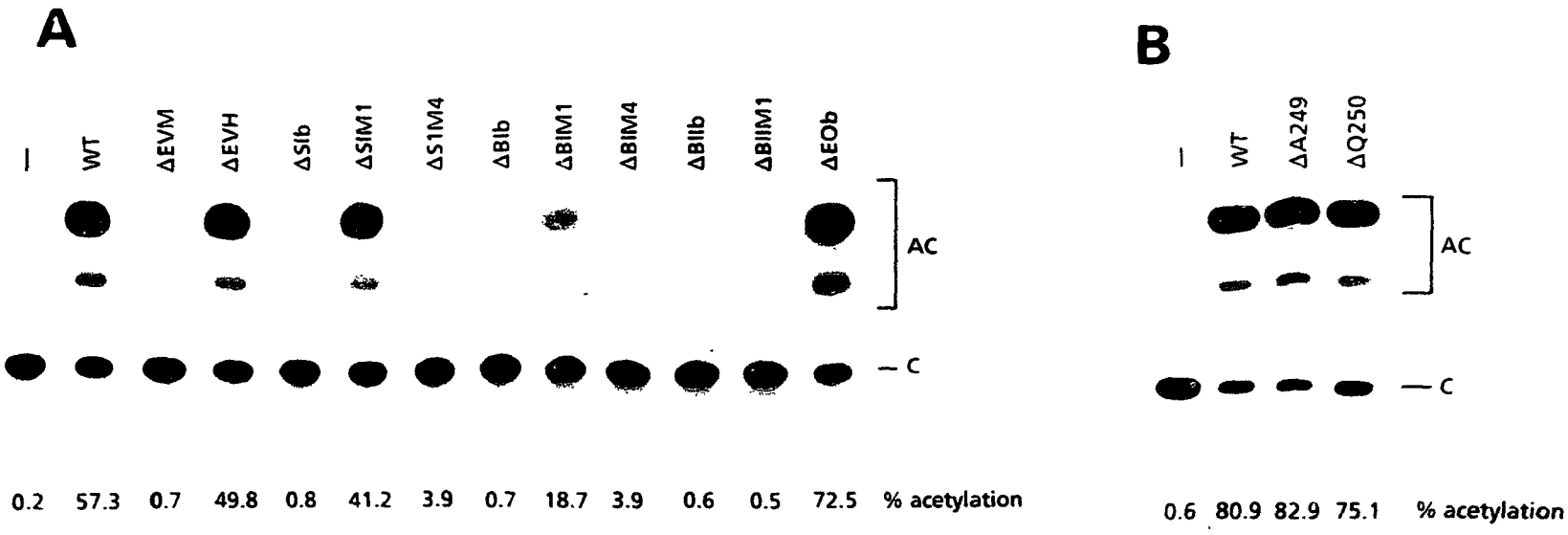

Figure 15 Transactivation of pP39CAT by NS-1 insertion (A) and point mutations (B)

Figure 16 Competitive inhibition of pSVNS1-1 induced transactivation of the p38 promoter.

COS-7 cells (100 mm^2 plates) were cotransfected with pSVNS1-1 (1μg), pP39CAT (5μg), and between 0 and 15 μg of competitor plasmid, (A) ΔBIIM1, (B) ΔEVM, or (C) pSVRNS1-1. The amount of transfected DNA was equalized to 21 μg with sheared calf thymus DNA. Samples were assayed for CAT enzyme activity as described in Materials and Methods. Percent acetylation is indicated. AC, acetylated chloramphenicol; C, chloramphenicol.

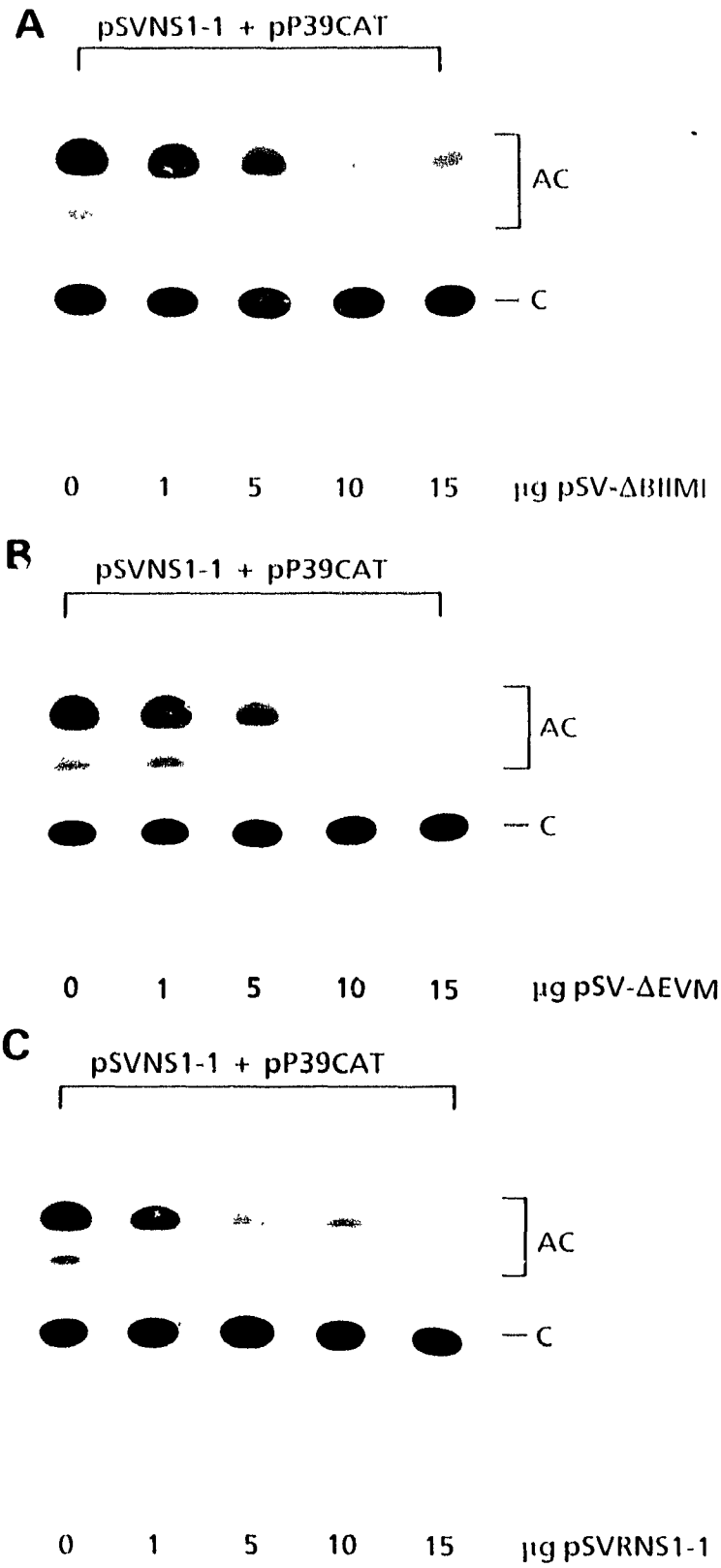

Figure 16 Competitive inhibition of pSVNS1-1 induced transactivation of the p38 promoter

pSVL in the opposite orientation, such that the NS-1 gene cannot be expressed. Figure 16 (B and C) shows that at equimolar amounts of wild type and competing plasmids there was no decrease in transactivation of p38. However, as the concentration of competing plasmid was increased, transcription from the p38 promoter was inhibited. It therefore appears that the decrease in transactivation is not due to a dominant negative phenotype of the mutations tested but perhaps is due to an uptake of transcription factors by the p38 promoter that is located within the competing plasmid DNA sequences.

3.9 Detection of mutant NS-1 proteins

The relative stability of several of the mutant NS-1 proteins in COS-7 cells was determined by immunoblot analysis as described in Methods. The rabbit anti-NS-1 antibody recognized a single major protein that was present in extracts of COS-7, A9 and EA cells that had been infected with MVM, whereas this protein was not detected in the mock-infected cells (Fig. 17 A). The relative abundance of three mutant proteins from transfected COS-7 cells as compared to transfected wild type NS-1 was determined. Figure 17 B shows that the levels of NS-1 for ΔEVH (lane 2), ΔBIM1 (lane 3) and ΔBIIM1 (lane 4) were approximately the same as compared to wild type NS-1 (lane 1), indicating that the these mutations do not adversely affect the stability of NS-1.

3.10 Mutagenesis of the conserved tyrosines of NS-1

3.10.1 Sequence comparison of related parvovirus NS-1 proteins

The NS-1 protein is covalently attached to the 5' end of DNA, most likely through a tyrosine residue. Computer assisted alignment of the predicted NS-1 amino acid sequences of feline parvovirus (Reed *et al.*, 1988), porcine parvovirus (Ranz *et al.*, 1989) and MVM reveals that there are four non-conserved tyrosines in the MVM NS-1 protein (Fig. 18). These include Tyr152 which is replaced by leucine in PPV , Tyr252 which is replaced by phenylalanine in PPV, Tyr374 which is replaced by tryptophan in FPV and Tyr595 which is replaced by histidine in FPV. These four tyrosines are therefore not likely to be involved in the formation of a tyrosyl-DNA linkage and hence mutations were not introduced at these

Figure 17 Immunoblot analysis of wild type and mutant NS-1 proteins.

Protein extracts from infected or transfected cells were prepared and were separated by SDS polyacrylamide gel electrophoresis and electroblotted onto a nitrocellulose filter. The blot was incubated with rabbit polyclonal anti-NS-1 antiserum followed by a swine anti-rabbit IgG peroxidase conjugate (see Methods). (A) Lanes 1. mock-infected COS-7 cells; 2. MVMp infected COS-7 cells; 3. mock infected A9 cells; 4. MVMp infected A9 cells. (B) Lanes 1. wild-type pSVNS1-1; 2. ΔEVH; 3. ΔBIM1; 4. ΔBIiMI; 5. MVM_p infected EA cells .

