

STUDIES ON THE 5' NON-CODING REGION OF THE GENOME OF

POLIOVIRUS

The work presented here was carried out by myself and does not include any work done in collaboration. This thesis has not been submitted for any other degree at any other university.

by

Michael Sullivan

M Sullivan 21/5/89

Submitted in 1989 for the degree of
Doctor of Philosophy at the University of Leicester.

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PREFACE

ABSTRACT

The work presented here was carried out by myself and does not include any work done in collaboration. This thesis has not been submitted for any other degree at any other university.

M. Sullivan 21/3/89

The last decade has seen widespread application of recombinant DNA technology. Using recombinant type 3 polioviruses it has been shown that a single point mutation located in this region dramatically reduces neurovirulence and inhibits the intracellular life-cycle of the virus. This mutation at this nucleotide contributes to the observed reversion to neurovirulence of the Sabin attenuated poliovirus type 3 vaccine strain currently used in vaccination programmes throughout the world. Knowledge concerning the function of the 5' non-coding region remains scant, and as a result, the mechanism whereby a single point mutation within this region results in alteration of the expressed phenotype of the virus remains unknown. Clearly, an understanding of the molecular mechanism(s) involved requires greater knowledge of the function of the 5' non-coding region.

This thesis describes the design and construction of vectors for analysis of the role of the 5' non-coding region in the control of viral translation, replication, and encapsidation of viral RNA. In the plasmid pRSV-5'polio-CAT₂(N'), the 5' non-coding region of poliovirus was fused to the coding region of the bacterial chloramphenicol acetyltransferase reporter gene. The presence of the 5' non-coding region resulted in the inhibition of CAT expression when this plasmid was introduced into eukaryotic cells in culture. Deletion analysis of the 5' non-coding region in this vector identified two regions that were responsible for the marked inhibition of expression of the reporter gene. It would appear from the results of these experiments that the poliovirus/CAT chimeric message is translated as a normal eukaryotic mRNA and is subject to the rules of the "scanning model". This observation suggests that the 5' non-coding region of poliovirus on its own does not possess features which enable a message containing it to be translated efficiently. It is concluded that a second factor, present in infected cells, is required for the efficient translation of poliovirus. A second plasmid was designed and constructed to investigate the role of the 5' non-coding region in replication and encapsidation of viral RNA. Preliminary data suggest that the product of this vector does undergo replication while its ability to be encapsidated has still to be tested.

STUDIES ON THE 5' NON-CODING REGION OF THE GENOME OF POLIOVIRUS.

M. SULLIVAN

ABSTRACT

The last decade has seen widespread application of recombinant DNA technology to the study of picornaviruses. Comparative sequence analysis has revealed that the most highly conserved region amongst many members of this family of viruses is the 5' non-coding region. Using recombinant type 3 polioviruses it has been shown that a single point mutation located in this region dramatically reduces neurovirulence and inhibits the intracellular life-cycle of the virus. Mutation at this nucleotide contributes to the observed reversion to neurovirulence of the Sabin attenuated poliovirus type 3 vaccine strain currently used in vaccination programmes throughout the world. Knowledge concerning the function of the 5' non-coding region remains scant, and as a result, the mechanism whereby a single point mutation within this region results in alteration of the expressed phenotype of the virus remains unknown. Clearly, an understanding of the molecular mechanism(s) involved requires greater knowledge of the function of the 5' non-coding region.

This thesis describes the design and construction of vectors that will allow analysis of the role of the 5' non-coding region in the control of viral translation, replication, and encapsidation of viral RNA. In the plasmid pRSV-5'polio-CATm2(N⁺), the 5' non-coding region of poliovirus was fused to the coding region of the bacterial chloramphenicol acetyltransferase reporter gene. The presence of the 5' non-coding region resulted in the inhibition of CAT expression when this plasmid was introduced into eukaryotic cells in culture. Deletion analysis of the 5' non-coding region in this vector identified two regions that were responsible for the marked inhibition of expression of the reporter gene. It would appear from the results of these experiments that the poliovirus/CAT chimaeric message is translated as a normal eukaryotic mRNA and is subject to the rules of the "scanning model". This observation suggests that the 5' non-coding region of poliovirus on its own does not possess features which enable a message containing it to be translated efficiently. It is concluded that a second factor, present in infected cells, is required for the efficient translation of poliovirus. A second plasmid was designed and constructed to investigate the role of the 5' non-coding region in replication and encapsidation of viral RNA. Preliminary data suggest that the product of this vector does undergo replication while its ability to be encapsidated has still to be tested.

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ABBREVIATIONS

A	adenine
Å	Angstrom
Ala	alanine
Amp ^r	ampicillin resistant
ATP	adenosine 5' triphosphate
BCIG	5-bromo-4-chloro-3-indoyl-β-galactoside
bp	base pair
BSA	bovine serum albumin
C	cytidine
°C	degree(s) Celcius
CAT	chloramphenicol acetyltransferase
cDNA	DNA complementary to RNA
CIP	calf intestinal phosphatase
cm, mm, nm	centimetre, millimetre, nanometre
cm ³	cubic centimetre(s)
CNS	central nervous system
cRNA	complementary RNA
dATP	2' deoxyribosyladenine 5' triphosphate
dCTP	2' deoxyribosylcytidine 5' triphosphate
dd, di-deoxy	2',3'-dideoxyribosyl-
DEPC	diethyl pyrocarbonate
dGTP	2' deoxyribosylguanine 5' triphosphate
DI	defective interfering
DNA	deoxyribonucleic acid
DNaseI	deoxyribonuclease I
dTTP	2' deoxyribosylthymine 5' triphosphate
dNTPs	2' deoxyribonucleotide 5' triphosphates
DTT	dithiothreitol
<u>E.coli</u>	<u>Escherichia coli</u>
eIF	eukaryotic initiation factor(s)
EM	electron microscope
EMCV	encephalomyocarditis virus
FCS	foetal calf serum
FMDV	foot-and-mouth disease virus
G	guanosine
g, mg, ug,	gramme, milligramme, microgramme
g	acceleration due to gravity
Gln	glutamine
Glu	glutamic acid
Gly	glycine
GTP	guanosine 5' triphosphate
h	hour(s)
HEPES	4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid
IAA	isoamyl alcohol
Ile	isoleucine
inv	inverted
IPTG	isopropyl β-D-thiogalactosidase
Kb	kilobase(s)
Kbp	kilobase pair(s)
Kcal	kilocalorie(s)
kD	kilodalton(s)
LB	Luria broth

LMP	low melting point agarose
LTR	long terminal repeat
Lys	lysine
M, mM, uM, nM	molar, millimolar, micro-, nano-
mA	milliampere
mCi	millicurie
MEM	minimal Eagles medium
Met	methionine
min	minute(s)
ml, ul	millilitre, microlitre
moi	multiplicity of infection
mRNA	messenger RNA
mRNP	mRNA/protein complex
NCS	new born calf serum
ng, pg	nanogramme, picogramme
nmole	nanomole
mol	mole
N	nucleotide
nm	nanometre
OD	optical density
oligo(U)	polymer of rUTP
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PEG	polyethylene glycol
pfu	plaque forming unit(s)
pmole	picomole
Pol I	<u>E.coli</u> DNA polymerase I
PVP	polyvinyl-pyrrolidone
rATP	riboadenosine 5' triphosphate
rCTP	ribocytosine 5' triphosphate
RF	replicative form
rGTP	ribogaunosine 5' triphosphate
RI	replicative intermediate
RNA	ribonucleic acid
RNase	ribonuclease
RSV	Rous sarcoma virus
rUTP	ribouridine 5' triphosphate
SDS	sodium dodecyl sulphate
sec	second(s)
ss	single-stranded
SV40	simian virus 40
T	thymidine
T4 pol.	phage T4 DNA polymerase
Tet ^S	tetracycline sensitive
Thr	threonine
TLC	thin layer chromatography
Tn	transposon
tRNA	transfer RNA
U	uridine
UV	ultraviolet
V	volt(s)
vol.	volume
vRNA	viral RNA
v\v	volume per unit volume

w\v weight per unit volume
W watt(s)
WHO World Health Organisation

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CHAPTER 1

Introduction

1.1 General Introduction

Poliomyelitis is characterised by severe and potentially fatal paralytic afflictions of the central nervous system. Towards the end of the last century epidemics of poliomyelitis first occurred in the developed world. Throughout the first half of this century such outbreaks became more frequent and affected an increasing number of people. Prior to the appearance of epidemics in the developed world, poliomyelitis was viewed primarily as a rare disease of infants but is now thought to have been endemic.

In 1908, Landsteiner and Popper successfully transmitted the disease to monkeys by intracerebral inoculation of a bacteria-free filtrate of spinal cord material taken from a poliovirus victim. These experiments provided the first indication that the causative agent of poliomyelitis was a virus. For the next thirty years research on poliovirus was limited because the monkey was the only system for virus growth and experimentation. Poliovirus was eventually adapted for growth in cotton rats (Armstrong, 1939).

In 1938, the National Foundation for Infantile Paralysis was founded in the USA. This organisation raised and managed funds, mainly donated by the general public, to finance research on poliomyelitis and poliovirus. The massive sums

donated allowed research to be carried out on an unprecedented scale and led, within two decades, to the development of vaccines and vaccination programmes which virtually eradicated poliomyelitis from the USA and the rest of the developed world. Although the number of lives claimed by poliovirus, even in the worst outbreaks, remained small in comparison with other diseases such as influenza or tuberculosis, the sight of the relatively young and often severely crippled survivors made poliomyelitis a terrifying disease. In the USA the problem of poliomyelitis was further highlighted by the presidential election of Franklin D. Roosevelt in 1932. Eleven years before his successful presidential election he contracted poliomyelitis and remained severely paralysed in both of his legs for the rest of his life. The National Foundation for Infantile Paralysis was founded in his name and throughout his presidency he gave his support to any measures that might lead to prevention or cure of the disease.

During this period much research was carried out on the treatment of those affected by the disease. The development of the "iron lung" allowed victims who would have died as a result of paralysis of their respiratory muscles to survive (See Paul, 1971). Many drugs and chemical agents were tested for antiviral activity, but none was of any therapeutic value (Lo-Grippo et al., 1949). It became apparent that the best prospect for controlling the disease would be the development

of an efficient vaccine. The first attempts to vaccinate humans against poliomyelitis were carried out in 1935 and 1936 using infected monkey spinal cord suspensions inactivated with formalin (Kolmer et al., 1935 ; Brodie and Park, 1936). Rather than acquiring protection against the virus, several vaccinees became infected due to incomplete inactivation of the virus.

The major step towards vaccine development came in 1949 when Enders and colleagues showed that poliovirus could be isolated and propagated in cell cultures of non-neuronal human or monkey tissue (Enders et al., 1949). This provided both a source of large quantities of virus and a rapid method to test for the inactivation of polioviruses.

In 1949 it was established that three distinct serotypes of polioviruses existed; types 1, 2, and 3 (Bodian et al., 1949). It was found that these were not effectively cross-neutralised by antibodies to any one serotype and that to be complete, immunological protection had to be gained against all three serotypes.

Within three years of the development of tissue culture for poliovirus, a formalin inactivated vaccine had been developed (Salk, 1953) and by 1955, large vaccination programmes using formalin inactivated viruses of all three serotypes, developed by Salk, were initiated in the USA and other developed countries. Throughout the 1950's live-attenuated viruses were being developed in tissue culture for

the preparation of live vaccines by several groups (Sabin, 1955; Koprowski et al., 1956; Cox et al., 1959). By 1956 Sabin had successfully produced live-attenuated viruses suitable as vaccines against all three serotypes (Sabin and Boulger, 1973). By 1955, America and most other developed countries had adopted the Salk vaccines and so Sabin had difficulty in carrying out substantial field trials and thus gain official acceptance of his live-attenuated vaccine strains. In 1956 Russia adopted the Sabin strains and in 1959 carried out what is probably the largest field trial ever undertaken (Smorodintsev, 1961). The results of the extensive Russian programme persuaded most countries to switch from the Salk to Sabin vaccines (exceptions being the Netherlands, Finland and, Sweden) in the early 1960's. The main advantages and attractions of the Sabin vaccines are the following; 1) they are relatively cheap to produce and administer (taken orally, usually on a sugar lump), 2) they confer high levels of both humoral and secretory immunity, 3) long-lasting protection removes the need for boosters.

The main failing of the Sabin vaccines, however, is the occurrence of vaccine-associated poliomyelitis caused by reversion to neurovirulence (Reviewed by Almond, 1987). Of the three serotypes, types 2 and 3 have been observed to revert, while type 1 has yet to be implicated in a case of vaccine-associated paralytic poliomyelitis. This problem means that the use of the current live-attenuated strains

will not lead to the total eradication of poliomyelitis worldwide, a stated goal of the World Health Organisation.

1.2 Classification

Poliovirus is classified as a member of the family Picornaviridae. This family has been divided into four genera on the basis of stability to acid pH, buoyant density and genome structure; Enterovirus, Rhinovirus, Cardiovirus, and Aphthovirus (see Table 1.2). Common features of picornaviruses, by which they are defined, are summarised in Table 1.1.

Although responsible for a wide range of diseases, advances in molecular virology, nucleotide sequencing and X-ray crystallography, have shown that all picornaviruses are remarkably similar in their particle structure and genome organisation. Members of the family Picornaviridae appear as small spherical particles (22-30nm in diameter) when visualised by electron microscopy. The capsid is non-enveloped, has icosahedral symmetry and is composed of sixty copies each of four viral proteins (VP1, 2, 3, and 4). The virus particle contains one linear single-stranded RNA molecule of approximate molecular weight 2.6×10^6 . This RNA is of positive sense, is infectious, and like most eukaryotic mRNAs, is polyadenylated at the 3' terminus. However, the 5' terminus lacks the 5'-5' triphosphate linkage or "cap" structure normally associated with eukaryotic mRNA (Shatkin,

Table 1.1 Common Features of Picornaviruses.

Virion

Coat	capsid (non-enveloped)
Diameter	22-30 nm
Conformation in EM	spherical
Capsid composition	4 major polypeptides, 60 copies of each
Structural symmetry	5:3:2
Dry molecular weight	$8-9 \times 10^6$ daltons
Buoyant density	$1.3-1.45 \text{ g/cm}^3$
Sedimentation coefficient	150-160S

Genome

Nucleic acid	single-stranded, positive sense RNA molecule (infectious) 5'-terminus is covalently linked to VPg 3'-terminus is polyadenylated
Molecular Weight	$2.5-2.6 \times 10^6$ daltons

Table 1.2

Genus Enterovirus

Main characteristics	stable at acid pH, buoyant density 1.33-1.34 g/ml
Members	Human polioviruses types 1-3 Human coxsackieviruses A1-22,24, B1-6 Human echoviruses 1-9, 11-27, 29-34 Human enteroviruses 68-72 Murine polioviruses Simian enteroviruses 1-18 Porcine enteroviruses 1-8 Bovine enteroviruses 1-7

Genus Rhinovirus

Main characteristics	unstable below pH 5-6, buoyant density 1.38-1.42 g/ml
Members	Human Rhinoviruses 1A, 1B, 2-115 Bovine rhinoviruses 1-2

Genus Aphthovirus

Main characteristics	unstable below pH 5-6 buoyant density 1.38-1.42 g/ml poly(C) tract close to the 5'- terminus of the genomic RNA
Member	FMDV

Genus Cardiovirus

Main characteristics	unstable below pH 5-6, buoyant density 1.33-1.34 g/ml poly(C) tract close to the 5'- terminus of the genomic RNA
Members	EMCV Mengovirus Murine encephalomyelitis virus

1976; Lee et al., 1977). Instead, picornaviruses have a small (20-24 amino acids) virus-coded, genome-linked protein (VPg) attached by a tyrosine-O⁴ phosphodiester bond to the 5' pUp of the RNA (Nomoto et al., 1976; Sangar et al., 1977; Rothberg et al., 1978).

Virus replication takes place in the cytoplasm of the host cell and can occur in cells lacking a nucleus. In all picornaviruses, other than the aphthoviruses, the translation product is derived from a single open reading frame. Mature viral proteins are all produced by progressive post-translational cleavage of this nascent precursor molecule.

The enteroviruses are primarily viruses of the alimentary tract where they cause little or no apparent illness (Melnick, 1976). They can spread from this site and replicate in other tissues, such as the heart (coxsackie), pancreas (coxsackie), liver (hepatitis A), or the CNS (polio), where they can cause severe damage.

The rhinoviruses are found in the nose and throat and are the major causative agents of the common cold in humans.

The cardioviruses are highly infectious for rodents, in which they normally induce fatal diseases. They have also been known to infect man and cause mild febrile illness.

The aphthoviruses are responsible for foot-and-mouth disease, one of the most important infectious diseases of cloven hooved farm animals. The disease is characterised by fever and vesicular eruptions in the mouth, tongue, and

hooves (foot and mouth disease virus, FMDV).

While there is only one serotype of cardiovirus and seven serotypes of the aphthoviruses, there are at least 120 serotypes of the rhinoviruses and 70 serotypes of the enteroviruses.

1.3 The Virus Particle

1.3.1 Composition and Physical Properties of the Virion

Mature poliovirus particles are spherical with a diameter of 28-30 nm. The virion has a dry molecular weight of 8.5×10^6 , sediments between 151 and 160S, and has a buoyant density of 1.34g/cm^3 in CsCl. Like those of other picornaviruses, poliovirus particles consist of approximately 70% by weight protein and 30% RNA. Purified poliovirus contains neither sugar nor lipid molecules (Drzeniek and Bilello, 1974). The proteins of the virion, with the exception of VPg, come together to form a coat or viral capsid for the genomic RNA. While the viral RNA is infectious, the viral capsid is essential for transmission and protection of the genome while outside the host cell.

1.3.2 Capsid Structure

Growth of poliovirus in cultured tissues produced sufficient quantities of the virus to allow crystallisation studies to be carried out (Schaffer and Schwerdt, 1955). In 1959, Finch and Klug produced X-ray diffraction patterns of

poliovirus crystals which indicated that the virion possesses 5:3:2 fold symmetry. It was concluded that the virion was composed of 60 or 60N identical asymmetric subunits arranged in an icosahedral lattice.

Electrophoresis of the poliovirus capsid revealed the presence of several non-identical polypeptide components (Maizel, 1963). Using SDS-PAGE, it was shown that poliovirus capsids contained four distinct proteins named VP1-4 in order of increasing electrophoretic mobility (Summers et al., 1965). It is now known that the virion contains 60 copies of each of the four capsid proteins. In addition to the four capsid proteins, another protein component is consistently observed for most picornaviruses. For poliovirus, this protein has a molecular weight of 4.1×10^3 and represents the uncleaved precursor of VP2 and VP4, termed VP0. VP0 is present at the level of one to two copies per virion. The functional significance of the uncleaved VP0 molecules remains unknown.

In 1985, the three dimensional structure of the Mahoney strain of type 1 poliovirus was determined at 2.9Å resolution by X-ray crystallographic methods (Hogle et al., 1985). The quality of the electron density map was such that it was possible to locate and identify most of the residues in all four capsid protein subunits, thus yielding the precise location, with respect to one another, of the four capsid proteins. The three-dimensional structures of VP1, VP2, and

VP3 are very similar, a core made up of eight antiparallel strands of β -pleated sheet forming a " β barrel" type structure, plus two short α -helical regions lying at right angles to one another. The major differences amongst these three proteins are the size and conformation of their termini and of the loops joining the domains that form the common core structures.

Nucleotide sequence analysis of many antigenic variants of all three serotypes of poliovirus, selected on the basis of their resistance to neutralising monoclonal antibodies, together with studies on synthetic peptides and proteins expressed in E.coli have revealed the major antigenic domains of the virus particles (Emini et al., 1983; Evans et al., 1983; Minor et al., 1983, 1986a; van der Werf et al., 1983). Correlation of this data with the three dimensional structure of poliovirus has located these sites to the exposed surface of the viral particle.

1.4 Primary Structure and Organisation of the Genome

The poliovirus genome is a single, linear, positive-sense RNA molecule of approximate molecular weight 2.6×10^6 . It has a poly(A) tract at the 3' terminus and is covalently linked to VPg at the 5' terminus. Advances in molecular biology led, in 1981, to the cloning of cDNA copies of poliovirus vRNA in E.coli plasmid vectors and the complete nucleotide sequence analysis of the genome of poliovirus type

1 (Kitamura et al., 1981; Racaniello and Baltimore, 1981a). Comparison of the complete nucleotide sequence with partial amino-acid sequence derived from the amino- and carboxy-termini of viral proteins provided precise information concerning the structure and organisation of the viral genome (Kitamura et al., 1981).

The genome consists of a 5' non-coding region of approximately 740 nucleotides. This is followed by a single open reading frame (ORF) of approximately 6.62 Kb, a small 3' non-coding region of approximately 70 nucleotides and a poly(A) tract of varying size.

1.4.1 The 5' Non-Coding Region

In the course of poliovirus infection, all the known viral gene products are derived by proteolytic processing from a polyprotein encoded by a single open reading frame. The initiation codon of this large ORF is located over 740 nucleotides downstream of the extreme 5' terminus of the viral genome (Dorner et al., 1982). Many AUG codons are contained within this 5' non-coding region (8 in poliovirus 1, 6 in poliovirus 2, and 7 in poliovirus 3- Kitamura et al., 1981; Stanway et al., 1983; Toyoda et al., 1984;) and this is not spliced out in viral mRNA (Dorner et al., 1982). The 5' non-coding region in all picornaviruses is unusually long when compared with the vast majority of eukaryotic cellular and viral mRNAs. Most vertebrate mRNAs have a 5' non-coding

region in the range of 20-100 nucleotides (Kozak, 1987), whereas those of picornaviruses vary from 610 to over 1100 nucleotides.

The aphthoviruses and cardioviruses contain a large poly(C) tract (the length varies from 100-250 nucleotides) in their 5' non-coding region which is absent from both the enteroviruses and rhinoviruses (Brown et al., 1974). The function of the poly(C) tract and the significance of its absence from some picornaviruses remain a subject for conjecture (Black et al., 1979).

Comparison of the sequences of the different strains of poliovirus reveals that the 5' non-coding region is highly conserved. The first 650 nucleotides share approximately 80% overall homology and contain several completely conserved blocks of nucleotides. The hundred nucleotides directly preceding the functional AUG represent the most divergent portion of the poliovirus genome (Toyoda et al., 1984). It has been shown that this region is non-essential for the life cycle of poliovirus when propagated in tissue culture (Kuge and Nomoto, 1987). Comparison of nucleotide sequence compiled for other enteroviruses and rhinoviruses (with the exception of hepatitis A) with that of poliovirus type 1 shows that the 5' non-coding region consistently represents the most highly conserved region.

The consensus of opinion is that the length and high level of conservation exhibited by the 5' non-coding region signify

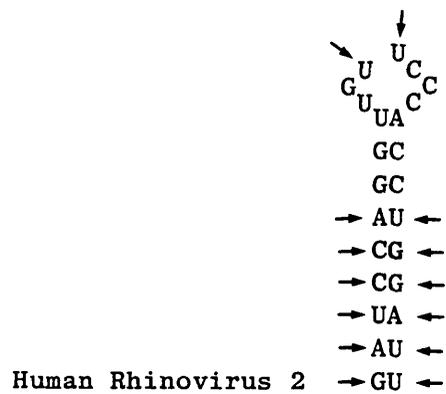
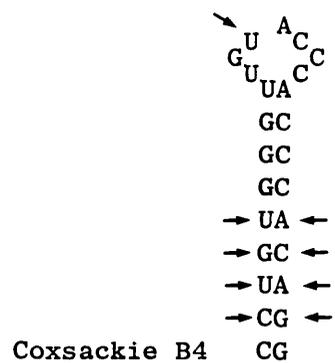
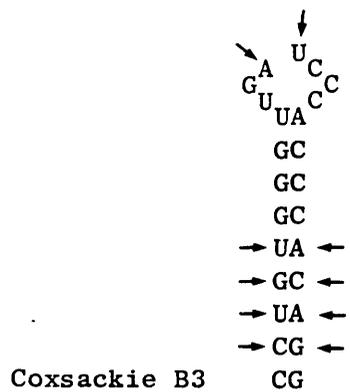
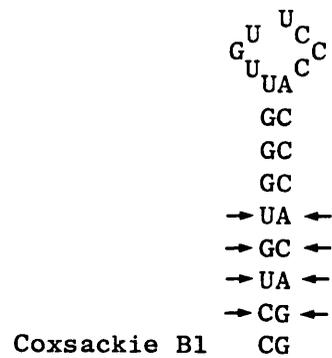
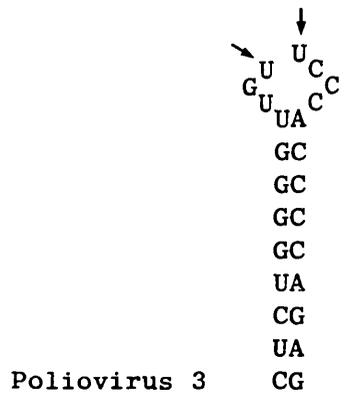
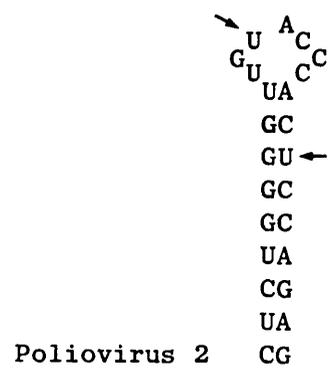
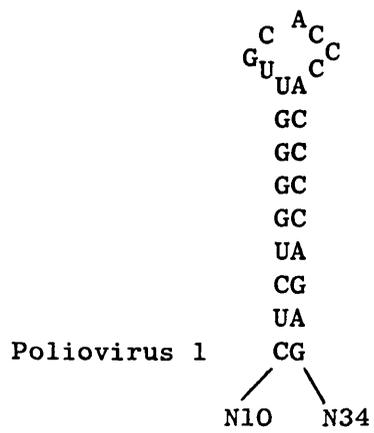
that this region plays a role in functions essential for the life-cycle of the virus. This theory is given credence by recent observations that mutations in this region alter neurovirulence, translational efficiency, and give rise to viruses exhibiting temperature sensitive phenotypes (Evans et al., 1985; Kuge and Nomoto, 1987; La Monica et al., 1986; Racaniello and Meriam, 1986; Svitkin et al., 1985; Semler et al., 1986). In addition, deletions within this region have been observed to destroy virus viability (Racaniello and Baltimore, 1982; Kuge and Nomoto, 1987). The fine mapping of the function of the 5' non-coding region has not been completed. Computer analyses of sequence data have predicted a number of very stable stem-loop structures for the 5' non-coding regions of several picornaviruses (Larsen et al., 1981; Vartapetian et al., 1983; Evans et al., 1985; Newton et al., 1985; Currey et al., 1986). The biological significance of such structures remains to be proven. However the presence of compensatory mutations in the stems of such structures in different viral strains suggests evolutionary pressure to maintain the structure (See Figure 1.1), thus implying the existence of a structure/function relationship (Newton et al., 1985).

1.4.2 The Coding Region

With the exception of FMDV, the initiation of translation of picornavirus RNA occurs at a single initiation site. In

Figure 1.1 Demonstrates the conservation of RNA secondary structure in the presence of sequence variation. "Larsen" loop found at the extreme 5' terminus of several picornaviruses.

Arrows indicate the nucleotides changed when compared with poliovirus type 1.



the case of FMDV, translation is initiated with equal efficiency at two in-frame AUG codons positioned 84 bases apart (Beck et al., 1983; Sangar et al., 1987). In poliovirus, the open reading frame contains about 6,600 nucleotides. Nucleotide sequence homology across the three serotypes is approximately 71%, and amino acid sequence homology is 88% (Stanway et al., 1983; Toyoda et al., 1984).

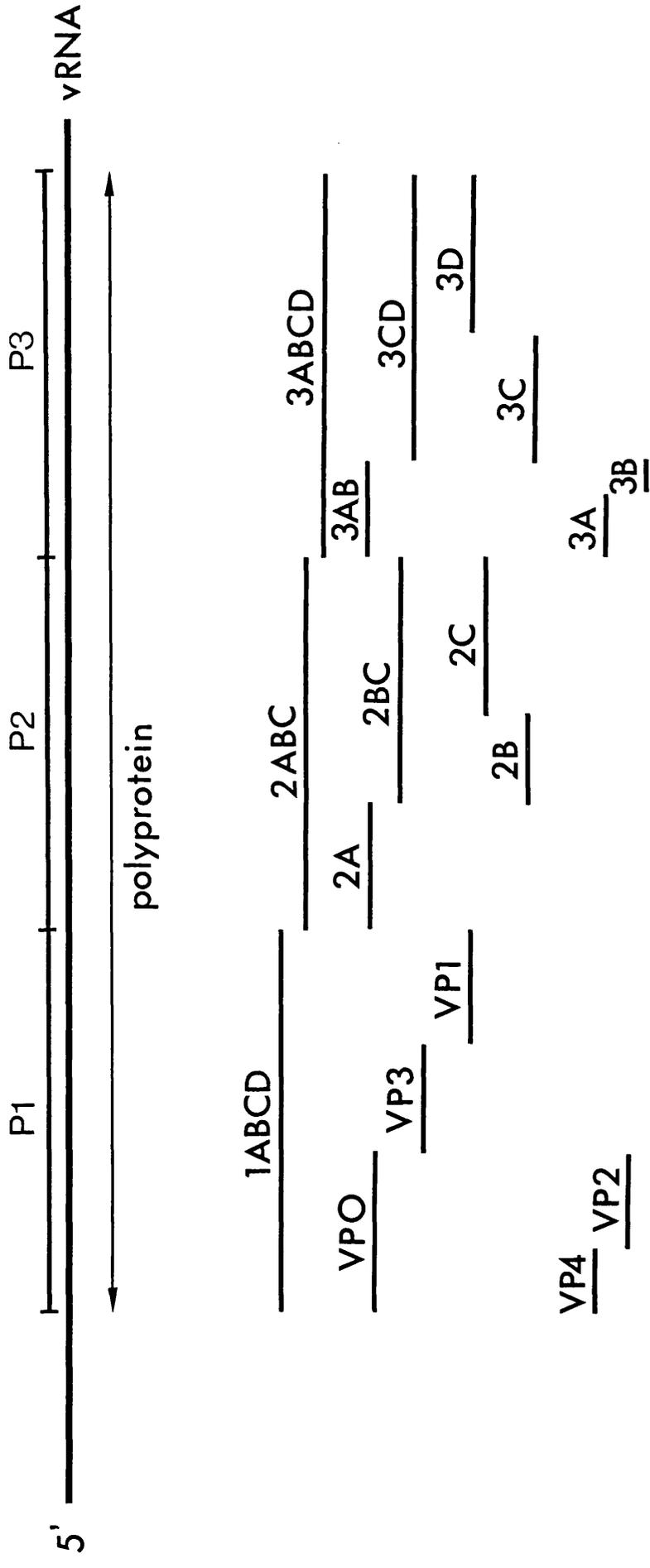
Using tryptic peptide mapping of viral proteins, pactamycin and hypertonic initiation block mapping, and genetic recombination, the gene order of poliovirus was determined (Jacobson and Baltimore, 1968a; Pallansch et al., 1980; Summers and Maizel, 1971; Taber et al., 1971; Saborio et al., 1974; Cooper, 1977). This map was confirmed and enhanced by correlation of nucleotide sequence data with partial amino acid sequence data obtained for poliovirus type 1 (See Figure 1.2).

1.4.3 The 3' Non-Coding Region

A small untranslated region lies between the termination codon of the long open reading frame and the poly(A) tract in all picornaviruses. This 3' non-coding region in poliovirus type 1 is 72 nucleotides long and is unusually short in comparison with the corresponding region of many eukaryotic cellular mRNAs. Between the three serotypes of poliovirus, this region is almost totally conserved (Stanway et al., 1983; Toyoda et al., 1984). The length and sequence homology

Figure 1.2

Structure and organisation of the poliovirus genome. Protein nomenclature, apart from the structural proteins VP1-4, is that adopted in 1983 by The European Study Group on the Molecular Biology of Picornaviruses.



exhibited by this region varies amongst genera but is highly conserved between closely related viruses.

1.4.4 The Poly(A) Tract

As with many eukaryotic mRNAs, picornaviruses possess a poly(A) tract at the 3' end of their genomic RNA. The length of the poly(A) tract varies from 40-90 nucleotides for poliovirus (Yogo and Wimmer, 1972; Ahlquist and Kaesberg, 1979). The poly(A) tracts in eukaryotic mRNAs are added by a post-transcriptional mechanism directed by a signal sequence (5'-AAUAAA-3'). However, those of picornaviruses appear to be transcribed from a poly(U) tract present at the 5' terminus of minus strand RNA (Yogo and Wimmer, 1973; Spector and Baltimore, 1975). It is interesting to note that EMCV and mengovirus both contain the polyadenylation signal, 5'-AAUAAA-3', present at the 3' terminus of polyadenylated eukaryotic mRNAs. Shortening the poly(A) tract of poliovirus has been shown to reduce the relative infectivity of the virus, while removal of the poly(A) tract has no effect on the efficiency of translation (Spector and Baltimore, 1974; Spector et al., 1975).

