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Nucleotide sequences of the trailer, nucleocapsid protein gene and intergenic regions of Newcastle disease virus strain Beaudette C and completion of the entire genome sequence

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The nucleotide sequences of the nucleocapsid protein (NP) gene, the intergenic regions in the nucleocapsid protein (NP)–phosphoprotein (P), P–matrix protein (M) and M–fusion glycoprotein gene junctions and the trailer region of a virulent Newcastle disease virus (NDV) strain Beaudette C were determined. The NP gene is 1747 nt long and encodes a protein of 489 amino acids. Each of the intergenic sequences determined is 1 nt long and, including the previously published intergenic sequences, the gene junction sequences varied in length from 1–47 nt and lacked any sequence identity. The 5′ trailer region is 113 nt in length. Comparison of the sequences of the terminal leader and trailer regions of Beaudette C strain with those of nonvirulent strain B1 showed a high level of conservation, indicating the likelihood of these elements not being a factor in virulence. Together with previously published data, this report completes the sequence of the 15 186 nt genomic RNA of NDV strain Beaudette C.

Newcastle disease virus (NDV) causes a highly contagious and fatal disease affecting all species of birds. The virus is found as a variety of strains which differ widely in virulence, from causing a mild asymptomatic infection to causing a highly fatal disease (Alexander, 1996). Based on the severity of disease produced in chickens, NDV strains have been categorized into three main pathotypes: lentogenic, mesogenic and velogenic strains. Lentogenic strains are avirulent and may cause mild or inapparent respiratory infection. Mesogenic strains are of intermediate virulence and cause respiratory symptoms with low mortality, whereas velogenic strains are highly virulent and cause high mortality.

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The nucleotide sequence data of the NP gene reported in this paper have been submitted to the GenBank database and assigned the accession number AF064091.

The virus is a member of the genus *Rubulavirus* of the family *Paramyxoviridae* and contains a single-stranded negative-sense RNA genome (Murphy *et al.*, 1995). The genomic RNA encodes at least seven proteins (Peeples, 1988; Stewad *et al.*, 1993): the nucleocapsid protein (NP), the phosphoprotein (P), the large protein (L), the fusion glycoprotein (F), the haemagglutinin–neuraminidase glycoprotein (HN), the matrix protein (M) and the V protein. Flanking the genes are the 3′ extracistronic region, known as the leader region, and the 5′ extracistronic region, known as the trailer region. These leader and trailer regions have been thought to be *cis*-acting regulatory elements in replication, transcription and packaging of the genomic and antigenomic RNA (Lamb & Kolakofsky, 1996).

Nucleotide sequences of several genes for different NDV strains are available, but the complete genomic sequence has not been established for any strain of NDV. In this respect, the Beaudette C strain of NDV, a neurotropic velogenic strain, has been the most well-characterized, with the majority of the nucleotide sequences already available. The complete nucleotide sequence of five genes, namely P (Daskalakis *et al.*, 1992), M (Chambers *et al.*, 1986*c*), F (Chambers *et al.*, 1986*b*), HN (Millar *et al.*, 1986) and L (Yusoff *et al.*, 1987), the sequences of the intergenic regions in the F–HN (Chambers *et al.*, 1986*b*) and HN–L (Chambers *et al.*, 1986*a*) junctions, the sequence of the leader region (Kurilla *et al.*, 1985), a partial sequence of the trailer region (first 49 nt; Yusoff *et al.*, 1987) and a partial sequence of the NP gene (first 192 nt; Kurilla *et al.*, 1985) have been reported. Only the availability of the complete sequence of the NDV genome will enable reverse genetic approaches to rescue infectious NDV from cloned cDNA. The potential for this application and many others should stimulate further research towards a complete understanding of this important veterinary pathogen. Bearing this perspective in mind, we report here the remaining unestablished sequences of the NDV Beaudette C strain and compare them to related viruses. These

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sequences include those of the complete NP gene, the entire trailer region and the intergenic regions in NP–P, P–M and M–F junctions.

