Characterization of the Mutations Responsible for the Electrophoretic Mobility Differences in the NS Proteins of Vesicular Stomatitis Virus New Jersey Complementation Group E Mutants

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SUMMARY

Temperature-sensitive (ts) mutants of vesicular stomatitis virus, New Jersey serotype, classified in complementation group E contain lesions in the NS gene, which manifest as marked electrophoretic mobility differences of the mutant NS proteins in SDS-polyacrylamide gels. We have cloned full-length cDNA copies of the mutant NS mRNAs, and have determined their nucleotide sequences. tsE1 and tsE3 had single nucleotide changes, and tsE2 had two nucleotide changes, compared to the wild-type NS gene. Three of the mutations were clustered in a region of 18 nucleotides. All the nucleotide differences resulted in amino acid substitutions, which in each case changed the charge of the amino acid concerned. Analysis of the wild-type and mutant NS protein sequences by the method of Chou & Fasman indicated that single amino acid substitutions can radically alter the predicted secondary structure, and these data are discussed in relation to the observed electrophoretic mobility differences.

INTRODUCTION

The negative-strand RNA genome of vesicular stomatitis virus (VSV) encodes five recognized proteins, L, G, N, NS and M. The L, N and NS proteins, together with the genomic RNA, constitute the ribonucleoprotein core of the virus particle, and the M and G proteins form the viral envelope (Wagner, 1975). Recently Herman (1986) has suggested that the NS mRNA may encode a second protein by internal initiation of translation. The NS protein itself is a component of the RNA polymerase complex (Banerjee et al., 1977) but its precise role(s) in transcription and replication is still uncertain. Complete nucleotide sequences of the NS mRNAs encoded by representative viruses of the two VSV serotypes Indiana (IND) and New Jersey (NJ) have been reported, and predict the mol. wt. of the NS protein of IND viruses to be 29900 (Gallione et al., 1981; Hudson et al., 1986) and that of NJ viruses to be 31400 (Gill & Banerjee, 1985; Rae & Elliott, 1986). However, the NS proteins migrate on SDS-polyacrylamide gels with apparent molecular weights of 40000 to 70000, depending on the degree of bisacrylamide cross-linking (Bell et al., 1984; Bell & Prevec, 1985; Kingsford & Emerson, 1980; Knipe et al., 1975; Obijeski et al., 1974; Wunner & Pringle, 1972). The aberrant mobility of NS is clearly seen when it migrates more slowly than the N protein, which has a molecular weight of 47300 (IND serotype; Gallione et al., 1981) or 47800 (NJ serotype; Banerjee et al., 1984); e.g. in the studies reported by Lesnaw et al. (1979), Maack & Penhoet (1980), Patton et al. (1984) and Pringle et al. (1981). The NS protein is phosphorylated but the phosphate groups are not responsible for the anomalous mobility; indeed, the more heavily phosphorylated NS molecules migrate faster in SDS-polyacrylamide gels (Hsu et al., 1982) while NS that has been dephosphorylated with bacterial alkaline phosphatase migrates more slowly (Hsu & Kingsbury, 1982). The NS protein contains many acidic residues (Gallione et al., 1981; Gill & Banerjee, 1985; Hudson et al., 1986; Rae & Elliott, 1986), and it is thought that these residues hinder SDS binding which results in the aberrant migration (discussed by Marnell & Summers, 1984).
Temperature-sensitive (ts) mutants of VSV-NJ were isolated by Pringle et al. (1971) following 5-fluorouracil treatment of infected cells. Three mutants, classified in complementation group E, were shown to have lesions in the NS gene (Evans et al., 1979; Lesnaw et al., 1979; Maack & Penhoet, 1980). These mutants form a heterogeneous group with respect to their phenotype, accompanied by marked differences in the electrophoretic migration of the NS protein. In order to characterize these mutants further, we have determined the nucleotide sequence of cloned full-length cDNA copies of their NS mRNAs. We report here that a single amino acid substitution in an NS protein can radically alter its electrophoretic mobility in SDS-polyacrylamide gels.

