ISOLATION AND CHARACTERIZATION OF CONDITIONAL LETHAL AMBER NONSENSE MUTANTS OF VESICULAR STOMATITIS VIRUS

by

BRIAN TEMPLETON WHITE

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INSTITUTE OF VIROLOGY
CHURCH STREET
GLASGOW G11 5JR
SCOTLAND

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NON STANDARD ABBREVIATIONS

ACR	5-azacytidine
Ad	adenovirus
Amb	amber
AMV	avian myeloblastosis virus
BMV	brome mosaic virus
BSA	bovine serum albumin
C (terminus)	carboxy terminus (of a protein)
CS	calf serum
DI	defective interfering particle
DMEM	Dulbecco's modification of Eagle's medium
EMS	ethyl methyl sulphonate
FCS	foetal calf serum
5-FU	5-fluorouracil
hr	host range
HSV	herpes simplex virus
Ind	Indiana (serotype of VSV)
kb	kilobase
MEM	minimal essential medium
M_r	relative molecular mass
MTX	methotrexate
NA	nitrous acid
NTE	NaCl, Tris, EDTA
N (terminus)	amino terminus (of a protein)
NDV	Newcastle disease virus
PBS	phosphate buffered saline
RNP	ribonucleoprotein
RT	room temperature
SDS	sodium dodecyl sulphate
su^+	genome with suppressor
su^-	genome without suppressor
SV	Sendai virus
TEMED	N,N,N',N'-tetramethylethylenediamine
TK	thymidine kinase
TMV	tobacco mosaic virus
TNP	transcribing nucleoprotein
uv	ultraviolet
VSV	vesicular stomatitis virus

SUMMARY

The work reported in this thesis is concerned with the isolation and characterization of conditional lethal amber nonsense mutants of the rhabdovirus vesicular stomatitis virus (VSV), Indiana serotype. Two murine cell lines were employed in this work. L39 (su^+) cells carried an amber suppressor tRNA gene, which was expressed, albeit at a relatively low level. These cells thus represented the permissive host for virus containing a conditional lethal amber mutation. The second cell line used was the cell line $LMTK^-$ (su^-). This was the parental line from which the L39 cell line was derived, and represented the non-permissive host for virus containing a nonsense mutation in an essential gene. A screen was set up to detect conditional lethal nonsense mutants of VSV, by assaying growth of mutagenized isolates on su^+ and su^- cells and retaining those which grew only on su^+ cells.

A wt stock of VSV (Indiana serotype) was mutagenized by the chemical mutagen 5-fluorouracil. The mutagenized virus was plated on the su^+ cells to produce well isolated plaques. Small plaques were picked to screen for a mutation affecting host range. Screening was performed by infecting monolayers of su^- cells and inspecting the monolayers for the presence or absence of cpe. Any isolates which did not give rise to cpe were subsequently used to infect monolayers of su^+ cells. The virus from productive infections was harvested and titrated on both cell lines to quantify the restriction in host range. Of 2614 plaques picked originally, 82 did not give rise to cpe on the su^- cells and of these 13 produced a difference in titre between the two cell lines of 30 fold or more. Viral protein synthesis in su+ and su- cells infected by each of these host range mutants was investigated by polyacrylamide gel electrophoresis. In comparison to the characteristic protein species synthesized by wt virus, seven mutants exhibited little virus specific protein synthesis in su^- cells.

Complementation analysis was carried out for each of these seven mutants with ts mutants representing each of the five VSV complementation groups. Five of the mutants were assigned to complementation group I (the L cistron), one to complementation group V (the G cistron) while a seventh mutant could not be complemented. No intragenic complementation was observed between any of the group I mutants.

The five group I mutants (retrospectively named AmbL1 to AmbL5) did not synthesize any detectable full length L protein in the su^+ cells. Four (AmbL1 to AmbL4) synthesized an additional protein species in the su^+ cells. An additional protein could not be identified for the fifth mutant, however. The molecular weights of the additional proteins were estimated to be 37000, 150000, 230000 and 227000 for AmbL1, AmbL2, AmbL3 and AmbL4 respectively (full length L protein has a molecular weight of 241012). The additional proteins were also detected by *in vitro* translation of mRNA isolated from su^+ cells infected by the mutants AmbL1 and AmbL2. These proteins were proposed to be fragments of the L protein produced by premature termination of translation within the L mRNA at an amber codon.

The mutants were found to be greatly restricted in the su^- cells. Analysis of the proteins synthesized in su^- cells revealed that the inhibition of host protein synthesis associated with wt infection of su^- cells was markedly reduced. The additional protein synthesized by these mutants in su^+ cells was only observed in su^- cells with some of the mutants (AmbL1, AmbL3 and AmbL4) after prolonged incubation. This is thought to be caused by outgrowth of revertants complementing the mutant late in infection. Analysis of the protein composition of the virion of these mutants showed that the shortened form of the L protein was not packaged.

The additional protein synthesized by AmbL1 and AmbL2 (and possibly AmbL4) in the su^+ cells was present in considerably higher abundance than either wild-type L protein or the truncated L protein found in AmbL3 infected cells. This is thought to reflect the action of a control

system on L protein synthesis.

Immunoprecipitation and immunoblotting analysis of the group I mutants with monospecific antisera directed against the N and C termini of the wt L protein provided direct evidence that the additional proteins produced by the group I mutants were derivatives of L protein and possessed N-terminal specific sequences but not C-terminal specific sequences. Immunoprecipitation and immunoblotting studies with AmbL5 each revealed a possible (but distinct) candidate for an L truncation protein. These results reinforce the interpretation that the mutants synthesize a truncated form of the L protein in the su^+ cells as a result of premature termination at an amber nonsense codon within the L mRNA.

Revertants of the group I mutants AmbL1, AmbL2, AmbL3 and AmbL4 were isolated by plating stocks of each mutant to give isolated plaques on the su^- cells. Such revertants were found at frequencies of 10^{-3} to 10^{-4} of the mutant titre and all had regained the normal plaque morphology of wt virus. All the revertants analysed synthesized an L protein of normal abundance and mobility in su^+ and su^- cells. The truncated version of L was not detected. The revertants are thus the product of back mutations in the same codon as the original mutation. No revertants of AmbL5 could be isolated, suggesting that AmbL5 may represent a multisite mutation within the L gene.

The sixth mutant (retrospectively named AmbG) assigned by complementation analysis to complementation group V, representing the G gene, synthesized a glycoprotein which possessed a slightly higher mobility than wt G protein. *In vitro* translation of mRNA isolated from AmbG infected cells showed that the altered mobility exhibited by the G protein was not the result of a difference in glycosylation. However, the mutant also synthesized this altered G protein after infection of the su^- cells, as did putative revertants isolated on the su^- cells. The difference in titre between the su^+ and su^- cells obtained for this mutant was only 30 fold. Thus it is proposed that this mutant is 'leaky' in the su^- cells.

A seventh mutant $hrTK^-7$ synthesized N and NS proteins

in $\underline{su}^+$ cells which possessed altered mobilities in polyacrylamide gels. This mutant is also thought to be a multiple mutant, since no revertants were isolated and attempts to assign the mutation to a specific complementation group were unsuccessful.

The aminoglycoside G418 which had been reported previously to facilitate readthrough of amber mutations in various systems, by affecting the fidelity of translation, failed to suppress the mutation (as judged by virus growth) in the group I mutant AmbL1.

In conclusion, short of actual nucleotide sequencing, the results obtained are entirely consistent with the interpretation that these are indeed conditional lethal amber mutants. They are thus the first such conditional lethal nonsense mutants to be isolated for an animal virus.

INTRODUCTION

INTRODUCTION

CHAPTER 1 NONSENSE MUTATIONS AND SUPPRESSION

Conditional mutants have proven to be an indispensable tool in the genetics and study of gene expression in bacteria and yeast. This approach has been at its most powerful in these systems with the employment of suppressible mutations. Among eukaryotic organisms however, due to the lack of suppressing cell lines, suppressible mutants have been studied extensively only in yeast and to a lesser extent in Aspergillus nidulans and in the nematode Caenorhabditis elegans. As a result, the development of animal virus genetics has instead relied on the use of temperature sensitive mutations for conditional lethal mutants. Now however, this situation is changing rapidly: advances in genetic construction techniques have allowed the development of a variety of suppressor tRNAs which, after introduction into the host cell, have been shown to suppress known nonsense mutations. Potentially the most useful development has been the construction of suppressor cell lines which contain a functional integrated suppressor tRNA gene. The objective of this thesis has been to investigate the use of a particular suppressor cell line in the isolation and characterization of conditional lethal amber nonsense mutants of the negative strand RNA virus vesicular stomatitis virus.

Since this study encompasses both the general fields of animal virology and nonsense mutations and suppression, the introduction to this thesis is divided into two chapters. Chapter 1 is entitled Nonsense mutations and suppression, and Chapter 2 is entitled The genetics of vesiculoviruses.

1.(a) NONSENSE MUTATIONS AND SUPPRESSION

The main aim of this chapter is to provide an overview of the field of translational suppression, specifically nonsense mutations and tRNA suppressors. The

chapter covers classic work on nonsense suppressors in bacteria and yeast as well as the latest developments in the search for nonsense mutations and tRNA suppressors in higher eukaryotes. Initially, however, I shall deal with the topic of nonsense mutations and their significance in gene expression. This will include related topics such as polarity and peptide chain termination. The second half of this chapter wil concentrate on the phenomenon of suppression in various organisms, ranging from prokaryotes (Escherichia coli), through lower eukaryotes (yeast, A. nidulans and the nematode C. elegans) and concluding with a section devoted to the relatively recent developments in mammalian cell suppression.

1.(a).1 NONSENSE MUTATIONS

In prokaryotes and eukaryotes the nonsense triplets UAA (ochre), UAG (amber) and UGA (opal) signal termination of translation and release of the polypeptide chain from the tRNA on the ribosome (Brenner et al., 1965; Weigart and Garen, 1965; Stretton and Brenner, 1965; Sambrook et al., 1967; Zipser, 1967; Beaudet and Caskey, 1971; Stewart and Sherman, 1972, 1973). However, when nonsense mutations occur within a polypeptide coding region, they cause premature chain termination at the point of mutation and release a fragment of the complete polypeptide (Sarabhai et al., 1964; Celis et al., 1973). Nonsense mutations were first discovered in bacteria when it was found that shortened forms of bacterial cell or bacterial virus proteins resulted from certain types of mutation. Nonsense mutations are considered conditional mutations since the mutant phenotype can be suppressed in the presence of a suppressor tRNA which inserts an amino acid at the nonsense codon allowing completion of the protein chain (suppression is dealt with in section 1.(b).1 below).

Nonsense mutations are classified according to the suppressor loci to which they respond. Amber (UAG) mutants were isolated by Benzer and Champe (1961) from a study of the rII mutants of phage T4. Brenner and Beckwith (1965) uncovered another class of chain termination mutation,

termed ochre (UAA), which were not suppressed by amber suppressors. However, suppressors of the ochre mutants also suppressed amber mutants implying that although the two classes of mutant were distinct, they must be related. Sambrook et al. (1967) and Zipser (1967) found a third class of nonsense mutant, termed opal (UGA). Opal suppressors do not suppress either amber or ochre codons.

Brenner et al. (1965) deduced the primary sequence of the amber and ochre codons by studying the production and reversion of rII amber and ochre mutants using the transition mutagens hydroxylamine and 2-aminopurine. Analysis of amber mutants of the head protein of the rII mutants of phage T4 defined the amino acids whose codons could be mutated to the amber triplet and comparison of the results with known amino acid codons helped to deduce the structures of the amber and ochre triplets. However, the normal termination triplets do not always have the same function. For example, in animal mitochondria the codon UGA is used as a Trp codon. In addition, the codons AGA and AGG (Arg) are stop signals in human mitochondrial DNA.

At a nonsense codon, in the absence of a suppressor, peptide chain termination occurs on the ribosome, and requires soluble protein factors in both prokaryotic and eukaryotic systems. Both mammalian and bacterial cells utilize the same terminator codons. In bacterial cells these codons are recognized by protein release factors (RFs) which are codon specific (Scolnick et al., 1968); RF-1 is specific for the codons UAA or UGA and RF-2 is specific for the codons UAG or UGA. In mammalian cells however, a single release factor recognizes all three terminator codons (Goldstein et al, 1970). A third protein factor, RF-3 identified in bacterial extracts (Milman et al., 1969) has the capacity to facilitate the binding of release factor to ribosomes (Goldstein and Caskey, 1970) and interacts with GDP and GTP. Since mammalian RF alone is stimulated by GTP, a separate RF-3 equivalent may not be involved. The hydrolysis of peptidyl-tRNA at chain teminators also requires the ribosomal enzyme peptidyl transferase.

1.(a).2 POLARITY

Bacterial operons encode polycistronic messengers which are translated sequentially into proteins. Because of this, a nonsense mutation in an early gene of an operon will affect not only the gene in which it resides but also the subsequent cistrons. Ames and Hartmann (1963) reported the existence of point mutations which as well as inactivating the enzyme encoded by the gene (by premature termination at the nonsense codon) also caused a reduction in the amount of protein produced from cistrons distal to the mutation. This phenomenon was termed polarity.

Newton et al. (1965) examined the effect of the position of nonsense mutations within the lactose operon of E. coli. They observed that mutations which were operator proximal were completely polar allowing virtually no synthesis of the subsequent proteins whereas mutations which were operator distal displayed little polarity. Yanofsky et al. (1971) found that deletion of a large part of the operator proximal gene between the site of mutation and the end of the gene restored activity of the neighbouring genes to previously strongly polar mutants. This provided evidence that it is the distance between the nonsense mutation and the following initiation site that is crucial in polarity. Thus double mutations constructed in consecutive cistrons reduce proportionally the synthesis of proteins distal to them.

Yanofsky and Ito (1966) observed that when two nonsense mutations reside in the same gene, the second mutation has no effect on the polarity resulting from the presence of the first mutation. This, however, is not always the case since if the first mutation is very close to the operator then there is a possibility that reinitiation may occur and the second mutation can then act as a chain terminator.

The strong coupling between transcription and translation in prokaryotic systems led to the proposal that the polar effects of a nonsense mutation are caused by the RNA polymerase encountering previously latent sites for termination of transcription that become active due to the

lack of translation (de Crombrugghe et al. 1973; Shimizu and Hayashi, 1974). This model for polarity includes two major premises: (1) that there are rho (transcription termination factor) dependent sites of transcription termination within operons, and (2) these sites are not susceptible to the influence of rho as long as the mRNA is concurrently translated (Adhya and Gottesman, 1978). Support for these features was provided by the discovery of a class of polarity suppressors that do not restore translation, and these mutations have been shown to affect rho factor (Ratner, 1976; Das et al., 1977)

1.(a).3 NONSENSE MUTATIONS IN HIGHER EUKARYOTES AND THEIR VIRUSES

An important property of nonsense mutations lies in their observed stringency, with a few exceptions, in both prokaryotes and eukaryotes. This is especially so since the application of traditional conditional lethal genetics (temperature-sensitive mutations) to mammalian cell genetics has not been without difficulty. The versatility of ts mutants has been limited because mammalian cells cannot be grown over as wide a temperature range as either bacteria or yeast. As a result, many ts mutants are `leaky' - that is, they exhibit measurable activity at the non-permissive temperature.

Some peripheral use of nonsense mutants has been made in animal virus genetics and in mammalian cell genetics (an account of these mutants is given below) but without access to suppressing host cells these mutations have all been in non-essential genes.

Several nonsense mutations in non-essential genes of eukaryotic viruses have been isolated. Cremer et al. (1979) showed that certain thymidine kinase (TK) deficient mutants of herpes simplex virus (HSV) were nonsense mutants. The mRNA from cells infected with the TK deficient mutants was in vitro translated in the presence of yeast nonsense suppressors. One of the mutants ($TK4^-$) was found to be an amber nonsense mutant since a serine inserting amber suppressor tRNA restored both the synthesis of normal length

protein and TK activity *in vitro*. Another TK nonsense mutant ($TK43^-$) could be suppressed by both leucine and serine opal suppressor tRNAs to produce normal length protein. However, neither succeeded in restoring an active gene product. Thus the insertion of leucine at the $TK43^-$ locus is presumably incompatible with TK function. More recently, Summers *et al*. (1983) showed, using a transient suppression system developed by Laski et al. (1982b and described in section 1.(b).6 below), in which a *Xenopus laevis* amber suppressor $tRNA^{Tyr}$ gene is inserted into an SV40 vector, that when cells are infected with this SV40 vector and subsequently infected with the TK deficient mutants described above, that the amber mutation in $TK4^-$ (but not the opal mutation in $TK43^-$) is suppressed to give a functional product.

Grodzicker *et al*. (1976) isolated nonsense mutants within the genome of the adenovirus type 2 (Ad2)-SV40 hybrid virus $Ad2^+ND1$ (see Lewis *et al*., 1969, 1973 for a description of the virus). This hybrid allows efficient growth of adenovirus in monkey cells (a non-permissive host) by insertion of the necessary SV40 helper sequences into the adenovirus genome. Unlike Ad2, which grows 1000 fold less well in African Green Monkey (AGMK) cells than in human cells, $AD2^+ND1$ grows equally well in both human and AGMK cells (Lewis *et al*., 1969) presumably by providing a helper function required for efficient adenovirus growth. This hybrid virus synthesizes a 30000 dalton (30K) protein encoded by both Ad2 and SV40 sequences. Host range mutants were generated, by nitrous acid mutagenesis, which were defective in growth on AGMK cells but which still retained the ability to grow on human cells. The mutants with the greatest host range restriction did not produce the $Ad2^+ND1$ hybrid 30K protein but instead they synthesized a novel fragment of this protein. Three of these host range mutants (140, 167 and 71) isolated by Grodzicker *et al*. (1976), which gave rise to a truncated 30K gene product, were shown to be nonsense mutants by Gesteland *et al*. (1977). In the presence of a yeast amber suppressor tRNA it was shown that two of the host range mutants (140 and 167) synthesized a

full-length 30K protein, as well as the fragment usually associated with these mutants, *in vitro*. The remaining host range mutant 71 yielded full length 30K protein only in the presence of an ochre suppressor tRNA *in vitro*.

Rawlins and Muzyczka (1980) described the construction of an amber mutation within the SV40 genome by site-specific mutagenesis. Nucleotide sequence analysis of the mutagenized region confirmed a C to T transition, resulting in the conversion of a glutamine codon to an amber stop codon. The position of this mutation within the SV40 genome should result in a T-antigen 24% shorter than the *wt* protein. It was suggested by the authors that the presence of nonsense mutations within what is a multfunctional protein should help identify the functional regions of the T-antigen.

More recently, Robertson *et al.* (1983) described the characterization of a *ts* mutant (*ts* 47) of fowl plague virus which contained a *ts* lesion in segment 8 (which encodes both the NS_1 and NS_2 proteins from overlapping reading frames) and which synthesized a NS_1 protein of higher electrophoretic mobility than that of the *wt* protein (Almond *et al.*, 1977). Nucleic acid sequence analysis of a 540 nucleotide portion from the 3' end of the message of segment 8 of *ts* 47 revealed a G to A change in the NS_1 reading frame. This resulted in the conversion of a UGG (Trp) codon to (UGA) opal stop codon resulting in premature termination of translation and the loss of 28 amino acid residues. This single base change was located in the overlapping coding region of the mRNA encoding the NS_1 and NS_2 proteins. The G to A base change in the NS_2 mRNA results in the conversion of a GGA (Gly) to a GAA (Glu) codon resulting in an increased negative charge of *ts* 47 NS_2.

Capecchi *et al.* (1977) isolated nonsense mutations within the mammalian cell gene which encodes the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT) which is non-essential for growth in tissue culture. This was accomplished by isolating a large number of mouse L cells deficient for this enzyme using the purine analogues

8-azaguanine and 6-thioguanine. Cell lines containing the enzyme HGPRT are killed by these compounds and thus select for HGPRT$^-$ cell lines. An HGPRT$^-$ cell line was isolated in which the HGPRT molecule was shown by tryptic peptide analysis to have a carboxy terminal tryptic peptide shorter than the corresponding wt peptide. After microinjection of a yeast ochre suppressor tRNA into this HGPRT$^-$ cell line, the mutant cells regained HGPRT activity. Microinjection of an amber suppressor tRNA did not restore this activity.

Amber and ochre nonsense codons have also been isolated in the human β-globin gene, from human patients with β-thalassaemia (Chang and Kan, 1979). Nucleotide sequence analysis of the non-functional mRNA from a patient with β-thalassaemia revealed that the lesion resulted from the mutation of an adenine to a uracil altering the lysine coding triplet AAG in the normal β-globin gene to an amber termination codon UAG.

1.(b) THE PHENOMENON OF SUPPRESSION

A nonsense mutation generates an in-phase polypeptide chain termination codon within the coding region of a gene, and as a consequence of a nonsense mutation an amino terminal polypeptide fragment, rather than the completed polypeptide chain, is synthesized (Sarabhai et al., 1964).

A second mutation may occur, which results in the production of a tRNA which can insert an amino acid at the point of mutation so suppressing the mutant phenotype by allowing completion of the protein (Hawthorne and Mortimer, 1963; Capecchi and Gussin, 1965; Engelhardt et al., 1965; Andoh and Ozeki, 1968; Goodman et al., 1968; Hawthorne and Mortimer, 1968; Gilmore et al., 1971; Sherman et al., 1973; Hawthorne and Leupold, 1974; Capecchi et al., 1975; Liebman et al., 1975; Gesteland et al., 1976; Piper et al., 1976; Brandriss et al., 1976).

Suppressor tRNAs with altered codon recognition might result in principle from two classes of mutation. The suppressor gene might code for a modifying enzyme so that the mutant enzyme fails to make some modification essential for proper codon recognition (e.g. a methylase). Such

mutations should be recessive. Alternatively, the suppressor locus may comprise a structural gene for the tRNA, in which case the mutation alters one of the bases in the anticodon. These mutations should be dominant. Base substitutions in the tRNA anticodon alter tRNA codon recognition so that the mutant suppressor tRNA can no longer translate its original codon. The amino acid inserted by the suppressor tRNA at the site of mutation must be compatible with the requirements of protein functionality to restore an active gene product.

In E. coli the majority of codons are translated by more than one tRNA species or by tRNAs coded by multiple identical genes so that a suppressor mutation in any one of these does not prove lethal by virtue of preventing the synthesis of any proteins which rely upon the translation of these codons. An anticodon change in a tRNA uniquely responsible for a coding function would be lethal to the cell. Such mutations are recessive lethals, since if the wt gene is lost the cell loses its capacity to respond to some codons and will die. This particular class of suppressor can only be isolated where the tRNA gene has been duplicated. Recessive lethal mutations have been isolated by Soll and Berg (1969a, 1969b) - Su7, a high efficiency glutamine inserter, and Su8, a much lower efficiency suppressor. In Salmonella typhimurium, Miller and Roth (1971) isolated recessive lethal UAG and UGA suppressors.

Most of the classical nonsense suppressors in E. coli result from mutations in tRNA genes. However a notable exception was characterized by Hirsh in 1971. He described a UGA suppressor (Su9) which turned out to be a mutant form of $tRNA^{Trp}$ and it contained the normal anticodon (CCA) corresponding to the Trp codon UGG. The structural alteration of this tRNA was found in the stem of the dihydrouracil loop where a G is replaced by an A. This implies a mechanism whereby the UGA triplet is read by a CCA anticodon and suggests A-C pairing in the third position. This result provided a striking indication that conformational aspects of tRNA structure may play a role in codon recognition. However, Yarus (1982) proposed that the

base change in the dihydrouracil stem leads to a change in translational specificity of the tRNA by increasing post-transcriptional modification of cytosine C34 in the anticodon wobble position. The enzyme postulated to do this normally modifies C34 of a minor Ile tRNA specific for AUA codons. This modification whould reduce reading of G in the third codon position, allowing the Hirsh suppressor to read UGA codons. Delamarche and Buckingham (1985), however, could not substantiate this theory and therefore found in favour of the former hypothesis.

In prokaryotes and eukaryotes all amber suppressors were found to translate exclusively the codon UAG and those sequenced have the anticodon CUA. Unlike the yeast and mammalian ochre suppressors, which are UAA specific, E. coli and phage T4 ochre suppressors translate both UAA and UAG codons. This is caused by reading imprecision (wobble) in the pairing of the third base of the codon (Crick, 1966)

1.(b).1 THE EFFECTS OF CODON CONTEXT ON SUPPRESSION

Apparent differences in the efficiency of suppression of a suppressor acting at different sites have been identified in a number of systems (Salser, 1969; Salser et al., 1969; Yahata et al., 1970; Akaboshi et al., 1976; Fluck et al., 1977; Feinstein and Altman, 1977, 1978; Fluck and Epstein, 1980; Bossi, 1983; Miller and Albertini, 1983). These reports have suggested that the reading of a given codon may be influenced by mRNA sequences external to this codon. This influence has been described as the effect of reading context on translation. Bossi and Roth (1980) studied the effect of the surrounding sequence on efficiency of suppression of the amber suppressor sup E at a particular amber site. The nucleotide adjacent to the 3' side of the nonsense codon was implicated in the observed alteration in suppression efficiency. The presence of an adenine residue (as opposed to a cytosine residue) at this position resulted in a tenfold increase of sup E efficiency in reading UAG. More recently Miller and Albertini (1983) and Bossi (1983) compared the surrounding sequence and resulting suppression efficiencies for a large number of nonsense codons in the

lac I gene of E. coli. A lac I-Z gene fusion permitted direct measurement of the suppressed levels of nonsense mutations in the lac I gene by assaying the β-galactosidase activity. This was possible since the lac I-Z gene fusion synthesized a hybrid repressor-β-galactosidase molecule which had normal β-galactosidase activity. Suppression efficiencies were shown to vary by 35 fold for amber suppression and 170 fold for UGA suppression. They observed that when the codon at the 3' side of the nonsense codon began with A or G the suppression efficiencies were high, whereas when the codon began with U or C suppression was low. Salser (1969) and Fluck et al. (1977) suggested that context effects result from variation in efficiency of termination at the nonsense site. However Bossi (1983) and Miller and Albertini (1983) argued that context effect results from variations in the efficiency of suppression. Several models were invoked to explain this phenomenon and are described here:

(1) The residue adjacent to the nonsense codon could form a fourth base pair with the suppressor tRNA. The presence of an invariant uracil residue (U33) at the relevant position in all known tRNAs and its proposed ability to form a fourth base pair would explain the observations that the presence of an A or a G on the 3' side of the nonsense codon resulted in efficient suppression whereas the presence of a U or C resulted in inefficient suppression. This explanation, however, does not fit with the observation that nonsense codons which were immediately followed by either the codons CUC and CUG were efficiently suppressed:

(2) Alternatively, the codon adjacent to the nonsense codon could be implicated in context effects. This hypothesis involves an interaction between the tRNA at the ribosomal P site and the tRNA at the ribosomal A site. Thus tRNAs that read codons starting with A or G might interact strongly with all amber suppressor tRNAs whereas tRNAs reading codons starting with C or U might interfere with the suppressor tRNA. The observation by Nishimura (1972) that tRNAs that read codons beginning with the same base have

similar modified bases on the 3' side of the anticodon reinforces this hypothesis.

(3) mRNA secondary structure has also been shown to affect efficiency of suppression. A potential secondary structure involving the amber site A26 was proposed by Miller and Albertini. It was conjectured that this secondary structure could possibly inhibit release factor action on nonsense sites, leading to elevated suppresssion levels noted at amber site A26 by the amber suppressors Su3 and Su6.

The results of Raftery et al. (1984), where the effect of context on a set of cloned termination suppressors was studied, also suggested that not only was the presence of a codon beginning with A following the stop codon a good context, but that features of the tRNA structure are important as well.

1.(b).2 THE EFFECT OF AMINOGLYCOSIDES ON SUPPRESSION

Aminoglycosides (in particular streptomycin) have been shown to cause non-genetic phenotypic suppression of auxotrophic mutants in bacteria (Gorini, 1974; Davies et al., 1964). Singh et al. (1979) reported the phenotypic suppression of all three nonsense codons in Saccharomyces cerevisiae by several aminoglycosides (including hygromycin B, paramomycin and neomycin). Palmer et al. (1979) also reported that paramomycin phenotypically suppressed all three types of nonsense codon in S. cerevisiae. In addition Roberts et al. (1979) and Martinelli and Roberts (1983) described the suppression of nonsense mutations in A. nidulans by aminoglycosides. The aminoglycosides have been shown to affect the fidelity of translation in vitro using E. coli ribosomes (Davies et al., 1965) and eukaryotic ribosomes (Palmer and Wilhelm, 1978; Wilhelm et al. 1978a, 1978b) and in vivo in E. coli (Edelmann and Gallant, 1977). Martinelli (1984) examined the effect of five ribosomal antibiotics on strains of A. nidulans containing informational suppressors. Strains carrying dominant (presumed tRNA) suppressors were only slightly sensitive to the effect of aminoglycosides whereas those carrying

recessive mutations (presumed ribosomal mutations) proved to be hypersensitive to the antibiotics. Burke and Mogg (1985) reported suppression of a nonsense mutation in mammalian cells by the aminoglycosides G418 (a neomycin derivative) and paromomycin. Both aminoglycosides promoted readthrough of an amber mutation in the chloramphenicol acetyl transferase (CAT) gene of the mammalian cell transfection vector pRSVcat^{amb38} in monkey cells (COS-7). The maximum level of suppression achieved by G418 was 19.6% and for paromomycin 5.4%. The observed difference in drug concentration needed to achieve maximum suppression was thought to reflect either a difference in permeability between the two drugs or different mechanisms of action on the ribosomes.

1.(b).3 CODON SPECIFIC AND NON-SPECIFIC SUPPRESSORS IN YEAST

Yeast nonsense suppressors were first isolated through their ability to reverse simultaneously the phenotype of as many as five mutations residing within the iso-1-cytochrome c gene (Hawthorne and Mortimer, 1963; Gilmore, 1967). These mutants designated *cyc1* mutants were easily identified since they were deficient in lactate metabolism and thus failed to metabolise the toxic analogue chlorolactate. Revertants of the *cyc1* mutants were selected by growth on lactate medium. Nonsense suppressors of the yeast *S. cerevisiae* have been divided into two groups, codon specific and codon non-specific.

The majority of suppressors in the former group arise by anticodon mutations of tRNA genes (Hawthorne and Leopold, 1974; Capecchi *et al.*, 1975; Gesteland *et al.*, 1976; Piper *et al.*, 1976; Olson *et al.*, 1977; Piper, 1978; Ono *et al.*, 1979a, 1979b, 1981; Liebman *et al.*, 1984). These suppressors have been shown to suppress defined UAA and UAG mutations in the iso-1-cytochrome c gene. The majority of UAG suppressors insert a tyrosine residue at the site of mutation in haploid strains (Liebman *et al.*, 1976). They restored production of iso-1-cytochrome c to approximately 50% of the *wt* activity. Leucine inserting UAA suppressors were isolated which suppressed the same set of amber

mutations as the tyrosine suppressors but at a much lower efficiency. These suppressors were distinguished from the tyrosine inserting amber suppressors by their reduced action on a specific amber allele cyc1-179 (about 15-20%, Liebman et al., 1977 and Ono et al., 1979b). Liebman et al. (1984) described the isolation and mapping of leucine inserting UAG suppressors. Genetic mapping placed them into nine complementation groups some of which were found not to be linked to the previously mapped leucine inserting ochre suppressors. Brandriss et al. (1975) examined suppression of amber mutations in diploid strains. Most of the suppressors isolated which suppressed two specific markers (trp-1 and aro-7), although stable in the heterozygous state, proved lethal in the homozygous condition. This suggested that the suppressors were coded for by a single gene in the haploid genome. One of the suppressors was shown to restore iso-1-cytochrome c levels to about 50% of the wt (Brandriss et al., 1976; Ono et al., 1981 and Etcheverry et al., 1982). Eight loci were identified which gave rise to suppressors which suppressed a set of UAA markers (Gilmore, 1967; Hawthorne and Mortimer, 1968; Ono et al., 1979a) by inserting a tyrosine residue. In contrast to the amber tyrosine inserters the ochre tyrosine suppressors restored cytochrome c levels to only about 5-10% of the wt.

The efficiency of suppression of UAA stop codons can be increased substantially by the presence of an extrachrosomal factor psi. Psi factor is probably a DNA plasmid. It enhances several fold the efficiency of tRNA ochre and some frameshift suppressors but is without effect on UAG or UGA suppressors. Psi factor is itself a weak UAA suppressor (Liebman and Sherman, 1979) rendering the tyrosine inserting ochre suppressors so efficient as to make them lethal to the cell. Its mode of action is unknown but Dujardin et al. (1985) suggested that psi is or affects a component of the translation machinery. Ono et al. (1979a) detected lower efficiency suppressors in psi^+ strains (the tyrosine inserters were isolated in psi^- strains) which could be mapped to two loci. These suppressors were shown to insert a serine residue in response to an ochre stop

codon. Additional serine inserting UAA suppressors were isolated in a study by Ono et al. (1981). These suppressors could be assigned to one or other of four different loci. In the study by Ono et al. (1979a) a minority class of UAA suppressors were discovered which were distinguished by a very low efficiency of suppression and their ability to suppress a marker (ura-4-1) which was not suppressed by the serine inserting suppressors. In addition, they did not suppress a marker (lys1-1) which was suppressed by both the tyrosine and serine inserting suppressors. This class of suppressor (SUP26) was shown to insert a leucine residue. Ono et al. (1979b) isolated additional examples of this class of suppressor and assigned them to six unlinked loci. Three of these suppressor loci were mapped to specific chromosomal locations. The remaining three were mapped by Ono et al. (1985).

Suppressors acting solely on UAG codons have been isolated in both yeast and bacteria. Piper et al. (1976) demonstrated that a modified base in the anticodon (pseudouridine) prevents wobble pairing - this is consistent with the situation in prokaryotes. However, although UAA suppressors have been observed to suppress both UAA and UAG nonsense codons in bacteria, UAA suppressors act solely on UAA nonsense codons in yeast. This was thought to result from a modification of the anticodon from UUA to SUA. The S could represent either a 2-thio-5-carboxymethyluridine derivative or a 5-carboxymethyluridine derivative (Yoshida et al., 1971; Kuntzel et al., 1975).

UGA specific suppressor tRNAs were first identified through reversion of the allele ade5,7-143. Hawthorne (1976) observed that this allele was not suppressible by either amber or ochre specific suppressors and that it was suppressed by a new class of allele specific suppressor. The ade5,7-143 allele could mutate to an ochre allele in the presence of an ochre suppressor. Conversely, ochre alleles can mutate to UGA suppressible alleles which are no longer susceptible to ochre specific suppressors. Hawthorne (1981) described the isolation of additional UGA suppressors and although the tRNA species involved was not identified there

appeared to be close linkages with either the serine inserting or leucine inserting UAA suppressors (Hawthorne, 1981).

Codon non-specific suppressors are not thought to be mutations in a tRNA gene since they lack codon specificity. They are generally recessive and capable of suppressing amber, ochre and opal termination codons and fall into two complementation groups. Dominant omnipotent suppressors have been identified which are thought to map to a separate locus from the recessive suppressors (Ono et al., 1981, 1984). They are thought to be ribosomal mutations (Smirnov et al., 1978 and Surguchov et al., 1980). Liebman and Allrobyn (1984) demonstrated that an extrachromosomal element eta^+ causes recessive omnipotent suppressors to become lethal genes. Unlike psi^+ which has a weak UAA suppresor activity, eta^+ has no such activity. In addition eta^+ does not act on UAA codons. Song and Liebman (1985) investigated the interaction of UAG tRNA suppressors and omnipotent suppressors in yeast. Previously, Liebman and Cavanagh (1980) had suggested that the strain lethality observed when dominant UAA suppressors and recessive omnipotent suppressors interacted in haploids was caused by excessive suppression. Song and Liebman, however, observed that only in some combinations did lethality occur and that in some combinations the interaction actually reduced the efficiency of suppression. This suggested that suppression in these cases involved some previously unsuspected type of interaction between the suppressor tRNA and the ribosome.

1.(b).4 SUPPRESSION IN ASPERGILLUS NIDULANS

Bal et al. (1978) described the reversion of two mutations at two different A. nidulans loci by informational suppressors. The first detailed report of suppressors in A. nidulans was by Roberts et al. (1979). They described the isolation of seven suppressor mutations by coreversion of alleles in the structural gene for allantoinase (alX), the sulphate permease gene (sB) and alcohol dehydrogenase (alcA). Two classes of suppressor were characterized: suaB111, suaD103 and D108 were semidominant in diploids and

did not have any pleiotropic effects. They were thus suggested to be informational suppressors with the mutation residing in the tRNA gene. Recessive suppressor mutations were also isolated which did have pleiotropic effects. Strains carrying these mutations exhibited slow growth, changes in colony morphology and, rarely, cold sensitivity. The recessive mutations probably map in genes coding for translation termination or in essential ribosomal genes. Martinelli (1984) investigated the effects of ribosomal antibiotics on informational suppressors in A. nidulans. As expected they observed that the antibiotics had a much greater effect on strains carrying the recessive suppressor mutations than those carrying dominant suppressors.

1.(b).5 SUPPRESSION IN CAENORHABDITIS ELEGANS

Two amber suppressor tRNA loci (SUP5 and SUP7) were initially identified in the nematode C. elegans (Waterson and Brenner, 1978; Waterson, 1981). Waterson (1981) isolated 25 suppressor mutations and mapped 19 to the SUP5 locus and 6 to the sex-linked locus SUP7. Nematodes which were homozygous for SUP7 (and to an extent SUP5) were found to be inviable at 15^{o}C (Waterson, 1981). This is known as cold sensitive lethality. Suppression efficiency was found to be elevated at this temperature. The SUP7 suppressor alleles were shown by Kimble et al. (1982) to suppress a nonsense mutation in the tra-3 allele which affects sex determination in the nematode. In the nematode sex is determined by the X:autosome ratio. Thus XX animals are hermaphrodites and XO animals are males (there are no females). A recessive mutation in any of the three autosomal transformer genes (tra1, tra2 and tra3) results in the generation of pseudomales (phenotypic males) instead of hermaphrodites.

SUP7 DNA was microinjected into the gonads of an animal bearing the tra-3 allele (e1107) and the offspring (F1) inspected for phenotypic suppression. 60% of the offspring gave rise to hermaphrodite offspring (F2). However, only a small number of the total offspring from each animal (F1) were hermaphrodites. This was thought to

be a result of few oocytes actually receiving the suppressor tRNA. As further proof that the hermaphrodite offspring arose as a direct result of suppression it was observed that no hermaphrodite offspring were detected when animals bearing the non-amber allele of tra-3 (e1767) were used.

Wills et al. (1983) attempted to determine the specificity and to define the nature of the SUP-5 and SUP-7 suppressors. In the 'allele first' approach they examined 6 null mutants of the unc54 gene in C. elegans. Macleod et al. (1977) had previously shown that these mutations were suppressible by SUP5 and SUP7 suppressors. The unc54 gene specifies a major myosin heavy chain present only in body wall muscle cells. Sequence analysis of the region containing the mutation in one of these mutants revealed a C to T substitution which resulted in the alteration of a glutamine codon to an amber terminator.

In the 'suppressor first' approach, extracted tRNA from suppressor carrying animals was added to a wheat germ translation system along with brome mosaic virus (BMV) RNA which contained a characterized suppressible amber stop codon at the 5' end of the coat protein gene (Dasgupta et al., 1980). Suppressor activity was detected as judged by the production of full length protein. There was no detectable suppressor activity when the BMV RNA was substituted by α-globin RNA which contained an ochre terminator. The DNA sequence of SUP7 was obtained (Bolten et al., 1984) and shown to be a tryptophan tRNA gene containing an alteration at the anticodon (CCA to CUA) which permitted the tRNA to read amber codons.