1.5 The Infectious Cycle

1.5.1 Virus Entry

The first stage of virus-cell interaction that leads to a productive infection requires the binding of a picornavirus to a cell-surface protein termed the virus receptor. This specific interaction with the cell determines, in many cases, the host range and tissue tropism of picornaviruses. Knowledge of the nature and identity of picornavirus receptors is scant but it has been shown that the three serotypes of poliovirus use the same receptor on the cell surface while other picornaviruses appear to bind to different cellular receptors (Minor et al., 1984). Human rhinoviruses can be classified into major and minor groups on the basis of receptor specificity. Monoclonal antibodies which bind to the cell surface and specifically block the attachment of poliovirus, coxsackie B virus and the major group of human rhinoviruses have been isolated (Minor et al., 1984; Campbell and Cords, 1983; Tomassini and Colonno, 1986). Putative receptor proteins for coxsackie B3 virus and the major group of human rhinoviruses have been identified (Mapoles et al., 1985; Tomassini and Colonno, 1986). However, the process of attachment and entry of picornaviruses remains unclear and the mechanisms employed undefined.

The initial step in this process is adsorption of the virus to the host cell. Adsorption is reversible under certain salt and pH conditions but leads rapidly, at 37°C, to

a tighter association between virus and host cell. This is called attachment. During attachment, or shortly after, the virus particle loses VP4 and the host cell's membrane fluidity is altered (Levanon and Kohn, 1978). The precise mechanism by which the virus attains internalisation following attachment is unknown but is believed to occur via either fusion-viropexis, or receptor mediated endocytosis. Following internalisation, the virus is uncoated and the viral RNA is released into the cytoplasm of the host cell where translation and replication occur.

1.5.2 Translation of the Viral Genome and Host Cell Shut-Off

Once the viral RNA reaches the cytoplasm, VPg is removed from the 5' terminus and viral protein synthesis occurs (Ambros et al., 1978). As has been stated previously, all the known viral gene products are derived from a polyprotein encoded by a single ORF. The initiation codon of this ORF is located 742 nucleotides downstream of the extreme 5' terminus of poliovirus type 3. Comparison of the primary structure of the 5' non-coding region of poliovirus with the corresponding region of the typical eukaryotic mRNA, coupled with the currently held theory regarding the mechanisms involved in the initiation of translation in eukaryotes, suggests that the "scanning model" proposed by Kozak does not extend to the initiation of picornavirus translation.

The initiation of eukaryotic translation is defined as the

process leading to the formation of an 80S initiation complex containing the 40S and 60S ribosomal subunits, initiator Met-tRNA, and mRNA. The assembly of an 80S initiation complex requires the action of at least thirteen different protein factors, ATP and GTP (Pain, 1986). The initial step regulating translation is the binding of mRNA to ribosomes.

The mRNA binding step in initiation is thought to occur in two stages; firstly, the association of the 40S ribosomal subunit with the 5' terminus of the mRNA, and secondly, the movement of the ribosomal subunit along the mRNA until it reaches an initiating AUG codon. This two step mechanism was originally proposed by Kozak (1978), and is known as the "scanning model".

Three eukaryotic initiation factors (eIF's) are required for the binding of mRNA to ribosomes; eIF-4A, eIF-4B, and eIF-4F (eIF-4F is also known as cap binding protein complex). eIF-4A is functional as a single polypeptide of 46 kD, eIF-4B appears to be a dimer of identical 80 kD peptide chains, and eIF-4F is composed of three subunits of 24 kD, 46 kD and 220 kD. The first initiation factor to bind mRNA is eIF-4F, which binds to the 5'm⁷G cap structure. It is not known whether eIF-4B binds to eIF-4F before or after eIF-4F binds the cap structure. eIF-4A then binds to this mRNP complex, which then interacts with a 40S complex to form the 40S preinitiation complex. The 40S preinitiation complex then moves along the mRNA (5' to 3') until it reaches the first AUG triplet. If

the first AUG codon occurs in the optimal context 5'-ACCAAUGG-3' (Kozak, 1981, 1984b, 1986a), all 40S subunits stop here to allow the 60S ribosomal subunit to bind, thus forming the 80S mRNA initiation complex and allowing translation to initiate. If the first AUG occurs in a suboptimal context, only a fraction of the 40S subunits will stop here (Kozak, 1984c; Liu et al., 1984), the remainder will move further downstream until they encounter the next AUG (most, but not all, mRNAs initiate translation at the first AUG codon).

The two structural features of picornavirus mRNAs which preclude them from the "scanning model" are, 1) the absence of a 5'-cap structure (Nomoto et al., 1976), and 2) the presence of many upstream AUG codons, some of which form the perfect Kozak consensus sequence (Toyoda et al., 1984).

During poliovirus infection, the translation of cellular mRNA is rapidly inhibited. This effect, called shut-off, is required for expression of the viral genome, but does not require replication of viral RNA (Penman and Summers, 1965). Cellular mRNA is not destroyed during poliovirus infection (Leibowitz and Penman, 1971; Koschel, 1974) and produces polypeptides when used as template in an in vitro translation system (Kaufmann et al., 1976). It has been shown that cellular mRNA translates efficiently in uninfected cell extracts but poorly in infected cell extracts. The converse is true for poliovirus RNA: it translates poorly in uninfected HeLa cell extracts and well in infected cell

extracts (Kaufmann et al., 1976; Helentjaris and Ehrenfeld, 1978).

Purification of eIF's from both poliovirus infected and uninfected cells showed that the 220 kD polypeptide of eIF-4F is apparently cleaved during poliovirus infection, and that addition of eIF-4F from uninfected cells to poliovirus infected extracts restored the ability to translate capped mRNAs (Etchison et al., 1982; Helentjaris et al., 1979; Lee et al., 1985). It was also shown that the inhibition of host translation that accompanies human rhinovirus 14 infection of HeLa cells also correlates with the proteolytic cleavage of p220 (Etchison and Fout, 1985), while EMCV and mengovirus infections do not lead to the cleavage of p220 (Mosenkis et al., 1985; Etchison and Etchison, 1987).

The observations that infection with a mutant poliovirus type 1 strain, containing an extra amino-acid in the viral protease 2A, does not result in inhibition of host cell translation or cleavage of p220 (Bernstein et al., 1985) and that functional 2A synthesised in vitro could direct the cleavage of crude preparations of p220 (Krausslich et al., 1987) suggest that host cell shut-off is mediated by 2A. In contrast though, experiments have shown that the proteolytic activity that cleaves p220 does not co-purify with 2A and that antibodies to 2A do not inhibit the processing of p220 in vitro (Lloyd et al., 1986). Taken together, the above observations have led to the suggestion that 2A induces the

cleavage of p220 rather than carrying out the cleavage directly (Krausslich et al., 1987).

The theory that poliovirus shuts off host protein synthesis and stimulates its own translation by inactivating p220, a subunit of the eIF responsible for cap binding, while poliovirus RNA lacks such a cap structure, seems attractive in its simplicity. There are doubts, however, as to whether inactivation of p220 is sufficient for inhibition of host cell protein synthesis (Bonneau and Sonenberg, 1987), while Kozak (1986c) has cast doubts on whether cleavage of p220 plays a role at all in the switch from cellular to viral protein synthesis.

Such controversy is likely to be settled when highly purified or recombinant DNA produced components of the eIFs become available and are used in in vitro reconstitution studies.

1.5.3 Products of Translation and Post-Translational Processing

30-60 minutes following poliovirus infection, host cell translation is shut-off with poliovirus protein synthesis reaching a peak 2-3 hours after infection. Mature viral proteins are derived by a set of post-translational cleavages, some of which occur while the peptides are still nascent on ribosomes. The comparison of partial amino-acid sequence data with nucleotide sequence data for poliovirus

type 1 led to the elucidation of the cleavage sites used in proteolytic processing, and allowed fine mapping of the location of the final products on the viral genome. In poliovirus type 1, nine cleavages occur between glutamine-glycine pairs, two between tyrosine-glycine pairs and one between an asparagine-serine pair (See Figure 1.3). Evidence suggests that the viral peptide 3C is responsible for the cleavages occurring between glutamine-glycine pairs, and that 3C acts autocatalytically to release itself from the polyprotein (Hanecak et al, 1982; Hanecak et al., 1984). Intramolecular cleavage for picornaviruses was originally suggested following experiments using EMCV (Palmenberg and Rueckert, 1982).

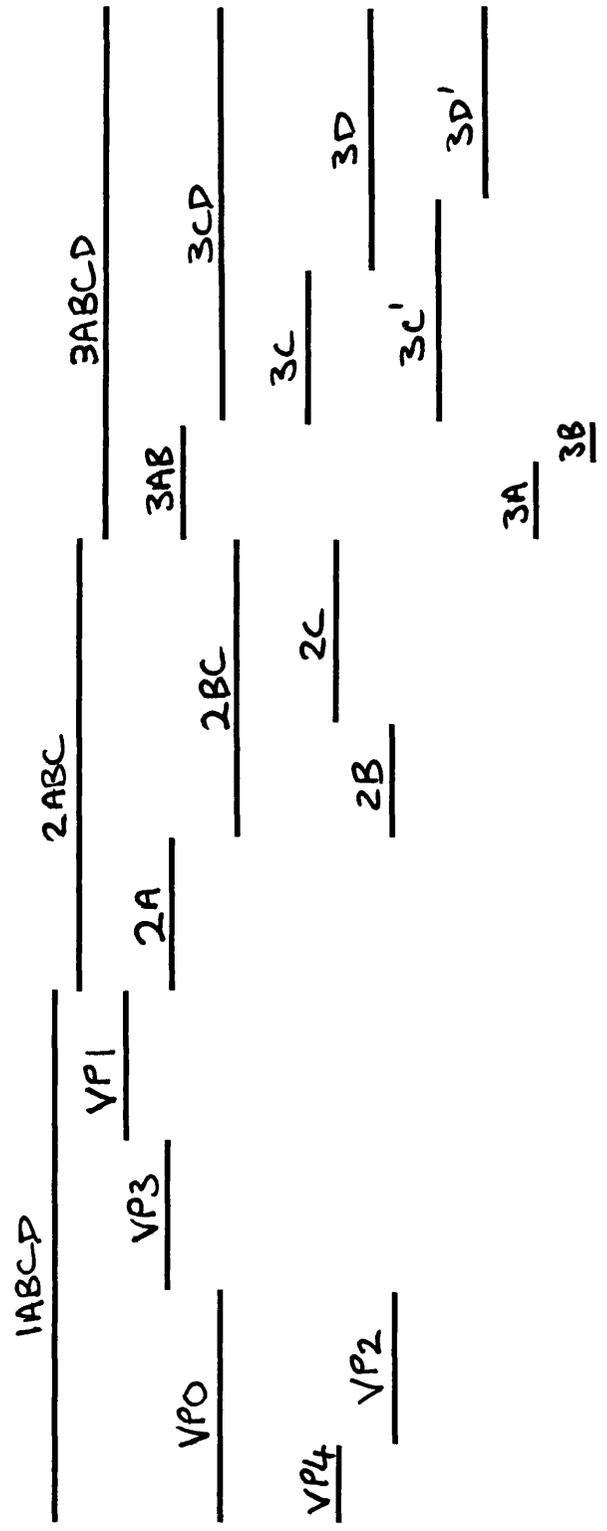
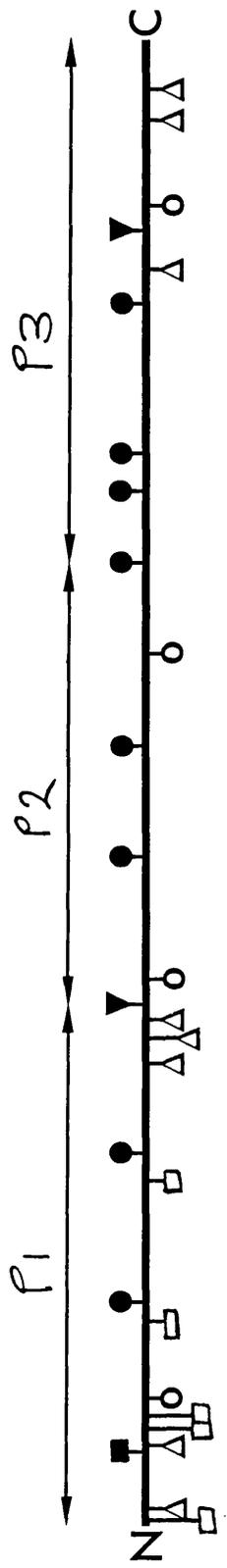
The first cleavage within the polyprotein of poliovirus occurs while the polypeptide chain is still nascent on a ribosome, at the junction between the structural and non-structural peptides. Cleavage at the P1-P2 junction occurs between a tyrosine-glycine pair (Kitamura et al., 1981) and is carried out autocatalytically by the virus peptide 2A (Toyoda et al., 1986). The second tyrosine-glycine cleavage occurs in the P3 region and gives rise to 3C' and 3D' whose functions and biological significance remain unknown.

The final cleavage within the poliovirus polyprotein occurs at the junction of VP4 (1A) and VP2 (1B) in VP0 (1AB) and occurs between an asparagine-serine pair. This cleavage takes place deep within the maturing particle and is believed

Figure 1.3

Protein processing map of poliovirus. The polyprotein is divided into three regions (P1, P2, and P3). Amino acid pairs known to be cleaved are represented by filled symbols, while apparently uncleaved pairs are represented by open symbols.

Glutamine-Glycine	○	●
Tyrosine-Glycine	▽	▼
Asparagine-Serine	□	■



to occur by autocatalysis, triggered by the encapsidation of viral RNA. This represents the final step in the assembly of progeny virions (Arnold et al., 1987).

Amongst the picornaviruses, poliovirus proteolytic processing has been the most thoroughly studied. The other picornaviruses appear to employ a similar mechanism to produce functional peptides, however some differences in the protein map and cleavage sites do exist. Both EMCV and FMDV produce leader peptides preceding the capsid precursor protein (Palmenberg et al., 1984; Beck et al., 1983). In addition, all FMDV serotypes initiate protein synthesis at two different AUGs (Sangar et al., 1987) and encode three VPg proteins, while other picornaviruses appear to initiate translation at a single AUG and encode only one VPg protein (Forss and Schaller, 1982). FMDV appears to produce only two proteins within the P2 region, seemingly lacking protein 2B (Grubman et al., 1984). Proteolytic processing of EMCV and HRV-14 occurs predominantly at glutamine-glycine pairs, though additional glutamine-tyrosine and glutamic acid-alanine cleavages appear to occur in HRV-14, while cleavages occur at glutamine-serine and tyrosine-proline pairs in EMCV (Stanway et al., 1984c; Palmenberg et al., 1984). During FMDV infection cleavages occur at a broader range of sites, including glutamic acid-serine, glutamic acid-glycine, and glutamic acid-threonine pairs (Boothroyd et al., 1982; Forss and Schaller, 1982; Carroll et al., 1984). Since not all

glutamine-glycine and tyrosine-glycine peptide pairs are used as substrates for cleavage during poliovirus infection this suggests that other factors determine protease specificity.

1.5.4 Viral RNA Synthesis

Following the initial rounds of translation of the infecting viral RNA, viral RNA synthesis begins. Replication of picornaviral RNA occurs exclusively in the cytoplasm of the infected cell, and appears to take place without any involvement of the cell nucleus (Follet et al., 1975). Baltimore et al. (1966) observed that viral RNA synthesis proceeds in three distinct phases; an early phase of exponential RNA synthesis, followed by a linear phase when the majority of RNA synthesis occurs, and finally a late phase of rapidly declining RNA synthesis. The precise events which lead to the switches in the rate of RNA synthesis remain undefined.

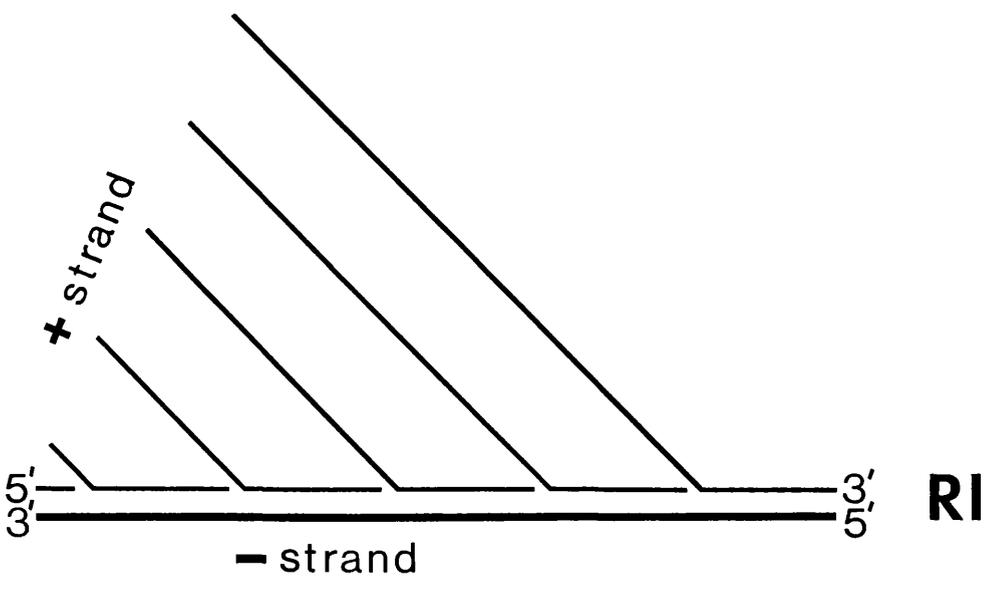
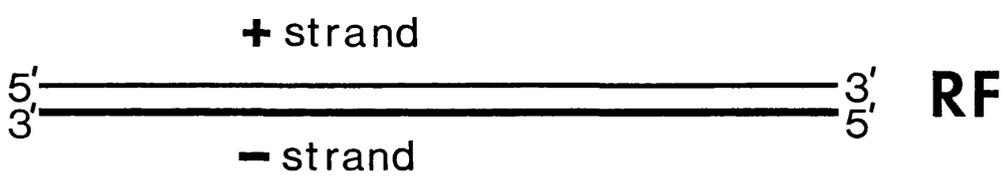
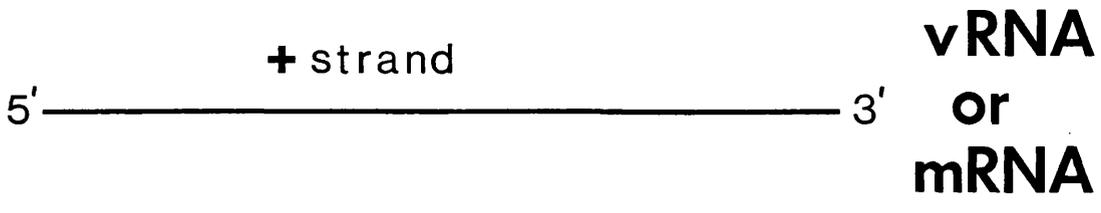
Three distinct species of virus related RNA are present in extracts of picornavirus infected cells; single-stranded, double-stranded, and partially double, partially single-stranded RNA (See Figure 1.4). The single-stranded RNA, the most abundant of the three species, is positive sense RNA, either vRNA or it's corresponding mRNA (Zimmerman et al., 1963). The double-stranded or replicative form RNA (RF; Montagnier and Sanders, 1963; Baltimore et al., 1964) consists of a full-length viral RNA molecule hydrogen bonded

Figure 1.4

Schematic representation of the three distinct species of virus related RNA present in extracts of cells infected with picornaviruses.

RF - replicative form.

RI - replicative intermediate.



to a full-length complementary minus strand RNA, cRNA (Baltimore, 1966). During the replicative cycle of the virus, this species increases in abundance and is believed to represent a short-lived intermediate between cRNA and positive sense RNA synthesis and an end product of RNA synthesis following termination of ssRNA synthesis. The third species, known as replicative intermediate (RI) RNA, consists of a full-length complementary minus strand in association with 5.5 to 6.5 nascent positive sense strands covalently linked to VPg at their 5'-termini (Baltimore, 1968; Girard et al., 1967; Nomoto et al., 1977a).

Replication of picornaviral RNA proceeds in two distinct steps. Initially the parental RNA is used as a template for the synthesis of complementary RNA (minus strand), the minus strand is then used as template for the synthesis of new viral RNA of the positive sense. Early in infection most of the new plus strands are processed (VPg removed), and used as mRNA for the production of viral proteins (Nomoto et al., 1977b). Later in infection more of the freshly synthesised plus strands are encapsidated to form progeny virions. Plus and minus strands are synthesised throughout the replication cycle (free minus strands have not been detected), but in unequal quantities. Plus strands are synthesised in great excess over minus strands. This agrees both with the occurrence of RI RNA containing a single complementary minus strand acting as template simultaneously for many plus

strands, and the absence of a converse RI structure, i.e. a single plus strand acting as template for many nascent negative strands. The molecular basis for such biased or asymmetric synthesis is unknown.

The RNA-dependent RNA polymerase of poliovirus was first isolated as a complex with its endogenous template (Baltimore et al., 1963). Subsequent attempts to purify the polymerase resulted in the complete loss of activity. Partial purification of active components of the poliovirus replication complex in a soluble and template dependent form required the development of a poly(A).oligo(U)-dependent poly(U) polymerase assay which identified a virus-induced enzyme (Dasgupta et al., 1979; Flanagan and Baltimore, 1977; Flanagan and van Dyke, 1979). Preparation of highly purified forms of the polymerase showed that 3D^{pol} was the only virus-specific protein to co-purify with the soluble polymerase (van Dyke and Flanagan, 1980). Antibodies raised against a synthetic peptide corresponding to the carboxy terminal region of 3D^{pol} were shown to inhibit the polymerase reaction in-vitro (Baron and Baltimore, 1982c).

The highly purified polymerase was capable of synthesising full-length copies of poliovirus RNA and other polyadenylated RNAs when oligo(U) was used as a primer in the reaction (Dasgupta et al., 1979; van Dyke et al., 1982; Baron and Baltimore, 1982a). Using the requirements of RNA bacteriophage replicases as a model for poliovirus replicase,

a cellular protein or host factor that played a role in the initiation of the polymerase reaction was isolated from the high salt wash of ribosomes from uninfected HeLa cells. The absolute requirement for oligo(U) could be overcome by the addition of the host factor (Dasgupta et al., 1980). The host factor was shown to co-purify with the polymerase during the early steps of polymerase purification thus explaining why partially purified polymerase could initiate RNA synthesis in the absence of added oligo(U) (Young et al., 1985; Dasgupta et al., 1982).

All nascent plus strands in RI RNA are linked to VPg at the 5' terminus, as is the minus strand in RI RNA (Nomoto et al., 1977a; Flanagan et al., 1977; Pettersson et al., 1978). Nascent RNA strands with a 5' terminal pppUp have not been found in-vivo (Nomoto et al., 1977a). The previous observations led to the proposal that VPg may act as a primer for the initiation of RNA synthesis (Wimmer, 1982). This theory was strengthened by reports that anti-VPg antibody inhibited RNA synthesis in-vitro and immunoprecipitated some product RNA synthesised in-vitro on a poliovirus RNA template by the polymerase and host factor (Baron and Baltimore, 1982b; Morrow and Dasgupta, 1983; Morrow et al., 1984). In addition, VPg-pUpU was detected in infected cells and shown to be produced by a crude membrane fraction isolated from poliovirus infected cells and was suggested to be part of the initiation complex formed during poliovirus RNA replication

(Crawford and Baltimore, 1983; Takegami et al., 1983).

The inability to chase VPg-pUpU into longer RNA strands cast doubts on the theory that VPg, VPg-pUpU, or a precursor of VPg acts as a primer in the initiation of the polymerase reaction (Takegami et al., 1983). More direct evidence suggests that VPg does not act as a primer or play another role in the initiation of the polymerase reaction. The RNA molecules immunoprecipitated with anti-VPg antibody were thought to represent nascent transcripts primed by VPg. It has now been shown however, that removal of VPg from the template RNA by pre-treatment with proteinase K results in no immunoprecipitation of labelled product RNA (Young et al., 1986; Andrews and Baltimore, 1986). Thus, VPg on the template RNA and not the product RNA was responsible for the immunoprecipitation of labelled RNA. These results argue that VPg is not required for initiation of RNA synthesis by 3D^{pol} and host factor in-vitro.

Andrews et al. (1985) have described a uridylyl transferase activity in highly purified preparations of host factor and suggest that the addition of several uridylate residues to the poly(A) tract at the 3' terminus of the poliovirus plus strand would allow formation of a hairpin which could prime the synthesis of minus strand RNA. This model for self-priming could explain the presence of products twice the length of viral template RNA in in-vitro reactions (Young et al., 1985; Hey et al., 1986). To date, most in-

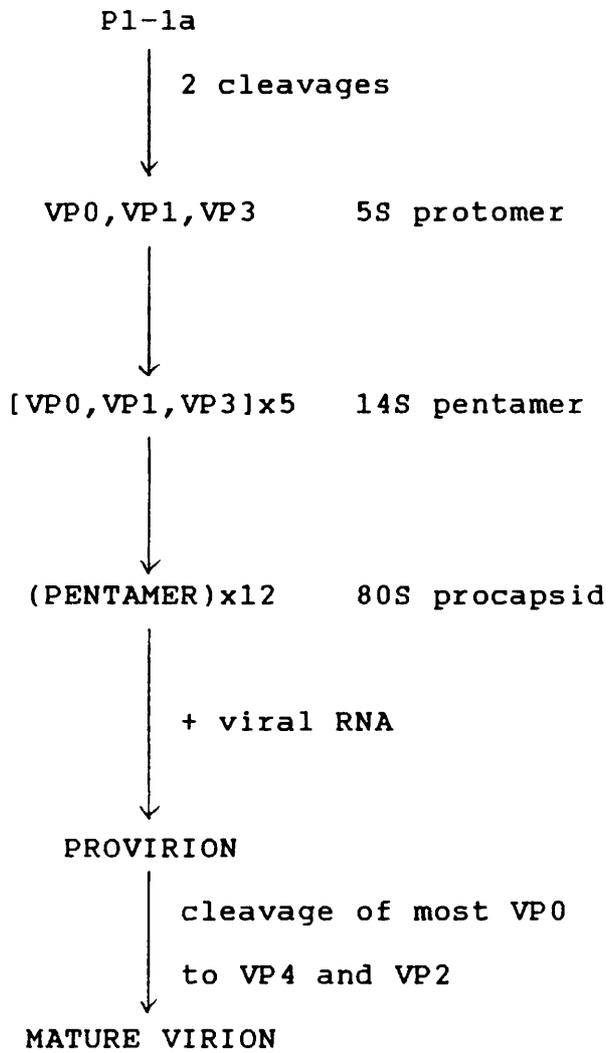
vitro work has concentrated on the initiation of negative strand synthesis and it is not known if the same strategy is used in the initiation of plus strand synthesis.

1.5.5 Virus Assembly

Although much is known about the synthesis and structure of the basic components of the poliovirus virion, little is known about the morphogenesis of the particle. The precise mechanisms which lead from the translation of the P1 region to assembly of the final capsid structure are not known. Factors likely to control the assembly of the viral components include proteolytic cleavages of the capsid proteins, hydrophobic and hydrophilic interactions between the capsid proteins, transport of capsid precursors within the infected cell, and the interaction of capsid proteins with viral RNA. The structure of some of the intermediates produced during the assembly process are known and have been used to create a model for the assembly of virus particles (See Figure 1.5).

The viral capsid proteins are synthesised as a large precursor protein P1-1a. This is released from the nascent polyprotein by cleavage at a Tyr-Gly pair, catalysed by protease 2A. P1-1A is subsequently cleaved by protease 3C at two Gln-Gly pairs to produce VP0, VP3, and VP1. These three proteins associate with one another to form 5S aggregates known as protomers (Phillips et al., 1968). It is not clear

Figure 1.5 Model for the assembly of mature virus particles.



whether the intermolecular bonding between these proteins exists as intramolecular bonds prior to cleavage, or whether cleavage is required to allow intermolecular bonding. The protomers then associate to produce a 14S pentamer (Phillips et al., 1968; Rueckert, 1976). Twelve pentamers then come together to form an 80S particle known as "top component" or the procapsid (Hummeler et al., 1962; Scharff and Levintow, 1963; Maizel et al., 1967; Jacobson and Baltimore, 1968b). Only viral RNA linked to VPg is inserted or taken into the procapsid, by an unknown process to produce provirions. The final step in the assembly of virions is the cleavage of VP0 to produce VP2 and VP4. This cleavage occurs between an asparagine-serine pair and is thought to occur by autocatalysis triggered by the encapsidation of viral RNA (Hogle et al., 1985; Arnold et al., 1987).

1.6 Genetics of Picornaviruses

1.6.1 Genetic Recombination

The occurrence of genetic recombination in picornaviruses, first suggested by Hirst (1962) and Ledinko (1963), was inferred from the observation of double mutants in the progeny of a cross (double infection) in significant excess over spontaneous mutation. Recombination was subsequently used to generate genetic maps of poliovirus and FMDV (Cooper, 1968; 1977; McCahon et al., 1977), which were in agreement with the physical map, i.e. structural proteins map in the 5'

half of the genome while non-structural proteins involved in RNA replication map to the 3' half of the genome (Summers and Maizel, 1971).

It was not until 1982 that recombination was demonstrated conclusively by fingerprinting progeny viral genomes produced by crossing different strains of FMDV (King et al., 1982). Recombination was subsequently shown to occur between different strains of poliovirus (Agol et al., 1984; 1985), and the importance of recombination, beyond its use as a laboratory tool, has been highlighted by the observation that the reversion to neurovirulence of poliovirus vaccine strains has been associated with intertypic recombination in some cases (Kew and Nottay, 1984).

Nucleotide sequence analysis of the recombinant junctions of poliovirus recombinants has shown that the process is random, not site-specific, and does not require extensive homology between the parental genomes at the crossover site (Kirkegaard and Baltimore, 1986). A copy choice model, in which the viral RNA polymerase switches templates during negative strand synthesis, has been proposed in preference to one of molecular exchange for picornavirus recombination (Kirkegaard and Baltimore, 1986).

1.6.2 Complementation

Mixed infection by viruses carrying defects in different genes can in many cases result in virus growth, as each

mutant virus contributes a functional gene product lacking in the other. This phenomenon is known as complementation or marker rescue. While recombination of picornaviruses occurs frequently (King, 1987), complementation of many ts mutants of poliovirus (Cooper, 1977), mengovirus (Bond and Swim, 1975), and aphthovirus (Lake et al., 1975) has not been demonstrated. Mutations that can be rescued have been described, though many of the experiments have shown that complementation with picornaviruses often appears to be asymmetric (Cords and Holland, 1964; Ikegami et al., 1964; Wecker and Lederhilger, 1964; Cooper, 1965). All the mutations that were rescued in the above experiments mapped in the structural region while mutations lying in the non-structural region were not complemented.

Complementation of more extensive lesions in the structural region is demonstrated by the recovery of defective interfering (DI) particles by serial passage of wild-type virus at high multiplicities of infection (Cole et al., 1971; Lundquist et al., 1979). These DI particles contain deletions within the capsid coding region, maintain the ability to produce all non-structural proteins, and require for propagation the presence of a helper virus capable of supplying the capsid proteins (Cole and Baltimore, 1973a; 1973b; 1973c; Kuge et al., 1986). These results, taken together, suggested that the non-structural region of picornaviruses formed a single complementation group, or that

the non-structural proteins were incapable of acting in cis, or that replication occurs within closed structures which block the trans action of the non-structural proteins (Lundquist et al., 1979; Agut et al., 1984; Kuge et al., 1986).

Recent experiments using characterised mutants derived from an infectious cDNA of poliovirus type 1 show that mutants bearing lesions in the 2A and 3A regions can be rescued by one another and by other mutants, though these other mutants, with lesions mapping in 3D^{pol}, 2B, and the 3' non-coding region, could not be rescued (Bernstein et al., 1986). These results demonstrate for the first time a two-way rescue of poliovirus mutants and imply that the non-structural region contains at least two complementation groups. They also provide evidence that some of the non-structural proteins are capable of acting in trans while others appear to act only in cis. Through the study of such well defined mutants it should be possible to construct a complementation map of poliovirus revealing functional features of the intermediate products of proteolytic cleavage.

1.7 Molecular Cloning and Nucleotide Sequencing of Picornaviruses

The last decade has seen widespread application of recombinant DNA technology to the study of picornaviruses. In

1981 the entire nucleotide sequence of the Mahoney strain of type 1 poliovirus was determined by two groups (Kitamura et al., 1981; Racaniello and Baltimore, 1981a). Comparison of the nucleotide sequence data with partial amino- and carboxy-terminal acid sequence of poliovirus proteins allowed the construction of a complete genetic map of the viral genome (Kitamura et al., 1981; Racaniello and Baltimore, 1981a). Many picornaviruses have now been successfully cloned and nucleotide sequence data is available for at least one member of each of the four genera within the family Picornaviridae (Cann et al., 1984; Carroll et al., 1984; Forss et al., 1984; Hughes et al., 1986; Iizuka et al., 1987; Jenkins et al., 1987; Kitamura et al., 1981; La Monica et al., 1986; Lindberg et al., 1987; Najarian et al., 1985; Palmenberg et al., 1984; Racaniello and Baltimore, 1981a; Skern et al., 1985; Stanway et al., 1983, 1984c; Toyoda et al., 1984; van der Werf et al., 1981; Vartapetian et al., 1983).