METHODS

Molecular cloning and nucleotide sequencing. Stocks of VSV-NJ tsE1, tsE2, tsE3, tsE1/R1 and tsE3/R1 were generously provided by J. F. Szilagyi and were used directly to infect cells for RNA preparation without further passage. BHK-C13 cells were infected in the presence of actinomycin D and total cellular RNA prepared by the guanidinium thiocyanate-CsCl method exactly as described previously (Chirgwin et al., 1979; Rae & Elliott, 1986). cDNA synthesis followed the method of Gubler & Hoffman (1983) as modified by Rae & Elliott (1986) and mutant NS gene-specific plasmids were identified by in situ colony hybridization (Grunstein & Hogness, 1975) using a 32P-labelled fragment (the large PstI fragment, bases 1 to 523) from the wild-type NS gene plasmid pNJNS1 (Rae & Elliott, 1986). Full-length cDNAs were identified by sizing in agarose gels and designated pNJE1, pNJE2 and pNJE3. Nucleotide sequences were determined by the dideoxy chain terminator method (Sanger et al., 1977) by subcloning restriction enzyme fragments into M13mp18 DNA as detailed previously (Rae & Elliott, 1986).

In vitro translation and SDS polyacrylamide gel electrophoresis. RNAs were translated in a rabbit reticulocyte lysate (Amersham) using 10 µg total RNA in a 25 µl reaction containing 50 µCi [35S]methionine. Radiolabelled translation products were separated on SDS–polyacrylamide gels (acrylamide : bisacrylamide 73 : 1; Watret et al., 1985) using the discontinuous buffer system of Laemmli.

Computer analysis. Nucleotide sequences were stored and manipulated with a DEC PDP11/44 computer using the programs devised by Staden (1982). Hydropathy profiles were generated using the SOAP algorithm of Kyte & Doolittle (1982). Protein secondary structure predictions used the method of Chou & Fasman (1978), and were graphically displayed using the PLOTCHOU program contained in the updated package of the University of Wisconsin Genetics Computer Group (originally described by Devereux et al., 1984). The graphic output was drawn on a Hewlett Packard HP7475A plotter in the Department of Molecular Biology, University of Edinburgh, Edinburgh, U.K. with the assistance of Dr A. Coulson.

RESULTS

Molecular cloning and nucleotide sequence determination of the NS mRNAs of tsE1, tsE2 and tsE3

Total cellular RNA was extracted from actinomycin D-treated infected cells (Rae & Elliott, 1986), and an aliquot was analysed by in vitro translation in a reticulocyte lysate. As seen in Fig. 1 the NS proteins of tsE1, tsE2 and tsE3 had different electrophoretic mobilities in an SDS–12% polyacrylamide gel compared to the wild-type NS protein. The in vitro products were also analysed on gels containing 10% and 15% polyacrylamide (data not shown), and the apparent mol. wt. of the NS proteins were determined relative to the G (62000), N (47 800) and M (26000) proteins. The averages of estimations from the three different concentration gels are given in Table 1.

No evidence of revertants, as would be indicated by the presence of an NS protein with wild-type migration pattern (Evans et al., 1979), was observed in any of the mutant virus mRNA preparations. Therefore, other aliquots of the same RNA preparations were used as templates for cDNA synthesis, which followed the method previously described for cloning cDNA to wild-type NS mRNA (Rae & Elliott, 1986). Mutant NS-specific recombinant plasmids were identified by in situ hybridization using a probe prepared from the wild-type NS plasmid, pNJNS1 (Rae & Elliott, 1986). Digestion with PstI revealed that the mutant NS gene inserts all contained an internal PstI restriction enzyme site, characteristic of the wild-type NS gene insert. Apparently full-length cDNA clones, as judged by the size of the insert, were obtained and designated pNJE1 (from tsE1), pNJE2 (from tsE2) and pNJE3 (from tsE3). Nucleotide
VSV NS protein mutants

Table 1. Summary of the mutations in the NS genes of vesicular stomatitis virus E group mutants