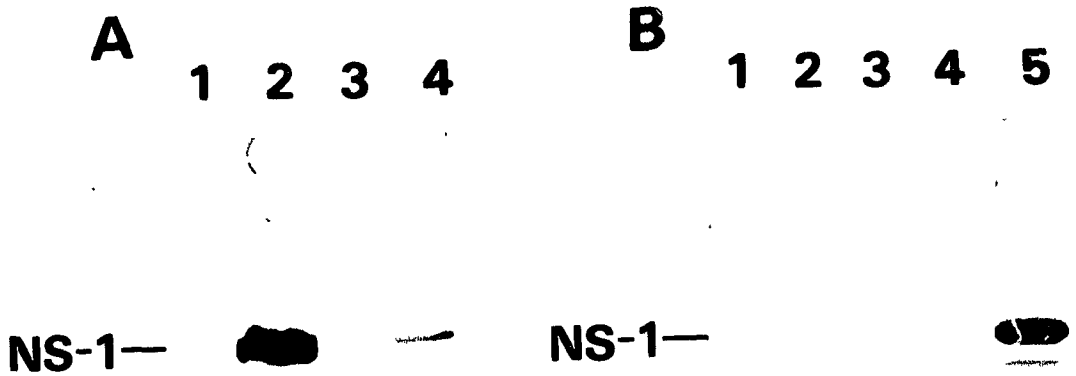

Figure 17 Immunoblot analysis of wild type and mutant NS-1 proteins

Figure 18 Computer assisted amino acid sequence alignment of mouse, porcine and feline parvovirus NS-1 proteins.

The amino acid sequences of the murine parvovirus (MVM), feline parvovirus (FPV) and porcine parvovirus (PPV) NS-1 proteins were aligned using the MULTILIN program (Corpet, 1988). Tyrosine residues are boxed. (*) indicates conserved residues; (:) indicates related residues.

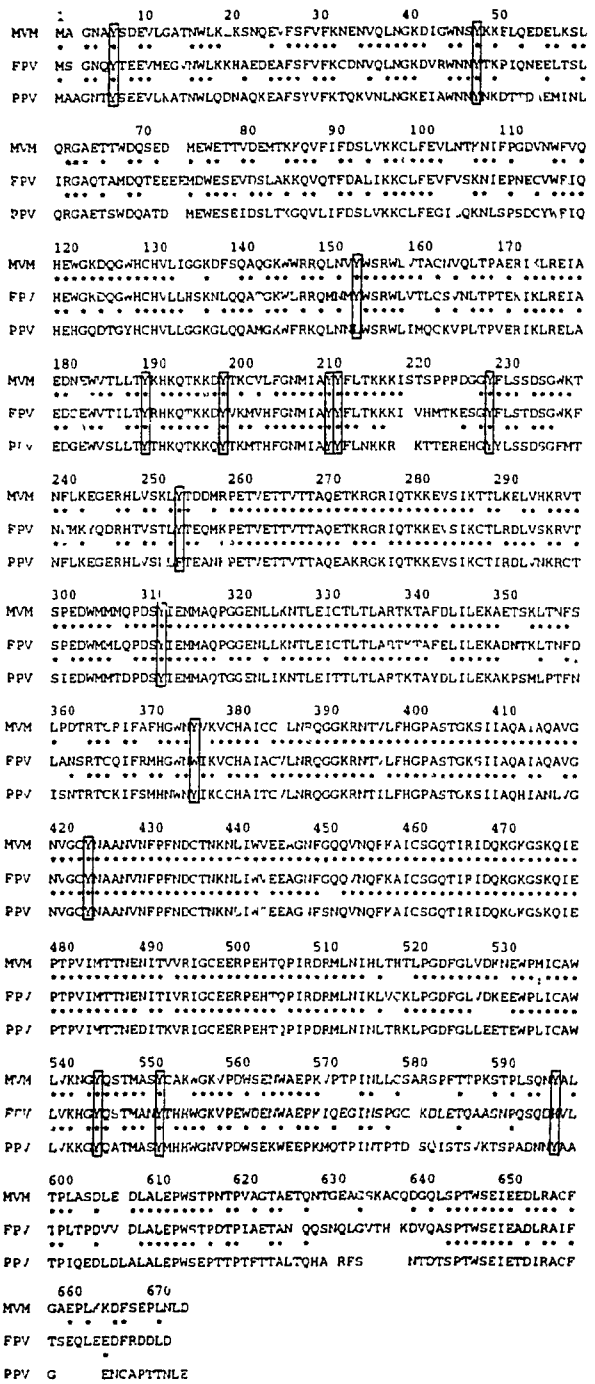

Figure 18 Computer assisted amino acid sequence alignment of mouse, porcine and feline parvovirus NS-1 proteins

positions in NS-1. The remaining 11 tyrosines that are conserved in the NS-1 proteins of all three viruses were mutated, and their ability to replicate viral DNA and transactivate the p38 promoter was determined (Skiadopoulos and Faust, 1993).

Eleven conserved tyrosine residues spanning the NS-1 protein (Fig. 19) were converted to phenylanines. The effect of Tyr to Phe substitution mutations on the DNA replication function of NS-1 was examined by cotransfecting the mutant or wild type NS-1 expression plasmid pSVNS1-1 into COS-7 cells with the reporter plasmid ins 20B. The efficiency of ins 20B replication promoted by mutant NS-1 proteins was compared with the activity of wild type pSVNS1-1.

3.10.2 Effect of F188, F197, F209 and F210 mutations on the replication activity of NS-1

Replacement of Tyr188, Tyr197 or Tyr210 with phenylanine reduced the *in vivo* DNA replication activity of NS-1 to less than 1% of the wild type level, as determined by comparing the level of monomer RF DNA produced in transfections of COS-7 cells to dilutions of viral DNA isolated from a wild type pSVNS1-1 and ins 20B cotransfection (Fig. 20). In contrast to these results, the F209 mutation had comparatively little effect, although this mutation reduced the DNA replication activity of NS-1 by approximately 2-fold, as determined by excision of the RF and ss bands and counting the radioactivity (data not shown). A double mutation in NS-1, namely F209/210, completely blocked its DNA replication function, as expected since the F210 mutation blocked DNA replication by itself.

3.10.3 F310 and F422 mutations also exhibit a rep- phenotype

The NS-1 protein contains a highly conserved region spanning approximately 250 amino acids that constitutes the central portion of the molecule, and contains a purine nucleotide binding motif (a.a. 402-406: STGKS) that is required for DNA replication (Li and Rhode, 1990; Nuesch et al., 1992) (Fig. 18). Replacement of Tyr310 and Tyr422 which are located in this highly conserved region of NS-1 with phenylalanine reduced its

Figure 19 Map of location of Tyr to Phe mutations in NS-1.

Diagram representing the relative location of the tyrosine to phenylalanine mutations in the NS-1 protein.

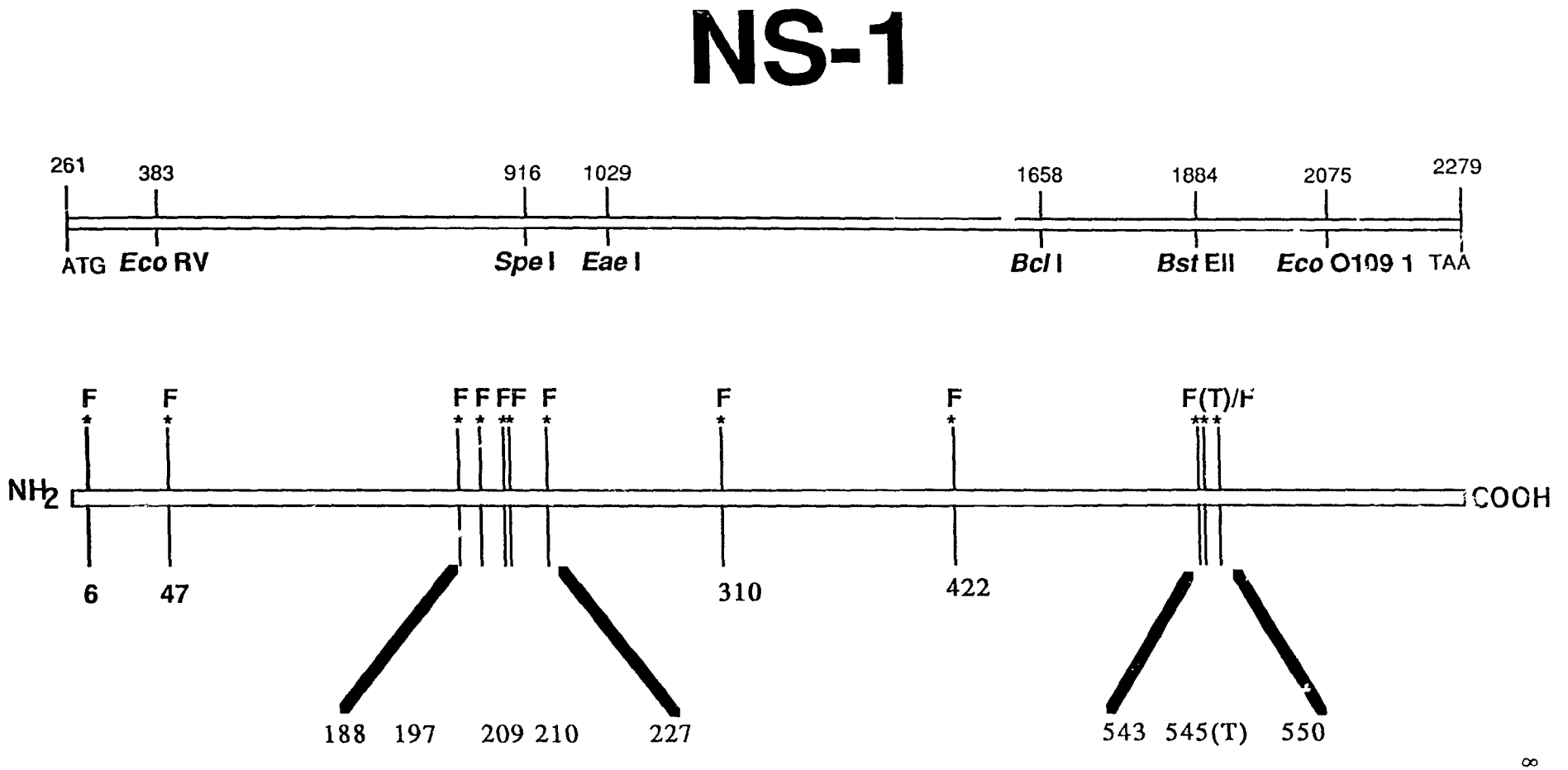

Figure 19 Map of location of Tyr to Phe mutations

Figure 20 Effect of mutations in pSVNS1-1 on viral DNA replication activity of NS-1.

