Location of a Neutralizing Epitope for the Haemagglutinin–Neuraminidase Glycoprotein of Newcastle Disease Virus

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SUMMARY

The binding site of a monoclonal antibody to the haemagglutinin–neuraminidase (HN) polypeptide of Newcastle disease virus (NDV) has been located. Complementary DNA or synthetic oligonucleotides corresponding to portions of the HN gene were cloned into the Escherichia coli vector pUC19 and fragments of the HN protein were thereby fused to the α-peptide of β-galactosidase. Western blot analysis of E. coli lysates containing expressed fragments of the HN cDNA or synthetic oligonucleotides identified an antibody-binding peptide (Asp-Glu-Gln-Asp-Tyr-Gln-Ile-Arg; amino acid residues 346 to 353). Nucleotide sequence analysis of an antibody-resistant mutant of NDV revealed a Glu (wild-type) to Lys (mutant) substitution within the above sequence. The methods described could be useful for the location of continuous epitopes of other polypeptides.

Newcastle disease virus (NDV) is a paramyxovirus that causes a severe and economically important respiratory disease in poultry (Lancaster & Alexander, 1975). The avian antibody response to the virus contains elements directed against both of the viral glycoproteins, the haemagglutinin–neuraminidase (HN) and the fusion (F) protein. Monospecific antisera raised against either purified glycoprotein can neutralize viral infectivity, although anti-F neutralization is only effective in the presence of fresh serum (Umino et al., 1984). Anti-HN serum, however, is no more effective than anti-F serum in prolonging the life of chickens after challenge with virulent NDV, but a combination of both is protective (Umino et al., 1987). Protective epitopes on paramyxovirus HN and F proteins may, however, be destroyed during the process of purification (Paterson et al., 1987). At present, therefore, it is not clear whether an antibody response to both glycoproteins is necessary for protection of chickens from challenge with virulent NDV.

Studies with murine monoclonal antibodies (MAbs) against NDV suggest that there may be four sites on the HN glycoprotein that stimulate the production of virus-neutralizing antibodies (Iorio & Bratt, 1983; Nishikawa et al., 1986; Le Long et al., 1986). A fifth site is formed by the overlap of two of these sites (Iorio et al., 1986). This communication describes the location of the epitope for a neutralizing MAb using recombinant plasmids which encode portions of the NDV HN protein and detection of antibody-binding polypeptides by Western blot analysis.