<table>
<thead>
<tr>
<th>Virus</th>
<th>Apparent mol. wt. of NS protein (× 10^-3)*</th>
<th>Nucleotide position and change</th>
<th>Amino acid position and change</th>
<th>Charge change</th>
<th>Phenotype†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>59</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Transcriptase +ve; RNA synthesis +ve</td>
</tr>
<tr>
<td>tsE1</td>
<td>50 104 G→A</td>
<td>32 Glu→Lys</td>
<td>–</td>
<td>–ve charged polar +ve charged polar</td>
<td>Transcriptase -ve; RNA synthesis -ve</td>
</tr>
<tr>
<td>tsE2</td>
<td>52 108 A→G</td>
<td>33 Asp→Gly</td>
<td>–</td>
<td>-ve charged polar +non-polar</td>
<td>Transcriptase +ve; RNA synthesis +ve</td>
</tr>
<tr>
<td></td>
<td>353 A→G</td>
<td>115 Lys→Glu</td>
<td>+ve charged polar</td>
<td>-ve charged polar +non-polar</td>
<td>Transcriptase +ve; RNA synthesis -ve</td>
</tr>
<tr>
<td>tsE3</td>
<td>54.5 93 A→G</td>
<td>28 Glu→Gly</td>
<td>+ve charged polar</td>
<td>-ve charged polar +non-polar</td>
<td>Transcriptase +ve; RNA synthesis +ve</td>
</tr>
</tbody>
</table>

* Average of estimations from 10%, 12% and 15% polyacrylamide gels.
† Pringle et al. (1971); Szilagyi & Pringle (1979).
sequence analysis confirmed that these three mutant NS cDNA clones were complete copies since they all contained the 5' and 3' consensus sequences found in all VSV mRNAs (McGeoch, 1979; Rose, 1980).

The complete nucleotide sequences of the mutant NS genes (Fig. 2) were determined by the dideoxy chain terminator method (Sanger et al., 1977). All the mutant NS mRNAs were the same length (876 bases excluding polyadenylic acid) and encoded the same length of polypeptide (274 amino acids) as the wild-type NS mRNA. 

tsE1 and tsE3 each had single nucleotide changes while tsE2 had two nucleotide substitutions. Portions of representative sequencing gels showing these changes are displayed in Fig. 3. Three of the nucleotide changes occurred within an 18-base region (nucleotides 93 to 108) while the second mutation in tsE2 was at position 413 (Fig. 2).

In all cases the nucleotide change resulted in an amino acid substitution with a change in the charge of amino acid concerned. These are summarized in Table 1.

Fig. 4 shows a hydropathy profile (Kyte & Doolittle, 1982) of the wild-type NS protein. The region marked ‘a’, the most hydrophilic domain of the protein, is where three amino acid substitutions are clustered, and ‘b’ indicates the position of the second mutation in tsE2.
The amino acid sequences of the wild-type and mutant NS proteins were analysed using the secondary structure prediction algorithm of Chou & Fasman (1978), and a graphic output was obtained (Fig. 5). Although these predictions were based solely on the primary amino acid chain, and did not take into account the effect of post-translation modifications such as phosphorylation, it is obvious that mutations occurring in amino acid residues 28 to 33 have a marked effect on the predicted secondary structure. The mutation in tsE1 predicted a loss of a region of α-helix and a change in the position of a β-turn. The mutation in tsE3 manifested as a loss of the same α-helical region, although no change in the β-turn was predicted. The mutation at amino acid 32 in tsE2 resulted in an additional region of α-helix and additional β-turns, whereas the mutation at amino acid 114 had a minor effect in establishing a small region of α-helix. No other effects of these mutations were predicted in the remainder of the NS protein.
Fig. 4. Hydropathy profile of the NS protein of VSV-NJ. 'a' indicates the region (amino acids 28 to 33) where three mutations are clustered, and 'b' indicates the position of the second mutation (amino acid 115) in tsE2.

Fig. 5. Predicted secondary structures of the NS proteins of wild-type and complementation E group mutants of VSV-NJ. The generation of these graphic representations is described in Methods. The complete plot of wild-type (wt) NS protein is given, and the relevant portions of the plots of the E group mutants. The arrows indicate differences in the predicted secondary structures due to amino acid substitutions. \( \alpha \)-helix, \( \beta \)-strand, represents undefined and a change of direction indicates a \( \beta \)-turn. The open ovals represent hydrophilic regions, and shaded ovals hydrophobic regions.
Nucleotide sequence determination of revertants of tsE1 and tsE3