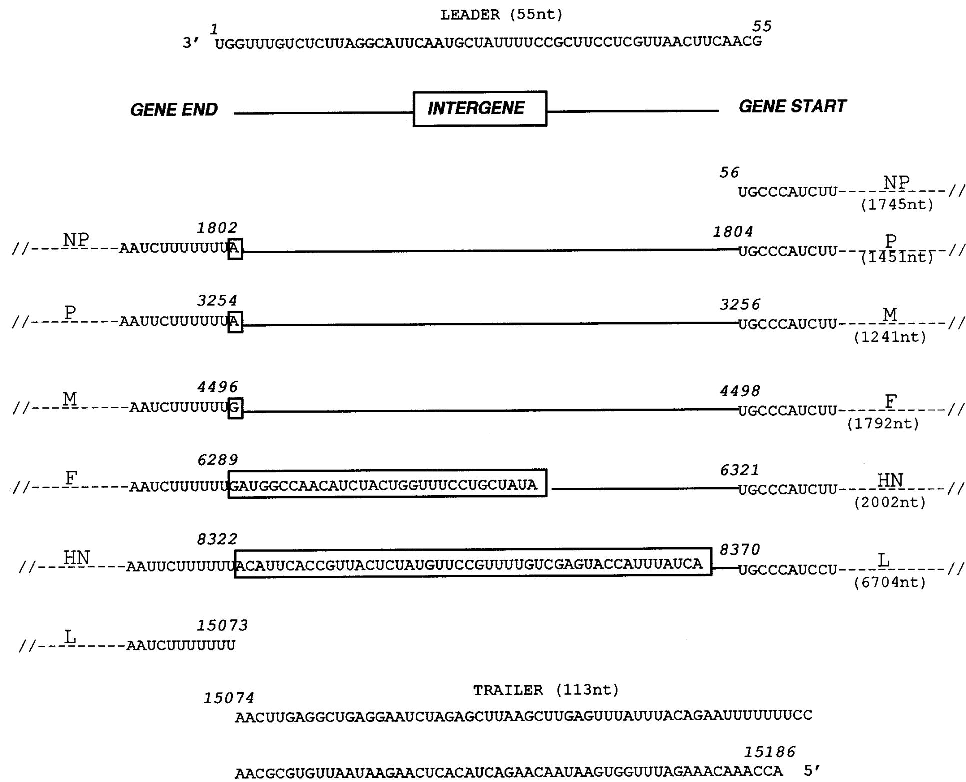
NDV strain Beaudette C was received from the National Veterinary Services Laboratory (Ames, IA, USA) and was propagated in the allantoic cavity of embryonated chicken eggs. The virus was purified as described previously (Kingsbury, 1966). The virion RNA was extracted using proteinase K and TRIzol reagent (Life Technologies). The NP gene, intergenic regions and 5′ trailer region were obtained by RT–PCR of the virion RNA. The cDNAs were synthesized using SuperScript II reverse transcriptase (Life Technologies). The cDNA corresponding to the NP gene was synthesized utilizing a positive-sense primer, 5′ GAAGGTGTGAATC-TCGAGTGCG, complementary to the established sequence at the start of the NP gene. This primer and a negative-sense primer corresponding to the 3′ end of the P gene, 5′ GCTCGTCGATCTCCGCATCTGT, were used in PCR with high fidelity *Pfu* DNA polymerase (Stratagene). The PCR product was cloned and sequenced by the dideoxynucleotide chain termination method. For obtaining cDNAs corresponding to the intergenic regions, the positive-sense oligonucleotide primer was derived from a sequence upstream of the respective gene junction. Likewise, the negative-sense primer was derived from a sequence downstream of that gene junction. The PCR product was cloned and sequenced by the dideoxynucleotide chain termination method.

The 5′ trailer region was cloned using the 5′ RACE method (Dorit, 1995). Briefly, a positive-sense primer, 5′ CACTA-AGGACATACTTGAAGC, complementary to the downstream end of the L gene was extended with reverse transcriptase and the resulting cDNAs were tailed with dCTP, and separately with dGTP, using terminal deoxynucleotidyl transferase. The cDNAs were then amplified by PCR by using the L gene-specific primer described above and either oligo(dG) primer for reactions tailed with dC, or oligo(dC) primer for reactions tailed with dG. The PCR products were then cloned and sequenced by the dideoxynucleotide chain termination method. Tailing reactions with C and G residues assured unambiguous determination of the 5′ terminal nucleotide. To sequence the 3′ leader region, virion RNA was ligated to a synthetic RNA, and cDNA was made using RT–PCR. The PCR product was cloned and sequenced by the dideoxynucleotide chain termination method.