Neutralizing MAbs to the NDV HN protein have been obtained from two sources (Russell et al., 1983; Le Long et al., 1986). Several of these bind HN in Western blots when the structure of HN has been disrupted by boiling with SDS and 2-mercaptoethanol (Le Long et al., 1986; Samson, 1986; Samson et al., 1988). These antibodies may, therefore, recognize epitopes that are continuous or are readily regenerated by refolding after denaturation. The binding sites of such
Fig. 1. Molecular clones used in the identification of the binding site of MAb 14. A scale in kb, numbered according to Millar et al. (1986), is shown along the top. The 5' (left) and 3' (right) ends of the HN mRNA are indicated by arrows. Beneath this, the bar represents the non-expressing cDNA insert of the clone containing the full HN open reading frame. This clone was constructed by simultaneous ligation to pUC19 cut within the multiple cloning site with SpH I and Sst I of three fragments from clones described by Chambers et al. (1986): a 0.8 kb SpH I–Nar I fragment from clone 7.44, a 0.45 kb Nar I–Acc I fragment from clone 4.68 and a 0.87 kb Acc I–Sst I fragment from clone 1.13. The open box indicates the location of the HN open reading frame, from N to C termini. Numbers down the left designate plasmids that express portions of HN that were used in the location of the antibody-binding site, with a designation (+) or (−) denoting positive or negative results in Western blots. Bars indicate the region of the HN gene contained in each plasmid. Polypeptides encoded by plasmids 1, 2 and 3 terminate at the NDV HN stop codon, whereas polypeptides encoded by plasmids 4 to 10 consist of NDV-specific peptides fused in phase to the α-peptide of β-galactosidase encoded by pUC19. Plasmids (1 to 10) were constructed as follows. (1) A 1.67 kb Nhe I–Sst I fragment was subcloned from the non-expressing HN plasmid described above into pUC19 cut with Xba I and Sst I. This recombinant was cut with Satt I, 50 μM dNTPs and 1 unit of Klenow polymerase were added to fill in the overhangs and plasmid 1 was formed by religation. (2) A 1.33 kb Nar I–Sst I fragment was subcloned to pUC19 cut with Acc I and Sst I. (3) A 0.64 kb Scal–Sst I fragment was subcloned from plasmid 2 to pUC19 cut with Hind III and Sst I. (4) Plasmid 2 was cut with Eco RI, thus excising a 0.62 kb fragment between the Eco RI site at base 1513 in the HN sequence and the Eco RI site of pUC19, which is adjacent to the Sst I site. Overhangs were filled and plasmid 4 was formed as for plasmid 1 above. (5) Plasmid 2 was cut with Sma I and Eco RI, excising a 0.74 kb fragment from the Sma I site at base 1386 in the HN sequence to the Eco RI site of pUC19. Overhangs were filled and plasmid 5 was formed as for plasmid 1 above. (6) Plasmid 2 was cut with Acc I and overhangs were filled as for plasmid 1 above. Klenow polymerase was inactivated by incubation at 75 °C for 10 min. DNA was then further cut with Hind III, and the 0.47 kb Hind III–filled Acc I fragment was subcloned into pUC19 cut with Hind III and Sma I. (7) A 0.10 kb Rsa I–Acc I fragment from plasmid 2 was subcloned to pUC19 cut with Hind III and Acc I (Acc I cuts at Sma I recognition sequences but generates a four-base 5' overhang rather than the blunt end generated by Sma I). Recombinant DNA was isolated (and also used in the preparation of plasmid 8), cut with Acc I, overhangs were filled and the plasmid was formed as for plasmid 1. (8) Recombinant DNA from plasmid 7 above was cut with Bal I and Sma I, thus excising a 0.02 kb fragment from the Bal I site at base 1366 in the HN gene to the Sma I site of pUC19, and the plasmid was formed by religation. (9) The
MAbs could possibly be located by synthesis of portions of the HN protein in *Escherichia coli* followed by Western blot analysis of bacterial lysates. MAb-resistant mutants have been isolated from the Beaudette C strain of NDV (Samson et al., 1985). Mutants raised against any of the antibodies that recognized reduced denatured HN were cross-resistant to the other antibodies that recognized reduced denatured HN (Samson et al., 1988). This suggests that the epitopes for these MAbs may comprise parts of one of the major antigenic sites on the HN protein detected by others (Iorio & Bratt, 1983; Nishikawa et al., 1983; Russell et al., 1983; Le Long et al., 1986). One of these MAbs, designated MAb 14, has been characterized for biological activities, and inhibits haemagglutination in most strains of NDV tested (Russell & Alexander, 1983). This MAb was chosen for investigation because the epitope recognized is common to most strains of NDV and because it gives a strong signal to HN protein in Western blots.

Details of the cDNA cloning and nucleotide sequence of the NDV (Beaudette C) HN gene have been reported previously (Chambers et al., 1986; Millar et al., 1986). A cDNA clone containing the complete HN gene was constructed by simultaneous ligation of three cDNAs, each containing part of the HN gene, into the *E. coli* plasmid vector pUC19 (Yanisch-Perron et al., 1985). Details of the construction of this and other plasmids used in this work are summarized in Fig. 1. This plasmid did not express NDV HN, but was used as a source of restriction fragments for the construction of other plasmids that did express portions of HN. The largest such expression plasmid (plasmid 1; Fig. 1) contained a 1.67 kb *NheI*-SstI fragment subcloned into pUC19 cut with *XbaI* and SstI. Expression was achieved after the cDNA was brought in phase by cutting recombinant DNA with *SalI*, filling in overhangs and religation. Inspection of the nucleotide sequence of the HN gene and the multiple cloning site of pUC19 revealed that the resultant hybrid polypeptide should contain the first 12 amino acids of the modified α-peptide of β-galactosidase encoded by pUC19, a three amino acid spacer (Ile-Asp-Ser), and the C-terminal 527 amino acids of NDV HN. This hybrid polypeptide did not contain the N-terminal 50 amino acids of HN. In NDV, the HN polypeptide is attached to the membrane near its N terminus, so the portion of HN on the carboxyl side of the transmembrane region is on the outside of the viral membrane and thus probably comprises the most antigenically significant part of the protein. The region of HN represented in plasmid 1 contains this potentially antigenic portion, including all of the most hydrophilic regions of the polypeptide which are likely candidates for stimulation of an immune response (Hopp & Woods, 1981).