COS-7 cells were transfected with 20 µg sheared calf thymus DNA (mock), 10 µg ins 20B DNA + 10 µg sheared calf thymus DNA (ins 20B), or ins 20B(10 µg) + wild type (WT) or mutant pSVNS1-1 (10 µg) as indicated. Hirt extracts were prepared and subjected to Southern blot analysis (see Methods). Dilutions of the extract from ins 20B + WT pSVNS1-1 cotransfection are indicated (1:10; 1:100). ss, single-stranded DNA; RF, monomer replicative form DNA.

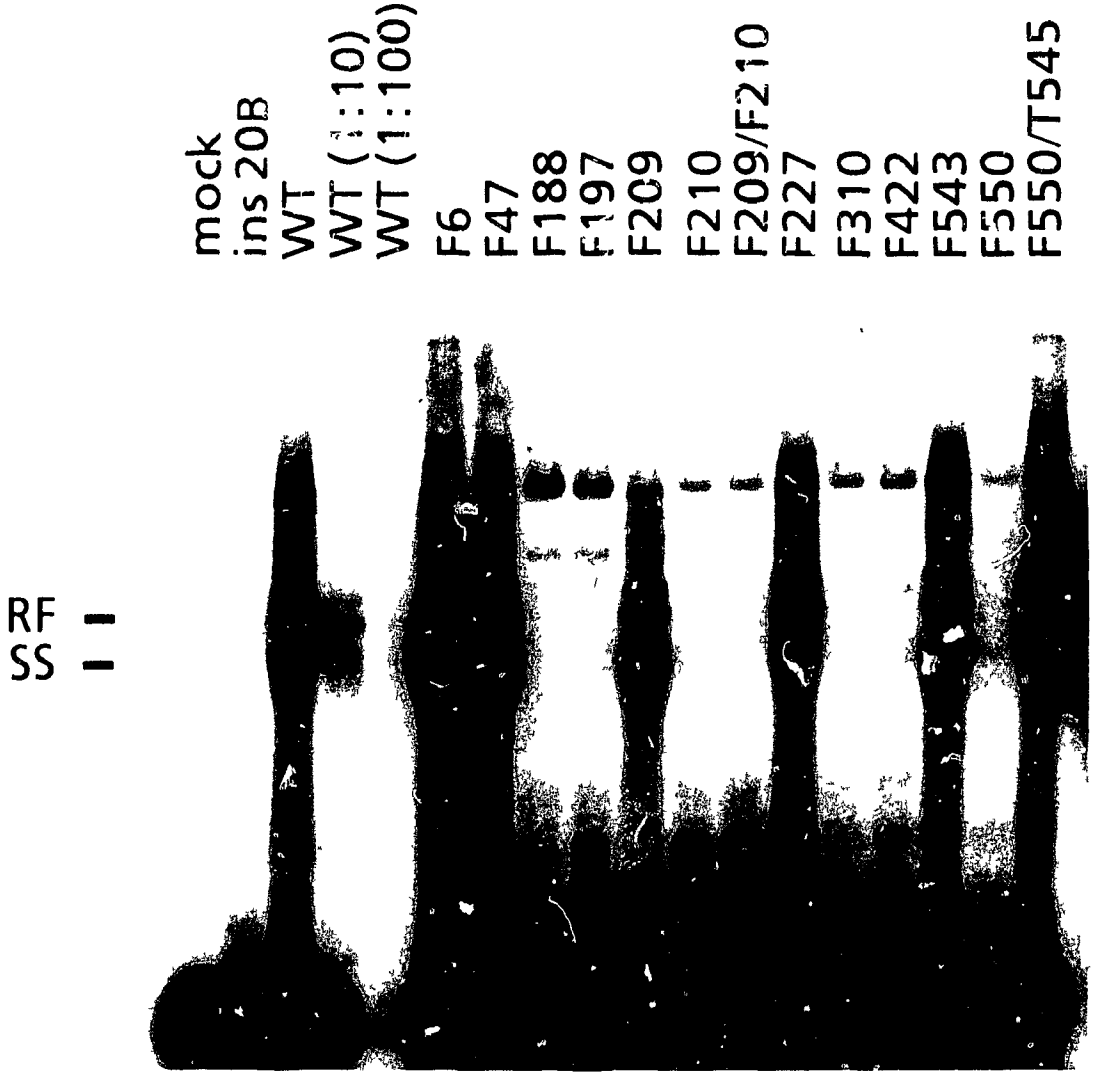

Figure 20 Effect of mutations in pSVNS1-1 on viral DNA replication

DNA replication activity by at least 100-fold (Fig. 20). These two tyrosines are therefore also essential for an aspect of NS-1 function that impacts ultimately on the efficiency of viral DNA replication.

3.10.4 Role for Tyr550 in the DNA replication function of NS-1

A Tyr550 to F550 mutation severely affected the DNA replication activity of NS-1 (Fig. 20). However, the effects of the F550 mutation were overcome by the fortuitous introduction of a second site mutation that changed Ser545 to T545. The F550/F545 double mutant displayed an approximately 3 to 5-fold enhanced replication activity as compared with wild type NS-1 (Fig. 20), in two out of three assays as determined by excision of the RF and ss bands and counting radioactivity (data not shown).

3.10.5 Phenylalanine mutations that do not block NS-1 replication function

The tyrosine at position 6 is the most amino terminal tyrosine in NS-1. When this tyrosine was changed to phenylalanine, NS-1 displayed a 5-fold greater activity in the *in vivo* DNA replication assay than did wild type NS-1 (Fig. 20), as determined by excision of the RF and ss bands and counting radioactivity (data not shown). Replacement of tyrosine 47, 227 or 543 with phenylalanine had no apparent effect on the extent to which NS-1 was able to complement the replication of ins 20B DNA in COS-7 cells (Fig. 20).

3.10.6 Immunoblot analysis of mutant NS-1 proteins

Since the DNA negative phenotype of the phenylalanine mutants described above could be explained by an effect on the turnover rate of NS-1, the level of expression of the replication defective mutant NS-1 proteins in COS-7 cells was examined using immunoblot analysis. NS-1 was detected at approximately wild type levels in all of the cases examined (Fig. 21). The F550/T545 mutation appeared to be present at levels less than wild type (Fig. 21), however this result was not reproducible in another immunoblot (data not shown).

Figure 21 Immunoblot analysis of wild type and mutant NS-1 proteins.

Protein from extracts of infected or transfected cells were prepared and separated by SDS-polyacrylamide gel electrophoresis and electroblotted onto a nitrocellulose filter. The blot was incubated with rabbit anti-NS-1 antiserum followed by a swine anti-rabbit IgG peroxidase conjugate (see Methods). Wild type pSVNS1-1 (WT) and mutant pSVNS1-1 are indicated. MVM, MVMp infected COS-7 cells; ΔBIIb, carboxyl-terminal truncation mutation of NS-1 (deletes residues 544-672).