The complete nucleotide sequence of the NP gene of NDV strain Beaudette C is 1747 nt long, including non-coding regions of 66 nt at the 3′ end and 211 nt at the 5′ end of the gene. The major open reading frame (ORF) of 1467 nt, extending from position 122 to 1588 of the genomic RNA sequence, contained a coding region of 489 amino acid residues. The predicted molecular mass of the polypeptide encoded in the ORF is 53 790 daltons. This sequence and amino acid length is consistent with the NP gene sequence of U2C (accession no. Z30084) and published sequence of D26 (Ishida *et al*., 1986). The NDV NP protein has a net charge of −14 at neutral pH, assuming histidine has a charge of +0·5, arginine and lysine each +1 and glutamic and aspartic acid each −1 at this pH. This unexpected characteristic of the RNA-binding protein has been reported for other paramyxoviruses (Parks *et al*., 1992; Sanchez *et al*., 1986).

A comparison of the NP gene sequences of the Beaudette C strain with those of the lentogenic strains D26 and U2C revealed 89·1% and 88·7% identity, respectively. The 3′ non-coding sequence of the NP mRNA showed considerable variation between the virulent and nonvirulent strains. The NP mRNA sequence of strain Beaudette C showed 31% variation in this region with strain D26 and 29% with strain U2C. One notable feature is that the NP proteins of the two lentogenic strains (D26 and U2C) revealed a close identity of more than 99% between the two, compared to a lower level of identity between the NP proteins of the virulent strain Beaudette C and the nonvirulent strain D26 (96·9%) or U2C (96·1%). It has been observed that the nucleocapsid proteins of many paramyxoviruses are much more conserved over the first 400 amino acids, with a very low level of identity in the last 125 amino acids (Sanchez *et al*., 1986; Rozenblatt *et al*., 1985). This variation has also been observed among the nucleocapsid proteins of four strains of rinderpest virus (Baron & Barrett, 1995). However, when the NP protein sequence of NDV strain Beaudette C was compared with those of the nonvirulent NDV strains, the variation in the level of identity at the C terminus (92% identity) and other regions of the protein (95–99% identity) was only marginal. Whether the changes in NP protein reflect, among other factors, a phenotypic change from extremely virulent to avirulent characteristics needs to be investigated.

The intergenic sequences and all the gene start and gene end signals with the consensus sequences of most paramyxoviruses are summarized in Fig. 1. Each of the three intergenic sequences determined here (NP–P, P–M and M–F junctions) has only one nucleotide. The two previously published intergenic sequences (F–HN and HN–L junctions) are 31 nt and 47 nt long (Chambers *et al*., 1986*a*, *b*). A previously determined sequence in the NP–P gene junction for NDV strain D26 is 2 nt (CA) long (Ishida *et al*., 1986). Thus, the intergenic regions in NDV vary in length from 1 to 47 nt. No generally conserved sequence is apparent among all these intergenic regions. Four out of the five sequences ended with adenosine, a common feature in the intergenic sequences of many paramyxoviruses (Collins *et al*., 1986; Crowley *et al*., 1988; Kawano *et al*., 1991). This lack of conservation with respect to length or sequence is in complete agreement with intergenic regions of respiratory syncytial virus (RSV; Collins *et al*., 1986) and other members of the genus *Rubulavirus*, namely parainfluenza virus type 2 (PIV2), mumps virus (MuV) and simian virus 5 (SV5) (Kawano *et al*., 1991; Elango *et al*., 1988; Sheshberadaran & Lamb, 1990), and in complete contrast with the conserved trinucleotide (3′ GAA or GGG) intergenic



Consensus sequence

	Gene end	Intergene	Gene start
NDV	$AAUCU_{6or7}$	Variable 1-47nt	$UGCCCAUC^{U}_{C}U$
PIV2	$UA^{G}_{A}AUUNU_{5-7}$	Variable 4-45nt	$^{UC}_{CU}N^{G}_{A}NN^{C}_{U}UNNNN$
SV5	$A^{GAU}_{AUA}UCU_{4-7}$	Variable 1-22nt	$U^{U}_{C}C^{GU}_{UG}GCUUG^{C}_{G}{}^{U}N$
MuV	$^{A}_{U}N^{U}_{A}A^{AU}_{UA}{}^{C}_{U}{}^{}_{A}U_{6or7}$	Variable 1-7nt	$U^{U}_{C}C^{GU}_{UG}N^{CU}_{UC}UNNN$
PIV3	$UUNAU^{AU}_{UC}U_{5}$	GAA	UCCUNNUUUC
SeV	$UNAUUCU_{5}$	GAA[GGG for HN-L]	UCCCANUUNC

