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Role of Untranslated Regions of the Hemagglutinin-Neuraminidase Gene in Replication and Pathogenicity of Newcastle Disease Virus[▽]

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To determine the role of untranslated regions (UTRs) in replication and pathogenesis of Newcastle disease virus (NDV), we generated recombinant viruses with deletions in 5′ and 3′ UTRs of the HN mRNA. Deletion of any HN UTR did not noticeably affect in vitro replication of these viruses. However, complete deletion of the 5′ UTR of the HN gene decreased the HN mRNA levels and HN protein contents in virus particles, resulting in attenuation of the virus in chickens. This indicates that the 5′ UTR of HN mRNA plays an important role in replication and pathogenicity of NDV in vivo.

Newcastle disease virus (NDV) causes a highly contagious respiratory and neurologic disease in chickens, leading to severe economic losses in the poultry industry worldwide (1). NDV is a member of the family *Paramyxoviridae* and has a nonsegmented, negative-sense RNA genome consisting of six genes (3′-NP-P-M-F-HN-L-5′) (9). Each gene is flanked by conserved transcriptional initiation and termination control sequences, known as gene start (GS) and gene end (GE), respectively (10). In addition, NDV contains 5′ and 3′ untranslated regions (UTRs) between the GS and ORF and between the ORF and GE, respectively. The sequences and lengths of the UTRs are variable among the different mRNAs. Little is known about the functions of UTRs in replication and pathogenesis of NDV and other paramyxoviruses.

Among the NDV proteins, the HN glycoprotein is a multifunctional protein, and its functions include recognition of sialic acid-containing receptors on cell surfaces and promotion of the fusion activity of F protein (6). The HN protein also induces a high level of NDV-specific neutralizing antibody in chickens. Therefore, we investigated the roles of UTRs of the HN mRNA in replication and pathogenicity of NDV. In the NDV intermediate virulent (mesogenic) strain Beaudette C (BC), 5′ and 3′ UTRs of the HN mRNA are 81 nucleotides (nt) and 166 nt, respectively. Since essential regions of the HN UTRs for virus replication are unknown, several partial and complete deletions in the HN UTRs in a full-length antigenomic cDNA of NDV strain BC (9) were constructed (Fig. 1A). The mutant UTRs included deletions of 6 or 78 nt in the 5′ UTR and 6, 24, or 162 nt in the 3′ UTR and double deletion of 78 nt in 5′ UTR and 162 nt in 3′ UTR. We also replaced the HN 5′ and 3′ UTRs with the corresponding UTRs of the NP mRNA. Recombinant NDVs were recovered by using our standard protocol (9). The in vitro replication of recovered viruses was determined by plaque assay in DF-1 cells that had been infected at a multiplicity of infection of 0.01 (6). All mutant viruses and the parental virus, rBC, grew to similar titers after 32 h postinfection (data not shown), indicating that neither the 5′ nor the 3′ UTR of the HN mRNA has an observable effect on in vitro replication of NDV.

The effect of HN UTR deletions on the HN transcription was determined by Northern blot hybridization. Total RNAs were isolated from the virus-infected DF-1 cells, electrophoresed, transferred to nitrocellulose membranes, and then hybridized with ^{32}P-labeled double-stranded cDNA probes specific to NDV HN, F, L, or NP mRNA (11) (Fig. 1B). The ratio of HN to NP mRNAs was determined by measuring radioactivity using a phosphorimager. A reduction in the ratio of HN to NP mRNA levels was detected in complete 5′ UTR deletion, complete 3′ UTR deletion, and complete 5′ and 3′ double-deletion viruses (Fig. 1C), suggesting that complete deletion of HN UTRs affected the level of HN mRNA transcription. However, quantification of the ratios of F to NP indicated that their deletions may not affect transcription of other viral mRNAs (Fig. 1D). In influenza A virus, mutation of nonconserved nucleotides in the 3′ and 5′ UTRs of the NA segment was also found to decrease levels of NA antigenome RNA and mRNA segments (12). Overall accumulation of viral mRNAs in infected cells can be impaired by the reduced binding of viral polymerase complex or host factors involved in transcription of viral mRNA (2). It is possible that the UTRs contain sites essential for binding of viral polymerase complex or host factors. Loss of these sites by the deletion of UTRs might decrease their efficiency of binding to the genome.