Nucleotide sequence data and the use of cDNA clones of picornaviruses has led to the expression of defined regions of these viruses in E.coli and the synthesis of antigenic peptides in-vitro which has shed light on the function of some viral proteins (Baron and Baltimore, 1982c, 1982d; Bittle et al., 1982; Emini et al., 1983; Hanecak et al., 1984; Kleid et al., 1981; Klump et al., 1984; Semler et al., 1982, 1987; Toyoda et al., 1986; van der Werf et al., 1983; Ypma-Wong and Semler, 1987).

A finding which has had a profound effect on picornavirology was the demonstration that transfection of susceptible cells in tissue culture with a full-length cDNA copy of poliovirus type 1/Mahoney produced infectious virus (Racaniello and Baltimore, 1981b). The viral RNA and proteins produced by this transfection-derived virus were found to have no gross changes when compared with those of the virus originally cloned (Racaniello and Baltimore, 1982). This observation has led to a surge in picornavirus genetics as it allows predetermined mutations to be incorporated into the RNA genome via site-directed mutagenesis of a cDNA clone and the generation of predetermined recombinant viruses. The effect of such alterations, if not lethal, can be studied by monitoring the phenotype of the transfection-derived virus. Such techniques are currently being used to study the function of viral proteins, the phenotype expressed by a given virus, and the development of novel vaccine strains. These advances have been used most successfully in the study of the attenuated and neurovirulent phenotypes of different strains of poliovirus (See Section 1.8).

1.8 Attenuation and Neurovirulence of Poliovirus

Vaccination programmes in the developed countries, using either the Salk or Sabin vaccines, have so effectively controlled poliomyelitis that infectious acute spinal paralysis is now extremely rare in these countries.

Poliomyelitis has not been eradicated though, and in countries using the Sabin attenuated vaccine strains a low level of the disease persists (Assad et al., 1972). Although the Sabin vaccine is regarded as being safe and highly effective many of these cases are attributable to vaccination (Assad and Cockburn, 1982), and are believed to be caused by reversion of an attenuated vaccine strain to neurovirulence. In many cases, when virus has been isolated it has been designated vaccine-like on the basis of T₁ oligonucleotide fingerprinting and by immunological criteria (Minor and Schild, 1981). As this association is circumstantial, there was some reluctance in the past to accept that the vaccines were responsible for paralysis (Sabin, 1981; Horstmann, 1982). Recently however, there has been a report of a clear case of reversion to neurovirulence of a vaccine strain and its involvement in a fatal case of paralytic poliomyelitis (Cann et al., 1984). Most vaccine-associated cases of paralysis have been caused by type 3 virus, some by type 2 virus , while vaccine-like type 1 virus has yet to be isolated from a case of vaccine-associated paralysis.

The live-attenuated vaccine strains developed by Sabin were derived from wild-type isolates by repeated passage in monkey tissue in-vitro and in-vivo (Sabin and Boulger, 1973). Strains were selected for use as vaccines on the grounds of low neurovirulence in monkeys. The precise mechanism by which neurovirulence was reduced in these strains has intrigued

researchers since their isolation.

The aim of recent work, carried out by several groups using recombinant DNA technology, has been the elucidation of the genetic basis of attenuation and the reversion of the Sabin vaccine strains to neurovirulence (Reviewed by Almond, 1987).

Comparative sequence analysis of type 1 strains revealed 57 nucleotide changes between the attenuated vaccine strain, P1/LS-c,2ab, and its neurovirulent progenitor, P1/Mahoney/41. All the changes represented single point mutations and 21 of the changes result in altered amino acids (Nomoto et al., 1982). To investigate the role these point mutations play in the attenuating process, recombinants between the attenuated and neurovirulent type 1 strains were constructed. Such recombinants were made via common restriction sites in the full-length cDNAs. Recombinant virus was produced by transfection of cells in culture and the phenotype of the progeny virus tested (Omata et al., 1985, 1986). This study produced some conflicting results but did show that many mutations scattered throughout the genome contributed to the attenuation of the vaccine strain, explaining the observed stability of this vaccine strain (Assad and Cockburn, 1982).

The poliovirus type 2 vaccine strain, P2/P712,Ch,2ab, has been sequenced but as yet sequence data from neither its progenitor nor a neurovirulent revertant is available (Toyoda et al., 1984). As the progenitor strain, P2/P712, is also

attenuated, work on type 2 will be restricted to a study of the basis for its reversion to neurovirulence. As mentioned previously many type 2 revertants appear to be intertypic recombinants (Kew and Nottay, 1984). Correlation of the crossover points in any type 2/type 3 recombinants with the knowledge of type 3 attenuation/neurovirulence could be used to map attenuating mutations in the type 2 vaccine strain.

Studies carried out to investigate the attenuation and reversion to neurovirulence of poliovirus type 3 have yielded clearer results than the work on type 1. The Sabin strain most frequently associated with paralysis in vaccinees is the type 3 strain, P3/Leon 12a₁b. In the past, several strains isolated from fatal paralytic cases have been shown to have T₁ oligonucleotide fingerprints very similar to the vaccine strain (Minor, 1980, 1982).

Comparative nucleotide sequence analyses of the attenuated vaccine strain, P3/Leon/12a₁b, and its neurovirulent progenitor, P3/Leon/37, revealed that they differed at only ten nucleotides throughout their genomes (Stanway et al., 1984b). Further insight was gained from the nucleotide sequence of strain P3/119, a vaccine-like strain that had been isolated from the brain and spinal cord of a victim of fatal paralytic poliomyelitis temporally associated with vaccination (Cann et al., 1984). The sequence of this strain revealed seven nucleotide differences from the vaccine strain. From these three sequences it was concluded that

attenuation was likely to be controlled by one of three mutations; a base change at position 472 which directly back mutates in P3/119; alteration of structural proteins; or a mutation lying directly adjacent to the poly(A) tract in the 3' non-coding region.

Further evidence for the involvement of the base change at position 472 was provided by sequencing studies carried out on viruses isolated from faecal samples collected from children following their vaccination (Evans et al., 1985; Minor et al., 1986b). Analyses of these viruses indicated that there was rapid selection for viruses which had reverted at position 472 during passage in the human gut. The vaccine strain has a uracil residue at position 472, while the neurovirulent progenitor, the neurovirulent revertant, and the excreted viruses (type 3 viruses shed after 36 hours post-vaccination) all have a cytidine. The secreted viruses possessing a cytidine at position 472 were found to have increased in neurovirulence.

To analyse the contribution of the other mutations in attenuation a series of recombinant viruses were constructed via cDNAs (Almond et al., 1985; Westrop et al., 1987). The phenotype of the recombinant viruses, generated by transfection, were examined by the standard WHO safety test of intraspinal inoculation in cynomolgus monkeys. These results suggested that two mutations were responsible for the attenuation of the Sabin type 3 vaccine strain. The mutations

are the cytidine to uracil at nucleotide position 472, and a cytidine to uracil at nucleotide position 2034 which results in a serine to phenylalanine substitution in VP3. Independently the mutation at 472 brings about a 70-80% reduction in the number of animals paralysed. The serine to phenylalanine substitution in VP3 is responsible for the ts phenotype expressed by the vaccine strain.

It has now been shown that the mutation at 472 can also attenuate a derivative of the mouse virulent strain P2/Lansing. Intertypic recombinants, in which the 5' non-coding regions of P3/Leon/37 and the type 3 vaccine strain, P3/Leon 12a₁b, were exchanged for the corresponding region of the type 2 virus, were constructed via cDNAs (La Monica et al., 1987). When tested in mice, the recombinant virus with the 5' non-coding region from P3/Leon/37 was found to be as neurovirulent as P2/Lansing, while the recombinant containing the 5' non-coding from the type 3 vaccine strain was attenuated. Moreover, the attenuated recombinant apparently replicates poorly, if at all, in the mouse brain (La Monica et al., 1987).

The mechanism, whereby a single point mutation in the 5' non-coding region of the genome of poliovirus dramatically reduces neurovirulence and inhibits the intracellular life-cycle of the virus is not known. An understanding of the molecular mechanisms involved requires greater knowledge of the function of the 5' non-coding region.

1.9 The Aim of This Project

Comparative nucleotide sequence analysis of many enteroviruses and rhinoviruses has revealed that the 5' non-coding region represents the most highly conserved region in the genome amongst these different viruses. The high degree of sequence homology exhibited by this region, together with its relative size when compared with the corresponding region of the average eukaryotic mRNA, suggest that the 5' non-coding region plays an essential role in the life-cycle of picornaviruses.

The observations of many groups give added weight to the argument outlined above (See section 1.4.1). The most dramatic and intriguing observation illustrating the importance of the 5' non-coding region is the ability of nucleotide 472 in type 3 poliovirus to alter virus neurovirulence.

The aim of the project presented here is the development of viral vectors to study in-vivo, using deletion analysis, the function of the 5' non-coding region of poliovirus. During the life-cycle of a given strain of poliovirus the products of translation, replication, and encapsidation appear and accumulate in a highly reproducible manner resulting in the production of progeny virions. The production of progeny virions requires the coordinated expression of the products of translation, replication, and

encapsidation. As translation and replication are interdependent, and encapsidation requires the products of both of these, it would appear that these three processes are tightly regulated with respect to each other in both timing and efficiency. Such control represents a key element in the productive viral life-cycle. It was decided to construct vectors that would allow analysis of the role played by the 5' non-coding region in these three processes. As a significant number of small deletions in this region rendered poliovirus cDNA incapable of producing virus (V. Racaniello and B. Semler personal communications), the development of a system not requiring the production of infectious virus from a cDNA was attempted. The goal was to construct plasmids which would allow the measurement of the three processes mentioned above via the products of transient expression.

To study the role of the 5' non-coding region in translation this region was to be fused directly with the coding region of the bacterial chloramphenicol acetyl transferase gene (CAT). The CAT gene has found widespread use as a marker gene in monitoring gene expression in many systems. Under the transcriptional control of a known eukaryotic promoter/enhancer element the poliovirus/CAT chimaera would be expressed following transfection of eukaryotic cells in culture, and levels of CAT measured. Deletion analysis would then be used to study the control of translation by the 5' non-coding region and identify elements

essential for control.

To study the involvement of the 5' non-coding region in viral replication and encapsidation a second vector was constructed. This plasmid was designed to direct the expression of a mRNA containing the poliovirus signal sequences required for replication. Together with the signal sequences, this mRNA also contained the coding information required for expression of the poliovirus proteins essential for the initiation of a normal replicative cycle. Replication of this specific mRNA molecule, following transfection of eukaryotic cells, could be measured by quantitative analysis of the message. Deletion analysis would allow the identification of sequences within the 5' non-coding region essential for viral replication.

It is suggested that the same vector could also be used to study the role of the 5' non-coding region in the encapsidation of viral RNA. Although the mRNA produced by this vector contains the coding information required for the expression of all the poliovirus non-structural proteins, the coding sequences for all four poliovirus capsid proteins are not present. In order to become encapsidated, the mRNA produced by this vector requires the provision, in-trans, of the full complement of viral capsid proteins. This could be accomplished by infecting transfected eukaryotic cells with wild type poliovirus. Capsid proteins produced by the wild type poliovirus would then be used to encapsidate both viral

RNA and the viral-like RNA produced by the vector. To differentiate between the viral and viral-like RNA in progeny, the viral-like RNA is smaller than viral RNA and also contains a foreign marker gene (the CAT gene inserted within the poliovirus sequences).

Chapter 2 describes the construction of the plasmid p12R-LEON which served as the basis for the construction of the vectors and series of deletions made in the 5' non-coding region of poliovirus cDNA.

Chapter 3 describes the construction of a poliovirus cDNA/CAT recombinant vector designed to allow the study of the translational control exerted by the poliovirus 5' non-coding region. In this vector, pRSV-5'polio-CATm2(N⁺), the DNA encoding the bacterial enzyme chloramphenicol acetyl transferase (CAT) is fused directly downstream of the poliovirus 5' non-coding region, and the CAT initiating codon plus flanking nucleotides are replaced with the corresponding region of poliovirus. Chapter 4 describes the construction and characterisation of deletions spanning the poliovirus 5' non-coding region and their subsequent incorporation into the plasmid pRSV-5'polio-CATm2(N⁺). In Chapter 5 the results of CAT assays carried out on extracts of eukaryotic cells transfected with the plasmids described in Chapters 3 and 4 are presented and discussed in detail.

Chapter 6 describes the construction of a poliovirus cDNA/CAT vector designed to allow the identification of cis-

acting regulatory sequences involved in viral replication and the encapsidation of viral RNA. Finally, Chapter 7 consists of a summary of the work presented in this thesis and discusses the future directions this work should take with reference to recently published reports of experiments carried out by several other groups using similar approaches to those described here.

CHAPTER 2

The Construction of p12R-LEON

2.1 Introduction

In 1981 it was reported that a full-length cDNA clone of poliovirus type 1, inserted in the bacterial plasmid pBR322, produced live-infectious virus following transfection of cultured mammalian cells (Racaniello and Baltimore, 1981b). A biochemical profile of the RNA and proteins produced by this virus demonstrated that the genome had not been grossly mutated during the cloning process or transfection. The phenotype and growth characteristics exhibited by the transfection derived virus were found to be identical to those of the virus originally cloned (Racaniello and Baltimore, 1982). The success of these experiments has resulted in the application of the powerful techniques of genetic engineering to the functional analysis of the poliovirus genome and its products.

The precise steps which lead from the introduction of a cDNA copy of a viral RNA genome to the production of infectious virus in transfected cells are unknown. However, this process must involve the production of a transcript of the cDNA and transport of this transcript into the cytoplasm where it can be translated. The translation products must then recognise and replicate viral sequences within transcripts present in the cytoplasm to produce viral RNA, and so initiate virus replication.

The initial transcripts from the transfected plasmids are

produced as a result of fortuitous transcription. An event most probably controlled by sequences within the plasmids bearing sequence homology with eukaryotic promoter elements as opposed to integration of the poliovirus cDNA downstream of a host promoter. It is likely that the "promoter" lies within the bacterial vector and not within the poliovirus cDNA as it has been observed that the orientation of the poliovirus cDNA within the plasmid determines whether infectious virus is produced (Stanway, unpublished).

As stated above, the ability of a cDNA of poliovirus RNA to produce infectious virus has allowed functional analysis to be carried out using site-specific mutagenesis of a given region of the genome. The efficiency of production of infectious virus from cDNA was so low that there was a danger that the failure of a cDNA to produce virus could be over interpreted. It was decided therefore to attempt to increase the efficiency of recovery of virus from transfection of cDNA by placing a full-length cDNA of poliovirus under the control of a defined eukaryotic transcriptional promoter and enhancer element.

This chapter describes the construction of p12R-LEON. This plasmid contains a cDNA copy of the P3/Leon/37 strain of poliovirus under the transcriptional control of the long terminal repeat (LTR) of Rous sarcoma virus (RSV).

2.2 The Construction of p12R-LEON

The bacterial plasmid used in the construction of p12R-LEON was the vector pUC12 (Vieira and Messing, 1982). This plasmid was chosen as it is relatively small, 2.7 Kbp, and contains a polylinker region with many unique restriction sites. Insertion of the poliovirus cDNA at the PstI site in the polylinker region would make it easier, at later stages, to move either the complete cDNA or specific terminal regions to other plasmids.

The LTR of RSV was chosen as the promoter/enhancer element as it had been shown to be the most active element yet, and was active in a variety of cells (Gorman et al., 1982b).

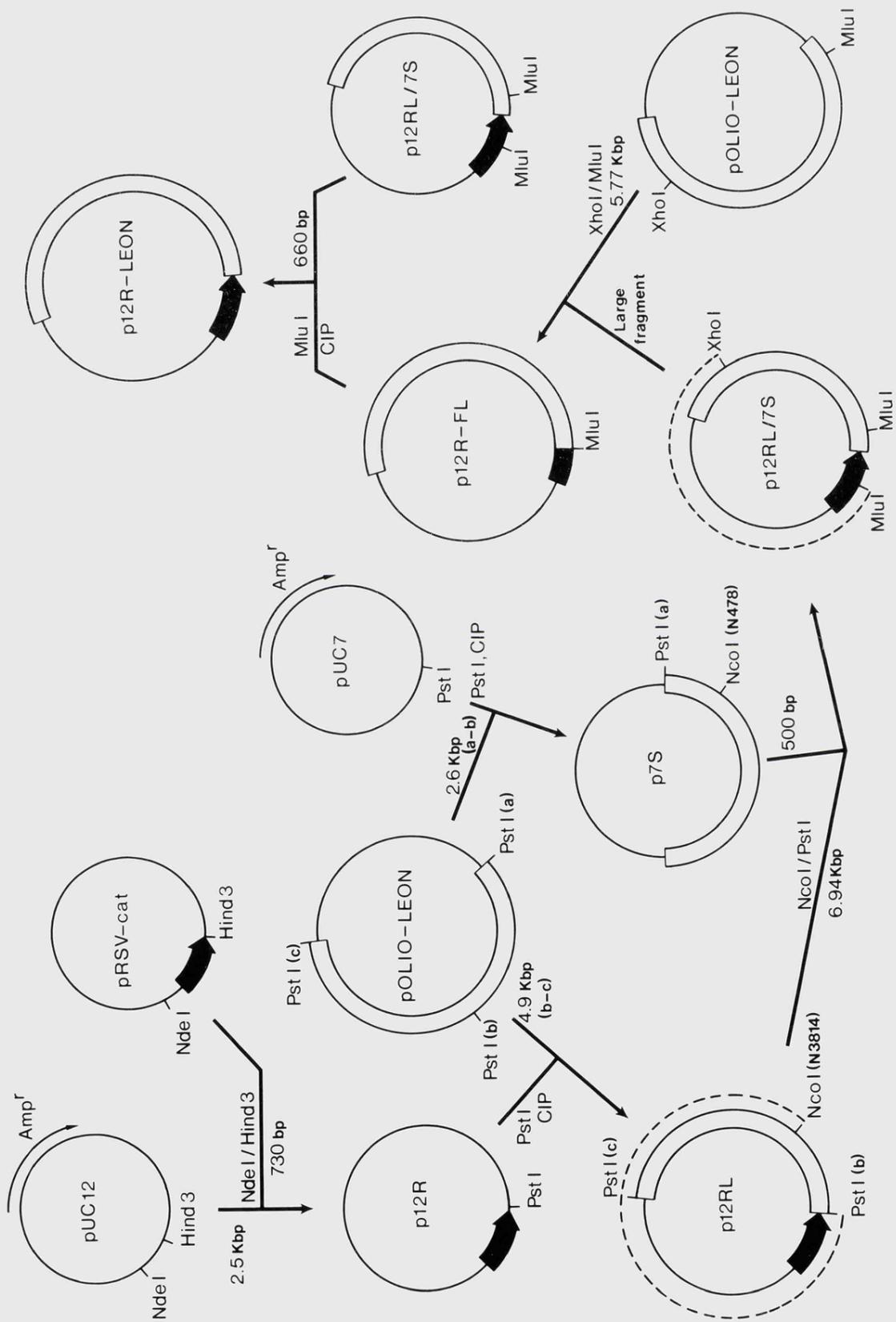
The source of poliovirus cDNA used in this work was the plasmid pOLIO LEON provided by Dr. G. Stanway. This plasmid contains a full-length cDNA copy of the genome of P3/Leon/37 inserted in the PstI site of the pBR322 derived plasmid pAT153 (Stanway et al., 1984a; Twigg and Sherrat, 1980). The complete nucleotide sequence of this poliovirus cDNA has been determined, and the plasmid pOLIO LEON produces live-infectious virus following transfection of HeLa cells in culture (Stanway et al., 1984b; 1986).

Prior to the sub-cloning of the poliovirus cDNA a plasmid designated p12R was generated (See Figure 2.1). The plasmids pUC12 and pRSV-cat were both digested to completion with the restriction enzymes Hind3 and NdeI. The resulting DNA fragments were separated by agarose gel electrophoresis. The 2.5 Kbp fragment from pUC12, and the 730 bp fragment from pRSV-cat,

Figure 2.1 Construction of p12R-LEON

Plasmids were digested with the restriction enzymes indicated. Following purification of the desired fragments, they were ligated and the products used to transform competent E.coli. All plasmids were selected for Amp^r.





containing the LTR, were isolated and purified (See Chapter 8). After ligation to one another the products were used to transform competent E.coli strain JM 83 and plated on agar plates containing ampicillin and the chromogenic indicator BCIG. Plasmid DNA from Amp^r "white" colonies was purified and characterised by restriction mapping. The plasmid designated p12R contains a single copy of the LTR positioned to direct transcription through the polylinker region.

The method used to clone P3/Leon/37 resulted in the creation of PstI sites at both ends of the cDNA (Stanway et al., 1984a). As only one internal PstI site is present in pOLIO LEON, partial digestion with PstI was attempted with the aim of isolating the complete poliovirus cDNA on a single PstI restriction fragment which could be inserted into the plasmid p12R at the unique PstI restriction site. Partial digestion of pOLIO LEON failed to produce sufficient quantities of the desired 7.5 Kbp fragment. The enzyme preferentially digested the plasmid at the internal PstI site. Such preferential cleavage with PstI has been observed by others and is apparently caused by resistance to cleavage at sites lying adjacent to runs of G-C base pairs (Armstrong and Bauer, 1982). As G-C tailing was used in the cloning of this cDNA, this explains the preferential cleavage observed.

To insert the poliovirus cDNA in the PstI site of p12R involved reconstruction of the full-length cDNA in this plasmid and required many more steps than originally anticipated. Initially, the 4.9 Kbp PstI fragment of pOLIO LEON, containing

the 3' end of the cDNA, was inserted in p12R at the unique PstI site. Colonies harbouring plasmids containing the 4.9 Kbp PstI fragment were identified by colony hybridisation using the 4.9 Kbp radio-labelled PstI fragment as probe. A plasmid containing the insert in the desired orientation (See Figure 2.1) was identified by restriction mapping. In this plasmid, p12RL, the internal poliovirus PstI site [labelled PstI (b) in Figure 2.1] lies adjacent to the LTR.

Plasmid p12RL was linearised by digestion with NcoI and then subjected to partial digestion with PstI. Following separation of the DNA fragments by agarose gel electrophoresis, the 6.94 Kbp NcoI/PstI fragment containing the 3' end of the poliovirus cDNA, pUC12 sequences, and the LTR, was isolated and purified. This fragment was ligated with the approximately 500 bp NcoI/PstI fragment produced by double digestion of the plasmid p7A. The plasmid produced by this ligation, p12RL/7S, contained both the extreme 5' and 3' ends of the poliovirus cDNA. The plasmid lacked an NcoI restriction fragment spanning nucleotides 478 to 3814 of the poliovirus cDNA. In an attempt to regenerate the full-length poliovirus cDNA in this plasmid, the missing 3.33 Kbp NcoI fragment from pOLIO LEON was ligated with NcoI digested p12RL/7S. Colonies produced upon transformation of competent E.coli with the products of this ligation reaction contained plasmids with gross rearrangements. The desired plasmid was not found with extensive screening and the reasons for the production of the observed rearrangements are unknown, though similar results have

been obtained by others working with poliovirus cDNA (Semler et al., 1984; G.D. Westrop, personal communication).

The problem encountered above was overcome by using an alternative strategy. The plasmid p12RL/7S was digested to completion with XhoI and MluI, and the large fragment isolated. Following purification, this fragment was ligated with the 5.77 Kbp XhoI/MluI fragment from pOLIO LEON to produce the plasmid designated p12R-FL. It had been anticipated, as its name suggests, that p12R-FL would contain the complete poliovirus cDNA under the transcriptional control of the intact LTR. Characterisation of the plasmid with restriction enzymes revealed that this was not the case. The plasmid was missing the first 270 bp from the poliovirus 5'-terminus and a small region of the LTR. A MluI site hitherto unnoticed and not present in the published sequence of RSV was the reason for the small deletion. Inspection of the published RSV nucleotide sequence revealed that a single point mutation could produce a MluI restriction site at the position observed.

The deletion in p12R-FL was corrected by insertion of the 660 bp MluI fragment from p12RL/7S at the unique MluI site present in p12R-FL. A plasmid with the MluI fragment in the correct orientation was identified by digestion with EcoRI which cuts asymmetrically within the insert. This plasmid, called p12R-LEON contains a full-length cDNA of P3/Leon/37 under the direct transcriptional control of the LTR of RSV.

2.3 Production of Infectious Virus From p12R-LEON

To test whether the reconstructed full-length cDNA could produce infectious virus, p12R-LEON was introduced into HeLa cells in culture using the calcium phosphate co-precipitation technique. Plasmid DNA was first purified by equilibrium centrifugation in cesium chloride-ethidium bromide gradients (See Chapter 8). Transfection of HeLa cells with the plasmid p12R-LEON gave rise to virus production and resulted in cell death in all of the three transfected monolayers. Addition of the media from these monolayers to fresh monolayers of HeLa cells caused cell death of the latter within 24 hours. Treatment of p12R-LEON with DNaseI prior to transfection resulted in no virus production.

Attempts to monitor and compare the specific infectivity of p12R-LEON and the parental plasmid POLIO LEON failed. This requires a direct plaque assay for measurement of the number of plaques produced on a cell monolayer following transfection with a given quantity of plasmid DNA. Although attempts were made to develop a direct plaque assay the transformation procedure damaged the cells to such an extent that monolayers failed to grow to confluence under agar (G.D. Westrop, personal communication). It is worth noting however that the time required for the production of virus did not differ for p12R-LEON and POLIO LEON (5-7 days), suggesting that the presence of the LTR does little to enhance the production of poliovirus.

During the construction of p12R-LEON, it was demonstrated that the production of poliovirus type 1 from cloned cDNA could be

dramatically increased by placing it under the control of SV40 transcription and replication signals and transfecting into COS cells (Semler et al., 1984). The prospect of reconstructing a similar full-length type 3 cDNA under the control of SV40 sequences was considered to be a poor investment of time in the light of difficulties encountered in the construction of p12R-LEON. It is worth noting that recovery of poliovirus from cDNAs under the transcriptional control of eukaryotic promoter and enhancer elements has now been superseded. The method commonly used now is the efficient recovery following transfection of susceptible cells with infectious transcripts. These transcripts are synthesised in-vitro, from full-length cDNAs cloned directly downstream from a phage T7 promoter (van der Werf et al., 1986).

The work described above however was of some benefit as p12R-LEON was to prove a valuable source of DNA for the construction of the vectors and deletions described in subsequent chapters.

CHAPTER 3

Construction of a Polio/CAT Chimaeric Gene

3.1 Introduction

This chapter describes the construction of the recombinant plasmid pRSV-5'polio-CATm2(N⁺). This plasmid contains the bacterial "marker" sequences encoding chloramphenicol acetyl transferase (CAT) fused directly downstream of a cDNA copy of the complete 5' non-coding region of the LEON/37 strain of type 3 poliovirus. This plasmid was designed to allow quantitative analysis of the translational control exerted by the 5' non-coding region of poliovirus. It would be possible to achieve this by measuring transient CAT expression following transfection of eukaryotic cells with pRSV-5'polio-CATm2(N⁺) and derivatives of this plasmid. It was hoped that deletion analysis of the 5' non-coding region in this plasmid would lead to the identification of elements involved in the control of poliovirus translation.

The origin of the CAT DNA used in this study was the plasmid pRSV-cat (Gorman et al., 1982b). In this plasmid, the CAT coding region from the E.coli transposable element Tn 9 is positioned downstream of the long terminal repeat (LTR) of Rous sarcoma virus (RSV), and upstream of both the intron of the small t antigen of SV40 and signals for polyadenylation and the termination of transcription (also from SV40). The LTR has been shown to be an extremely strong eukaryotic promoter and enhancer element (Gorman et al., 1982b), and by placing the CAT encoding

region under transcriptional control of the LTR this chimaeric gene has produced, in eukaryotic cells, levels of CAT which are easily measured. Since endogenous CAT activity is not present in mammalian cells, and rapid and sensitive assays for CAT activity exist, this vector offers a very attractive system for monitoring the expression of foreign DNA in eukaryotic cells (Gorman et al., 1982a). Widespread use of the CAT system, with derivatives of pRSV-cat, has led to the identification and analysis of many controlling elements involved in eukaryotic gene expression.

To investigate translational control with the 5'-end of poliovirus, the CAT 5' non-coding sequences present in pRSV-cat were removed and replaced with the poliovirus noncoding region. Moreover, to give such analysis added biological significance, the CAT AUG initiating codon, plus flanking nucleotides were replaced with the corresponding region from poliovirus (Kozak, 1984b, 1986a).

3.2 Mutagenesis to Create a Common Restriction Site

Comparison of the published nucleotide sequences of CAT (Alton and Vapnek, 1979) and poliovirus type 3 (Stanway et al., 1983) around their initiation codons revealed that no common restriction sites existed which would facilitate the construction of the ideal fusion. However, downstream of the initiation codon of the polyprotein of poliovirus, a recognition site for the restriction enzyme SstI, 5'-GAG CTC-3'(position N752), was identified. The corresponding region of the CAT gene contains

part of this recognition sequence; the second codon represents the first half of the palindromic site (see Figure 3.1). It was therefore decided to mutate the third codon of the CAT gene from 5'-AAA-3' to 5'-CTC-3', thus creating a common SstI site which could be used to fuse the 5' non-coding region of poliovirus directly onto the CAT coding region. It was also necessary, in order to bring the CAT coding region in frame with the poliovirus AUG in the chimaeric gene, to further mutate the CAT DNA by deleting the A residue corresponding to the first base of the fourth codon (See Figure 3.1). These mutations would result in a change of the first four amino acids of CAT from Met-Glu-Lys-Lys to Met-Gly-Ala-Gln (See Figure 3.4B).

These specific alterations were made in the CAT DNA using oligonucleotide-mediated site-directed mutagenesis. Following digestion of pRSV-cat with Hind3 and EcoRI, the 250 bp fragment containing the extreme 5'-end of the CAT encoding region was sub-cloned into M13mp18 and the nucleotide sequence of the target region verified (See Figure 3.2B). This clone, M13mp18-CAT, was used as template for oligonucleotide mutagenesis. The oligonucleotide 5'-AGG AAG CTA AAA TGG AGC TCA AAT CAC TGG-3' (MS-9) was designed to introduce the three changes and the deletion of a residue in the CAT DNA. Computer analysis was carried out on the DNA sequences of the M13 vector and the cloned fragment to ensure that no region, other than the specified target sequence, exhibited extensive complementarity with MS-9. To demonstrate that MS-9 efficiently primed from the target site,

Figure 3.1 Creation of a common restriction site.

The nucleotide sequence proceeding the AUG initiation codons of poliovirus type 3 and the CAT gene is shown. The mutational target site and the desired mutagenesis in the CAT sequence are shown.

polio 5'-untranslated region 

CAT coding region 

CREATION OF A COMMON RESTRICTION SITE

5' [] ATGG ^{Sst I} GAGCTC AA POLIO 3

CAT GENE

ATG GAG AAA AAA 

MUTATED,
TO CREATE
AN Sst I
SITE.

DELETED

CTC

the purified oligonucleotide was used as a primer to sequence M13mp18-CAT template DNA (Sanger et al., 1977) and the product analysed by PAGE and autoradiography.

MS-9 was phosphorylated at its 5'-terminus and used to prime mutagenesis of the CAT DNA using the gapped duplex method (Kramer et al., 1984). Following termination of the reaction, a fraction of the product was used to transform competent E. coli (mismatch repair deficient strain BMH:mutL) and plated in the presence of a lawn of E. coli (BMH strain). Plaques were picked, phage grown, and single-stranded DNA purified.

3.3 Screening for Mutants

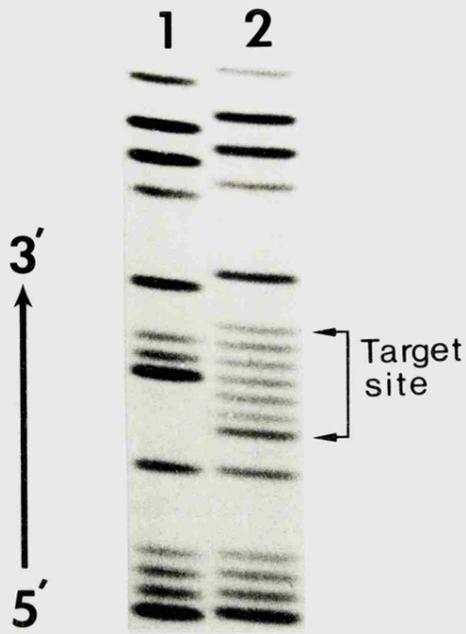
Due to the proximity of the mutations to the binding site of the universal M13 sequencing primer, potential mutants were screened by single-track sequencing. Because of the nature of the changes, mutation of three A residues and deletion of a fourth in a track of seven adjacent A residues, dideoxy-A was used in the sequencing reaction (See Figure 3.2A). Following identification of potential mutants, these were plaque purified prior to complete nucleotide sequence analysis. Plaque purification is necessary as it has been shown that potential mutant clones, generated by the above procedure, can contain up to 50% wild-type molecules (Zoller and Smith, 1983). Such heterogeneity amongst DNA molecules purified from a single plaque results from transformation and plating being carried out without a cycle of replication and reinfection (Kramer et al., 1984). Sequence

Figure 3.2A Identification of mutant CAT gene.