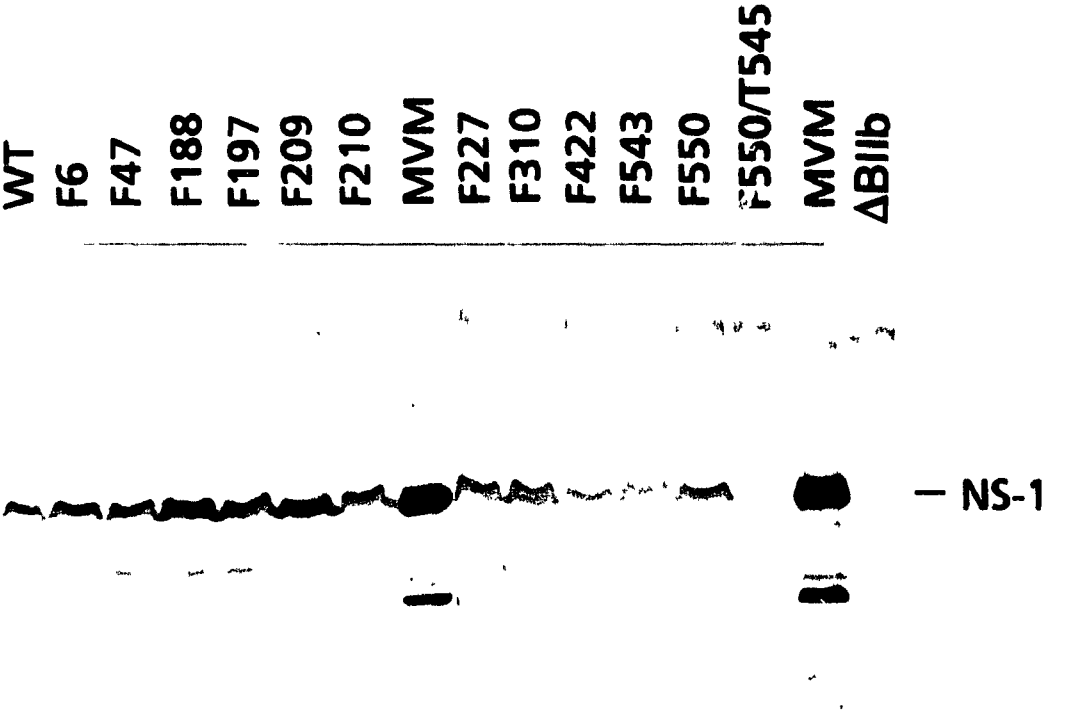

Figure 21 Immunoblot analysis of wild type and mutant NS-1 proteins

3.10.7 Effect of Tyr to Phe mutations on the transcriptional activation function of NS-1

Immunoblot analysis indicated that the mutant phenotypes were not due simply to enhanced rates of degradation of NS-1. However these data did not rule out the possibility that the phenylalanine mutations affected the DNA replication function of NS-1 by disrupting its tertiary structure. CAT assays were conducted, in order to determine whether these mutations retained their ability to stimulate transcription of the chloramphenicol acetyl transferase gene under the control of the MVM capsid gene promoter.

In this study, two point mutations that affected the ability of NS-1 to stimulate replication of ins 20B also affected the transactivation function of NS-1. The transactivation function of F197 was reduced to 7 percent of wild type, while F188 was virtually completely blocked in its ability to transactivate the capsid gene promoter (Fig. 22). All the other phenylanine mutations including the replication defective F210 mutant were approximately wild type for transactivation of the p38 promoter in CAT assays, with the notable exception of F550 and F550/T545 which transactivated the p38 promoter at 200 and 50 percent of WT, respectively (Table 5). The F550/T545 mutation was more active than WT by approximately 2-fold when assayed at 32 ^{o}C and therefore appears to be temperature sensitive for transactivation (Fig. 22). The F188 and F197 mutations did not appear to be temperature sensitive in this assay (Fig. 22).

3.10.8 F188 and F197 display a dominant negative phenotype

The transactivation defective mutations (F188, F197) were assayed for their ability to block transactivation of the p38 promoter by wild type NS-1, in transient expression assays. Cotransfection of equimolar amounts of wild type NS-1 and F188 or F197 resulted in approximately a 5-fold and 4-fold reduction, respectively, in the ability of wild type NS-1 to transactivate p38 (Fig. 23). A similar competition assay using EVM, a truncation mutant of NS-1 and BIIM1 (ins 543-544) which is a replication and transactivation defective mutant (Table 4), showed that these latter mutations did not block wild type NS-1 transactivation of p38 (Fig. 23).

Figure 22 Transactivation of pP39CAT by Tyr to Phe mutations in NS-1.

COS-7 cells were cotransfected with pP39CAT (10 μg) and 10 μg of sheared calf thymus DNA (—), or with wild type (WT) or mutant pSVNS1-1 as indicated. Crude extracts were prepared and CAT enzyme was assayed as described under Materials and Methods. 32°C, wild type (WT) or mutant pSVNS1-1 transfected COS-7 cells were incubated for 40-44 hrs at 32 °C in the same experiment. The percentage of acetylation is indicated at the bottom of the figure. C, chloramphenicol; AC, acetylated chloramphenicol.

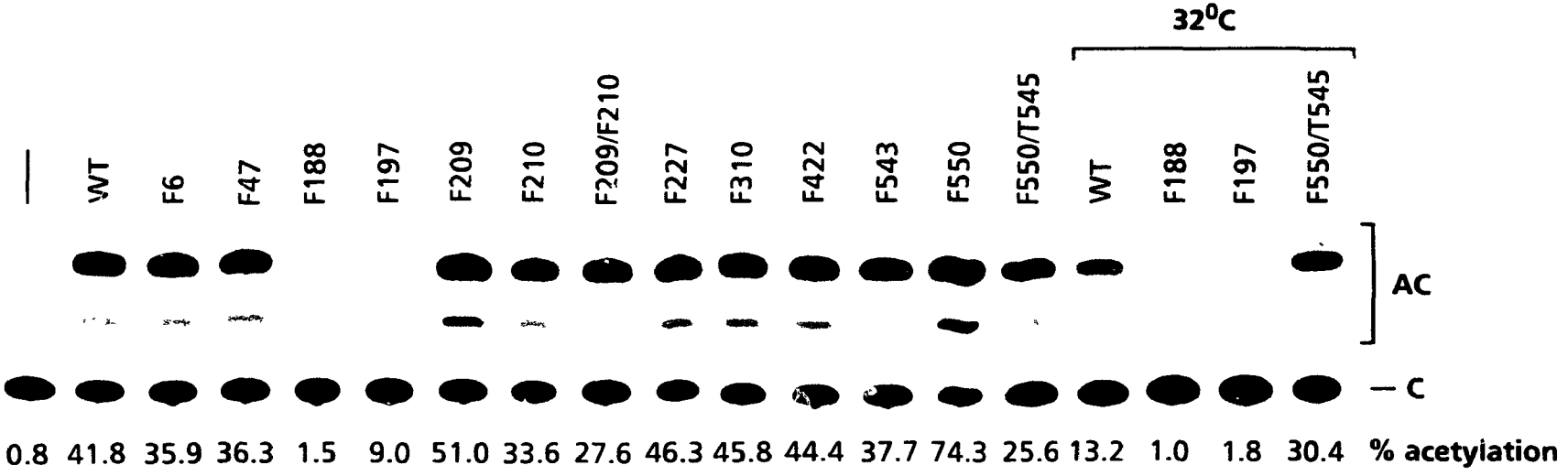

Figure 22 Transactivation of pP39CAT by Tyr to Phe mutations in NS-1

Figure 23 Competitive inhibition of pSVNS1-1 induced transactivation of pP39CAT by NS-1 mutations.

COS-7 cells were cotransfected with pSVNS1-1 (1 μg), pP39CAT (5 μg) and 1 mg of sheared calf thymus DNA (CT) or mutant pSVNS1-1 plasmid DNA as indicated. CAT enzyme was prepared 40-44 hrs after transfection and was analyzed for activity as described.

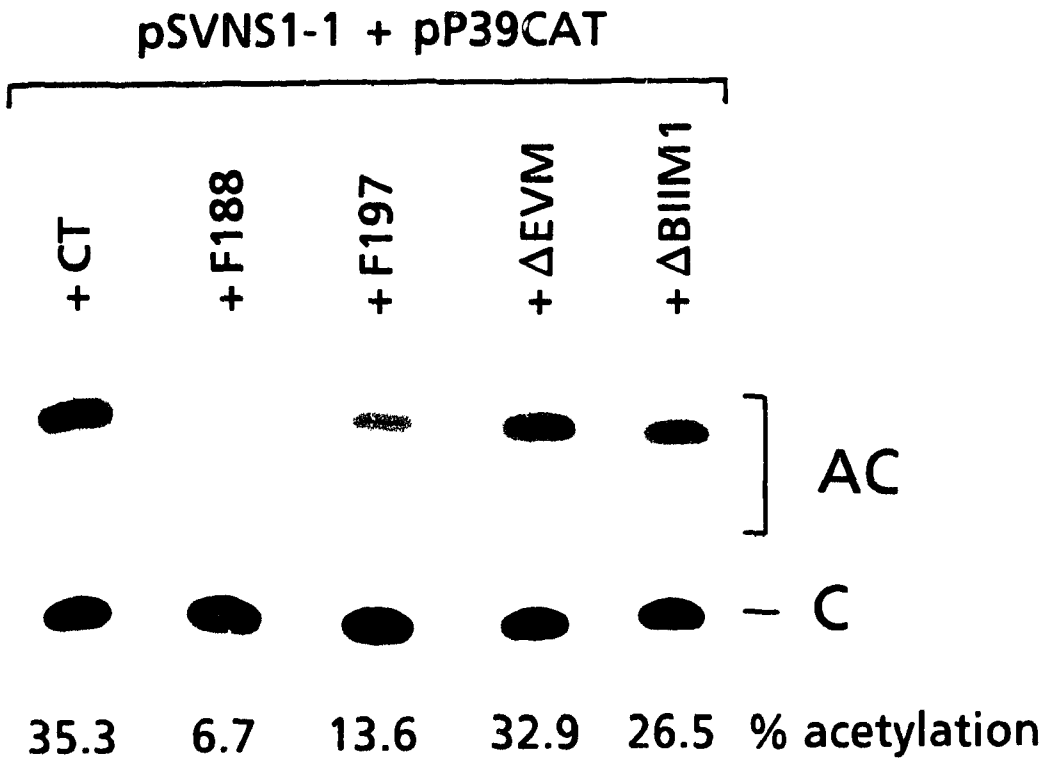

Figure 23 Competitive inhibition of pSVNS1-1 induced transactivation of pP39CAT by NS-1 mutations

Table 5. Summary of effects of Tyr to Phe conversions on DNA replication and transcriptional activation activities.

