Glycoconjugate Journal 18, 283–289, 2001

Membrane glycoproteins of Newcastle disease virus: Nucleotide sequence of the hemagglutinin-neuraminidase cloned gene and structure/function relationship of predicted amino acid sequence

Ana Sagrera[†], César Cobaleda[†], Jose M. González De Buitrago[†], Adolfo García-Sastre[‡], Enrique Villar[†,*]

[†]*Departamento de Bioquímica y Biología Molecular Universidad de Salamanca, Plaza Doctores de la Reina s/n, Edificio Departamental, lab 109. E-37007, Salamanca, Spain,* [‡]*Department of Microbiology, Mount Sinai School of Medicine, One Gustave Levy Place, New York, NY, 10029-6574, USA*

The nucleotide sequence of the glycoprotein hemagglutinin-neuraminidase (HN) gene of the Newcastle disease virus (NDV) strain Clone-30 has been determined. The open reading frame of the HN gene contains 1731 nucleotides and encodes a protein of 577 amino acids. Three highly conserved patterns among all paramyxovirus HN glycoproteins, and one additional conserved species-specific region are present. The protein contains five potential *N*-glycosylation sites, all but one located in the C-terminal external domain. The secondary structure prediction shows that the C-terminal external domain is mostly arranged in β-sheets, while α-helices are predominantly located in the N-terminal domain. The nucleotide sequence data of the HN gene reported in this paper has been deposited in the GenBank database, under accession number AF098289.

Keywords: Newcastle disease virus, hemagglutinin-neuraminidase glycoprotein, viral attachment protein, gene sequence, structure/function prediction

Introduction

Newcastle disease virus (NDV) is an avian enveloped single-stranded RNA virus and a member of the genus Rubulavirus of the family *Paramyxoviridae* [1]. The virus consists of an outer lipoprotein membrane that encloses an internal helical nucleocapsid. The membrane is composed of a lipid bilayer, which is acquired from the plasma membrane of infected cell, and three membrane-bound proteins: two transmembranal glycoproteins–the hemagglutinin-neuraminidase glycoprotein (HN), the fusion glycoprotein (F)- and an inner non glycosylated matrix protein (M). The outer domain of both transmembranal glycoproteins, projecting from the external surface of the lipid envelope, play a central role in the infection by the virus since they are essential in the early events occurring in the invasion of host cells: the two consecutive steps of attachment and membrane fusion. The HN glycoprotein is a type II [2] multifunctional glycoprotein, and is the major antigenic determinant of paramyxoviruses [3]. Its ectodomain is responsible for the attachment of the virus to sialic acids in the plasma membrane of target cells, thus acting as a receptor through hemagglutinating activity. In addition, its sialidase (neuraminidase) activity hydrolyses sialic acid-containing macromolecules, which is likely to be crucial in the release of the progeny virions from the remains of the host cells preventing the aggregation of newborn viral particles. Finally, a fusion-promoting activity has been described for the HN glycoprotein [4,5]. The fusion glycoprotein induces viral-host cell membrane fusion but a significant membrane fusion activity is only found when the HN and F glycoproteins interact [6,7]. It has also been shown that the HN and F glycoproteins have a conformation that only exists when both are present within the same viral membrane [8]. Although the transmembrane glycoproteins of NDV and other paramyxoviruses play a crucial role in the early events of the viral infection cycle and although their conformation and physical interaction are of paramount importance for their biological activities, so far little information is available about their three-dimensional structure [9,10].

*To whom correspondence should be addressed: Prof. Enrique Villar, Depto. de.Bioquímica y Biología Molecular, Universidad de Salamanca, Salamanca, Spain. Tel.: +34-923-294465; Fax: +34-923-294579; E-mail: evillar@usal.es

The nucleotide sequences of several genes for different NDV strains have been reported [reviewed in ref. 3]. However none of the sequences of the Clone-30 lentogenic strain genes has been reported previously. Clone-30 strain deserves particular attention since it is obtained from the lentogenic La Sota strain, one of the NDV strains used for efficacious live-virus vaccine [11] and Clone-30 strain itself is also now widely used as a vaccine. In addition, it is known that NDV strains differ in their sialidase and fusigenic activities, specially among lentogenic (*e.g.* Clone-30) and velogenic strains. A relationship between changes in the predicted amino acid sequence of HN with an increase in thermostability of NDV vaccine has been reported [12], supporting the idea that knowledge about the residues directly involved in pathogenicity and stability and about the conformation of the HN glycoprotein is crucial for the design of new vaccines and therapies against NDV infections. In this paper we describe for the first time the complete nucleotide sequence of the hemagglutinin-neuraminidase gene from the lentogenic Clone-30 strain of NDV. Also, the structure of the HN glycoprotein is discussed from the predicted amino acid sequence in terms of the predicted secondary structures. These data may be important for the future design of NDV mutants for the development of new vaccines for use in the poultry industry. Also, knowledge of HN sequence may provide a tool for the analysis of the involvement of specific regions in the biological functions of the protein, as well as in the evolutionary relationships between NDV and other members of the *Paramyxoviridae* and other families such as *Orthomyxoviridae* and *Rhabdoviridae*.