The hybrid polypeptide encoded by plasmid 1 was largely degraded, but some was detected in Western blot analysis of *E. coli* lysates by MAb 14. The polypeptide encoded by plasmid 2 was relatively stable and was detected by both MAb 14 (Fig. 2, lane 8) and mouse polyclonal anti-NDV sera (not shown). It was therefore feasible to attempt to locate the epitope by subcloning progressively smaller portions of the HN gene into the expression vector pUC19. Small peptides of less than about 100 amino acids would not, however, be readily resolved in SDS-polyacrylamide gels and might not be sufficiently stable in *E. coli* for the purpose of detection. It was therefore considered necessary to link a carrier peptide to the HN moieties for detection by Western blotting. The carrier chosen was the α-peptide of β-galactosidase, since cDNA fragments subcloned into the multiple cloning site of pUC19 are located very near the amino terminus of this fragment.

Strains harbouring pUC19 generate blue colonies on indicator plates containing the *lac* inducer isopropyl-β-D-thiogalactopyranoside (IPTG) and substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) by complementation between the plasmid-encoded α-peptide and an *E. coli* chromosome-encoded defective β-galactosidase. Active β-galactosidase produced by oligonucleotide 5'-AGCTACAAGGGATACATGACACATGCCCCTGCA-3' encoding the NDV peptide YKRYNDTCP was annealed to its partial complement 5'-GGGGCATGTGTCATTGTTATCCCTTGT-3' and ligated into pUC19 cut with *HindIII* and *PstI*. (10) The oligonucleotide 5'-AGCTCCGATGAGCAAGACTACCAGATCCGCTGCA-3' encoding the NDV peptide DEQDY-QIR was annealed to its partial complement 5'-GCGGATCTGGTAGTCTTGCTCATCGG-3' and ligated into pUC19 cut with *HindIII* and *PstI*.
Fig. 2. Western blots of *E. coli* lysates. Plasmids were transformed into *E. coli* strain JM105 (Maniatis *et al.*, 1982) for lanes 1 to 8 or into PAR35 (ts*^{+}^\circ\text{C}^\circ$ protease strain, Kan*R, F lacI*) for lanes 9 and 10. For lanes 1 to 8, blue or white colonies, as appropriate, were picked from indicator plates (Luria agar containing 50 µg/ml ampicillin, 150 µg/ml X-gal and 125 µg/ml IPTG) into 2YT medium containing 50 µg/ml ampicillin for overnight growth at 37 °C on a rotary shaker. For lanes 9 and 10, colonies were picked from Luria agar plates supplemented with 50 µg/ml ampicillin into 2YT medium containing 50 µg/ml ampicillin for overnight growth at 30 °C on a rotary shaker. The following day, NDV-specific polypeptides were induced as follows. (i) For lanes 1 to 8, 0.1 ml of overnight culture was transferred to 10 ml of 2YT medium containing 240 µg/ml IPTG, and cultures were grown at 37 °C in aerated tubes until the optical density at 650 nm reached 0.5. Growth of cultures that expressed larger HN-specific polypeptides was noticeably slower than those which expressed small or no HN-specific polypeptides. (ii) For lanes 9 and 10, 0.1 ml of overnight culture was transferred to 10 ml of 2YT medium and cultures were grown at 30 °C until the OD had reached 0.4, whereupon 240 µg/ml IPTG was added and cultures were grown at 42 °C until the OD had reached 0.7. All cultures were cooled on ice for 10 min before bacteria were harvested by centrifugation. The cell pellets were resuspended by vortexing into 100 µl (lanes 1 to 8) or 500 µl (lanes 9 and 10) SDS sample buffer (Laemmli, 1970) and immediately boiled at 100 °C for 2 min. Samples were stored at -20 °C before electrophoresis on 11% polyacrylamide gels (Laemmli, 1970), and were blotted onto nitrocellulose as described by Samson (1986) for lanes 1 to 8 or by Dunn (1986) for lanes 9 and 10. Lane S contains phosphorylase b (M, 92K), bovine serum albumin (68K), alcohol dehydrogenase (41K), α-chymotrypsinogen A (25K) and cytochrome c (12.4K) stained with Indian ink (Hancock & Tsang, 1983). The remaining nitrocellulose was stained with peroxidase-conjugated rabbit anti-mouse sera after incubation with MAb 14 as described by Samson (1986). Lane N contains purified NDV (Chambers & Samson, 1980) in which the HN polypeptide at 74K is prominent. Lanes 1 to 10 contain lysates of *E. coli* containing (1) pUC19, (2 and 9) plasmid 10, (3 and 10) plasmid 9, (4) plasmid 8, (5) plasmid 3, (6) plasmid 5, (7) plasmid 6 and (8) plasmid 2. Lanes (2), (4), (6), (8) and (9) contain hybrid polypeptides that bind MAb 14 (approximate M, values 11K, 12K, 32K, 49K and 11K, respectively). The use of PAR35 in conjunction with the blotting procedure of Dunn (1986) gave better visualization of the polypeptide encoded by plasmid 10 than the other protocol (compare lanes 2 and 9).
recombinant plasmid can give rise to blue colonies on indicator plates as complementation to produce active β-galactosidase is still possible. It is, however, quite unusual to clone cDNA into pUC19 in the correct phase at both junctions between plasmid and cDNA and, therefore, most recombinants give rise to white colonies on indicator plates. These properties of recombinant DNA in the vector pUC19 were exploited to generate a series of plasmids that synthesize portions of the NDV HN linked to the α-peptide in a two-stage procedure. In the first stage, a DNA fragment was subcloned to pUC19 in phase at one junction only (the amino-proximal junction in plasmids 4 to 8 of this communication), and white colonies containing recombinant plasmids were selected from indicator plates. In the second stage, the out-of-phase junctions were modified to generate recombinants in phase at both termini, and blue colonies containing the desired plasmids were selected from indicator plates. Each stage in the constructions was monitored by restriction enzyme analysis of small scale plasmid DNA preparations (Birnboim & Doly, 1979) to verify that the correct recombinants had been selected.