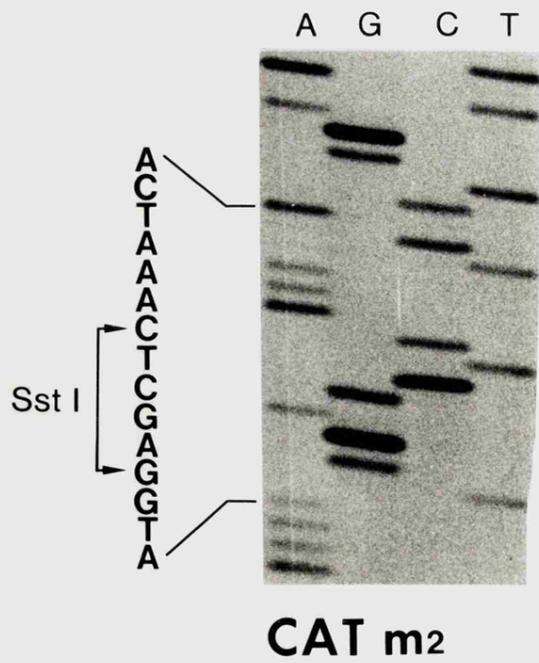
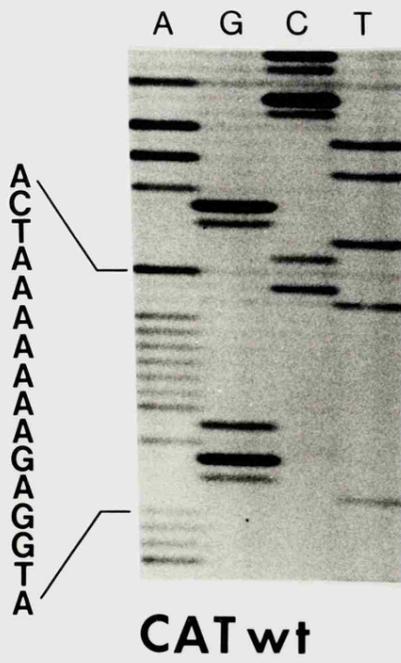
An autoradiograph image of single-track nucleotide sequence analysis of M13mp18-CATm2 (lane 1) and M13mp18-CAT (lane 2) using ddATP.

Figure 3.2B Nucleotide sequence comparison of wild type CAT with mutant CAT (CATm2).

A



B



analysis of plaque purified mutants confirmed that the correct mutations had been introduced into the CAT DNA (See Figure 3.2B). This phage was designated M13mp18-CATm2.

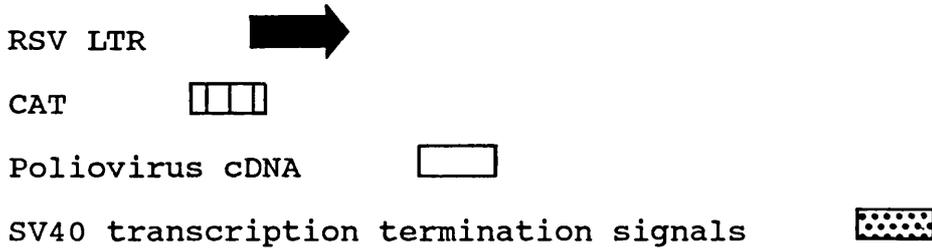
3.4 Construction of pRSV-5'polio-CATm2(N⁺)

With reference to Figure 3.3A, double-stranded replicative form DNA from a plaque purified mutant, M13mp18-CATm2, was prepared and digested with Hind3 and EcoRI. The 250 bp fragment containing the mutated CAT DNA was ligated to the large Hind3/EcoRI fragment of pBR322. Following transformation, plasmid DNA was purified from Amp^r/Tet^S colonies, and screened for the presence of the unique SstI site introduced by the mutagenesis. This plasmid, pBR-CATm2, was digested with EcoRI, treated with calf intestinal phosphatase, and purified by agarose gel electrophoresis. After ligation with the 2.1 Kbp EcoRI fragment of pRSV-cat, the products were used to transform competent E. coli. Colonies harbouring plasmids containing the 2.1 Kbp fragment were identified by the colony hybridisation procedure of Grunstein and Hogness (1975) using the 2.1 Kbp radio-labelled EcoRI fragment as a probe.

A recombinant plasmid containing the insert in the desired orientation was identified by restriction mapping. This plasmid, pBR-CATm2/337, contains the complete CAT coding region and the SV40 transcription signals. The LTR and complete poliovirus 5' non-coding region were introduced on a 1.48 Kbp NdeI/SstI fragment generated by digestion of p12R-LEON. The resulting

Figure 3.3A Construction of pRSV-5'polio-CATm2(N⁺)

Following sub-cloning of the 250bp EcoRI/Hind3 fragment from pRSV-cat into M13mp18 it was subjected to site-directed mutagenesis to create an SstI site (see text). Mutant phage were identified and characterised. mpl8-CATm2 RF DNA was digested with the restriction enzymes indicated. Following purification of the desired fragments they were ligated and the products used to transform competent E.coli. All plasmids were selected for Amp^r.



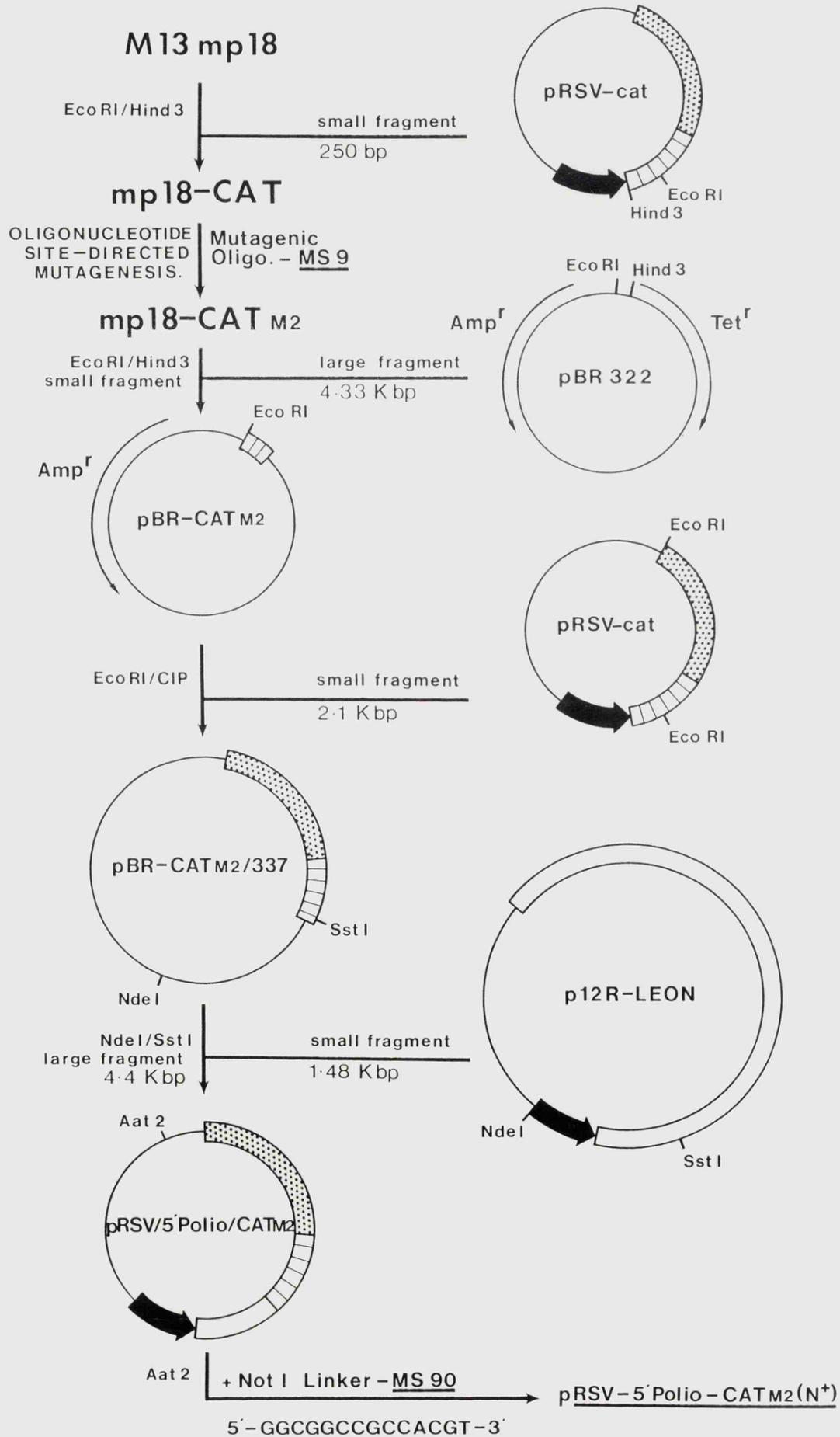
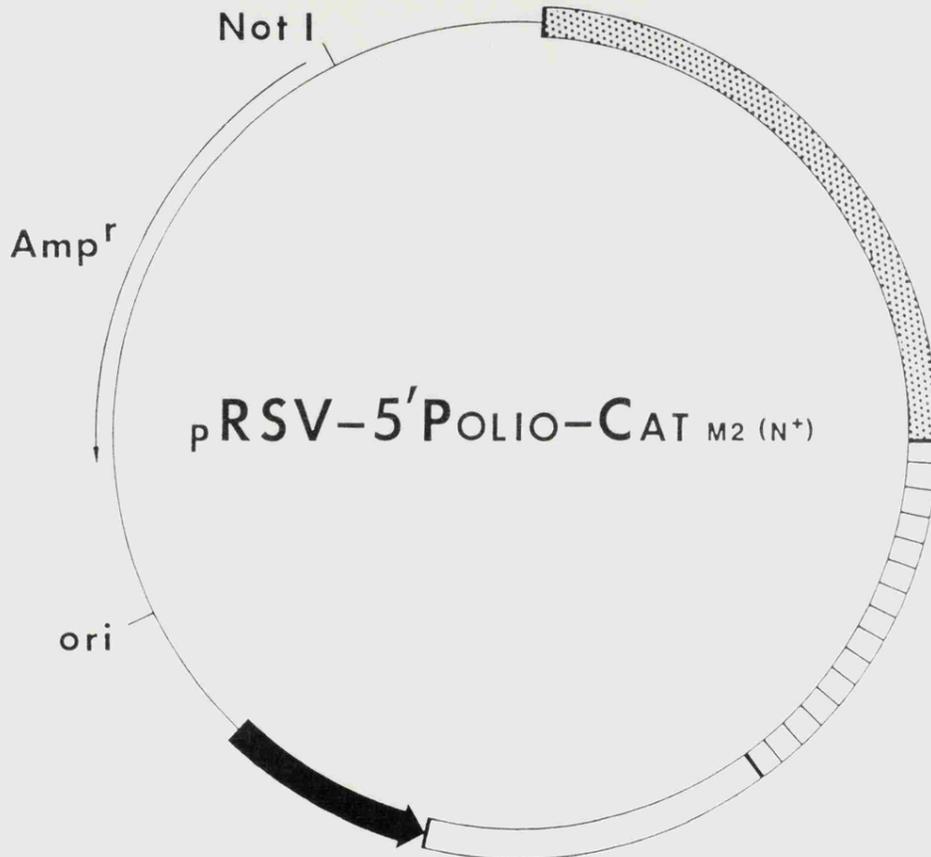
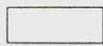


Figure 3.3B Structure of pRSV-5'polio-CATm2(N⁺).

POLIO - CAT FUSION GENE



RSV Long Terminal Repeat.



5' Untranslated Region of Polio 3.



Chloramphenicol Acetyl Transferase GENE.



SV40 Intron, Polyadenylation and Termination of Transcription Signals.

plasmid, pRSV-5'polio-CATm2, was further modified by replacing the unique Aat2 site present in the pBR322 region with a unique NotI site (the reason for this replacement is discussed in Chapter 6). This was accomplished by digesting the plasmid with Aat2 and ligating it with the self-complementary NotI linker 5'-GGC GGC CGC CAC GT-3' (MS-90). MS-90 was designed to anneal with the DNA termini generated by Aat2 digestion without reforming the Aat2 site. Prior to transformation, the ligation products were digested with Aat2 to remove self-ligated vector molecules. Following transformation and plating in the presence of ampicillin, plasmid DNA was purified from individual colonies and screened for the presence of a unique NotI site and absence of the Aat2 site. Finally, this plasmid, pRSV-5'polio-CATm2(N⁺) (See Figure 3.3B), was partially sequenced to ensure that the CAT coding region was in frame with the poliovirus AUG. A 290 bp BamHI/EcoRI fragment was sub-cloned into M13mp18 to generate M13mp18-5'polio/CATm2 which was then sequenced across the polio/CAT junction (See Figure 3.4A). Comparison of the predicted amino acid sequence for wild type CAT with that of the polio/CAT chimaera shows that the second, third, and fourth amino acids at the amino-terminus have been altered from Glu-Lys-Lys to Gly-Ala-Gln.

Figure 3.4A Sequence analysis of the polio/CAT junction.

Verification that the AUG of poliovirus is in-frame with the CAT coding region.

Figure 3.4B N-terminal amino acid sequence comparison.

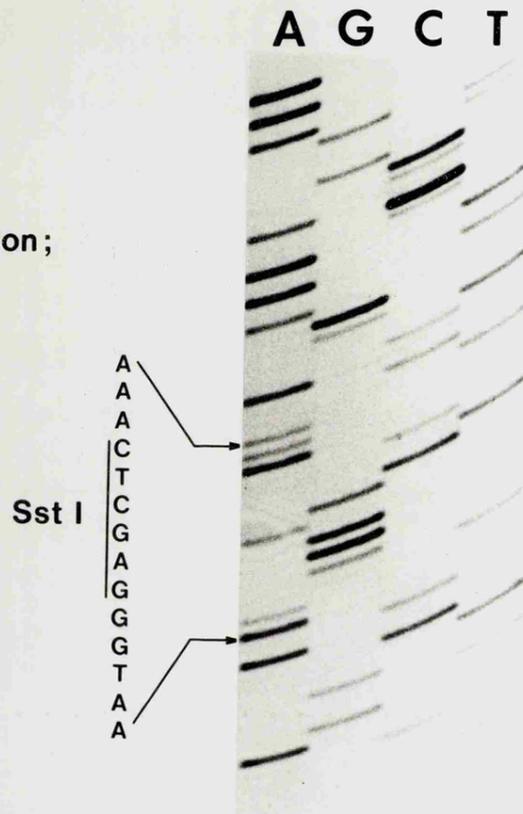
Comparison of the amino acid sequence at the amino termini of wild type CAT the polio/CAT fusion.

A

SEQUENCE ANALYSIS OF M13mp 18-5' POLIO-CAT-M2

Shows sequence at junction of fusion;

ATG GGA GCT CAA
Sst I ↑
5' Polio / CAT gene



B

COMPARISON OF THE N-TERMINI OF POLIO-CAT AND WILD-TYPE CAT

MET	GLY	ALA	GLN	ILE	THR	GLY	TYR	
ATG	GGA	GCT	CAA	ATC	ACT	GGA	TAT	<u>POLIO-CAT</u>
MET	GLU	LYS	LYS	ILE	THR	GLY	TYR	
ATG	GAG	AAA	AAA	ATC	ACT	GGA	TAT	<u>CAT</u>

→

3.5 Activity of the Polio-CAT Fusion Protein in Eukaryotic Cells

CAT inactivates chloramphenicol by formation of mono- and diacetylated derivatives (Shaw, 1967). A number of assays have been developed to measure this activity and the one most widely used is that adopted by Gorman et al., (1982b), in which acetylation of [¹⁴C]-chloramphenicol is measured by autoradiography following silica gel thin-layer chromatography (TLC) (See Figure 3.5). This assay for CAT activity is highly specific, easily separating the parent and acetylated forms of chloramphenicol, and is also very sensitive. Following transfection of eukaryotic cells (48 hr.), cell extracts are prepared and assayed for CAT activity (See Chapter 8).

Figure 3.6A shows an autoradiograph measuring CAT activity produced by HeLa cells transfected with pRSV-5'polio-CATm2(N⁺) and the parental plasmid, pRSV-cat. It is obvious that very little CAT activity is produced by cells transfected with the polio-CAT recombinant plasmid (over-exposure of the TLC plate did show that monoacetylated derivatives of chloramphenicol were produced). It was concluded that such low activity might be the result of one of two possibilities, or a combination of both; i.e. 1) loss of CAT function due to the changes which were introduced into the amino-terminus of the chimaeric protein (See Figure 3.4B), or 2) inefficient translation of the fusion mRNA due to the presence of the poliovirus 5' non-coding region.

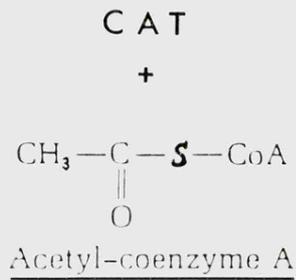
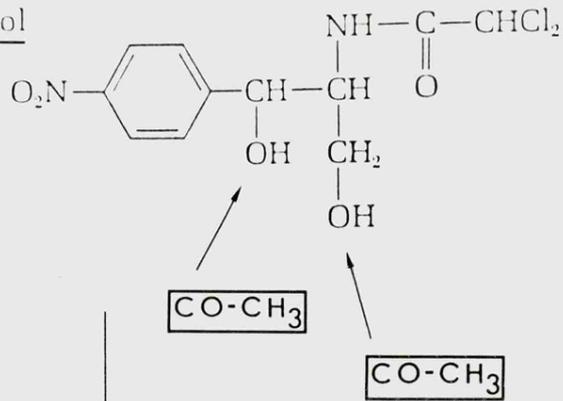
To investigate this further, most of the poliovirus 5' non-

Figure 3.5A Schematic representation of the acetylation of chloramphenicol by CAT.

Figure 3.5B Autoradiograph of a TLC plate following separation, using ascending chromatography, of the [^{14}C]-labelled products and starting material, following termination, of the reaction depicted in Figure 3.5A. The reaction was carried out as described in Chapter 8.

Lanes: 1) 1 unit of CAT.
2) 0.1 unit of CAT.
3) 0.01 unit of CAT.
4) No CAT added.

Chloramphenicol



- 1-ACETATE CHLORAMPHENICOL
- 3-ACETATE CHLORAMPHENICOL
- 1, 3-DIACETATE CHLORAMPHENICOL.

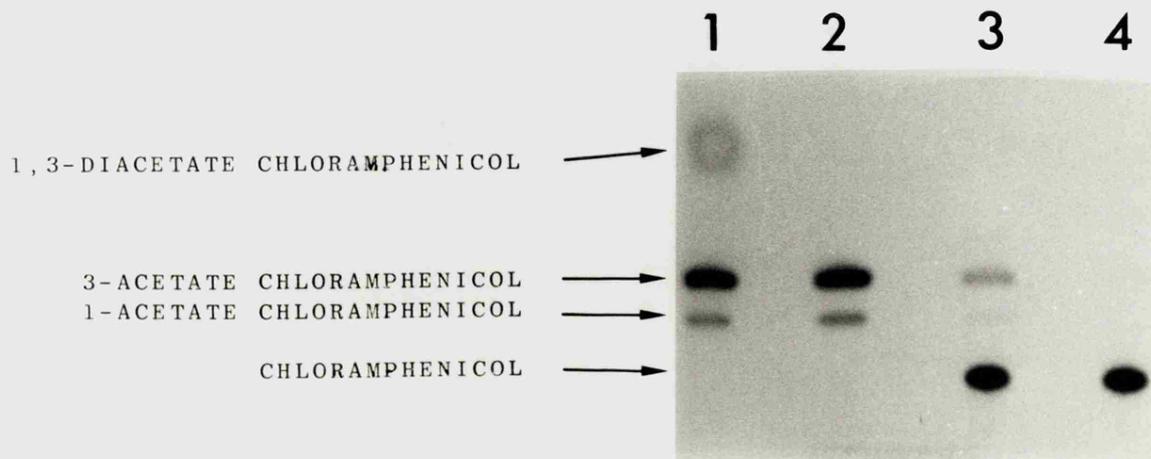


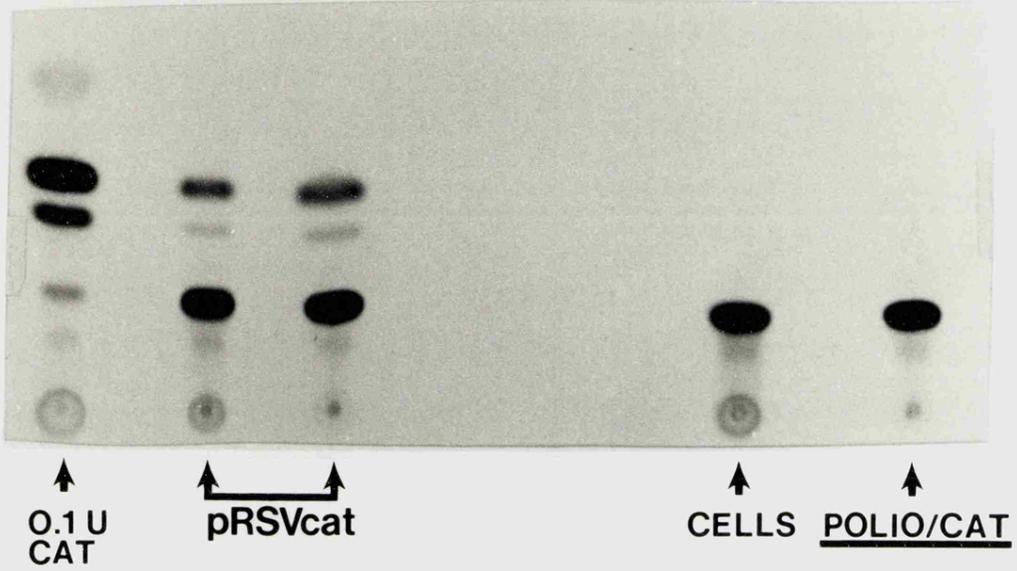
Figure 3.6A Measurement of CAT activity expressed by pRSV-5'polio-CATm2(N⁺).

Following transfection of HeLa cells with the plasmids pRSVcat and pRSV-5'polio-CATm2(N⁺), cell monolayers were harvested and cell extracts prepared. Equivalent amounts of protein were assayed for CAT activity (See Chapter 8). Pure CAT enzyme (1 unit) and extract from untransfected cells (cells) were included as positive and negative controls. An autoradiograph of the TLC plate is shown.

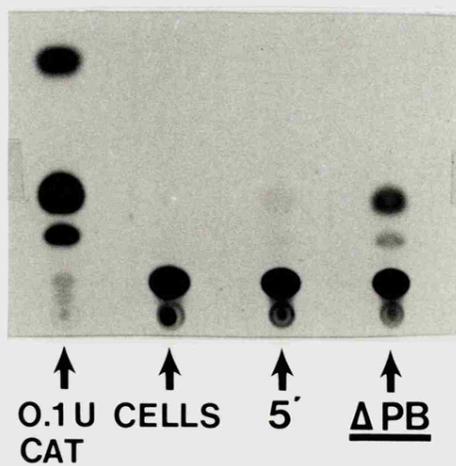
Figure 3.6B Measurement of CAT activity expressed by pRSV-CATm2(N⁺) ΔPB.

- 1 unit of CAT - positive control.
- Cells - extract from untransfected cells.
- 5' - extract from HeLa cells transfected with pRSV-5'polio-CATm2(N⁺).
- ΔPB - extract from HeLa cells transfected with pRSV-CATm2(N⁺) ΔPB.

A CAT ACTIVITY FROM pRSV-5'POLIO-CAT_{M2}(N⁺)



B CAT ACTIVITY FROM pRSV-CAT_{M2}(N⁺) ΔPB



coding region was deleted. The plasmid pRSV-CATm2 Δ PB is a derivative of pRSV-5'polio-CATm2(N⁺) which has had the first 673 nucleotides of the poliovirus 5'-end removed, leaving approximately 70 nucleotides before the AUG of the fusion protein (details of the construction of this plasmid are discussed in Chapter 4). Following transfection of HeLa cells with this plasmid and pRSV-5'polio-CATm2(N⁺), CAT assays were carried out on cell extracts (See Figure 3.6B). Deletion of the first 673 nucleotides of the 5' non-coding of poliovirus results in high levels of CAT activity. The low level of CAT activity produced by pRSV-5'polio-CATm2(N⁺) must therefore be due to the presence of the 5' non-coding region of poliovirus and not the changes introduced at the amino-terminus of the fusion protein.

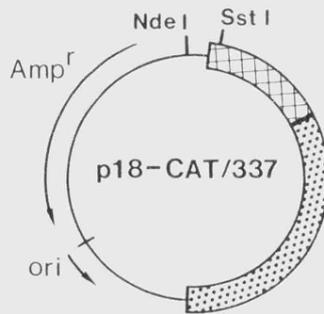
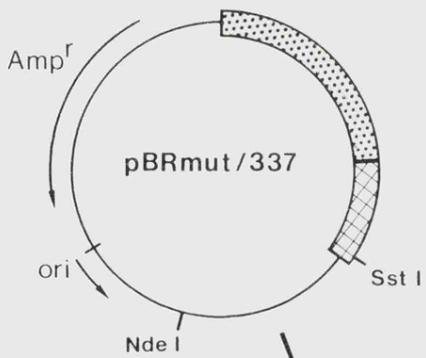
3.6 Orientation of Insert DNA

The experiments described in this chapter led to the successful construction of a CAT/polio recombinant vector. Initial attempts to construct a similar vector in the plasmid pUC18 failed repeatedly. Double stranded replicative form DNA of the CAT mutant M13mp18-CATm1 (one base change from CATm2) was prepared and digested with Hind3 and EcoRI. The 250 bp Hind3/EcoRI fragment was sub-cloned into Hind3/EcoRI digested pUC18 to generate p18-CATm1. This plasmid was linearised with EcoRI, treated with CIP and ligated with the 2.1 Kbp EcoRI fragment from pRSV-cat (As in Figure 3.3A for CATm2). Colonies harbouring plasmids with the 2.1 Kbp insert in the correct

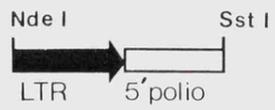
orientation were identified by restriction mapping following colony hybridisation. The resulting plasmid, p18-CAT/337, contains the entire CAT coding region, the intron of the small t antigen of SV40, plus signals for the termination of transcription and polyadenylation. The final step involved the introduction of the LTR and the 5' non-coding region of poliovirus on a SstI /NdeI fragment (1.48 Kbp). This ligation was attempted several times and failed to produce colonies upon transformation. Separation of the DNA in the ligation reaction by agarose gel electrophoresis showed that DNA of high molecular weight had been generated during the reaction. Separate digestion of p18-CAT/337 with either NdeI or SstI, followed by self-ligation and re-digestion showed that neither enzyme was contaminated with exonuclease activity. It was clear that the failure to obtain transformants was not due to a block in the ligation reaction, but must result from the formation of either a non-viable plasmid or the introduction of a "poison" sequence lethal to the E.coli.

To investigate this phenomenon further and to generate a viable polio/CAT recombinant vector, the orientation of the insert DNA was reversed with respect to the Amp^r gene and origin of replication (See Figure 3.7). This reversal was achieved by carrying out a complete Hind3, partial EcoRI digest of p18-CAT/337 and ligating the resulting 2.3 Kbp fragment to the 4.33 Kbp Hind3/EcoRI fragment of pBR322. The plasmid generated by this ligation, pBRmut/337, was digested with NdeI and SstI, and the

Figure 3.7 Plasmids pBRmut/337 and p18-CAT/337 were both digested with Nde I and Sst I. The large fragments were purified and ligated with the 1.48 Kbp Nde I/Sst I fragment containing the LTR from RSV and the cDNA of the 5'-untranslated region of poliovirus type 3. Following transformation of E. coli with the ligation products, colonies were produced by the ligation mix containing fragment from pBRmut/337 while no colonies were produced by the ligation mix containing fragment from p18-CAT/337. These results determined the strategy used in the construction of pRSV-5'polio-CATm2(N⁺).



Nde I / Sst I
Large fragment
Ligated to;



TRANSFORMANTS

NO
TRANSFORMANTS

4.4 Kbp fragment was ligated to the 1.48 Kbp NdeI/SstI fragment, which contained both the LTR and the 5' non-coding region of poliovirus. Transformation of competent E.coli with the products of this ligation reaction did result in the recovery of transformants containing the plasmid designated pRSV-5'polio-CATm1 (See Figure 3.7). Due to this apparent orientation problem encountered with CATm1, when CATm2 was built back into plasmid DNA, it was sub-cloned into pBR322 and no problems arose in the subsequent construction of pRSV-5'polio-CATm2.

3.7 Summary

A poliovirus cDNA/CAT recombinant vector has been successfully constructed. The recognition site for the restriction enzyme SstI was engineered into the DNA that codes for CAT and was used to fuse this region directly downstream of the poliovirus 5' non-coding region. The novel protein expressed by this chimaeric plasmid in eukaryotic cells exhibits detectable levels of CAT activity. It is interesting to note that deletion of most of the 5' non-coding region of poliovirus results in a dramatic increase in CAT expression (this will be discussed in detail in Chapter 5).

CHAPTER 4

Synthesis of Deletions Spanning the 5' Non-Coding Region

4.1 Introduction

This chapter describes the construction and sequence analysis of several deletions and an inversion made in a cDNA copy of the 5' non-coding region of the Leon/37 strain of type 3 poliovirus and their incorporation into the plasmid pRSV-5'polio-CATm2(N⁺).

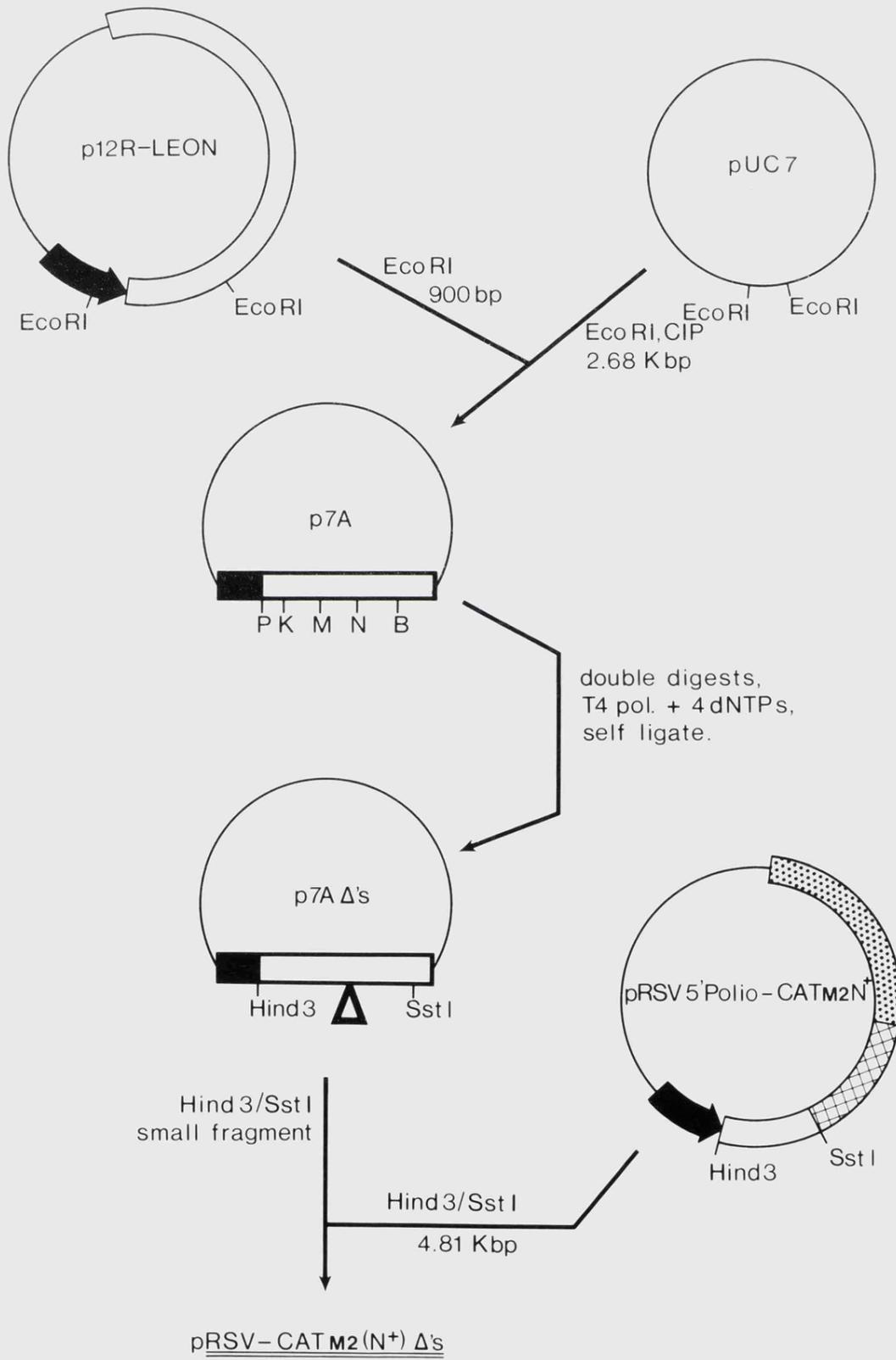
Firstly, the 5' non-coding region was sub-cloned from p12R-LEON, on a 900 bp EcoRI fragment, into pUC7 to generate the plasmid p7A (See Figure 4.1). Sub-cloning this fragment into pUC7 rendered the following restriction sites, in p7A, unique; Hind3 and PstI (both flank the extreme 5'-end of poliovirus), KpnI (N71), MluI (N279), NcoI (N478), BamHI (N674) and SstI (N752). Using these unique restriction sites, defined deletions in the poliovirus cDNA were created and substituted for the cDNA copy of the complete 5' non-coding region of poliovirus in pRSV-5'polio-CATm2(N⁺). These plasmids were then used to determine the effect of the deletions on the expression of CAT.