Materials and methods

The lentogenic strain Clone-30 of NDV was grown at 37°C for 48 h in the allantoic cavity of 11-day-old specific-pathogen-free chick embryos. The virus was purified exactly as previously described [13]. Virion RNA was extracted using proteinase K (Boehringer-Mannheim) and purified as described [14]. The open reading frame (ORF) of the HN gene was obtained by RT-PCR of the virion RNA. The cDNA was synthesized using SuperScript™ RNase H^- reverse transcriptase (Life-Technologies), and the positive-sense primer, 5′CCGGCCATGGACCGCGCAGTTAAGA, complementary to the established sequence to the start of the ORF of the HN gene of NDV strain Beaudette C [15]. For PCR, this primer and a negative-sense primer corresponding to the start of the L gene (contiguous in viral RNA to the 3′ end of the HN gene) of NDV strain Beaudette C [16], 5′GCGCGTCGACGGACCGGAGCTCGCCAT, were used and the reaction was performed with the Expand™-High Fidelity PCR system (Boehringer-Mannheim). The PCR product was cloned into pGEM®-T Easy vector system (Promega) and automatically sequenced in an ABIPRISM Model 377 sequencer (Figure 1).

The amino acid sequence was predicted at http://embl-heidelberg.de/predictprotein Physicochemical parameters such as molecular mass (M_r), isoelectric point and charge were estimated using the ProtParameter program. As previously described [17], the hydropathy profile was obtained with the ProtScale program at http://www.expasy.ch/tools/protpar-ref.html:window (#9); relative weight of the windows edges compared to the window center (100%); weight variation modes (linear); normalized dats 0–1 (yes). The prediction of transmembrane segments was accomplished using the program HMMTOP [18]. All these programs are available from the web at http://www.expasy.ch/. The secondary structure prediction was estimated using the information in the "Protein Analysis Server" available at http://bmerc-www.bu.edu/psa.

Results and discussion

The ORF of the NDV "Clone-30" HN gene has a length of 1731 nt and contains a coding region of 577 amino acid residues, with a predicted M_r of 63.3 kDa for the translated polypeptide. The estimated M_r is in agreement with the previously predicted M_r of 67 kDa [19] for the unglycosylated HN form in the Australia-Victoria strain NDV-infected cells in the presence of tunicamycin. The addition of oligosaccharides to HN molecule ocurrs only by *N*-glycosylation [20]. Glycosylation is an extremely important event for viral glycoproteins since it may affect their biological properties and the antigenicity of the virus itself which can alter the host-cell tropism of the virus and its degree of virulence [21]. Analysis of the sequence of the NDV Clone-30 HN revealed five potential asparagine-linked *N*-glycosylation sites at positions 119, 341, 433, 481, and 538. All of them but site 119 are in the C-terminal external region, as has been described for HN in other paramyxoviruses [22]. The presence of an aspartic acid residue at position 342 makes site 2 less favourable for glycosylation [23]. In the Beaudette strain of NDV [15] there is one additional potential glycosylation site, NPT (Asparagine-Proline-Threonine), at positions 500–502, which is the only site conserved between NDV, Sendai virus and SV5. In our case, the Clone-30 strain, residue 502 is alanine instead of threonine, meaning that the additional glycosylation site should not exist.