CONSTRUCT	REPLICATION [a]	TRANSACTIVATION [b]
WT	++	100
F6	+++	113.0 ± 15.4
F47	++	78.2 ± 0.1
F188	-	3.9 ± 0.3
F197	-	7.4 ± 1.5
F209	+	109.5 ± 16.2
F210	-	81.0 ± 0.6
F209/F210	-	92.5 ± 23.2
F227	++	108.9 ± 2.0
F310	-	101.4 ± 8.3
F422	-	104.9 ± 13.7
F543	++	87.4 ± 2.9
F550	-	175.9 ± 25.1
F550/T545	++(+)	46.2 ± 15.6

[a] rep activity of NS-1 as determined with*in vivo* transient replication assays. The lower limit of detection in this assay was between 1-5% of wild type. [b] transactivation activity as determined with CAT assays. Values indicate the percent transactivation (± standard error) normalized to wild type NS-1 which is set at 100%. pP39CAT alone: 2.3 ± 0.7 % with respect to wild type.

3.11 Construction of other expression vectors

It has been reported that the expression of NS-1 or fragments of NS-1 is lethal to *E. coli* cells carrying plasmids with such sequences (Carlson et al., 1987; Ozawa et al., 1988). In this study several other expression vectors designed for high level expression of NS-1, for the purpose of purification and characterization of enzymatic activities of NS-1, were constructed. Strains carrying these plasmids were grown without difficulty. One of the bacterial expression vectors (pANS-1) was used to overproduce an NS-1 fusion protein. The B19 VP proteins have been expressed in a similar fusion vector (Morinet et al., 1989)

3.11.1 Construction of baculovirus vectors

3.11.1.1 Construction of pVLNS1

The coding sequence for the NS-1 protein was inserted into the unique *Bam*HI site of pVL941 (see Materials and Methods). This vector can be used in cotransfections with wild type baculovirus DNA to create recombinant baculoviruses that express NS-1 protein.

3.11.1.2 Construction of pP10NS-1

The *Bam*HI fragment containing the coding sequence for NS-1 was excised from pVLNS-1 and *Nhe* I linkers were added to the ends as described in Materials and Methods. This fragment was inserted into the unique *Nhe* I site of pJVP10. This vector provides a selectable β-galactosidase marker that can be used for selecting recombinants in cotransfections with wild type baculovirus DNA.

3.11.2 Construction of pETNS1

The pET series of plasmid vectors allow the expression of toxic genes in the appropriate *E. coli* strain, by virtue of a tightly regulated T7 promoter. High level transcription and translation can be induced by the addition of IPTG to the culture medium of log phase *E. coli* carrying the expression vector. The NS-1 coding sequence was placed downstream of the T7 promoter in pET-11d as described in Materials and Methods.

Figure 24 Elution profile of protein A/NS-1 fusion polypeptide from IgG Sepharose.

Crude protein extract from heat induced *E. coli* strain N4830-1 bearing the pANS-1 expression vector was prepared as described and placed onto an IgG Sepharose Fast Flow column, and was washed with TST , followed by 5 mM ammonium acetate (pH 5.0). Bound protein was eluted with 0.1 M acetic acid (pH 3.4). Fractions (0.75 ml) were collected and immediately neutralized with the addition of 500 ul 0.5 M potassium phosphate buffer (pH 12.4). Protein concentration was quantitated by determining the OD at 280 nm. The protein concentration in each fraction is shown.

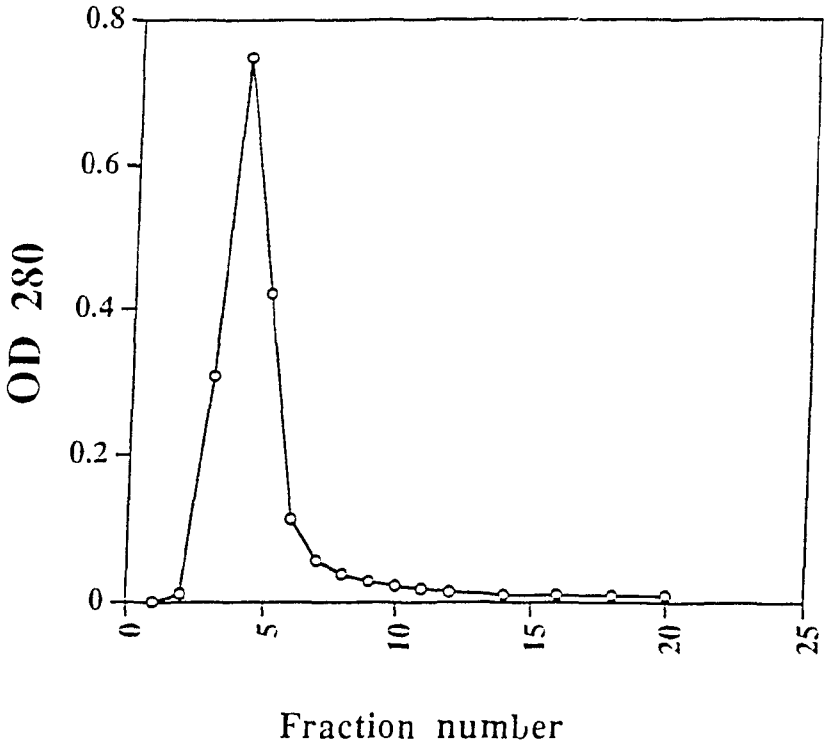

Figure 24 Elution profile of protein A/NS-1 fusion polypeptide from IgG Sepharose

Similar plasmid constructs have been successfully used to overproduce the AAV proteins (Hunter and Samulski, 1992).

3.11.3 Construction pANS-1 and expression of a protein A/NS-1 fusion protein

The MVM NS-1 coding sequence was placed downstream of the coding sequence for the antibody binding region of the *Staphylococcus aureus* protein A. This vector contains a thermoinducible λ P_R promoter. The *E. coli* strain N4830-1 used to carry the pANS-1 expression vector contains the thermolabile λ cI_{857} repressor integrated into the genome. Shifting this *E. coli* strain from 30 °C to 42 °C inactivates the λ cI_{857} repressor and allows high level transcription to proceed from the λ P_R promoter.

A crude lysate was prepared from a heat induced culture and placed onto an IgG Sepharose 6 Fast Flow column (see Materials and Methods). After several washes, protein was eluted with a low pH buffer. The eluted protein showed as a sharp peak (Fig. 24), and yields were typically 2.5 to 3.5 mg/ 300 ml culture. Immunoblot analysis of the eluted fractions reveals a high molecular weight band migrating at the approximate position expected for the predicted size (110 kDa) of the fusion product (Fig. 25). The peak of the immunoreactive product coincided with the peak of the eluted protein. SDS-PAGE analysis of the eluted fractions shows that a protein of the predicted molecular weight was expressed (Fig. 26 A). Additional bands of lower molecular weight were also detected by immunoblot analysis and Coomassie staining (Fig. 25, Fig. 26). These bands may be proteolysis products of the proteinA/NS-1 fusion protein, or may be contaminants that bound to the IgG Sepharose. Comparison of several fractions around the peak of eluted protein A/NS-1 (fraction 10) and a non-fusion protein A (fraction 10) shows that the 110 kDa band is not present in fractions collected from the cells expressing protein A alone (Fig 26 B). The 110 kDa band is barely visible in unpurified crude protein extracts (Fig. 25 and Fig. 26, lysate), and so is enriched by affinity chromatography.

Figure 25 Immunoblot analysis of eluted Protein A/NS-1 fractions.

Fractions shown in Fig. 24 were subjected to discontinuous SDS-PAGE and were transfered onto nitrocellulose as described. The blot was incubated with rabbit anti-NS-1 antiserum followed by swine anti-rabbit IgG peroxidase conjugate as described in Materials and Methods.

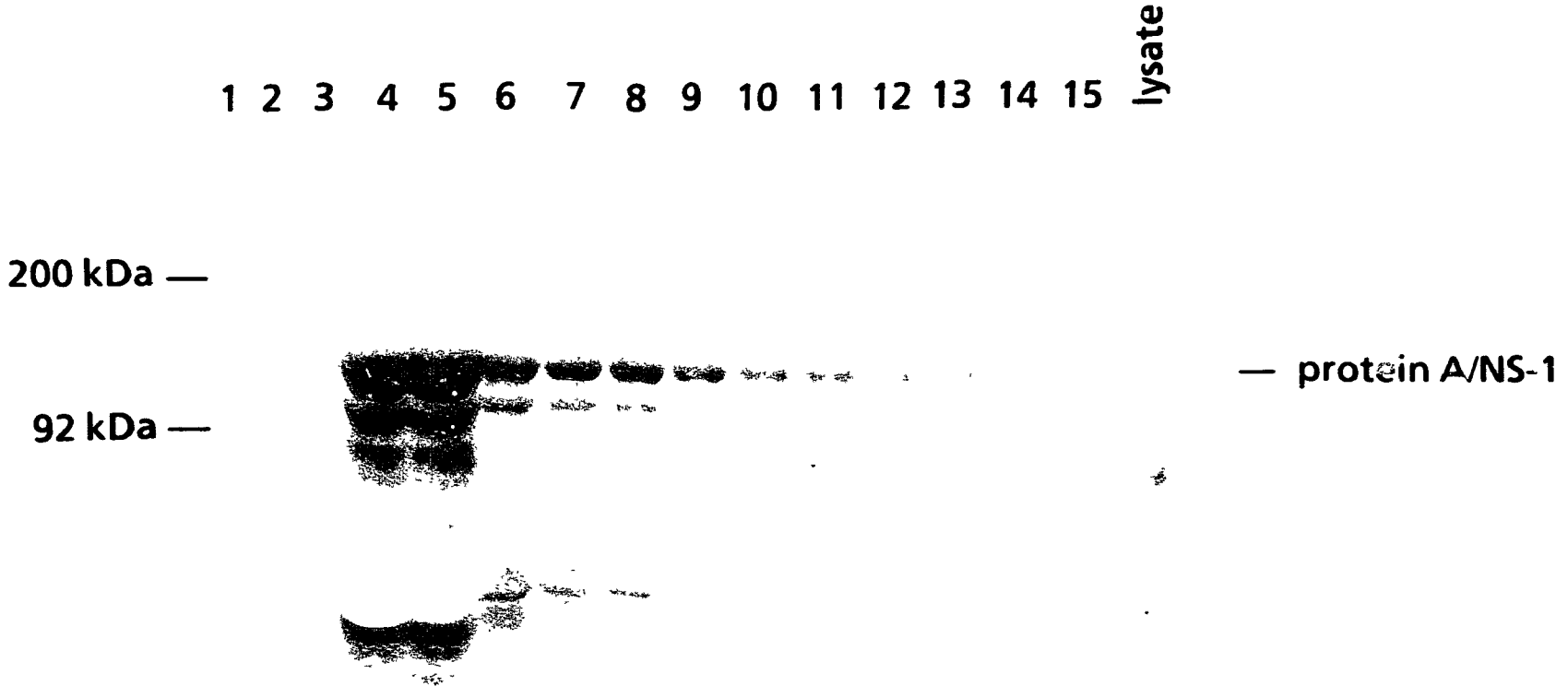

Figure 25 Immunoblot analysis of eluted Protein A/NS-1 fractions

Figure 26 SDS-PAGE analysis of protein A/NS-1 and protein A.