Hodgkin (1985) identified three additional amber suppressor tRNAs (SUP21, SUP22 and SUP23) in C. elegans by isolating revertants of amber mutants of the tra-3 gene. The efficiency of suppression of a variety of amber alleles in the nematode was measured for comparison. A marked heterogeneity in the suppression level of alleles from the SUP21 and SUP22 loci was discovered. Thus, it was suggested that the different isolates could represent tRNA genes from different families or tRNA genes from the same family expressed at different levels. An allele of SUP21 (e1957)

was found to be a good suppressor of hypodermal (dpy) amber mutations, a poor suppressor of amber mutations in muscle genes (unc-54) and a very poor suppressor of amber mutations in neuronal genes (unc-13). This suggested tissue specificity for this allele. In addition, the suppressor loci SUP21 encoded a weaker suppressor than either SUP5 or SUP7. This meant that animals homozygous for this allele were viable at 15°C unlike SUP5 or SUP7 homozygotes.

1.(b).6 SUPPRESSOR tRNAs IN MAMMALIAN CELLS

The first account of suppressor tRNAs in a mammalian cell system was reported by Laski et al. (1982b). Prior to this, Laski et al. (1982a) had described the cloning of a Xenopus laevis $tRNA^{tyr}$ gene into an SV40 vector and its subsequent introduction into a mammalian cell line (CV-1). The mature $tRNA^{tyr}$ was found to be overproduced by transcription of high copy numbers of the SV40-$tRNA^{Tyr}$ recombinant. The RNA was also fully processed and modified. This involved removal of a 13 bp intervening sequence and appropriate modification of some of the nucleotides. They then described the generation of an amber suppressor tRNA from this $tRNA^{Tyr}$ gene by site specific mutagenesis using a synthetic oligonucleotide containing a single base pair mismatch in the anticodon region of the $tRNA^{Tyr}$ gene (Laski et al., 1982b). This changed the anticodon sequence of the tRNA gene from GTA to CTA. The suppressor $tRNA^{Tyr}$ gene was then inserted into the late region of an SV40 vector. After infection of CV-1 cells by the SV40-suppressor $tRNA^{Tyr}$ recombinant the su^+ tRNA was found to be produced at only 20% of the level of the wt $tRNA^{Tyr}$. This was thought to be due to inefficient splicing of the intervening sequence - thought to be essential for suppressor tRNA gene expression (Johnson and Abelson, 1983). However, the su^+ $tRNA^{Tyr}$ was shown to promote readthrough of amber mutations in the hybrid virus $Ad2^+$ ND1 (see section 1.(a).3 above) which apparently yielded suppressed levels of the protein comparable to wt $Ad2^+$ ND1.

Hudziak et al. (1982) reported the first description of mammalian cell lines to express a suppressor tRNA. Mouse

L cell lines were isolated which could suppress an amber mutation in three conditional lethal genes. This was accomplished initially by isolating nonsense mutations in the HSV TK gene, the E. coli xanthine-guanine phosphoribosyl transferase (eco-gpt) gene and the aminoglycoside 3' phosphotransferase (npt-11) gene, in E. coli. These mutations were then transferred into cultured mammalian cells. It was important to isolate mutations in these genes and to subsequently transfer them into mammalian cells because their gene products confer a selectable phenotype on the cell. Thus the acquisition of an integrated amber suppressor tRNA would allow the cell to grow in the appropriate selection medium. The amber suppressor $tRNA^{Tyr}$ gene (described by Laski et al., 1982b) was then introduced into the cell lines containing the multiple amber mutations. Transformants expressing thymidine kinase activity were selected on HAT medium and those expressing npt-11 on G418 medium. Transformants were isolated and shown to have phenotypically suppressed the nonsense mutations, presumably by acquisition of the amber suppressor $tRNA^{Tyr}$ gene. Three of the transformed cell lines were maintained for over 70 generations in their respective selection medium without loss of suppressor activity. One of the transformants characterized (designated L39) is the suppressor cell line used in this Ph.D. project report. Young et al. (1983) measured the suppression level of both the L39 suppressor cell line and the SV40-$su^{+}tRNA^{Tyr}$ recombinant. They made use of a field isolate of influenza virus (A/CAM/46) which proved to have a suppressible NS1 gene amber termination codon. This phenomenon is not restricted to this field isolate. Parvin et al. (1983) reported several field isolates of influenza virus with a possible suppressible NS1 mRNA. The suppression level could be estimated from the amount of radiolabelled suppressed readthrough product detected by polyacrylamide gel electrophoresis. The L39 cells were found to suppress the NS1 amber codon at a low level (about 3.5%) whereas the SV40-$su^{+}tRNA^{Tyr}$ recombinant suppressed the nonsense codon relatively efficiently (about 22.5%). The

low level of suppression observed for the L39 cell line may be the maximum that a cell line constitutively expressing a suppressor tRNA can tolerate.

Laski et al. (1984) generated an ochre suppressor tRNA gene by site specific mutagenesis of a X. laevis $tRNA^{Tyr}$ gene. This was the same tyrosine tRNA gene that had been used previously to produce an amber $tRNA^{Tyr}$ gene (Laski et al., 1982b). The suppressor gene is efficiently expressed as mature spliced tRNA after introduction into the cell as part of a replicating SV40 recombinant. However, the ochre suppressor tRNA was produced at only 20% of the level of the wt $tRNA^{Tyr}$. The amber $tRNA^{Tyr}$ was also produced at this low level (Laski et al., 1982b) possibly as a result of inefficient splicing. An ochre codon separating the Ad2 hexon gene from a 23kd downstream gene was successfully suppressed as was an ochre termination codon in the NS1 gene of the influenza isolate A/TEX/1/68. The ochre suppressor did not suppress the amber termination codon of the A/CAM/46 isolate. This is in direct contrast to ochre suppressors in prokaryotes which suppress both amber and ochre suppressors. Ochre suppressors in yeast are also specific for ochre codons only. A modification of the U in the first position of the UAA anticodon to a base (pseudouridine) which cannot recognise amber codons by 'wobble' pairing was thought to be responsible for the observed specificity in yeast and mammalian cells.

Unless tyrosine inserting suppressors act on a nonsense site which contained a tyrosine codon in the wt it is possible that the insertion of a tyrosine could be incompatible with the structure or function of the suppressed protein. It should be noted however that tyrosine inserting suppressors are the most efficient suppressors yet isolated in yeast (Liebman et al., 1976).

Capone et al. (1985) generated amber, ochre and opal suppressor tRNA genes from a human serine tRNA gene by oligonucleotide directed site specific mutagenesis to change one or two bases in the anticodon. After infection of CV-1 cells as part of an SV40 recombinant both the amber and ochre suppressor tRNAs were produced at almost the same

level as wt serine tRNA. In contrast to the tyrosine inserting amber and ochre suppressor tRNAs described by Laski et al. (1982b, 1984) which were present at only 20% of the wt $tRNA^{Tyr}$ level in CV-1 cells. This was attributed to a lower rate of splicing of the su^+ gene in these cells. The human serine tRNA gene does not contain an intron and as such its behaviour is consistent with this hypothesis (Capone et al., 1985). The amber and ochre suppressors were shown to promote readthrough of their respective nonsense codons. The amber suppressor promoted readthrough of the amber termination codon of the A/CAM/46 isolate at a level comparable to the tyrosine inserting suppressor (about 25-30%). The ochre suppressor suppressed the termination codon of the Ad2 hexon gene at a 1.5 fold higher efficiency than that achieved by the ochre tyrosine suppressor used by Laski et al. (1984). Attempts to produce an opal serine inserting suppressor tRNA were unsuccessful. It was suggested that possibly the presence of an opal suppressor is deleterious to CV-1 cellular metabolism and/or SV40 propagation. The major capsid protein VP1 of SV40 terminates with an opal codon. Readthrough of this termination codon results in a VP1 protein 40 amino acids longer than normal. The authors suggested that since Tooze (1981) had shown that mutants in VP1 were defective in virus assembly it was possible that the presence of an opal suppressor interfered with virus assembly. Capone et al. (1986) constructed amber, ochre and opal mutations in the CAT gene of the mammalian cell transfection vector pRSVCAT by oligonucleotide-directed site specific mutagenesis, to facilitate rapid and sensitive testing of the level of suppression of cell lines containing su^+ tRNA genes. Significantly, they tested the opal suppressor (as well as the amber and ochre suppressors) derived from a human serine tRNA gene (Capone et al., 1985). This opal suppressor had been found previously (Capone et al., 1985) not to function in mammalian cells for some reason. However, in this case pronounced readthrough of the opal termination codon within the CAT gene was found resulting in the production of active enzyme. Thus, the opal suppressor was shown to be effective

in short term transfection experiments. Their results also showed that in mammalian cells, as in bacteria and yeast, opal suppressor tRNAs are specific for UGA termination codons.

Recently Ho et al. (1986) described the establishment of permanent cell lines (derived from CHO cells) containing different copies of amber suppressor $tRNA^{Lys}$ genes (constructed by Temple et al. (1982). The suppressor tRNA gene was linked to the dihydrofolate reductase (DHFR) gene and the resulting recombinant plasmid was used to transform (CHO-K1) $DHFR^-$ cells. This was especially useful since genes linked to DHFR can be coamplified during the selection of methotrexate (MTX) resistance after transforming them into $DHFR^-$ cells. Transformants were exposed to increasing concentrations of methotrexate to select resistant colonies. The level of suppression in the CHO cell lines was determined by measuring the readthrough NS1 protein after infecting them with the influenza isolate A/CAM/46 (described above) and was found to be only 3.6% in cells isolated at the highest concentration of MTX, in spite of the increase in the tRNA level as the gene copy number increased with increasing concentration of MTX. This is comparable to the level observed by Hudziak et al. (1982) for the L39 cells. This low level of suppression was thought to be caused by the low level of aminoacylated $tRNA^{Lys}$ found in vivo.

1.(b).7 NATURAL SUPPRESSION IN EUKARYOTES

Suppressor activity can be of two types: (1) naturally occurring and (2) induced through mutagenesis. It has been suggested that natural suppression may be a mechanism for producing proteins which, while related to other proteins, nonetheless perform distinct and essential functions. Naturally ocurring suppressors have been largely associated with viral infection in prokaryotic as well as eukaryotic systems. It appears that natural suppression does not affect each of the three termination codons equally. In fact Geller and Rich (1980) went as far as to suggest that the termination codons may have different

meanings in the cell; namely, that perhaps the ochre codon (UAA) means that translation must always stop and that the codons UGA and UAG may mean it should usually stop. This interpretation resulted from the observation that, in several systems, natural suppression of UGA and UAG stop codons (especially UGA in phage and bacteria) has been found. For instance, Pelham (1978) showed for tobacco mosaic virus (TMV) that a 160000 (160K) dalton protein is generated by partial readthrough of the amber termination codon of a 110000 (110K) dalton protein gene. After addition of an amber suppressor tRNA the *in vitro* synthesis of the 160K protein is increased whereas the yield of the 110K protein is diminished. Bienz and Kubli (1981) purified a $tRNA^{Tyr}$ (anticodon G-pseudo U-A) that stimulated this readthrough in Xenopus oocytes which apparently translated UAG using a G/G wobble base pair.

Natural suppression has also been documented in the case of the retroviruses Moloney murine leukaemia virus and Rous sarcoma virus. It has been known for some time (Philipson *et al.*, 1978) that although an amber nonsense codon separates the *gag* and *pol* genes of MuLV, translation of the genome size mRNA, results in the synthesis of a *gag-pol* polyprotein as well as the *gag* protein (at a much reduced level). Yoshinaka *et al.* (1985) purified the protease comprising the gag proximal product of the *pol* gene, and carried out partial amino acid sequence analysis. They found that the first four amino acids are encoded by the end of the *gag* gene and that the fifth is glutamine which is inserted by suppression of the UAG termination codon at the *gag-pol* junction, presumably by misreading by the normal $tRNA^{Gln}$.

In Mo-MuLV the *gag* and *pol* genes are in the same reading frame, however this is not the case in other retroviruses such as Rous sarcoma virus. The actual mechanism responsible for suppression in Rous sarcoma virus was reported by Jacks and Varmus (1985). They found that translation of an mRNA produced *in vitro* gave a level of *gag-pol* polyprotein, relative to the *gag* protein, similar to that found *in vivo*. The authors concluded that ribosomal

frameshifting was responsible for the synthesis of the gag-pol polyprotein.

More recently, Chambers et al. (1986) reported an intriguing example of natural suppression in which the selenocysteine residue in the active site of the mouse glutathione peroxidase protein was shown to be encoded by the triplet UGA (normally the opal stop codon). In addition, it had been shown previously that selenocysteine was introduced into the enzyme during translation (as opposed to post-translationally), and that a specific selenocysteyl-aminoacyl-tRNA had been identified (see Chambers and Harrison, 1987). This result suggested that, since UGA evidently has more than one function, some other unspecified factor was responsible for its different usage.

Possible uses for natural readthrough were provided by Geller and Rich (1980). They suggested that natural suppression could provide a system with an additional control of gene expression at the level of translation and more speculatively, that perhaps natural suppression was a less complexalternative toanother method of joining proteins; namely RNA splicing.

CHAPTER 2 THE GENETICS OF VESICULOVIRUSES

The aim of this chapter is to provide a review of the more relevant (to this study) areas of vesiculovirus virology. This chapter will include topics such as VSV taxonomy, virion structure, genome structure, growth cycle and genetics. Due to the confines in space, important topics including pathogenesis, persistent infection and virus cell interactions are not covered.

2.(a) TAXONOMY

Rhabdoviruses are widely distributed among vertebrates and are also found in invertebrates and plants. They are grouped into the Lyssavirus and the Vesiculovirus genera. However, only a few of more than 75 named rhabdoviruses are included in the two recognized genera (Brown et al., 1979). The prototype Vesiculovirus is vesicular stomatitis virus (VSV) of which various strains have been isolated from cattle and horses as well as flies and mosquitoes (Brown and Crick, 1979). The prototype Lyssavirus is rabies virus - a virus which still represents a major health hazard.

VSV is predominantly a disease of the New World, but it also exists in Europe and possibly South Africa. The clinical manifestation of VSV infection is similar to foot and mouth disease, though it is much milder. In man, VSV infection may be inapparent or resemble influenza (Howatson, 1970; Andrewes et al., 1978).

The genus Vesiculovirus comprises nine viruses. These are VSV Indiana, VSV New Jersey, Chandipura virus, Isfahan virus, Piry virus, Jurona virus, Keuraliba virus, La Joya virus and Yug Bugdanovac virus. The Indiana serotype comprises the subtypes Indiana, Cocal, Argentina and Brazil. The New Jersey serotype comprises the Concan and Hazelhurst subtypes.

2.(b). VIRION STRUCTURE AND COMPOSITION

2.(b).1 MORPHOLOGY

The family Rhabdoviridae are typified by a bullet shaped or bacilliform morphology (figure 1). The virus particles range in size from 130-380nm in length and 60-75nm in diameter (Brown et al., 1979). The major constituents of the virus particle are a surface projection layer, a membrane envelope layer and a helically wound nucleocapsid. The mass of the native virion was calculated, by dark field scanning transmission electron microscopy, to be 256.6± 13.3 MDa (Thomas et al., 1985).

2.(b).2 PROTEINS

The five recognized VSV proteins are the L (large), N (nucleoprotein), NS (non-structural), G (glycoprotein) and M (matrix) proteins. The N, L and NS proteins associate to form the internal ribonucleoprotein core (RNP) along with the genomic RNA. The G and M proteins associate with the outer membrane of the virus (Kang and Prevec, 1969; Wagner et al., 1969; Cartwright et al., 1970b; Bishop and Roy, 1972; Emerson and Wagner, 1972).

Until recently, the weight of the available evidence suggested that, in infected cells, there was no virus encoded protein which was not present in the mature virion (Mudd and Summers, 1970; Kang and Prevec, 1971; Wagner et al., 1972; Wunner and Pringle, 1972a,1972b). However, a recent report by Herman (1986) suggested the existence of a small non-structural polypeptide present in the cytoplasm of cells infected with the Indiana serotype of VSV, encoded by the 3' one-third of the NS gene. It has also been suggested that a small protein could be encoded by the second open reading frame of both the New Jersey and Indiana serotypes (Hudson et al., 1986).

A quantitative analysis of the structure and assembly of the VS virion of the Indiana serotype was undertaken by Thomas et al. (1985). They determined the molecular composition of the virion using a scanning transmission electron microscope. The results of this study are included

Figure 1

(a) Schematic model of an idealized rhabdovirus particle.

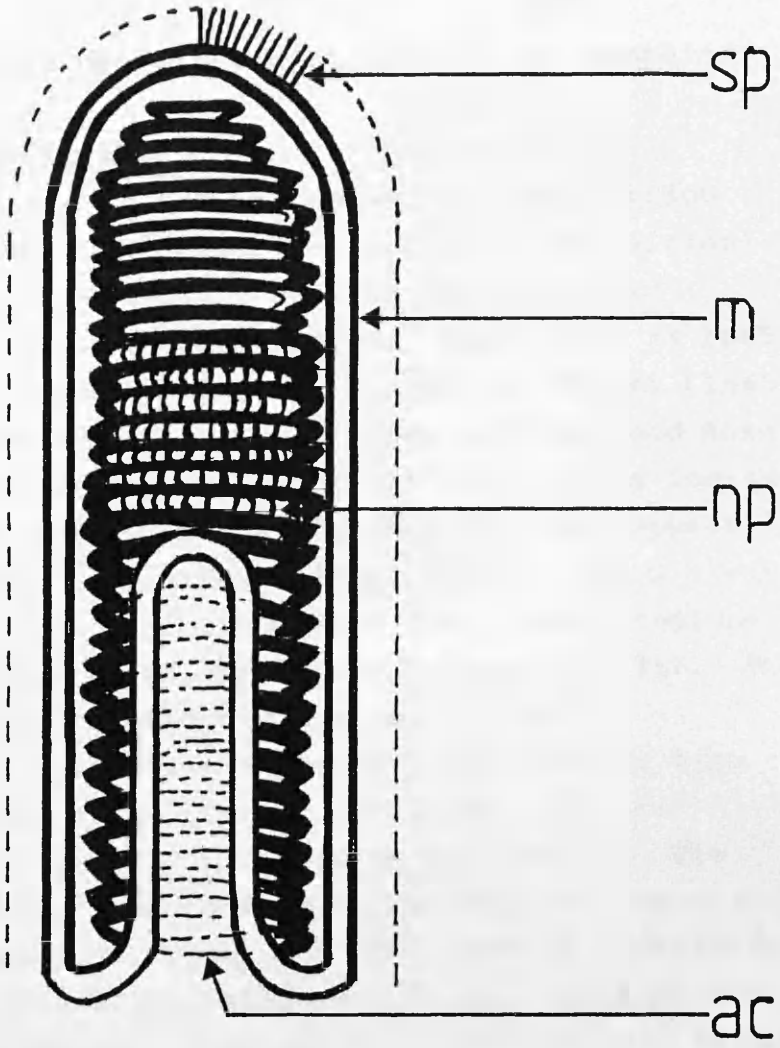
Illustrated features include the surface projection layer (sp), the membrane envelope (m), the helical nucleoprotein (np) and an invagination giving rise to an axial channel (ac).

(b) Purified preparation of VSV Indiana serotype negative stained with sodium silico tungstate.

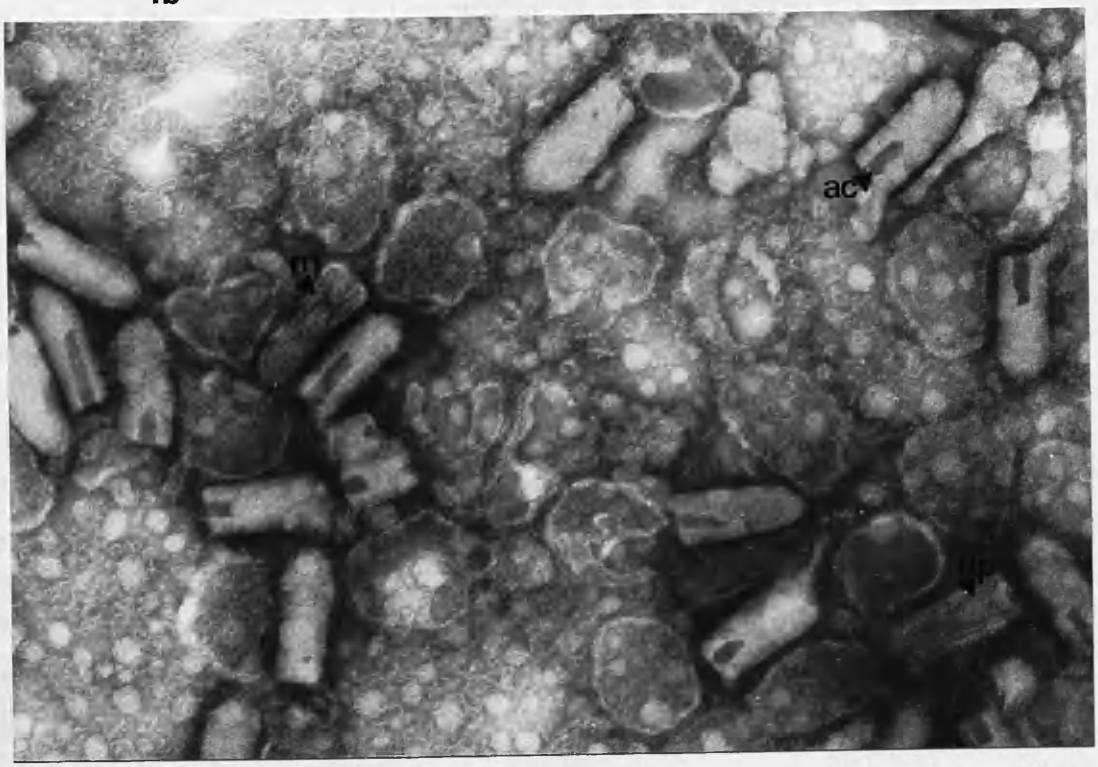
The characteristic bullet shape is clearly evident as well as the helical nucleoprotein (np). The membrane layer (ml) and an axial channel (ac) are also visible. (Magnification x 150,000)

Negative kindly provided by Jim Aitken.

1a



1b



within the detailed description of each virion protein.

2.(b).2.1. THE G PROTEIN

The G protein is the third most abundant virion protein (in terms of the number of molecules per virion) comprising 33.8% of the total protein (estimated to represent 1205 molecules per virion). The G gene is 1665 nucleotides (excluding polyadenylic acid) in length (1565 nucleotides in the New Jersey serotype; Gallione and Rose, 1983) and encodes a polypeptide of 511 amino acids including a signal peptide of 16 amino acids that does not appear in the mature virion (Rose and Gallione, 1981). The G protein forms the spikes which protrude from the virion envelope (Cartwright et al., 1970a, b; McSharry et al., 1971). The G protein is the major antigenic determinant and is responsible for neutralization by antibody and for type specificity of the virus (Cartwright et al., 1970a; Cartwright and Brown, 1972; Kelley et al., 1972). The glycoprotein can be removed from the surface of the virion by proteolytic enzymes, resulting in a loss of infectivity (Cartwright et al., 1970b; McSharry et al., 1971; Bishop et al., 1975). It has also been shown to mediate cell fusion in vitro (Florkiewicz and Rose, 1984).

The G protein is synthesized on membrane bound ribosomes (Morrison and Lodish, 1975) and is inserted as a nascent chain into the membrane of the rough endoplasmic reticulum (RER) (Rothman and Lodish, 1977; Toneguzzo and Ghosh, 1978). A hydrophobic signal sequence is cleaved from the N-terminus of the growing polypeptide chain (Rose, 1977; Lingappa et al., 1978; Irving et al., 1979). After synthesis, the G protein remains anchored in the membrane of the RER by a carboxy terminal hydrophobic segment (Chatis and Morrison, 1979; Rose et al., 1980; Rose and Gallione, 1981). It is subsequently transported to the Golgi apparatus (Bergmann et al., 1981) where fatty acid acylation takes place (Capone et al., 1982). The fatty acid chain (palmitate) is linked to the single cysteine residue in the cytoplasmic domain in the Indiana serotype but not in the New Jersey serotype (Rose et al., 1984). G is then

transported to the plasma membrane, where it facilitates the assembly of the mature virion.

The Indiana serotype contains two identical asparagine linked complex oligosaccharides (Reading et al., 1978). The exact positions of these glycosylation sites were determined by Rose and Gallione (1981) by sequence analysis. From the sequence analysis it has been suggested that the protein could be divided into four domains. These are the signal sequence (16 amino acids), the main body of the protein (446 amino acids), the transmembrane domain (20 amino acids) and the cytoplasmic domain of 29 amino acids (Rose and Gallione, 1981).

Several authors have reported that a derivative of G protein can be identified in the culture medium from VSV infected cells as one of the major soluble antigens (Kang and Prevec, 1970; Little and Huang, 1977). This G_S protein is thought to arise from the viral G protein by proteolytic cleavage at the cell surface (Little and Huang, 1978; Chatis and Morrison, 1982; Irving and Ghosh, 1982). Little and Huang (1978) suggested a role for this protein in viral pathogenesis - the G_S protein could serve to neutralize antibody.

Garreis-Wabnitz and Kruppa (1984) demonstrated that after VSV infection of BHK cells, a short form of the viral glycoprotein (G_{Si}) is formed intracellularly which lacks a carboxy terminus region. As already mentioned, the carboxy terminal region of the G protein contains the hydrophobic sequence which is essential for membrane integration. This G_{Si} protein is missing the membrane anchoring sequence and is therefore secreted into the culture medium.

2.(b).2.2. THE M PROTEIN

The M (matrix) protein is the most abundant virion protein which represents 24% of total protein per virion (estimated to be 1826 molecules per virion; Thomas et al., 1985). The M gene is 833 nucleotides in length (excluding polyadenylic acid) and encodes a protein of M_r 26064 (Rose and Gallione, 1981). M is the most basic of the VSV proteins (Rose and Gallione, 1981). It lines the inner

surface of the lipid bilayer in close association with the RNP core (Newcomb et al., 1982). The M protein appears to be synthesized as a soluble protein, but is then directly incorporated into membranous structures. It was found to enter extracellular virus very quickly, as though it moved directly from a soluble state into budding virus (Knipe et al., 1977a).

The M protein is thought to play a regulatory role in virus directed RNA synthesis in infected cells and to be an inhibitor in vitro of viral transcription (Carroll and Wagner, 1979; Wilson and Lenard, 1981; De et al., 1982; Pinney and Emerson, 1982). Pal et al. (1985) investigated the effects of monoclonal antibodies directed against three epitopes on the M protein on the capacity of the M protein to regulate transcription of VSV RNP cores. Antibody directed against epitope 1 greatly enhanced transcription by the inhibited RNP/M core complex. Antibodies to epitopes 2 and 3 had the opposite effect of considerably enhancing the inhibitory effect of the M protein. The ts mutant ts 023, which was defective in inhibition, did not bind antibody against epitope 1. A revertant which had fully recovered its inhibitory properties did bind antibody against epitope 1 whereas three revertants which had not recovered any inhibitory properties did not bind antibody against epitope 1. This suggested that exposure of epitope 1 on the surface of M protein is essential for inhibition of transcription by VSV RNP cores.

2.(b).2.3. THE N PROTEIN

The N protein is the second most abundant virion protein contributing 30.1% of the total protein (estimated to represent 1258 molecules per virion; Thomas et al., 1985) and is the group specific antigen (Cartwright and Brown, 1972). The N gene is 1326 nucleotides in length (excluding polyadenylic acid) and encodes a polypeptide of M_r 47355 (Gallione et al, 1981).

The N protein is found closely associated with the virion RNA and also with the replicative intermediate and mRNA species in infected cells (Soria et al., 1974; Grubman

and Shafritz, 1977; Rubio et al.,1980; Naeve and Summers, 1980; Adam et al., 1986). As a result of the close association of the N protein and virion RNA, the virion RNA is rendered resistant to ribonuclease digestion (Cartwright et al., 1970a).

2.(b).2.4. THE NS PROTEIN

The NS protein of VSV in association with the L protein constitutes the RNA polymerase complex (Emerson and Yu, 1975; Naito and Ishihama, 1976). The NS gene of the Indiana serotype is 814 nucleotides long (excluding polyadenylic acid) and encodes a 265 amino acid polypeptide of M_r 30000, while the NS gene of the New Jersey serotype is 856 nucleotides long encoding a 274 amino acid polypeptide (Gallione et al., 1981; Gill and Banerjee, 1985; Hudson et al., 1986). It contributes 5.9% of the total protein (estimated to be 466 molecules per virion) and is therefore only a minor component of the virion (Thomas et al., 1985). Several reports have suggested that the degree of phosphorylation of the NS protein both in vitro and in vivo plays a regulating role in the transcription process (Clinton et al., 1978; Hsu et al., 1982; Kingsford and Emerson, 1980; Testa et al., 1980; Kingsbury et al., 1981; Sinacore and Lucas-Lenard, 1982).

Isoelectric focusing, electrophoresis and chromatography have revealed a variety of phosphorylated forms of the NS protein which can be separated into two classes (NS1 and NS2) which, in addition to differing markedly in their degree of phosphorylation, have different transcriptase activating ability (Clinton et al., 1979; Kingsford and Emerson, 1980; Kingsbury et al., 1981; Hsu and Kingsbury, 1982).

Comparison of the location of the phosphorylated regions by chymotryptic cleavage in both NS1 and NS2 has revealed a conserved group of phosphorylated residues, which may be required for a basal level of activity. Hsu and Kingsbury (1982) defined the constitutively phosphorylated regions close to the amino terminus and suggested that they may be embedded within the tertiary structure of the

protein. The amino acids which are phosphorylated secondarily, and which make NS more active in RNA synthesis, reside in a more exposed region of the protein molecule. The NS protein is alone (amongst VSV proteins) in its ability to withstand mutations. Unlike the N, M and G proteins of the two serotypes (Indiana and New Jersey), which are highly conserved in amino acid sequence, the NS proteins of these two serotypes exhibit only 30% homology (Gill and Banerjee, 1985). Nevertheless, the NS proteins share certain structural features: a negatively charged N-terminal region, 18 conserved potential phosphorylation sites (serine and threonine) spanning the polypeptide (Rae and Elliott, 1986) and a highly conserved (90%) 21 amino acids at the C terminus.

2.(b).2.5. THE L PROTEIN

The L protein is the largest VSV protein. Its gene is 6380 nucleotides in length and codes for a basic 2109 amino acid protein with a M_r 241012 (Schubert *et al.*, 1984). Thomas *et al.* (1985) observed that the L protein is the least abundant protein in the virion contributing only 6.1% of the total protein (estimated to represent 50 copies/virion). The L protein is known to be located in the nucleocapsid in the virion and to form at least part of the transcription complex (Emerson and Wagner, 1973; Emerson and Yu, 1975; Szilagyi *et al.*, 1977; Naito and Ishihama, 1976; Mellon and Emerson, 1978). Ongradi *et al.* (1985b) however conjectured that L is the transcriptase itself. Sanchez *et al.* (1985) described a protein kinase activity associated with the purified L protein that preferentially phosphorylates the NS protein on serine residues *in vitro*. Blocking NS phosphorylation resulted in the cessation of RNA synthesis indicating that the phosphorylation of the NS protein by the L-protein-associated kinase plays a direct role in transcription. Thus the L protein is not only the polymerase but also mediates auxiliary reactions essential for transcription (and conceivably replication) and in addition L is thought to perform functions such as capping, methylation and polyadenylation of the messages. Attempts

to identify domains within the L protein and relate them to specific functions using recombinant DNA technology have been hampered by the large size and instability of the L protein.

2.(c). THE ORGANIZATION OF THE VSV GENOME

2.(c).1. GENERAL

The genome of VSV Indiana consists of the leader region (Colonno and Banerjee, 1978a; Rose et al., 1980; McGeoch and Dolan, 1979; Rowlands, 1979), the leader-N intertranscript region (McGeoch and Dolan, 1979), the five VSV genes; N, NS (Gallione et al., 1981), M, G (Rose and Gallione, 1981) and L (Schubert et al., 1984) and the 5' terminal trailer region (Schubert and Lazzarini, 1981b). The genome including the leader region, the five genes, the four intercistronic regions and the terminal tail region comprises 11162 nucleotides (Figure 2). The genes are transcribed sequentially in the order 3'-N-NS-M-G-L-5'. Villarreal et al. (1976) observed that the five mRNA's are not present in equimolar amounts in vivo and that they are present in a decreasing order of abundance which is inversely related to their distance from the 3' terminus. N mRNA is thus the most abundant mRNA and L mRNA is the least abundant. This abundance gradient is thought to be a result of partial termination of transcription (attenuation) at or near the intergenic boundaries (Iverson and Rose, 1981) since both the efficiency of translation and the rate of degradation are similar for each mRNA (Pennica et al., 1979; Lodish and Froshauer 1977; Villarreal et al., 1976).

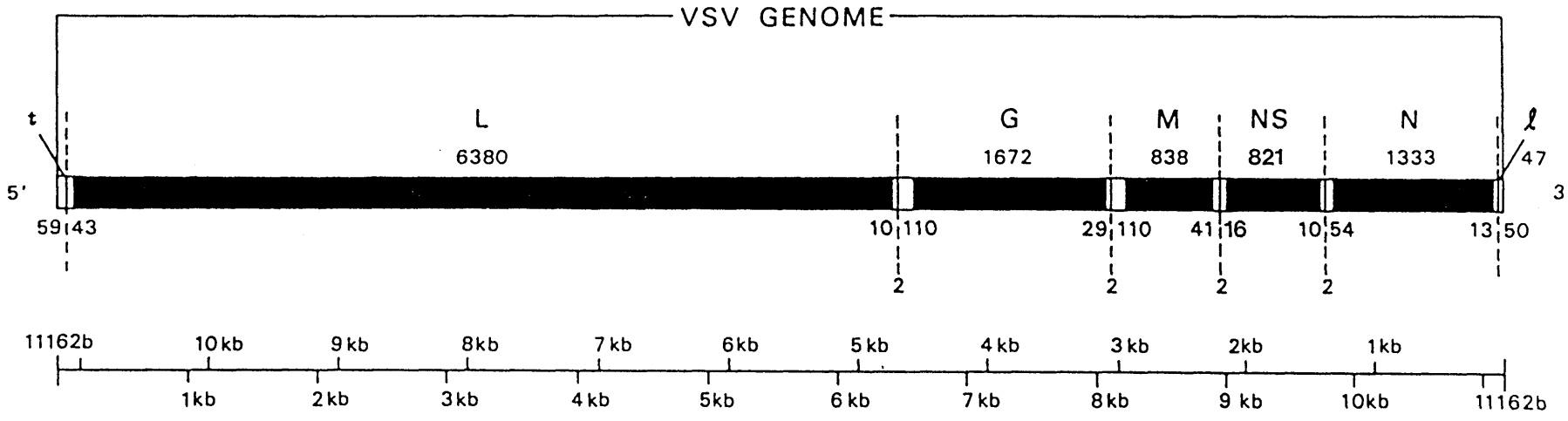
Until recently, there was no evidence of any polycistronic mRNA in VSV, although the occurrence of small non-structural polypeptides encoded both in the same reading frame and in overlapping reading frames of the viral P protein (a phosphoprotein thought to correspond to NS) have been reported for other non-segmented negative strand viruses such as Newcastle disease virus (Collins et al., 1982), mumps virus (Simpson et al., 1984), simian virus 5 (Paterson et al., 1984), measles virus (Bellini et al.,

Figure 2

A schematic diagram of the VSV genome constructed from the nucleotide sequence data.

The coding regions of each gene are marked by filled boxes. The terminal trailer region is denoted 't' and the leader region 'l'. The four two-nucleotide intercistronic regions are also shown. The sizes of the mRNAs without poly(A) tails and the sizes of the terminal untranslated regions of each mRNA are indicated above and below.

Adapted from Schubert et al. (1984)


VSV GENOME
t
L
6380
G
1672
M
838
NS
821
N
1333
l
47
5'
3'
59
43
10
110
29
110
41
16
10
54
13
50
2
2
2
2
11162b
10kb
9kb
8kb
7kb
6kb
5kb
4kb
3kb
2kb
1kb
1kb
2kb
3kb
4kb
5kb
6kb
7kb
8kb
9 kb
10kb
11162b

1985) and Sendai virus (Giorgi et al., 1983). The report by Herman (1986) of a small polypeptide encoded in the same open reading frame as the NS protein of VSV New Jersey was the first of a possibly bicistronic message in VSV. The additional protein was shown to be synthesized both in an in vitro translation system and in vivo in intracellular infected cell extracts. Immunoprecipitation with monoclonal antisera directed against the NS protein showed that the additional protein was encoded in the same open reading frame as the NS protein. Translational mapping of the NS mRNA indicated that the additional polypeptide was encoded by the 3' one-third of the sequence probably by initiation at an internal AUG.

The five mRNAs are capped and methylated at the 5' terminus and polyadenylated at the 3' terminus (Abraham et al., 1975b; Rose, 1975; Rose and Knipe, 1975).

2.(c).2 THE LEADER RNA

The leader RNA is 47-48 nucleotides in length and unlike the viral mRNAs is not capped, polyadenylated or translated (Colonno and Banerjee, 1976, 1978a, 1978b). It is transcribed from the exact 3' end of the genome. Leppert et al. (1979) have shown the presence of both plus and minus leader RNAs in VSV infected cells, the minus strand leader being transcribed from the 3' end of the plus strand antigenomic RNA. The plus strand leader has been implicated in the inhibition of host RNA synthesis (Weck and Wagner, 1978, 1979; McGowan et al., 1982; Grinnell and Wagner, 1984). Blumberg et al. (1983) noted the existence of a five times repeating array of adenine residues in nascent plus and minus leader RNA and proposed that this sequence repetition constitutes at least part of the specific assembly sequence of VSV. Kurilla et al. (1982) observed that the leader RNA is transiently associated with the nucleus. In addition Leppert et al. (1979) and Blumberg et al. (1981) proposed that the plus and minus leader RNAs may function as decision points for switching between transcription and replication.

Kurilla and Keene (1983) proposed that the plus

strand leader RNA could be divided into three domains;

(1) the 3' terminus: This region is conserved between both VSV serotypes and at the 3' end of DI's (Keene et al., 1979). It has been suggested that this region is probably essential for polymerase initiation on the template and for N protein nucleation on the leader RNA transcript (Blumberg et al, 1983).

(2) the middle 1/3: This region contains a TATA like sequence (Rose and Iverson, 1979) and it has been shown that the viral RNA polymerase interacts with this region on the template (Keene et al., 1981) and it therefore may function as part of a promoter sequence for recognition by virion polymerase components (Keene et al., 1981).

(3) the terminal 1/3: Kurilla and Keene (1983) proposed that the interaction of this region of the leader RNA with the cellular La protein raises the possibility of the involvement of La protein in the viral inhibition of host macromolecular synthesis. La protein is normally found transiently bound to unprocessed cellular RNAs that are products of RNA polymerase III. It has also been suggested that the binding of La protein to plus and minus leader may serve as a modulator of virus transcription versus replication (Kurilla and Keene, 1983; Wilusz et al., 1983).

2.(c).3 INTERGENIC REGIONS

VSV has four intergenic junctions. They are similar in structure and contain the conserved sequence 3'-AUAC$(U)_7$-5'. There is a further poly(A) addition site downstream of the distal L gene (McGeoch, 1979; Schubert et al., 1980; Rose, 1980). The above sequence is thought to represent the signal for polyadenylation by `slippage polymerization'. The sequence is followed by the dinucleotide 3'-GA-5', or 3'-CA-5' in the case of the NS:M junction (Rose, 1980) which is not transcribed and marks the true intergenic junction. This dinucleotide is followed by sequence with the general form 3'-UUGUCNNUAG-5' (where N represents a variable site) which is complementary to the 5' end of the mRNA (McGeoch, 1979; McGeoch et al., 1980). There is no evidence of any potential secondary structure at

the intergenic junctions that might correlate with the observed partial termination of transcription, thought to occur at or near the intergenic boundaries (Iverson and Rose, 1981).

2.(d). THE REPLICATIVE CYCLE

Virus entering the cell is uncoated and releases its nucleocapsid into the cytoplasm. This incoming nucleocapsid contains the negative polarity RNA strand which serves as a template for the five subgenomic mRNAs (Morrison et al., 1974; Both et al., 1975), the leader RNA (Leppert et al., 1979; Blumberg et al., 1981; Kurilla et al., 1982) and a sequence complementary to the 5' terminal region (Schubert and Lazzarini, 1981a).