It has been predicted that for at least two different paramyxoviruses the structure of HN is organized as a so-called β-sheet propeller [24,25]. This structure has been confirmed from X-ray data [10]. The folding consists of six similarly folded antiparallel β-sheets of four strands each, which are connected by loops. The loops connecting the last strand of one β-sheet with the first strand of the next β-sheet are located on the top of the molecule. The sheets are arranged cyclically around an axis through the center of the molecule. According to the three-dimensional model for the HN protein of bPIV-3 [24] the glycosylation sites are located on the surface of the molecule and mostly on the loops of the top.

```
ATGGACCGCGCAGTTAGCCAAGTTGCGTTAGAGAATGATGAAAGAGAGGCAAAAAATACATGGCGCTTGATATTCCGGATT
  M  D  R  A  V  S  Q  V  A  L  E  N  D  E  R  E  A  K  N  T  W  R  L  I  F  R  I 27
GCAATCTTATTCTTAACAGTAGTGACCTTGGCTATATCTGTAGCCTCCCTTTTATATAGCATGGGGGCTAGCACACCTAGC
  A  I  L  F  L  T  V  V  T  L  A  I  S  V  A  S  L  L  Y  S  M  G  A  S  T  P  S 54
GATCTTGTAGGCATACCGACTAGGATTTCCAGGGCAGAAGAAAAGATTACATCTACACTTGGTTCCAATCAAGATGTAGTA
  D  L  V  G  I  P  T  R  I  S  R  A  E  E  K  I  T  S  T  L  G  S  N  Q  D  V  V 81
GATAGGATATATAAGCAAGTGGCCCTTGAGTCTCCGTTGGCATTGTTAAATACTGAGACCACAATTATGAACGCAATAACA
  D  R  I  Y  K  Q  V  A  L  E  S  P  L  A  L  L  N  T  E  T  T  I  M  N  A  I  T 108
TCTCTCTCTTATCAGATTAATGGAGCTGCAAACAACAGTGGGTGGGGGGCACCTATCCATGACCCAGATTATATAGGGGGG
  S  L  S  Y  Q  I  N  G  A  A  N  N  S  G  W  G  A  P  I  H  D  P  D  Y  I  G  G 135
ATAGGCAAAGAACTCATTGTAGATGATGCTAGTGATGTCACATCATTCTATCCCTCCGCATTTCAAGAACATCTGAATTTT
  I  G  K  E  L  I  V  D  D  A  S  D  V  T  S  F  Y  P  S  A  F  Q  E  H  L  N  F 162
ATCCCGGCGCCTACTACAGGATCAGGTTGCACTCGAATACCCTCATTTGACATGAGTGCTACCCATTACTGCTACACCCAT
  I  P  A  P  T  T  G  S  G  C  T  R  I  P  S  F  D  M  S  A  T  H  Y  C  Y  T  H 189
AATGTAATATTGTCTGGATGCAGAGATCACTCACATTCATATCAGTATTTAGCACTTGGTGTGCTCCGGACATCTGCAACA
  N  V  I  L  S  G  C  R  D  H  S  H  S  Y  Q  Y  L  A  L  G  V  L  R  T  S  A  T 216
GGGAGGGTATTCTTTTCTACTCTGCGTTCCATCAACCTGGACGACACCCAAAATCGGAAGTCTTGCAGTGTGAGTGCAACT
  G  R  V  F  F  S  T  L  R  S  I  N  L  D  D  T  Q  N  R  K  S  C  S  V  S  A  T 243