A) Fractions shown in Fig. 24 were subjected to discontinuous SDS-PAGE. Protein bands were visualized by staining with Coomassie Blue as described in Materials and Methods. MVM VP1 (83 kDa) and VP2 (64 kDa) proteins (gift of E. Faust) were used as markers (MVMe). lysate, total protein extract.

B) Crude proteins extracts from heat induced *E. coli* N4830-1 cells bearing the expression vector pANS-1, or pRIT2T (produces only the IgG binding portion of protein A: protein A/-) were prepared and fusion (protein A/NS1-) or non-fusion (protein A/-) proteins were isolated by affinity chromatography as described. Protein A/NS-1 (lanes 9-12) and protein A/- (lanes 9-11) representing several fractions around the peak (fraction 10 in both examples) were subjected to discontinuous SDS-PAGE. Protein bands were visualized with Coomassie Blue. lysate, total protein extract from heat induced *E. coli* N4830-1 cells bearing the expression vector pANS-1.

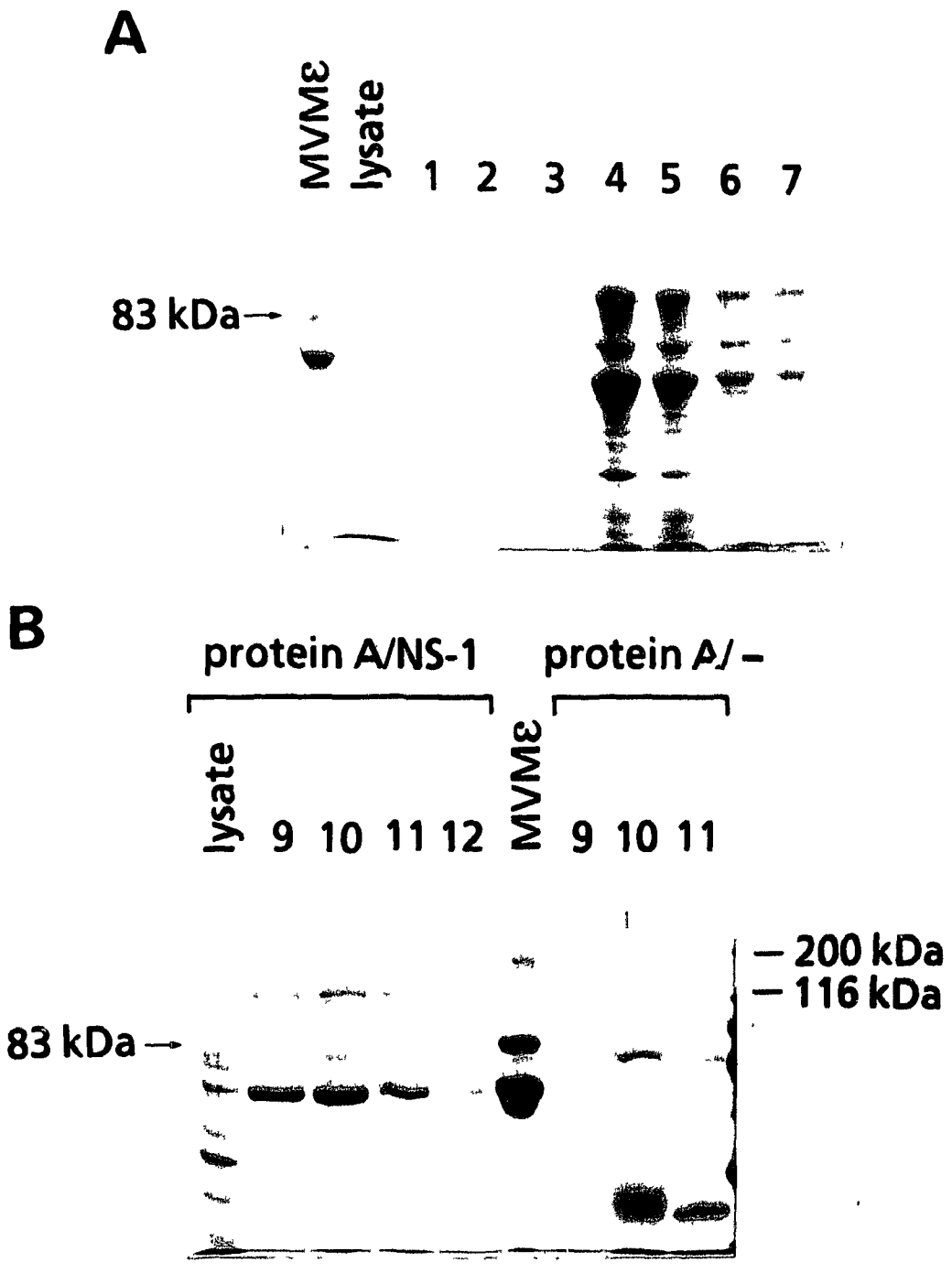

Figure 26 SDS-PAGE analysis of protein A/NS-1 and protein A.

CHAPTER 4. DISCUSSION

4.1 Mutations in a C-terminal region of NS-1 affect transactivation

A mutational analysis of the NS-1 gene was undertaken to further define the domain structure of NS-1 and thus to provide additional information on its role in transcriptional regulation and viral DNA replication. Mutations located in C-terminal and N-terminal regions of NS-1 were shown to affect the transactivation function. Transactivation was completely blocked by a C-terminal truncation of 129 amino acids, in confirmation of a previous study of a similar mutation (Brandenburger et al., 1990), and by a 5 amino acid insertion between Tyr543 and Gln544. An insertion of 4 or 12 amino acids between Asp467 and Gln468 also reduced the ability of NS-1 to transactivate, but did not eliminate it. In a previous study of the NS-1 protein of H-1, transactivation was abolished by a deletion of approximately 70 amino acids that included Tyr543 and Gln544 (Rhode III and Richard, 1987) and by a Lys405 to Ser405 point mutation (Li and Rhode, 1990). Transactivation was also blocked by a relatively large internal deletion of amino acids 180-560 in the MVM NS-1 protein (Brandenburger et al., 1990).

Because of the large size of the deletion mutations studied previously in H-1 and MVM NS-1, it is difficult to ascertain the basis for their effect on transactivation. In this study a relatively small perturbation of NS-1 at Tyr543/Gln544 blocked transactivation, whereas similar sized insertions at other locations in NS-1 had only a relatively small inhibitory effect. Significantly, the ΔBIIMI mutation did not affect the stability of NS-1 which provides support for the idea that the mutation directly affects a transactivation domain. However, it is still possible that the Tyr-543/Gln-544 insertion mutation abolishes transactivation and/or DNA replication due to an effect on the tertiary structure or nuclear localization of NS-1. In a recent study, Legendre and Rommelaere (1992) described C-terminal truncation mutations that were still able to transactivate, albeit at a reduced efficiency. The largest of these was a deletion of a.a. 565 to 672, which retained some transactivation ability, but was unable to repress transcription of the RSV LTR promoter. A mutant NS-1 protein containing an insertion of a valine residue between Lys469 and

Gly470 was defective for replication but transactivated at wild type level. These results taken together with data presented here suggest that there is a domain or part of a domain required for transactivation encompassing residues 544 and 565.

The acidic nature of transactivation domains in other transcriptional regulatory proteins encoded by DNA viruses has been noted previously and, as in the case of the adenovirus E1A protein, such domains may contain as few as 49 amino acids (Lillie et al., 1987). Insertion of an aspartic acid residue at position 606-607 had no effect on transcriptional *trans*-activation. The amino acid sequence corresponding to positions 603-610 of wild type NS-1 is DLEDLALE, and contains four negatively charged amino acids as well as 4 neutral amino acids and could therefore form a surface with a relatively high net negative charge. It is possible that the insertion of an aspartic acid residue in this peptide as occurs in the ΔEOb mutant would increase its acidic nature and could explain why transactivation remains unaffected. Alternatively, this region may not be required for transactivation, as suggested by the C-terminal truncation of Legendre and Rommelaere (1992). The peptide inserted between Tyr543 and Gln544 in the ΔBIIM1 mutant contains two arginine residues that would be expected to significantly reduce the overall net negative charge of this region of NS-1, which may affect its ability to transactivate the p38 promoter.