Deletion of the 5′ UTR of the HN mRNA affected not only the levels of HN mRNA but also HN protein production. The expression of HN protein by the mutant NDVs was analyzed by Western blotting (7). Total cell lysates were collected from the virus-infected DF-1 cells at 24 h postinfection, electrophoresed, transferred to a nitrocellulose membrane, and immunostained using a monoclonal antibody against the NDV HN protein or a monospecific antibody against the M protein. Among the mutant viruses, complete 5′ UTR deletion and complete 5′ and 3′ double-deletion viruses showed the lowest ratio of HN to M (5% for both viruses), compared to that of the parental virus (50%) (Fig. 2). Consequently, we observed less incorporation of the HN protein into these two mutant

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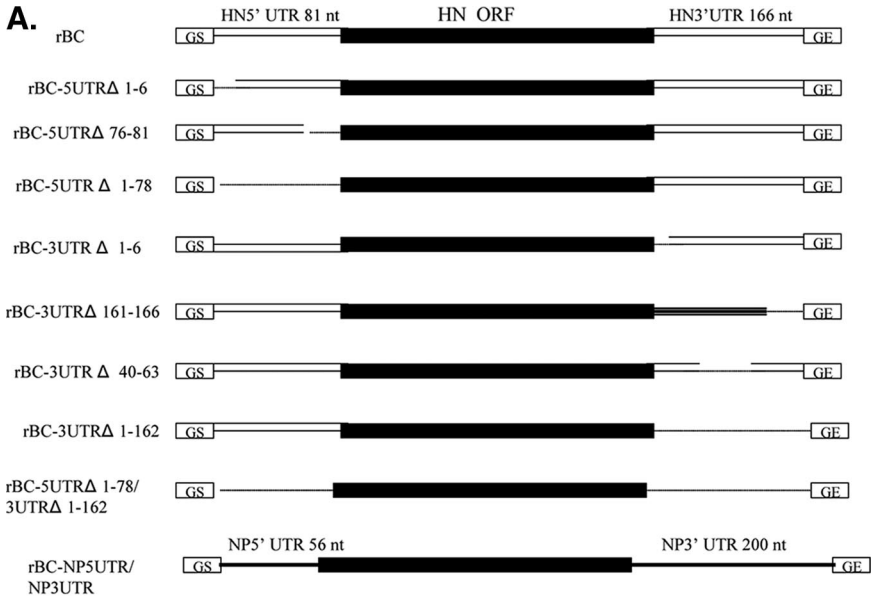
A.
HN5' UTR 81 nt
HN ORF
HN3'UTR 166 nt
rBC
GS
GE
rBC-5UTRΔ 1-6
rBC-5UTRΔ 76-81
rBC-5UTR Δ 1-78
rBC-3UTR Δ 1-6
rBC-3UTRΔ 161-166
rBC-3UTR Δ 40-63
rBC-3UTRΔ 1-162
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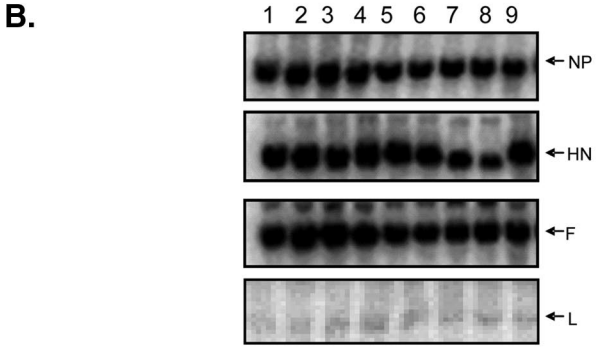
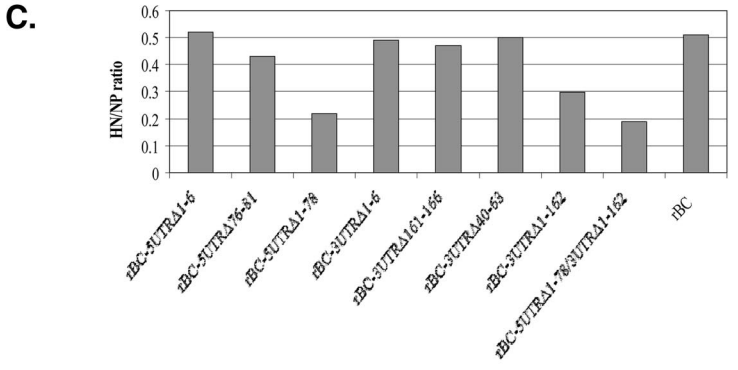
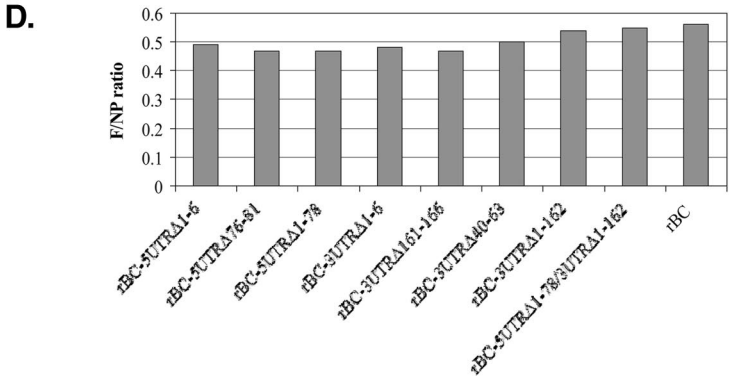
3UTRΔ 1-162
NP5' UTR 56 nt
NP3' UTR 200 nt
rBC-NP5UTR/
NP3UTR