CCCCTGGGTTGTGATATGCTGTGCTCGAAAGTCACGGAGACAGAGGAAGAAGATTATAACTCAGCTGTCCCTACGCGGATG
  P  L  G  C  D  M  L  C  S  K  V  T  E  T  E  E  E  D  Y  N  S  A  V  P  T  R  M 270
GTACATGGGGGGTTAGGGTTCGACGGCCAGTACCACGAAAAGGACCTATATGTCACAACATTATTCGGGGACTGGGTGGCC
  V  H  G  G  L  G  F  D  G  Q  Y  H  E  K  D  L  Y  V  T  T  L  F  G  D  W  V  A 297
AACTACCCATTAGTATGGGGTGGATCTTTTATTGACAGCCGCGTATGGTTCTCAGTCTACGGAGGGTTAAAACCCAATTCA
  N  Y  P  L  V  W  G  G  S  F  I  D  S  R  V  W  F  S  V  Y  G  G  L  K  P  N  S 324
CCCAGTGACACTGTACAGGAAGGGAAATATGTGATATACAAGCGATACAATGACACATGCCCAGATGAGCAAGACTACCAG
  P  S  D  T  V  Q  E  G  K  Y  V  I  Y  K  R  Y  N  D  T  C  P  D  E  Q  D  Y  Q 351
ATTCGAATGGCCAAGTCTTCGTATAAGCCTGGACGGTTTGGTGGGAAACGCATACAGCAGGCTATCTTATCTATCAAGGTG
  I  R  M  A  K  S  S  Y  K  P  G  R  F  G  G  K  R  I  Q  Q  A  I  L  S  I  K  V 378
TCAACATCCTTAGGCGAAGACCCGGTACTGACTGTACCGCCCAACACAGTCACACTCATGGGGGCCGAAGGCAGAATTCTC
  S  T  S  L  G  E  D  P  V  L  T  V  P  P  N  T  V  T  L  M  G  A  E  G  R  I  L 405
ACAGTAGGGACATCTCATTTCTTGTATCAACGAGGGTCATCATACTTCTCTCCCGCGTTATTATATCCTATGACAGTCAGC
  T  V  G  T  S  H  F  L  Y  Q  R  G  S  S  Y  F  S  P  A  L  L  Y  P  M  T  V  S 432
AACAAAACAGCCACTCTTCATAGTCCTTATACATTCAATGCCTTCACTCGGCCAGGTAGTATCCCTTGCCAGGCTTCAGCA
  N  K  T  A  T  L  H  S  P  Y  T  F  N  A  F  T  R  P  G  S  I  P  C  Q  A  S  A 459
AGATGCCCCAACTCGTGTGTTACTGGAGTCTATACAGATCCATATCCCCTAATCTTCTATAGAAACCACACCTTGCGAGGG
  R  C  P  N  S  C  V  T  G  V  Y  T  D  P  Y  P  L  I  F  Y  R  N  H  T  L  R  G 486
GTATTCGGGACAATGCTTGATGGTGTACAAGCAAGACTTAACCCTGCGTCTGCAGTATTCGATAGCACATCCCGCAGTCGC
  V  F  G  T  M  L  D  G  V  Q  A  R  L  N  P  A  S  A  V  F  D  S  T  S  R  S  R 513
ATTACTCGAGTGAGTTCAAGCAGTACCAAAGCAGCATACACAACATCAACTTGTTTTAAAGTGGTCAAGACTAATAAGACC
  I  T  R  V  S  S  S  S  T  K  A  A  Y  T  T  S  T  C  F  K  V  V  K  T  N  K  T 540
TATTGTCTCAGCATTGCTGAAATATCTAATACTCTCTTCGGAGAATTCAGAATCGTCCCGTTACTAGTTGAGATCCTCAAA
  Y  C  L  S  I  A  E  I  S  N  T  L  F  G  E  F  R  I  V  P  L  L  V  E  I  L  K 567
GATGACGGGGTTAGAGAAGCCAGGTCTGGCTAGTTGAGTCAATTATAAAGGAGTTGGAAAGATGGCATTGTATCACCTATC
 D  D  G  V  R  E  A  R  S  G
TTCTGCGACATCAAGAATCAAACCGAATGCCGGCGCGTGCTCGAATTCCATGTTGCCAGTTGACCACAATCAGCCAGTGCT

CATGCGATCAGATTAAGCCTTGTCAATAGTCTCTTGATTAAGAAAAAA
```