Primary transcription of the incoming genome utilises the relevant proteins present in the virion particle including the proteins necessary for replication (N, NS, L). The polymerase has a single entry site at the 3' end of the genome (Emerson, 1982). In the replication mode, the polymerase proceeds to the 5' end, ignoring all internal termination and polyadenylation signals along the RNA. The resulting plus strand antigenome then serves as the template for full length negative strand genomes which in turn take part in secondary transcription to generate more mRNAs.

The remainder of this section will concentrate on a more detailed description of the entry of virus into the cell, the viral proteins involved in transcription, and the mechanisms of transcription and replication.

2.(d).1 VIRUS ATTACHMENT AND PENETRATION

VSV infects host cells via adsorptive endocytosis (Dahlberg, 1974; Matlin et al., 1982; Schlegel et al., 1981, 1983). This process involves the interaction of the VSV G protein with specific receptors on the plasma membrane of the cell. VSV binds preferentially to the acidic phospholipid phosphatidyl serine (Schlegel et al., 1983). After binding VSV is rapidly translocated into plasma membrane 'coated pits' and then transferred to intracellular vesicles (endosomes).

Evidence for the involvement of G protein in this process was provided by Kelley et al. (1972) and Bussereau et al. (1975) who effectively prevented any G mediated interaction by proteolytic hydrolysis of the external spikes (Kelley et al., 1972) and by specific neutralization of the G protein by antibody (Bussereau et al., 1975). More recently, several reports have demonstrated the direct involvement of the G protein in membrane interactions. Firstly, White et al. (1981) observed that mammalian cells can be fused by exogenous VSV at low pH. Secondly, Florkiewicz and Rose (1984) and Riedel et al. (1984) demonstrated that cells expressing cloned G protein are fusogenic at low pH. Thirdly, Eidelman et al. (1984) and Bailey et al. (1984) observed that G protein can promote liposome membrane fusion. Finally, Schlegel and Wade (1984, 1985) demonstrated that G protein can haemolyse erythrocytes at low pH and that the haemolytic domain resides within the terminal six amino acids at the mature NH_2 terminus.

Schlegel et al. (1982) reported the existence of specific receptors for VSV on Vero cells. Approximately 4000 sites were detected per cell. In addition to these so called 'saturated sites', 'non-saturated sites' were also found which bound VSV at low multiplicities and which were thought to possibly represent a route of infectivity.

2.(d).2 VSV PROTEINS INVOLVED IN TRANSCRIPTION

The transcribing ribonucleoprotein (RNP) of VSV consists of linear, single stranded genome RNA of negative polarity closely associated with approximately 1258 molecules of nucleocapsid protein (N), 50 molecules of L protein and 466 molecules of NS protein (Thomas et al., 1985). In the presence of the four NTPs, this RNP synthesizes the five VSV mRNAs in vitro.

Using in vitro reconstitution experiments Schubert et al. (1982) and Patton et al. (1984) demonstrated a requirement for both L and NS protein for mRNA synthesis. Because of the lability of the L protein, investigations into the roles played by both L and NS proteins in the transcription process have met with some difficulty. In

addition, there is still some controversy as to the exact roles of the L and NS proteins in both the initiation and elongation steps of transcription. However, it has been established that the requirement for L protein, for optimal RNA synthesis, is catalytic whereas the requirement for NS protein is stoichiometric (De and Banerjee, 1985; Schubert et al., 1985).

The results of De and Banerjee (1984, 1985) with VSV Indiana suggest that while L protein is involved in the initiation step, the NS protein is involved in the RNA chain elongation step. However, Ongradi et al. (1985a, 1985b) in their studies of transcription using transcriptase negative ts mutants of VSV New Jersey, proposed that polypeptide L is the transcriptase itself and that the NS protein exerts some control over transcription. More recently, Banerjee (1987) suggested that the NS protein comprises three distinct functional regions directly involved in transcription. Domain I contains the negatively charged N-terminal half of the protein which it is thought may interact with the N protein, thereby making the template RNA accessible to the L protein transcriptase. Domain II has been shown by deletion mapping experiments and by use of an in vitro transcription and translation system (Gill et al., 1986) to contain a 34 amino acid stretch (residues 213-247) that is involved in binding NS protein to the L protein. The L protein in turn interacts with the N-RNA complex to initiate RNA chains. In addition, Chattopadhyay and Banerjee (1987) showed, in an investigation of the functional significance of phosphoserine residues in Domain II, that site directed substitution of serine residues at positions 236 and 232 (which are conserved between the Indiana and New Jersey serotypes) by alanine abolished RNA synthesis catalysed by the NS-L complex in vitro. It was suggested therefore that phosphorylation of these two serine residues regulates NS binding to L protein and the N-RNA template and that it is essential for activation of viral RNA synthesis (Chattopadhyay and Banerjee, 1987). Domain III comprises the 21 amino acids at the C-terminus. This domain is not required for transcription but since it is the most

conserved between the two VSV serotypes, it was suggested that it might interact with the N protein that is associated with the genomic RNA template.

2.(d).3 TRANSCRIPTION

Studies on the mechanism of transcription *in vitro* by the RNP of VSV have demonstrated that RNA synthesis is sequential and takes place in the order leader-N-NS-M-G-L (Abraham and Banerjee, 1976a, 1976b; Ball and White, 1976; Iverson and Rose, 1981, 1982). Two models have been proposed for this sequential mode of VSV mRNA transcription *in vitro*.

In the single initiation model, it is suggested that the transcriptase begins transcription at the 3' end of the genome and that the leader plus five mRNAs are produced by sequential transcription followed by sequential cleavage of the growing precursor chain. Evidence for such a model is provided by the detection both *in vitro* (Rose *et al.*, 1977; Herman *et al.*, 1978, 1980) and *in vivo* (Masters and Samuel, 1984) of polycistronic transcripts. However, there appears to be a major difference between *in vivo* and *in vitro* synthesized polycistronic messenger. The *in vitro* synthesized mRNAs are polyadenylated at intercistronic junctions (Masters and Samuel, 1984) whereas the *in vivo* synthesized polycistronic messengers are only polyadenylated at the 3' molecular termini (Herman *et al.*, 1978, 1980).

In the multiple initiation model, it is suggested that the RNA polymerase initiates transcription at multiple promoter sites, but that chain elongation from a given site only occurs after transcription of its upstream gene. Thornton *et al.* (1984) provided evidence for this model using a uv irradiated template reconstituted with soluble transcriptase. They showed that the synthesis of leader RNA and other small initiated mRNA sequences continued while full length mRNA synthesis decreased by 90%. Also, under partial reaction conditions phosphorylated oligonucleotides which represent 5' terminal sequences transcribed from the leader template and genes coding for mRNAs were detected. In addition, in the presence of arabinosyl ATP (an inhibitor of

RNA synthesis in vitro) the synthesis of leader RNA was found to be initiated considerably more than other small initiated RNA sequences but under conditions in which leader RNA synthesis is abolished initiation at the N gene continued demonstrating that an internal site is available for binding of transcriptase.

With a view to addressing the respective roles of the L and NS proteins in the transcription process Banerjee (1987) proposed a model for VSV transcription in vitro based on the structure and function of the L and NS proteins (figure 3). It had previously been established that the requirement for L protein for optimal RNA synthesis is catalytic whereas the requirement for NS protein is stoichiometric (De and Banerjee, 1985; Schubert et al., 1985). Banerjee proposed that NS protein is bound uniformly along the RNP, whereas the L protein is located at specific sites on the template (to be able to initiate transcription of individual genes) and is also probably associated with a subset of NS molecules. During transcription as the L protein associates with previously bound NS protein, it displaces the tightly bound N protein, allowing access to the template by the L protein transcriptase.

2.(d).4 CAPPING AND POLYADENYLATION OF mRNAs

The in vitro synthesized mRNA species, in common with typical eukaryotic mRNAs, contain a 5'-terminal cap structure (GpppA) (Abraham et al., 1975b; Hefti and Bishop, 1976) and a 3'-polyadenylic acid [poly(A)] tract (Banerjee and Rhodes, 1973; Galet and Prevec, 1973; Villarreal and Holland, 1973: Ehrenfeld, 1974). In the presence in vitro of the methyl donor S-adenosyl-L-methionine, specific methylation of the 5' cap structure occurs with the formation of the dimethylated cap 5'-$_{m}$GpppA^{m} (where $_{m}$G represents 7-methyl guanine and A^{m} represents 2-O-methyl adenine; Abraham et al., 1975a). In contrast, the mRNAs isolated from infected cells contain, in addition to methylated G, a doubly methylated penultimate A and variable degrees of methylation at the second A residue (Rose, 1975; Moyer and Banerjee, 1976). In addition, uncapped full

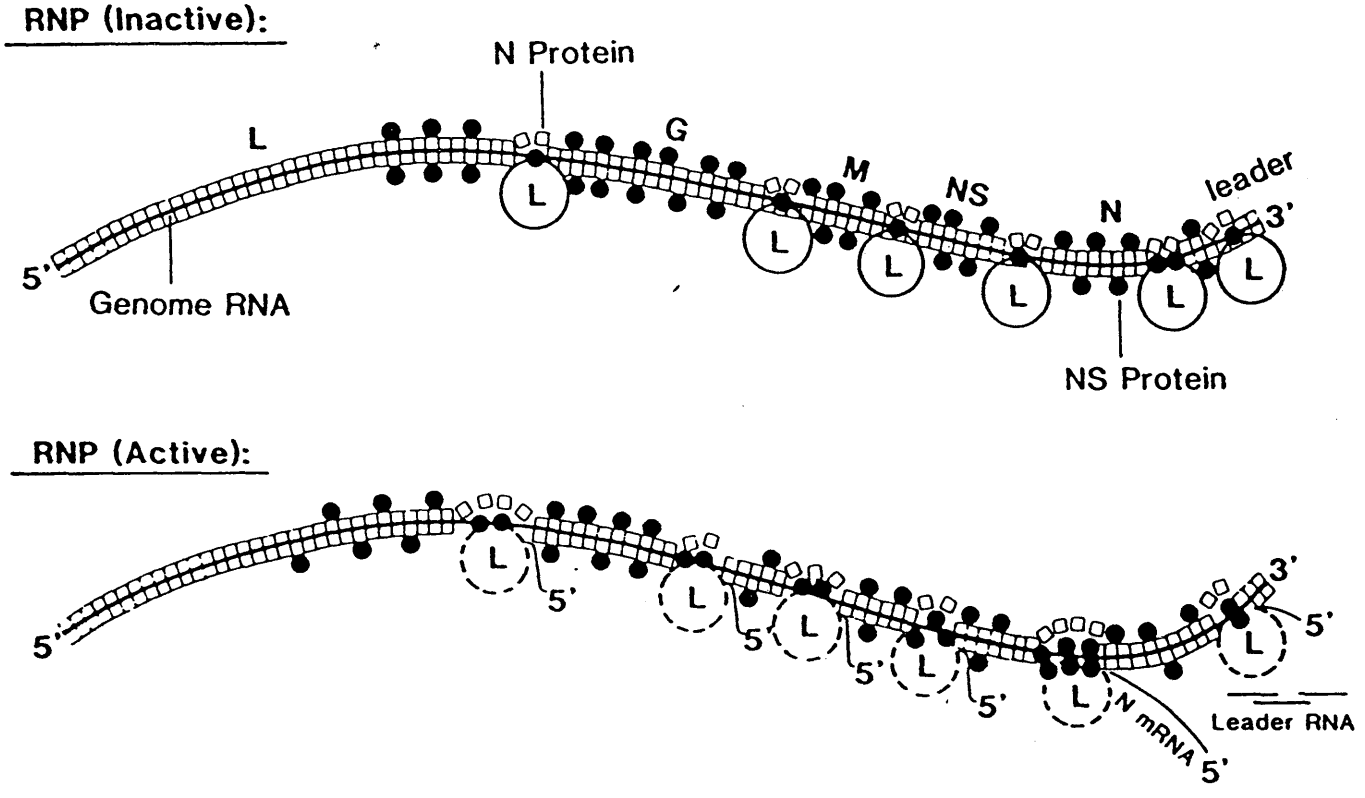
Figure 3

A possible model for VSV transcription.

The top half of the model shows the proposed positions of the N and NS proteins when the ribonucleoprotein is in an inactive state. NS is bound uniformly along the RNP whereas L protein is located at specific sites on the template, to be able to initiate transcription of individual genes, and is probably associated with a subset of NS molecules.

During active transcription (bottom half of model), as the L protein moves to each new region of the template, it binds to NS protein already bound so displacing tightly bound N protein and allowing access to the template by the L protein transcriptase.

From Banerjee (1987).


RNP (Inactive):
N Protein
L
G
M
NS
N
leader
3'
5'
Genome RNA
NS Protein
RNP (Active):
5'
3'
5'
N mRNA
Leader RNA

length transcripts containing 5'-triphosphate A and G have been found in low levels *in vivo* (Rose, 1975) and *in vitro* (Hefti and Bishop, 1976; Toneguzzo and Ghosh, 1976). The unique feature of the cap structure of the VSV mRNAs is that the α and β phosphates of the blocking GTP and the α phosphate of the penultimate A are incorporated into the 5'-5'-triphosphate moiety G(5')$\alpha\beta/\alpha$(5')AA (Abraham *et al.*, 1975a, 1975b). This feature is quite different from those of other viral systems (such as reovirus and vaccinia) where the α phosphate of the blocking GTP and both α and β phosphates of the penultimate base are incorporated into the triphosphate bridge (Banerjee, 1980).

The *in vitro* synthesized mRNAs contain approximately 200 adenine residues at their 3' end, indicating that post-transcriptional polyadenylation is carried out by one or more virus specified proteins (Banerjee and Rhodes, 1973; Galet and Prevec, 1973; Villarreal and Holland, 1973; Ehrenfeld, 1974). Curiously, S-adenosyl-L-homocysteine, an inhibitor of methylation, greatly increases the length of the poly(A) tails made *in vitro* (Rose *et al.*, 1977). Although the role of S-adenosyl-L-homocysteine has not been elucidated, the results seem to indicate that methylation and polyadenylation are interdependent and both may have roles in the transcription process.

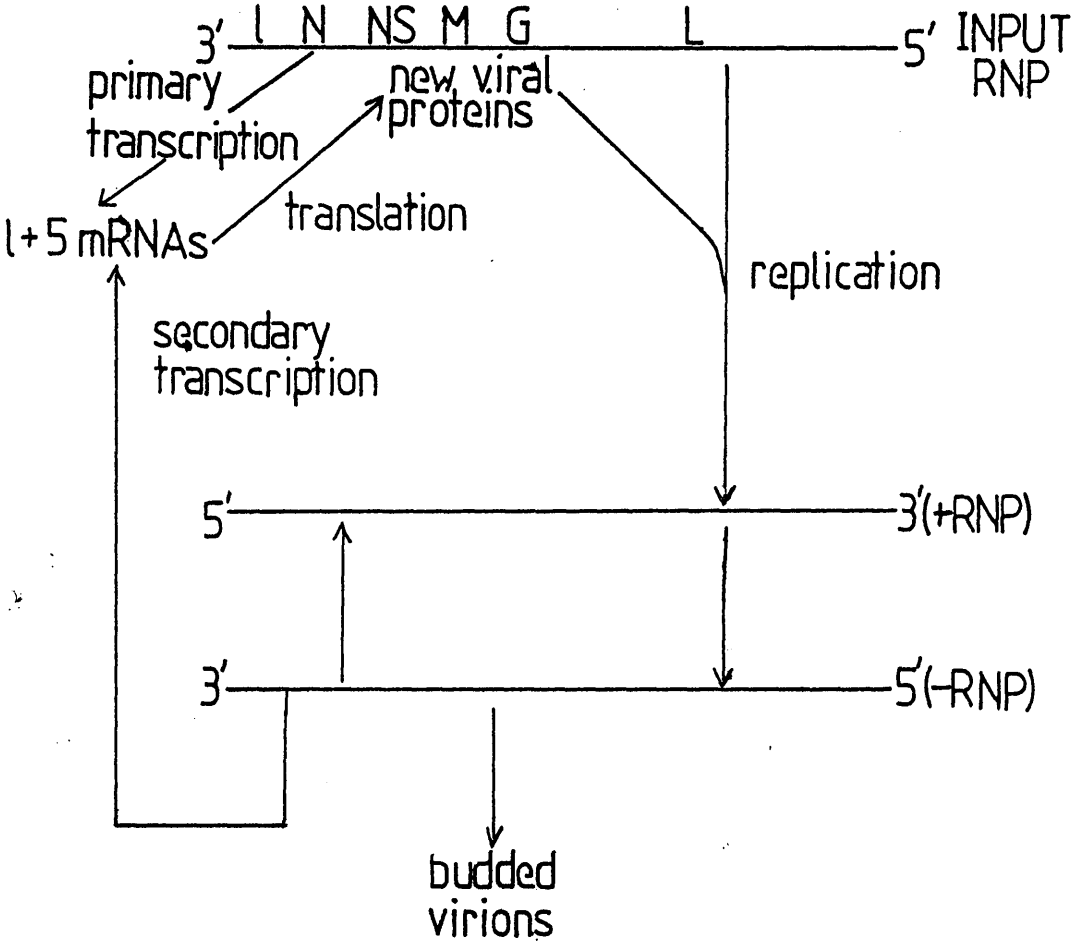
2.(d).5 REPLICATION OF VSV RNA

VSV nucleocapsids serve as templates for both transcription and replication (figure 4). The negative sense RNP serves as the template for the synthesis of full length negative strand RNA. This plus strand complexed with N protein serves as the template for the synthesis of negative sense RNP's which are employed in secondary transcription and progeny virus assembly. The synthesis of both full length genomic and antigenomic RNA is thought to be catalysed by the same replicase (Emerson and Yu, 1975; Perlman and Huang, 1973; Pringle, 1978), perhaps in conjunction with unidentified host factors (Simpson *et al.*, 1979; Morrongiello and Simpson, 1979; Mukherjee and Simpson, 1984).

Figure 4

A schematic diagram of the replication cycle of VSV

Replication of rhabdoviruses takes place in two stages. Primary transcription involves transcription of the negative sense genomic RNA from the extreme 3' end to generate the five viral mRNAs wich are subsequently translated. Plus sense RNA is synthesized when viral protein synthesis decreases. The plus sense RNA then acts as a template for synthesis of full length negative sense RNA which is packaged into budding virions or used as a template for subsequent mRNA synthesis (secondary transcription).


3' l N NS M G L 5' INPUT RNP
primary transcription
new viral proteins
l+5 mRNAs
translation
replication
secondary transcription
5'
3'(+RNP)
3'
5'(-RNP)
budded virions

Using a coupled transcription/translation system, Patton et al. (1984) employed both intracellular and virion derived nucleocapsids in conjunction with purified individual mRNAs to examine the protein synthesis requirements for VSV RNA replication. Their results showed that VSV replication required only a new source of N protein and that it did not require the synthesis of new L or NS protein.

Arnheiter et al. (1985) suggested that specific binding of the nucleocapsid protein to the 5' end of the leader RNA, so initiating encapsidation of the growing RNA strand (Blumberg et al., 1983), might posibly serve to protect the RNA from processing or cause the readthrough of the transcriptional signals thus allowing synthesis of genome size RNA. Arnheiter et al. (1985) investigated the role of the N protein in the regulation of VSV RNA synthesis using two monoclonal antibodies directed against different epitopes of the N protein. Antibody 1 bound to nucleocapsids and possibly the pool of cytoplasmic free N protein. It interfered with both the transcriptional and replicative activity of the nucleocapsid - possibly by arresting the progress of the polymerase. Antibody 2 did not inhibit transcription and bound only poorly to nucleocapsids. However, it did inhibit replication and it bound to the free pool of cytoplasmic N protein. They suggested that this antibody inhibited replication by neutralizing the pool of free N protein. The results obtained using these antibodies suggest a mechanism of regulation in which the availability of free N protein determines the balance between replicative and transcriptional RNA synthesis in VSV infected cells (Arnheiter et al., 1985).

2.(d).6 VIRAL PROTEIN SYNTHESIS

All newly synthesized viral proteins can be detected by 1-2 h post infection in VSV infected cells (Mudd and Summers, 1970; Wunner and Pringle, 1972a, 1972b). The first viral protein to be detected is the N protein, which can be detected about 30 minutes before the other four viral

proteins. Later in infection, N protein is found associated with a large number of nucleocapsids (Hsu et al., 1979). N is synthesized on free ribosomes (as are the rest of the VSV proteins except G protein) and is first detected as a soluble protein before it is incorporated into the nucleocapsid (Knipe et al., 1977a; Hsu et al., 1979). NS protein is found in abundance in the cytoplasm of infected cells (Wagner et al., 1976). L protein is also found free in the cytoplasm after synthesis as well as becoming associated with the plasma membrane.

Early in infection M protein is distributed diffusely throughout the cytoplasm of infected cells. Later however, N, NS and M accumulate close to the membrane and it is thought that M only interacts with the nucleocapsid close to the assembly site at the membrane.

G protein is synthesized on membrane bound ribosomes. The nascent protein is inserted into the membrane of the rough endoplasmic reticulum where the 16 amino acid signal sequence is proteolytically cleaved (Chatis and Morrison, 1979; Irving et al., 1979). The co- and post-translation glycosylation and transport of G protein to the cell surface have been well documented (Rothman and Lodish, 1977; Rothman and Fine, 1980; Lodish and Rothman, 1979; Etchison and Summers, 1979; Morrison, 1980; Bergmann et al., 1981; Rose and Bergmann, 1982; Wehland et al., 1982; Guan et al., 1985; Puddington et al., 1986). Additional post-translational modification occurs before or when G arrives at the plasma membrane in VSV Indiana, but not in VSV New Jersey. This consists of the addition of fatty acids which are thought to act as an auxiliary lipophilic anchor of G protein to the membrane (Schmidt and Schlesinger, 1979; Capone et al., 1982). The fatty acid chain (palmitate) is linked to the single cysteine residue in the cytoplasmic domain (Rose et al., 1984). The findings of Villarreal et al. (1976) and Lodish and Froshauer (1977), which revealed that the VSV mRNAs are all translated with equal efficiencies and that the molar ratios of the proteins reflect the molar ratios of the mRNAs, suggest that control of viral protein synthesis in vivo occurs at the level of transcription.

2.(d).7 VIRUS MATURATION AND BUDDING

The ultimate step in the replicative cycle of VSV is the assembly of the virus, which involves the interaction of the nucleocapsid with virus modified host membranes at the site of budding. The respective roles of the G and M proteins in promoting attachment of the nucleocapsid to the membrane have been long debated. Several observations support the role of the G protein in virus assembly and budding. Exposure of VSV infected cells to monensin (which stops the transport of G protein to the cell surface) prevents virus budding at the cell surface (Johnson and Schlesinger, 1980). In differentiated epithelial cells, G protein is transported mainly to the basal pole, and VSV buds predominantly from this domain (Rodriguez-Boulan and Prendergast 1980; Rodriguez-Boulan and Sabatini, 1978). Roth et al. (1979) and Roth and Compans (1980) observed that the polarity of budding is maintained after tunicamycin treatment, suggesting that glycosylation of G protein is not a major determining factor in the site of virus budding. However, several reports have concluded that the G protein does not have an essential role in budding, in particular the observation that spikeless virions were formed at a relatively high yield from cells infected by the G mutant ts 045. The ts 045 glycoprotein is retarded in the rough endoplasmic reticulum (RER) due to a transport block and no protein is found at the cell surface (Knipe et al., 1977a; Zilberstein et al., 1980). However, more recently a report by Metsikko and Simons (1986) showed that the spikeless virions formed from ts 045 infected cells contain a full complement of membrane anchors derived from the C-terminus of the G protein. This clearly favours the idea that an interaction between the G protein carboxy terminal domain with the (M-protein coated) nucleocapsid is an essential event in viral assembly.

Zakowski and Wagner (1980) suggested that since the M protein is located on the inside of the envelope, it may form a bridge between the G protein and the nucleocapsid. Evidence for this association was provided by Newcomb and

Brown (1981) who demonstrated that M protein remains complexed with the tightly coiled nucleocapsid skeleton after removal of both G protein and the viral membrane from virions by treatment with the detergent octyl glucoside. Odenwold et al. (1986) examined the role of the M protein in the budding process by stereo electron microscopy. From their results they postulated that the process of budding starts with the attachment of nucleocapsids to the cell membrane (presumably by interacting with G protein on the cytoplasmic side of the membrane) so resulting in the initiation of nucleocapsid coiling. This conformational change allows M protein to specifically bind to the nucleocapsid and induce a conformation favourable for coiling or cross link adjacent turns in the tight coil or both (Odenwold et al.,1986). Moreover, Ono et al. (1987) have recently reported that no nucleocapsid coiling is seen in cells infected by the M mutant ts G33, supporting this view of VSV assembly.

2.(e). GENETICS

2.(e).1 MUTANTS

Mutants of VSV can be divided into two general categories (Pringle, 1977). These categories consist of;

(1) mutants which have a specific phenotype. Examples of this type of mutant are the plaque morphology mutants described by Wagner et al. (1963) and the Rif^+ mutants (Moreau, 1974).

(2) mutants which are conditionally lethal. Mutants which fall into this category can be of three distinct forms. They can be temperature sensitive (ts), temperature dependent host range (tdCE) or host restricted (hr) mutants. A fourth class of conditional lethal mutant can now be included in this category. These are suppressible nonsense mutants, which were isolated from mutagenized VSV and are the subject of this thesis.

Of the mutants described above, ts mutations have been used more extensively in VSV genetic analysis than any other type of mutant because of the ease with which they can

be isolated from mutagenized virus and also because of the ease with which they can be assayed.

The mutagen which has been most effective in the generation of ts mutants of VSV is the base analogue 5-fluorouracil (5-FU; Pringle, 1970). Several other mutagens have been used, such as ethyl methane sulfonate (EMS) (Pringle, 1970; Holloway et al., 1970), 5-azacytidine (ACR) (Pringle, 1970), and nitrous acid (NA) (Holloway et al., 1970; Rettenmeier et al., 1975) but with considerably less success. In a comparison of the mutagenic abilities of different mutagens (EMS, 5-FU and ACR), Pringle (1970) reported that 5-FU was the most potent mutagen of the three tested. However, Flamand (1970) reported the isolation of ts mutants from an unmutagenized stock of VSV Indiana. This must surely reflect the low fidelity of RNA genome replication resulting from the lack of any proofreading mechanisms.

Ts mutants of VSV Indiana and New Jersey have been classified into six complementation groups. In the case of the New Jersey serotype the six groups are designated A-F and in the case of the Indiana serotype I-VI. The anomaly of six complementation groups and only five gene products is thought to result from intragenic complementation. Although no intragenic recombination was observed between the 5-FU induced mutants of the Glasgow strain of VSV Indiana, intragenic complementation has been detected in group ChI (polymerase gene) of Chandipura virus (Gadkari and Pringle, 1980). The extent of the intragenic complementation in Chandipura virus was thought to suggest that the virion polymerase exists in a multimeric form (Gadkari and Pringle, 1980).

No direct evidence of recombination has been observed for complete VSV genomes, although the structures of fusion defective interfering particles (DI's, which are described in a separate section) suggest the presence of a RNA breakage-reunion process in VSV infected cells. In addition, no recombination has been detected for any other negative strand RNA virus with an unsegmented genome. Genome rearrangements have been detected within negative

strand RNA viruses with segmented genomes, however this is more correctly reassortment and not recombination. Although the possibility that recombination occurs cannot be excluded, it must be an extremely rare event if it does occur.

Complementation however, has been observed widely within vesiculoviruses and classification of ts mutants into complementation groups has proven an effective method of examining the functional properties of the genome of these viruses.

Host range (hr) mutants of VSV Indiana have been isolated which were restricted in Hela cells, but not in CE cells (Obijeski and Simpson, 1974; Simpson and Obijeski, 1974). In a comparison of thirty cell types Simpson et al. (1979) reported a gradient in permissiveness of these mutants in which cells of human origin were the least permissive. In addition a difference in permissiveness was observed between individual sublines of Hela cells (Simpson et al., 1979) and it was concluded that host factors were involved in viral replication.

Conditional (tdCE) and non-conditional (hrCE) host range mutants of VSV New Jersey, Indiana and Cocal have also been described (Szilagyi and Pringle, 1975; Pringle 1977, 1978). The tdCE mutants are conditionally ts mutants which grow normally in BHK21 (clone 13) cells at either 31°C or 39°C, but only at 31°C in CE cells. A reversible inhibition of in vitro polymerase activity at 39°C was exhibited by several of these mutants (Szilagyi and Pringle, 1975). Dissociation and reconstitution experiments revealed that the mutation in these mutants lay in the L protein (Szilagyi et al, 1977). Szilagyi and Pringle (1975) observed that this phenotype was expressed irrespective of the cells in which the virus had been grown. The host range involvement in the phenotype of these cells suggested that host factors, absent in CE cells, were involved with the polymerase in some way maintaining it in an active conformation at the non-permissive temperature (Szilagyi and Pringle, 1975; Pringle, 1978).

In a study involving different cell types (picked

because they represented cell types from different stages in differentiation) Pringle (1978) suggested that the putative host factors may not be expressed until the host cell has undergone an undefined degree of differentiation.

2.(e).2 GENE ASSIGNMENT OF VSV INDIANA

This section summarizes the data presently available linking complementation groups to specific genes. Some of the evidence is in fact quite weak but this probably does not affect the validity of the gene assignments derived from such data. The application of additional nucleotide sequence analysis promises to clarify this area.

2.(e).2.1. COMPLEMENTATION GROUP I

The defect in complementation group I was identified by Hunt *et al*. (1976) as residing in the L protein. Mutants in group I exhibit restricted RNA synthesis (RNA^- phenotype) at the non-permissive temperature. Hunt and Wagner (1974) demonstrated that the transcription defect in three group I mutants was due to thermosensitivity of the solubilized transcriptase enzyme. Hunt *et al*. (1976) distinguished between the two components of the transcriptase complex (L and NS) by demonstrating that the *in vitro* transcriptase activity of three group I mutants was restored by the addition of L protein fractions derived from *wt* and not by NS protein fractions.

2(e).2.2. COMPLEMENTATION GROUP II

The defect in complementation group II was localized both by partial proteolysis of *wt* and *ts* mutants of VSV (Metzel and Reichmann, 1981) and also by tryptic peptide mapping (Lafay and Benejean, 1981).

Metzel and Reichmann (1981) compared the partial digests of VSV proteins derived from *wt* and the group II *ts* mutants by partial proteolysis with *Staphylococcus aureus* V8 protease. Mutants of complementation group II differed from the *wt* virion in peptide profiles of their NS protein. Lafay and Benejean (1981) analysed tryptic digests by ion-exchange chromotography of NS protein from mutants and

revertants of group II. Since a ts defect is correlated with a specific protein modification, it follows that revertants of this mutation should posess a new modification in the same protein. In the case of the group II mutant studied (ts 052) the modified peptide was found in the NS protein.

2.(e).2.3. COMPLEMENTATION GROUP III

The defect in mutants of complementation group III was also identified by Metzel and Reichmann (1981) and Lafay and Benejean (1981) by the same method. Partial proteolysis revealed that a mutant of complementation group III (ts G31) differed from wt in the peptide profile of its M gene (Metzel and Reichmann, 1981). Lafay and Benejean (1981) revealed by tryptic peptide mapping that the modified peptide in the group III mutant ts 023 was detected in the M protein. In addition, Freeman and Huang (1981) used heteroduplex mapping to identify the defect in group III mutants. The genomic RNA was annealed to small complementary mRNAs and trimmed with ribonucleases. Should a base mismatch occur between the mutant gene and mRNA isolated from revertant infected cells, the duplex containing the lesion would be expected to migrate differently on a polyacrylamide gel. Heteroduplex mapping of the mutant ts G31 assigned this mutation to the gene coding for the M protein. More recently Gopalakrishna and Lenard (1985) determined the nucleotide sequence of the M gene of four ts M-protein mutants (ts G31, ts G33 of the Glagow strain and ts 023 and ts 089 of the Orsay strain) and of their respective wt M-protein genes. It was found that the mutants differed from the wt by one or two point mutations in the M gene.

2.(e).2.4. COMPLEMENTATION GROUP IV

The defect in complementation group IV of VSV was determined in the same manner as mutants of complementation group II. Metzel and Reichmann (1981) compared the partial digests, produced by partial proteolysis, of ts G41(IV) and wt virus. The peptide profile of ts G41(IV) differed from

the wt in the peptide profile of the N protein. Lafay and Benejean (1981) identified the mutation in ts 011(IV) by tryptic peptide analysis. The modified peptide was found in the N protein.

2.(e).2.5. COMPLEMENTATION GROUP V

Zavada (1972) identified the defect in complementation group V by rescuing the ts 045 phenotype by pseudotype formation. The mutant ts 045 grown at 45°C rapidly lost infectivity when plated for assay in chicken, L or BHK-21 cells. When ts 045 was grown in avian myeloblastosis virus (AMV) preinfected cells, heated, and then cultured in chick cells, the resultant virus had about 100 times higher activity. All thermostable chick specific virus was neutralized with anti-AMV serum. Thus pseudotype formation had rescued the ts 045 phenotype. In addition, Knipe et al. (1977b) showed that the mutant ts M501(V) did not undergo the final sialylation at 39°C resulting in a G protein with a slightly different electrophoretic mobility as detected on polyacryamide gels. Also the mutant could not be complemented by coinfection with virus strains coding for G protein that migrated normally (Knipe et al., 1977b). Gallione and Rose (1985) determined the site of the ts 045 mutational lesion, responsible for the conditional transport of G protein, by determining the sequence of cloned cDNA copies of G protein mRNA of ts 045(V), a spontaneous revertant of ts 045(V) and wt virus. This revealed that any one of three mutations could be responsible for the temperature sensitivity of the mutant. Recombinant plasmids containing these mutations were transfected into COS-1 cells and the temperature sensitivity of each recombinant investigated. This revealed that the recombinant molecule containing a mutation which substituted phenylalanine by serine was sufficient to prevent transport of G protein to the cell surface at 39.5°C.

2.(e).2.6. COMPLEMENTATION GROUP VI

The defect in complementation group VI was identified by Deutsch et al. (1979). The sole member of group VI,

ts 082, was shown by uv inactivation studies to have similar rescue characteristics to group II (NS) mutants. This implies that the NS protein comprises two domains. Complementation observed between this mutant and a group II mutant (ts 052) would result therefore from intragenic complementation between two separate domains of the NS protein.

2.(f). DEFECTIVE INTERFERING PARTICLES

Defective interfering particles (DI's) are viral particles that contain only a portion of the genetic material of the parental virus and are equivalent to deletion mutants. They are presumed to arise from aberrant replicative events during which the polymerase changes template position without releasing the nascent strand. They are constantly generated at low levels by infectious virus and can accumulate during replication at high multiplicities of infection. They are unable to self replicate in host cells but can utilize the functions of a helper virus to multiply. This results in the phenomenon of autointerference when the replication of the helper virus is greatly suppressed. DI particles are thought to suppress replication of the helper virus by effectively competing for the L or N proteins supplied by the helper virus. DI particles need only cis-acting replication functions such as initiation sites at their 3' terminus, the complement of an initiation site at the 5' terminus and the nucleocapsid encapsidation site. Trans-acting functions are supplied by the helper virus.

DI's have been implicated in the establishment and/or maintenance of persistent infection in cell culture (Holland et al., 1980; Holland and Villarreal, 1974; Horodoyski and Holland, 1980). Other factors have been observed to play a role, such as interferon (Sekellick and Marcus, 1979) and ts mutants, but clearly not to the same extent as the attenuating properties of DI's in moderating an otherwise cytocidal infection. Interestingly, DI's appear to provide a selective pressure for rapid and extensive mutational drift of standard virus during long term persistent

infections (Holland et al., 1979).

Four types of DI particle genome have been characterized and have been designated (1) fusion, (2) panhandle (3) snapback and (4) compound (Lazzarini et al., 1981; Perrault, 1981).

The first class (fusion) consists of simple deletion mutants, of which DI-LT is the prototype. They represent internal deletions of the L gene leaving the terminal regions intact (Leamnson and Reichmann, 1974; Perrault and Semler, 1979; Epstein et al., 1980). This type of DI was observed to predominate in early passages and were subsequently replaced by genomes with compound complementary ends suggesting that the latter may have a selective advantage (Keene et al., 1981). Fusion DI's can be transcribed both in vivo and in vitro and complement ts mutants because the parental 3' terminus along with the G, M, NS and N genes are undeleted (Colonno et al., 1977; Johnson et al.,1979).

The second class of DI particles (panhandle) have lost the entire N, NS, M and G coding regions in addition to the 3' end of the L gene and thus only contain information from the 5' half of the VSV parental genome. Unlike fusion DI's they can neither transcribe functional mRNA nor complement ts mutants from any of the VSV complementation groups.

The third class (snapback) also only contain sequences from the 5' half of the genome and have been shown to contain RNA with extensive self-complementarity (Perrault and Leavitt, 1977; Keene et al., 1978; Schubert et al., 1978, 1979; Schubert and Lazzarini, 1981a). If DI-011 is deproteinized, the DI particle snaps into a perfect duplex of about 1000bp (Schubert and Lazzarini, 1981a).

The fourth class (compound) consists of DI particles with compound initiation sites at the 3' terminus of their RNAs and have about 6000 nucleotides of the L mRNA coding region deleted. The prototype is DI-LT_2 which was discovered in seed stocks of the simple deletion DI particle DI-LT, and it has been suggested that DI-LT_2 may be derived from DI-LT (Perrault and Semler, 1979; Epstein et al.,

1980). Unlike DI-LT, DI-LT_2 is not transcriptionally active and the leader region (now located internally) is muted.

There are three major factors which influence the generation of DI particles:

(1) The host cell type; Holland et al. (1976) observed that different cell types varied in the amount of DI's they produced. In addition, when a mixed stock of DI's was used to infect different cell types, the predominant DI species amongst the progeny was shown to vary with cell type.

(2) The time of harvest; Khan and Lazzarini (1977) observed that harvesting virus shortly after infection results in a virus preparation which contains fewer DI's than virus harvested late in infection. The explanation for this observation is thought to be a consequence of the slower rate of release of DI particles in comparison to standard virus.

(3) Multiplicity of infection; Faulkner and Lazzarini (1980) showed that infecting cells at low multiplicity (i.e. less than 1 pfu/cell) resulted in fewer DI particles in the resulting virus preparation than infecting cells at high multiplicity.

MATERIALS AND METHODS

MATERIALS AND METHODS

CHAPTER 3.(a) MATERIALS

3.(a).1 CELLS

Baby hamster kidney (BHK-21) clone 13 cells (Macpherson and Stoker, 1962) were provided by the cytology department, Institute of Virology.

LMTK$^-$ cells, a line derived from mouse fibroblasts were a gift from M. Capecchi of the Department of Biology, University of Utah, Salt Lake city, Utah.

(L39) cells, also obtained from M. Capecchi were derived from LMTK$^-$ cells and carried an amber suppressor $tRNA^{Tyr}$ gene.

3.(a).2 VIRUS

Wild-type and ts stocks of vesicular stomatitis virus Indiana serotype were obtained from C.R. Pringle, Institute of Biological Sciences, University of Warwick, Coventry, England. (Pringle, 1970, Pringle et al., 1971).

3.(a).3 CHEMICALS

Chemicals and biochemicals were obtained from the following suppliers.

Acrylamide (BDH); agar (Difco); aminopterin (Sigma); ammonium persulphate (Bio-Rad); bovine serum albumin (BSA) (Sigma); caesium chloride (Koch-Light); 5-fluorouracil (Sigma); Geneticin (G-418; Sigma); glutaraldehyde 2% (Arbrook Products); glycerol (Koch-Light); hypoxanthine (Sigma); Lyovac Cosmegen (actinomycin-D; Merck, Sharpe and Dohme); N,N'-methylene bisacrylamide (BDH); protein-A-sepharose (Sigma); trypsin (Difco).

All other chemicals were obtained, where possible, in Analar form, from BDH and Sigma.