Fig. 1. Complete gene map of the Beaudette C strain of NDV (in genome RNA-sense). The last nucleotide of the gene end, the first nucleotide of the gene start and the first and last nucleotides of the leader and trailer are numbered. The sequences of the leader are derived from Kurilla *et al.* (1985); the intergenic regions in the F–HN junction from Chambers *et al.* (1986*b*) and in the HN–L junction from Chambers *et al.* (1986*a*). The gene end and gene start sequences of NDV are derived from the published sources of the respective genes (mentioned in the text). The consensus sequences are from the following sources: PIV2, Kawano *et al.* (1991); Sendai virus and PIV3, Lamb & Kolakofsky (1996); MuV, Elango *et al.* (1988); and SV5, Sheshberadaran & Lamb (1990).

sequences described for Sendai virus (SeV; Gupta & Kingsbury 1984), measles virus (MeV; Crowley *et al.*, 1988) and canine distemper virus (Sidhu *et al.*, 1993).

No trailer sequence for any NDV strain was reported before, but it has been determined in strain Beaudette C to be 113 nt long (Fig. 1). The trailer region of NDV was compared with those available for other members of the family *Paramyxoviridae* (Fig. 2*c*). In length, the NDV trailer is intermediate between an unusually long (155 nt) RSV trailer (Mink *et al.*, 1991) and the typical 40–60 nt long trailer of most paramyxoviruses (Shioda *et al.*, 1986; Blumberg *et al.*, 1988; Galinski *et al.*, 1988). NDV shares only 4 or 5 nt identity at the 5′ terminus with other members of the genus *Rubulavirus* (PIV2 and MuV), compared to 8 to 10 nt identity with PIV3, SeV and MeV. In NDV, 11 of the first 12 terminal nucleotides are exact complements with a single mismatch at position 9, and 20 of the terminal 30 nt are exact complements (Fig. 3). This result indicates that the promoters for genome and antigenome synthesis probably lie within the first 30 nt from both ends.

Comparison of terminal sequences between the lentogenic and velogenic strains of NDV has not been reported before,

(*a*)

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                                                                                  55
         D26 Leader  3'  UGGUUUGUCUCUUAGACAUUCCAUGCUAUUUUCCGCUUCCUCGUUAGCUUCAGCAUGCCCAUCUU
                         *************** ***** ************************ ***** *
Beaudette C Leader   3'  UGGUUUGUCUCUUAGGCAUUCAAUGCUAUUUUCCGCUUCCUCGUUAACUUCAACGUGCCCAUCUU
                         ***************** ********************************* ***
          B1 Leader  3'  UGGUUUGUCUCUUAGGCACUCAAUGCUAUUUUCCGCUUCCUCGUUAACUUCAGCGUGCCCAUCUU
                                                                                   NP gene start
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(*b*)

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                                                                            58
Beaudette C Trailer 3'  _AACUUGAGGCUGAGGAAUCUAGAGCUUAAGCUUGAGUUUAUUUACAGAAUUUUUUC
                         **** *** **********  **************************** *******
         B1 Trailer 3'  CAACUCGAGACUGAGGAAUCCUGAGCUUAAGCUUGAGUUUAUUUACAGAAAUUUUUUC

                        59
Beaudette C Trailer     CAACGCGUGUUAAUAAGAACUCACAUCAGAACAAUAAGUGGUUUAGAAACAAACCA  5' 113
                        ****************************** ** **********************
         B1 Trailer     CAACGCGUGUUAAUAAGAACUCACAUCAGAGCAGUAAGUGGUUUAGAAACAAACCA  5' 114
```

(*c*)

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                                         50       40        30         20         10
NDV                                    .......AAUAAGAACUCACAUCAGAACAAUAAGUGGUUUAGAAACAAACCA
PIV2                                                AAAUUCUUUUUGUAUAACUAAAAGGGGAACCA
MuV                                            AAAAUUCUUUUUUAACUAAAAUGAAAGAGGGGAACCN
PIV3                        AUUCUUUUUGUACAUUAUUAUAUAUAUAUGGUUUGUCUCAAGAAGAGAACAAACCA
SeV               AUUCUUUUUGAAUCUUCUUCUGUUCUUUUAAAUUUUCCUAUGUAUAGAGAAUUUGAGAACAGACCA
MeV                                   UUCUUUUGAAACUUUUAUGCUUCAAAGAUAAAGGGUCGAAACAGACCA
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Fig. 2. Nucleotide sequences of the 3′ and 5′ terminal regions of NDV and their comparison with related viruses. All sequences are shown in genome RNA-sense. (*a*) Comparison of leader sequences from NDV strains Beaudette C, D26 and B1. The leader sequence of strain B1 is compared to the previously published sequences of strain D26 (Ishida *et al.*, 1986) and Beaudette C (Kurilla *et al.*, 1985). Identical nucleotides are indicated by asterisks. Nucleotides in the leader sequence of strain D26 that are different from those of strain B1 are underlined. (*b*) Comparison of the trailer sequences from NDV strain B1 and Beaudette C. Identical nucleotides are indicated by asterisks. (*c*) Comparison of the trailer region of NDV strain Beaudette C and those of some of the other nonsegmented negative-sense viruses. Only the 5′ terminal 45 nt of the NDV trailer region are shown. The gene end sequences of the L genes of the other viruses are underlined. The sequences are from the following sources: PIV2, Kawano *et al.* (1991); MuV, Okazaki *et al.* (1992); PIV3, Galinski *et al.* (1988); SeV, Shioda *et al.* (1986); and MeV, Blumberg *et al.* (1988).