Figure 1. Nucleotide sequence and deduced amino acid sequence of Clone-30 strain NDV HN gene. Residues conserved among all paramyxovirus HN proteins are in boldface. Potential glycosylation sites are underlined.

It has been described [26] that glycosylation sites at positions 341, 433 and 481 could be accomodated in this structure and could be located at the side or the bottom of the tetramer structure, while site 500 (conserved in several NDV strains, as well as Sendai virus and SV5) remains hidden in the interior of the structure. The last glycosylation site in the C-terminal region, at position 538, must be located in a region involved in the tetramerization process [27].

The oligomerization of HN is not common to all NDV strains [28,29] and to date it is not clear which domain is

involved in tetramer formation. In most cases NDV HN glycoproteins have a residue at position 123, located in the predicted stalk region in the external domain, which is believed to form an intramolecular disulfide with another HN ectodomain to form a homodimer [29–31]. Also, it has been proposed that the cysteine residue at position 6 in the cytoplasmic domain must be involved in the process [30] although studies with the Kansas NDV strain [32] suggest that the transmembrane and/or the external domains must be involved in the process, rather than the cytoplasmic domain. This is in agreement with the importance given [26] to the transmembrane domain in the tetramerization of Australia-Victoria NDV HN protein. A variability analysis of 25 viral HN aligned sequences, including NDV [33], revealed that the highest rate of variability was found near to the transmembrane domain, which seemed to be involved in the formation or stabilization of the functional tetramer structure. Recently, a detailed study [34] elucidated the disulfide bond arrangement of the Queensland strain NDV HN glycoprotein, confirming that HN ectodomains dimerize via cysteine 123. We found that the sequence of NDV Clone-30 HN protein contains 12 cysteine residues at positions 172, 186, 196, 238, 247, 251, 344, 455, 461, 465, 531 and 542, which are conserved in all NDV strains [26]. However, cysteine residues at position 6 and 123 are not present, suggesting that NDV Clone-30 HN glycoprotein do not form disulfide-linked oligomers. In fact, our data from polyacrylamide gel electrophoresis analysis under reducing and non-reducting conditions showed no evidence of the presence of any disulfide-linked oligomers (data not shown). If any oligomerization does occur, this process may not be linked to intramolecular disulfide bond formation. Previous data [35] from our lab, using sucrose gradient ultracentrifugation also support the idea that Clone-30 HN behaves as a monomer. However, we are currently investigating this topic. It has been described that the oligomerization state affects the biological properties of the viral glycoproteins [36]. In a previous work we reported [37] that the kinetics of the sialidase activity of HN in NDV strain Clone-30 followed a substrate-inhibition model instead of the classical hyperbolic model. So far it is not clear whether the possible absence of an oligomeric structure in Clone-30 NDV HN could be involved in the above-mentioned different sialidase kinetic mechanism.

Unless crystallographic data are available, it is not possible to assign specific regions of the sequence to the biological activities of the HN glycoprotein. Most studies conducted in this field mainly rely on homologies obtained from comparison analysis with HN glycoproteins from other paramyxoviruses and with the NA and HA proteins of influenza viruses [24,38,39]. It has been described that in proteins with functional similarity a convergent evolution might be operating, thus resulting in conservation of the residues involved in the maintenance of active site. This leads to a similar three-dimensional clustering of these active elements. Furthermore the framework elements (amino acid residues) that do not play a direct role in the active site (*i.e.*, those that do not contact with the substrate) are not necessarily conserved [39]. A description of the NDV Kansas strain HN (23xxx), from crystallographic studies has been published recently. However, this HN from Kansas strain behaves as a homodimer. Crystallographic data show for the globular region an overall structure consisting of a six-bladed β-propeller fold, supporting the accuracy of the studies based on sequence homologies.

There are three highly conserved regions in paramyxovirus HN proteins [10,40] that are also present in our protein. The first one, NRKSCSI/V/L, is located at positions 234–240 [38], and has been identified as a part of the active site of the neuraminidase activity of the NA protein of influenza virus. It has also been suggested to be involved in the same activity in the HN of paramyxoviruses [39]. This region in bPIV-3 is located on the loop that connects strand 4 of β-sheet 6 with strand 1 of β-sheet 1, at the top of the molecule [24]. The other two conserved regions seem to be involved in hemagglutinating activity: the residues at positions 314 to 320, FXXYGGV/L/M, at the start of the sequence predicted to be the hemagglutinat-

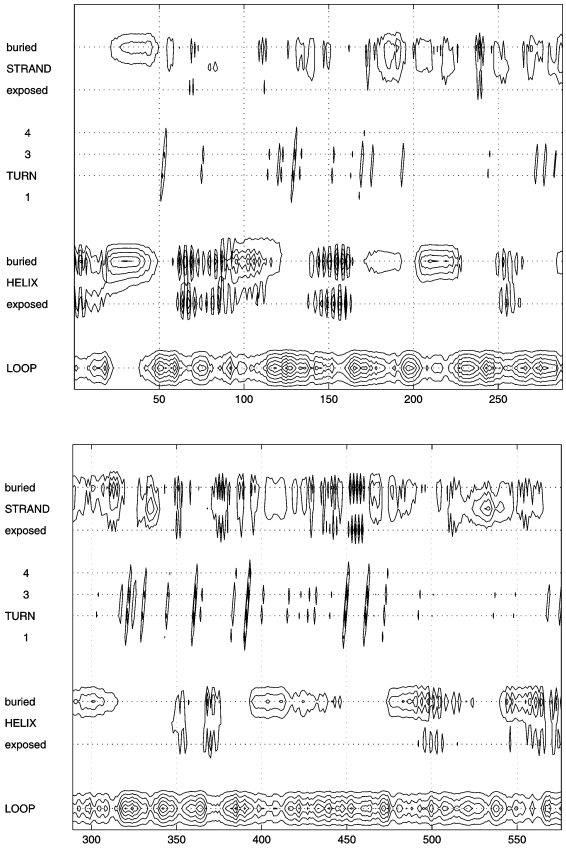

Figure 2. Topographic maps of the secondary-structure probabilities for Clone-30 strain NDV HN. Areas of high probability in the contour plot correspond to dark regions with closely spaced contour lines. The contour lines show probability increments of 0.1.