3.(a).4 RADIOCHEMICALS

L-[^{35}S]-methionine (1150-1500 Ci/m mol), and Na^{125}I were supplied by The Radiochemical Centre, Amersham. Na^{125}I

was used in the preparation of ^{125}I-protein A, which was obtained from C. Maclean.

3.(a).5 TISSUE CULTURE MATERIALS

Flasks, petri dishes, and 24-well Linbro trays were manufactured by Nunc and were supplied by Gibco-Biocult Limited.

3.(a).6 TISSUE CULTURE REAGENTS

Tissue culture reagents were supplied by Flow Laboratories and Gibco-Biocult.

Calf serum, Giemsa, Neutral red, Eagle's overlay, trypsin and versene were all prepared in the Institute of Virology.

The various tissue culture reagents used are described below.

DMEM/10%FCS comprised 500 ml 1x Dulbecco's minimal essential medium (Dulbecco and Freeman, 1959), supplemented with 5 mM L-glutamine and 10% calf serum.

MEM/10%CS comprised 10x Glasgow modification of Eagle's medium, diluted in distilled water and supplemented with 5 mM L-glutamine, 2.75 g/l $NaHCO_3$ and 10% calf serum.

EC/2%CS low methionine comprised 140 ml Eagle's A, 15 ml Eagle's B without L-methionine, 5 ml Eagle's B, supplemented with 2% calf serum.

PBS/2%CS comprised 8 parts PBS A, 1 part PBS B, 1 part PBS C supplemented with 2% calf serum.

Agar overlay comprised 310 ml Eagles A, 60 ml Eagles B without phenol red, 125 ml 3.6% Difco agar, 7 ml calf serum.

Neutral red overlay comprised 310 ml Eagles A, 60 ml Eagles B without phenol red, 125 ml 3.6% Difco agar, 10 ml neutral red.

3.(a).7 MISCELLANEOUS MATERIALS

Miscellaneous materials were obtained from the following suppliers. En^{3}Hance (New England Nuclear); Kapton tape (R and J Wood); Kodak X-Omat S film (Kodak); Nitrocellulose paper (Schleicher and Schuell).

3.(a).8 BUFFERS AND SOLUTIONS

PBS 170mM NaCl, 3.4mM KCl, 1mM Na_2HPO_4 pH 7.2.

NTE 100mM NaCl, 10mM Tris HCl pH 7.5, 1mM EDTA.

3.(b) METHODS

3.(b).1 GROWTH OF CELLS

BHK-21 C13 cells were grown in 2 litre glass or plastic burrler bottles containing 250 ml of Eagle's/10%CS in an atmosphere of 95% air and 5% CO_2. LMTK$^-$ (su$^-$) and L39 (su$^+$) cells were grown in 175 cm^2 tissue culture flasks containing 75 ml of DMEM/10%CS in an incubator containing 5% CO_2 at 37^{o}C. L39 cells were propagated in HAT medium (DMEM/10%FCS supplemented with 100 uM hypoxanthine, 0.4 uM aminopterin, 16 uM thymidine and 3 uM glycine). Cells were harvested by washing twice with versene and once with a trypsin/versene mix. 90 mm plates of su$^+$ cells for use in plaque picking and plaque purification, were seeded as required - usually three from a large tissue culture flask. The plates were then incubated at 37^{o}C until confluency was reached five days later. Linbro wells of su$^+$ cells, used to titrate virus and for protein analysis, were prepared by seeding five, 24 well multiwells from a large tissue culture flask. The monolayers were incubated for 24 h at 37^{o}C.

90 mm plates of su$^-$ cells for use in isolating revertant plaques were prepared from large tissue culture flasks by seeding four plates per flask and incubating until confluency 48 h later. Eight 24 well multiwells, to be used for titration of virus and for protein analysis, could be seeded from a large tissue culture flask of su$^-$ cells with confluency reached 16-24 h later. 25 cm^2 tissue culture flasks were prepared for complementation analysis by seeding from a single large tissue culture flask of su$^-$ cells and incubating for 24 h at 37^{o}C.

3.(b).2 PURIFICATION OF VIRUS

VSV stocks were grown in monolayers of BHK-21 C13 cells in 2 litre burrler bottles.

Monolayers were infected at low multiplicity with a 10 ml inoculum containing 0.01 pfu/cell in PBS/2%CS. The virus was allowed to absorb for 60 min at 31^{o}C and 40 ml of Eagle's/2%CS added. The cells were incubated at 31^{o}C until extensive cpe was observed. The supernatant was decanted

and clarified by centrifugation for 30 min in a Sorvall RC-5B superspeed centrifuge using a GSA rotor. Virus was pelleted from the clarified medium through a 9 ml cushion consisting of 30% (v/v) glycerol, 20 mM Tris-HCl pH8.0, 1 mM EDTA and 100 mM NaCl. Centrifugation was for 90 min at 21000 rpm and 4°C using the Du Pont 10x100 rotor in the Sorvall OTD-50 ultracentrifuge. The pellet was resuspended in 1 ml NTE. Virus was further purified by banding the virus in a 15-45% sucrose gradient. The gradient was centrifuged for 90 min at 22000 rpm and at 4°C using the TST41 rotor in the Sorvall OTD50 ultracentrifuge. The band was collected, diluted to 10 ml with NTE, and pelleted through a 30% glycerol cushion as before. The final pellet was resuspended in 1 ml of NTE and stored at -70°C.

3.(b).3 5-FLUOROURACIL MUTAGENESIS

Confluent monolayers of *su*$^+$ cells were infected in 90 mm plates, with a 0.6 ml inoculum containing 0.02 pfu/cell of *wt* VSV in PBS/2%CS. The virus was absorbed for 1 h at 31°C and 10 ml of DMEM/2%CS containing either 0, 50, 100, or 200 ug/ml of the chemical mutagen 5-FU. Infection was continued until cpe was observed in the cells infected in the absence of the mutagen. The supernatant was harvested and clarified by centrifugation as descibed above. The mutagenized virus preparation was aliquoted into bijoux and stored at -70°C.

3.(b).4 ISOLATION OF HOST RANGE MUTANTS

Confluent monolayers of the *su*$^+$ cells in 90 mm plates, were infected with a 0.6 ml inoculum of 10 fold serial dilutions of the mutagenized virus in PBS/2%CS. The plates were incubated for 1 h at 31°C. The inoculum was removed and the plates washed twice with PBS/2%CS. 10 ml of agar overlay was then added. The plates were incubated for 2 days at 31°C. The plates were overlayed again with 4 ml of neutral red overlay. The plates were then returned to 31°C until plaques were clearly visible, usually 3-4 h later. Small plaques were picked and dispersed into vials containing 1.5 ml of Eagle's medium supplemented with 20%

calf serum. The plaques were stored at -70°C.

3.(b).5 SCREENING PLAQUES

Confluent monolayers of su^- cells in Linbro wells were infected with 0.1 ml of the dispersed plaque preparation. The cells were incubated for 1 h at 31°C, the inoculum removed and the monolayers washed twice with PBS/2%CS. 1 ml of DMEM/2%CS was added and the infections continued at 31°C. The cell monolayers were examined twice daily for any sign of VSV infection, such as translucent areas on the cell sheet, rounding up of cells or cpe. This process was continued for 14 days after infection at which point any remaining monolayers were grossly overgrown.

Isolates which did not give rise to cpe in su^- cells after 14 days were used to infect monolayers of su^+ cells in Linbro wells. Not all isolates then gave rise to cpe on su^+ cells. This appeared to be caused by the occurrence of syncytia on the su^+ cell monolayer. These syncytia do not stain as efficiently as normal cells and it was not always possible to distinguish them from small plaques.

3.(b).6 CLONAL PURIFICATION

In order to plaque purify the mutants isolated, the original isolate was plated by serial dilution to produce well isolated plaques on a monolayer of su^+ cells in a 90 mm petri dish. 0.6 ml of the inoculum was plated on the su^+ cells and the plates incubated at 31°C for 1 h. The inoculum was removed, 6 ml of agar overlay was added and the cells returned to 31°C for 2 days at 31°C. The plates were then overlayed again with 4 ml of agar overlay containing neutral red and returned to 31°C until plaques were visible. 24 well isolated plaques were picked, dispersed into 1.5 ml of Eagle's/20%CS and stored at -70°C. Monolayers of su^- cells in Linbro wells were infected with 0.1 ml of the dispersed plaque preparation. The cells were incubated for 1 h at 31°C after which the inoculum was removed and the monolayers washed twice with PBS. 1 ml of DMEM/2%CS was added and incubation continued at 31°C. The monolayers were inspected twice daily as before for any sign of viral

infection. After 14 days none of the infections had given rise to cpe and in fact the monolayers were grossly overgrown indicating that each of the isolates had retained the host range phenotype of the original isolate. A single isolate was taken and used to infect monolayers of su^+ cells in 90 mm petri dishes. 1 h post infection, the inoculum was removed and the monolayers washed twice with PBS. 10 ml of DMEM/2%CS was added and the infections returned to 31°C until advanced cpe was observed. The supernatant was decanted and clarified by centrifugation for 30 min at 2,500 rpm in an MSE Coolspin centrifuge. The virus was aliquoted and stored at -70°C. This virus was designated passage 1 and was used throughout.

3.(b).7 QUANTITATION OF HOST RANGE RESTRICTION

Passage 1 virus (see previous section) was used to quantify any restriction in host range between the su^+ and su^- cell lines. Monolayers of both cell lines in Linbro wells were inoculated with 0.1 ml of a serial dilution of the virus preparation in PBS/2%CS. Absorption was carried out at 31°C for 1 h, the inoculum removed and the monolayers washed twice with PBS/2%CS and 1 ml of agar overlay added. The plates were incubated for 2 days at 31°C. The agar overlay was removed and the cells fixed with 10% Cidex in PBS for 2 h. Plaques were counted after staining with Giemsa for 30 min and the titre of the virus obtained for each cell line. Comparison of the titre attained by each isolate gave a measure of the restriction in host range.

3.(b).8 ISOLATION OF REVERTANTS

Revertants of the host range mutants were isolated by plating 10 fold serial dilutions of the passage 1 stock of each mutant to produce isolated plaques on monolayers of su^- cells. 0.6 ml of virus in PBS/2%CS was added to confluent monolayers of su^- cells in 90 mm petri dishes. The virus was allowed to absorb for 1 h at 31°C. The inoculum was removed and the cell monolayer washed twice with PBS/2%CS and 6 ml of agar overlay added. The plates were incubated at 31°C for 2 days. 4 ml of agar overlay containing neutral

red stain was added and the plates returned to 31°C for 3-4 h. Well isolated plaques were picked and dispersed into glass vials containing 1.5 ml Eagle's/20%CS.

3.(b).9 SCREENING OF REVERTANTS

Confluent monolayers of *su*$^-$ cells in Linbro wells were infected with 0.1 ml of the dispersed plaque preparation (see previous section) and incubated for 1 h at 31°C. The inoculum was removed and the monolayers washed twice with PBS. 1 ml of DMEM/2%CS was added and the infected cells incubated at 31°C. When cpe was observed, usually within 48 h of infection, the supernatant medium was harvested, clarified by centrifugation at 1500 rpm for 15 min. The supernatant was decanted and stored in bijoux at -70°C.

3.(b).10 PROTEIN SYNTHESIS IN VIRUS INFECTED CELLS

Monolayers of *su*$^+$ and *su*$^-$ cells were infected in Linbro wells with an inoculum consisting of 0.1 ml PBS/2%CS which also contained 10 ug/ml actinomycin-D and 10 pfu/cell virus (either wild-type, mutant or revertant virus). After 1 h absorption at 31°C, the inoculum was removed and replaced with DMEM/2%CS containing 10 ug/ml actinomycin-D for either 7 h or 23 h at which point the medium was removed and 0.1 ml PBS containing 10 ug/ml actinomycin-D and 100 uCi/ml [^{35}S]-methionine was added to each well and incubation continued at 31°C for 1 h.

After this period of incubation the supernatant medium was discarded and the monolayers washed once with cold PBS. 60 ul of protein dissociation mix (0.125 M Tris.HCl pH 6.8, 4% SDS, 10% mercaptoethanol, 20% glycerol, 0.1% bromophenol blue) was added to each well. The cells were then scraped into the boiling mix with a piece of silicon tubing. The cells were then aspirated slowly by pasteur pipette into a 0.5 ml Eppendorf tube and heated at 100°C for 2 min. The labelled cell extracts were either stored at -20°C or loaded directly on to a SDS-polyacrylamide gel for fractionation. Labelled species were detected by autoradiography.

3.(b).11 GROWTH OF RADIOLABELLED VIRUS

Confluent monolayers of su$^+$ cells were infected in Linbro wells with a 0.1 ml inoculum containing 0.02 pfu/cell of either wild-type virus or one of the mutants. The cells were incubated for 1 h at 31°C after which the inoculum was removed and the monolayers washed twice with PBS. 1 ml of MEM/2%CS was added and the cells returned to 31°C for 6 h at which time the medium was decanted and replaced by EC/2%CS deficient in methionine containing 100 uCi/ml of [^{35}S]-methionine. 24 h post infection the supernatant was decanted and clarified by centrifugation for 30 min at 2500 rpm in a MSE coolspin centrifuge. The labelled virus was pelleted through a 5 ml cushion consisting of 30% (v/v) glycerol, 20 mM Tris.HCl pH 8.0, 1 mM EDTA and 100mM NaCl. Centrifugation was for 90 min at 21000 rpm and at 4°C using the Sorvall AH650 rotor in the Sorvall OTD-50 ultracentrifuge. The pellet was resuspended in 200 ul of protein dissociation mix (0.125 M Tris.HCl pH 6.8, 4% SDS, 10% mercaptoethanol, 20% glycerol, 0.1% bromophenol blue) and heated at 100°C for 2 min. The labelled proteins were analysed by polyacrylamide gel electrophoresis.

3.(b).12 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

Vertical slab gels were cast in a sandwich consisting of two glass plates separated by 1.5 mm thick plastic spacers and sealed with Kapton tape. Single concentration gels were used throughout. The resolving gel contained acrylamide crosslinked with 1 part in 40 (w/w) N,N'-methylenebisacrylamide in 375 mM Tris pH 8.9, 0.4% SDS. The resolving gel was polymerised with the aid of 0.006% ammonium persulphate and 0.004% TEMED. In order to achieve a smooth interphase, a few ml of butan-2-ol was layered over the resolving gel.

Stacking gels were buffered with 122 mM Tris pH 6.7 containing 0.4% SDS. They contained 5% acrylamide cross linked with N,N'-methylenebisacrylamide at the same ratio as was used in the resolving gel. The stacking gel was polymerized with ammonium persulphate and TEMED.

After loading of samples the gels were electrophoresed at either 60 mA for 3-4 h at 4°C or 10 mA for 18 h at room temperature (RT) using a buffer containing 52 mM Tris, 53 mM glycine and 0.1% SDS.

3.(b).13 FLUOROGRAPHY

To increase the sensitivity of detection of [^{35}S]-methionine labelled proteins, gels were fixed in 45% methanol; 5% acetic acid for 45 mins, soaked and shaken in En^{3}Hance for 60 mins and washed in water for 30 mins. The gels were then dried down under vacuum using a Bio-Rad gel drier, placed against Kodak X-Omat-S film and stored at -70°C.

3.(b).14 COMPLEMENTATION ANALYSIS

Temperature sensitive mutants representing each of the five complementation groups were given by Professor C.R. Pringle, Department of Biological Sciences, University of Warwick, England. The ts mutants comprised ts 11 (group I), ts 22 (group II), ts 31 (group III), ts 41 (groupIV) and ts 045 (group V). The host range mutants comprised AmbL1, AmbL2, AmbL3, AmbL4, AmbL5, AmbG and hrTK$^-$7.

Confluent monolayers of LMTK$^-$ cells in five 25 cm^2 tissue culture flasks were infected with 0.2 ml of an inoculum containing 5 pfu/cell of host range virus and 5 pfu/cell of ts virus in PBS/2%CS. Virus was allowed to adsorb for 20 min at 4°C. The inoculum was removed and the monolayer washed twice with PBS. 5 ml of prewarmed (39°C) DMEM/2%CS was added, the flasks were then sealed with Lasso tape and submerged in a Grant waterbath for 8 h at the non-permissive temperature (39°C). The supernatant was decanted into bijoux and stored at -70°C.

The supernatant was subsequently analysed for growth of the ts mutants by titrating the virus on su$^-$ cells at the permissive temperature (31°C).

For singly infected controls, monolayers of su$^-$ cells were infected with 0.2 ml of an inoculum containing 5 pfu/cell of either host range virus or ts virus in PBS/2%CS. The protocol used for analysis of single

infections was identical to that employed for mixed infections. Single and mixed infections were carried out in parallel using the same virus preparations and the same waterbath.

3.(b).15 IMMUNOPRECIPITATION

Monospecific antisera raised in rabbits against the amino (N) and carboxy (C) termini of the VSV (Indiana) L protein were a gift from Dr. Manfred Schubert (Institute of Communicative Disorders and Stroke, National Institutes of Health, Bethesda, USA), and were desribed in Schubert et al. (1985).

Monolayers of su^+ cells in Linbro wells were infected with 0.1 ml of an inoculum containing 10 pfu/cell of a group I mutant in PBS/2%CS. Virus was absorbed for 1 h at 31°C, the inoculum removed and the cells washed twice with PBS. 1 ml of DMEM/2%CS containing 10 ug/ml of actinomycin-D was added and incubation continued for 24 h at 31°C.

The supernatant was decanted and 0.1 ml of PBS/2%CS containing 250 uCi/ml of [^{35}S]-methionine and 10 ug/ml of actinomycin-D added. The cells were incubated for 1 h at 31°C after which the supernatant was removed. The monolayers were washed twice with PBS/2%CS and then scraped into 1 ml of PBS/2%CS and transferred to a 1.5 ml Eppendorf tube. The cells were pelleted by a 1 min spin in an MSE microfuge. The pellet was resuspended in 100 ul of cell lysis buffer (015 M NaCl, 1% Na deoxycholate, 1% triton x-100, 0.1% SDS, 0.01 M tris.HCl pH 7.4 and 1 mM PMSF) and incubated on ice for 30 min. After brief vortexing, the nuclei and debris were pelleted with a 5 min spin.

A 50 ul aliquot was taken and 5 ul of either N or C-terminal specific antibody added. After incubation on ice for 3 h, 60 ul of protein-A-Sepharose beads was added. Incubation proceeded for 30 min and the immunecomplex pelleted by a 20 sec spin in an MSE microfuge. The pellet was washed three times in 200 ul 500 mM LiCl, 100 mM Tris.HCl pH 8.5 and dissolved in 50 ul protein dissociation mix. The samples were heated at 100°C for 2 min and stored at -20°C. Prior to loading on a polyacrylamide gel the

samples were again heated at 100°C for 2 min, and 25 ul analysed on a 7.5% polyacrylamide gel.

3.(b).16 IMMUNOBLOTTING

The immunoblotting technique was based on the method by Towbin et al. (1979), with several modifications. [^{35}S]-methionine labelled polypeptide extracts from su$^+$ cells infected by wt virus or one of the five group I mutants were fractionated by gel electrophoresis on a 7.5% SDS-polyacrylamide gel. The separated proteins were transferred from the gel to a sheet of nitrocellulose using a BioRad 'transblot' apparatus. The gel was placed on a sheet of Whatman grade 182 filter paper on top of a foam pad- both presoaked in transfer buffer (25mM Tris HCl pH 8.3, 192mM glycine, 20% methanol). The sheets of nitrocellulose, also pre-soaked, were placed face down on the gel. A further sheet of pre-soaked filter paper and then another foam pad were placed over the gel and the sandwich was placed in the electrophoresis tank in a plastic holder. Proteins were transferred to the nitrocellulose sheets by electrophoresis in transfer buffer at 250 mA for 3 h at RT.

Following transfer, the nitrocellulose sheets were incubated overnight at 65°C in wash buffer (1 mM Tris.HCl pH 7.4, 15.4 mM NaCl, 0.01% sodium azide) containing 0.05% Tween 20. The sheets were then immersed in approximately 100 ml of appropriately diluted antiserum in plastic tubs. This was incubated for 2 h at 37°C. The sheets were then washed thoroughly in wash buffer containing 0.05% Tween 20 by gently shaking at 37°C, with at least three changes of buffer over 2 h. To detect bound antibody, the sheets were covered with a solution of wash buffer containing 3% BSA and $5x10^5$ cpm of iodinated protein A. This was gently shaken at 37°C for 2 h, and the sheets removed and washed twice for 1 h in wash buffer containing 1 M potassium iodide, followed by a 15 min wash in PBS. The sheets were placed on tissues to dry and taped to card prior to autoradiography.

To determine with which of the [^{35}S]-methionine labelled proteins the iodinated protein A had interacted,

the sheets were exposed to three superimposed sheets of film with a sheet of black paper between the first and second sheets and with an image intensifying screen directly on top of all three films (Haarr et al., 1985). The first film detects the [^{35}S]-methionine label with the black sheet preventing further penetration of this isotope. The high energy emission from the ^{125}I passes through all the films with little absorption and interacts with the image intensifying screen producing photons which are detected only on the second and third films. ^{14}C and ^{125}I radioactive ink was spotted onto the card to allow the three films to be aligned.

3.(b).17 EXTRACTION OF TOTAL CELLULAR RNA

Confluent monolayers of *su*$^+$ cells were infected at 10 pfu/cell with mutant and *wt* virus as described previously for viral protein analysis. At 8 h post infection the cells were trypsinized and centrifuged at 1500 rpm for 15 min at 4^{o}C. The cell pellet was resuspended in cold PBS and pelleted at 1500 rpm for 15 min at 4^{o}C. The cell pellet was lysed in 5 volumes of 6 M guanidinium isothiocyanate, 5 mM Na-citrate pH 7.0, 100 mM β-mercaptoethanol, 0.5% sarcosyl. To each 2.5 ml of cell lysate, 1 g caesium chloride was added and dissolved by vortexing. The mixture was layered onto a 1.2 ml cushion of 5.7 M caesium chloride in 100 mM EDTA and centrifuged at 35000 rpm for 12 h at 20^{o}C in an AH650 rotor. The supernatant was decanted and the RNA pellet resuspended in 10 mM Tris HCl pH 7.4, 5 mM EDTA, 1% v/v SDS and extracted once with an equal volume of a 4:1 mixture of chloroform and 1-butanol. The organic phase was reextracted once more with an equal volume of the Tris/EDTA/SDS buffer. The aqueous phases were combined and the RNA precipitated in the presence of 0.3 M Na acetate and 2.5 volumes of ethanol. After centrifugation at 12000 rpm for 10 min the RNA was resuspended in water and reprecipitated with ethanol. The RNA was stored at -70^{o}C.

3.(b).18 IN VITRO TRANSLATION OF INFECTED CELL RNA

RNA prepared as described above was used to programme the following *in vitro* translation system (New England Nuclear). The reaction comprised: 2-4 ul RNA, 2.5 ul [^{35}S]-methionine, 5 ul rabbit reticulocyte lysate, 2.75 ul *in vitro* translation cocktail (containing spermidine, creatine phosphate, dithiothreitol and GTP in concentrations optimized by the manufacturers), 1 ul 1 M potassium acetate, 0.25 ul 50 mM magnesium acetate. The reactions were incubated for 1 h at 37^{o}C and terminated by the addition of 15 ul protein dissociation mix (0.125 M Tris-HCl pH 6.8, 4% SDS, 10% mercaptoethanol, 20% glycerol, 0.1% bromophenol blue). Samples were heated at 100^{o}C for 2 min and stored at -20^{o}C. Prior to loading onto the gel, samples were heated at 100^{o}C for 5 min. The proteins were fractionated on a 7.5% SDS-polyacrylamide gel, and the labelled species detected by autoradiography.

3.(b).19 ANALYSIS OF VIRAL PROTEIN SYNTHESIS IN THE PRESENCE OF G418

Monolayers of *su*$^{-}$ cells in Linbro wells were infected with 0.1 ml of PBS/2%CS containing 10 pfu/cell of virus, 10 ug/ml of actinomycin-D and 0, 50, 100 or 200 ug/ml of G418. The cells were incubated for 1 h at 31^{o}C to allow virus to absorb. The inoculum was removed and the monolayers washed twice with PBS. DMEM/2%CS containing 10 ug/ml of actinomycin-D and the same concentration of G418 as the inoculum was then added and incubation continued for 7 h at 31^{o}C. 0.1 ml of PBS containing 100 uCi/ml of [^{35}S]-methionine, 10 ug/ml of actinomycin-D and the appropriate concentration of G418 was then added and the cells incubated for a further hour at 31^{o}C. The label was removed and the monolayers washed twice with PBS. 50 ul of protein dissociation mix was added and the samples heated at 100^{o}C for 2 min. The proteins were fractionated on a 7.5% SDS-polyacrylamide gel, and the labelled species detected by autoradiography.

3.(b).20 INVESTIGATION OF VIRAL GROWTH IN THE PRESENCE OF G418

Monolayers of *su*$^-$ cells were grown in Linbro wells and infected with 0.1 ml of PBS/2%CS containing 0.02 pfu/cell of virus and 0, 50, 100 or 200 ug/ml of G418. Virus was allowed to absorb for 1 h at 31^{o}C. The inoculum was removed and DMEM/2%CS added which contained the same concentration of G418 as did the inoculum. The cells were incubated for 8 h at 31^{o}C. The supernatant was removed and titrated in the usual way on the *su*$^+$ cells.

RESULTS

CHAPTER 4 RESULTS

4.(a) ISOLATION OF MUTANTS

4.(a).1 VIRUS PURIFICATION

The wild-type strain of vesicular stomatitis virus (Indiana serotype) used was the attenuated derivative Indiana C (provided by C.R. Pringle). Virus was purified by infecting BHK (C-13) cells at a moi of 0.02 pfu/cell. When cpe was observed approximately 48 h later the supernatant was harvested and virus purified in a 14-45% sucrose gradient. The presence of a single band after centrifugation, as opposed to two separate bands, indicated an absence of defective interfering particles in the virus preparation.

The purified virus was then titrated on LMTK$^-$ (su$^-$) cells and a titre of 1.1×10^{10} pfu/ml obtained.

4.(a).2 5-FLUOROURACIL MUTAGENESIS

Confluent monolayers of the suppressor carrying cell line (L39; su$^+$) were infected by wt virus at a moi of 0.02 pfu/cell in the presence of the chemical mutagen 5-fluorouracil (5-FU). Virus was grown in the presence of 0, 50, 100 and 200 ug/ml of 5-FU. The virus was harvested when cpe was observed (about 48 h later) in cells infected in the absence of 5-FU. The virus from each infection was then titrated on the su$^+$ cells (Table 1). It was found that concentrations of 50 and 100 ug/ml of 5-FU reduced the titre three fold and five fold, while 200 ug/ml reduced it thirty fold. Virus treated with 100 ug/ml of 5-FU offered the best compromise between maximum mutagenesis and viability of the virus.

4.(a).3 ISOLATION OF MUTANTS

Mutagenized virus stocks were plated on su$^+$ cells to give 50-60 plaques per 90 mm plate. These plaques were visualized 3 days post infection by neutral red staining. Plaques were then picked to screen for a mutation affecting host range. After mutagenesis the majority of plaques produced were 3-6 mm in diameter. In addition to these

Table 1

The effect of various concentrations of the mutagen 5-FU on the titre of a wild-type stock of VSV.

Concentration of 5-FU (ug/ml)	Titre (pfu/ml)
zero	1.1×10^{10}
50	3.4×10^{9}
100	2.2×10^{9}
200	4.0×10^{8}

Confluent monolayers of su^+ cells were infected with wt virus in the presence of different concentrations of 5-FU. Mutagenized virus was harvested when cpe was observed in the control plate (no mutagen) and then titrated on the su^+ cells.

plaques there was a small number of plaques (about 10% of all plaques) which were approximately 1 mm in diameter. To increase the possibility of picking up an amber mutant, only the small plaques were picked for screening. This selection strategy presumes that virus containing an amber mutant will not grow as well as wt in a weak suppressor system (about 4% suppression in the su^+ cells; Young et al., 1983). From a single 90 mm plate only 6-10 plaques were picked. In total 2614 plaques were picked. Isolates were screened for a mutation affecting host range by infecting monolayers of su^- cells in Linbro wells and scoring the infections for the presence or absence of cpe. The majority of plaques which gave rise to cpe did so within 48 h. The remaining monolayers were examined twice daily until 7 days post infection for any evidence of virus growth. In the case of plaques which did not give rise to cpe after 7 days the monolayers were substantially overgrown, indicating that there was no virus growth. 82 plaques did not give rise to cpe on su^- cells. These isolates were then used to infect su^+ cells to determine if there was any viable virus. Growth of the su^+ cells on plates resulted in the formation of syncytia. However these syncytia did not stain as efficiently as normal cells and it was not always possible to distinguish them from small plaques. Thirty isolates gave rise to productive infections after infection of the su^+ cells, and the supernatant from each was then titrated on both the su^+ and su^- cells to quantify any difference in host range. Of these, 13 revealed a difference in titre between the two cell lines of at least 20 fold (Table 2, and Figure 5). These isolates were thus considered to represent conditional lethal nonsense mutants. Subsequent analysis revealed that the mutations affect a specific gene product in 6 of the isolates and these were therefore named accordingly. The remainder of the mutants which could not be characterized were classified simply as host range mutants ($hrTK^-x$, where x represents the number of the mutant).

The isolates which exhibited the most marked restriction were AmbL3 and AmbL4 which differed in titre between the su^+ and su^- cells by a factor of 7.3×10^4 and

Table 2

Plating efficiencies of the putative host range mutants on the su^+ and su^- cells respectively.

Mutant	Titre L39 (su+)	Titre LMTK$^-$ (su^-)
Ind/AmbL1/84	1.0×10^7	2.5×10^4
Ind/AmbL2/85	1.7×10^7	3.0×10^3
Ind/AmbL3/85	2.2×10^7	3.0×10^2
Ind/AmbL4/85	3.0×106	2.0×10^2
Ind/AmbL5/85	3.0×10^5	8.9×10^1
Ind/AmbG/85	1.0×10^7	3.0×10^5
hrTK$^-$7	1.9×10^5	2.0×10^2
hrTK$^-$8	2.0×10^7	3.2×10^5
hrTK$^-$9	1.4×10^7	4.0×10^5
hrTK$^-$10	5.4×10^5	2.4×10^4
hrTK$^-$11	7.0×10^7	4.0×10^6
hrTk$^-$12	1.0×10^6	2.4×10^3
hrTK$^-$13	1.0×10^4	5.0×10^1

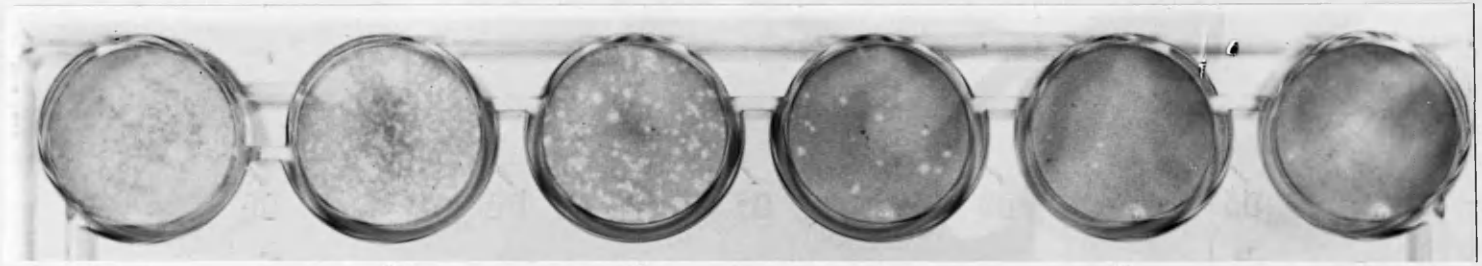
Titres in pfu/ml of a passage 1 stock of each host range VSV mutant were measured on monolayers of su^+ and su^- cells. Subsequent analysis has revealed that the mutations affect a specific gene product in six of the isolates and these were therefore named accordingly. The remainder of the mutants which could not be characterized were classified simply as host range mutants.

Figure 5

An example of the restricted host range exhibited by the group I mutant AmbL4.

Monolayers of su^+ and su^- cells in Linbro wells were infected with a passage 1 stock of AmbL4 virus. The monolayers were fixed three days post infection and stained with Giemsa. In 5a plates 1 to 6 show 10 fold serial dilutions (10^{-1} to 10^{-6}) of the virus stock on su^+ cells. In 5b plates 1 to 6 show the same dilutions plated on su^- cells.

5a



1 2 3 4 5 6

5b

1 2 3 4 5 6

$1.5x10^4$ respectively. The isolates AmbL2, AmbL5 and $hrTK^-7$ showed a $5.7x10^3$, $3.4x10^3$ and $1x10^3$ fold difference in titre. AmbL1 and $hrTK^-13$ exhibited a 400 and 200 fold difference in titre while the isolate AmbG exhibited a 30 fold difference in titre between the two cell lines. The remaining isolates $hrTK^-8$ to $hrTK^-12$ gave rise to a difference ranging from 20 fold ($hrTK^-11$) to 60 fold ($hrTK^-8$).

4.(a).4 CLONAL PURIFICATION OF THE MUTANTS

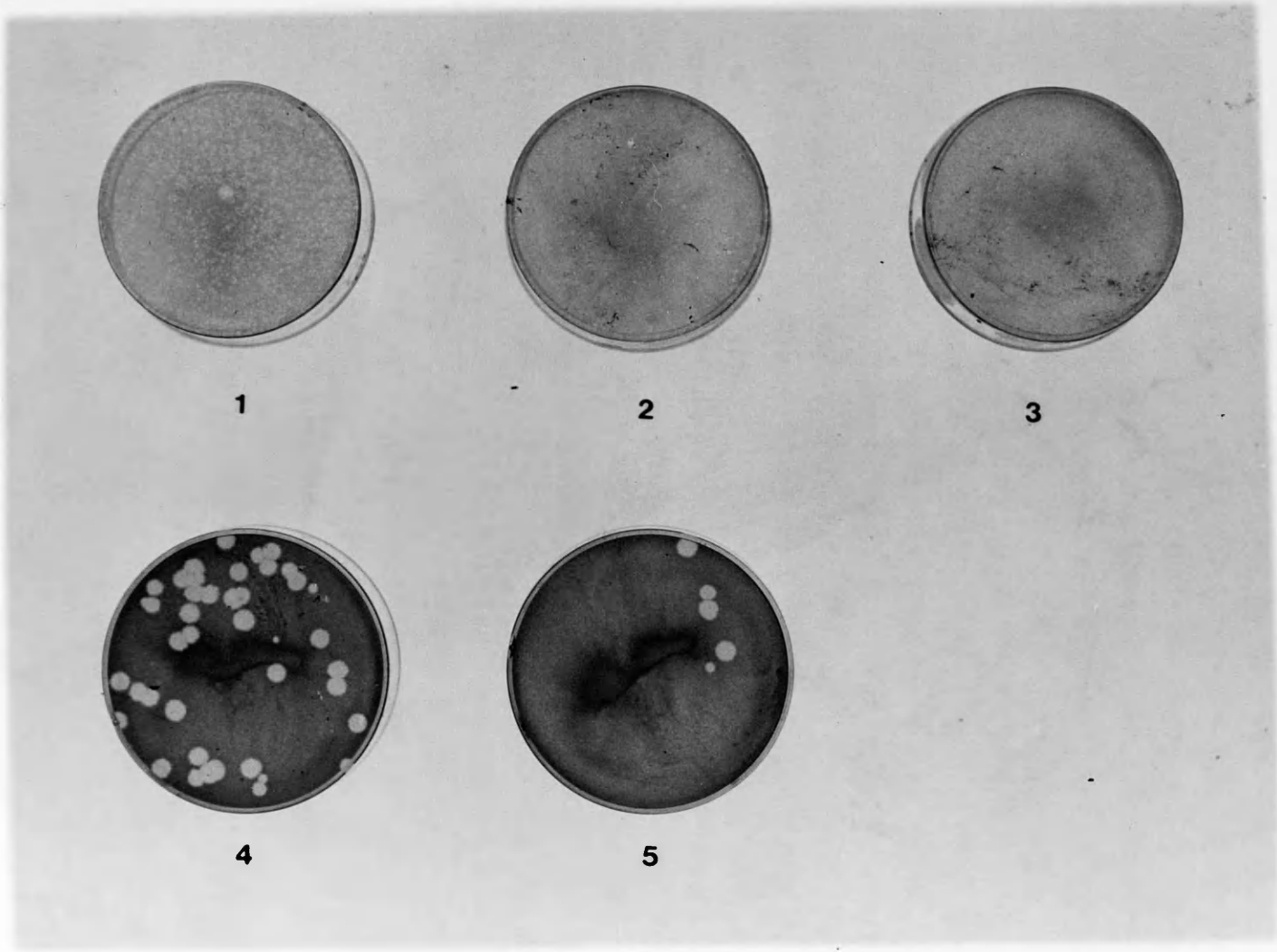
The stock used in the characterization of the mutant AmbL1 was obtained by plating the original plaque to give well isolated plaques on a 90 mm plate. 24 well isolated plaques were picked. Each plaque was then checked for retention of its host range phenotype by infecting su^- cells in Linbro wells and scoring each infection for the presence or absence of cpe. None of the plaques gave rise to cpe after 7 days and in fact the cells in each Linbro well were substantially overgrown after this period. This indicated that all the plaques picked represented virus which had retained its host range restriction. A single plaque was used to infect monolayers of su^+ cells. A single plaque from these plates was then taken and used to infect 90 mm plates of su^+ cells. The supernatant was harvested when advanced cpe was observed in each of the plates. The virus was aliquoted and stored at -70^oC. This passage (designated passage 1) was used throughout. This precaution was taken to ensure homogeneity of the virus preparation and to keep the level of revertants in a mutant stock to a minimum. The small plaque morphology exhibited by AmbL1 (passage 1 stock) on the su^+ cells, in comparison to the normal plaque morphology of its revertants on the su^- cells, is shown in Figure 6.

The identical procedure was performed for purification of each of the twelve remaining isolates. In each case the orginal isolate was used to plaque purify the mutant on su^+ cells. The virus was plated to produce well isolated plaques on a 90 mm plate of the su^+ cells. The resulting plaques all retained their small plaque morphology and 24 well isolated plaques were picked. Each plaque was

Figure 6

An example of the different plaque morphologies of the group I mutant AmbL1 and its revertants.

Monolayers of *su*$^+$ and *su*$^-$ cells infected with serial dilutions of the same stock of the group I mutant AmbL1, fixed three days post infection and stained with Giemsa. Plates 1, 2 and 3 show the small plaques produced by AmbL1 on the *su*$^+$ cells at the 10^{-3}, 10^{-4} and 10^{-5} dilutions respectively. Plates 4 and 5 show the normal plaque morphology of revertants of AmbL1 at the 10^{-1} and 10^{-2} dilutions respectively on *su*$^-$ cells.


1
2
3
4
5

then screened on *su*$^-$ cells in Linbro wells to check that all plaques represented mutant and not *wt* virus. For each mutant a single plaque was used to infect monolayers of *su*$^+$ cells. The virus was harvested and aliquoted into ampoules and stored at -70^{o}C. Only passage 1 virus was used throughout.

4.(b) CHARACTERIZATION OF THE MUTANTS

4.(b).1 VIRAL PROTEIN SYNTHESIS IN SU$^+$ AND SU$^-$ INFECTED CELLS

The object of this experiment was to examine viral protein synthesis in suppressor and non-suppressor cells infected with the host range mutants isolated, and to determine whether the mutation which caused the restriction in host range resulted in the generation of a novel polypeptide species representing a fragment of the complete polypeptide, produced by premature termination of translation at an amber codon.