```
           10        20         30         40         50
3'  UGGUUUGUCUCUUAGGCAUUCAAUGCUAUUUUCCGCUUCCUCGUUAACUUCAACGUGCCCAUCUU ............
                                                        NP gene start
                                                                          L gene end
5'  ACCAAACAAAGAUUUGGUGAAUAACAAGACUACACUCAAGAAUAAUUGUGCGCAA-----------//UUUUUUUCUAA...
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Fig. 3. Complementarity between the 3′ and 5′ ends of NDV (strain Beaudette C) genomic RNA. The nucleotide sequences are in genome RNA-sense and the complementary nucleotides are marked by vertical lines. The sequences of the NP gene start and L gene end are indicated. The leader end of the sequence is derived from Kurilla *et al.* (1985).

probably due to the unavailability of the trailer sequence. We have determined the sequences of the leader and trailer regions of lentogenic NDV strain B1 with an intention to see if there is any difference between the ends of a nonvirulent and virulent NDV genome. The trailer region of strain B1 is 114 nt long, 1 nt longer than the trailer sequence of strain Beaudette C (Fig. 2*b*). The two sequences, however, showed a high level of identity, with only 7 nt differences throughout. One notable feature was that the 5′ terminal 22 nt were identical in the two sequences, indicating a critical role in antigenome synthesis. Similarly, the comparison of leader sequences of strains B1, Beaudette C and another lentogenic strain, D26, revealed minimal variation (Fig. 2*a*). The leader sequences of strains Beaudette C, B1 and D26 share 15 nt at their 3′ termini. Interestingly, the leader sequence of lentogenic strain B1 showed only a 2 nt difference with that of the velogenic strain Beaudette C, but a 5 nt difference with the lentogenic strain D26. Thus, the terminal regions do not show any remarkable divergence in sequence between the virulent and nonvirulent strains and probably do not play a role in determining the virulence of NDV. Similar observations also have been reported for rinderpest virus (Baron & Barrett, 1995).

The length of the genome of NDV strain Beaudette C is 15 186 nt, which is similar to the genome length of some of the other members of the genus *Rubulavirus*, namely MuV (15 384 nt; Okazaki *et al*., 1992), SV5 (15 244 nt; Parks *et al*., 1992) and SV41 (15 450 nt; accession no. X64275). One implication of the genome length is the fact that RNA replication by certain paramyxoviruses is efficient only if the nucleotide length of the genome is a multiple of six. This rule, termed the 'rule of six', is thought to arise as a result of association of the NP protein monomer with exactly six nucleotides of the RNA. The rule holds for SeV (Calain & Roux, 1993), MeV (Sidhu *et al*., 1995) and PIV3 (Durbin *et al*., 1997), but does not appear to hold for rabies virus and vesicular stomatitis virus and has been demonstrated not to hold for RSV (Samal & Collins, 1996). The rule has thus far been noted as a feature of viruses which have RNA editing. It would be interesting to see if NDV, the genome length of which is an exact multiple of six and which possesses RNA editing, follows the rule. Completion of the genome sequence of NDV should allow the development of a reverse genetic system for rescuing synthetic minigenomes or full-length antigenome RNA analogues. Recovery of infectious NDV from cDNA will be useful for studying the molecular biology of NDV and for the development of better vaccines for this important poultry pathogen.

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