Perhaps transactivation of the p38 promoter by NS-1 occurs by a mechanism that does not require acidic residues, as suggested for transactivation by the tat protein of the human immunodeficiency virus (Green and Lowenstein, 1988). It is also possible that amino acids Tyr543 and Gln544 are part of a transactivation domain in NS-1 that includes Lys405. Lys405 lies within a consensus sequence believed to be important for binding purine nucleotides (Anton and Lane, 1986; Li and Rhode, 1990). Failure of the Ser405 mutant to transactivate the p38 promoter could reflect a role for Lys405 in binding ATP rather than participation in a transactivation domain. ATP-binding transactivating proteins such as the hepatitis B virus Hbx protein have been described previously (Wu et al., 1990), and NS-1 may turn out to transactivate by a similar mechanism, as suggested by McCarty et al. (1992). Also, a cytotoxic activity of NS-1 appears to require DNA helicase or ATPase activity since a mutation in the purine nucleotide consensus region abolished cytotoxicity along with viral DNA replication and transactivation (Li and Rhode, 1990). Nevertheless, it has been suggested that cytotoxicity could be separated from the ability to

support viral DNA amplification (Brandenburger et al., 1990) and transactivation (Legendre and Rommelaere, 1992).

4.2 N-terminal dominant negative mutations

Results presented here also point to an N-terminal domain required for transactivation. While insertions at Asp41/Ile42, Thr219/Ser220 and a 6 a.a. insertion (GTGTGP) between Pro258 and Gln259 (Salvino, 1990; Skiadopoulos et al., 1992) inhibited transactivation to some extent, these mutations as well as the point mutations ΔA249 and ΔQ250, failed to abolish transactivation. The effects exerted by these insertion and point mutations therefore may result from changes in the secondary and/or tertiary structure of NS-1 as opposed to direct alteration of a transactivation domain. In a previous study, a truncation mutant of H-1 missing the N-terminal half of NS-1 failed to transactivate the p38 promoter (Rhode and Richard, 1987) possibly reflecting the loss of an N-terminal transactivation domain. However, fusion to the DNA binding domain of GAL4 was necessary to maintain activity of the transactivation domain in truncated forms of the EBNA-1 antigen of Epstein-Barr virus and the adenovirus E1A protein (Ambinder et al., 1991; Lillie and Green, 1989) and it is possible that such large truncations of the NS-1 protein also render it unstable.

The results presented here point to Tyr188, Tyr197 as well as Tyr550 as being important for the transactivation activity of NS-1 and demonstrate directly that eight other tyrosines in NS-1 are dispensable for this function, at least with respect to the tyrosyl hydroxyl group. The F188 and F197 mutations also conferred a dominant negative phenotype on NS-1 which suggests that they retain the ability to interfere with wild type NS-1 mediated transactivation, and provides evidence for the existence of at least two separate domains that are important for transactivation. The mechanism by which this interference occurs remains unknown, but it is possible that NS-1 is binding to cellular transcription factors, which then become unavailable for promoter transactivation. Mutagenesis of Tyr550 and Ser545 also appears to have an effect on the level of transactivation. These results are therefore consistent with a 2-domain model for the transactivation function of NS-1 in which one domain is located in the amino-terminal half of NS-1 and the other is located towards the carboxyl-terminus (Fig. 27). Such bipartite domain structures for transactivating proteins have been described previously for the RelB

Figure 27 Putative transactivation and replication domains of NS-1.

The domain map of NS-1 as determined by effects of insertion and point mutations on the DNA replication and transactivation functions. ▨ , required for activity. ■ , not absolutely required for activity. This diagram does not necessarily represent the absolute boundaries of these domains. The relative positions of point and insertion mutations are indicated at the top.

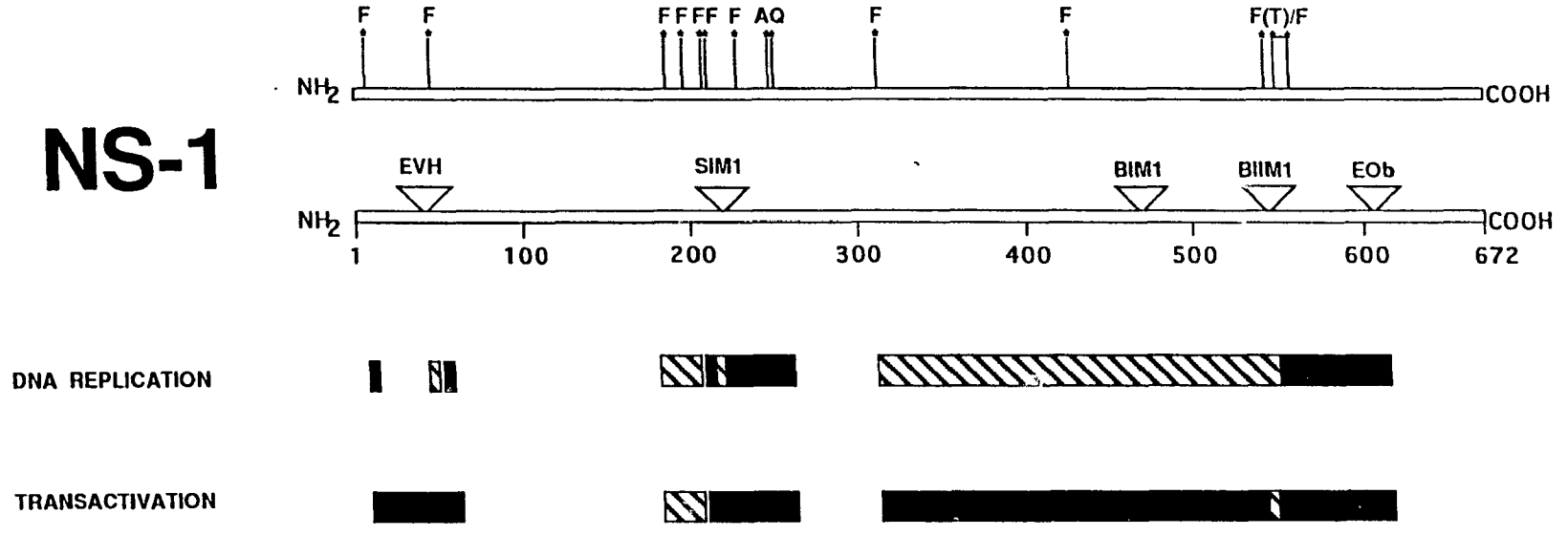

Figure 27 Putative transactivation and replication domains of NS-1

protein, in which domains located in the N and C-terminal ends act together to transactivate (Dobrzanski et al., 1993). However, these domains also contain acidic (C-terminal) and leucine zipper (N-terminal) motifs which have not been described for NS-1.

A 2-domain model for transcriptional regulation by NS-1 is also supported by studies of transcriptional repression of the LTR promoter of Rous sarcoma virus by NS-1 in which active domains for this function were mapped to both amino- and carboxyl-terminal regions (Legendre and Rommelaere, 1992). It is not clear if the repression and activation domains are related, but Legendre and Rommelaere (1992) reported that one mutation which was able to repress the RSV LTR was unable to transactivate the p38 promoter or replicate MVM DNA. Previous studies indicated that NS-1 mediates transactivation of the p38 promoter by interacting with cellular transcription factors rather than by binding to viral DNA, and so Tyr188 and Tyr197 may be critical for maintaining the appropriate protein-protein interactions that permit recognition of the p38 promoter.

The mechanism of transactivation by NS-1 may be different from that used by other transcriptional activating proteins in which acidic protein surfaces seem to play a central role, since rather than bearing a preponderance of acidic amino acids, the region around Tyr188 and Tyr197 in NS-1 is highly positively charged. However, this region of NS-1 also contains an unusually high concentration of serine, threonine and tyrosine residues which may potentially be involved in the transactivation mechanism. The essential domain in the transactivating tat protein of HIV-1 has similar structural features to those described here for NS-1 including a conserved tyrosine, serine and threonine (Green and Lowenstein, 1988). Phosphorylation of hydroxyl amino acids, as suggested for the tat protein (Green and Lowenstein, 1988), could therefore be a mechanism for creating an acidic protein surface on NS-1 that allows this viral protein to act as a transcriptional regulator.

Phosphorylation of serine, threonine or tyrosine residues might also be a mechanism by which active or inactive states of the protein are modulated, similarly to the SV40 large T-antigen (Prives, 1990). Recently the importance of acidic domains and α-helices in transactivating proteins has been challenged (Leuther et al., 1993; Van Hoy et al., 1993). These workers created mutations in the GAL4 transactivating domain and showed

that neither acidic residues nor an α helix were absolutely required for transactivation. It therefore seems that other factors are also important for these functions. It has been suggested that viral transactivating proteins act as adapter proteins, bringing together transcription factors into an active complex or conformation, which may activate transcription. Many of these viral transactivating proteins appear to have similar effects on heterologous promoters, suggesting common mechanisms of transactivation. Finally with regard to the transactivation negative phenotype, it is noted that all the transactivation negative mutations described thus far are also replication negative. The viral DNA replication model does not invoke a step that would necessitate a transactivation function. However, a 2 a.a. insertion at position 400-401 in AAV rep78 had a replication-positive, transactivation-negative phenotype (Yang et al., 1992), indicating that a rep^+ 78 kDa protein is not required for transactivation.

4.3 Effect of NS-1 mutations on viral DNA replication activity

Several in-frame insertion mutations representing relatively minor perturbations in the structure of NS-1 severely affected its rep activity, in support of the idea that there may be multiple rep domains in NS-1. In a previous study, codon insertion mutations in various parts of the NS-1 gene were shown to severely inhibit the plaque-forming ability of MVM including one mutation consisting of an Ile insertion which was temperature sensitive for viral DNA amplification (Tullis et al., 1988). DNA binding, DNA helicase, and DNA topoisomerase (site-specific endonuclease) are physical and enzymatic properties that have been attributed to NS-1, consistent with the idea that NS-1 contains multiple rep domains.