Previously, in a study of amber head protein mutants of the rII collection of phage T4 mutants, Benzer and Champe (1961) showed that in *su*$^+$ strains both the fragment and a completed polypeptide were detectable whereas only the fragment itself was detectable in *su*$^-$ strains. This has also proved to be the case in a eukaryotic suppressor system. Young *et al.* (1983) studied the effect of suppression on a strain of influenza virus (A/CAM/46) which contained a characterized amber nonsense mutation within its NS1 gene. Although this resulted in a shorter than usual NS1 protein, it was stable and functional. Sequence analysis revealed an in-frame opal stop codon twenty amino acids downstream from the amber termination codon. Readthrough of the amber termination codon would therefore result in an NS1 protein equivalent in length to other influenza field isolates (see Parvin *et al.*, 1983 for a description of A/CAM/46 and other influenza nonsense mutants). Using virus stocks of SV40 recombinants containing active amber *su*$^+$tRNA^{Tyr} genes introduced into COS-1 cells, Laski *et al.*, (1982b) showed that both the

suppressed and non-suppressed forms of the NS1 protein were synthesized after superinfection with A/CAM/46. The suppressed form of the NS1 protein could be detected in significant amounts after polyacrylamide gel electrophoresis and the level of suppression was calculated by estimating the quantity of readthrough product. Significantly, they also examined suppression of the amber termination codon with the suppressor cells employed in this study (L39; $\underline{su}^+$). This resulted in a relatively weak suppression of the amber codon, estimated at about 4% compared to 22.5% with the SV40 system.

Synthesis of viral proteins by $\underline{wt}$ and the 13 mutants isolated was investigated by infecting $\underline{su}^+$ and $\underline{su}^-$ cells in the presence of Actinomycin D, labelling with [^{35}S]-methionine, then gel electrophoresing the extracted proteins and detecting labelled proteins by autoradiography.

4.(b).1.1 IN VIVO PROTEIN SYNTHESIS BY WILD-TYPE VIRUS

When $\underline{su}^+$ and $\underline{su}^-$ cells were infected by $\underline{wt}$ virus and the proteins analysed by the above procedure, the characteristic VSV protein profile was observed - host cell protein synthesis was reduced compared with mock infected cells, and labelled bands corresponding to the five VSV polypeptides L, G, NS, N and M were easily identified (refer to Figure 19).

4.(b).1.2 IN VIVO PROTEIN SYNTHESIS BY IND/AMBL1/84

Viral protein synthesis by the mutant AmbL1 in $\underline{su}^+$ and $\underline{su}^-$ cells was studied (Figure 7a). A comparison of the [^{35}S]-methionine labelled proteins synthesized in $\underline{su}^+$ cells by $\underline{wt}$ and AmbL1 showed no detectable differences in the electrophoretic mobilities of the G, NS, N and M intracellular proteins. The L protein however, was not present in the mutant infected extract until late in infection (24 h post-infection). In addition, there was a novel viral specific polypeptide which migrated between the nucleocapsid protein (N) and the matrix protein (M). Since the mutant did not synthesize any full length L protein to a significant level up to 24 h post-infection, it was proposed

Figure 7

Comparison of the polypeptides synthesized in su^+ and su^- cells by wild-type and AmbL1 virus.

Su^+ and su^- cells were infected at a moi of 10 pfu/cell. Infected monolayers were incubated at 31°C and labelled from 5 to 6 h post infection, 7 to 8 h post infection or 23 to 24 h post infection. Cells were harvested and the polypeptides analysed on a 7.5% polyacrylamide gel.

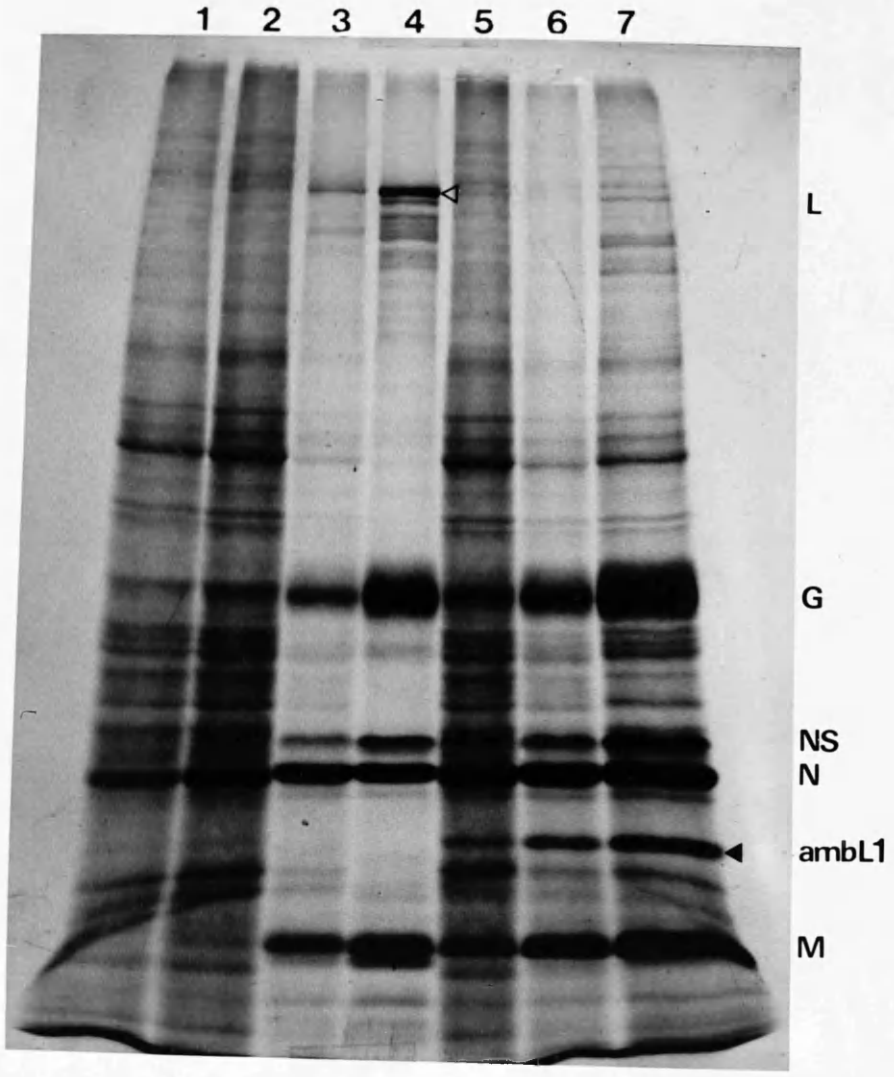
(a) su^+ cells

The polypeptides synthesized in [^{35}S]-methionine labelled su^+ cells are shown for mock infected cells (track 1), wt infected cells at 6, 8 and 24 h post infection (tracks 2, 3 and 4 respectively) and AmbL1 infected cells at 6, 8 and 24 h post infection (tracks 5, 6 and 7 respectively). The L protein fragment is indicated by a closed arrowhead and named after the mutant, in this case ambL1. Full-length L protein is indicated by an open arrowhead.

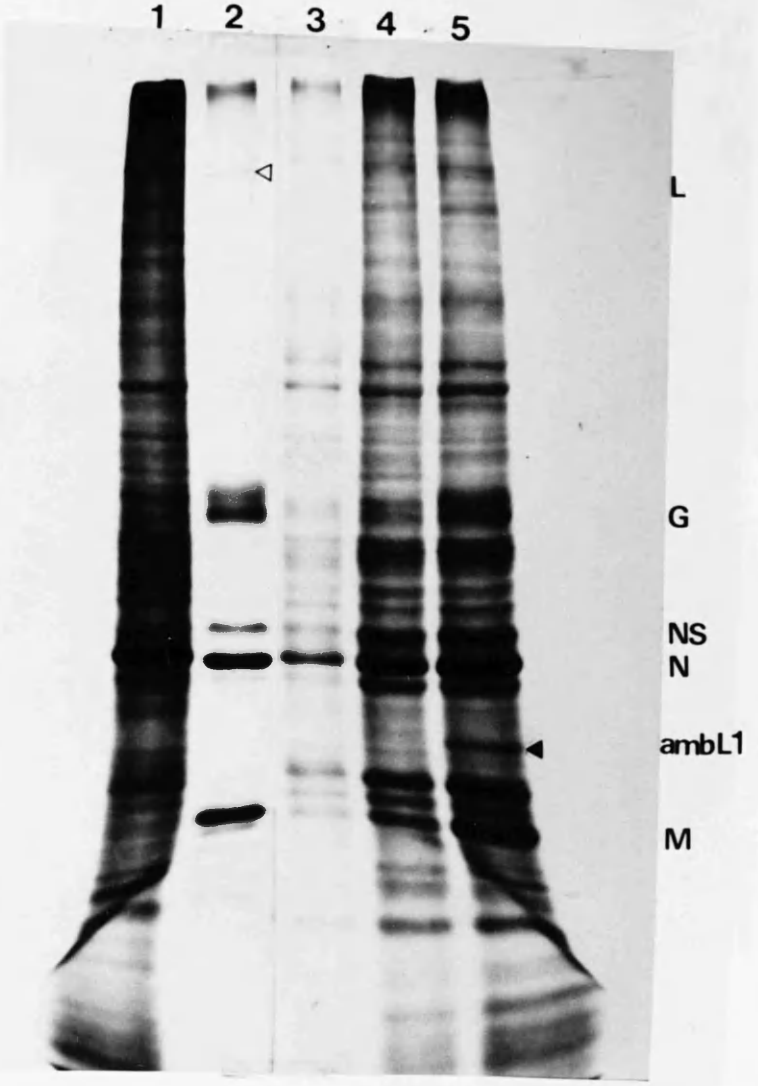
(b) su^- cells

The polypeptides synthesized in [^{35}S]-methionine labelled su^- cells are shown for mock infected cells (tracks 1 and 3), wt infected cells 8h pi (track 2) and AmbL1 infected cells at 8 and 24 h post infection (tracks 4 and 5 respectively). The bands are named and marked in the same way as above.

(a) suppressor cells



(b) non suppressor cells



that this new viral polypeptide is a truncated form of the L protein, produced by premature termination at an amber codon. The nucleocapsid gene encodes a polypeptide of 422 amino acids with a M_r of 47355 (Gallione et al., 1981) while the M protein encodes a polypeptide of 229 amino acids with a M_r of 26064 (Rose and Gallione, 1981). The truncated form of the L protein migrated slightly closer to the N protein than it did to the M, consequently its M_r could be estimated at approximately 37000 by comparing the observed mobility of the putative truncated L protein on polyacrylamide gels with the mobility and known M_r of the N and M proteins.

The L (polymerase) protein is the least abundant protein in wild-type infected cells. However it was evident that the proposed shortened form of the L polypeptide was more abundant in su^+ infected cells, than L protein present in wt infected cells. This apparent increase in the abundance of some parts of L protein may be the result of partial termination of transcription along the L RNA, resulting in an abundance of short L message over full length L message. Alternatively, translational attenuation could explain this result.

In contrast to infection of the su^+ cells by this mutant, viral protein synthesis in su^- cells was found to be less prominent and 'switch-off' of host-cell proteins was less marked (Figure 7b). This suggested that viral protein synthesis was itself much reduced. Negligible amounts of viral proteins were synthesized at the 8 h time point and only after 24 h were they clearly in evidence. The G, N, and M proteins were visible although no full length L protein could be identified. It was possible to identify the proposed shortened version of L after 24 h, although repeated analysis did not always reveal this polypeptide. Subsequent analysis has revealed that different preparations of the mutant contain differing proportions of revertants. This was not unexpected since the proportion of revertants in a preparation is dependent upon the time during infection at which the initial reversion event occurs. Mutant stocks containing higher numbers of revertants were associated with

the detection of the novel polypeptide species after infection of the su$^-$ cells. A possible interpretation of this result is that a high proportion of revertants present in the original inoculum could complement the mutant, late in infection, in cells multiply infected with mutant and revertant virus.

4.(b).1.3 IN VIVO PROTEIN SYNTHESIS BY IND/AMBL2/85

Comparison of the polypeptides synthesized in su$^+$ cells by wt and AmbL2 virus showed that although the G, NS, N and M proteins were present, there appeared to be no L protein produced (Figure 8a). Also, the G, NS, N and M proteins synthesized by the mutant did not exhibit any anomalous electrophoretic mobility. A novel viral specific polypeptide could be identified which migrated between the full length L protein and the glycoprotein G. This polypeptide was thought to represent a truncated form of the L protein. The L protein has a M_r of 241012 consisting of 2109 amino acids (Schubert et al., 1984) while the G protein has a M_r of 75416 encoded by 511 amino acids (Rose and Gallione, 1981). Thus, the M_r of the putative truncated L polypeptide could be estimated at approximately 150000 by using the standard viral proteins as markers. On comparison of the observed abundance of the truncated L protein in su$^+$ cells with the level of full length L protein in wt infected cells, it was evident that the shortened L is present intracellularly in significantly higher quantities.

The proteins synthesized by AmbL2 and wt virus in su$^-$ cells are compared in Figure 8b. It can be seen that little or no viral proteins were synthesized in mutant infected cells in comparison to the wt result.

4.(b).1.4 IN VIVO PROTEIN SYNTHESIS BY IND/AMBL3/85

Analysis of the polypeptides synthesized by AmbL3 in su$^+$ cells (Figures 9a, b) showed that this mutant behaved in a similar fashion to the two host range mutants described previously; namely, the G, NS, N and M proteins migrated normally, no full length L protein could be detected and a novel polypeptide species was evident. However, in this

Figure 8

Comparison of the polypeptides synthesized in su^+ and su^- cells by wild-type and AmbL2 virus.

Su^+ and su^- cells were infected at a moi of 10 pfu/cell. Infected monolayers were incubated at 31°C and labelled from 7 to 8 h post infection in the case of mock and wt infected cells or 7 to 8 h and 23 to 24 h post infection in the case of AmbL2 infected cells. Cells were harvested and the polypeptides analysed on a 7.5% polyacrylamide gel.

(a) su^+ cells

The polypeptides synthesized in [^{35}S]-methionine labelled su^+ cells are shown for mock infected cells (track 1), wt infected cells (track 2) and AmbL2 infected cells at 8 and 24 h post infection (tracks 3 and 4 respectively). The L protein fragment (ambL2) is marked by a closed arrowhead. Full-length L protein is indicated by an open arrowhead.

(b) su^- cells

The polypeptides synthesized in [^{35}S]-methionine labelled su^- cells are shown for mock infected cells (track 1), wt infected cells (track 4) and AmbL2 infected cells at 8 and 24 h post infection (tracks 2 and 3 respectively). The bands are named and marked in the same way as above.

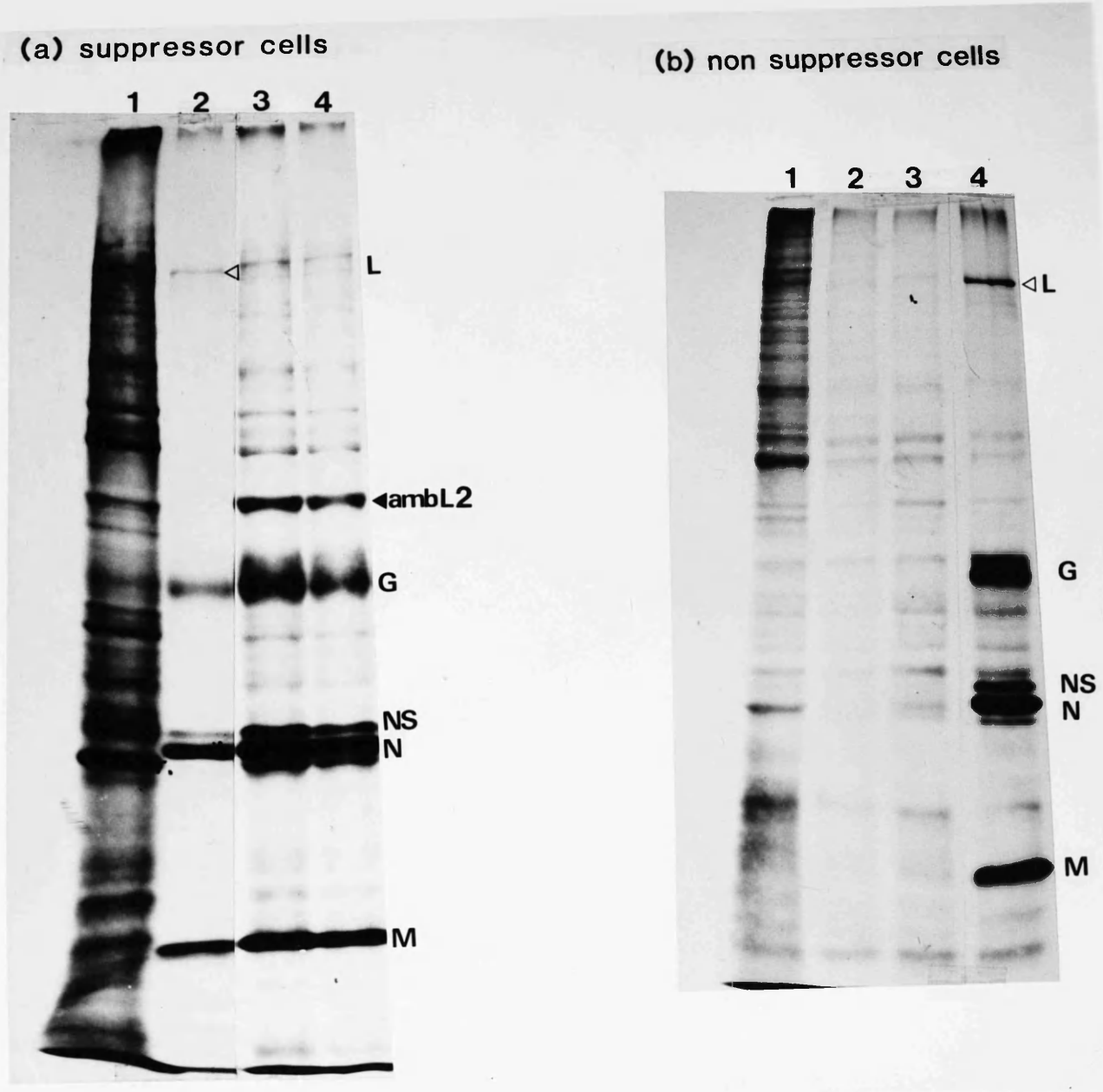

(a) suppressor cells
1
2
3
4
L
ambL2
G
NS
N
M
(b) non suppressor cells
1
2
3
4
L
G
NS
N
M

Figure 9

Comparison of the polypeptides synthesized in su^+ and su^- cells by wild-type and AmbL3 virus.

Su^+ and su^- cells were infected at a moi of 10 pfu/cell. Infected monolayers were incubated at 31°C and labelled from 7 to 8 h in the case of mock and wt infected cells or from 7 to 8 h and 23 to 24 h post infection in the case of AmbL3 infected cells. Cells were harvested and the polypeptides analysed on a 7.5% polyacrylamide gel (a) and (c) or a 4.5% polyacrylamide gel (b).

(a) su^+ cells

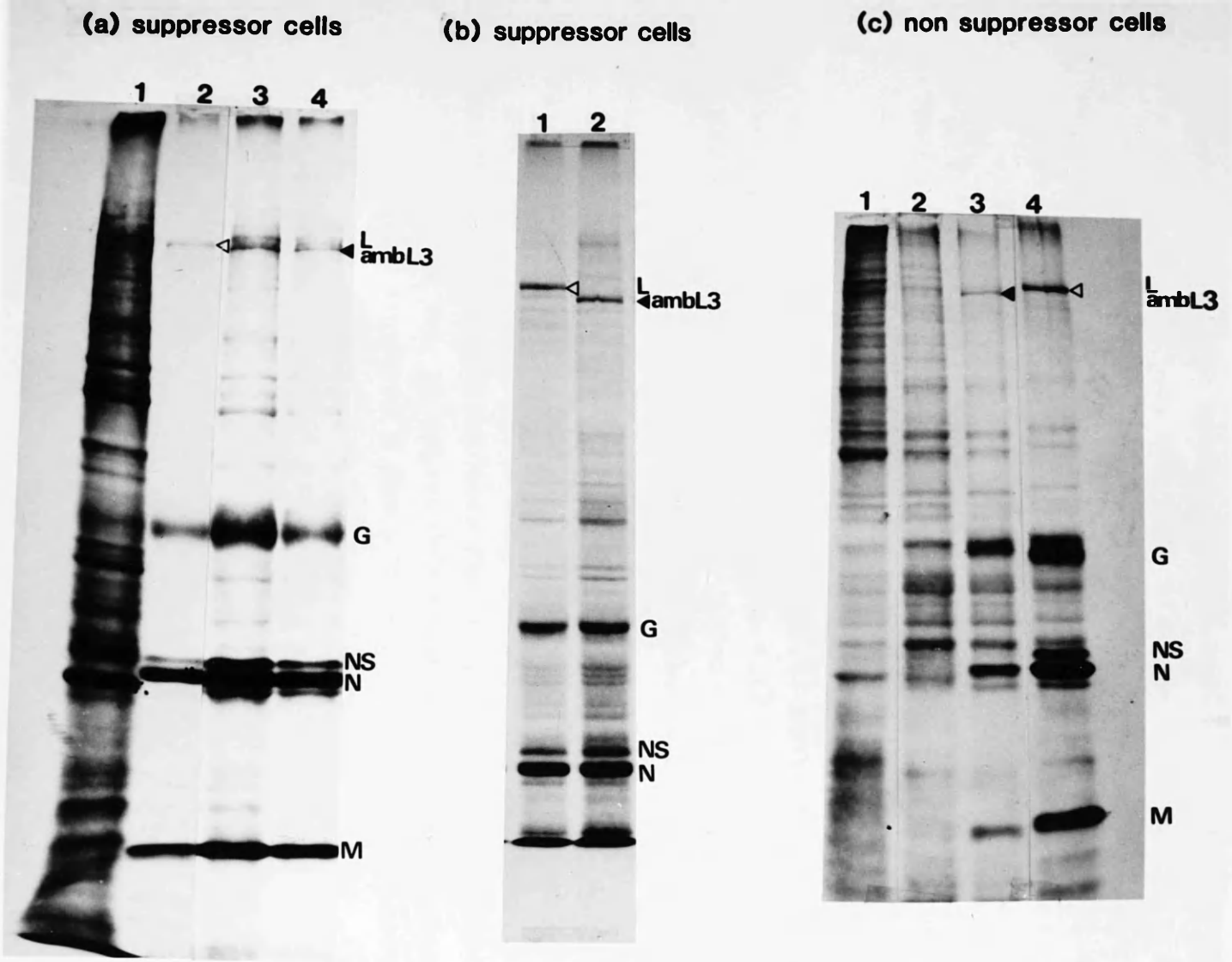
The polypeptides synthesized in [^{35}S]-methionine labelled su^+ cells are shown for mock infected cells (track 1), wt infected cells (track 2) and AmbL3 infected cells at 8 and 24 h post infection (tracks 3 and 4 respectively). The L protein fragment (ambL3) is indicated by a closed arrowhead. Full-length L protein is indicated by an open arrowhead.

(b) su^+ cells

The polypeptides synthesized in [^{35}S]-methionine labelled su^+ cells are shown for wt infected cells at 8 h post infection (track 1) and AmbL3 infected cells at 8 h post infection (track 2). The bands are named and marked in the same way as above.

(c) su^- cells

The polypeptides synthesized in [^{35}S]-methionine labelled su^- cells are shown for mock infected cells (track 1), wt infected cells (track 4) and AmbL3 infected cells at 8 and 24 h post infection (tracks 2 and 3 respectively). The bands are named and marked in the same way as above.


(a) suppressor cells
(b) suppressor cells
(c) non suppressor cells
1
2
3
4
L
ambL3
G
NS
N
M

case, the putative truncated L protein migrated only slightly faster than the authentic wt L protein. Thus, if this faster migrating L polypeptide is a result of premature termination of translation then the mutation must reside close to the downstream end of the message. In contrast to the mutants AmbL1 and AmbL2, the L protein fragment is present intracellularly at a level comparable to wild-type.

In Figure 9c, the proteins synthesized by AmbL3 and wt virus in su^- cells are compared. Although AmbL3 synthesized viral protein in readily detectable amounts at 8 h post infection in the su^+ cells, there was little evidence of viral protein synthesis at this time point in the su^- cells. There was however, evidence of viral protein synthesis after 24 h. The G, N and M proteins were detectable as well as small quantities of the additional polypeptide species. This may have resulted from revertants in the original inoculum reaching a significant level and complementing the mutant in multiply infected cells late in infection.

4.(b).1.5 IN VIVO PROTEIN SYNTHESIS BY IND/AMBL4/85

The [^{35}S]-methionine labelled proteins synthesized by this mutant in su^+ cells were studied. In Figure 10a the proteins synthesized in wt, AmbL4 and mock infected cells are compared. Each of the five viral proteins could be identified both at 8 h and at 24 h post infection. The L protein synthesized by AmbL4 migrated slightly faster than the full length protein and thus probably represents a truncated form of the L protein. The M_r of the faster migrating L protein could be estimated to be about 227000 using the standard viral proteins as markers. In addition, it appears that the proposed truncated version of the L protein may be more abundant than the full length protein, and in this respect, the mutant behaves similarly to AmbL1 and AmbL2.

Examination of the proteins synthesized by AmbL4 in su^- cells is shown in Figure 10b. Inhibition of host cell protein synthesis, a characteristic of wt infection, was markedly reduced in su^- cells infected by the mutant. As a

Figure 10

Comparison of the polypeptides synthesized in su^+ and su^- cells by wild-type and AmbL4 virus.

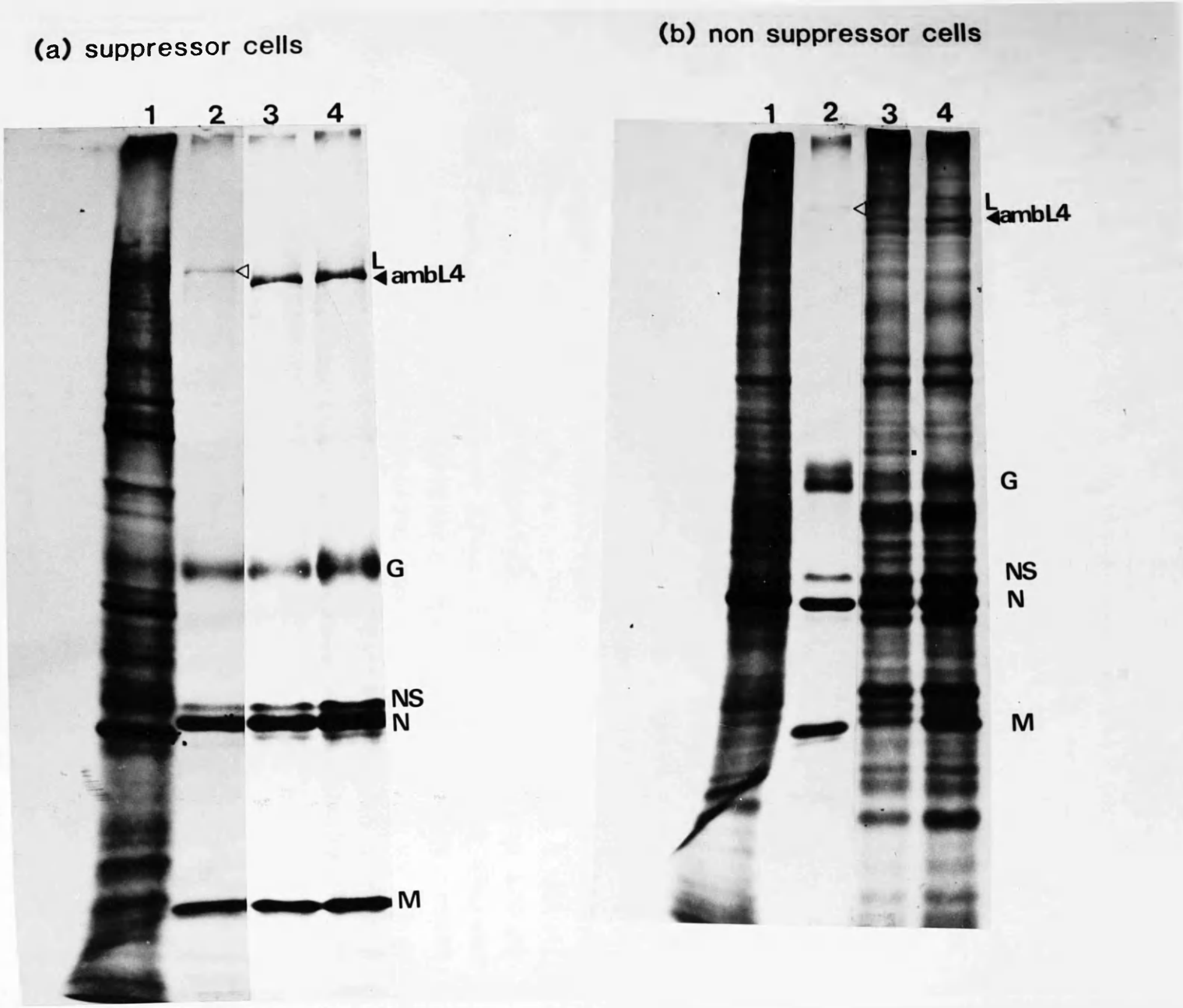
Su^+ and su^- cells were infected at a moi of 10 pfu/cell. Infected monolayers were incubated at 31°C and labelled from 7 to 8 h in the case of mock and wt infected cells or 7 to 8 h and 23 to 24 h in the case of AmbL4 infected cells. Cells were harvested and the polypeptides analysed on a 7.5% polyacrylamide gel.

(a) su^+ cells

The polypeptides synthesized in [^{35}S]-methionine labelled su^+ cells are shown for mock infected cells (track 1), wt infected cells (track 2) and AmbL4 infected cells at 8 and 24 h post infection (tracks 3 and 4 respectively). The L protein fragments are indicated by a closed arrowhead and named after the mutant (ambL4). Full-length L protein is indicated by an open arrowhead.

(b) su^- cells

The polypeptides synthesized in [^{35}S]-methionine labelled su^- cells are shown for mock infected cells (track 1), wt infected cells (track 2) and AmbL4 infected cells at 8 and 24 h post infection (tracks 3 and 4 respectively). The bands are marked and named in the same way as above. (•) signifies a host band which is present in AmbL4 infected cells at 8 h but not at 24 h post infection.


(a) suppressor cells
1
2
3
4
L
ambL4
G
NS
N
M
(b) non suppressor cells
1
2
3
4
L
ambL4
G
NS
N
M

result, the full extent of viral protein synthesis was somewhat obscured. However, some viral proteins were present, albeit at a reduced level. The M protein was detectable both at 8 h and 24 h post infection and probably the NS protein also. The truncated L protein could be identified at 24 h post infection although there was a high background of host proteins. One of these host proteins was absent at the 24 h time point, suggesting increased growth of the virus (Figure 10b). It is unclear whether this represented growth of mutant and/or revertant virus.

4.(b).1.6 IN VIVO PROTEIN SYNTHESIS BY IND/AMBL5/85

A comparison of the [^{35}S]-methionine labelled proteins synthesized in mock, wt and AmbL5 infected cells is shown in figure 11a. After infection of su$^+$ cells with AmbL5, the G, NS, N and M proteins were not observed to exhibit any anomalous migratory properties. However, the mutant did not appear to synthesize any L protein. No full length L protein was detectable, nor was any detectable truncated form. Since inhibition of cellular protein synthesis by this mutant was incomplete, it is possible that a truncated version may co-migrate with a host protein, or even another viral protein.

Analysis of the proteins synthesized by this mutant after infection of su$^-$ cells is shown in Figure 11b. There was no apparent inhibition of host cell protein synthesis. As a result viral specific proteins could not be identified easily. This observation in itself suggested that growth of the mutant in these cells was severely restricted. However the M and possibly NS proteins were detectable, particularly at the 24 h time point. Not unexpectedly, there was no evidence of either full length or truncated L protein. In accord with the result obtained with the mutant AmbL4 (Figure 10b), one of the host proteins was absent at 24 h (Figure 11b).

Figure 11

Comparison of the polypeptides synthesized in su^+ and su^- cells by wild-type and AmbL5 virus.

Su^+ and su^- cells were infected at a moi of 10 pfu/cell. Infected monolayers were incubated at 31°C and labelled from 7 to 8 h post infection in the case of mock and wt infected cells or 7 to 8 and 23 to 24 h post infection in the case of AmbL5. Cells were harvested, extracts prepared and the polypeptides analysed on a 7.5% polyacrylamide gel.

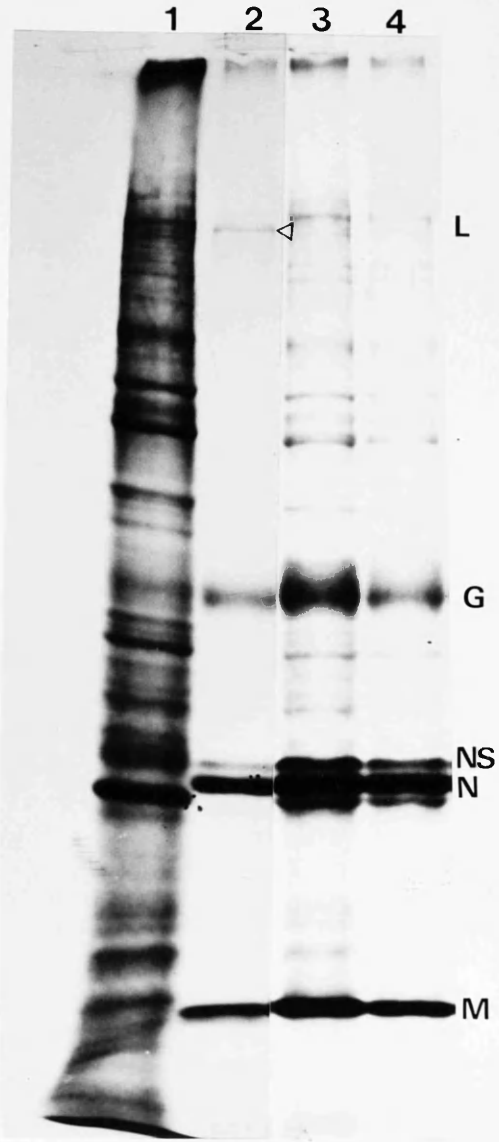
(a) su^+ cells

The polypeptides synthesized in [^{35}S]-methionine labelled su^+ cells are shown for mock infected cells (track 1), wt infected cells (track 2) and AmbL5 infected cells at 8 and 24 h post infection (tracks 3 and 4 respectively). Full length L protein is indicated by an open arrowhead.

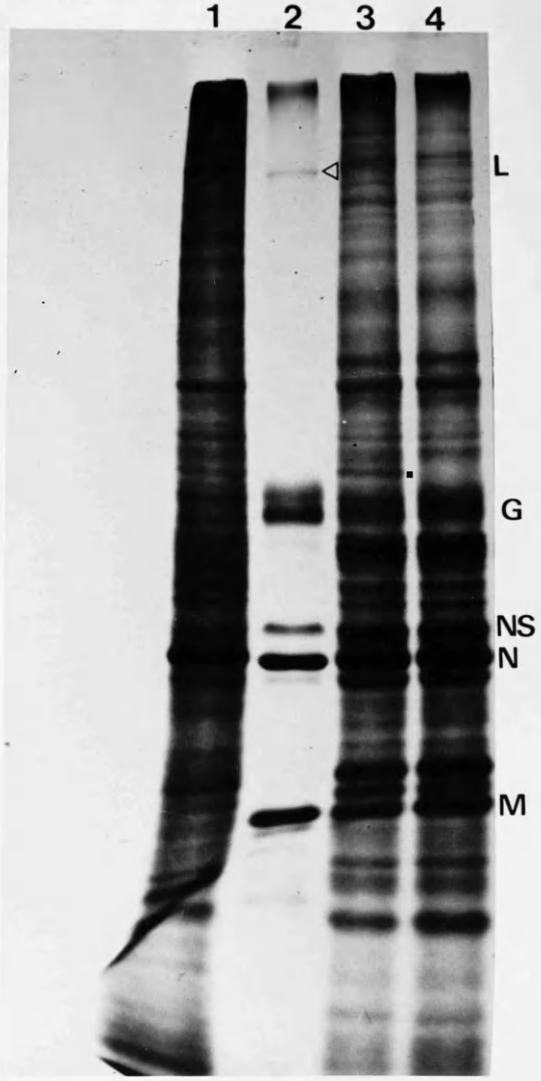
(b) su^- cells

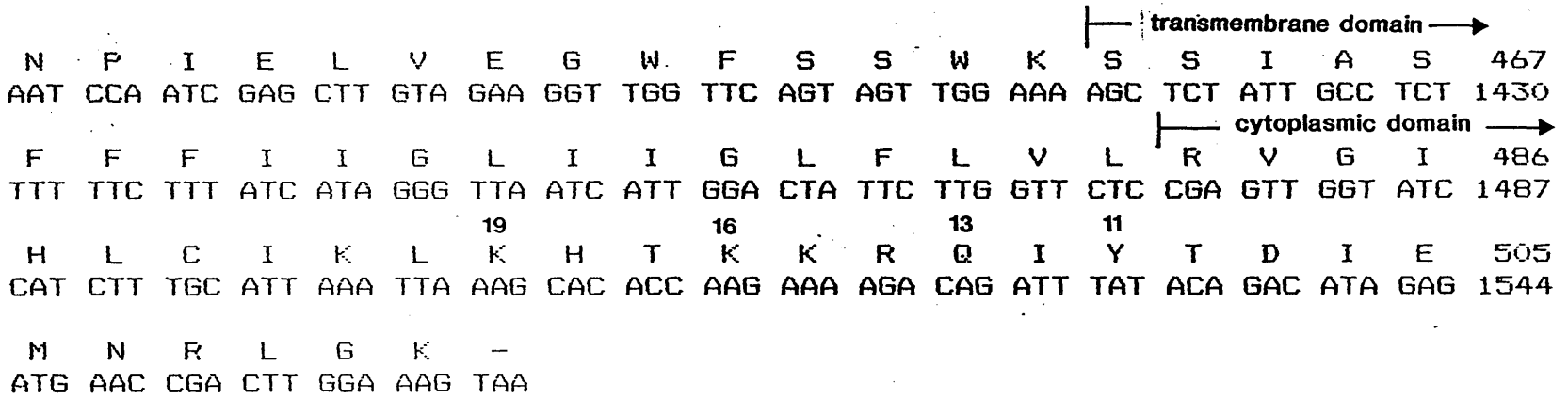
The polypeptides synthesized in [^{35}S]-methionine labelled su^- cells are shown for mock infected cells (track 1), wt infected cells (track 2) and AmbL5 infected cells at 8 and 24 h post infection (tracks 3 and 4 respectively). Full length L protein is indicated by an open arrowhead. (•)signifies a host band present in AmbL5 infected cells at 8 h but not at 24 h post-infection.

(a) suppressor cells



(b) non suppressor cells





Four amino acid codons which, by a single base change within the cytoplasmic domain of the G protein, could be changed to an amber codon are shown above (11, 13, 16 and 19).

4.(b).1.7 IN VIVO PROTEIN SYNTHESIS BY THE MUTANT IND/AMBG/85

The [^{35}S]-methionine labelled proteins synthesized by this mutant as well as wt virus in su$^+$ cells is shown in Figure 12a. All five viral proteins were present, both at 8 h and 24 h post infection. The glycoprotein produced by AmbG migrated slightly faster than wt G protein. The M_r of the mutant glycoprotein could be estimated at about 74500 from its mobility relative to the known M_r of full length G protein (75416; Rose and Gallione, 1981). The G protein synthesized by AmbG may therefore represent the product of premature termination, at an amber stop codon, close to the downstream end of the G message. On comparing the mobilities of the putative truncated G protein and full-length G protein, it was calculated that approximately 10 amino acids were absent from the C-terminus of the AmbG glycoprotein. If the change in mobility does indeed result from an amber mutation, then the mutation probably resides within the 29 amino acid cytoplasmic domain of G. Examination of the amino acid sequence which encodes the cytoplasmic domain of the G protein revealed four codons which, by a single base change, could be mutated to an amber stop codon. These codons lie, with respect to the C-terminus of the G protein gene, at codon positions 11, 13, 16 and 19. # see diagram opposite Three involve transition mutations (13, 16 and 19) and one a transversion mutation (11). Interestingly, the base change at position 11 would result in the alteration of a tyrosine specifying codon to an amber codon. Suppression of an amber mutation at this position by this particular suppressor (tRNA^{Tyr}) would be expected to result in the production of an active gene product.