4.2 Synthesis of Single Deletions

Following complete digestion of p7A with KpnI and PstI, the resulting termini were rendered flush by treatment with T4 polymerase in the presence of all four dNTPs. After purification, the DNA was self-ligated, digested with KpnI and used to transform competent E.coli. Plasmid DNA was purified from

Figure 4.1 Creation of Deletions in the 5' Non-Coding Region

The poliovirus 5' non-coding region was sub-cloned on a 900bp EcoRI fragment into pUC7. The resulting plasmid, p7A, was then subjected to double digestion with pairs of the enzymes; PstI (P), KpnI (K), MluI (M), NcoI (N), and BamHI (B). Following digestion the DNA was treated with T4 polymerase in the presence of all four dNTPs to render the ends flush. Treated DNA was then self-ligated and used to transform competent E.coli. Following characterisation of the plasmids to identify those carrying the desired deletions, these were digested with Hind3 and SstI, and the small fragment purified. This was then ligated with the 4.1 Kbp Hind3/SstI fragment of pRSV-5'polio-CATm2(N⁺). Following transformation, plasmids carrying the desired deletions were identified and characterised. To map the precise deletion point, the small Hind3/SstI fragments from the plasmids pRSV-CATm2(N⁺)Δ's were subcloned into M13mp18 or mp19 and subjected to sequence analysis (see Figure 4.2).



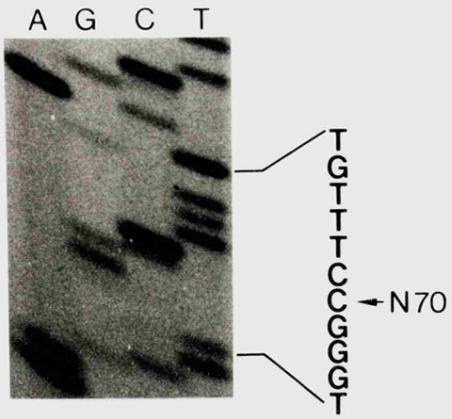
individual colonies and shown to have lost both the PstI and KpnI sites. This plasmid, p7A Δ PK, was then digested with Hind3 and SstI, and the small fragment was purified from agarose and ligated to the large fragment generated by Hind3/SstI digestion of pRSV-5'polio-CATm2(N⁺). The products of this ligation reaction were used to transform E.coli. Plasmid DNA was purified from transformants and characterised. The resulting plasmid, designated pRSV-CATm2(N⁺) Δ PK was partially sequenced to map the termini of the deletion. The junction of the deletion was sub-cloned, on the small Hind3/SstI fragment, into M13mp18 and sequenced. This showed that the first 69 bp of poliovirus cDNA had been deleted (See Figure 4.2).

The general procedure used to create Δ PK was repeated using the corresponding restriction enzyme sites to create the following deletions; Δ PM (PstI, MluI), Δ PN (PstI, NcoI), Δ PB (PstI, BamHI), Δ KM (KpnI, MluI), Δ KN (KpnI, NcoI), Δ KB (KpnI, BamHI), Δ MN (MluI, NcoI), Δ MB (MluI, BamHI), Δ NB (NcoI, BamHI). The junction of the deletions were all sub-cloned into either Hind3/SstI digested M13mp18 or mp19 and sequenced (See Figure 4.2.). The nucleotide position of the junction of each of the deletions is shown in Table 1.

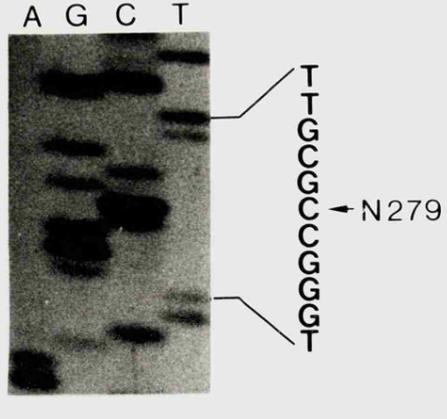
4.3 Deletion and Inversion of the "Larsen" Loop

To investigate the role of the stable hairpin loop located at the extreme 5'-end of the poliovirus genome (Larsen et al., 1981,

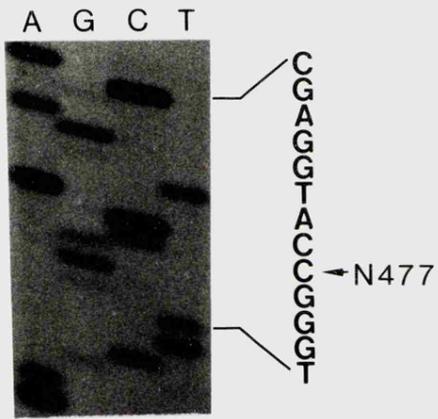
Figure 4.2 Sequence analysis of the deletions made in the poliovirus 5' non-coding region. Restriction fragments (EcoRI/SstI) spanning the deletion junctions were subcloned into M13mp18 or mp19 and subjected to sequence analysis using the universal primer. The precise nucleotide position of the deletion is signified by arrows.



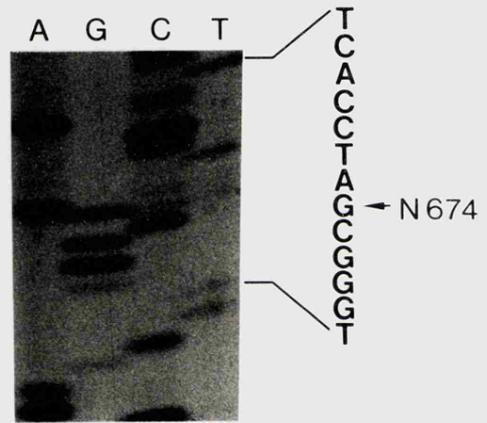
Δ PK



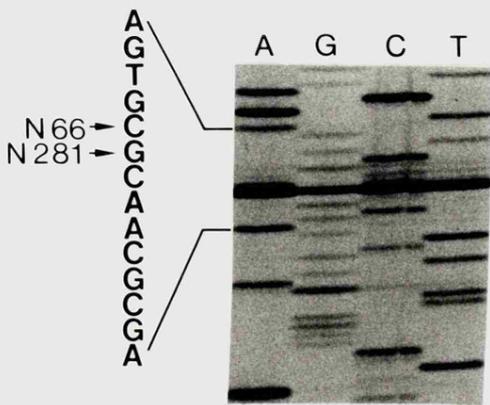
Δ PM



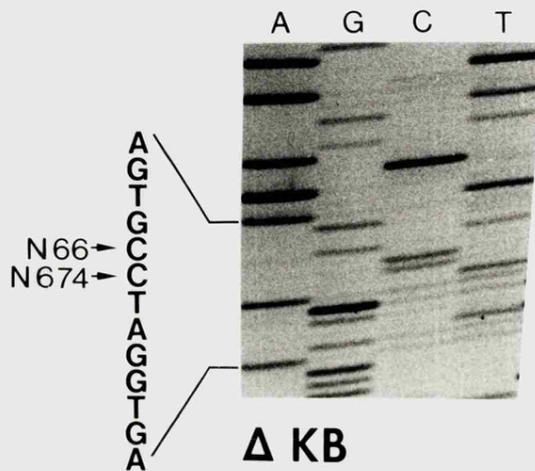
Δ PN



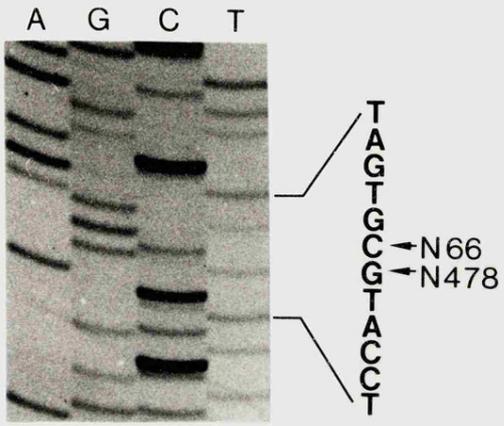
Δ PB



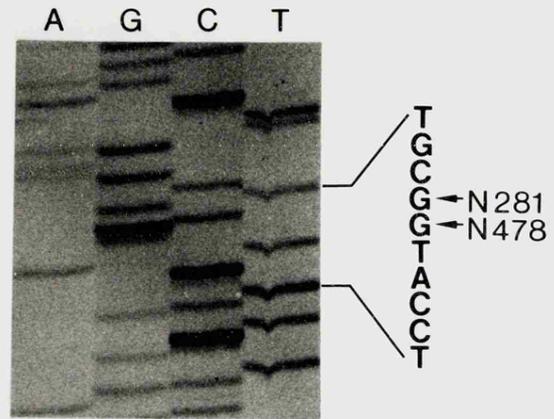
Δ KM



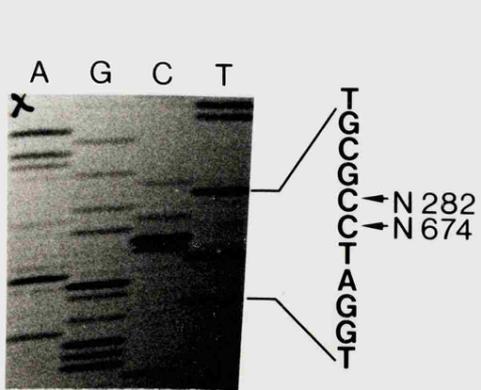
Δ KB



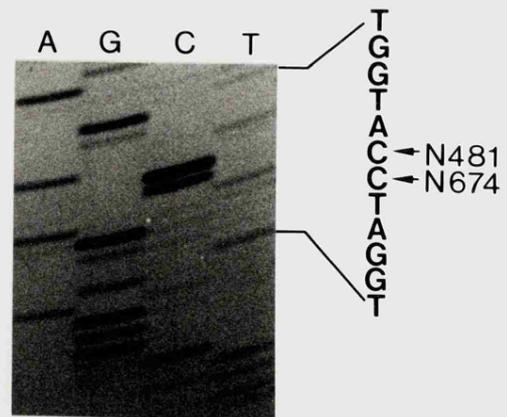
ΔKN



ΔMN



ΔMB



ΔNB

TABLE 4.1

<u>CLONE</u>	<u>POLIOVIRUS NUCLEOTIDES DELETED</u>
Δ PK	5'---N69
Δ PM	5'---N278
Δ PN	5'---N476
Δ PB	5'---N673
Δ KM	N67---N280
Δ KN	N67---N477
Δ KB	N67---N673
Δ MN	N282---N477
Δ MB	N283---N673
Δ NB	N482---N673
Lar ⁻	N11---N33
Lar ⁻ Δ KB	N11---N33 and N67---N673
Lar ^{inv} Δ KB	N67---N673

Racaniello and Meriam, 1986), this region was specifically altered. In one construct the hairpin was deleted, while in a second, the nucleotides which make up the hairpin structure were inverted (See Figure 4.3).

It was hoped that these mutations would answer the following questions; 1) does the highly conserved stem-loop structure play an essential role in the viral life-cycle, and if so, could its role be identified ?, 2) does it function via secondary structure and independently of specific primary sequences ?

4.3.1 Deletion of the "Larsen" Loop

The stable hairpin loop was removed by deleting nucleotides N11 to N33 (See Figure 4.3). This was achieved by digesting pRSV-5'polio-CATm2(N⁺) with Hind3 and KpnI and ligating the large fragment to the oligonucleotide linkers MS 60 and MS 61. These two oligonucleotides were annealed to form a Hind3/KpnI fragment containing poliovirus nucleotides N1 to N10 then N34 to N71. Following transformation of E.coli, plasmid DNA was purified from transformants and characterised by digestion with PstI. Insertion of MS 60/61 resulted in the loss of the PstI site located between the Hind3 site and the extreme 5'-terminus of the poliovirus cDNA. This plasmid, designated pRSV-CATm2(N⁺)Lar⁻, was digested with Hind3 and KpnI and the resulting small fragment sub-cloned into Hind3/KpnI digested M13mp19 and sequenced (See Figure 4.4). To monitor the effect that this deletion had on translation it was necessary to delete most of the 5' non-coding region. Two

Figure 4.3 Sequence at the extreme 5' terminus of poliovirus is folded to show the secondary structure known as the "Larsen" loop. Below this, the sequences of the two constructs made to investigate the function of the "Larsen" loop are shown. In the first of these, the nucleotides which form the stable stem-loop are removed. In the second the nucleotides which form the stem-loop are inverted.

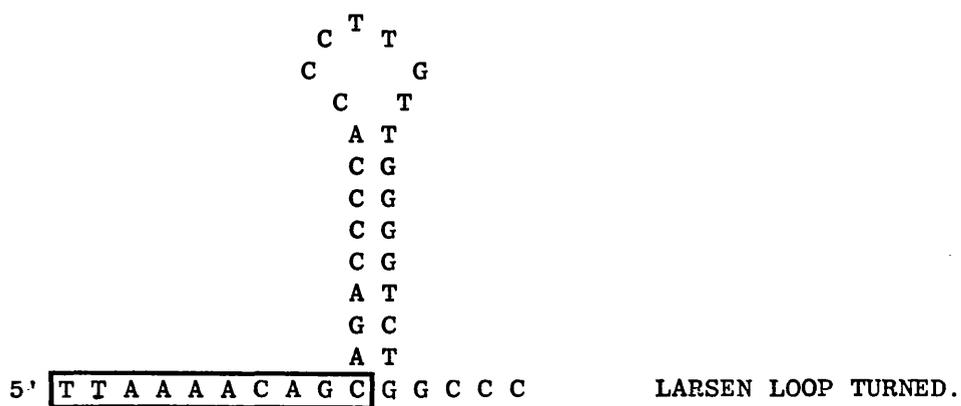
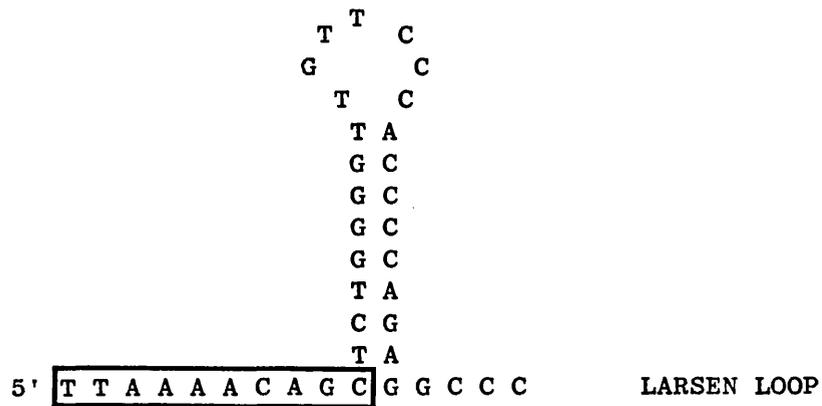
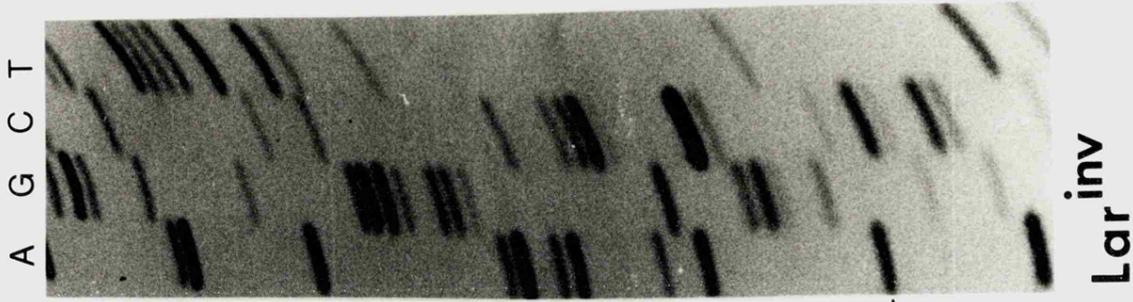
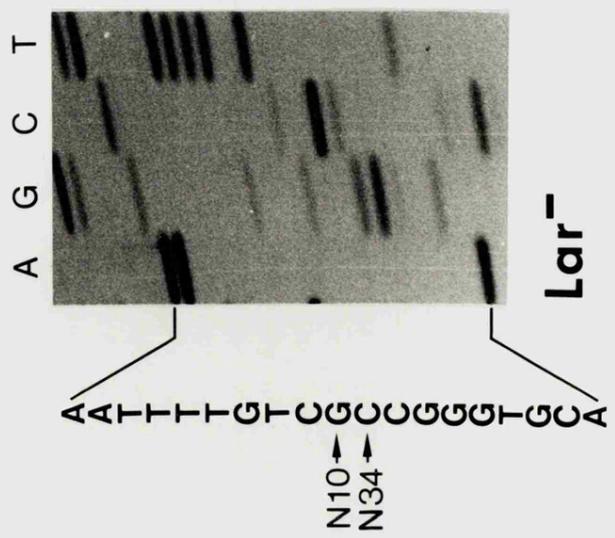


Figure 4.4 Sequence analysis of Lar⁻ and Lar^{inv}. The plasmid, pRSV-CATm2(N⁺)Lar⁻, was digested with Hind3 and KpnI. The small fragment released by this digestion was sub-cloned into Hind3/KpnI digested M13mp19 and subjected to sequence analysis using the universal primer. The precise junction point is signified by arrows. This template DNA carrying Lar⁻ was used for site-directed mutagenesis to create M13mp19Lar^{inv}. This mutant was plaque purified and sequenced using the universal primer. This shows that nucleotides 10 to 34 have been inverted.



AATTTTGTCTCTGGGGTGGGAAACAACUUUAGACUUGGGT-GCA
 inverted



AATTTTGTCTGGGGTGGGAAACAACUUUAGACUUGGGT-GCA
 N10
 N34

double deletions were made; pRSV-CATm2(N⁺)Lar⁻/ΔKB and pRSV-CATm2(N⁺)Lar⁻/ΔMB. The first, Lar⁻ΔKB was made by linearising pRSV-CATm2(N⁺)Lar⁻ with KpnI and subsequently digesting partially with BamHI. The termini of the DNA were rendered flush by treatment with T4 polymerase in the presence of all four dNTPs. The DNA fragments generated by these reactions were separated by agarose gel electrophoresis and a 5.2 Kbp fragment was purified and self-ligated. Following ligation and transformation of *E.coli*, individual colonies were picked, grown and plasmid DNA characterised. The junction of the deletion in the resulting plasmid, pRSV-CATm2(N⁺)Lar⁻ΔKB, was sub-cloned into M13mp19 and sequenced (See Table 4.1).

pRSV-CATm2(N⁺)Lar⁻/ΔMB was generated by digesting both pRSV-CATm2(N⁺)Lar⁻ and pRSV-CATm2(N⁺)ΔMB with NotI and KpnI. The small fragment from Lar⁻ was ligated with the large fragment from ΔMB to produce the plasmid pRSV-CATm2(N⁺)Lar⁻/ΔMB.

4.3.2 Inversion of the "Larsen" loop

Inversion of the hairpin was achieved by oligonucleotide site-directed mutagenesis. Firstly, the small Hind3/KpnI fragment from pRSV-CATm2(N⁺)Lar⁻, representing the extreme 5'-end of the poliovirus cDNA was sub-cloned into Hind3/KpnI digested M13mp19. Single-stranded template was prepared from "white" plaques and subjected to site directed mutagenesis using the mutagenic oligonucleotide MS 21 (See Figure 4.5). MS 21 contains the first 48 bases of poliovirus with the "Larsen" loop inverted. One

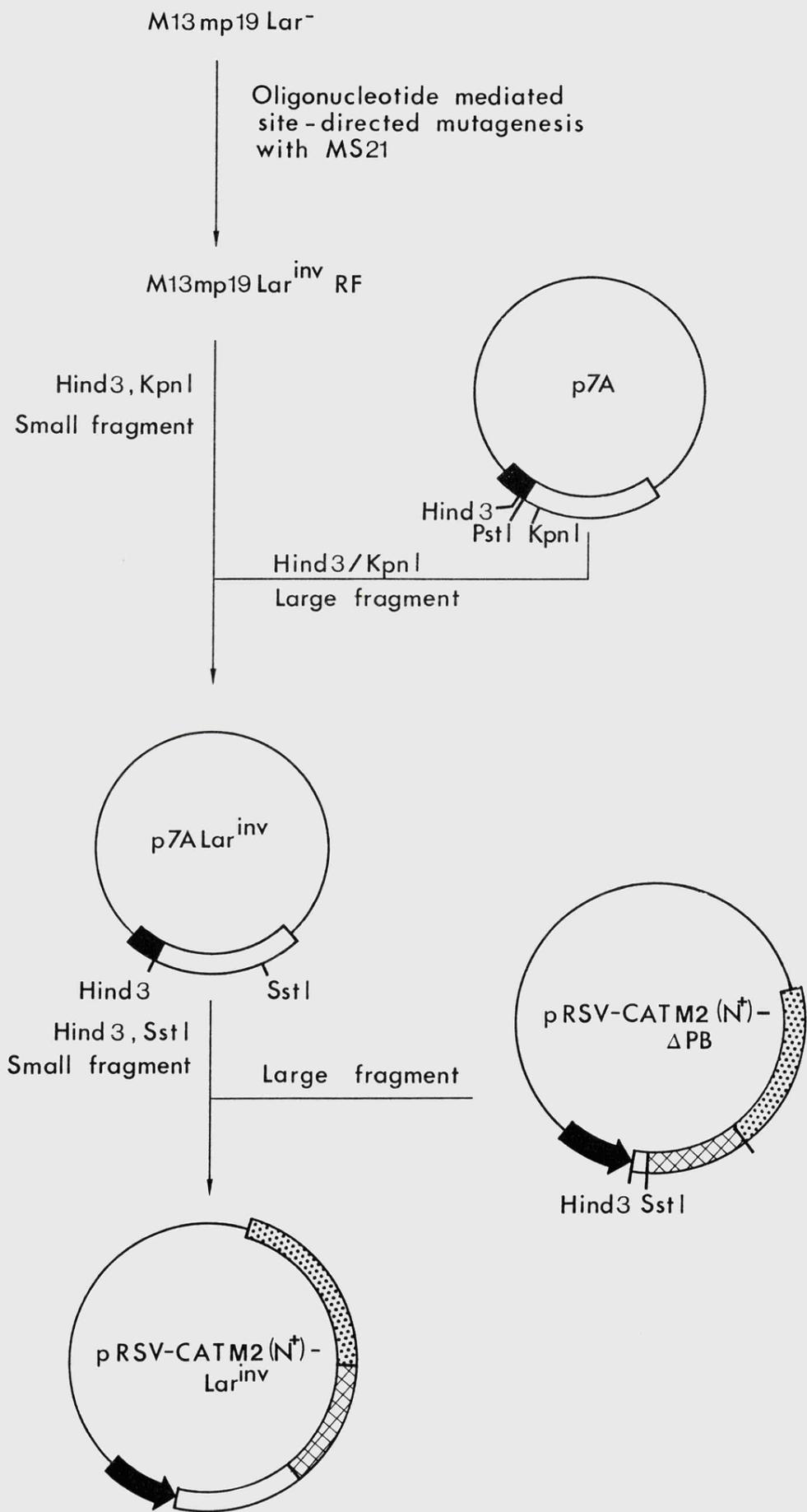
Figure 4.5 Construction of pRSV-CATm2(N⁺)Lar^{inv}

The small Hind3/KpnI fragment from pRSV-CATm2(N⁺)Lar⁻ representing the extreme 5' terminus of poliovirus cDNA was sub-cloned into Hind3/KpnI digested M13mp19. Single-stranded template was prepared and subjected to site-directed mutagenesis primed with the mutagenic oligonucleotide MS21. MS21 contains the first 48 nucleotides of poliovirus with the nucleotides that form the "Larsen" loop inverted. One mutant was identified and characterised (see Figure 4.4). RF DNA of this mutant, mp19Lar^{inv}, was prepared and digested with Hind3 and KpnI. This was mixed and ligated with Hind3/KpnI digested p7A. The products of this ligation reaction were digested with PstI, to remove any background of p7A, prior to transformation. The small Hind3/SstI fragment of p7ALar^{inv} was purified and ligated with the large Hind3/SstI fragment of pRSV-CATm2(N⁺)ΔPB to generate pRSV-CATm2(N⁺)Lar^{inv}.

CAT 

5' non-coding region 

SV40 polyadenylation signals 



mutant was identified, plaque purified and sequenced (See Figure 4.4). This mutant was found to contain one extra base change; N10 had mutated from a C to an A residue. This mutation though, does not disrupt the potential for base-pairing within the "Larsen" hairpin in the positive sense RNA. The U at position N10 can base-pair with the G residue at position N34. Recent evidence indicates that the secondary structure adopted by this region is functional in the positive sense RNA and not the negative strand (Racaniello and Meriam, 1986) and so it was decided to use this mutant in this study.

Double-stranded replicative form DNA of the mutated M13 clone, mp19 Lar^{inv}, was prepared and digested with Hind3 and KpnI. This was mixed with Hind3/KpnI digested p7A and ligated. The ligation reaction was digested with PstI prior to transformation to remove any background of p7A (as the Hind3 and KpnI sites are approximately 80 bp apart it is not possible to separate the DNA fragments resulting from single and double digestion). The resulting plasmid, designated p7ALar^{inv}, was digested with Hind3 and SstI and the small fragment was ligated with the large Hind3/SstI fragment of pRSV-CATm2(N⁺)ΔPB to generate pRSV-CATm2(N⁺)Lar^{inv}.

To monitor the effect of inverting the "Larsen" loop on translation most of the 5'-end of the poliovirus cDNA was removed. Using p7ALar^{inv} instead of p7A, the procedure used to construct p7AΔKB was repeated to generate p7ALar^{inv}ΔKB. The small Hind3/SstI fragment of this plasmid was ligated with the large

Hind3/SstI fragment of pRSV-5'polio-CATm2(N⁺). The junction of the deletion in the resulting plasmid, pRSV-CATm2(N⁺)Lar^{inv}/ΔKB, was sub-cloned on the small Hind3/SstI fragment into M13mp19 and sequenced to determine the exact junction point (See Table 4.1).

4.4 Summary

Deletions spanning most of the 5' non-coding region of the poliovirus genome were created via cDNA and substituted for the complete 5' non-coding region present in the plasmid pRSV-5'polio-CATm2(N⁺). Following transfection of eukaryotic cells, the level of CAT activity produced by this series of plasmids was used to measure the influence of the 5' non-coding region on translation. These results are discussed in Chapter 5.

CHAPTER 5

Translational Control Exerted by the 5' Non-Coding Region of Poliovirus

5.1 Introduction

The plasmid pRSV-5'polio-CATm2(N⁺) and its derivatives, described in Chapter 4, were introduced into HeLa cells in culture, using the technique of calcium phosphate co-precipitation. The results of the CAT assays carried out on extracts of these transfected cells are presented and discussed in detail in this chapter.

For the sake of clarity in this chapter, pRSV-5'polio-CATm2(N⁺) and its derivatives will not be referred to by their full name. Instead, a shortened title corresponding to the poliovirus sequence present in a given plasmid will be employed. For example, the plasmid pRSV-CATm2 Δ PB will simply be referred to as Δ PB.

As described in Chapter 3, the plasmid containing the entire 5' non-coding region of poliovirus, pRSV-5'polio-CATm2(N⁺), produced low levels of CAT while the plasmid with the first 673bp of poliovirus sequence removed, Δ PB, produced significantly higher levels of CAT activity. To study this inhibition further, levels of CAT activity expressed by plasmids with smaller deletions spanning the poliovirus 5' non-coding region were measured.

5.2 Deletions Extending From The Extreme 5' Terminus

Figure 5.1 compares the levels of CAT expressed by the plasmids Δ PK, Δ PM, Δ PN, Δ PB, and 5'polio. There is little if any difference in the levels of CAT activity expressed by the plasmids 5'polio and Δ PK, while Δ PM and Δ PN produce higher levels of CAT activity. The plasmid producing the highest level of CAT activity is Δ PB, which has had the first 673bp of the poliovirus 5' non-coding region deleted.

It would appear from Figure 5.1 that progressive deletion of poliovirus sequences from the extreme 5' terminus results in the expression of increasing levels of CAT activity. The plasmids Δ PM, Δ PN, and Δ PB contain deletions of 278, 476, and 673bp respectively. This suggests that the corresponding increase in expression of CAT activity is not determined solely by the size of the deletion but that specific sequences play a role in the inhibition of CAT expression.

5.3 Deletions Extending From N674 Toward The Extreme

5' Terminus

The plasmids discussed in the section above contain deletions which extend from the extreme 5' terminus, while the plasmids discussed in this section; Δ NB, Δ MB, Δ KB, and Δ PB, contain deletions in the poliovirus 5' non-coding region extending from N674 towards the extreme 5' terminus.

As shown in Figure 5.2, Δ NB produces slightly higher levels of CAT activity than the undeleted 5'polio. Subsequent deletion to

Figure 5.1 Expression of CAT from a series carrying deletions in the 5' non-coding region of poliovirus in the vector pRSV-5'polio-CATm2(N⁺). Human HeLa cells in culture were transfected with plasmid DNA using the calcium phosphate co-precipitation technique. At 48h after transfection, extracts from transfected cells were prepared and analysed for CAT activity. Chloramphenicol and its acetylated products were separated by thin layer chromatography and visualised by autoradiography.

CM - chloramphenicol

CM-AC₁ - 1-acetate chloramphenicol

CM-AC₃ - 3-acetate chloramphenicol

Dotted line corresponds to deleted DNA

Arrows represent the upstream initiation codons

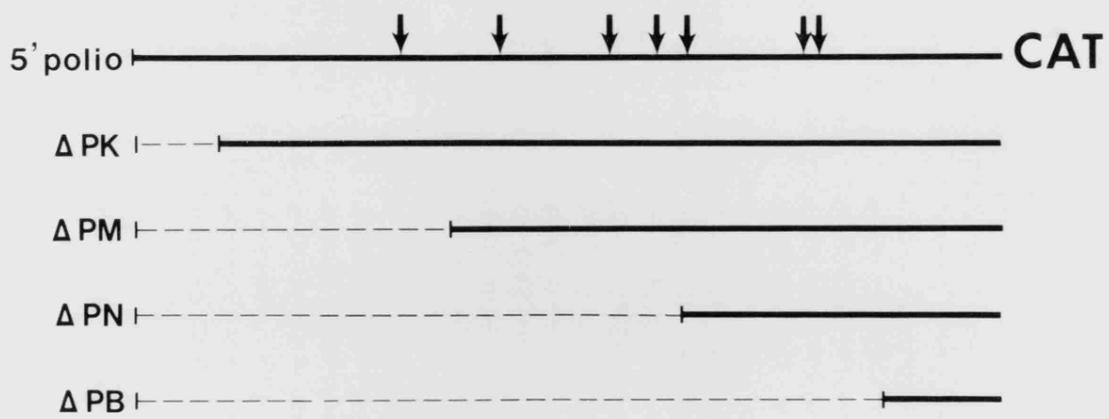
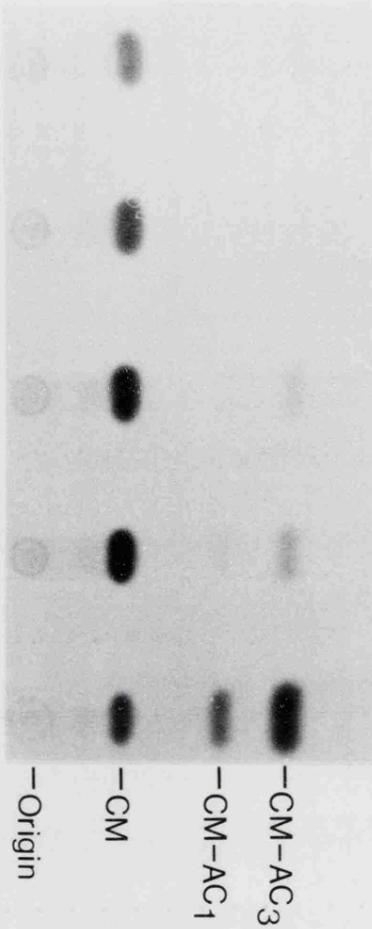
5' polio

Δ PK

Δ PM

Δ PN

Δ PB



N282 (Δ MB) resulted in elevated levels of CAT activity; compare Δ NB and Δ MB. Further deletion to N66 (Δ KB) had little if any effect on CAT activity, while deletion to the PstI site lying 5' to the poliovirus 5' terminus (Δ PB) produced the largest increase and highest level of CAT activity. These results suggest again that the size of the deletion is not solely responsible for the level of CAT activity expressed, but that specific sequences contained within the poliovirus 5' non-coding region determine the level of expression.