4.4 Tyrosine residues required for replication map to two regions of NS-1

A central role postulated for NS-1 in the MVM DNA replication mechanism is that of a site-specific DNA nicking-closing enzyme. This derives support from the existence of a covalent association of NS-1 with the 5' ends of viral DNA although additional direct evidence delineating which amino acid(s) is linked to the DNA is lacking. The stability of the NS-1-DNA linkage to both alkali and mild acid treatment argues against a role for the involvement of either a serine or threonine residue and strongly implies that a tyrosine residue is involved. The AAV rep68 protein was shown to be covalently attached to the 5' end of DNA at the nicking site through a tyrosine residue (Im and Muzyczka, 1992). To

obtain support for this possibility and to map the location of the covalently linked amino acid, mutations were created in which the conserved tyrosines in NS-1 were replaced by phenylalanine. The effects of these mutations on the *in vivo* DNA replication activity of NS-1 were determined. This approach was used previously to successfully map the active site tyrosine for the DNA topoisomerase I of vaccinia virus. The vaccinia virus and Shope fibroma virus DNA topoisomerase I proteins retain 60 % sequence similarity, while 2 of 14 tyrosines are not conserved. Two additional tyrosines were similarly ruled out as being part of the active site on the basis of a comparison with DNA topoisomerase I of *Saccharomyces cerevisiae*, *S. pombe* and human (Eng *et al.*, 1989; Lynn *et al.*, 1989). Only one of the remaining 10 tyrosines, when converted to phenylalanine, had a deleterious effect on enzyme activity and was therefore designated the active site tyrosine (Shuman *et al.*, 1989).

The results obtained in this study showed that replacement of any one of six tyrosines in NS-1 with phenylalanine had deleterious effects on its *in vivo* DNA replication activity while identical substitution mutations at five other locations had no detrimental effects. The tyrosine to phenylanine mutations described here that are absolutely required for replication map to two domains. The results are therefore consistent with the involvement of a tyrosine residue in the covalent linkage between NS-1 and viral DNA. Although tyrosine to phenylalanine conversions are conservative, it cannot be ruled out that the rep$^-$ phenotypes described here are not due to a perturbation of the tertiary structure. Nevertheless, at least one of these tyrosines must be involved in covalent binding of the protein to the 5' ends of viral DNA, and is therefore also essential for the active site of the site-specific endonuclease activity postulated for NS-1.

4.4.1 N-terminal replication domain

Immunoprecipitation experiments with antibodies raised against defined portions of the NS-1 polypeptide have indicated that the putative active site tyrosine involved in the DNA-protein linkage is probably located in the N-terminal portion of NS-1 (Cotmore and Tattersall, 1988), consistent with the finding that mutations in this part of NS-1 severely inhibit viral DNA replication. The amino terminal portion of NS-1 spanning Tyr188 to Tyr210 contains four closely spaced tyrosines. The peptide sequence between Tyr188 and Lys199 contains a relatively high proportion of basic amino acids making it a relatively

hydrophilic portion of NS-1 which potentially could be important for DNA binding, whereas the peptide from Cys200 to Tyr210 consists almost exclusively of hydrophobic amino acids, including tyrosines 209 and 210 (Fig. 18). Replacement of three conserved tyrosines (Tyr188, Tyr197 and Tyr210) in this region of NS-1 with phenylalanine proved to be detrimental to its DNA replication function, while the fourth tyrosine (Tyr209) was not absolutely required. This result may be significant in regard to mapping the covalently bound tyrosine since Cotmore and Tattersall (1989) showed that antibodies directed against N-terminal portions of NS-1 selectively precipitated viral DNA that remained bound to NS-1 following partial trypsin digestion of DNA protein complexes, suggesting that NS-1 is bound to DNA via an amino acid located at an N-terminal portion of NS-1.

4.4.2 The highly conserved domain tyrosines are required for replication

The results also show that tyrosines 310 and 422 are essential for a DNA replication function of NS-1. The fact that mutagenesis of these two tyrosines, which are situated in a highly conserved region of NS-1, affects replication may simply point to the overall functional importance of this region, at least with respect to the DNA replication function of NS-1. However it is also possible that one or both of these tyrosines are critical for the postulated nicking activity of NS-1. A recently reported comparison of the highly conserved region of NS-1 for several parvoviruses revealed that Tyr422 is conserved in MVM, H-1, CPV, FPV, PPV and mink enteritis virus (MEV) (Dumas *et al*, 1992). This tyrosine residue is not conserved in *Junonia coenia* DNV, B19, AAV-2 and ADV, although there is a Tyr at position 419 next to an invariant Gly residue. In BmDNV, the equivalent of the MVM Tyr422 is replaced by Thr. The degree of conservation of Tyr422 is therefore not as strict as it is for some other amino acids in this region of NS-1, such as Lys405. The Phe422 mutation may affect ATP -binding since it is located just downstream of the ATP-binding consensus motif which includes Lys405. Mutations at Lys405 have been shown to adversely affect DNA replication, transactivation and cytotoxicity (Ti and Rhode, 1990; Nuesch et al., 1992).

Aside from these tyrosine residues, one other conserved tyrosine, namely Tyr550, was found to be essential for the DNA replication activity of NS-1. However, a second mutation that changed Ser545 to Thr545 restored the replication activity of the F550 mutant to at least wild type levels, indicating that Tyr550 is not absolutely required and is

probably not involved in a covalent linkage with viral DNA. This result may also point to a regulatory role, possibly as a phosphorvlation substrate, for Ser545 in the DNA replication activity of NS-1.

The phenylalanine substitution mutation at Tyr6 also displayed a DNA replication enhancement phenotype, suggesting the existence of a negative regulatory mechanism that controls the level of viral DNA replication. This mechanism could involve phosphorylation of Tyr6. Although phosphoserine and phosphothreonine are clearly the major phosphoaminoacids in NS-1 (Cotmore and Tattersall, 1986), a relatively low level of phosphotyrosine may have gone undetected in previous studies.

Previous work has established the location of the active site tyrosines for DNA topoisomerases from humans, yeast and vaccinia virus (Eng *et al.*, 1989; Lynn *et al.*, 1989; Shuman *et al.*, 1989) and a motif, S K X_2 Y, containing the active site tyrosine has been conserved in each of these proteins. The only sequence resembling such a motif in MVM NS-1 includes Tyr252. A Phe252 substitution mutation in NS-1 introduced into a pMM984 background replicated normally and at wild type levels in mouse A9 cells (Salvino, 1990). This particular tyrosine residue is also not conserved in PPV NS-1. The active site tyrosine motif described for DNA topoisomerase I therefore appears to have no obvious functional significance for NS-1.

4.5 Multiple domains for nickase activities ?

Since several tyrosines essential for the DNA replication function of NS-1 have been identified, these results could signify the involvement of multiple tyrosines in the postulated nicking-closing reactions predicted for NS-1. Recent reports of the *in vivo* and *in vitro* 3' and 5' terminal hairpin resolution assays have shed some light into the nature of the cleavage reactions (Cotmore et al., 1992; Cotmore and Tattersall, 1992; Cotmore et al., 1993). It is clear from these studies that the nickase recognizes different substrates in different contexts. It is therefore possible that more than one active site exists for these reactions, and that multiple tyrosines may be involved. Support for this mechanism also comes from previous studies of the covalent linkage between the gene A protein of ϕX174 and viral DNA. The mechanism of nicking and resealing by the A protein involves covalent linkage of the 5' end of the DNA through a tyrosine residue (located in the sequence Tyr -

Val - Ala- Lys - Tyr - Val - Asn - Lys) after the nicking reaction. The DNA strand is then nicked in another location and is resealed by possibly using the adjacent tyrosine. In any case, both tyrosines are found covalently attached to viral DNA at an equal frequency (van Mansfield et al., 1986). The region around the ϕX174 A protein active site also contains two positively charged lysine residues, a structural feature which may be significant for NS-1 function as well.

Beginning with Tyr-188, the amino acid sequence of a 35 amino acid peptide in NS-1 encompassing the tyrosines mentioned above is YKHKQTKKDYTKCVLF GNMIAYYFLTKKKISTSPP. This region of NS-1 would be expected to bear a high net positive charge due to the presence of multiple lysine residues. The analogy of NS-1 to the ϕX174 gene A protein is also strengthened by the recent observation that parvoviruses retain so called rolling circle replication (RCR) motifs in common with many other replicons encoding site-specific DNA nicking enzymes and may therefore have evolved from eubacterial replicons (Ilyina and Koonin, 1992). The approximately 2-fold reduction in DNA replication activity of NS-1 caused by the Phe substitution mutation at Tyr209 could also be due to an effect of this mutation on the postulated nicking-closing reaction, although it is clearly not the active site tyrosine. Another possibility is that the F188 and F197 mutations adversely affect the transport of NS-1 to the nucleus although the DNA negative phenotype of the F210 mutation cannot be explained in this way since it is wild type for transcriptional transactivation.

4.6 Domain structure of NS-1

Mutational analysis of the NS-1 protein suggests that there are multiple domains required for the various functions attributed to this protein. In this study, two regions required for transactivation have been identified. However, further proof will require the isolation of specific fragments that can act alone or in conjunction with a heterologous protein such as GAL4 to activate transcription. Tyrosine residues absolutely required for viral DNA replication also mapped to two domains. The purification of these mutant proteins, by using one or more of the expression vectors described here, and assaying for *in vitro* biochemical activities will help to further define the functional significance of these mutations. The definitive proof will be the isolation of specific peptide fragments that are covalently attached to the viral DNA through tyrosine residues. Finally it should be pointed

out that with respect to the domain structure of NS-1, one region appeared to be overall less sensitive to mutagenesis. This region between Ser219 and Q250 may not contain any functional domains, but could in fact be a hinge or spacer region separating N-terminal domains from the rest of the polypeptide. Additional mutations and assays for promoter repression, DNA binding, protein binding and cytotoxicity will be necessary to complete the domain map of NS-1.

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