At 24h post infection two forms of the G protein were detectable, one of which probably represented full length G protein (synthesized by revertants), the other, the truncated form of G protein (synthesized by AmbG).

Viral protein synthesis in su$^-$ cells infected either with mock, AmbG or wt virus is shown in Figure 12c. At 8 h there was little evidence of viral protein synthesis in mutant infected cells. However, after 24 h significant

Figure 12

Comparison of the polypeptides synthesized in su^+ and su^- cells by wild-type, AmbG and $hrTK^-7$ virus.

Su^+ and su^- cells were infected at a moi of 10 pfu/cell. Infected monolayers were incubated at 31°C and labelled from 7 to 8 h post infection and from 23 to 24 h post infection. Cells were harvested, extracts prepared and the polypeptides analysed on a 7.5% polyacrylamide gel.

(a) su^+ cells

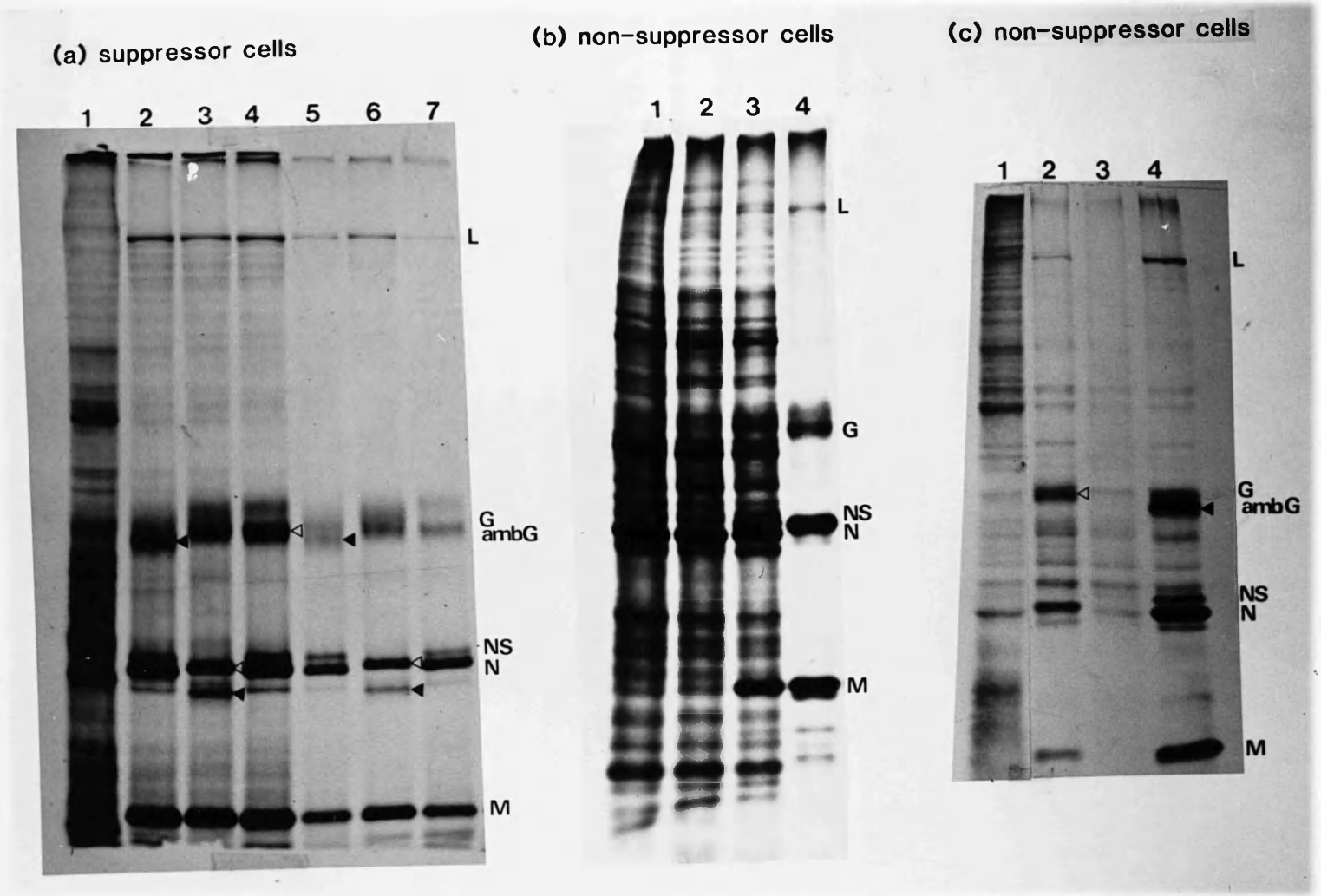
The polypeptides synthesized in [^{35}S]-methionine labelled cells are shown for mock infected, AmbG infected, $hrTK^-7$ infected and wt infected cells at 8 h post infection (tracks 1, 2, 3 and 4 respectively). Tracks 5, 6 and 7 show the polypeptides from AmbG infected, $hrTK^-7$ infected and wt infected cells at 24 h post infection respectively. In tracks 2 and 5, a closed arrowhead marks the position of the faster migrating G protein (named ambG). Normal G protein is indicated by an open arrowhead in track 4. In tracks 3 and 6, the faster migrating NS protein is indicated by a closed arrow and the slower migrating N protein by an open arrowhead.

(b) su^- cells

The polypeptides synthesized in [^{35}S]-methionine labelled su^- cells are shown for mock and wt infected cells at 8 h post infection (tracks 1 and 4 respectively) and for $hrTK^-7$ infected cells at 8 and 24 h post infection (tracks 2 and 3 respectively).

(c) su^- cells

The polypeptides synthesized in [^{35}S]-methionine labelled su^- cells are shown for mock and wt infected cells at 8 h post infection (tracks 1 and 2 respectively) and for AmbG infected cells at 8 and 24 h post infection (tracks 3 and 4 respectively). Wt G protein is indicated by an open arrowhead and the faster migrating version (ambG) by a closed arrowhead.


(a) suppressor cells
1 2 3 4 5 6 7
L
G
ambG
NS
N
M
(b) non-suppressor cells
1 2 3 4
L
G
NS
N
M
(c) non-suppressor cells
1 2 3 4
L
G
ambG
NS
N
M

amounts of viral specific proteins were visible. The faster migrating G polypeptide was readily detectable as well as some full length protein. The observation that AmbG synthesizes the mutant G protein at such a high level in the su$^-$ cells would suggest that AmbG is slightly 'leaky' in these cells. In addition, AmbG grew to a higher titre than any of the previously described mutants on su$^-$ cells (Table 2) and, although some, if not all, of this may have represented growth of revertants, it is possible that this may have resulted from growth of the mutant itself.

Several studies have examined the effect of an altered cytoplasmic domain on transport of the VSV glycoprotein (Rose and Bergmann, 1983; Rose et al., 1984; Puddington et al., 1986). The most directly relevant study by Rose and Bergmann (1983) examined the effect of deletions introduced into a cDNA clone encoding G protein. Three of the deletions lacked a portion of the cytoplasmic domain. The deletion which resulted in a G protein nearest in size to the AmbG glycoprotein (lacking approximately 10 amino acids) lacked 16 terminal amino acids of its cytoplasmic domain. It was observed that this deleted G protein was transported to the Golgi apparatus at a markedly reduced rate in comparison to full length protein, although it was eventually transported to the cell surface. Thus, it is feasible that the shortened glycoprotein synthesized in su$^-$ cells by AmbG, which is thought to lack 10 amino acids from its carboxy terminus, is also retarded, resulting in a decreased rate of transport of G to the cell surface, or even fewer G molecules on the cell surface at a given time.

From this model, one might conclude that an amber mutation, which resides within the cytoplasmic domain, and which results in the synthesis of a truncated version of G protein, as I propose is the case with AmbG, would have a deleterious but not lethal effect in the absence of the suppressor. This interpretation would explain why AmbG appears to be 'leaky' on the su$^-$ cells.

4.(b).1.8 IN VIVO PROTEIN SYNTHESIS BY IND/HRTK$^-$7/85

Analysis of the [^{35}S]-methionine labelled proteins synthesized by hrTK$^-$7 in su$^+$ cells is shown in figure 12a. Comparison of the polypeptides synthesized after infection of su$^+$ cells by wt and hrTK$^-$7 shows that the mutant produces an NS polypeptide which migrates faster than the wt polypeptide in polyacrylamide gels. The N protein also migrates anomalously, migrating slightly slower than wt N protein. Possibly the faster migrating NS polypeptide is a consequence of an amber mutation within the NS RNA. However, the anomalous migratory properties of this phosphoprotein have been well documented (Wunner and Pringle, 1972; Obijeski et al., 1974; Knipe et al., 1975; Kingsford and Emerson, 1980; Bell et al., 1984; Bell and Prevec, 1985). It has been reported that 5-FU induced mutants belonging to complementation group E (the NS gene) of VSV New Jersey (tsE1, tsE2 and tsE3) possess an NS protein with altered mobility in polyacrylamide gels (Evans et al., 1979). Nucleotide sequence analysis of a cDNA clone containing a full length copy of the NS gene revealed in each case that the basis of the mutant phenotype was a single nucleotide change, resulting in substitution of an amino acid of altered charge (Rae and Elliott, 1986b). Analysis of the predicted secondary structure of the wt and mutant NS proteins using the sequence data obtained indicated that the mutations might have a marked effect on the conformation of the protein. The authors postulated that the predicted alterations to the secondary structure of the NS protein were responsible for the observed electrophoretic mobility differences (Rae and Elliott, 1986b). Thus, it is possible that the altered migration of the NS protein specified by hrTK$^-$7 is the result of a point mutation possibly changing the charge of the affected amino acid so affecting the conformation of the protein. The reason for the slower migrating N protein is unknown, although it may also be caused by a point mutation altering the charge of the amino acid affected, possibly resulting in the altered mobility of the protein. Alternatively, the aberrant migration of the N protein could be the result of a

mutation altering the normal stop codon to an amber codon. Readthrough of the amber codon in the su$^+$ cells, followed by termination at an in-frame ochre or opal stop codon further downstream, would result in a higher molecular weight N protein. However, examination of the nucleotide sequence data for this region (Gallione et al., 1981) shows that there are no in-frame stop codons within 30 nucleotides of the termination codon.

Viral protein synthesis after infection of su$^-$ cells by hrTK$^-$7 was much reduced (Figure 12b) in comparison to the proteins synthesized by the mutant in su$^+$ cells. No viral proteins were detectable 8 h post infection and the 8 h preparation was very similar to mock infected. At 24 h post infection, the L, N and M proteins were easily detecable. The N protein migrated mormally and the faster migrating form of the NS protein could not be detected.

In conclusion, it is apparent that hrTK$^-$7 growth is restricted in su$^-$ cells. However, it is not clear whether the mutations affecting the mobilities of the N and NS proteins contribute singly or in conjunction towards the observed restriction. The NS protein specified by this mutant may possibly have a shorter chain length than normal and as such could conceivably be the product of a premature termination event. The N protein, on the other hand, appeared to possess a higher M_r than the normal protein and as such is unlikely to result from premature termination of translation at an amber codon.

4.(b).1.9 IN VIVO PROTEIN SYNTHESIS BY THE HOST RANGE MUTANTS HRTK$^-$8 TO HRTK$^-$12

Examination of the [^{35}S]-methionine labelled proteins synthesized by these mutants after infection of su$^+$ cells by polyacrylamide gel electrophoresis showed that each synthesized all five viral proteins normally. In addition, viral protein synthesis in su- cells infected by these mutants did not appear to be restricted. The mutant hrTK$^-$13 could only be grown to a titre of 10^4 and so any attempts at analysing the proteins synthesized intracellularly by this mutant would require concentration of the virus by

centrifugation. Although these mutants may merit further study it was decided to concentrate on a more complete characterization of the seven mutants described previously. Therefore, the remainder of this thesis relates only to these seven mutants.

4.(b).2 COMPLEMENTATION ANALYSIS

4.(b).2.1 INTERGENIC COMPLEMENTATION

Complementation analysis has been used extensively in the assignment of ts mutants of the Glasgow, Massachusetts, Orsay and Winnipeg strains of VSV Indiana (Flamand, 1970; Holloway et al., 1970; Pringle, 1970; Rettenmeier et al., 1975), VSV Cocal (Pringle and Wunner, 1973), VSV New Jersey, Hazelhurst subtype (Pringle et al., 1971), Concan subtype (Byrd et al., 1984), and Chandipura virus (Gadkari and Pringle, 1980). However, ts mutants isolated from rabies virus have not exhibited convincing complementation, even though they have their own distinct phenotypic properties, and thus remain unclassified (Bussereau and Flamand, 1978).

Ts mutants of VSV Indiana were employed to assign the mutations specifying the restriction in host-range to a specific complementation group. The ts mutants used for this purpose were ts11 (a group I mutant), ts22 (group II), ts31 (group III), ts41 (group IV) and ts045 (group V).

Initial attempts to assign the mutants were by a qualitative method. This consisted of infecting su^- cells in 50 mm dishes with an amber and a ts mutant, incubating the infections for 24 h at the non-permissive temperature and scoring each plate for the presence or absence of cpe. However, this method, although successfully employed in the assignment of ts mutations (Pringle et al., 1971), consistently resulted in a failure to restrict growth of the singly infected ts controls.

A quantitative analysis was obtained by infecting monolayers of su^- cells at the non-permissive temperature with pairs of mutants (a ts mutant plus an amber mutant) for a period equivalent to twice the latent period of the virus

(8 h) and then titrating the supernatant on *su*$^-$ cells to test for growth of the *ts* mutants. Attempts to test for growth of the amber mutants at the restrictive temperature on the *su*$^+$ cells were unsuccessful due to the inviability of the *su*$^+$ cells at this temperature.

Of the mutants tentatively assigned as L gene mutants from the results of the protein analysis, all five have been shown by means of negative complementation (Table 3) to have a mutation in complementation group I (the L cistron). The mutants *ts*22, *ts*31, *ts*41 and *ts*045 all successfully complemented AmbL1, AmbL2, AmbL3, AmbL4 and AmbL5 whereas the mutant *ts*11 failed to do so. Thus this analysis is consistent with the results obtained from the protein analysis, assigning the mutations to complementation group I (the L cistron). As a control, the group I mutant (*ts* 11) was tested in complementation with the four other *ts* mutants. Ts 11 was found to complement each of the representative *ts* mutants.

The mutant AmbG, which exhibited a slightly faster migrating glycoprotein in polyacrylamide gels after infection of the *su*$^+$ cells (Figure 12a), appeared to complement all *ts* mutants apart from the mutant *ts*045, although the result was not completely convincing (Table 3). Since *ts*045 is a group V mutant this mutant can be tentatively assigned to complementation group V, the G gene.

The mutant hrTK$^-$7 failed to complement any of the *ts* mutants and could not be allocated to a specific VSV complementation group. Thus this is probably a multiple mutant, and as a result it was classified simply as a host range (hr) mutant.

4.(b).2.2 INTRAGENIC COMPLEMENTATION

The occurrence of intragenic complementation has been observed in the analysis of *ts* mutants of the Orsay collection of spontaneous *ts* mutants (Flamand, 1970) and *ts* mutants of the Winnipeg strain (Wong *et al.*, 1972) of VSV Indiana. Intragenic complementation has also been reported between *ts* mutants of VSV New Jersey (Pringle *et al.*, 1981) and Chandipura virus (Gadkari and Pringle, 1980a). However

Table 3

Complementation analysis of host range mutants.

(a) Ind/AmbL1

Singly infected	Titre	Doubly infected	Titre
AmbL1	2.0×10^4	AmbL1 + ts 11	2.1×10^4
ts 11	1.8×10^3	AmbL1 + ts 22	5.8×10^6
ts 22	1.6×10^3	AmbL1 + ts 31	4.0×10^6
ts 31	1.8×10^3	AmbL1 + ts 41	7.0×10^5
ts 41	2.0×10^3	AmbL1 + ts 0/45	1.0×10^7
ts 0/45	2.1×10^3		

(b) Ind/AmbL2

Singly infected	Titre	Doubly infected	Titre
AmbL1	1.5×10^2	AmbL2 + ts 11	2.0×10^2
ts 11	1.8×10^3	AmbL2 + ts 22	6.4×10^5
ts 22	2.0×10^3	AmbL2 + ts 31	5.0×10^5
ts 31	1.5×10^3	AmbL2 + ts 41	1.0×10^4
ts 41	1.5×10^3	AmbL2 + ts 0/45	2.0×10^6
ts 0/45	1.9×10^3		

(c) Ind/AmbL3

Singly infected	Titre	Doubly infected	Titre
AmbL3	3.0×10^2	AmbL3 + ts 11	5.0×10^2
ts 11	1.2×102	AmbL3 + ts 22	7.1×10^5
ts 22	2.4×10^2	AmbL3 + ts 31	6.4×10^5
ts 31	1.8×103	AmbL3 + ts 41	1.1×10^4
ts 41	1.8×10^2	AmbL3 + ts 0/45	2.0×10^6
ts 0/45	3.0×10^2		

(d) Ind/AmbL4

Singly infected	Titre	Doubly infected	Titre
AmbL4	2.8×10^2	AmbL4 + ts 11	7.2×10^2
ts 11	2.0×10^2	AmbL4 + ts 22	3.0×105
ts 22	1.0×10^3	AmbL4 + ts 31	4.6×10^5
ts 31	2.5×10^2	AmbL4 + ts 41	1.1×10^5
ts 41	2.7×10^2	AmbL4 + ts 0/45	4.1×10^6
ts 0/45	6.0×103		

(e) Ind/AmbL5

Singly infected	Titre	Doubly infected	Titre
AmbL5	1.6×10^2	AmbL5 + ts 11	2.4×10^2
ts 11	2.2×10^2	AmbL5 + ts 22	4.6×10^5
ts 22	8.4×10^2	AmbL5 + ts 31	1.0×10^5
ts 31	2.0×10^2	AmbL5 + ts 41	3.0×10^5
ts 41	1.0×10^3	AmbL5 + ts 0/45	2.1×10^6
ts 0/45	2.4×10^3		

(f) Ind/AmbG

Singly infected	Titre	Doubly infected	Titre
AmbG	3.2×10^3	AmbG + ts 11	3.0×10^4
ts 11	1.0×10^2	AmbG + ts 22	3.0×10^5
ts 22	2.1×10^2	AmbG + ts 31	6.2×10^5
ts 31	1.8×102	AmbG + ts 41	6.0×104
ts 41	2.0×10^2	AmbG + ts 0/45	7.4×10^3
ts 0/45	1.8×10^3		

(g) hrTK$^-$7

Singly infected	Titre	Doubly infected	Titre
hrTK$^-$7	4.2×10^2	hrTK$^-$7 + ts 11	5.2×10^2
ts 11	4.0×10^3	hrTK$^-$7 + ts 22	4.0×10^3
ts 22	5.4×10^3	hrTK$^-$7 + ts 31	8.2×10^3
ts 31	1.7×10^4	hrTK$^-$7 + ts 41	3.4×10^3
ts 41	1.1×10^3	hrTK$^-$7 + ts 0/45	2.2×10^4
ts 0/45	1.0×10^4		

Monolayers of su$^-$ cells were infected either singly or with a mixture of a host range mutant and a ts mutant. After 8 h at 39°C, the supernatants were recovered and the yield of virus titred of su$^-$ cells at 31°C. Yields are given in pfu/ml.

no such intragenic complementation was reported within the 5-FU induced mutants of the Glasgow strain of VSV Indiana (Pringle, 1987). Its occurrence between ts mutants of Chandipura virus resulted in the sub-division of complementation group ChI (the L gene) into ChIA and ChIB (Gadkari and Pringle, 1980a). Thus it has been suggested that the gene product specified by this complementation group forms a multimeric protein (Gadkari and Pringle, 1980a). For two mutations to complement, the conformation of the mutated region on one monomer must be corrected by the unmutated region of the other monomer. However, in cells multiply infected by two group I amber mutants, both viruses are defective in the same region since they all lack a portion of the carboxy terminus of their L protein.

The results of the intragenic complementation analysis are shown in Table 4. Of the ten possible pairwise combinations of these viruses none exhibit any significant complementation. These results are thus consistent with the previous interpretation that each of the host range group I mutants are defective in the same region, namely the C terminus of the L protein.

4.(b).3 ISOLATION OF REVERTANTS

4.(b).3.1 PLAQUE PURIFICATION

Revertants of the host range mutants were isolated by plating the passage 1 stock of each isolate on the su^- cell line to give well isolated plaques. In the case of four of the group I mutants (AmbL1 to AmbL4) isolated plaques were produced at the 10^{-2} to 10^{-3} dilution. The plaques exhibited a wt plaque morphology with plaques ranging in size from 3-6 mm. The plaques produced by these mutants on su^+ cells were consistently about 1mm in diameter. 24 well isolated plaques were picked for each of the mutants and used to infect Linbro wells of su^- cells. Without exception, each plaque gave rise to cpe within two days of infection. The virus was harvested and stored at $-70^{o}C$.

Isolation of revertants of the group V mutant, AmbG, followed a slightly different pattern. Although plaques

Table 4

Intragenic complementation analysis of the group I mutants.

(a) AmbL1 + AmbL2

Singly infected	Titre	Doubly infected	Titre
AmbL1	3.4×10^2	AmbL1 + AmbL2	5.4×10^2
AmbL2	1.6×10^2		

(b) AmbL1 + AmbL3

Singly infected	Titre	Doubly infected	Titre
AmbL1	3.4×10^2	AmbL1 + AmbL3	4.1×10^2
AmbL3	2.7×10^2		

(c) AmbL1 + AmbL4

Singly infected	Titre	Doubly infected	Titre
AmbL1	3.4×10^2	AmbL1 + AmbL4	5.0×10^2
AmbL4	3.7×10^2		

(d) AmbL1 + AmbL5

Singly infected	Titre	Doubly infected	Titre
AmbL1	3.4×10^2	AmbL1 + AmbL5	7.3×10^2
AmbL5	1.1×10^2		

(e) AmbL2 + AmbL3

Singly infected	Titre	Doubly infected	Titre
AmbL2	1.6×10^2	AmbL2 + AmbL3	2.0×10^2
AmbL3	2.7×10^2		

(f) AmbL2 + AmbL4

Singly infected	Titre	Doubly infected	Titre
AmbL2	1.6×10^2	AmbL2 + AmbL4	3.6×10^2
AmbL4	3.7×10^2		

(g) AmbL2 + AmbL5

Singly infected	Titre	Doubly infected	Titre
AmbL2	1.6×10^2	AmbL2 + AmbL5	4.0×10^2
AmbL5	1.1×10^2		

(h) AmbL3 + AmbL4

Singly infected	Titre	Doubly infected	Titre
AmbL3	2.7×10^2	AmbL2 + AmbL4	6.4×10^2
AmbL4	3.7×10^2		

(i) AmbL3 + AmbL5

Singly infected	Titre	Doubly infected	Titre
AmbL3	2.7×10^2	AmbL3 + AmbL5	4.3×10^2
AmbL5	1.1×10^2		

(j) AmbL4 + AmbL5

Singly infected	Titre	Doubly infected	Titre
AmbL4	3.7×10^2	AmbL4 + AmbL5	6.0×10^2
AmbL5	1.1×10^2		

Monolayers of su$^-$ cells were infected either singly or with a mixture of two host range mutants. After 8 h at 39^{o}C, the supernatants were recovered and the yield of virus titred on su$^-$ cells at 31^{o}C. Yields are given in pfu/ml.

were produced at the 10^{-4} dilution, they took 24 h longer to appear than revertant plaques of the group I mutants and were smaller in size (about 2-3mm in diameter). 24 well isolated plaques were picked and used to infect monolayers of *su*$^-$ cells in Limbro wells. 17 out of 24 plaques gave rise to cpe, but only after incubation for 3-4 days. This virus appeared to give rise to a less virulent infection of the su$^-$ cells than either *wt* virus or revertants of the group I mutants.

When the mutants AmbL5 and hrTK$^-$7 were used to infect *su*$^-$ cells only very small hazy plaques could be seen at the 10^{-1} dilution on inspection, even after seven days post infection. A further incubation period of seven days failed to increase the size of the plaques appreciably. At this point 10 of the 'plaques' were picked, dispersed into 1.5 ml of MEM/20%CS and used to infect monolayers of *su*$^-$ cells in Linbro wells. However, none of the plaques gave rise to cpe even after seven days, although at two days post infection there was some evidence of viral infection, in that small areas of the cell monolayer appeared translucent. This usually occurred during a *wt* infection and invariably led to extensive cpe. However, in this case no cpe was forthcoming. This phenomenon was consistently reproducible. One possible interpretation of this is that the hazy plaques were simply the result of reasonably high titre virus being plated on cells (albeit non-permissive cells) and not from actual growth of either mutant.

4.(b).3.2 IN VIVO PROTEIN SYNTHESIS OF REVERTANTS OF THE GROUP I AND GROUP V HOST RANGE MUTANTS

Revertants isolated for four (AmbL1 to AmbL4) of the group I mutants and for the group V mutant (AmbG) were used to infect both *su*$^+$ and *su*$^-$ cells, and the [^{35}S]-methionine labelled intracellular proteins were then analysed at 8 h post infection by fractionation on a polyacrylamide gel.

Examination of the proteins synthesized by revertants of the group I mutants AmbL1, AmbL2, AmbL3 and AmbL4 (figures 13, 14, 15 and 16 respectively) showed that of the isolates examined, all exhibited a *wt* profile. In

Figure 13

Intracellular polypeptides synthesized in su^+ and su^- cells by wt virus and revertants of AmbL1.

Su^+ and su^- cells were infected at a moi of 10 pfu/cell. Infected monolayers were incubated at 31°C and labelled from 7 to 8 h post infection. Cells were harvested and extracts prepared. The polypeptides were analysed by gel electrophoresis on a 7.5% polyacrylamide gel.

The polypeptides synthesized in [^{35}S]-methionine labelled su^+ cells are shown for mock infected (track 1), wt infected (track 2) and three revertants of AmbL1 (tracks 3, 4 and 5).

The polypeptides synthesized in [^{35}S]-methionine labelled su^- cells are shown for wt infected (track 6), three revertants (the same three as above) of AmbL1 (tracks 7, 8 and 9) and mock infected (track 10).

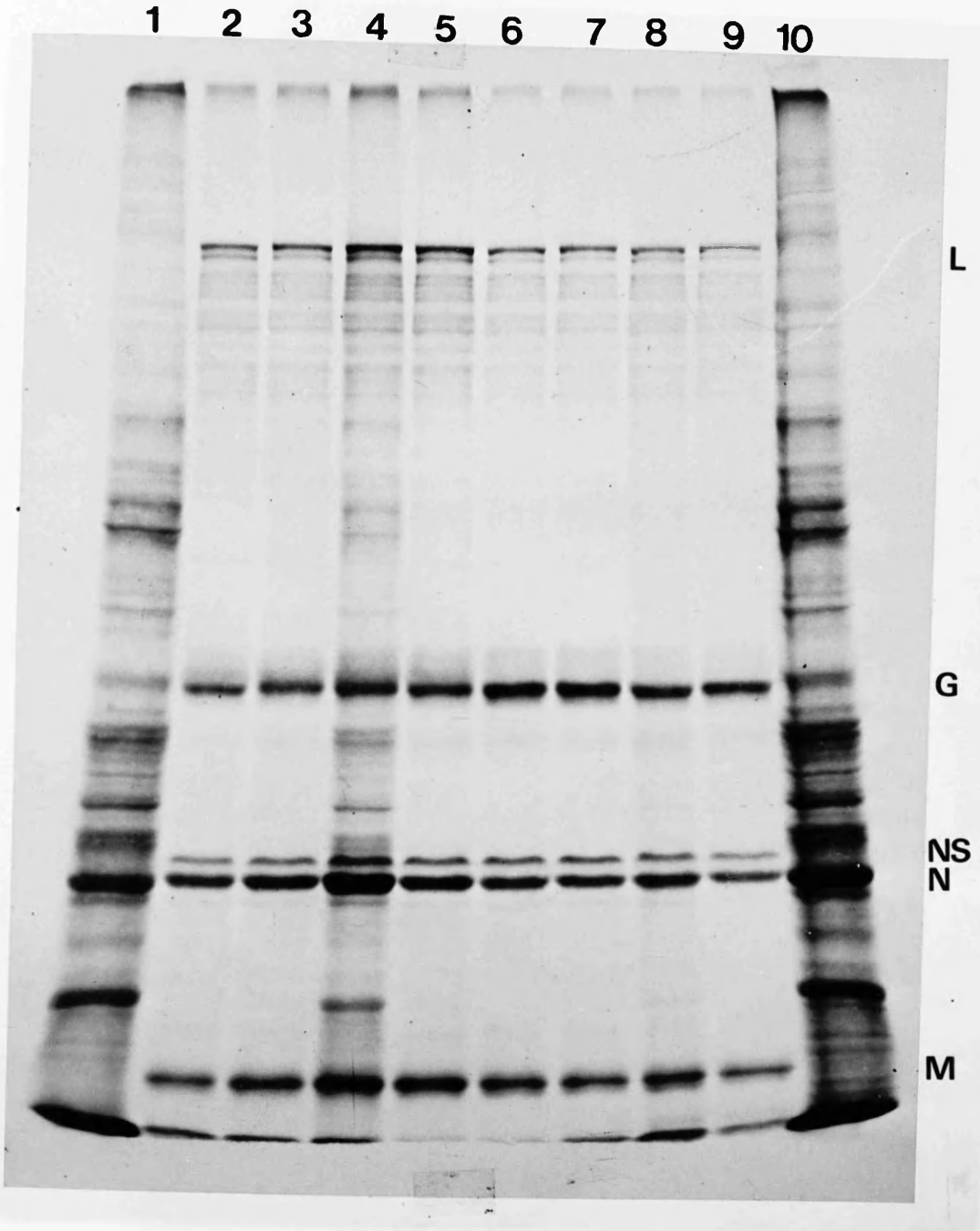

1
2
3
4
5
6
7
8
9
10
L
G
NS
N
M

Figure 14

Intracellular polypeptides synthesized in su^+ cells by wt virus and revertants of AmbL2.

Su^+ cells were infected at a moi of 10 pfu/cell. Infected monolayers were incubated at 31°C and labelled from 7 to 8 h post infection. Cells were harvested and extracts prepared. The polypeptides were analysed by gel elecrophoresis on a 7.5% polyacrylamide gel.

The polypeptides synthesized in [^{35}S]-methionine labelled su^+ cells are shown for mock infected (track 1), wt infected (track 2), AmbL2 infected (track 3) and five revertants of AmbL2 (tracks 4 to 8 respectively). The L protein fragment (ambL2) is indicated by a closed arrowhead. Full length L protein is indicated by an open arrowhead.

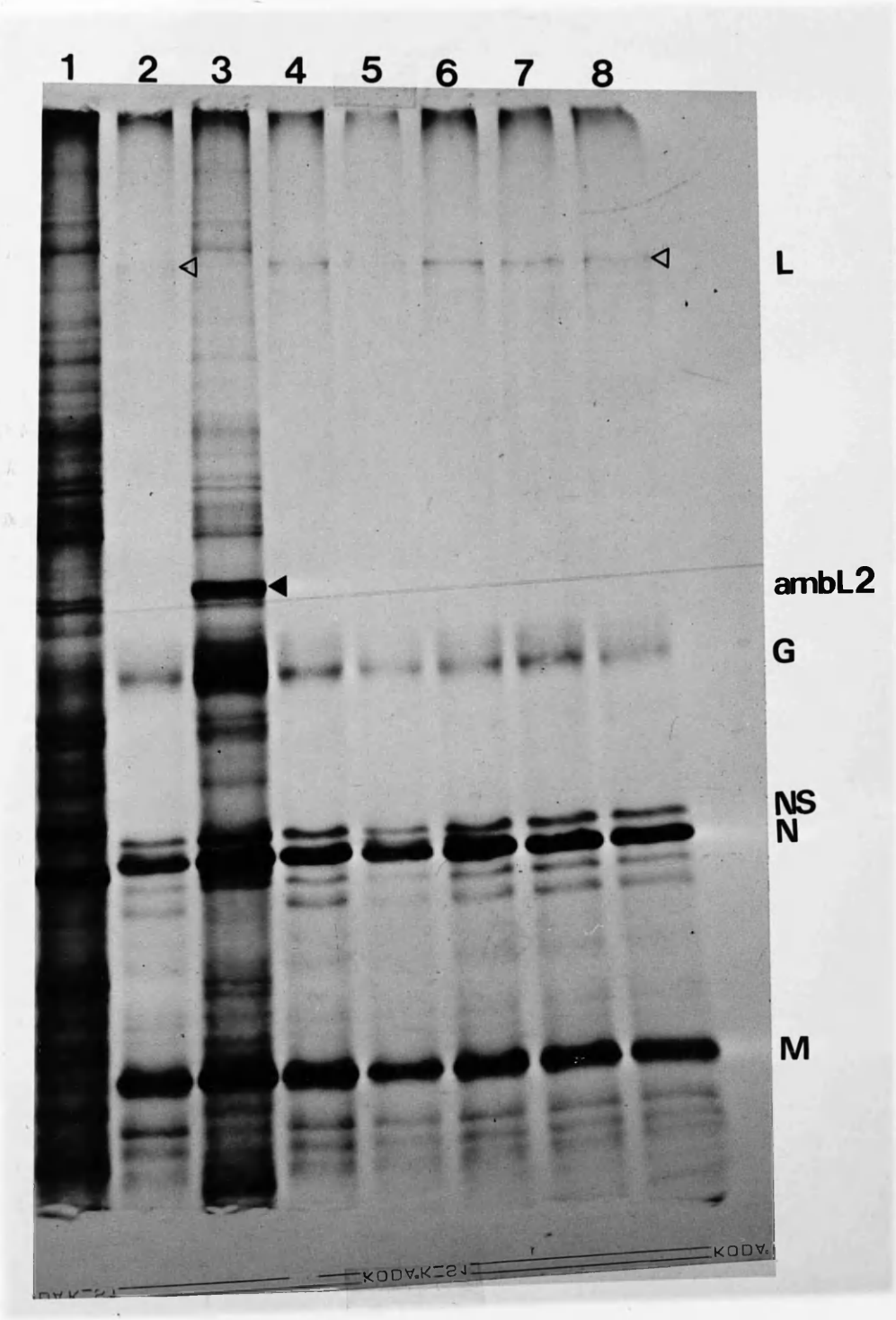
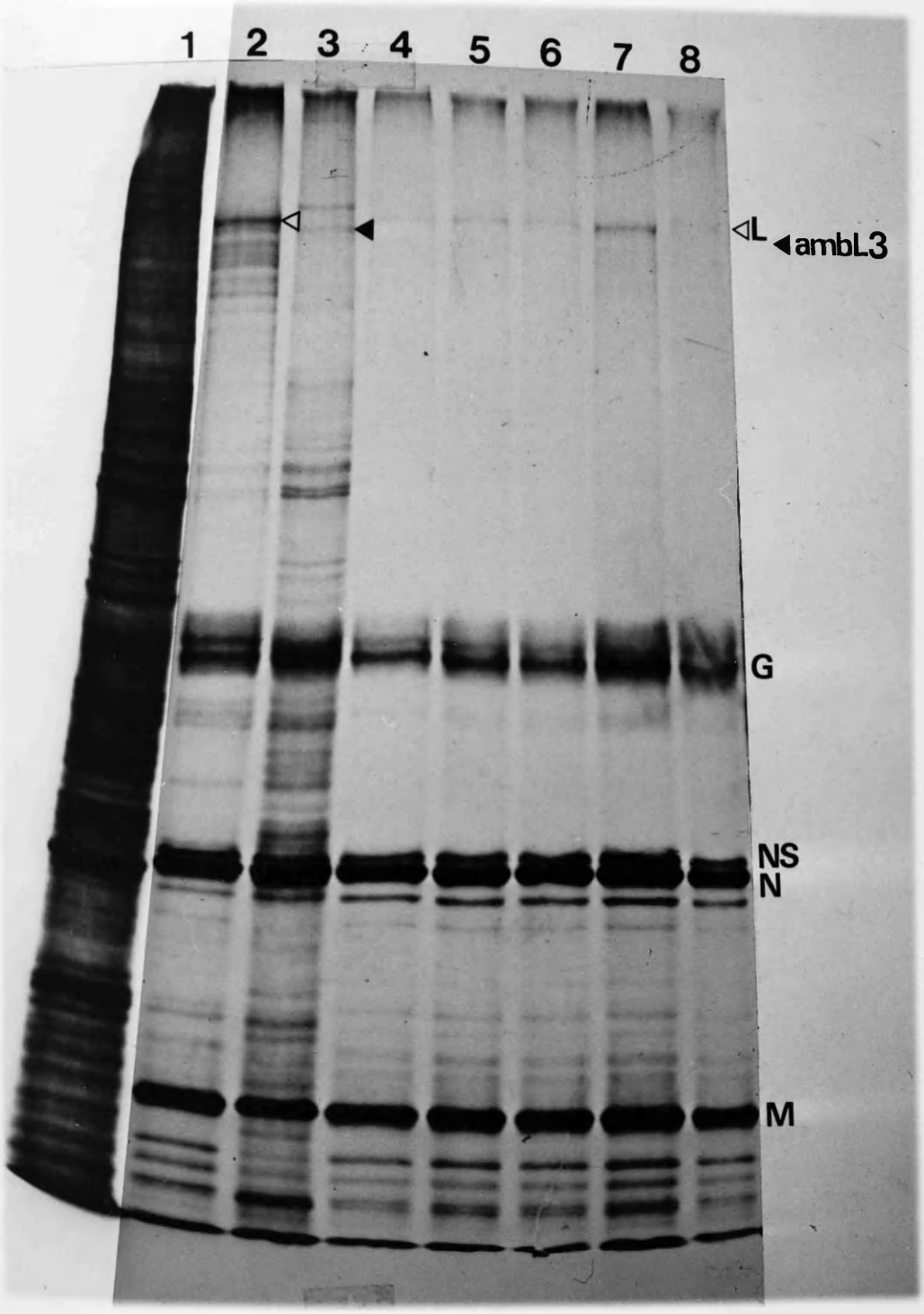

1
2
3
4
5
6
7
8
L
ambL2
G
NS
N
M

Figure 15

Intracellular polypeptides synthesized in su^+ cells by wild-type virus and revertants of AmbL3.

Su^+ cells were infected at a moi of 10 pfu/cell. Infected monolayers were incubated at 31°C and labelled from 7 to 8 h post infection. Cells were harvested and extracts prepared. The polypeptides were analysed by gel electrophoresis on a 7.5% polyacrylamide gel.

The polypeptides synthesized in [^{35}S]-methionine labelled su^+ cells are shown for mock infected (track 1), wt infected (track 2), AmbL3 infected (track 3) and five revertants of AmbL3 (tracks 4 to 8 respectively). The L protein fragment (ambL3) is indicated by a closed arrowhead. Full length L protein is indicated by an open arrowhead.