5.4 Inhibition of CAT Expression By Specific Sequences

Taken together, the results discussed in sections 5.2 and 5.3 suggested that sequences within two areas of the 5' non-coding region of the mRNA produced by the plasmid pRSV-5'polio-CATm2(N⁺) severely inhibit CAT translation. The first of these lies at the extreme 5' terminus and extends from the PstI site to N66; compare Δ KB and Δ PB in Figure 5.2. The second region extends from N477 to N674; compare Δ PN and Δ PB in Figure 5.1. This conclusion is strengthened by the observation that deletion of the sequences lying between N66 and N477 has little effect on the level of CAT activity expressed, see Δ KM and Δ KN in Figure 5.2.

What is the basis for the inhibition observed with the 5' non-coding region of poliovirus? While it is well known that eukaryotic mRNAs are translated with unequal efficiencies, the basis for such inequality is not completely understood (Alton and Lodish, 1977; Cordell et al., 1982). The only elements so far

Figure 5.2 Expression of CAT from a series of plasmids carrying deletions in the 5' non-coding region of poliovirus in the vector pRSV-5'polio-CATm2(N⁺). See legend for Figure 5.1.

5' polio

Δ KM

Δ KN

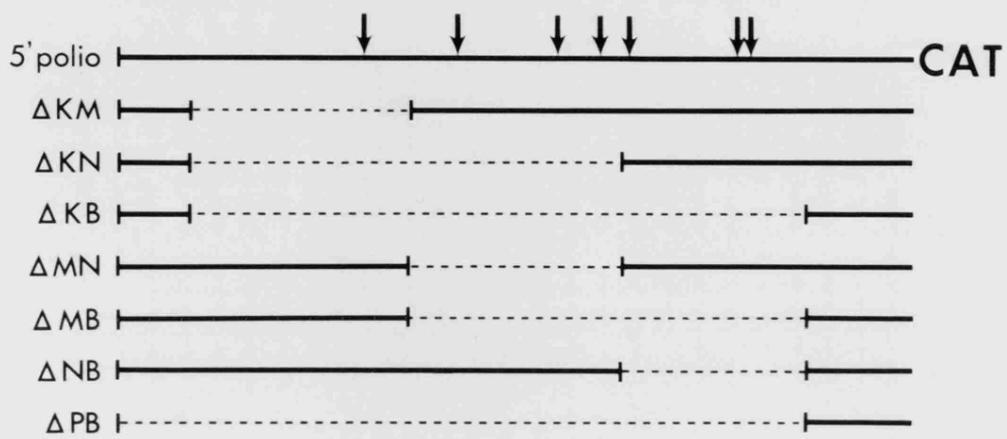
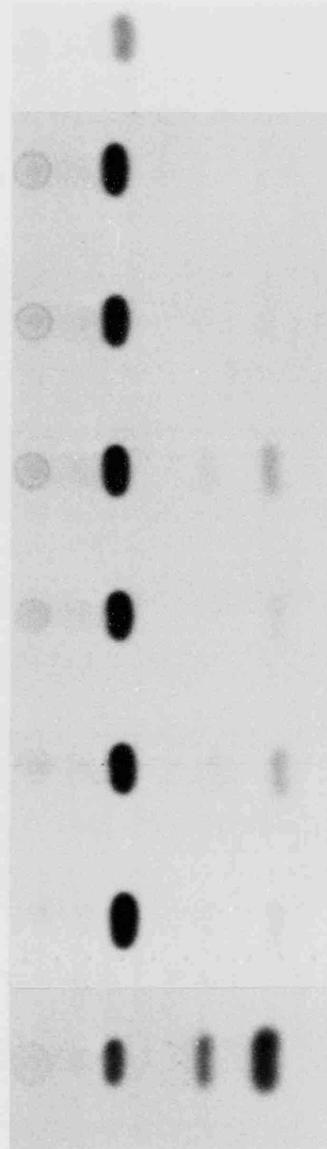
Δ KB

Δ MN

Δ MB

Δ NB

Δ PB



identified which act in a positive manner to enhance the translational efficiency of a eukaryotic mRNA are nucleotides which flank the initiation codon and secondly, the m⁷G cap structure located at the 5' terminus of all eukaryotic mRNAs and most animal virus mRNAs (Shatkin, 1976; Kozak, 1986a). In recent years several elements have been identified within the 5' non-coding region of eukaryotic mRNAs that reduce the efficiency of translation of the mRNA. These elements include stable secondary structure, upstream AUG initiation codons, and poly(G) tracts lying close to the extreme 5' terminus (Jobling and Gehrke, 1987; Johansen et al., 1984; Kozak, 1984c and 1986b; Liu et al., 1984; Lomedico and McAndrew, 1982; Mueller and Hinnebusch, 1986; Pelletier and Sonenberg, 1985). Do any of these elements reside in the 5' non-coding region of poliovirus and if so, are they responsible for the poor translation observed for mRNA containing this region?

The 5' non-coding region of the mRNA produced by the plasmid pRSV-5'polio-CATm2(N⁺) contains all three of the elements described above. Inspection of the primary sequence of the 5' non-coding region of poliovirus type 3 reveals the presence of seven upstream initiation codons (Stanway et al., 1983). Computer analyses of sequence data predicts a number of stable stem-loop structures in the 5' non-coding region of poliovirus and other picornaviruses (Currey et al., 1986; Evans et al., 1985; Larsen et al., 1981; Newton et al., 1985; Vartapetian et al., 1983). As discussed in Chapter 2, the method used to clone poliovirus

P3/Leon/37, the 5' non-coding region of which was used in this study, resulted in the introduction of a poly(G) tail, on the positive strand, i.e the strand produced as mRNA from the LTR in the series of plasmids studied here, between the PstI site used to clone the virus and the extreme 5' terminus of poliovirus (Stanway et al., 1984a).

Are these three elements responsible for the observed inhibition of CAT expression? The potential contribution of each of these elements to the inhibition of translation and correlation with the results given above will be discussed below.

5.4.1 Upstream Initiation Codons

The presence of upstream initiation codons has been shown to inhibit expression of downstream coding sequences (Liu et al., 1984; Kozak et al., 1984c). This inhibition is best understood in the context of the "scanning model" for initiation of translation in eukaryotes, and remains one of the strongest pieces of evidence supporting this model. The "scanning model" (Kozak, 1978, 1986a), states that 40S ribosomal subunits with associated factors bind mRNA at the extreme 5' terminus, via the cap structure, and then migrate along the message until they encounter an initiation codon in a favourable context. At this point the 40S ribosomal subunit stops to allow binding of the 60S ribosomal subunit, thus forming the 80S mRNA initiation complex and allowing translation to begin. If the first initiation codon is not in a favourable context a proportion of the 40S subunits

will pass this initiation codon and migrate further downstream until they reach the next initiation codon. The percentage of 40S subunits that stop at a given initiation codon is determined by the nucleotides that flank it. Analysis of single base mutations flanking the ATG initiation codon identified ACCATGG as the optimal sequence for initiation by eukaryotic ribosomes, and is identical to the consensus sequence derived by comparison of the initiation codons used in eukaryotic mRNAs (Kozak, 1981; 1984a; 1986a).

Upstream initiation codons inhibit translation from a downstream ORF by halting a percentage of migrating 40S subunits which then bind the 60S subunit and initiate translation at this point, thus allowing less than 100% of the available 40S subunits to reach a second ATG lying further downstream. The degree of inhibition caused by an upstream ATG is determined by its flanking nucleotides. If the upstream initiation codon consists of the optimal sequence given above, then translation of a downstream ORF will be severely blocked, while one with a suboptimal sequence has less effect on translation of the downstream ORF (Liu *et al.*, 1984).

Figure 5.3 shows the nucleotides flanking the initiation codons found in the 5' non-coding region of poliovirus P3/Leon/37, and compares them with the optimal sequence utilised by eukaryotic ribosomes during the initiation of translation. One of the initiation codons, located at nucleotide position N479, is identical to the optimal sequence, while the rest exist in a

Figure 5.3 Comparison of nucleotides flanking the upstream initiation codons present in the 5' non-coding region of P3/Leon/37 with those that form the Kozak consensus sequence.

suboptimal context. Are these upstream initiation codons responsible for the observed inhibition?

As stated in section 5.4, two areas of the mRNA produced by the plasmid containing the full length 5' non-coding region of poliovirus severely inhibit production of CAT. The first extends from the PstI site at the 5' terminus to N66. Since this region contains no initiation codons it will not be discussed here. The second region extends from N477 to N674, and contains three upstream initiation codons, found at nucleotide positions N479, N579, and N589. As shown in Figure 5.3 the ATG at N479 matches the consensus sequence perfectly while the other two do not. There is now evidence from several different mRNAs that when an upstream ATG is found in the optimal context then initiation from a downstream ATG is suppressed almost completely (Kozak, 1983, 1984b; Liu *et al.*, 1984). It seems likely therefore, that the upstream ATG at position N479 plays a dominant role in the inhibition of CAT expression observed with constructs containing the region located between N477 and N674 of the poliovirus 5' non-coding region, though site-directed mutagenesis is required to confirm this hypothesis.

5.4.2 Secondary Structure and the Poly(G) Tract

Insertion of oligonucleotides, capable of forming stable hairpin loops, in the 5' non-coding region of the thymidine kinase gene of herpes simplex virus 1, has been shown to reduce the translational efficiency of the thymidine kinase message,

both in-vivo and in-vitro (Pelletier and Sonenberg, 1985). From the results of the above study it was not possible to determine whether the engineered secondary structure inhibited translation by blocking the binding of ribosomes to the message or by inhibiting the migration of ribosomes bound to the message.

In a similar experiment, designed to study the effect that secondary structure has on the translational efficiency of preproinsulin mRNA, it was concluded that stable secondary structure in the 5' non-coding region of a eukaryotic mRNA can inhibit translation by blocking the migration of ribosomal subunits bound to the mRNA (Kozak, 1986b). The stability of the introduced secondary structure determined the level of inhibition. While hairpin structures with a calculated Gibbs energy of formation approaching -30 kcal/mol caused little inhibition, structures in the range of -50 kcal/mol inhibited translation by 85-95% (Kozak, 1986b).

Although extensive secondary structure can be inferred from primary sequence data for the poliovirus 5' non-coding region, and their conservation during sequence divergence amongst different picornaviruses provides evidence that they do exist, their stability suggests that they are incapable of inhibiting translation by interfering with migration of 40S ribosomal subunits (Evans et al., 1985; Larsen et al., 1981; Dr. Michael Skinner, personal communication).

Interestingly, the most stable stem-loop structure found in the 5' non-coding region of poliovirus, the "Larsen" loop, is

located close to the extreme 5' terminus (N10 to N34) in one of the regions lacking initiation codons responsible for the inhibition of CAT expression (Larsen et al., 1981). While the stability of this structure suggests that it would not inhibit the progress of bound 40S ribosomal subunits, its proximity to the 5' terminus may interfere with the binding of ribosomes to the message, thus inhibiting translation. Although such a mechanism has been discussed, there is as yet no clear evidence either to support or rule out such a mechanism (Pelletier and Sonenberg, 1985; Kozak, 1986b; Spena et al., 1986).

As stated above, the method used to clone this strain of poliovirus resulted in the incorporation of a poly(G) tract at the 5' terminus. When expressed in eukaryotic cells from the series of plasmids discussed here, this poly(G) tract is transcribed and is located at the extreme 5' terminus. It has been demonstrated recently that such a poly(G) tract located at the extreme 5' terminus of a message can, in some cases, inhibit its translation quite severely (Jobling and Gehrke, 1987; Gough et al., 1985). By monitoring the levels of CAT expressed by constructs specifically altered at the 5' terminus, it was possible to measure the effect of the "Larsen" loop, and the poly(G) tract separately, and determine which of the two was responsible for the marked inhibition observed with constructs containing the region extending from the PstI site to N66 in the poliovirus sequence (see below).

5.5 Inversion and Removal of the "Larsen" Loop

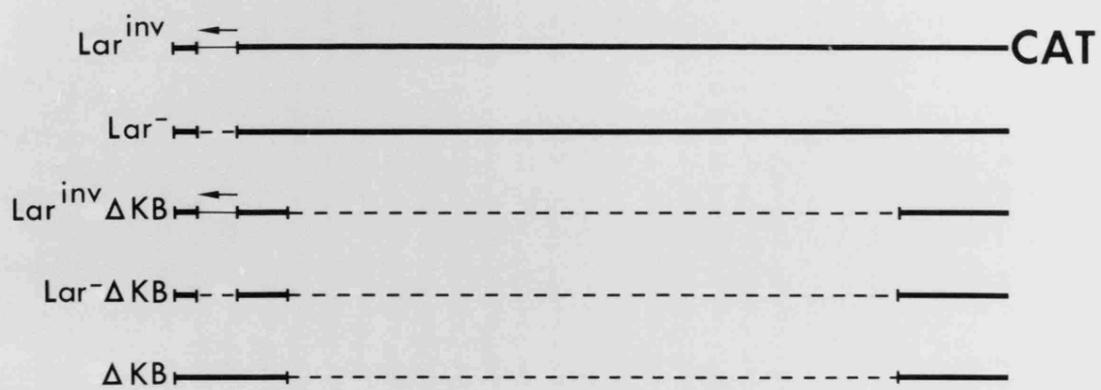
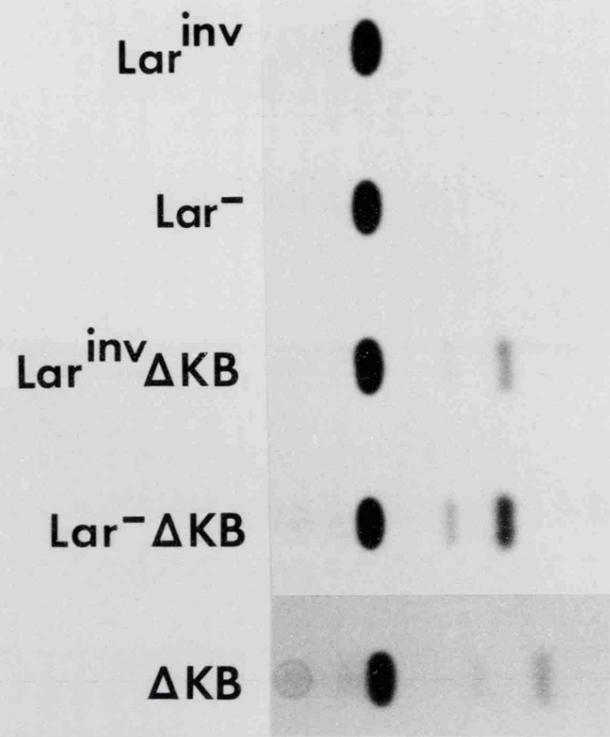
In this section, the inhibition caused by the sequences located between the PstI site and N66 was studied further in an attempt to determine the contribution made by the poly(G) tract and the "Larsen" loop. Deletion of the sequences that form the "Larsen" loop created the plasmid Lar⁻. This was accomplished as described in Chapter 4 (section 4.3.1.). However it is important to mention here that deletion of the "Larsen" loop was carried out by replacing the Hind3/KpnI fragment at the extreme 5' terminus by complementary oligonucleotides with the "Larsen" loop sequences deleted. The Hind3 site used in this construct lies 5' to the PstI site and the poly(G) tract (see Chapter 4, Figure 4.1) and so both the PstI site and the poly(G) tract were deleted in the construction of the plasmid Lar⁻. As a result of this the Lar⁻ plasmids on their own do not reveal the individual effects of the poly(G) tract and the "Larsen" loop: this required comparison with the Lar^{inv} plasmids. Using site-directed mutagenesis of Lar⁻ it was possible to insert the sequences that form the "Larsen" loop in the reverse orientation (see Chapter 4, section 4.4.b), thus allowing direct comparison with Lar⁻ and separation of the poly(G) tract and the "Larsen" loop.

As shown in Figure 5.4, deletion or inversion of the "Larsen" loop, on their own, brings about no change in the level of CAT expressed (Lar⁻ and L^{inv}). Again, this is explained by the presence of the downstream inhibiting region still present in the plasmids Lar⁻ and Lar^{inv}. Deletion of most of the 5' non-coding

Figure 5.4 Expression of CAT from plasmids carrying alterations in the 5' non-coding region of poliovirus in the vector pRSV-5'polio-CATm2(N⁺). See legend for Figure 5.1.

Dotted line represents deletion.

← represents inversion of the "Larsen" loop.



region (from the KpnI site to the BamHI site) from these two plasmids allows one to monitor the effect of deleting the "Larsen" loop and the poly(G) tail on CAT expression.

Comparison of the levels of CAT activity produced by cells transfected with the plasmids, Lar⁻ΔKB, Lar^{inv}ΔKB, ΔKB, and ΔPB, suggests the following; 1) the poly(G) tract does not appear to be involved in inhibition (compare ΔKB with Lar^{inv}ΔKB), 2) deletion of the "Larsen" loop brings about an increase in the level of CAT activity (compare Lar⁻ΔKB with Lar^{inv}ΔKB), and 3) the "Larsen" loop is not solely responsible for the inhibition apportioned to the PstI/KpnI fragment at the 5' terminus of the poliovirus noncoding region (compare ΔPB with Lar⁻ΔKB and ΔKB). It is possible that sequences lying between N34 and N66 of poliovirus either form or contribute to a hairpin structure capable of inhibiting translation, and that this is disrupted by deletion of the first 66 nucleotides of the poliovirus 5' non-coding region.

5.6 Control of Poliovirus Translation

The absence of the normal cap structure at the extreme 5' terminus of poliovirus and the presence of many, apparently untranslated, open reading frames upstream from the open reading frame used in poliovirus expression, suggest that the "scanning model", currently used to explain the process of translation initiation in eukaryotes, is inadequate in the case of poliovirus. It was therefore reasonable to think that there was

some property inherent in the 5' non-coding region of poliovirus, be it primary sequence or secondary structure, which conferred cap independent translation on this virus.

The above experiments reveal that a chimaeric gene containing the 5' non-coding region of poliovirus located directly upstream of the CAT coding sequences is translated in a manner fully in step with the "scanning model". It would appear therefore that inclusion of the 5' non-coding region of poliovirus on a eukaryotic mRNA is insufficient to circumvent cap dependent translation. This suggests that poliovirus is not translated efficiently by the host translational machinery solely by virtue of having the 5' non-coding region, but requires another factor. Soon after infection of a susceptible cell by poliovirus, translation of host cell mRNA is completely inhibited. The inhibition of host mRNA translation by poliovirus is thought to be mediated by cleavage of eIF4a, a factor involved in the cap binding activity of 40S ribosomal subunits. As the genome of poliovirus remains uncapped and translated it can be concluded that intact eIF4a is not required for its translation, and that perhaps this cleavage or another product of infection is required to stimulate the efficiency of poliovirus translation.

To make the study of the 5' non-coding region of poliovirus more valid the plasmids were allowed to express CAT under less artificial conditions by infecting transfected cells with poliovirus and then measuring levels of CAT expressed. For this experiment only two of the polio/CAT plasmids were used, 5'polio

and Δ PB. It was hoped that infection of cells transfected with the plasmid 5'polio would lead to elevated levels of CAT expression, while infection of cells transfected with the plasmid Δ PB would inhibit CAT expression. This would then allow one to study the role of the 5' non-coding region in the control of poliovirus translation and identify essential elements in this region.

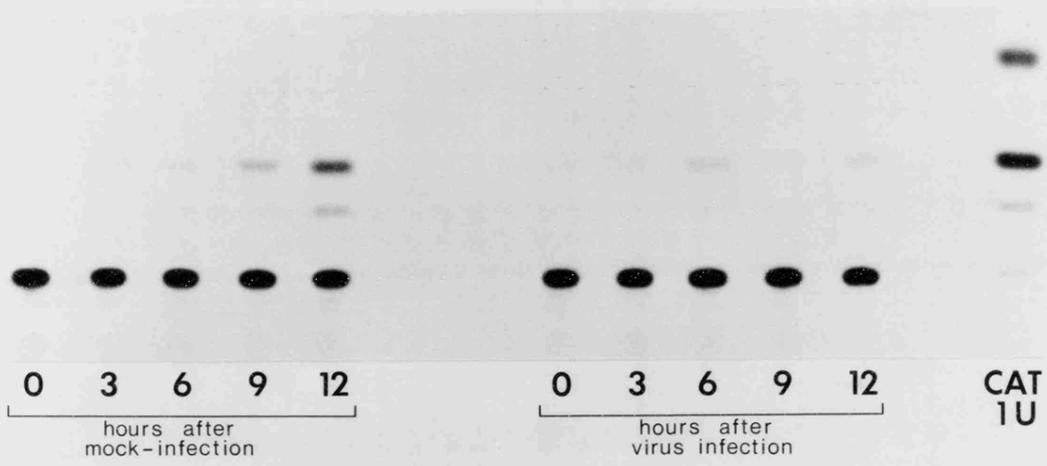
In these experiments, twelve hours after transfection with the two plasmids, cells were infected with poliovirus type 3 at an input moi of 50 pfu/cell and the cells harvested for measurement of CAT activity every 3h until 12h post infection (poliovirus type 3, strain P3/Leon/37 was generously provided by Dr. P. Minor, NIBSC, London, and Miss Karen Hadingham). The results of these experiments are shown in Figure 5.5. Panel A shows the effect infection has on the levels of CAT expressed by cells transfected with Δ PB. The right side of Figure 5.5.A shows the levels of CAT expressed by cells infected with poliovirus, while the left side shows the levels of CAT expressed by mock infected cells. Comparison of the right with left side suggests that expression of CAT by cells transfected with Δ PB is inhibited by infection with poliovirus, presumably as a result of the inhibition of cap-dependent translation.

Figure 5.5.B shows the levels of CAT activity expressed by cells transfected with the plasmid 5'polio following infection (right side), or mock infection (left side). The level of CAT expression is unaffected by infection with poliovirus. While the

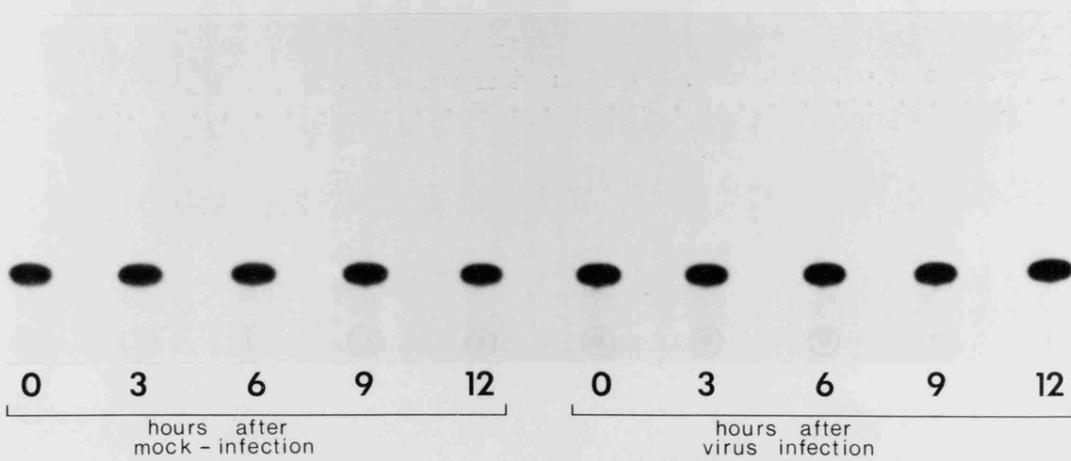
Figure 5.5A Expression of CAT activity from the plasmid pRSV-CATm2(N⁺)ΔPB in poliovirus infected cells (right panel) and mock infected cells (left panel). 12h after transfection with plasmid DNA, cells were infected with poliovirus type 3 at an input moi of 50 pfu/cell. Cells were then harvested every 3h until 12h post infection and assayed for CAT activity.

Figure 5.5B Expression of CAT activity from the plasmid pRSV-5'polio-CATm2(N⁺) in poliovirus infected cells (right panel) and mock infected cells (left panel).

A Δ PB



B 5'polio



experiment carried out using Δ PB gave the expected result, i.e. inhibition of expression, no stimulation of expression was seen in the case of infection of cells transfected with 5'polio. The failure of infection to stimulate CAT expression from 5'polio may be explained by one or a combination of the following; 1) the input moi of virus was too low, and 2) the infection and sampling were initiated too soon after transfection. These two points would explain the following observations; 1) although expression from Δ PB was inhibited following infection, this inhibition does not appear to be complete, 2) 12h after infection total cpe was not observed, and 3) in the mock infected Δ PB transfection the level of CAT expressed at the last time point (24h following transfection) does not reach the level previously recorded for Δ PB at 48h post transfection, suggesting that expression from the transfected plasmids is not optimal during the time course of this experiment (see Figure 5.1). The initial timing of infection was chosen because at that time, 12h after transfection, only low levels of CAT were expressed by Δ PB, while it was a possibility (with no knowledge of the half-life of CAT) that at later times, higher initial levels of CAT would mask any inhibition, thus complicating interpretation. It is possible that increasing the input moi of poliovirus and delaying the timing of infection may result in the stimulation of CAT expression from the plasmid pRSV-5'polio-CATm2(N⁺) to significant levels.

5.7 Summary

Deletion analysis of the 5' non-coding region of poliovirus in the plasmid pRSV-5'polio-CATm2(N⁺) identified two regions that were capable of markedly inhibiting expression of the marker gene. The first lies at the extreme 5' terminus, extending from the PstI site to N66 of the poliovirus sequence, while the second extends from N477 to N674. The "Larsen" loop appears to be partially responsible for inhibition caused by the first region, while a second element present in the first 66 nucleotides of poliovirus, and as yet unidentified, also contributes to the inhibition caused by this region. Inhibition by the second region is presumed to be caused by the initiation codon at N479 which matches Kozak's consensus sequence perfectly, though site-directed mutagenesis is required to prove that this is the case.

It is clear from the results of the above experiments that the poliovirus/CAT chimaeric message is translated as a normal eukaryotic mRNA and is subject to the rules of the "scanning model". This observation suggests that the 5' non-coding region of poliovirus alone is not sufficient to circumvent cap-dependent translation, i.e. it does not possess features which allow a message containing it to be translated efficiently. It was concluded that another factor, a product of poliovirus infection, is required for the efficient translation of poliovirus.

As a result of the conclusion reached above it was decided that monitoring of CAT expression following infection of transfected cells with poliovirus would be a more useful system

for identifying elements located in the 5' non-coding region that play a key role in the translational control of poliovirus. The outcome of these experiments was not a complete success, though the initial results are promising enough to warrant further development of this system. Infection of cells transfected with the plasmid pRSV-CATm2(N⁺)ΔPB resulted in a reduction in the level of CAT expressed, while the levels expressed by the plasmid pRSV-5'polio-CATm2(N⁺) during infection did not change. The first result was expected while the second was not, though this deviation from the predicted results could probably be corrected by minor alterations in the experimental design.

CHAPTER 6

The Design and Construction of a Vector to Probe the Sequence Requirements of Viral Replication and Encapsidation

6.1 Introduction

As stated in Chapter 1, the role of the 5' non-coding region in three processes essential to the intracellular life-cycle of poliovirus was to be investigated by means of transient expression from non-infectious viral vectors. The previous chapter dealt with the control of translation, while this chapter describes the construction of a vector designed to allow the identification of sequences essential for viral replication and encapsidation.

The genome of a virus encodes proteins essential for viral replication and packaging, and contains cis-acting regulatory sequences that are recognised by and thereby direct the action of some of these proteins, thus enabling the virus to become an infectious entity. During the course of infection many viruses produce deletion mutants termed defective-interfering (DI) particles (Huang, 1973). DI particles contain varying amounts of coding sequence (for some viruses all coding capacity may be deleted) but retain the cis-acting regulatory sequences. For successful replication DI particles require helper viruses to provide essential proteins capable of acting in trans. Thus they compete with helper genomes for a limited pool of proteins and, in so doing, interfere with the replication and lower the yield

of helper virus. These DI particles represent a group of viral mutants that offers an insight into the strategy of viral replication and packaging.

DI particles of poliovirus have been identified and studied in some detail, revealing aspects of the viral life-cycle unique to picornaviruses. These had to be taken into account in the design of vectors to study the cis-acting requirements of replication and packaging. DI particles of poliovirus were first identified for the Mahoney strain after passage at high moi (Cole et al., 1971). Infection of cells with purified poliovirus DI particles resulted in the initiation of the normal replication cycle. While non-structural proteins were produced, not all the capsid proteins were synthesised and so progeny virions were not produced (Cole and Baltimore, 1973a, 1973b, 1973c). Propagation of poliovirus DI particles requires the production of capsid proteins by helper virus, suggesting that cis-acting encapsidation signals are present in the genomes of these DI particles.

The deletions contained in DI particles have been mapped to the region of the viral genome encoding the capsid proteins (Lundquist et al., 1979; Nomoto et al., 1979). It would appear that most, if not all, of the capsid region can be deleted, though individual mutants have deletions consisting of no more than 10-20% of the viral genome (Lundquist et al., 1979; Kajigaya et al., 1985). It has been suggested that this size limitation may reflect the inefficient encapsidation of smaller genomes.

Exact mapping of deletions contained in DI particles was accomplished by cloning and sequencing their genomes (Kuge et al., 1986). This study revealed that the size of the deletions varied from 4.2%-13.2% of the total genome length and that all the deletions resulted in the maintenance of the reading frame, allowing production of the non-structural proteins located downstream from the deletion point. The latter observation suggests that DI particles must provide some, if not all, of their own non-structural proteins for replication, implying, in agreement with earlier complementation studies, that some non-structural proteins can only function in-cis or that their ability to function in-trans is very inefficient.

6.2 Implications for the Design of Vectors

The demonstration that non-structural proteins had to be provided in-cis to function in replication meant that any vector developed to study viral replication in-vivo must produce an mRNA molecule containing intact cis sequences required for replication and must also direct the synthesis of the non-structural proteins required for replication. It was decided to mimic as closely as possible the structure of a DI particle and produce one vector which, under different conditions, could be used to study both replication and encapsidation of viral RNA.

The aim was to construct a plasmid that could produce viral-like mRNA capable of replication in tissue culture cells. The mRNA would contain the complete 5' and 3' poliovirus non-coding

regions, both of which are involved in the process of replication (Racaniello and Meriam, 1986; Sarnow et al., 1986). This replicating RNA would also, upon infection by poliovirus, be capable of being encapsidated to produce progeny virions by virtue of the capsid proteins provided by the infecting virus. To differentiate between poliovirus and the DI, and allow the unambiguous identification and measurement of DI RNA encapsidated in the progeny virus the DI RNA was tagged by incorporating a marker gene. The chosen marker sequence, the CAT coding sequence, was inserted into the poliovirus cDNA in place of a portion of capsid protein coding sequences. The substitution resulted in the deletion of less than 13% of the genome size of poliovirus, in keeping with the observations outlined above.

6.3 Vector Construction

The starting material used in the construction of the replication/packaging vector was the plasmid pRSV-5'polio-CATm2(N⁺). By inserting most of the poliovirus coding region and 3'-noncoding region downstream of, and in frame with, the CAT coding sequences would create a plasmid capable of producing the desired viral-like mRNA capable of replication and encapsidation. It was necessary of course to remove the termination codon from the CAT sequences, thus allowing the translation of all the poliovirus non-structural proteins essential for viral replication (see Figure 6.1).

With reference to Figure 6.2, removal of the CAT termination

Figure 6.1 Structure of RNA to be produced by the replication vector. The RNA produced by this plasmid contains the entire 5' and 3' non-coding regions of poliovirus. The termination codon of the CAT gene will be removed to allow in frame fusion between CAT and the P1 region of poliovirus. This in frame fusion allows expression of all the viral non-structural proteins in the P2 and P3 regions.