We have also cloned and sequenced full-length cDNA copies of the NS mRNAs of revertants of tsE1 (tsE1/R1; Szilagyi & Pringle, 1979) and tsE3 (tsE3/R1; Evans et al., 1979). (It is worth noting that no revertants have yet been isolated from the double mutant tsE2.) These revertants have a wild-type pattern of RNA synthesis and electrophoretic mobility of the NS protein (Evans et al., 1979). The revertant NS genes were identical to the wild-type sequence (Rae & Elliott, 1986) at the site of mutation, but two additional point mutations were noted: a silent T to C change at position 471 and a T to G change at position 513 which results in a Met to Arg substitution (amino acid 168; Fig. 2). Both revertant NS gene sequences were identical. The region encompassing the additional changes in the NS gene (i.e. nucleotides 471 and 513) was sequenced in three other independent plasmid isolates containing the tsE1/R1 NS insert, and confirmed the substitutions given above. The amino acid substitution at residue 168 did not affect the predicted secondary structure of the revertant NS protein compared to wild-type (data not shown).

DISCUSSION

The complementation group E mutants of VSV-NJ exhibit a variety of phenotypes and different electrophoretic mobility patterns of the NS protein (Pringle et al., 1971; Evans et al., 1979). By determining the nucleotide sequences of cloned cDNA copies of the mutant NS mRNAs we have precisely mapped the lesions in the NS genes. Our data show that the altered migration of the mutant NS proteins is not due to the synthesis of truncated proteins but rather to the effects of amino acid substitutions.

The relative electrophoretic mobility of proteins in polyacrylamide gels containing SDS is a widely used method of estimating their molecular weights (Shapiro et al., 1967). The migration of the protein depends on a high level of SDS binding (1.4 g of SDS per 1 g of protein) so that an approximately constant negative charge per unit mass is achieved (Creighton, 1983); under these conditions the electrophoretic mobility in gels is proportional to the logarithm of the length of the polypeptide chain. However, the amino acid composition or conformation of the protein may perturb the stoichiometric binding of SDS, resulting in aberrant electrophoretic migration. De Jong et al. (1978) reported that the mobility of α-crystallin A chains was altered by single amino changes, and Carstens et al. (1986) reported that a single amino acid substitution in a mutant baculovirus polyhedral protein affected its electrophoretic migration. In addition, the latter workers showed that the single amino acid change grossly affected the predicted secondary structure of the mutant protein. Our data also demonstrate that single amino acid substitutions can radically influence the predicted secondary structure of a protein.

A potential criticism of our approach is that we have sequenced cloned cDNAs rather than obtaining a consensus sequence derived from dideoxy sequencing on an RNA template. However, our results are in complete agreement with the data of D. McGeoch and A. Dolan who have obtained sequence information for approximately 200 nucleotides at the 5' end of the NS genes of tsE1, tsE2, tsE3, tsE1/R1 and tsE3/R1 by primer extension on genomic RNA (D. McGeoch, personal communication; and data cited in Pringle, 1986). Therefore we feel that the sequences presented here are an accurate reflection of the differences in the wild-type, mutant and revertant NS genes.

We do not think that the presence of additional changes in the NS genes of the revertant viruses is due to a cloning artefact, since the recombinant plasmids were isolated in different cloning experiments which were performed on separate occasions. Furthermore, four individual clones of the tsE1/R1 NS genes contained the additional base substitutions. It also seems unlikely that these changes represent a variant wild-type virus which had somehow contaminated the revertant selection procedures, since these were also carried out at different times (C. R. Pringle, personal communication). A further possibility is that one of the stocks was mislabelled at some time and though we also feel this to be very unlikely it cannot be unequivocally excluded. The occurrence of other changes in the NS genes of revertants is implied from the data of Maack & Penhoet (1980) who isolated revertants of tsE1 and found different mobilities of the NS protein compared to both tsE1 and wild-type viruses. We suggest
that this could be due to a different amino acid substitution at position 32, or additional amino acid changes elsewhere in the NS protein, any of which could influence the conformation of the protein.

In contrast to the situation in VSV M gene mutants, where point mutations have been located throughout the M protein (Gopalkrishna & Lenard, 1985), three of the four mutations in the NS mutants are clustered around the most hydrophilic region of the NS protein (Fig. 4). This region of the NS protein represents an obvious area to explore by in vitro mutagenesis in order to correlate active sites in the protein with the different functions ascribed to NS.

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REFERENCES


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