1
2
3
4
5
6
7
8
L
ambL3
G
NS
N
M

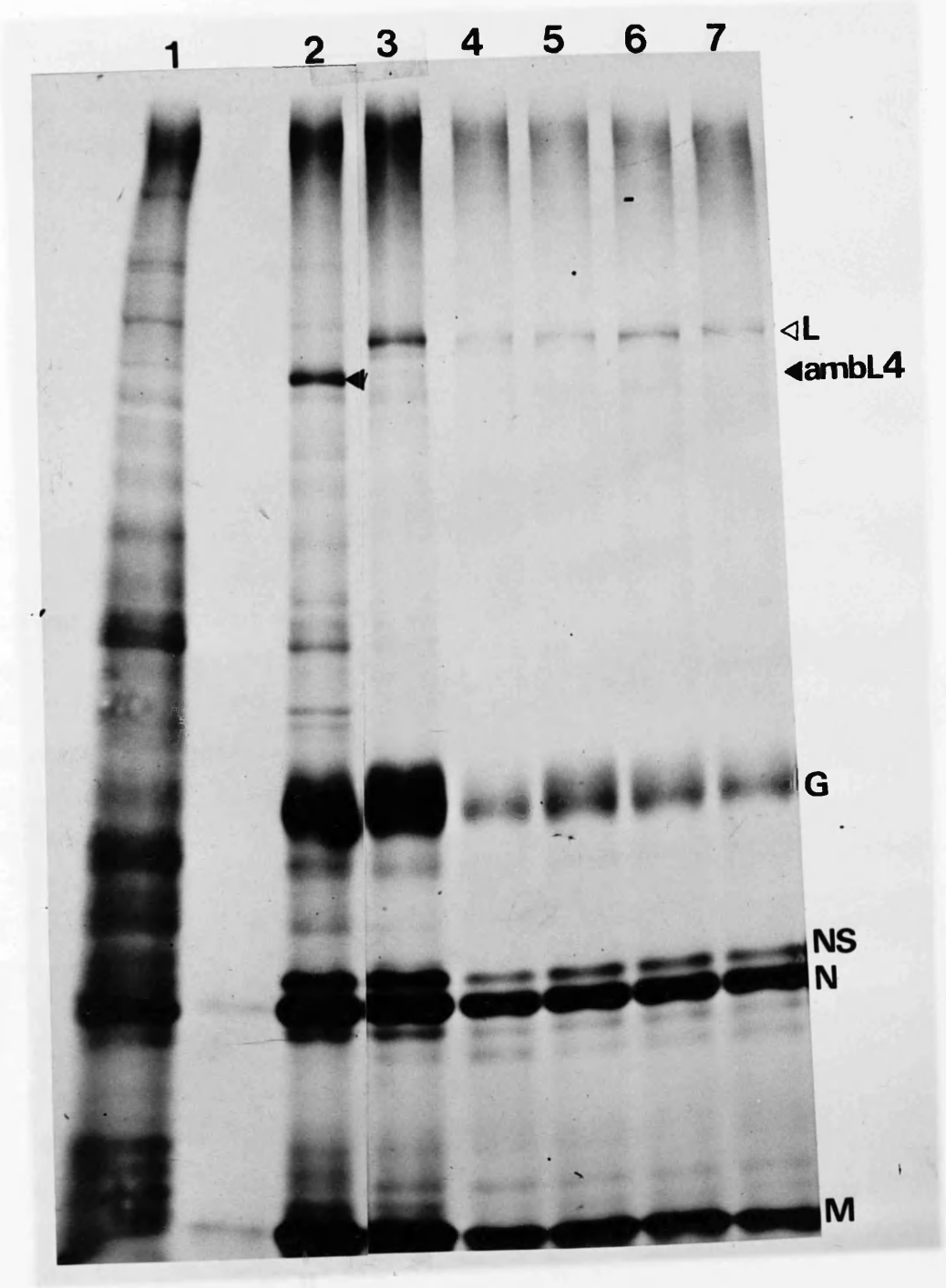

1
2
3
4
5
6
7
L
ambL4
G
NS
N
M

Figure 16

Intracellular polypeptides synthesized in su^+ cells by wild-type virus and revertants of AmbL4.

Su^+ cells were infected at a moi of 10 pfu/cell. Infected monolayers were incubated at 31°C and labelled from 7 to 8 h post infection. Cells were harvested and extracts prepared. The polypeptides were analysed by gel electrophoresis on a 7.5% polyacrylamide gel.

The polypeptides synthesized in [^{35}S]-methionine labelled su^+ cells are shown for mock infected (track 1), AmbL4 infected (track 2) and *wt* infected (track 3). The polypeptides synthesized in su^+ cells infected by four revertants of AmbL4 are shown in tracks 4 to 7 respectively. The L protein fragment (ambL4) is indicated by a closed arrowhead. Full length L protein is indicated by an open arowhead.

particular, the L protein band possessed both a normal mobility and a normal intensity. The characteristic novel polypeptide species observed after infection of $\underline{su}^+$ cells, and proposed to represent a truncated form of the L protein, were not present. This suggested that the revertants were all products of a back mutation in the same codon as the original mutation.

Comparison of the polypeptides synthesized in $\underline{su}^+$ cells by revertants of AmbG, $\underline{wt}$ virus and AmbG virus revealed that the putative revertants did not exhibit a $\underline{wt}$ protein profile (Figure 17). Instead, all synthesized a faster migrating glycoprotein similar in size to the faster migrating G protein synthesized by AmbG in the $\underline{su}^+$ cells. Thus, it appears that this so called 'revertant' virus does not represent true revertants of AmbG but instead the original AmbG inoculum. Evidence to support this interpretation was provided by titrating these 'revertants' on both the $\underline{su}^+$ and $\underline{su}^-$ cells. Whereas revertants of the four group I mutants grew to a similar titre in both cell types, 'revertants' of AmbG exhibited the same restricted host range as the mutant itself. It seems likely that true revertants of AmbG are present in the passage 1 stock, but that many more plaques need to be picked on the $\underline{su}^-$ cells to isolate any.

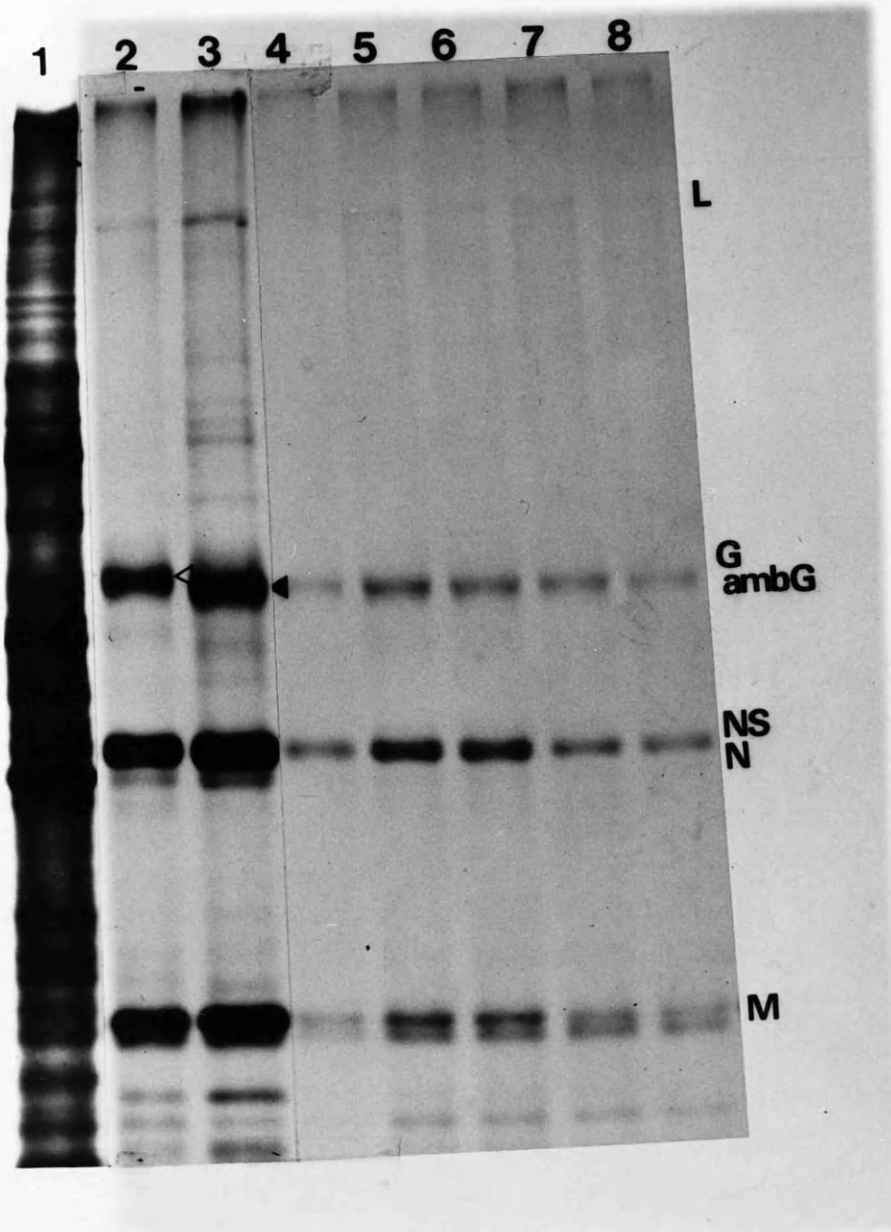
These results suggest that AmbG is a leaky mutant and will grow in the $\underline{su}^-$ cells, albeit at a reduced level when compared to growth in the $\underline{su}^+$ cells. This is consistent with the results obtained in the analysis of AmbG viral protein synthesis in the $\underline{su}^-$ cells. Here the mutant was observed to synthesize significant amounts of the putative truncated G protein in the $\underline{su}^-$ cells (section 4.(b).1.7),. In conclusion, it appears that although AmbG probably contains an amber mutation within the cytoplasmic domain of its G protein, the mutant can still produce viable virus, albeit at a reduced level, in cells lacking the suppressor tRNA needed to allow production of full length protein.

Figure 17

Intracellular polypeptides synthesized in su^+ cells by wild-type virus and revertants of AmbG.

Su^+ cells were infected at a moi of 10 pfu/cell. Infected monolayers were incubated at 31°C and labelled from 7 to 8 h post infection. The cells were harvested and extracts prepared. The polypeptides were analysed by gel electrophoresis on a 7.5% polyacrylamide gel.

The polypeptides synthesized in [^{35}S]-methionine labelled su^+ cells are shown for mock infected (track 1), wt infected (track 2), AmbG infected (track 3) and for five revertants of AmbG (tracks 4 to 8 respectively). Normal G protein is indicated by an open arrowhead. The faster migrating G protein (ambG) is indicated by a closed arrowhead.


1
2
3
4
5
6
7
8
L
G
ambG
NS
N
M

4.(b).4 ANALYSIS OF THE PROTEIN COMPOSITION OF THE VIRION OF WILD-TYPE VIRUS AND THE FIVE GROUP I MUTANTS

In order to determine whether the truncated versions of the L protein were packaged, the protein compositions of the virion of the group I mutants were analysed. *Su*$^+$ cells infected either with *wt* or one of the group I mutants were cultured in methionine-free medium containing [^{35}S]-methionine. The supernatant was removed and partially purified virions obtained by centrifugation through a glycerol cushion. Comparison of the virion protein composition of the five group I mutants with the *wt* result showed that none of the mutants packaged the truncated L protein (Figure 18). It was not possible to detect any full length L protein either hoewever, so it would be necessary to employ more sensitive methods before one could conclude that only full length L is packaged. Possibly further concentration of the virion preparation will allow detection of a readthrough product.

4.(b).5 IMMUNOPRECIPITATION STUDIES OF THE NOVEL POLYPEPTIDE SPECIES SYNTHESIZED BY THE GROUP I MUTANTS

It has been proposed from the analysis of the proteins synthesized in *su*$^+$ cells by the group I mutants, that the observed aberrant migration of the L polypeptide resulted from premature termination of translation at an amber stop codon within the L mRNA. Premature termination at a nonsense codon results in the synthesis of a fragment of the complete polypeptide chain which contains an intact N terminus but lacks the C terminal portion present in the normal protein. The presence and/or absence of these regions in the L protein specified by these mutants (proposed to be the novel polypeptide) was examined by immunoprecipitation analysis using monospecific antisera which were directed against the N and C terminus of the *wt* L protein.

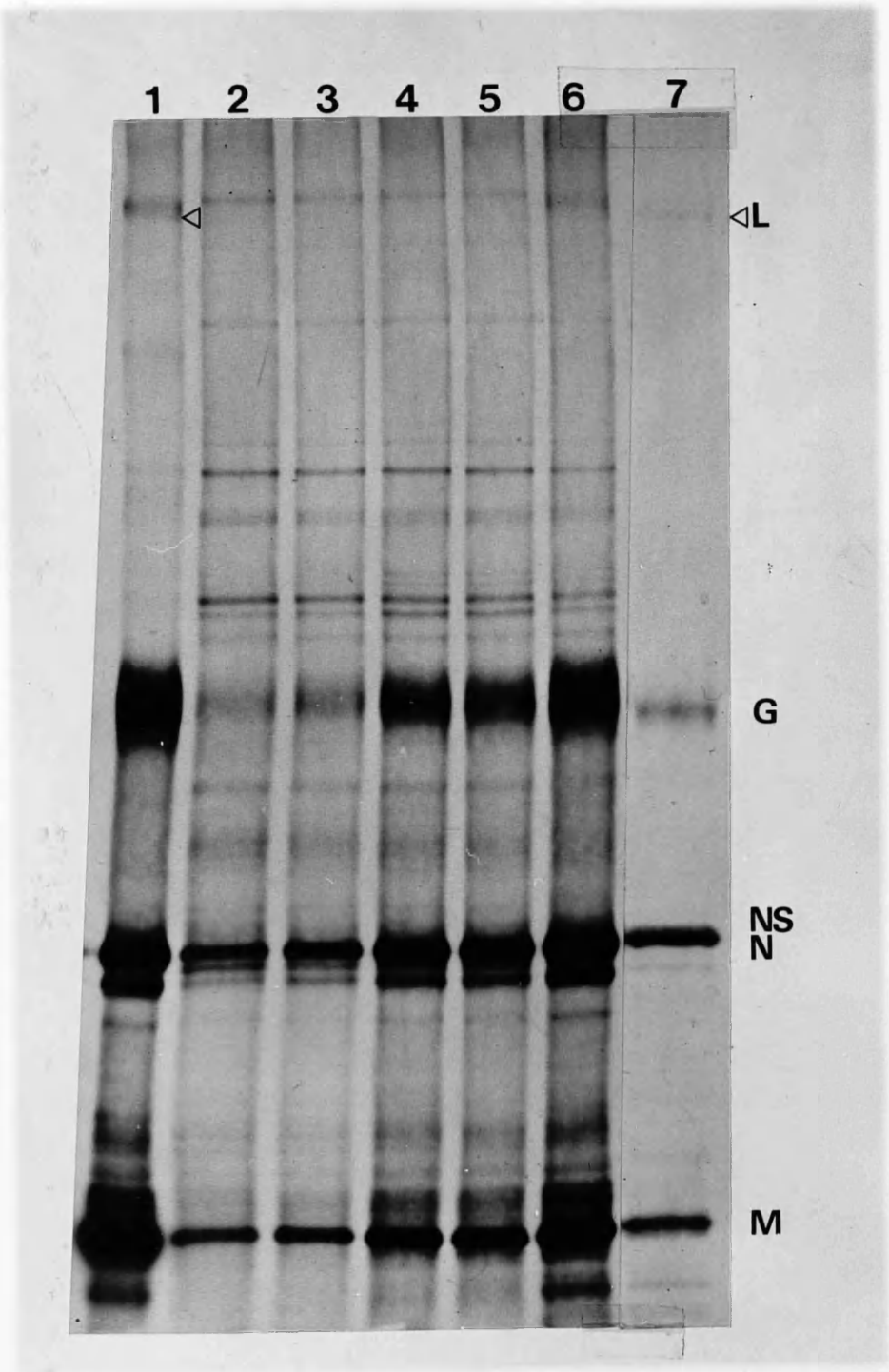
The monospecific antisera used in both this analysis and in the immunoblotting analysis were the gift of Manfred Schubert of the National Institute of Neurological diseases and Stroke, National Institutes of Health, Bethesda, USA.

Figure 18

Protein composition of the virion of the group I mutants

Radiolabelled virus was prepared by growing *su*$^+$ cells, infected at a moi of 0.02 pfu/cell by either *wt* virus or one of the group I mutants, in methionine-free medium containing [^{35}S]-methionine. Labelled virus in the culture medium was partially purified by centrifugation through a glycerol cushion.

The [^{35}S]-methionine labelled proteins present in the virion of wild-type virus is shown in track 1. The [^{35}S]-methionine labelled proteins present in the virion of the five group I mutants (AmbL1 to AmbL5 respectively) are shown in tracks 2 to 6 respectively. As a marker, the [^{35}S]-methionine labelled proteins synthesized by *wt* virus in *su*$^+$ cells are shown in track 7. Full-length L protein is indicated by an open arrowhead.


1
2
3
4
5
6
7
L
G
NS
N
M

Schubert et al. (1985) described the synthesis of these antisera in rabbits by injection with oligopeptides which represented the sequences from the exact amino and carboxy termini of the wt VSV Indiana L protein.

Su$^+$ cells were infected with wt virus or with one of the group I mutants. At 24 h post infection the cells were labelled with [^{35}S]-methionine for 1 h and cell extracts prepared. Antibodies directed against the N and C termini of the L protein were added and the immunecomplexes isolated by binding to protein-A-Sepharose beads.

As shown in Figure 19, both antibodies specifically precipitated the L protein from cells infected with wt virus. Non-specific precipitation of other viral proteins was also observed, in particular the N and M proteins. However, subsequent analysis has shown that this non-specific binding was independent of the antibody or the cell extract used.

These antisera were then used to study the novel polypeptides produced by the group I mutants in the su$^+$ cells. With cell extracts prepared from su$^+$ cells infected with AmbL1, AmbL2, AmbL3 and AmbL4 the N terminal specific antiserum precipitated the novel polypeptide whereas the C terminal specific antiserum failed to do so (Figure 20a, b, c, and d respectively). Some non-specific binding of the G, NS and M proteins was again observed.

Immunoprecipitation of AmbL5 infected cell extract revealed a polypeptide species, where none had been observed before (see section 4.(b).1.6), of estimated M_r 187000 which was specifically precipitated only by the N-terminal specific antiserum and not by the C-terminal specific antiserum (Figure 20e).

These results are consistent with my proposal that the novel polypeptide species synthesized by these mutants in su$^+$ infected cells is a result of premature termination within the L mRNA at an amber codon.

Although the results obtained by immunoprecipitation analysis are reasonably convincing, it should be noted that it was difficult to get sufficient label into the L protein fragment. This resulted in the relatively weak

Figure 19

Immunoprecipitation of protein extracts from su^+ cells infected by wt virus with monospecific antisera directed against the N or C termini of the VSV L protein.

Su^+ cells were infected at a moi of 10 pfu/cell. Infected monolayers were incubated at 31°C and labelled from 23 to 24 h post infection. Cell extracts were prepared and reacted with either the N-terminal specific antisera or the C-terminal specific antisera. Immunecomplexes were isolated by binding to protein-A-Sepharose beads. The precipitated proteins were analysed on a 7.5% polyacrylamide gel.

The polypeptides synthesized in su^+ cells infected by wt virus are shown in track 1. Tracks 2 and 3 show the polypeptides precipitated from the wt extract by the N-terminal and C-terminal specific antisera respectively. L protein is indicated by an open arrowhead.

extracts from mock-infected cells gave rise to no specific bands after immunoprecipitation with either the N or C-terminal specific antisera.

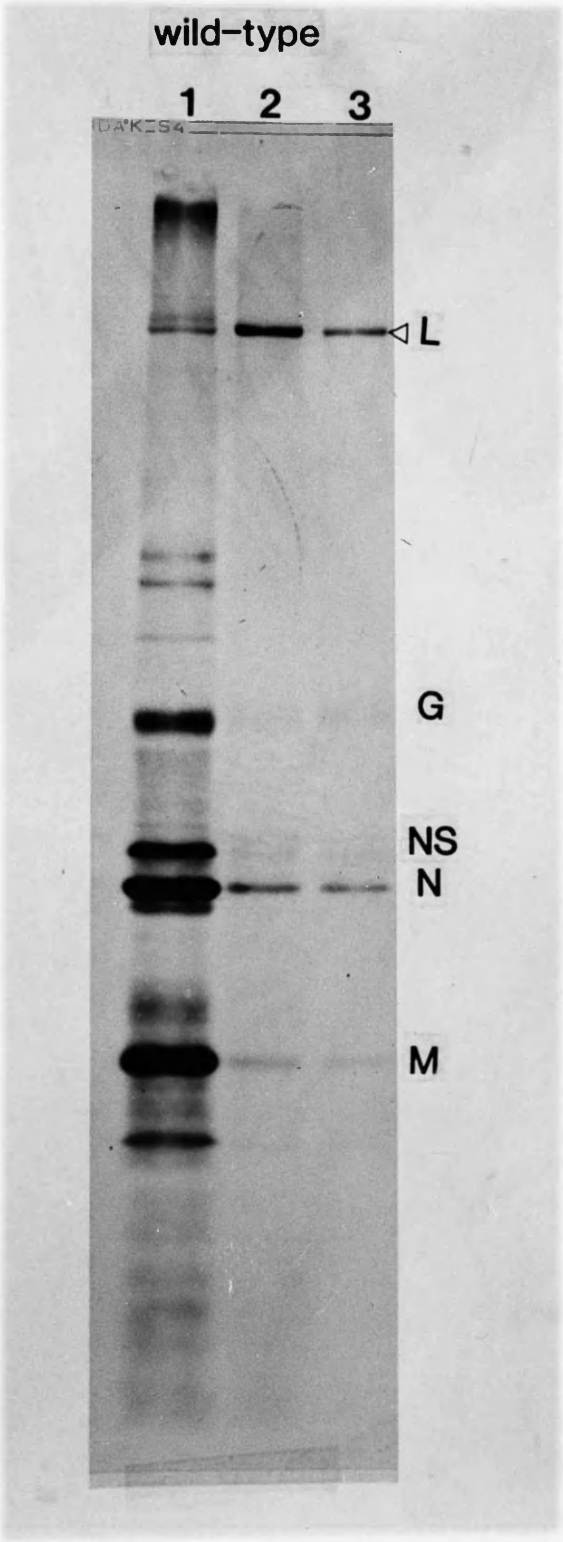

wild-type
1
2
3
L
G
NS
N
M

Figure 20

Immunoprecipitation of protein extracts from su^+ cells infected by the mutants AmbL1, AmbL2 and AmbL3 with monospecific antisera directed against the N or C-termini of the L protein.

Su^+ cells were infected at a moi of 10 pfu/cell. Infected monolayers were incubated at 31°C and labelled from 23 to 24 h post infection. Cell extracts were prepared and reacted with either the N or C-terminal specific antiserum. Immunecomplexes were isolated by binding to protein-A-Sepharose beads and the precipitated proteins analysed on a 7.5% polyacrylamide gel.

(a) ind/ambL1

The polypeptides synthesized in [^{35}S]-methionine labelled su^+ cells infected by AmbL1 are shown in track 1. Tracks 2 and 3 show the polypeptides precipitated from the AmbL1 infected cells extract by the N-terminal and C-terminal specific antisera respectively. The L protein fragment (ambL1) is indicated by a closed arrowhead.

(b) ind/ambL2

The polypeptides synthesized in [^{35}S]-methionine labelled su^+ cells infected by AmbL2 are shown in track 1. Tracks 2 and 3 show the polypeptides precipitated from the AmbL2 infected cell extract by the N-terminal and C-terminal specific antisera respectively. The L protein fragment (ambL2) is indicated by a closed arrowhead.

(c) ind/ambL3

The polypeptides synthesized in [^{35}S]-methionine labelled su^+ cells infected by wt virus and AmbL3 are shown in tracks 1 and 2 respectively. Tracks 3 and 4 show the polypeptides precipitated from the AmbL3 extract by the N-terminal and C-terminal specific antisera respectively. The L protein fragment (ambL3) is indicated by a closed arrowhead. Full length L protein is indicated by an open arrowhead.

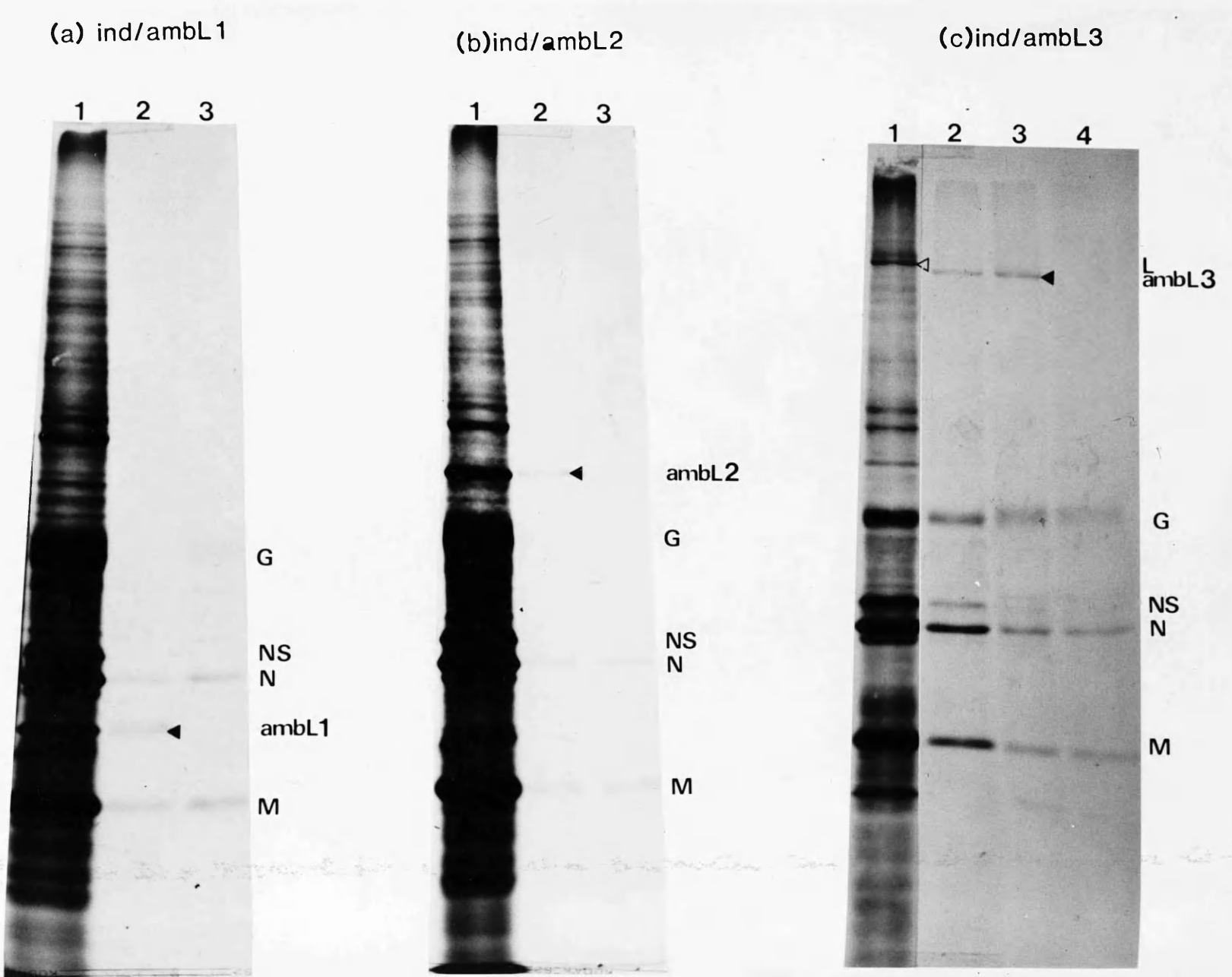

(a) ind/ambL1
1
2
3
G
NS
N
ambL1
M
(b)ind/ambL2
1
2
3
ambL2
G
NS
N
M
(c)ind/ambL3
1
2
3
4
L
ambL3
G
NS
N
M

Figure 20 (continued)

Immunoprecipitation of protein extracts from su^+ cells infected by the mutants ind/ambL4 and ind/ambL5 with monospecific antisera directed against the N or C-termini of the L protein.

Su^+ cells were infected at a moi of 10 pfu/cell. Infected monolayers were incubated at 31°C and labelled from 23 to 24 h post infection. The cells were harvested, extracts prepared and reacted with either the N or C-terminal specific antiserum. Immunecomplexes were isolated by binding to protein-A-Sepharose beads and the precipitated proteins analysed on a 7.5% polyacrylamide gel.

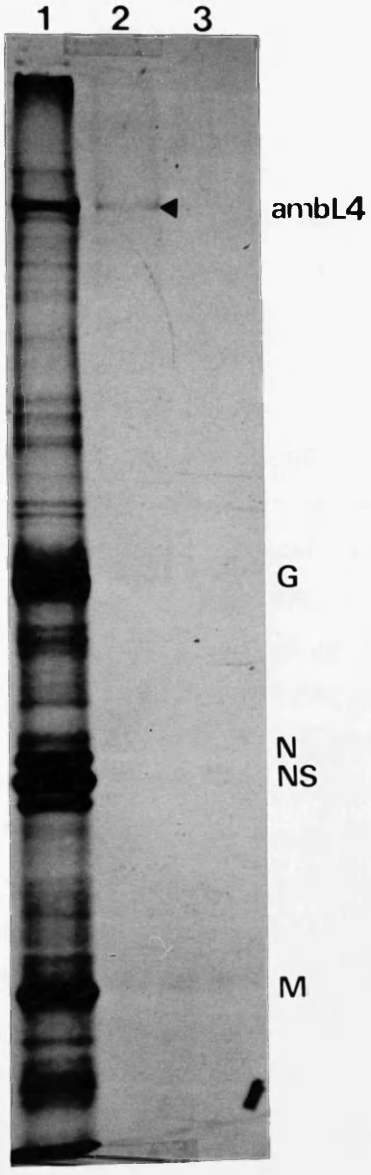
(d) ind/ambL4

The polypeptides synthesized in [^{35}S]-methionine labelled su^+ cells infected by AmbL4 are shown in track 1. Tracks 2 and 3 show the polypeptides precipitated from the AmbL4 extract by the N-terminal and C-terminal specific antisera respectively. The L protein fragment (ambL4) is indicated by a closed arrowhead.

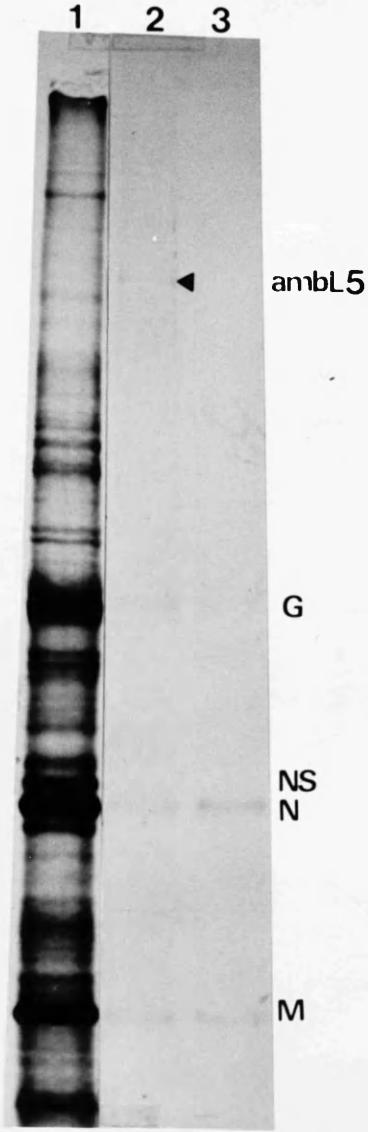
(e) ind/ambL5

The polypeptides synthesized in [^{35}S]-methionine labelled su^+ cells infected by AmbL5 are shown in track 1. Tracks 2 and 3 show the polypeptides precipitated from the AmbL5 extract by the N-terminal and C-terminal specific antisera respectively. The L protein fragment (ambL5) is indicated by a closed arrowhead.

(d) ind/ambL4



(e) ind/ambL5



precipitation of the L protein fragment with the N-terminal specific antisera in comparison to the wt result.

4.(b).6 WESTERN BLOT ANALYSIS OF THE TRUNCATED VERSION OF L PROTEIN SYNTHESIZED BY THE GROUP I MUTANTS

In order to confirm the results obtained by immunoprecipitation analysis, [^{35}S]-methionine labelled extracts of su^+ cells infected with each of the group I mutants were electrophoretically transferred from an SDS-polyacrylamide gel to nitrocellulose (Figure 21). Proteins which contained L protein N or C specific sequences were detected by autoradiography after reaction with the relevant antibody and iodinated protein-A (Figure 22). Firstly, the L protein synthesized by wt virus was shown to contain N and C specific sequences. The shortened version of the L protein produced by AmbL1, AmbL2, AmbL3 and AmbL4 were then shown to contain N but not C specific sequences. With respect to the AmbL3 result, although the original autoradiograph does show the presence of a band representing the AmbL3 protein fragment, the processes of duplication and photography have almost eliminated it.

These results provide further proof that the novel polypeptide species synthesized by the group I mutants AmbL1 to AmbL4 represent a fragment of the full length protein, produced by premature termination of translation.

The putative truncation product synthesized by AmbL5 and detected only by immunoprecipitation analysis could not be identified. However, inspection of the AmbL5 result showed that an additional protein, present in AmbL5 infected cells, is bound strongly by the N-terminal specific antiserum only. Thus, it is even conceivable that there are two versions of the L protein synthesized in AmbL5 infected cells, one which has an M_r of about 187000 detected by immunoprecipitation and another with an estimated M_r of about 48000 detectable by immunoblotting. It is probably significant that AmbL5 appears to be a double mutant since it is not known to revert. However, a corresponding band is present in each of the remaining tracks (if at a reduced level) which appears to represent non-specific binding to

Figure 21

Immunoblotting analysis of the polypeptides synthesized in su$^+$ cells by wild-type virus and the five group I mutants with monospecific antisera directed against the N and C-termini of the L protein.

Su$^+$ cells were infected at a moi of 10 pfu/cell. [^{35}S]-methionine labelled cell extracts were prepared and fractionated by gel electrophoresis. The separated proteins were transferred to nitrocellulose sheets by electrophoresis. The blotted proteins were then reacted with either the N or C-terminal specific antiserum. Immunecomplexes were detected by binding to iodinated protein-A.

The polypeptides synthesized in [^{35}S]-methionine labelled su$^+$ cells, after transfer to nitrocellulose sheets are shown for AmbL1, AmbL2, AmbL3, AmbL5, AmbL4 and wt virus (tracks 1 to 6 respectively) in Figure 21(a) and 21(b).

Wt L protein is indicated by an open arrowhead. The shortened versions of L (named ambL1 to ambL4) are indicated by a closed arrowhead.

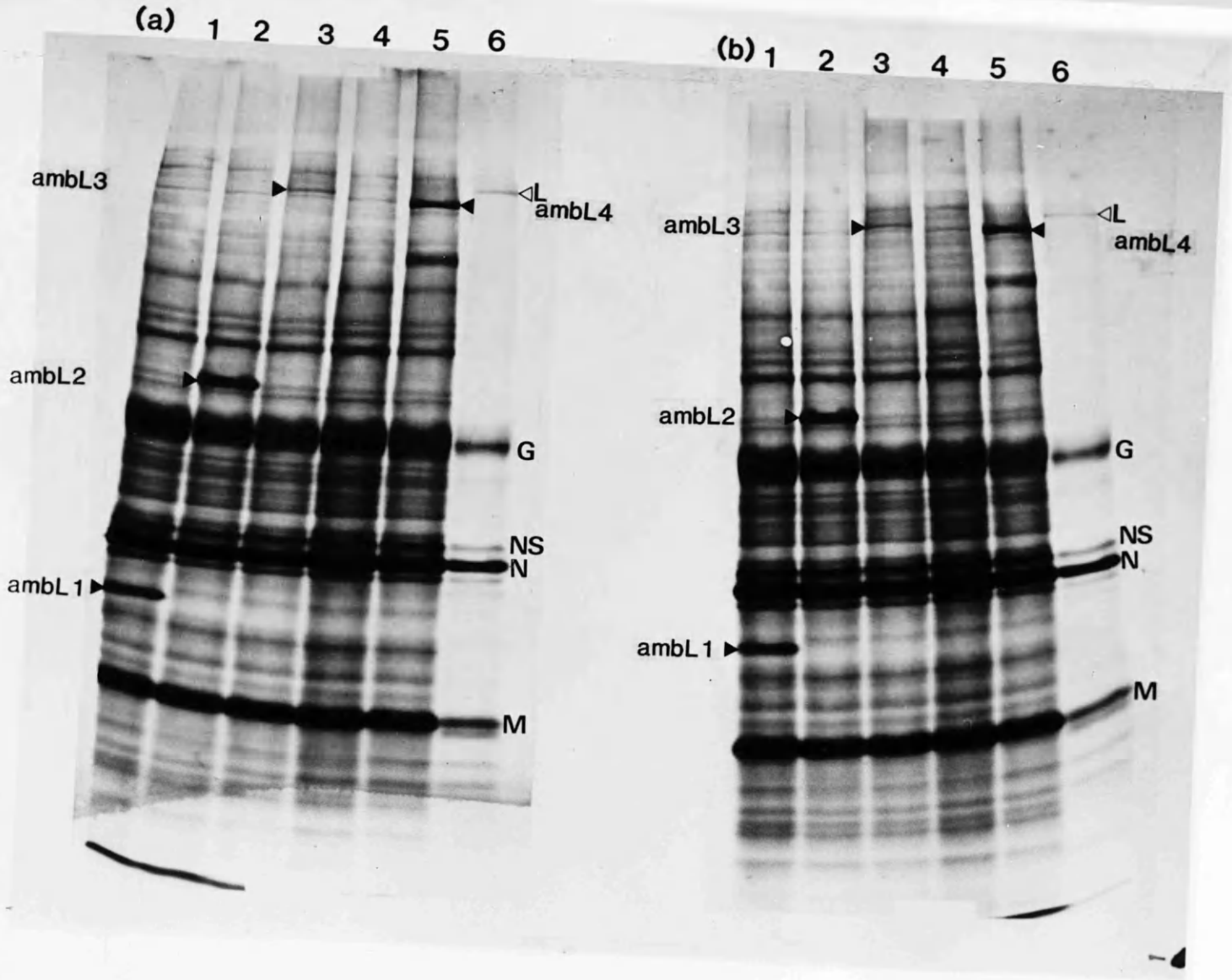
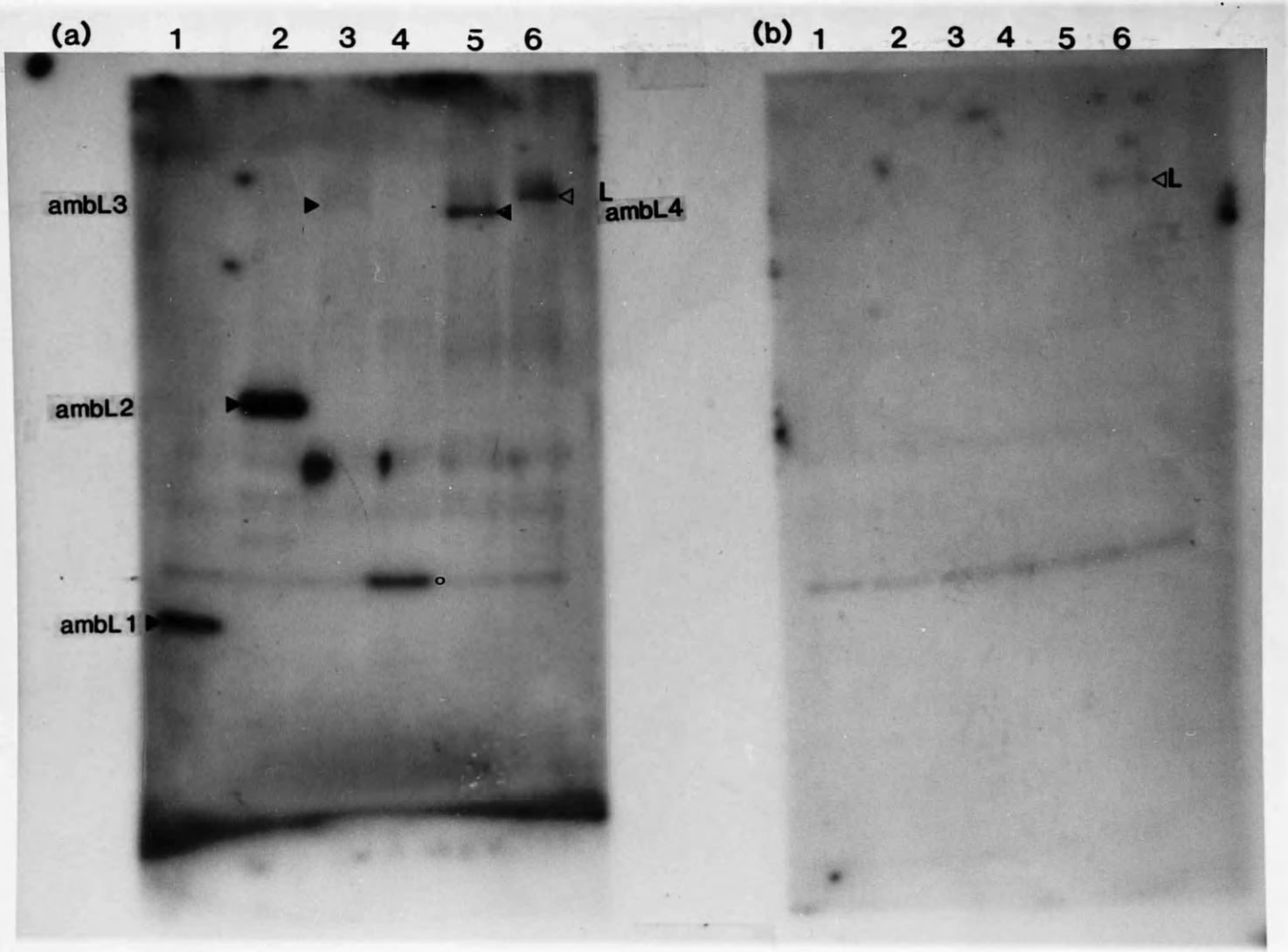

(a)
1
2
3
4
5
6
ambL3
ambL4
L
ambL2
G
NS
N
ambL1
M
(b)
1
2
3
4
5
6
ambL3
L
ambL4
ambL2
G
NS
N
ambL1
M

Figure 22

Immunoblotting analysis of the polypeptides synthesized in su^+ cells by wt virus and the five group I mutants with the monospecific antisera directed against the N and C-termini of the L protein.