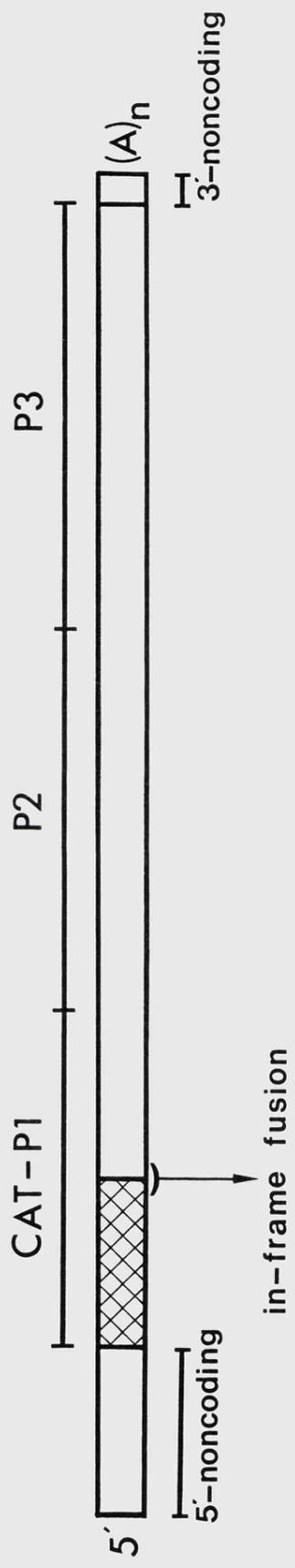
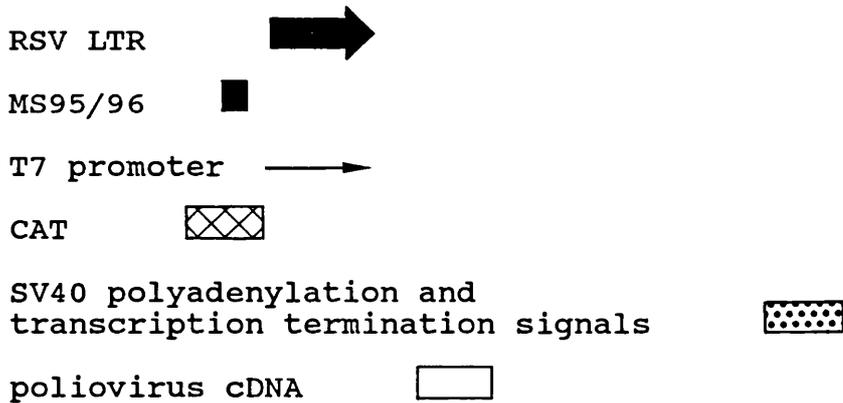
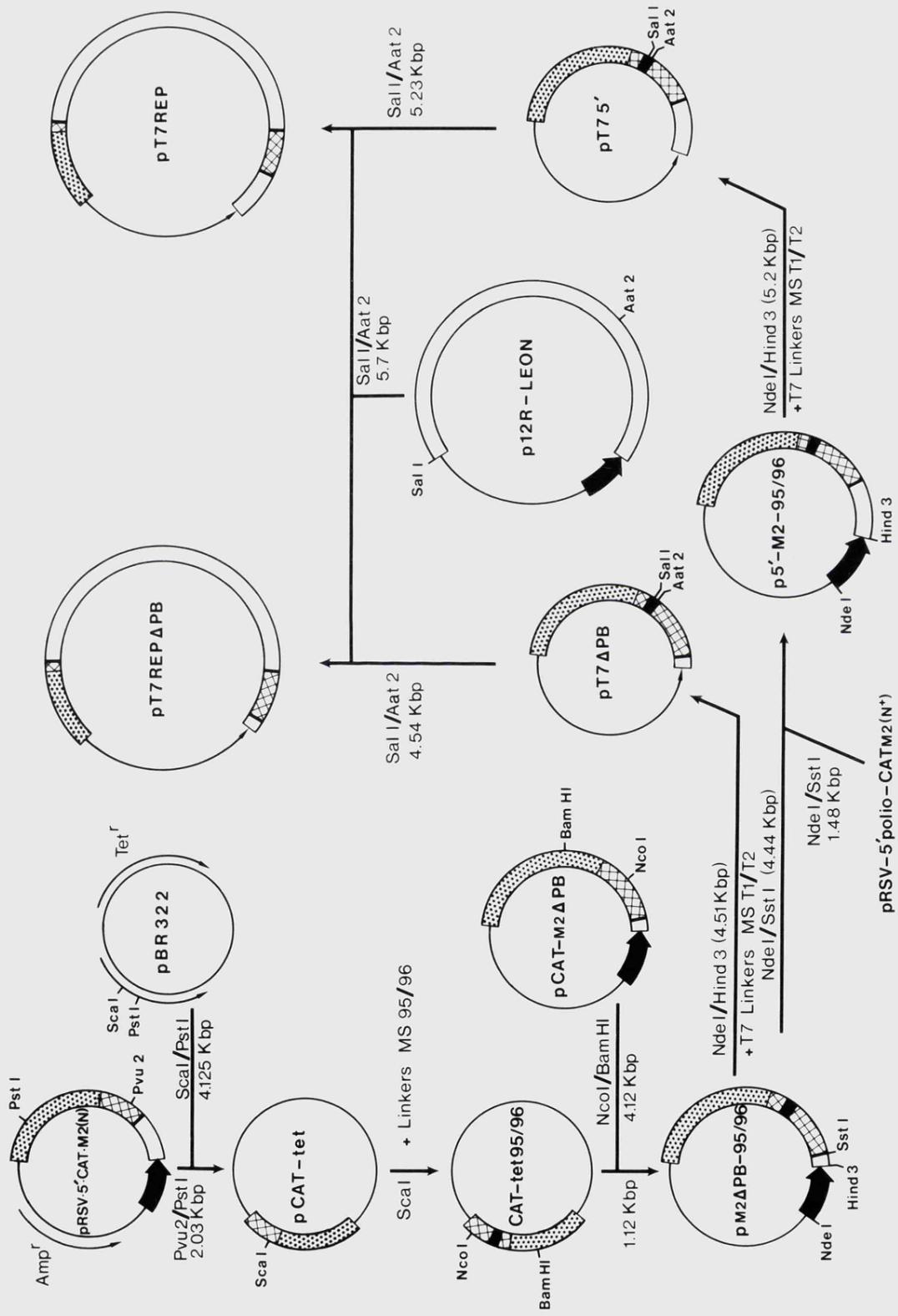


Figure 6.2 Construction of pT7REP and pT7REP Δ PB

Initially, the 2.03Kbp Pvu2/PstI fragment, from pRSV-5'polio-CATm2(N⁺), spanning the 3' terminus of the CAT gene was sub-cloned into ScaI/PstI digested pBR322. Transformants were selected on the basis of Tet^r/Amp^s. Ligation of the Pvu2 and ScaI blunt ends destroyed the pBR322 ScaI site, leaving the ScaI site near the CAT termination codon unique in pCAT-tet. This plasmid was digested with ScaI and ligated with the annealed complementary oligonucleotides, MS95 and MS96 to create pCAT-tet95/96. Insertion of these linkers removed the CAT termination codon and allowed the eventual in frame insertion of the poliovirus coding region, from N1809 (Aat2), and the 3' non-coding region. For other details see text.





codon and in frame fusion of the poliovirus coding sequence was made possible by inserting a synthetic oligonucleotide linker at the extreme 3'-terminus of the CAT coding region. Initially, the plasmid pRSV-5'polio-CATm2(N⁺) was digested with Pvu2 and PstI, the resulting 2.03 Kbp fragment containing the 3'end of the CAT gene and signal sequences from SV40 was purified and ligated with the large fragment created by ScaI/PstI double digestion of pBR322. Following transformation of competent E.coli with the ligation products, tetracycline resistant ampicillin sensitive colonies were screened for and their plasmid DNA purified and characterised. The resulting plasmid, pCAT-tet, by virtue of ligating the Pvu2 and ScaI blunt ends, contains a unique ScaI site located within the CAT coding region, 26 nucleotides from the termination codon. Two complementary oligonucleotides (MS95 and MS96) were designed to be inserted at this unique ScaI site, to destroy the ScaI site, and to recreate the carboxy-terminus of the CAT protein while removing its termination codon. Immediately downstream of the CAT coding sequence contained within this linker three restriction sites were engineered. These three sites, Aat2, PstI, and SalI, were incorporated to allow firstly, the identification of plasmids containing the linker in the correct orientation, and secondly, the in frame fusion of the poliovirus coding region (see Figure 6.3).

The plasmid pCAT-tet was digested with ScaI and ligated with annealed MS95/96. As the ScaI digested pCAT-tet was not treated with phosphatase, the ligation products were digested with ScaI

Figure 6.3 Removal of the CAT termination codon using the complementary oligonucleotides MS95 and MS96. Using the unique SalI and Aat2 sites, the complete poliovirus cDNA 3' to the Aat2 site at N1809 was inserted to produce an in frame fusion of CAT and poliovirus VP3.

Sca I
 5'-CAA CAG TAC TGC GAT GAG TGG CAG GGC GGG GCG TAA-3' 3' TERMINUS OF CAT CODING REGION

Digested with Sca I.
 Ligated with the annealed complementary oligos, MS 95 and MS 96.

CAA CAG TAT TGT GAC GAA TGG CAA GGA GGT GCG ACG TCT GCG A G TCG ACC GA ACT
 MS 96
 Aat 2 Pst I Sal I

Digested with Aat 2 and Sal I.
 Ligated with the 5.7 Kbp Aat 2 - Sal I fragment from p12R-LEON.

5'-CAA GGA GGT GCG ACG TCA GAC AAC CTC-3' + ve strand
 Aat 2
 CAT in-frame fusion VP3

prior to transformation of competent E.coli to linearise any self-ligated pCAT-tet molecules, and thus reduce the background of pCAT-tet transformants. Transformants were screened for plasmids carrying the linker in the desired orientation, by restriction analysis of plasmids with PstI, which cuts asymmetrically within the linker, and EcoRI. A plasmid containing the linker in the desired orientation, pCAT-tet95/96 was then used as the source for the altered CAT 3'-terminus. This plasmid was digested with both NcoI and BamHI to release a 1.12 Kbp fragment, which was ligated to the 4.12 Kbp NcoI/BamHI fragment of pRSV-CATm2(N⁺) Δ PB. The plasmid Δ PB was used in preference to the plasmid containing the full 5' non-coding region as the latter contains a second NcoI site and would have required partial digestion. Following ligation and transformation, colonies harbouring the correct construct, pM2 Δ PB-95/96, were screened for and identified.

During the course of this study it was shown by others that full-length cDNA copies of poliovirus, positioned downstream of bacteriophage T7 promoters, could produce poliovirus transcripts, using commercially available T7 RNA polymerase in a simple in-vitro reaction. Transfection of susceptible eukaryotic cells in culture with these transcripts produced infectious poliovirus; the efficiency of infection was 5% of that obtained for purified viral RNA, while infectivity of DNA was 0.1% (van der Werf et al., 1986). As a result of the increase in infectivity obtained with the transcripts synthesised in-vitro, it was decided to use

RNA transcripts in this study in preference to cDNA constructs under the transcriptional control of a eukaryotic promoter. This required the insertion of a T7 promoter in the replication/packaging vector.

Complementary oligonucleotides, MST1 and MST2, 40 and 42 nucleotides in length respectively, were synthesised and purified. Following annealing, MST1 and MST2 form a double-stranded DNA fragment, with Hind3 and NdeI cohesive ends, containing a T7 promoter (Dunn and Studier, 1983, see Figure 6.4). The plasmid pM2 Δ PB-95/96 was digested with Hind3 and NdeI to release the LTR of RSV, and the large fragment (4.51 Kbp) purified and ligated to the annealed MST1/MST2. The resulting plasmid, pT7 Δ PB was isolated and used to test whether the T7 promoter was functional. As the promoter was functional the plasmid was then used to construct pT7REP Δ PB. This plasmid, containing a large deletion in the 5' non-coding region, was to be used as the negative control alongside the replication/packaging vector pT7REP which would contain the complete 5' non-coding region of poliovirus.

Using the unique SalI and Aat2 sites introduced at the 3'-terminus of the CAT coding sequence, via the linker MS95/96, it was possible to fuse, in frame, the bulk of the poliovirus coding region along with the 3'-noncoding region and poly(A) tail. The plasmid pT7 Δ PB was digested with Aat2 and SalI and ligated with the 5.7 Kbp Aat2/SalI fragment from p12R-LEON. The SalI site of p12R-LEON lies just beyond the 3'-terminus of the poliovirus

Figure 6.4 Nucleotide sequence of the complementary oligonucleotides MST1 and MST2 which form a promoter recognised by T7 RNA polymerase.

MS T1
5' -TAT GTC GCG ACT TTC GAA ATT AAT ACG ACT CAC TAT AGG A-3'
3' -A CAG CGC TGA AAG CTT TAA TTA TGC TGA GTG ATA TCC TTC GA-5'
MS T2

NDEI OVERHANG

HIND3 OVERHANG

cdNA, while the Aat2 site is located at nucleotide position N1809 of poliovirus, 45 nucleotides downstream of the VP2/VP3 junction. Fusion with the 3'-terminus of CAT at this Aat2 site results in a CAT/VP3 fusion, the loss of VP4 and VP2 coding sequence, and the loss of approximately 470 nucleotides (just over 6% of the poliovirus genome). Following transformation of competent E.coli with the ligation products, plasmid DNA was isolated from transformants and the correct construct identified by restriction analysis. This plasmid, named pT7REP Δ PB, as stated above, was to be used as the negative control in the development of assays to monitor replication and packaging.

As the Aat2/SalI fragment of the poliovirus cdNA had been chosen for this construct, to simplify the manipulations at this stage the Aat2 site in the linker of pCAT-tet95/96 needed to be unique. This is why in the final step of the construction of pRSV-CATm2(N⁺) (see Chapter 3, Section 3.4) the Aat2 site lying just outside the promoter region of the β -lactamase gene was destroyed. It was replaced by a NotI site that would be unique in the plasmid pT7REP, thus allowing easy replacement of the 5' non-coding region by altered versions using the NotI and SstI sites flanking this region in pT7REP.

To construct pT7REP, the plasmid pM2 Δ PB-95/96 first had the deletion in the 5' non-coding region repaired. This was achieved by digesting pM2 Δ PB-95/96 with NdeI and SstI, the 4.44 Kbp fragment was isolated, purified and ligated with the 1.48 Kbp NdeI/SstI fragment of pRSV-5'polio-CATm2(N⁺). After

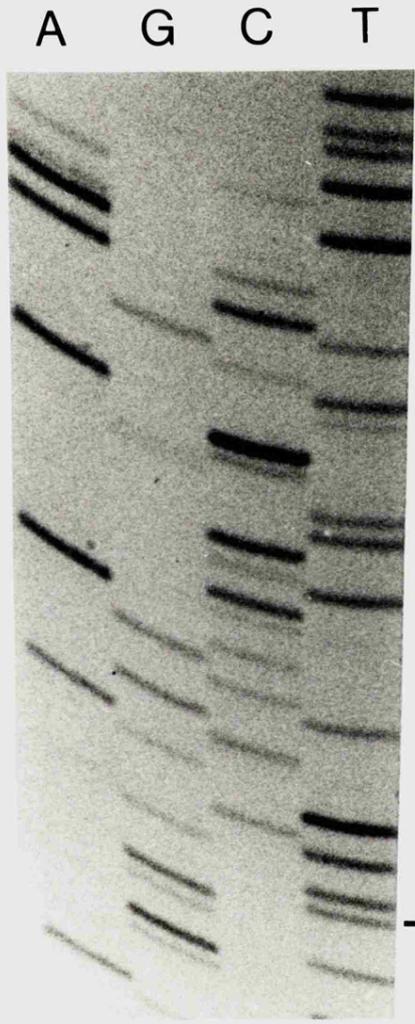
transformation, plasmid DNA was isolated and the desired plasmid identified by restriction analysis. The LTR from this plasmid, p5'-M2-95/96, was removed and replaced by the T7 promoter, as described above for the construction of pT7 Δ PB. The final step in the construction of pT7REP was the insertion of the poliovirus SalI/Aat2 fragment isolated from p12R-LEON. This step was performed as described above for pT7REP Δ PB. Finally, these two plasmids, pT7REP and pT7REP Δ PB, were partially sequenced to ensure that the poliovirus coding region was in frame with the CAT coding sequence. A 475 bp SstI/EcoRI fragment, containing the junction point, was sub-cloned into M13mp19 and sequenced (see Figure 6.5). From this it was clear that the constructions were correct and the two coding regions were in frame.

6.4 Expression from pT7REP

Time did not allow the development of the assay conditions to measure the ability of pT7REP to produce a message capable of replication and encapsidation, though it is clear from work carried out since the completion of the experimental work described here that pT7REP produces functional CAT, while pT7REP Δ PB does not. The production of CAT by the plasmid carrying the full 5' non-coding region, and the failure of the plasmid containing the 5' deletion, Δ PB, to do so, was measured following transfection of a eukaryotic cell line producing functional T7 RNA polymerase, and suggests that the message from pT7REP is undergoing replication and has initiated the normal replicative cycle while the corresponding message from pT7REP Δ PB does not.

Figure 6.5 Sequence analysis of the CAT/VP3 fusion. Verification that the fusion is in frame. The 475bp EcoRI/SstI fragment spanning the junction point was sub-cloned into EcoRI/SstI digested M13mp19 and subjected to sequence analysis using the universal primer. The sequence shown represent the negative strand in relation to the poliovirus plus strand.

3'
↑
5'



REP

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

CAT

← CAT/Polio
Junction.

Polio

Negative strand

Work is currently being carried out to confirm this interpretation and further develop the system. The ability to produce functional CAT may be of use in measuring the presence of CAT/poliovirus DI particles in studies of encapsidation. It would be possible to infect a fresh monolayer of cells with the progeny virus and then carry out a CAT assay to determine the efficiency of encapsidation.

CHAPTER 7

Discussion

7.1 Translation

In an attempt to study the translational control of poliovirus, the complete 5' non-coding region from poliovirus was fused directly upstream of the bacterial CAT gene. Using site-directed mutagenesis of the CAT sequence it was possible to create a poliovirus/CAT fusion in which the CAT initiation codon and flanking nucleotides were replaced by those normally utilised during expression of the poliovirus genome. This resulted in the mutation of three amino acids in the CAT protein encoded by this chimaera, though CAT activity was retained. To ensure expression of this fusion in eukaryotic cells in culture the fused cDNA was placed under the transcriptional control of the LTR of RSV. The plasmid containing this chimaera was labelled pRSV-5'polio-CATm2(N⁺).

A series of internal and terminal deletions spanning most of the 5' non-coding region of poliovirus were made and inserted into pRSV-5'polio-CATm2(N⁺) in place of the complete 5' non-coding region. Following transfection of eukaryotic cells the levels of CAT activity expressed by the different plasmids were measured and compared. From these results it would appear that by fusing a marker gene downstream from the 5' non-coding region of poliovirus this marker gene is translated in a manner fitting the "scanning model", as proposed by Kozak (1986a). This suggests

that the 5' non-coding region, on its own, is incapable of circumventing cap-dependent translation and conferring cap-independent translation on a message containing this region upstream from its coding region.

Due to the results of the experiments outlined above, it was decided to study the expression of CAT following poliovirus infection of the transfected cells, thereby mimicking the conditions under which poliovirus is translated. Under such conditions, cap-independent translation would prevail while cap-dependent translation would be inhibited. This would allow one to study the control of poliovirus translation by measuring the levels of CAT expressed by the poliovirus/CAT chimaeras in infected cells. It is suggested that during infection, levels of CAT expressed by plasmids containing the entire 5' non-coding region would increase, while levels of CAT expressed by plasmids lacking this region would decrease. This would occur as a result of the switch from cap-dependent to cap-independent translation.

When the levels of CAT activity expressed by cells transfected with the plasmid carrying the largest deletion in the poliovirus 5' non-coding region, pRSV-CATm2(N⁺)ΔPB, were measured, it was clear that expression of CAT activity was inhibited during poliovirus infection. This result was as predicted. As ΔPB had had most of the 5' non-coding region deleted, its message would only be translated while cap-dependent translation occurred, during poliovirus infection this would be inhibited, resulting in lower levels of CAT expression.

For the plasmid containing the entire 5' non-coding region of poliovirus, the results were not so satisfactory. Following infection of cells transfected with this plasmid no change in the level of CAT activity was detected. It was anticipated that infection of cells transfected with pRSV-5'polio-CATm2(N⁺) would bring about a significant increase in CAT expression. This increase would be due to the switch from cap-dependent to cap-independent translation and the presence of the entire 5' non-coding region at the 5' terminus of the mRNA produced by this plasmid. As discussed in Chapter 5, the failure of this experiment may result from the timing of the poliovirus infection following transfection, or may have been caused by infecting with a low level of virus.

After completion of the practical work presented here several reports concerning the control of poliovirus translation and the role of the 5' non-coding region were published. The first of these reported the results of experiments designed to measure the in-vitro translational efficiencies of a series of synthetic mRNAs carrying deletions in the 5' non-coding region of poliovirus type 2 (Pelletier et al., 1988). Efficiency was determined by measuring specific products, following translation of the synthetic mRNAs in cellular extracts of mock-infected and poliovirus-infected HeLa cells. It is clear from the results measuring translation in mock-infected HeLa cell extracts that translational efficiency is dramatically increased when the first 631 nucleotides of the 5' non-coding region of poliovirus is

removed. This result is in agreement with the observation of Nicklin et al. (1987), studying the in-vitro translation of poliovirus type 1, and the results discussed in Chapters 3 and 5, dealing with the in-vivo translation of poliovirus type 3.

Pelletier et al. (1988) concluded from comparison of the results obtained for mock-infected and poliovirus-infected cell extracts, that a cis-acting element, mapping between nucleotides 320 and 631, allows poliovirus to translate in a cap-independent manner. It is interesting to note that the mRNA carrying the entire 5' non-coding region of poliovirus was translated with equal efficiency in extracts of the mock-infected and poliovirus-infected HeLa cells. The presence of the cis-acting element did not lead to an increase in translation in the infected cell extract, its presence was inferred from a decrease in translation, in the infected cell extract, of mRNAs carrying deletions in the 5' non-coding region. A similar result for the infected cell extract was obtained when the entire 5' non-coding region and several deletions were fused to the CAT gene. Measurement of the CAT gene product in mock-infected cell extracts though, provided odd results. The increase in translation previously seen to accompany deletions made in the 5' non-coding region of poliovirus was not apparent when production of the CAT gene product was measured in this system (Pelletier et al., 1988).

In a second paper, Trono et al. (1988b) reported the results of experiments designed to study the role of the 5' non-coding

region of poliovirus type 1 in the in-vitro translation of a polio/CAT chimaera similar to that described in Chapter 3. In this case, the chimaera was placed under the transcriptional control of the early promoter of SV40. Following electroporation of COS cells, production of CAT activity was monitored in both mock-infected and poliovirus-infected cells.

As before, the presence of the 5' non-coding region of poliovirus severely inhibited the production of CAT activity in non-infected cells. In this paper though, it was shown, using a construct that contained the entire wild type poliovirus type 1 5' non-coding region, that the production of CAT activity increased more than twofold upon infection with poliovirus. When a construct lacking the entire 5' non-coding region of poliovirus was used, production of CAT was found to decrease following infection of the electroporated cells with poliovirus. It was concluded that the 5' non-coding region of poliovirus was required to confer cap-independent translation upon infection.

This study went on to show that lesions mapping to the 5' non-coding region, of previously identified mutant viral strains deficient in viral translation (Trono et al., 1988a), when fused to the CAT gene, failed to give an increase in CAT expression following infection with poliovirus. From the position of these lesions it was concluded that the region of the 5' non-coding region required for cap-independent translation extends from nucleotide 130 to around nucleotide 600 (Trono et al., 1988b).

It is worth noting that the conditions used in the study

described above differed from those used for the experiments described in this thesis. Forty hours after electroporation cells were infected with poliovirus at a moi of 100 pfu/cell in the presence of guanidine and actinomycin D. Guanidine was added to prevent early cell death by inhibiting poliovirus replication. Actinomycin D was added to block accumulation of cellular mRNA. These two steps were not considered for the experiments carried out in this thesis and may explain the unexpected result observed in Chapter 5, section 5.6, i.e. the apparent failure of the 5' non-coding region to give an increase in the level of CAT produced during poliovirus infection of cells transfected with pRSV-5'polio-CATm2(N⁺).

Pelletier and Sonenberg (1988) went on to determine the process by which the 5' non-coding region of poliovirus circumvents cap-dependent translation and confers cap-independent translation. Two obvious possibilities existed, either poliovirus promotes the internal binding of ribosomes, or ribosomes bind to the extreme 5' terminus of the poliovirus mRNA as normal but, due to the effects of poliovirus infection, their ability to scan and recognise initiation codons is altered in a manner that allows efficient translation of the poliovirus mRNA.

Using elegant plasmid constructions that produce bicistronic mRNAs, with two marker genes separated with the poliovirus 5' non-coding region as the intercistronic spacer, it was shown that during poliovirus infection of transfected cells, internal binding by ribosomes occurred (Pelletier and Sonenberg, 1988).

The thymidine kinase gene of herpes simplex virus formed the first cistron while second cistron was the bacterial CAT gene. A control plasmid contained the CAT 5' non-coding region as the intercistronic spacer. In the absence of poliovirus infection both of the plasmids produced the protein products of both cistrons. However, during poliovirus infection, the plasmid containing the CAT intercistronic spacer produced neither of its protein products. Under the same conditions, the plasmid containing the poliovirus 5' noncoding region as the intercistronic spacer only produced the gene product of its second cistron. As the first cistron was not translated during poliovirus infection, a result of the inhibition of cap-dependent translation, translation of the second cistron is unlikely to result from re-initiation by ribosomes. It is concluded that this results from internal binding by ribosomes mediated by an internal sequence in the 5' non-coding region of poliovirus (Pelletier and Sonenberg, 1988).

7.2 Replication and Encapsidation

Chapter 6 describes the design and construction of a plasmid, pT7REP, designed to probe the sequence requirements of viral replication and encapsidation. This vector was designed to produce an mRNA that resembled the genome of a DI particle, which would thus be capable of replicating and being encapsidated. The plasmid pT7REP produces a viral-like mRNA containing the entire 5' and 3' poliovirus non-coding regions, and the ability to

produce all the poliovirus non-structural proteins. The CAT coding sequences were inserted into the poliovirus genome as a foreign marker gene, in place of a segment of the P1 coding region, but in-frame with the major ORF of poliovirus.

Unfortunately time did not allow for the functional analysis of this vector, though from experiments carried out since the completion of the work presented here, it would appear that pT7REP produces a mRNA capable of replication and produces significant levels of CAT activity. This is as expected for recently it was reported that in-vitro synthesised transcripts of poliovirus cDNAs, with deletions spanning the poliovirus sequences deleted in pT7REP, are capable of replicating after transfection into HeLa cells (Kaplan and Racaniello, 1988). It remains to be seen whether the mRNA produced by pT7REP is encapsidated when the full complement of viral capsid proteins are provided in trans.

When initiated, the experiments described in this thesis represented the first attempt to comprehensively characterise the function of the 5' non-coding region of poliovirus. Vectors have been constructed which should, in the future, allow one to investigate the role of the 5' non-coding region in viral replication and the encapsidation of viral RNA. Furthermore, fine-mapping of the primary sequences or secondary structures that control these processes should be possible. Some of the plasmids described here are currently being used to probe the involvement of the 5' non-coding region in viral replication

(N.Percy, personal communication).

Greater knowledge of the function of the 5' non-coding region of poliovirus is required if we are to fully understand the replicative cycle of poliovirus and its role in the disease process. Armed with such knowledge it would be possible to design live-attenuated vaccine strains of poliovirus which, on the grounds of safety, would surpass those currently used.

CHAPTER 8

Materials and Methods

8.1 Materials

8.1.1 Reagents

Acrylamide	Serva Feinbiochemica, Heidelberg, GDR.
Agar (Bacto agar)	Difco Laboratories, Detroit, USA.
Ampicillin	Sigma Chemical Company, Poole, UK.
BSA (DNase/RNase free)	Pharmacia.
Diethylaminoethyl-Dextran	Sigma.
Diethyl pyrocarbonate	Sigma.
Dimethyldichlorosilane	British Drug Houses Ltd., Poole, UK.
Dithiothreitol	Sigma.
Ficoll (Mr 400,000)	Sigma.
Formaldehyde	B.D.H.
Formamide	B.D.H.
Nitrocellulose filters	Schleicher and Schull Ltd., Dassel, GDR.
3-(N-Morpholino) propane- sulphonic acid (MOPS)	B.D.H.

N,N'-methylenebisacrylamide	B.D.H.
Nucleoside triphosphates	Boehringer-Mannheim, Lewes, UK., Sigma, and Pharmacia.
Di-deoxynucleotides	Pharmacia.
Phenol	Fisons, Loughborough, UK.
Polyethylene glycol (6000)	Fisons and Sigma.
Polyvinyl-pyrrolidone (PVP-40)	Sigma.
Ribonucleotides	Pharmacia.
Sodium dodecyl sulphate (SDS)	B.D.H.
Tetracycline	Sigma.
Tris-(hydroxymethyl) amino- methane-acetate	Sigma.
Tris-(hydroxymethyl) methyl-amine	Fisons.
Urea	Fisons.

8.1.2 Enzymes

Calf intestinal phosphatase (CIP)	Boehringer-Mannheim.
DNaseI (RNase free-DPRF grade)	Lorne Laboratories, Berkshire, UK.
DNase	Sigma.
<u>E.coli</u> DNA polymease (Pol I)	Boehringer-Mannheim.
Large fragment of <u>E.coli</u> DNA Pol I (Klenow fragment)	Boehringer-Mannheim.
Lysozyme	Sigma.

Restriction endonucleases	BRL, Pharmacia, NEBL, NEN.
Ribonuclease A	Sigma.
RNase inhibitor (RNA guard)	Pharmacia.
SP6 RNA polymerase	New England Biolabs.
T4 DNA ligase	BRL.
T4 polynucleotide kinase	New England Biolabs.
T7 RNA polymerase	New England Biolabs.

8.1.3 Radioisotopes

Amersham.

[α -³²P] dATP (2000-3000 Ci/mmole)
 [α -³²P] dCTP (2000-3000 Ci/mmole)
 [γ -³²P] dATP (2000-3000 Ci/mmole)
 [α -³⁵S] dATP (>400 Ci/mmole)
 D-threo-[dichloroacetyl-1-¹⁴C] chloramphenicol (53 mCi/mmole)

8.1.4 Solutions and Buffers

Denhardt's solution (50x): BSA (1%, w/v), ficoll (1%, w/v),
 polyvinylpyrrolidone (1%, w/v).

SSC (20x): NaCl (17.53%, w/v), sodium citrate (8.82%, w/v),
 adjusted to pH 7.0 with NaOH.

Tris-acetate buffer (10x): Tris base (400mM), EDTA (20mM),
 glacial acetic acid (200mM), adjusted to pH 8.1.

Tris-acetate 5x loading buffer: bromophenol blue (0.25%, w/v) and

sucrose (40%, w/v) in Tris-acetate buffer (5x).

TBE (10x): Tris base (10.9%, w/v), boric acid (5.5%, w/v), EDTA (0.93%, w/v).

8.2 Methods

8.2.1 Preparation of Competent Cells

E.coli strains were made competent for the uptake of plasmid DNA by the following method. Luria broth (1 vol., routinely 10-30ml) was inoculated with 0.01 vol. of an overnight culture of E.coli and grown with vigorous aeration at 37°C until the OD at 650 nm = 0.5 (2-3 h). The culture was then chilled on ice for 5 min and cells pelleted in round-bottomed, sterile, plastic universal tubes. The pellet was gently resuspended in 0.5 vol. calcium chloride solution (100mM, 0°C). After incubation on ice for 40 min cells were again pelleted and resuspended in 0.05 vol. calcium chloride solution (100mM, 0°C). Cells were stored on ice and used between 1-24 h after preparation.

8.2.2 Transformation of E.coli

E.coli strain JA 221 (F', hsdM, hsdR, lacY, leuB6, trpE6, del, recA1) was used in all plasmid vector transformations, E.coli strain JM101 (lac, pro), supE, thiF', traD36, proAB, lacI^q, lacZ, delta M15) was used in all M13 vector transformations and E.coli strain JM83 (lacZ, delta M15, srI, recA56, RK , MK+) was used in initial pUC vector transformations. E.coli strains BMH 71-18 (lac-proAB), thi, supE; F', lacI^q, lacZ, delta M15,

proA+B+) and BMH 71-18 mutL (BMH 71-18, mutL::Tn10) were used for all site-directed mutagenesis transformations.

E.coli transformation protocols were essentially the same for all strains used. Ligation mixtures were incubated on ice with competent cells (200ul) for 45 min, heat-shocked at 42°C for 2 min and returned to ice for 40 min. Subsequent steps were as follows:

a) JA 221 cells Antibiotic selection:

Cells challenged with antibiotic selection were allowed to express transformed phenotypes by incubation in L.B. (1ml) for 30 min. Cells were pelleted and gently resuspended in L.B.(100ul) and plated onto L.B. agar plates containing ampicillin (100ug/ml) or tetracycline (10ug/ml). Plates were incubated overnight at 37°C.

b) JM 101 cells B-galactosidase phenotype selection:

The cells are added to "soft-top" agar (3.5ml, 42°C) containing IPTG (5ug/ml), BCIG (7ug/ml) and 130ul of an exponential phase culture of JM 101. The mixture was quickly vortexed and poured onto 2xTY agar plates. The soft-top overlay was allowed to solidify and plates incubated overnight at 37°C.

c) JM 83 cells phenotype selections a) and b):

Plasmids in the pUC series confer ampicillin resistance and may complement the defective B-galactosidase gene in JM 83. Cells are therefore plated onto L.B. agar plates containing ampicillin and BCIG (concentrations as above). IPTG inducer is not required as B-galactosidase is constitutively expressed in JM 83. Plates

were incubated at 37°C overnight.

d) BMH 71-18 mutL cells:

The cells were added to "soft-top" agar (3.5ml, 42°C) containing 130ul of an exponential phase culture of BMH 71-18. The mixture was vortexed and poured onto 2xTY agar plates. The "soft-top" overlay was allowed to solidify and plates were incubated overnight at 37°C.

8.2.3 Plasmid Isolation

8.2.3a Rapid Isolation

Luria broth (10ml) with added antibiotic was inoculated with a single colony of E.coli from an agar plate and grown overnight at 37°C. Cells were pelleted and resuspended in 630ul of Tris-HCl (50mM, pH 8.0), EDTA (50mM), sucrose (15%, w/v). Following transfer to Eppendorf tubes, 70ul of a lysozyme solution (10mg/ml, made fresh) were added and left at room temperature for 10 min. SDS (30ul, 10%) and potassium acetate (75ul, 4M) were added, and mixed by inverting several times. Reaction tubes were kept on ice for 30 min and centrifuged in an Eppendorf centrifuge for 15 min. The supernatant was carefully decanted into a new tube and digested with RNase A (1ul, 1mg/ml) at 37°C for 30 min. Protein was removed by extraction with an equal volume of phenol/chloroform (1/1, v/v). Nucleic acids were precipitated from the aqueous phase by filling the tubes with cold ethanol, mixing and centrifugation for 5 min. The pellet was dissolved in sterile distilled water (100ul).