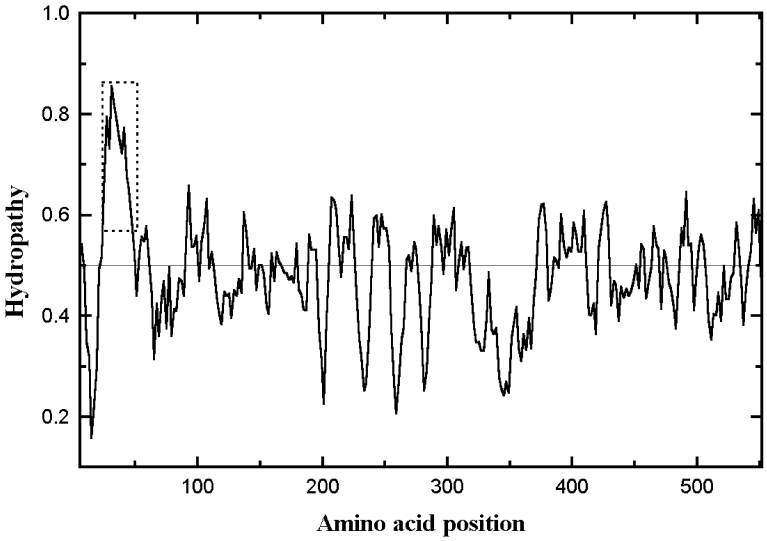

Figure 3. Hydropathy profile of the HN amino acid sequence, using the procedure of Kyte and Doolittle (1982). A window of 11 amino acids was used in the calculation. The residues corresponding to the transmembrane region are boxed. Hydrophobic and hydrophilic regions are indicated respectively by peaks above and below the horizontal line, which represents the relative average hydropathy of the sequenced proteins.

ing domain, and the residues GA/SEGRI/V/L, 399 to 405, at the end of the region. Point mutations in this sequence [5] cause alterations in the maturation process of the HN protein by affecting intracellular processing, which could interfere with the correct folding of the molecule. Finally, the residues at positions 15 to 23, REAKNTWRL, are consistent [33] with the species-specific pattern for NDV.

Figure 2 shows the probability of secondary structures predicted for the HN protein from NDV strain Clone-30. The β-sheets are mostly in the C-terminal domain, while in the N-terminal domain α-helices are predominant and are in aggrement with those previously described [25]. Comparison of the HN protein sequences from NDV strain Clone-30 described here with those of D26/76 [41] revealed 94% homologies (results not shown). Our results, according to the three-dimensional model described for the HN proteins from NDV strains D26/76 [25] and Kansas [10] and bPIV-3 [24], suggest that the HN protein from NDV strain Clone-30 is organized as a so-called β-sheet propeller.

The contribution of basic and acidic residues of the Clone-30 strain NDV HN protein was similar (9%) although they were asymetrically distributed within the protein, with their positive charge located in the C-terminal region, and their negative charge in the N-terminal region. The isoelectric point, 7.3, was practically neutral. The hydropathy profile of the HN protein (Figure 3) showed the presence of a short hydrophilic region at the N-terminus, corresponding to the cytoplasmic domain (14x), and a major hydrophobic region including residues 27 to 48, which contained the only transmembrane helix in the molecule, corresponding to the membrane-spanning segment.

Acknowledgments

This work was partially supported by Grants PM97-0160 from the Dirección General de Enseñanza Superior (DGES) of the Spanish Government and SA62/99 from the Junta de Castilla y Leon to E.V. We thank E. Diaz de Espada and J.A. Rodriguez Hernández, from Intervet Laboratories, Salamanca, Spain, for providing the lentogenic "Clone-30" strain. A.S. and C.C*. are fellowship holders of the *Plan de Formación del Profesorado Universitario y Personal Investigador*, Ministerio de Educación y Cultura, Spain. Thanks are also due to Mr. N.S.D. Skinner for proof-reading the manuscript.

References

1 Murphy FA, Fauquet CM, Bishop DHL, Ghabrial SA, Jarvis AW, Marticelli GP, Mayo MA, Summers MD (eds), *The Classification and Nomenclature of Viruses: Sixth Report of the ICTV*, (Springer-Verlag, Vienna & New York, 1995).