Su^+ cells were infected at a moi of 10 pfu/cell. [^{35}S]-methionine labelled infected cell extracts from wt and the group I mutants were blotted onto nitrocellulose sheets (Figure 21). The blotted proteins were then reacted with either the N (Figure 22a) or C-terminal (Figure 22b) specific antiserum. Immunecomplexes were detected by binding to iodinated protein-A. The ^{125}I-labelled proteins are shown for AmbL1, AmbL2, AmbL3, AmbL5, AmbL4 and wt (tracks 1 to 6 respectively) in Figure 22(a) and 22(b). Wt L protein is indicated by an open arrowhead. The shortened versions of the L protein (named ambL1 to ambL4) are indicated by a closed arrowhead. A possible candidate for an AmbL5 truncation protein is indicated by a (∘).


(a)
1
2
3
4
5
6
ambL3
ambL2
ambL1
L
ambL4
(b)
1
2
3
4
5
6
L

the cellular actin protein and, because of this, any conclusions drawn based on the specificity of the N-terminal antisera to this 45000 dalton protein in AmbL5 infected cell extracts, must remain speculative.

In summary, a schematic diagram has been constructed showing the proposed approximate positions for each of the group I mutations within the L gene (Figure 23).

4.(b).7 ISOLATION AND IN VITRO TRANSLATION OF RNA FROM WILD-TYPE AND MUTANT INFECTED CELLS

With a view to precisely mapping the position of the mutations and as final proof of their nature, it would be of interest to determine the nucleic acid sequence of the RNA segment containing the mutation.

As a preparatory step RNA was isolated from su^+ cells infected either by wt virus or one of the mutants. Su^+ cells were infected by 10 pfu/cell of virus and the cells harvested 8 h post infection. RNA was isolated using the guanidinium isothiocyanate method. RNA isolated from infected cells was used to prime an *in vitro* translation system. The [^{35}S]-methionine labelled proteins were then fractionated by polyacrylamide gel electrophoresis and detected by autoradiography.

In vitro translation of wt, AmbL1 and AmbL2 RNA is shown in Figures 24a and 24b respectively. *In vitro* translation of wt RNA showed that the G, NS, N and M proteins were translated but not the L protein. The same pattern of proteins was seen after *in vitro* translation of AmbL3, AmbL4 and AmbL5 RNA. The inability to translate L message is well documented (Preston and Szilagyi, 1977), and it is thought to be the result of RNase degradation. L RNA would be more sensitive than the RNAs encoding the G, NS, N and M proteins because of its considerably greater length. *In vitro* translation of the RNA isolated from AmbL1 and AmbL2 infected cells showed that, in addition to G, NS, N and M proteins, the additional polypeptide species synthesized by these mutants were also present.

In vitro translation of RNA isolated from cells infected with wt and AmbG viruses is shown in Figure 24c.

Figure 23

A schematic diagram of the approximate position of the amber mutations within the L mRNA of the group I mutants.

The approximate positions of the amber mutations within the L mRNA of the group I mutants were calculated using the VSV proteins as known molecular weight markers on SDS-polyacrylamide gels. The position of each mutation in the L mRNA is shown by an arrow.

In the case of the mutant AmbL5, the solid arrow shows the position of a mutation that would give rise to the truncated protein which was detected by immunoprecipitation analysis. The broken arrow shows the position of a mutation that would give rise to the truncated protein which was detected by immunoblotting analysis (Figure 22a).

The coding region is shown by an open box.

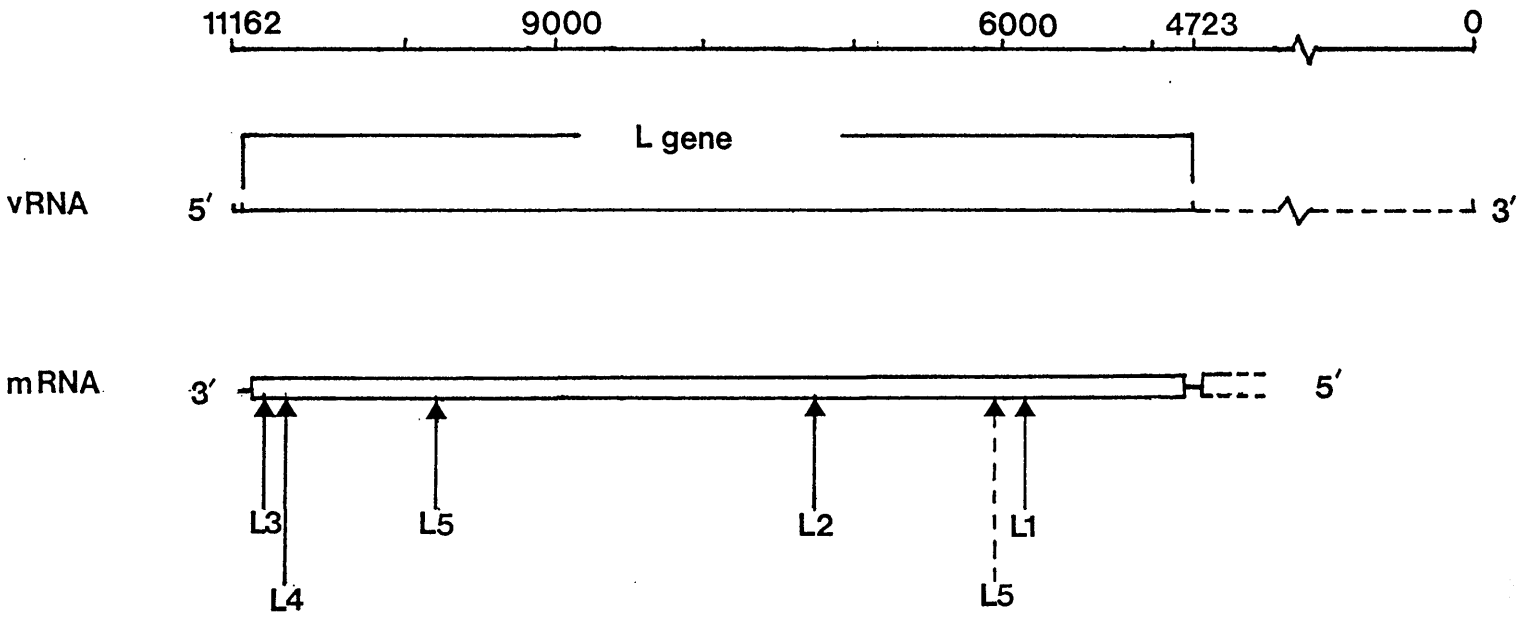

11162
9000
6000
4723
0
L gene
vRNA
5′
3′
mRNA
3′
5′
L3
L4
L5
L2
L5
L1

Figure 24

In vitro translation of RNA isolated from su^+ cells infected by the group I mutants AmbL1 and AmbL2 and the group V mutant AmbG.

Su^+ cells were infected at a moi of 10 pfu/cell. Infected monolayers were incubated for 8 h at 31°C. The cells were harvested and RNA isolated using the guanidinium isothiocyanate method. RNA was used to prime a reticulocyte lysate *in vitro* translation system. The proteins were analysed on a 7.5% polyacrylamide gel.

(a) in vitro translation of RNA isolated from AmbL1 infected cells.

The polypeptides synthesized in [^{35}S]-methionine labelled su^+ cells are shown for mock infected cells (track 1) and *wt* infected cells (track 2). The [^{35}S]-methionine labelled polypeptides synthesized *in vitro* using RNA isolated from AmbL1 infected su^+ cells are shown in track 3. The L protein fragment (ambL1) is indicated by a closed arrowhead.

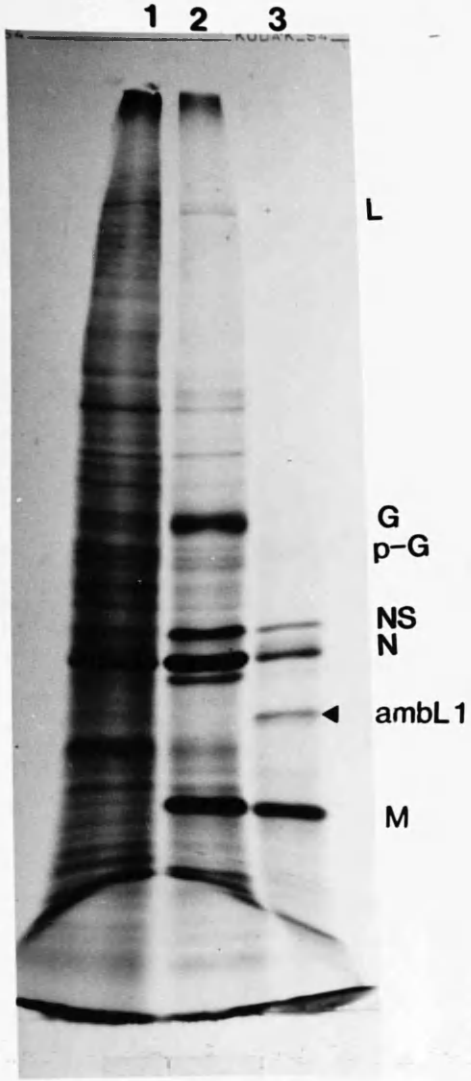
(b) in vitro translation of RNA isolated from AmbL2 infected cells.

The polypeptides synthesized in [35S]-methionine labelled su^+ cells are shown for mock infected cells (track 1) and AmbL2 infected cells (track 2). The [^{35}S]-methionine labelled polypeptides synthesized *in vitro* using RNA isolated from AmbL2 infected su^+ cells are shown in track 3. The L protein fragment (ambL2) is indicated by a closed arrowhead.

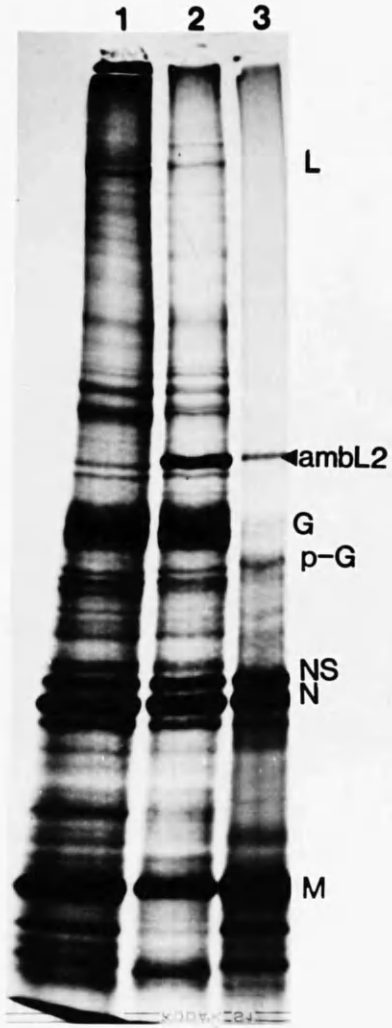
(c) in vitro translation of RNA isolated from AmbG infected cells.

The polypeptides synthesized in [^{35}S]-methionine labelled su^+ cells are shown for mock infected cells (track 1) and *wt* infected cells (track 2). The [^{35}S]-methionine labelled polypeptides synthesized *in vitro* using RNA isolated from AmbL2 infected cells are shown in track 3, from AmbG infected cells in track 4 and from *wt* infected cells in track 5. The faster migrating pre-G (named ambp-G) is indicated by a closed arrowhead.

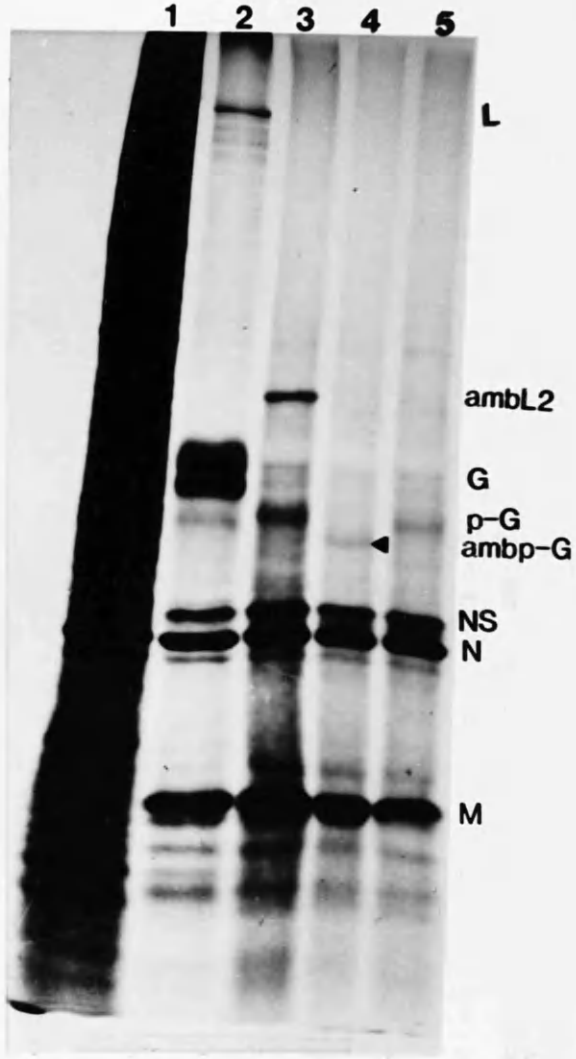
(a)ind/ambL1



(b)ind/ambL2



(c)ind/ambG



The M_r of the pre-G protein synthesized by wt and AmbG viruses could be estimated (by comparing their mobility to the known Mr of other VSV proteins, in this case the G and NS proteins) as approximately 71000 and 70000 respectively. This is consistent with the results obtained in the analysis of the viral proteins synthesized in su^+ cells infected with either AmbG or wt virus (section 4.(b).1.7) and confirms that the difference in migration between the two G polypeptides is not simply the result of a difference in glycosylation.

4.(b).8 THE EFFECT OF THE AMINOGLYCOSIDE G418 ON THE MUTANT AMBL1

Aminoglycosides have been shown to suppress nonsense mutations in bacteria (Gorini, 1974), yeast (Singh et al., 1979; Palmer et al., 1979), and A. nidulans (Roberts et al., 1979; Martinelli and Roberts, 1983) and have been shown to facilitate suppression by affecting the fidelity of translation (Davies et al., 1965; Palmer and Wilhelm, 1978; Wilhelm et al., 1978a, 1978b).

Burke and Mogg (1985) reported the suppression of an amber mutation in the chloramphenicol acetyl transferase gene of the mammalian cell transfection vector $pRSVCAT^{amb38}$. Readthrough of the nonsense mutation was assayed by the appearance of active enzyme. The optimum concentration required to facilitate suppression was found to be 50ug/ml. The efficiency of suppression by G418 was estimated to be approximately 19.6% (Burke and Mogg, 1985). If the proposed amber mutation in the L gene of AmbL1 was suppressed at such an efficiency, one might expect that AmbL1 grown in the presence of the optimum concentration of G418 would grow to a higher titre than it might normally do on su- cells. In addition, it would be of interest to see if analysis of the proteins synthesized by AmbL1 in su^- cells with G418 present revealed any viral protein synthesis which might be associated with suppression of the amber mutation. Accordingly, two experiments were carried out: (1) su^- cells in Linbro wells were infected with either wt or AmbL1 in the presence of 0, 25, 50, 100 and 200 ug/ml of G418.

8 h post infection the virus was harvested and titrated on su^+ cells for growth of the mutant. (2) monolayers of su^- cells in Linbro wells were infected with wt or AmbL1 and with either 0, 25, 50, 100 or 200 ug/ml of G418 present. 8 h post infection the intracellular proteins were labelled with [^{35}S]-methionine for 1 h, then extracted into protein dissociation mix and fractionated by polyacrylamide gel electrophoresis.

In both cases there was no evidence of suppression of the amber mutation. In fact, the yield of virus decreased with increasing concentration of G418 and the presence of G418 had no appreciable effect on the proteins synthesized by AmbL1 in infected su^- cells.

In conclusion, it is likely that the deleterious effect that G418 had on growth of the virus negated any possible effects of readthrough of the amber codon and, as such, this failure should not conflict with the previous interpretation that AmbL1 is an amber mutant.

DISCUSSION

CHAPTER 5 DISCUSSION

5.(a) DISCUSSION

The object of this study has been to investigate the use of suppressor cell lines in the isolation of nonsense mutations within an animal virus genome. The suppressor cell line used contained a *X. laevis* tyrosine transfer RNA gene altered by site specific mutagenesis within the anticodon to read amber codons. The suppression level of this suppressor cell line was estimated to be about 4% (Young *et al.*, 1983).

The use of vesicular stomatitis virus as a model has clearly contributed to the success of this work, in the following ways: VSV has a very broad host range and the virus readily grows to high titre on a large number of cell lines. (The only documented mammalian cell types in which VSV will not grow include some types of human lymphoblastoid cells, rabbit kidney (DRK3) cells and rabbit corneal (RC60) cells). Thus there is a greater possibility that virus containing a nonsense mutation may, in spite of the low level of suppression exhibited by the suppressor cells used in this study, still produce enough virus to permit analysis. In addition, VSV has been reasonably well characterized genetically and *ts* mutants representing each of the VSV complementation groups are readily available. Finally, determination of the nucleotide sequence of the mRNAs of VSV Indiana has shown that the five natural translation terminators are either ochre (UAA) or opal (UGA) codons (Gallione *et al.*, 1981; Rose and Gallione, 1981; Schubert *et al.*, 1984), thus the presence of an amber suppressor tRNA should not adversely affect natural termination by suppressing the natural stop codons.

A stock of *wt* VSV Indiana was mutagenized by the chemical mutagen 5-fluorouracil, which favours transition mutations. This had been shown by the work of Pringle (1970) to be a potent mutagen of VSV. However, there were serious limitations to this strategy of mutagenesis. Ideally, to ensure that an active gene product is restored after suppression of the mutation, the amino acid inserted

at the point of mutation by the suppressor tRNA should be identical to the amino acid present in the wt protein. To mutate a tyrosine codon (UAU and UAC) to an amber stop codon (UAG) requires a transversion mutagen. However, as far as I am aware, no applicable transversion mutagen is available. Thus suppression by the tyrosine inserting suppressor tRNA present in the L39 cell line will not result in replacement of the wt amino acid at any nonsense codon produced by 5-FU. However, the production of amber codons by mutation from other, non-tyrosine specifying codons should be increased. In addition, it has been established that the frequency of spontaneous mutations in laboratory stocks of vesiculoviruses is high suggesting an inherent genetic instability. Thus, there is the additional possibility that the mutants described in this study arose spontaneously.

Because the L39 cell line was observed to suppress amber nonsense mutations at a low level, it might be expected that virus containing a nonsense mutation would not grow as well as wt virus in su^+ cells. Thus the potential phenotypes of prospective nonsense mutants were limited by selecting only small plaque variants to screen for a mutation in host range. Whether or not this selection strategy excludes any class of viable mutant in practice is not known although it does seem unlikely.

Thirteen suppressible host range mutants were isolated using this method, of which seven have been partially characterized. Five were assigned by complementation analysis using ts mutants, representing the five VSV complementation groups, to complementation group I of VSV, representing the L gene. Another mutant was tentatively assigned to complementation group V, the G gene, while the remaining host range mutant analysed ($hrTK^-7$) could not be assigned to a specific VSV gene.

5.(b) CHARACTERIZATION OF THE GROUP I MUTANTS

Since the L gene comprises 2109 codons out of a total of 3536 codons representing the five genes (Gallione et al., 1981; Rose and Gallione, 1981; Schubert et al., 1984), this gene presents by far the largest target for mutagenesis.

This is borne out by the observation that the majority of ts mutants isolated, whether spontaneous or induced, belonged to the L gene (Pringle, 1975). The results obtained in this study, where five out of the 7 mutants isolated were assigned to the L gene, are in accord with this observation.

The enzymatic role of the L protein in VSV infected cells may also be a factor in the isolation of mutants defective in this function. It has been shown that L is required in catalytic amounts during transcription (Schubert et al., 1985) as opposed to NS which is required in stoichiometric amounts during transcription (Banerjee, 1987). It may therefore be the case that the catalytic/enzymatic role of the L protein puts it in a more favourable class than structural proteins for recovery of a conditional lethal mutant.

The observed host range phenotype exhibited by these mutants, where the mutants have been shown to grow on cells containing a suppressor tRNA but not on non-suppressor parental cells, suggests that these mutants are conditional lethal mutants. One presumes that this host range restriction is caused by the mutation of an amino acid specifying codon to an amber stop codon within the L gene of the group I mutants and the G gene of the group V mutant. These are thus the first conditional lethal nonsense mutants described for an animal virus.

Characterization of the group I mutants has provided supportive evidence that these are indeed suppressible nonsense mutants. Firstly, none of the group I mutants (with the possible exception of AmbL1) synthesizes detectable amounts of full length L protein in either the su^+ or su^- cells. In prokaryotic and yeast systems, where the suppression level can be as high as 60%, both the suppressed and the truncated form of the affected protein are detectable in suppressor strains. The level of suppression in the suppressor cells used here is such that the failure to detect a readthrough product, by suppressing the nonsense codon in the group I mutants, was not unexpected. In addition, the fact that L protein is the least abundant viral protein in infected cells further

reduces the possibility of detecting a readthrough product. Secondly, each mutant directs the synthesis of a characteristic novel polypeptide species after infection of su^+ cells. Thirdly, this additional polypeptide species has been shown by immunoprecipitation and western blot analysis, using antisera directed against the N and C termini of the VSV L protein, to contain N but not C-terminal specific sequences. This novel polypeptide species thus represents a fragment of the full length polypeptide chain produced by premature termination at an amber codon. Finally, revertants of four of the group I mutants (AmbL1 to AmbL4) all synthesized full length L but not the novel truncated polypeptide species. This suggests that the revertants represent back mutations in the same codon as the original mutation.

Prior to the advent of nucleic acid sequencing any virus that could satisfy the above criteria would certainly be considered to be a suppressible amber mutant. However, I recognize that the use of sequence analysis would provide definitive proof of the nature of these mutants.

In vitro translation of RNA isolated from su^+ cells infected by the group I mutants AmbL1 and AmbL2 resulted in the synthesis of the novel polypeptide species as well as the G, NS, N and M proteins. The failure to detect either full length or truncated L protein after in vitro translation of the RNA from *wt* or the remaining group I mutants respectively was in accord with expectation. A previous study by Preston and Szilagyi (1977) also reported a failure to detect L protein after *in vitro* translation of VSV mRNA. A possible explanation for this could be provided by the action of RNases. The L mRNA would be more sensitive to RNases than the other VSV mRNAs since it is almost four times longer than the next longest RNA, encoded by the G gene.

Revertants of AmbL5 could not be isolated on su^- cells, suggesting that this may be a double mutant. This might also explain why the virus could be amplified only to 10^5 pfu/ml when the other mutants (apart from $hrTK^-7$) grew to 10^6 to 10^7 pfu/ml on the su^+ cells. Since the

restriction in host range can be complemented by the ts mutants representing the G, NS, N and M genes but not by ts 11 representing the L gene, the mutational lesion(s) must reside within this complementation group. There is a precedent for such a supposition since Evans et al. (1979) reported that revertants of the complementation group E mutant ts E2 had not been isolated. Rae and Elliott (1986b) showed by sequence analysis of the affected gene that the NS gene of ts E2 contained a double mutation and suggested that this could account for the unsuccessful isolation of revertants.

The polymerase complex of VSV performs diverse functions such as capping (Banerjee, 1980), methylation (Testa and Banerjee, 1977; Horikami and Moyer, 1982; Horikami et al., 1984) and polyadenylation (Schubert et al., 1980; Herman et al., 1980; Schubert and Lazzarini, 1981; Hunt et al., 1984) of the messages, as well as chain initiation and elongation. It also contains the machinery to facilitate the replication/transcription choice. Investigations using the L nonsense mutants described here should allow the study of the organization of the multiple functional domains of the polymerase complex of which the L protein, as well as the NS protein, is a component. For example, it would be of interest to determine whether any of the mutants produce in su^- cells L fragments which are defective in say, methylation or polyadenylation while the elongation function is unaffected.

A recent report by Yusoff et al. (1987) described the nucleotide sequence of the L gene of the paramyxovirus Newcastle disease virus (NDV) and compared the amino acid sequence of the L protein with the L proteins of another paramyxovirus, Sendai virus (SV) and with VSV. Regions of conserved amino acid sequence were found between the three viruses within the N-terminal two-thirds of the L protein. These consisted of two conserved regions separated by a variable hydrophilic region and it was suggested that these could thus represent separate domains of the L protein which were involved in most of the RNA synthetic and modification activities. The most variable region of the protein,

located at the C-terminal one-third of the sequence, was postulated to have virus specific functions such as interactions with the NS polypeptide. From the estimated position of the amber mutations within the group I mutants described here, these comparisons suggest that two of the mutants (AmbL1 and AmbL2) contain an amber mutation within the N-terminal two-thirds of the L protein while AmbL3 and AmbL4 contain an amber mutation within the C-terminal one-third of the protein. Potentially then, these mutants could provide information on the putative domains and their respective functions, postulated by Yusoff et al. (1987) to reside within the L protein.

5.(c) CONTROL OF L PROTEIN SYNTHESIS IN INFECTED CELLS

An unexpected and interesting observation arising from the analysis of the proteins synthesized by the mutants AmbL1 and AmbL2 (and possibly AmbL4) in su^+ cells, was that the novel polypeptide species synthesized by these mutants was present at a much higher abundance than full length L protein in wt infected cells. Also, the novel polypeptide species produced by the mutant ambL3 in su^+ cells was present at the normal wt level. This observed overabundance may possibly reflect an extension of the control mechanism which directs the differential synthesis of the five VSV gene products. The recognized model which accounts for differential synthesis of the VSV proteins involves attenuation of transcription at or near the intergenic boundaries (Iverson and Rose, 1981). This results in mRNA species being made in successively lower amounts with respect to distance from the 3' terminus of the genomic RNA (Villarreal et al., 1976). Additional attenuation sites within the L gene probably provide the best explanation for the overabundance of the truncated version of the L protein. Support for this interpretation was provided by Iverson and Rose (1981), who suggested that in view of the apparent conflict between the results of Villarreal et al. (1976) and Rose (1978), that possibly some attenuation occurs within the L gene. Rose (1978) observed that the yield of the L mRNA ribosome binding site was about 50% lower than that of

the G mRNA suggesting that the level of transcription attenuation at the G-L gene junction was similar to the level of attenuation at the other gene junctions. Villarreal *et al*. (1976), however, on measuring the molar ratios of VSV induced RNA species, found only a low level of L mRNA.

Further support for this interpretation is not available however since no measurement of the level of attenuation at the G-L gene boundary or within the L gene was provided by Iverson and Rose (1981).

Alternatively, it is conceivable that attenuation of VSV transcription might occur by a mechanism similar to that found for the amino acid biosynthetic operons where, in the case of the tryptophan operon (Bertrand *et al*., 1975) many polymerases terminate shortly after initiation of transcription. This interpretation is supported by the observation by Testa *et al*. (1980) that, during *in vitro* synthesis of full length positive strand genome RNA, small *in vitro* transcripts (30-60 bases) were made which appeared to represent the 5' terminal portion of individual mRNA species.

Alternatively, but I believe less appealing, is the possibility that translational attenuation could also account for this phenomenon.

5.(d) CHARACTERIZATION OF THE GROUP V MUTANT AMBG

The group V mutant AmbG is a less convincing candidate for being a nonsense mutant than the group I mutants described above, although it was isolated in exactly the same way. Quantitation of the restriction in host range revealed a difference in titre between the two cell lines of only 30 fold. This is at least 10 fold lower than that obtained for any of the group I mutants. Thus, it appears that AmbG is slightly leaky in the su^- cells. Several observations support this hypothesis. Analysis of the proteins synthesized by this mutant in su^- cells showed that the mutant was not restricted to the same extent as the group I mutants since the putative truncation product, observed after infection of su^+ cells, was present in

significant amounts 24 h post infection. There was also a marked inhibition of host cell protein synthesis at this time, in contrast to the infection of su$^-$ cells by the group I mutants. In addition, virus isolated from su$^-$ cells and initially thought to represent revertants all appeared to synthesize the faster migrating glycoprotein. Finally, these so-called 'revertants' exhibited the same restricted host range as the original mutant from which they were derived, suggesting that these were not true revertants but in fact the original mutant AmbG. Due to the leakiness of this mutant, it would appear that many more plaques need to be picked on the su$^-$ cells to pick up revertants. Several reports provide a possible explanation for the observed leakiness of AmbG in the su$^-$ cells.

It has been shown that alterations to the amino acid sequence of the cytoplasmic domain of the G protein has a deleterious effect on the rate of transport of G to the cell surface (Rose and Bergmann, 1983; Puddington et al., 1986). Specifically, Rose and Bergmann generated deletions within the cytoplasmic domain of a plasmid borne cDNA encoded G protein gene. Two of these deletions which lacked 16 amino acid residues were transported at a significantly lower rate to the Golgi apparatus (possibly accumulating in the rough endoplasmic reticulum). One could extend these observations and postulate that the shortened G protein specified by AmbG, which has been calculated to have lost approximately 10 terminal amino acids of its cytoplasmic domain (results section 4.(b).1.7), may also have an altered rate of transport to the Golgi apparatus and hence the cell surface in su$^-$ cells. The results of Lodish and Porter (1980) showed that the presence of G protein on the cell surface (although not a prerequisite for virus maturation) may facilitate the extent of budding and one might suspect that G protein, which has an altered rate of transport to the plasma membrane, might alter the extent of budding of progeny virions. Alternatively, an altered rate of transport may result in fewer G molecules at the plasma membrane at a given time resulting in a corresponding reduction in the number of G molecules on the virion

envelope. It is conceivable that either of these explanations might contribute to the observed leakiness of this mutant in *su*$^-$ cells.

RNA was isolated from *su*$^+$ cells infected with *wt* and mutant virus. Preliminary analysis of the RNAs involved *in vitro* translation using a rabbit reticulocyte system. An interesting result arising from the *in vitro* translation of the RNA isolated from *su*$^+$ cells infected by AmbG was that the pre-G protein synthesized by the mutant AmbG possessed a greater mobility than the pre-G synthesized by *wt* virus. This confirms that the aberrant mobility of this protein is not the result of a defect in glycosylation.

5.(e) CHARACTERIZATION OF THE HOST RANGE MUTANT HRTK$^-$7

The mutant hrTK$^-$7 appeared more complex than any of the other suppressible host range mutants characterized. Analysis of the viral proteins synthesized in *su*$^+$ cells showed that the N protein migrated slower than *wt* N. In addition, no full length NS protein was detectable and an additional polypeptide species, thought to represent a shortened form of the NS protein, was present, migrating between the N and M proteins. However, unequivocal evidence that this is a truncated version of the NS protein is lacking. The observation that this mutant failed to complement the defect in any of the representative *ts* mutants suggests that the defects in this mutant lie in more than one cistron. In addition, although very small plaques were visible on the *su*$^-$ cells they did not grow appreciably even after extended incubation and consistently could not be amplified. The available evidence thus suggests that this may be a multiple mutant. It is conceivable that the mutation affecting the NS protein, resulting in the detection of a faster migrating version in polyacrylamide gels, may be a constituent of the multiple mutation. However, the mutation affecting the mobility of the N protein could only be regarded as suppressible if the mutation resided within the termination codon, perhaps altering the normal ochre stop codon to an amber. Termination would then occur further downstream.

Examination of the sequence for this region however does not reveal any stop codons within 30 nucleotides of the termination codon. Alternatively, it has been reported that a sixth complementation group has been identified for the Orsay (Deutsch *et al.*, 1979) and Massachusetts (Rettenmeier *et al.*, 1975) strains of VSV Indiana which has been tentatively assigned to the NS gene (or possibly the L gene of the Massachusetts strain) suggesting that the NS gene may contain two cistrons. In addition, Herman (1986) reported the existence of an additional small polypeptide in the Indiana serotype, both *in vivo* and *in vitro* encoded by the C-terminal portion of the NS protein gene. Thus it is possible that the proposed multiple mutation affecting this mutant resides within the NS gene but in different complementation groups. A simple charge difference might then explain the aberrant mobility of the N protein synthesized by this mutant. These offered explanations can only be regarded as speculative however. At this time it is not possible to be more definitive regarding the nature of the mutation conferring the restriction in host range to this mutant and as a result this mutant must remain unclassified.

Although the results obtained by complementation analysis, in which the mutants were assigned to a specific complementation group, did not take into account any possible effect of recombination between the mutants, the validity of the gene assignments should not be affected. This is because attempts to detect *wt* recombinant virus in the progeny of mixed infections of *ts* mutant parents have all been unsuccessful. In a study by Pringle *et al.* (1981) no *wt* recombinant virus was recovered in a cross of two complementing *ts* mutants of VSV New Jersey which could also be scored for four independent non-*ts* markers affecting the G, NS, N and M proteins, Therefore, although the occurrence of genetic recombination cannot be excluded, it must be a rare event, if it occurs at all.

5.(f) THE EFFECT OF THE AMINOGLYCOSIDE G418

The aminoglycosides have been reported to promote readthrough of nonsense codons in various systems by affecting the fidelity of translation and, in consequence, the accuracy of chain termination. The recent report by Burke and Mogg (1985) showed that the antibiotics G418 and paromomycin suppressed a characterized amber mutation in the CAT gene of the mammalian cell transfection vector pRSVCAT^{amb38}. The level of readthrough promoted by G418 was calculated to be 19.6% (Burke and Mogg, 1985).

It seemed appropriate to investigate the effect of G418 on the suppressible host range mutants described herein. Wt and AmbL1 virus was grown on the su$^-$ cells in the presence of varying concentrations of G418 and either (1) the supernatant titrated on the su$^+$ cells to assay for growth of the mutant or (2) the intracellular proteins were labelled and analysed for detection of a readthrough product in a polyacrylamide gel presumably promoted by the action of G418. However, it was found that G418 failed to facilitate readthrough of the mutation affecting AmbL1, since neither an increase in growth of the mutant in su$^-$ cells nor the synthesis of a readthrough product in su$^-$ cells could be detected. It is possible, however, that the deleterious effect that G418 has on growth of the virus negates any effect resulting from suppression of the mutation. Also, since the L protein is the least abundant protein in wt infected cells, a 19.6% suppression level might still not be enough to allow detection of a readthrough product. Although the optimum level of suppression promoted by G418 was obtained at 50 ug/ml this was achieved using COS cells and not the LMTK$^-$ cells which were used here. A difference in permeability between the two cell lines might affect the concentration of drug needed to promote optimum suppression. It may have been more appropriate to use COS cells for this analysis. LMTK$^-$ cells, however, were used because they represented the non-permissive host used throughout this study.

5.(g) FUTURE DIRECTIONS

As mentioned previously, nucleic acid sequencing would provide conclusive proof that these are indeed nonsense mutants. In addition, it would reveal the complex nature of the mutation specifying the restriction in host range of the proposed double mutant AmbL5. A sequence can be obtained either by directly sequencing the RNA template or cloned cDNAs. Nonsense mutants lend themselves to sequencing to the extent that the length of the truncated polypeptide should correlate with the position of the mutation in the affected gene. With respect to the mutant AmbG, it would be of interest to compare the rate of transport of the glycoprotein synthesized by AmbG with that of wt G protein. This could be accomplished by investigating the time taken for the respective glycoproteins to acquire resistance to endoglycosidase (endo H). It has been shown by Rose and Bergmann (1983) that when G protein is transported to the Golgi apparatus the high mannose simple oligosaccharides attached to the protein are converted to complex oligosaccharides which are endo H resistant. Work by Rose and Bergmann (1983) in which G protein containing deletions in the cytoplasmic domain exhibited a retarded rate of transport to the Golgi apparatus, suggests that the putative shortened G protein produced by AmbG will also exhibit an altered rate of transport.

It is envisaged that VSV nonsense mutants will be used in the study of VSV gene expression. In particular the L gene mutants may facilitate the assignment of sub-functions of what is a multifunctional protein to different regions of the polypeptide chain.

It is important to register that the utility of these mutants is restricted in two ways. Firstly, their utility will be restricted by the low suppression level of the already existing suppressor cells. This low level of suppression is probably the maximum that a mammalian cell line expressing a suppressor tRNA can tolerate without having a deleterious effect on the physiology of the cell. The second restriction is the high reversion rate which

probably reflects the low fidelity of replication caused by the lack of a proofreading system in VSV RNA replication.

Extension of this suppression system to other viruses will be dependent on two factors. Firstly it will be dependent on the host range of the virus and secondly on the nature of the viral termination codons. The accumulating sequence data will show which, if any, gene products might be adversely affected by readthrough of the natural translation terminators in the presence of the particular suppressor.

NOTE ADDED

Following completion of the text of this thesis, a paper has appeared which describes two significant advances in the field of suppressible nonsense mutants of animal viruses, by essentially the same group that constructed the L39 suppressor cell line used in this Ph.D. report (Sedivy, J.M., Capone, J.P., RajBhandary, U.L. and Sharp, P.A. (1987). An inducible mammalian suppressor: Propagation of an amber mutant. Cell 50, 379-389). First, this paper describes the construction of an inducible mammalian suppressor system. A human amber suppressor $tRNA^{Ser}$ gene (Capone *et al.*, 1985) was linked to the SV40 origin of replication and to a gene encoding a selectable marker (*neo*, which confers resistance to G418). This plasmid was cotransfected into monkey cells along with a vector encoding a *ts* mutant of the SV40 large T-antigen. Cells were selected for G418 resistance at the non-permissive temperature (39.5°C), which resulted in the stable integration of a few copies per cell of each plasmid. When these cells were shifted down to 33°C, production of active T-antigen resulted in the amplification of the tRNA gene to about 2000-4000 copies per cell. The level of suppression achieved by such cells ranged from 10-70%.

The second important advance described in this paper was the generation of a conditional lethal amber mutant of poliovirus. The mutant was generated by *in vitro* site directed mutagenesis which changed a serine residue to an amber stop codon in the RNA replicase gene of an infectious cDNA clone. The alteration was confirmed by nucleotide sequence analysis. The amber mutant plaqued on the suppressor cells only after induction of the suppressor phenotype, but not on the non-suppressor parental cells.

The major advantage that this type of inducible suppression system has over the constitutive system used in this thesis is the elevated suppression level which may permit the isolation of classes of nonsense mutants where larger amounts of readthrough protein are needed for successful production of useable amounts of infectious virus.

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