8.2.3b Large Scale Isolation

This method gives high yields of plasmid DNA which is purified from contaminating chromosomal DNA and cellular RNAs by caesium chloride equilibrium density centrifugation.

Luria broth (10ml) with added antibiotic was inoculated with the desired bacterial colony and grown for 5 h with vigorous shaking (37°C). This culture was then used to inoculate 500ml of Luria broth containing antibiotic. Following growth overnight at 37°C, the bacterial cells were pelleted (4°C, 3000g, 10 min), resuspended in TES (4.5ml; 50mM Tris-HCl, pH 7.5, 40mM EDTA, 25% sucrose (w/v) and chilled on ice. All subsequent steps in the lysis procedure were carried out on ice. Firstly, lysozyme (0.9ml, 10mg/ml in TES) was added, mixed and left for 5 min. Then EDTA (0.925ml, 0.5M, pH 8.0) was added and left for a further 5 min. Finally, Triton mix (7.25ml; 0.1% Triton, 50mM Tris-HCl, pH 7.9, 7.9mM EDTA) was added, mixed thoroughly and left for 10 min. Following cell lysis, the cell debris was pelleted by centrifugation (30000g, 4°C, 45 min). To the supernatant (13ml), CsCl (13g) was added and dissolved, followed by ethidium bromide (2.21ml, 10mg/ml). This mixture was placed in centrifuge tubes and centrifuged at 128000g (20°C, 16.5 h). Following centrifugation, plasmid DNA was visualised by UV illumination and removed with a syringe after first piercing the side of the tube with a needle. Ethidium bromide was removed from the gradient fraction by repeated extraction with an equal volume of

isopropylalcohol saturated with a solution of 5M NaCl, 10mM Tris-HCl, 1mM EDTA. The clear aqueous phase was then dialysed against sterile distilled water for several hours to remove CsCl. Plasmid DNA was then precipitated by the addition of sodium acetate (0.1 vol, 3M) and ethanol (2 vol) and left at -20°C overnight. DNA was pelleted by centrifugation (7600g, 4°C, 15 min). Following removal of the supernatant and all traces of ethanol, the DNA was dissolved in sterile distilled water (0.5ml) and quantified by measuring the absorbance at 260 nm.

8.2.4 Restriction Endonuclease Digestion of DNA

Restriction enzyme digestions were carried out using the following buffer conditions:

Aat2 10mM Tris-HCl(pH 7.5), 50mM KCl, 10mM MgCl₂, 1mM DTT.

BamHI 50mM Tris-HCl(pH 8.0), 50mM NaCl, 10mM MgCl₂, 1mM DTT.

ClaI 10mM Tris-HCl(pH 8.0), 10mM MgCl₂, 100ug/ml BSA.

DdeI 50mM Tris-HCl(pH 8.0), 50mM NaCl, 10mM MgCl₂, 1mM DTT.

EcoRI 50mM Tris-HCl(pH 8.0), 50mM NaCl, 10mM MgCl₂, 1mM DTT.

Hind3 50mM Tris-HCl(pH 8.0), 50mM NaCl, 10mM MgCl₂, 1mM DTT.

KpnI 6mM Tris-HCl(pH 7.5), 6mM NaCl, 6mM MgCl₂, 1mM DTT.

MluI 10mM Tris-HCl(pH 7.5), 50mM NaCl, 7mM MgCl₂, 100ug/ml

BSA, 6mM 2-mercaptoethanol.

NcoI 50mM Tris-HCl(pH 8.0), 50mM NaCl, 10mM MgCl₂, 50mM KCl.

NdeI 50mM Tris-HCl(pH 8.0), 50mM NaCl, 10mM MgCl₂, 1mM DTT.

NotI 10mM Tris-HCl(pH 7.9), 150mM NaCl, 10mM MgCl₂, 100

ug/ml BSA, 0.01% Triton X-100.

PstI 50mM Tris-HCl(pH 8.0), 50mM NaCl, 10mM MgCl₂, 1mM DDT.

SalI 50mM Tris-HCl(pH 8.0), 100mM NaCl, 10mM MgCl₂, 1mM DDT.

ScaI 6mM Tris-HCl(pH 7.4), 100mM NaCl, 6mM MgCl₂, 1mM DDT.

SmaI 5mM Tris-HCl(pH 8.0), 15mM KCl, 6mM MgCl₂.

SstI 50mM Tris-HCl(pH 8.0), 50mM NaCl, 10mM MgCl₂, 1mM DDT.

XbaI 50mM Tris-HCl(pH 8.0), 10mM MgCl₂, 1mM DDT.

XhoI 50mM Tris-HCl(pH 8.0), 50mM NaCl, 10mM MgCl₂, 1mM DDT.

Reactions were terminated in one of two ways; a) addition of loading buffer, if the digests were to be analysed by agarose gel electrophoresis, or b) phenol extraction followed by ethanol precipitation, if the DNA was to be digested with a second enzyme requiring different buffer conditions.

Restriction endonuclease buffers were prepared as 10x solutions and stored at -20°C. One unit of enzyme was required for every ug of DNA to be digested. For complete digests, reactions were incubated at 37°C for 1 h (except for digests with SmaI which were incubated at room temperature).

8.2.5 Dephosphorylation of Vector DNA

In some sub-cloning experiments the vector DNA was prepared for ligation by a single enzyme digest. In order to minimise re-ligation of such vector DNA molecules, it was necessary to remove the 5'-terminal phosphate groups using the enzyme calf intestinal alkaline phosphatase (CIP).

Vector DNA was digested to completion before addition of the required amount of CIP (0.01 unit/pmole of 5'-ends) to the restriction enzyme reaction. The dephosphorylation was then carried out at 37°C for 30 min and the vector DNA was subjected to agarose gel electrophoresis prior to purification and use in ligation reactions.

8.2.6 Agarose Gel Electrophoresis of DNA

Agarose gel electrophoresis was carried out using a commercially available horizontal sub-merged gel apparatus (Bio-Rad Labs.).

All gels were run at 100V for several hours at room temperature. Agarose gels of the required percentage (0.6-2%) were prepared in Tris-acetate buffer (40mM Tris-OH, 2mM EDTA, 20mM glacial acetic acid [pH 8.1]) with 1ug/ml ethidium bromide. The agarose (Seakem HGT) was mixed with the buffer and heated until the agarose went into solution. After cooling for 10 min the gel was poured and allowed to set. Once the agarose had set the well-formers were removed and the gel sub-merged in running buffer (40mM Tris-OH, 2mM EDTA, 20mM glacial acetic acid [pH 8.1]).

DNA samples were mixed with 1/5 vol. of 5x loading buffer and loaded into the sample wells. The DNA marker used was 1kb ladder (BRL).

If DNA fragments were to be purified following gel electrophoresis, then a preparative gel, using low melting point

agarose (BRL) was prepared and run as described for a standard analytical agarose gel.

8.2.7 Purification of DNA Fragments from LMP Agarose

Restriction fragments of DNA were separated on a low melting point agarose gel (% of agarose [w/v] varied according to the size of the fragment to be isolated) and purified according to the method of Wieslander (1979). Following separation by gel electrophoresis, DNA fragments were visualised using a long wave ultraviolet transilluminator. The desired fragment was excised from the gel with a sterile scalpel blade and transferred to a sterile Eppendorf tube. The volume of the agarose slice containing the DNA fragment was quantified and 0.1 vol. of 5M NaCl was added before heating at 65°C for 10 min. The melted agarose was vortexed and extracted with two vol. of phenol (saturated with 0.5M NaCl). Following centrifugation (5 min, Eppendorf centrifuge, room temperature), the aqueous phase was transferred to a fresh tube and extracted with two volumes of phenol as above. The aqueous phase was then extracted with isobutanol and the DNA was precipitated by the addition of 2.5 vol. of ethanol. The DNA was pelleted by centrifugation for 10 min at room temperature in an Eppendorf centrifuge, the ethanol was removed with a Pasteur pipette. The pellet was dried under vacuum and resuspended in 10ul of sterile distilled water.

8.2.8 Ligation Reactions

Ligation reactions were carried out in 1.5ml Eppendorf tubes using a 3:1 molar ratio of insert:vector DNA in a final reaction volume of 25ul (50mM Tris-HCl, pH 7.5, 10mM MgCl₂, 10mM DTT, T4 DNA ligase-2 units) and left at room temperature for 1-3 h. The products of the ligation reactions were then used to transform competent E.coli.

8.2.9 Grunstein-Hogness Colony Hybridisation

Colonies were grown overnight on nitrocellulose filters overlain onto ampicillin plates. Cells were lysed and DNA bound to the nitrocellulose filters by the following method: nitrocellulose filters were carefully placed (colonies up) onto filter paper soaked in NaOH (0.5M) for 8 min, neutralised by transferring the nitrocellulose to filter paper soaked in Tris-HCl (0.5M, pH 7.4), for 2 min. This last step was repeated on a second filter paper soaked in Tris-HCl as above, before transferring the nitrocellulose onto filter paper soaked in Tris-HCl (0.5M, pH 7.4), NaCl (1.5M), for 4 min. Nitrocellulose filters were then dried and baked at 80°C for 2 h.

All further steps in the procedure were performed at 65°C using solutions pre-warmed and degassed.

Filters were pre-treated by incubation in hybridisation buffer (12ml, 5x Denhardt's solution, 6x SSC) for 2-3 h. During this period, sites on the nitrocellulose filter that bind single or double stranded DNA non-specifically become saturated with components of the solution. Filters were transferred to fresh

hybridisation solution (12ml, 5x Denhardt's solution, 6x SSC) containing the freshly denatured radio-labelled probe (see section 8.2.10). This reaction was incubated overnight. Filters were then washed in hybridisation wash buffer (12ml, 3x SSC, 0.1% SDS or 1x SSC, 0.1% SDS) for 1 h with several changes of wash buffer. Filters were then dried, covered with cling-film, and the distribution of radio-label determined by autoradiography.

8.2.10 Preparation of Hybridisation Probes by Nick-

Translation

Restriction endonuclease fragments, isolated from agarose gels have strand discontinuities in the DNA duplex introduced by DNase I, and are nick-translated by E.coli DNA Pol. I which adds nucleotides to the 3'-OH termini created by the DNase I and removes residues from the 5'-termini. The "nick" thereby moves along the DNA and radio-labelled nucleotides in the reaction mixture are incorporated into the newly synthesised DNA strands (Rigby et al., 1977).

Nick-translations were performed in a reaction mixture containing DNA (10-50ug/ml), Tris-HCl (50mM, pH 7.8), MgCl₂ (5mM), 2-mercaptoethanol (10mM), dNTPs (50uM), if [³²P-dATP] label was used then dATP was omitted from the nucleotide pool, if [³²P-dCTP] was used then dCTP was omitted), DNase I (50 ng/ml) and E.coli DNA Pol. I (50u/ml). The reaction mixture was incubated at room temperature for 1 h, boiled for 7 min to denature the DNA, then added to hybridisation buffer (12ml, 5x

Denhardtts, 6x SSC).

8.2.11 Oligonucleotide Purification

After the synthesis of a deoxyoligonucleotide on Applied Biosystems Model 381A DNA synthesiser, the DNA produced is not biologically active, but is protected to prevent side reactions. The protecting groups have to be removed to produce biologically active DNA, which then has to be released by hydrolysis from the solid support. The three types of protecting groups are; a) 5' protecting group dimethoxytrityl (DMT), b) phosphate protecting groups methyl, c) base protecting groups benzoyl on A and C residues and isobutyryl on G residues.

The 5' protecting group, the dimethoxytrityl group, is attached to each nucleoside phosphoamidite and is cleaved from the growing oligonucleotide during each cycle. The last DMT group is removed as part of the synthetic cycle.

The methyl groups are removed from the phosphates with a thiophenol treatment which transforms the neutral triester phosphates to charged diester phosphates. Cleavage of DNA from the solid support is carried out with ammonia or ammonium hydroxide.

The method used for deprotection and cleavage of the oligonucleotides was originally developed by Gait and Sheppard (1977), and has been adapted for use with Applied Biosystems synthesis columns.

After the synthesis is complete, the column was removed and fitted onto the syringe Luer. The other end of the column was

fitted with the male to male Luer connector which was in turn fitted with the syringe needle.

With the syringe plunger inserted in the syringe barrel to the 0.5ml mark, thiophenol/triethylamine/dioxane was drawn up to fill the column. The needle was then inserted into the rubber stopper and left for 30 min. The thiophenol solution was expelled into bleach to inactivate it and the column was then washed 10x with methanol. Finally, the column was filled with ammonia (room temperature), the needle inserted in the rubber stopper and left at room temperature for 30 min. The ammonia was expelled into a 1.5ml Eppendorf tube. The ammonia step was repeated three more times.

To remove the base protecting groups, the Eppendorf tube of DNA in ammonia was heated to 55°C for at least 5 h. The oligonucleotide was now biologically active and was purified by PAGE.

Prior to PAGE, oligonucleotides of greater than 40 bases were subjected to desalting by gel exclusion chromatography. This eliminates the low molecular weight failure sequences and concentrates the product in the first few UV absorbing fractions. A Sephadex G-50 column was packed in a drawn out Pasteur pipette plugged with a small amount of polyallomer wool. Vacuum dried oligonucleotide was redissolved in water (200ul) and loaded onto the column. Fractions (200ul) were obtained by loading 200ul of water and collecting until the column stopped dripping water. The largest oligomers will elute first, usually in the seventh to

ninth fractions. 10ul of each fraction was added to 490ul of water, the absorbance of which was measured at 260 nm. The fractions with high UV absorption were pooled and vacuum dried before being subjected to PAGE.

Following vacuum drying, a quarter of the product (in 15ul) was added to formamide loading dye and heat denatured (3 min, 100°C) before being loaded on the gel (0.5 mm x 5 cm x 2 cm well). Electrophoresis was carried out at 40 W for several hours. Oligonucleotide was visualised by ultra-violet "shadowing", and a slice of the gel containing the oligonucleotide was cut from the gel with a sterile scalpel blade. To UV "shadow", the gel plates were separated carefully leaving the gel adhering to one of the plates. Saran wrap was stretched over the gel and then, grasping the gel plus the Saran wrap, the gel was peeled off the glass plate. The gel plus Saran wrap were placed on top of a sheet of Whatman 3MM chromatography paper impregnated with a liquid scintillant, such that the Saran wrap was sandwiched between the gel and the 3MM paper. The oligonucleotide bands were observed and marked under a handheld UV lamp (short wavelength). The desired band is usually the darkest band, positioned at the top of the ladder. The shortwave ultra-violet light makes the liquid scintillant fluoresce but the DNA absorbs the UV light to produce a "shadow" or dark band on the gel.

The gel slice was placed in an Eppendorf tube, allowed to dry for several hours then roughly mashed up. Water (200ul) was added to the tube and left at 4°C overnight. The oligonucleotide was

then desalted using a 1ml spinning column packed with Sephadex G-25.

8.2.12 Desalting Oligonucleotides

The final step in the purification of oligonucleotides was desalting by spinning column chromatography. The column was prepared as follows; after removing the plunger from a 1 cm³ syringe, the syringe was plugged with sterile polyallomer wool and packed with 1 cm³ of Sephadex G-25 (equilibrated with 10mM Tris-HCl, 1mM EDTA, pH 8.0). The syringe was inserted into a de-capped Eppendorf tube within a 17ml corex tube. This was then briefly centrifuged to pack the column. Sephadex G-25 was added and packed until the volume of the column was 1 cm³. The packed column was then washed with distilled water. Finally, the oligonucleotide solution (400ul) was loaded onto the column and centrifuged (1400g, 30 sec). The eluate, containing the purified oligonucleotide was collected in a sterile Eppendorf tube and the concentration of the oligonucleotide was then measured by the absorbance at 260 nm (OD of 1 = 20ug/ml) and diluted as required.

8.2.13 Phosphorylation of Purified Oligonucleotides

Prior to its use in mutagenesis the purified oligonucleotide was phosphorylated at the 5'-end with T4 polynucleotide kinase. This allows ligation rather than strand displacement to take place in the mutagenesis reaction.

Purified mutagenic oligonucleotide (20-50 pmole) was added to

a mixture containing : Tris-HCl (50mM, pH 7.5), MgCl₂ (10mM), DTT (1mM), rATP (1mM) and T4 polynucleotide kinase (1 unit), (total reaction volume = 10ul), and incubated at 37°C for 60 min.

8.2.14 Oligonucleotide-Directed Mutagenesis of DNA Fragments

Cloned Into M13 Vectors

The method used was essentially the gapped duplex DNA method of Kramer et al., (1984).

In a 1.5ml Eppendorf tube, 0.1 pmole (0.5ug) of M13mp8 (digested with Hind3 and EcoRI) were mixed with 0.5 pmole (1.25ug) of single stranded template DNA in a total volume of 40ul of KCl (200mM), Tris-HCl (12.5mM, pH 7.5). This annealing mixture was boiled for 3 min and then incubated at 65°C for 5 min. An 8ul aliquot of this hybridisation mixture was then mixed with 4-10 pmole (2ul) of 5'-phosphorylated mutagenic oligonucleotide and incubated at 65°C for 3 min, then allowed to cool at room temperature for 20 min. The mixture was then adjusted to the following; 100mM KCl, 30mM Tris-HCl (pH 7.5), 15mM MgCl₂, 2mM DTT, 125um ATP and 125um of each of the four dNTPs with DNA polymerase I Klenow fragment (1 unit) and T4 DNA ligase (2 units) added (final reaction volume: 40ul). The reaction was incubated at room temperature for 45 min before being stopped by the addition of EDTA (1ul, 0.5M, pH 8.0) and heating to 65°C for 10 min. The mixture was then extracted with an equal volume of phenol/chloroform (1/1, v/v) followed by three extractions with diethylether (100ul). Residual traces of ether

were evaporated at 65°C and the mixture was then used for transformation of mismatch repair deficient strain of E.coli (BMH 71-18 mutL).

8.2.15 Preparation of M13 Template ss DNA

M13 phage plaques were picked using a sterile Gilson tip and used to infect 1ml cultures of Luria broth seeded with an overnight culture of JM 101 cells (1/100 dilution). M13 infected cultures were grown in Bijoux bottles at 37°C for 5 h. Cultures were transferred to 1.5ml Eppendorf tubes and cells pelleted by centrifugation for 5 min. The supernatant was transferred to fresh Eppendorf tubes and M13 phage particles were precipitated by the addition of 200ul of a solution of PEG (10% [w/v]) and NaCl (2.5M). After mixing, the tubes were left at room temperature for 30 min and then M13 phage was pelleted by centrifugation for 5 min. The supernatant was removed with a Pasteur pipette (tubes without pellets were discarded at this stage), the tubes re-spun, and any remaining supernatant was removed using a drawn out Pasteur pipette. Sodium acetate (100ul, 1.1M, pH 7.4) was then added to the phage pellet and left at 4°C overnight. Capsid protein was removed by vortexing with an equal volume of phenol/chloroform (1/1, v/v). The aqueous phase was extracted with 75ul of chloroform/IAA (24/1, v/v) and the ss DNA template was then precipitated from the aqueous phase by the addition of ethanol (250ul) and chilling at -70°C for 30 min. The DNA was then pelleted by centrifugation for 10 min, the supernatant removed and the pellet vacuum dried then dissolved in

20ul Tris-HCl (10mM, pH 8.0), EDTA (0.1mM).

8.2.16 Single Track Screening of Templates

Following an oligonucleotide-directed mutagenesis reaction, in the absence of any selection, only a small percentage of the plaques obtained are mutants. To distinguish between wild-type and mutant phage a screening procedure is required. In the case of the CAT mutagenesis single track sequencing with di-deoxy A was used. This method was chosen as the mutations are close to the universal primer binding site and the desired mutation reduces a stretch of seven consecutive A residues to four (see Chapter 3), which is easily visualised on a gel. Since transformation and plating were carried out without a cycle of replication and reinfection, the plaques obtained contain a mixture of wild-type and mutant phage. Therefore, any potential mutant identified by the initial screening was plaque purified and sequenced, to verify that the desired mutation had been produced.

The single track and DNA sequencing reactions are both based on the dideoxynucleotide chain-termination method of Sanger et al., (1977) with some modifications (Biggin et al., 1983).

Template DNA (1ul) was mixed in an Eppendorf tube with 1ul of priming mix; Tris-HCl (10mM, pH7.4), MgCl₂ 10mM) and universal primer (0.2 ng). The tubes were sealed and placed in a boiling water bath for 3 min then allowed to cool at room temperature for 20 min. The tubes were briefly centrifuged and the lids removed.

1.5ul of reaction mixture was added; 1ul dideoxynucleotide/deoxynucleotide mix (see Table 8.1), DTT (0.5ul, 3mM), [³⁵S-dATP] (1uCi.) and Klenow (0.3 units). The reaction was left at room temperature for 20 min then chased for 20 min with the addition of chase mix (1ul, 0.25mM dATP, dCTP, dGTP, dTTP). Finally, formamide dye (2ul, 0.1% xylene cyanol FF, 0.1% bromophenol blue, and 10mM EDTA, in formamide) was added, the mixture boiled for 3 min and reaction products were analysed by PAGE.

Polyacrylamide gels were formed between glass plates (20 cm x 50 cm), separated with plasticard spacers (1 cm x 50 cm x 0.4 mm), and sealed with adhesive tape. Gels contained acrylamide (5.7%, w/v), bisacrylamide (0.3%, w/v), urea (7.66M), Tris (90mM), boric acid (90mM), and EDTA (25mM). Electrophoresis was carried out at 40 W (approximately 20 mA, 2000 V) for 2 h. Gels were fixed in 10% acetic acid (v/v) for 15 min the gels were then transferred to Whatman 3MM paper, covered in Saran wrap and dried on a Bio Rad 1125B gel drier (Bio Rad Laboratories Ltd, Watford, U.K.) before being autoradiographed.

8.2.17 DNA Sequencing

All the DNA fragments that were sequenced were sub-cloned into the phage M13 vectors and sequenced using the dideoxynucleotide sequencing method (Sanger et al., 1977) with some modifications (Biggin et al., 1983).

Priming of template DNA was carried out in 1.5ml Eppendorf

tubes, in a 5ul mixture containing; 2.5ul of template DNA, 1 ng primer, Tris-HCl (10mM, pH 8.5), MgCl₂ (5mM). The tubes were sealed, placed in a boiling water bath for 3 min then allowed to cool at room temperature for 15 min. After priming, DTT (4ul, 3mM), [³⁵S-dATP] (2uCi.) and E.coli DNA polymerase I Klenow fragment (0.4 units) were added to each tube. For each of the primed template mixes, four 2ul aliquots were transferred to four decapped 1.5ml Eppendorf tubes, each containing 1ul of dideoxy A/G/C/T nucleotide mixes respectively (see Table 8.1). After 20 min at room temperature, sequence chase (1ul, 0.25mM dATP, dGTP, dCTP, dTTP) was added, and after a further 20 min the tubes were either frozen or prepared for electrophoresis as in single tracking. On short sequencing runs 6% acrylamide TBE gradient gels containing 8M urea were used (Biggin et al., 1983). Electrophoresis was carried out at a constant 40 W (approximately 20 mA and 2000 V) with 0.5 x TBE in the upper chamber and 1 x TBE in the lower. After electrophoresis the gel was fixed and dried as for single tracking gels before being subjected to autoradiography.

TABLE 8.1

Composition of Nucleotide Sequencing Mixes

	A	G	C	T
	(ul)	(ul)	(ul)	(ul)
0.5mM dGTP	250	12.5	250	250
0.5mM dCTP	250	250	12.5	250
0.5mM dTTP	250	250	250	12.5
5mM ddATP	1			
10mM ddGTP		8		
10mM ddCTP			4	
10mM ddTTP				25
T.E.*	250	500	500	500

* Tris-HCl (10mM, pH 8.0), EDTA (1mM).

8.2.18 Preparation of Double-Stranded M13 Replicative Form

DNA

M13mp8 (Messing and Vieira, 1982) double-stranded replicative form (RF) DNA, for use in gapped duplex mutagenesis, was prepared as follows; 1ml of Luria broth seeded with a 1:100 dilution of an overnight culture of E.coli JM 103 was infected with a single phage plaque and incubated with vigorous aeration at 37°C for 5 h, 100ul of this was then added to 10ml of Luria broth seeded with a 1:100 dilution of an overnight culture of E.coli JM 103 and incubated, as above, overnight. The cells were harvested from this culture and double-stranded RF DNA was extracted and

purified using the 10ml rapid isolate method described previously.

8.2.19 In-vitro Synthesis of RNA

In-vitro synthesis of RNA from cDNA templates was performed using T7 RNA polymerase to test the viability of T7 promoters inserted in plasmids (see Chapter 6). Reaction mixtures (20ul) contained linearised plasmid DNA (1ug), Tris-HCl (40mM, pH 7.5), MgCl₂ (6mM), spermidine (2mM), NaCl (10mM), DTT (10mM), RNA guard (20 units), rATP, rCTP, rGTP, rUTP, (1mM each), BSA (RNase, DNase free, 0.5ug/ul), and T7 RNA polymerase (10 units). Following incubation for 30-60 min at 40°C, product RNA was examined by electrophoresis in an agarose gel (1%).

8.2.20 Growth of HeLa Cells in Culture

The human HeLa cell line was maintained by continuous passage in MEM (Eagle) supplemented with 5% NCS and 0.22% NaHCO₃. Cells were routinely grown in 175 cm³ disposable flat-bottomed flasks. To passage cells, confluent monolayers were washed twice with PBS (calcium and magnesium free). After removal of the PBS wash, 10ml of a trypsin suspension (0.25% in Puck's saline) was added to each flask and incubated at room temperature for 2 min. Trypsin was then removed and the flask incubated at 37°C for several minutes. After this time, medium was added to each flask and the cells dislodged by violent shaking. Aggregates of cells were disrupted by repeated pipetting. Aliquots of these cells were

then dispensed into flasks and pre-heated medium was added to a final volume of 75ml. Cells were incubated at 37⁰C in a humid, 5% CO₂ atmosphere and split when monolayers reached confluence.

8.2.21 DNA Transfection

a) Buffers and solutions. HEPES-buffered saline was prepared at 2x strength and stored at 4⁰C in polyethylene tubes until required. 2x HEPES-buffered saline consisted of 1% (w/v) HEPES and 1.6% (w/v) NaCl, adjusted to pH 7.1 with NaOH. 100x phosphate was prepared by mixing equal volumes of 70mM NaH₂PO₄ and 70mM Na₂HPO₄. 2M CaCl₂ was stored at -20⁰C in polyethylene tubes for up to one month. All solutions were prepared in Nanopure distilled water and filter sterilised.

b) Preparation of Cell Monolayers. Monolayers were seeded the day before transfection. A single 175 cm³ flask was split into eight 10 cm petri dishes to give monolayers of between 70% and 90% confluence the following day. Cell monolayers to be transfected were grown in MEM + 2% FCS.

c) Calcium Phosphate/DNA Co-precipitates. Transfection solutions were equilibrated to room temperature. Solution A and solution B were prepared in 10ml test-tubes as follows; solution A, 50ul of 100x phosphate and 2.5ml of 2x HEPES-buffered saline, solution B contained 300ul of 2M CaCl₂, 100ug of supercoiled plasmid DNA and sterile distilled water to a final volume of 2.5ml. Each solution was vortexed and solution B was added dropwise to solution A. B was added to A while a continuous stream of air was bubbled

through solution A. After all of solution B was added, the solution was vortexed for 30 sec and left at room temperature for 30-40 min. A fine chalky-white precipitate is formed during this time.

d) Transfection. The medium was removed from each of four sub-confluent monolayers. 1ml of the calcium phosphate/DNA co-precipitate was added to each monolayer. After incubation at 37°C for 20 min, 9ml of MEM + 10% FCS was added to each plate. The monolayers were then incubated for a further 4 h at 37°C.

e) Glycerol Shock. Medium containing the DNA precipitate was removed from each plate and the monolayers were washed with MEM + 2% FCS. 3ml of glycerol solution (20%, v/v, in HEPES-buffered saline) was then added to each plate. After incubation at 37°C for 3 min, the glycerol solution was removed and the monolayers were washed three times with MEM + 2% FCS. Finally, 10ml of MEM + 2% FCS was added to each plate and the transfected monolayers were incubated for 5-7 days at 37°C. For a viral plaque assay 3ml of agar overlay was added to the cells instead of the 10ml of MEM + 2% FCS. The agar overlay consisted of MEM + 2% FCS + 1% Noble agar and was prepared by mixing equal volumes of 2x MEM + FCS and 2% Noble agar at 45°C. Monolayers were incubated at 37°C in an inverted position.

When transfections were carried out for CAT assays no glycerol shock was included in the transfection protocol. The DNA precipitate was added to the medium, and this was incubated with the cells overnight at 37°C. The medium containing the

precipitate was then removed and the cells were washed with fresh medium as described above.

8.2.22 CAT Assays

After transfection (48 h) with plasmids expressing the CAT gene, cell monolayers were harvested, cell extracts prepared and assayed for CAT activity following the method of Gorman et al., (1982a).

Cell monolayers were washed three times with PBS before being harvested in PBS (2ml) using a rubber policeman. Cells were transferred to Eppendorf tubes and pelleted by centrifugation (1 min, Eppendorf centrifuge). The supernatant was removed and cells resuspended in Tris-HCl (0.1ml, 0.25M, pH 7.8), pelleted and resuspended in fresh Tris-HCl (as above). Cell extracts were then prepared by repeated cycles of rapid freeze-thawing (frozen in liquid nitrogen and thawed at 37°C). Following lysis, the cell debris and nuclei were pelleted by centrifugation (15 min, Eppendorf centrifuge), the supernatants removed (can be stored at -20°C at this point) and a sample assayed for total protein content. Protein concentration was determined with a colorimetric assay kit purchased from Bio Rad, using known concentrations of BSA as standards. Equivalent amounts of protein were then assayed for CAT activity. The assay reaction mixture contained the following; Tris-HCl (70ul, 0.25M, pH 7.8), H₂O (35ul), 20ul diluted cell extract (in Tris-HCl, 0.25M, pH 7.8), [¹⁴C] chloramphenicol (1ul, 40-50 Ci/mmol). These were mixed and

allowed to equilibrate (37°C, 5 min) before starting the reaction by adding acetyl CoA (20ul, 4mM). The reaction was left at 37°C for 90 min before being stopped, and the chloramphenicol extracted, by the addition of ethyl acetate (1ml). After vortexing (30 sec) and centrifugation (2 min, Eppendorf centrifuge), the organic phase, containing chloramphenicol and the reaction products, was transferred to a fresh Eppendorf tube and dried under vacuum (30 min, 55°C). Samples were then resuspended in ethyl acetate (15ul) and spotted onto silica gel thin layer chromatography plates (Merck).

Plates were then subjected to ascending chromatography with a solvent mixture of chloroform:methanol (95:5, v/v) for 13 min. After air drying, the chromatography plate was exposed to X-ray film overnight.

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STUDIES ON THE 5' NON-CODING REGION OF THE GENOME OF POLIOVIRUS.

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ABSTRACT

The last decade has seen widespread application of recombinant DNA technology to the study of picornaviruses. Comparative sequence analysis has revealed that the most highly conserved region amongst many members of this family of viruses is the 5' non-coding region. Using recombinant type 3 polioviruses it has been shown that a single point mutation located in this region dramatically reduces neurovirulence and inhibits the intracellular life-cycle of the virus. Mutation at this nucleotide contributes to the observed reversion to neurovirulence of the Sabin attenuated poliovirus type 3 vaccine strain currently used in vaccination programmes throughout the world. Knowledge concerning the function of the 5' non-coding region remains scant, and as a result, the mechanism whereby a single point mutation within this region results in alteration of the expressed phenotype of the virus remains unknown. Clearly, an understanding of the molecular mechanism(s) involved requires greater knowledge of the function of the 5' non-coding region.

This thesis describes the design and construction of vectors that allow analysis of the role of the 5' non-coding region in the control of viral translation, replication, and encapsidation of viral RNA. In the plasmid pRSV-5'polio-CATm2(N⁺), the 5' non-coding region of poliovirus was fused to the coding region of the bacterial chloramphenicol acetyltransferase reporter gene. The presence of the 5' non-coding region resulted in the inhibition of CAT expression when this plasmid was introduced into eukaryotic cells in culture. Deletion analysis of the 5' non-coding region in this vector identified two regions that were responsible for the marked inhibition of expression of the reporter gene. It would appear from the results of these experiments that the poliovirus/CAT chimaeric message is translated as a normal eukaryotic mRNA and is subject to the rules of the "scanning model". This observation suggests that the 5' non-coding region of poliovirus on its own does not possess features which enable a message containing it to be translated efficiently. It is concluded that a second factor, present in infected cells, is required for the efficient translation of poliovirus. A second plasmid was designed and constructed to investigate the role of the 5' non-coding region in replication and encapsidation of viral RNA. Preliminary data suggest that the product of this vector does undergo replication while its ability to be encapsidated has still to be tested.