2 Schuy W, Garten W, Linder D, Klenk HD, The carboxyterminus of the hemagglutinin-neuraminidase of Newcastle disease virus is exposed at the surface of the viral envelope, *Virus Res* **1**, 415–26 (1984).

3 Lamb RA, Kolakofsky D, Paramyxoviridae: the viruses and their replication. In *Fields Virology*, 3rd edition, edited by Fields BN, Knipe DM, Howley PM, (Lippincot-Raven, Philadelphia, 1996), pp. 1177–204.

4 Lamb RA, Paramyxovirus fusion: a hypothesis for changes, *Virology* **197**, 1–11 (1993).

5 Sergel T, McGinnes LW, Peeples ME, Morrison TG, The attachment function of the Newcastle disease virus hemaggluti-

nin-neuraminidase protein can be separated from fusion promotion by mutation, *Virology* **193**, 717–26 (1993).
6 Horvath CM, Paterson RG, Shaughnessy MA, Wood R, Lamb RA, Biological activity of paramyxovirus fusion proteins: factors affecting formation of syncitia, *J Virol* **66**, 4564–9 (1992).
7 Stone-Hulslander J, Morrison TG, Detection of an interaction between the HN and F proteins in Newcastle disease virus-infected cells, *J Virol* **71**, 6287–95 (1997).
8 Citovsky V, Yanai P, Loyter A, The use of circular dichroism to study conformational changes induced by Sendai virus envelope glycoproteins: a correlation with the viral fusogenic activity, *J Biol Chem* **261**, 2235–9 (1986).
9 Takimoto T, Taylor GL, Crennell SJ, Scroggs RA, Portner A, Crystallization of Newcastle disease virus hemagglutinin-neuraminidase glycoprotein, *Virology* **270**, 208–14 (2000).
10 Crennell S, Takimoto T, Portner A, Taylor G, Crystal structure of the multifunctional paramyxovirus hemagglutinin-neuraminidase, *Nature Structural Biology* **7**, 1068–74 (2000).
11 Alexander DJ, In *Diseases of Poultry, 9th ed.*, edited by Calnek BW, Barnes HJ, Beard CW, Reid WM and Yoder HW (Iowa State University Press, Ames, Iowa, 1991), pp. 486–519.
12 Seal BS, King DJ, Bennet JD, Characterization of Newcastle disease virus vaccines by biological properties and sequence analysis of the hemagglutinin-neuraminidase protein gene, *Vaccine* **14**, 761–6 (1996).
13 Shnyrov VL, Zhadan GG, Cobaleda C, Sagrera A, Muñoz-Barroso I, Villar E, A differential scanning calorimetric study of Newcastle disease virus: identification of proteins involved in thermal transitions, *Arch Biochem Biophys* **341**, 89–97 (1997).
14 Sambrook J, Fristsch EF, Maniatis T, *In Molecular Cloning. A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989.
15 Millar NS, Chambers P, Emmerson P, Nucleotide sequence analysis of the hemagglutinin-neuraminidase gene of Newcastle disease virus, *J Gen Virol* **67**, 1917–27 (1986).
16 Yusoff K, Millar NS, Chambers P, Emmerson PT, Nucleotide sequence analysis of the L gene of Newcastle disease virus: homologies wirh Sendai and Vesicular stomatiti viruses, *Nucleic Acids Res* **15**, 3961–76 (1987).
17 Kyte J, Doolittle RF, A simple method for displaying the hydropathic character of a protein, *J Mol Biol* **157**, 105–32 (1982).
18 Túsnady GE, Simon I, Principles governing amino acid composition of integral membrane proteins: application to topology prediction, *J Mol Biol* **283**, 489–506 (1998).
19 Morrison T, Simpson D, Synthesis, stability and cleavage of Newcastle disease virus glycoproteins in absence of glycosylation, *J Virol* **36**, 171–80 (1980).
20 Nakamura K, Homma M, Compans RW, Effect of tunicamycin on the replication of Sendai virus, *Virology* **119**, 474–87 (1982).
21 Schulze IT, Manger ID, Viral glycoprotein heterogeneity-enhancement of functional diversity, *Glycoconj J* **9**, 63–6 (1993).
22 Shioda T, Iwaski K, Shibuta H, Determination of the complete nucleotide sequence of the Sendai virus genome RNA and the predicted amino acid sequences of the F, HN and L proteins, *Nucleic Acids Res* **14**, 1545–63 (1986).
23 Ronin C, Bouchilloux S, Garnier C, van Rietschoten J, Enzymatic *N*-glycosyation of synthetic Asn-X-Thr containing peptides, *FEBS Lett* **96**, 179–82 (1978).