Recombinants that contain successively smaller C-terminal portions of the HN gene were also generated. For expression, the cDNA need only be in phase at the N-terminal junction with pUC19, as the hybrid polypeptides terminate at the normal NDV HN stop codon and do not reach the body of the α-peptide coding sequence (Fig. 1, plasmids 1 to 3).

The procedure used to induce synthesis of hybrid polypeptides for Western blot analysis (Fig. 2) was to induce transcription at the lac promoter by adding IPTG to liquid cultures of bacteria, harvest cells at an appropriate optical density and resuspend cell pellets by boiling in sample buffer that contained SDS and 2-mercaptoethanol (Laemmli, 1970). Samples were run on SDS-polyacrylamide gels, blotted onto nitrocellulose (Dunn, 1986; Samson, 1986) and stained with antibodies and peroxidase as previously described (Samson, 1986).

In general, it was considered that positive results on Western blots were of greater predictive value than negative results, as false negatives due to cloning artefacts could not be ruled out without extensive DNA sequencing, even if restriction enzyme analysis and colony colour on indicator plates were consistent with the production of the desired hybrid polypeptide.

Deletion of cDNA towards the C-terminal end of the HN gene suggests that the antibody-binding site was encoded between NarI and ScaI sites (i.e. between amino acids 166 and 387 in the protein sequence; Millar et al., 1986). Progressive deletions of cDNA towards the NarI site (plasmids 4 to 6) suggested that a smaller part of the same region, between the AccI and SmaI sites (residues 317 and 361 in the HN protein sequence) was involved in the binding. Examination of the hydropathy profile of HN (Millar et al., 1986; Fig. 4b) revealed that this region is the most hydrophilic part of the HN polypeptide.

The DNA representing this major hydrophilic peak was subcloned on a RsaI–BalI fragment (amino acid residues 330 to 354) in plasmid 8 and its product gave a positive result in Western blots (Fig. 2, lane 4). This region did not contain convenient restriction sites for further subdivision, so the two most hydrophilic peptides in this region of HN (YKRYNDTCP and DEQDYQIR, amino acid residues 337 to 345 and 346 to 353 respectively) were fused in phase with the α-peptide by the use of synthetic oligonucleotides. Oligonucleotides were synthesized to encode both DNA strands of each of these peptides such that the double-stranded oligonucleotide could be cloned into the plasmid pUC19 cut with HindIII and PstI, but would not regenerate the HindIII site. It was thus possible to screen plasmid DNA from blue colonies for the presence of this oligonucleotide insert by digestion with PvuII to detect an increase in the size of the smaller PvuII restriction fragment relative to that generated from pUC19, and also to screen for the loss of the HindIII site of the smaller PvuII restriction fragment of pUC19. Recombinants with the oligonucleotide encoding the peptide DEQDYQIR gave positive results on Western blots (Fig. 2, lanes 2 and 9). This peptide is also found in HN of NDV strains B1, AV, Italien, D26 and Ulster 2C (Jorgensen et al., 1987; McGinnes et al., 1987; Wemers et al., 1987; Sato et al., 1987; Millar et al., 1988), and is within the region identified previously (Samson et al., 1988). In contrast, recombinants with the oligonucleotide encoding the peptide YKRYNDTCP were negative on Western blots (Fig. 2, lanes 3 and 10).

The entire coding region of the HN gene of a mutant of NDV resistant to MAb 14 (designated 14R-P6) was sequenced directly on genomic RNA by the dideoxynucleotide chain termination
method using reverse transcriptase. A portion of the sequence determined for the wild-type Beaudette C strain of NDV is compared to that of the mutant 14R-p6 (Fig. 3). The only base change detected in the HN sequence was at the second base of the codon for amino acid residue 347 indicating a substitution in the wild-type protein sequence DEQDYQIR to DKQDYQIR in the mutant. Such an amino acid substitution (Glu to Lys) would change the charge of the HN protein and could prevent binding of MAb 14. This result correlates well with the Western blotting data and we believe we have located at least the major part of the epitope for MAb 14.

Two different approaches were used to locate the epitope for MAb 14. The cloning and expression proceeded rapidly once the use of the α-peptide as a carrier had been developed, and most DNA manipulations were performed on small scale plasmid preparations. The plasmids constructed to map the epitope for MAb 14 will be used to map epitopes of other MAbs to the NDV HN protein (Samson et al., 1988). This approach could, in theory, be applied to the location of continuous epitopes in any polypeptide and is not limited to analysing MAbs that neutralize viruses. Selection and sequencing of antibody-resistant mutants gives information on both continuous and discontinuous epitopes but, by definition, its use is limited to polypeptides involved in the neutralization of viruses or other organisms. The largest hybrid polypeptide we constructed was encoded by plasmid 1 and was probably degraded in E. coli since many faint bands were seen beneath a small amount of full-length polypeptide (not shown), but the smaller hybrid polypeptide encoded by plasmid 2 was relatively stable (Fig. 2, lane 8). Plasmid 2 encodes the C-terminal 71% of the HN polypeptide, which includes the highly basic regions present in the stable gp40 fragment produced by breakdown of HN of many strains of NDV (Chambers & Samson, 1982; Millar et al., 1986). Perhaps the stability of the polypeptide encoded by plasmid 2 reflects some folding in E. coli of the C-terminal portion of HN, which we have earlier suggested may form a protease-resistant domain (previously detected as gp40) that might be analogous in structure to the head of the influenza virus haemagglutinin (Millar et al., 1986; Wilson et al., 1981).

Chicken anti-NDV sera inhibit the binding of MAb 14 to HN (Russell & Alexander, 1983), but it remains to be determined whether the antigenic region represented by the MAb 14 binding site plays a major role in the protective immune response of birds to NDV. Pigeons immunized with live NDV B1 vaccine are not well protected against pigeon paramyxovirus isolates which caused recent outbreaks of Newcastle disease in Europe (Alexander et al., 1985) although inactivated NDV Ulster 2C was protective (Alexander et al., 1986). It may be significant that pigeon paramyxovirus isolates do not bind MAb 14 (Alexander et al., 1984), and
the apparent loss of the epitope for this antibody may be a contributory factor in the failure of live NDV B1 vaccine to protect fully against the pigeon paramyxovirus.

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