24 Langedijk JP, Daus FJ, Oirschot JT, Sequence and structure alignment of Paramyxoviridae attachment proteins and discovery of enzymatic activity for a morbillivirus hemagglutinin, *J Virol* **71**, 6155–67 (1997).
25 Epa VC, Modeling the paramyxovirus hemagglutinin-neuraminidase protein, *Proteins Struct Funct Genet* **29**, 264–81 (1997).
26 McGinnes LW, Morrison TG, Modulation of the activities of HN protein of Newcastle disease virus by nonconserved cysteine residues, *Virus Res* **34**, 305–16 (1994).
27 McGinnes LW, Morrison TG, The role of individual oligosaccharide chains in the activities of the HN glycoprotein of Newcastle disease virus, *Virology* **212**, 398–410 (1995).
28 Markwell MAK, Fox CF, Protein–protein interactions within paramyxoviruses identified by native disulfide bonding or reversible chemical cross-linking, *J Virol* **33**, 152–66 (1980).
29 Sheehan JP, Iorio RM, Sydall RJ, Glickman RL, Bratt MA, Reduced agent-sensitive dimerization of the hemagglutinin-neuraminidase glycoprotein of Newcastle disease virus correlates with presence of cysteine at residue 123, *Virology* **161**, 603–6 (1987).
30 Mirza AM, Sheehan JP, Hardy LW, Glickman RL, Iorio RM, Structure and function of a membrane anchor-less form of the hemagglutinin-neuraminidase glycoprotein of Newcastle disease virus, *J Biol Chem* **268**, 21425–31 (1993).
31 Thompson SD, Portner A, Localization of functional sites on the hemagglutinin-neuraminidase glycoprotein of Sendai virus by sequence analysis of antigenic and temperature sensitive mutants, *Virology* **160**, 1–8 (1987).
32 Takimoto T, Bousse T, Coronel EC, Scroggs RA, Portner A, Cytoplasmic domain of Sendai virus HN protein contains a specific sequence required for its incorporation into virions, *J Virol* **72**, 9747–54 (1998).
33 Zenteno-Cuevas R, Hernández J, Espinosa B, Reyes J, Zenteno E, Secondary structure prediction of the haemagglutinin-neuraminidase from a porcine rubulavirus, *Arch Virol* **143**, 333–52 (1998).
34 Pitt JJ, Da Silva E, Gorman JJ, Determination of the disulfide bond arrangement of Newcastle disease virus hemagglutinin neuraminidase, *J Biol Chem* **275**, 6469–78 (2000).
35 García-Sastre A, Cabezas JA, Villar E, Proteins of Newcastle didease virus: interaction between the outer hemagglutinin-neuraminidase glycoprotein and the inner non-glycosylated matrix protein, *Bichim Biophys Acta* **999**, 171–5 (1989).
36 McGinnes LW, Morrison TG, The role of individual cysteine residues in the formation of the mature, antigenic HN protein of Newcastle disease virus, *Virology* **200**, 470–83 (1994).
37 Sagrera A, Cobaleda C, Muñoz-Barroso I, Shnyrov V, Villar E, Modulation of the neuraminidase activity of the HN protein from Newcastle disease virus by substrate binding and conformational change: kinetic and thermal denaturation studies, *Biochem Mol Biol Int* **37**, 717–27 (1995).
38 Jorgensen ED, Collins PL, Lomedico PT, Cloning and nucleotide sequence of Newcastle disease virus hemagglutinin-neuraminidase mRNA: identification of a putative sialic acid binding site, *Virology* **156**, 12–24 (1987).
39 Colman PM, Hoyne PA, Lawrence MC, Sequence and structure alignment of paramyxovirus hemagglutinin-neuraminidase with influenza virus neuraminidase, *J Virol* **67**, 2972–80 (1993).
40 Morrison TG, Portner A, Structure, function and processing of the glycoproteins of paramyxoviridae. In *The Paramyxoviruses*,

edited by Kingsbury, DW, (Plenum Publishing Co, New York, 1991), pp. 347–82.

41 Gotoh B, Sakaguchi T, Nishikawa K, Inocencio NM, Hamaguchi M, Toyoda T, Nagai Y, Structural features unique to each of the three antigenic sites on the hemagglutinin-neuraminidase protein of Newcastle disease virus, *Virology* **163**, 174–82 (1988).

Received 21 June 2000; revised 3 August 2001; accepted 14 August 2001