B.
1 2 3 4 5 6 7 8 9
NP
HN
F
L


C.
HN/NP ratio
0.6
0.5
0.4
0.3
0.2
0.1
0
rBC-5UTRΔ1-6
rBC-5UTRΔ76-81
rBC-5UTRΔ1-78
rBC-3UTRΔ1-6
rBC-3UTRΔ161-166
rBC-3UTRΔ40-63
rBC-3UTRΔ1-162
rBC-5UTRΔ1-78/3UTRΔ1-162
rBC


D.
F/NP ratio
0.6
0.5
0.4
0.3
0.2
0.1
0
rBC-5UTRΔ1-6
rBC-5UTRΔ76-81
rBC-5UTRΔ1-78
rBC-3UTRΔ1-6
rBC-3UTRΔ161-166
rBC-3UTRΔ40-63
rBC-3UTRΔ1-162
rBC-5UTRΔ1-78/3UTRΔ1-162
rBC

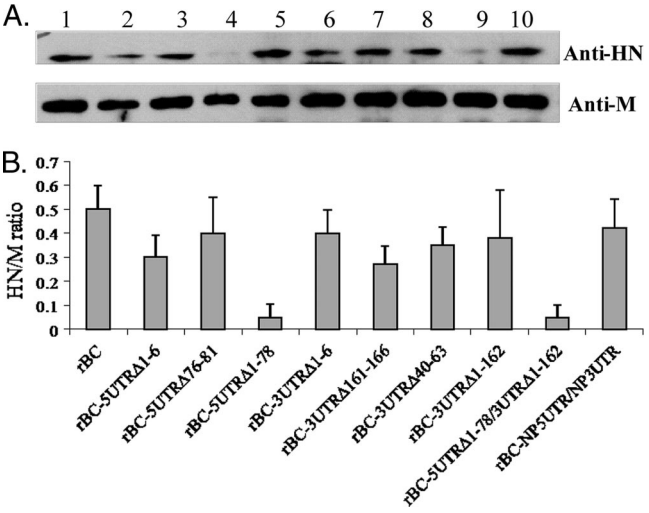


FIG. 2. Protein synthesis in DF1 cells infected with recombinant viruses. (A) Western blot of HN and M proteins from parental and HN UTR mutant virus-infected cells. DF1 cells were infected at 5 PFU/cell, and total proteins were collected at 24 h postinfection. Lanes: 1, rBC; 2, rBC-5′UTRΔ1-6; 3, rBC-5′UTRΔ76-81; 4, rBC-5′UTRΔ1-78; 5, rBC-3′UTRΔ1-6; 6, rBC-3′UTRΔ161-166; 7, rBC-3′UTRΔ40-63; 8, rBC-3′UTRΔ1-162; 9, rBC-5′UTRΔ1-78/3′UTRΔ1-162; and 10, rBC-NP5′UTR/NP3′UTR. (B) Ratios of quantified HN protein to M protein levels from the parental virus, rBC, and the HN UTR mutant viruses. Each bar shows the mean and standard error for the mean of triplicate samples.

particles than other virus particles. The parental and mutant viruses harvested from allantoic fluids were purified through a 30% sucrose cushion, and the viral proteins were separated on an 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel (3). Complete 5′ UTR deletion and 5′ and 3′ double-deletion viruses showed relatively low ratios of HN to M proteins (Fig. 3A). We also confirmed that smaller amounts of the HN protein were expressed on the surfaces of these mutant virus particles than on those of the parental and complete 3′ UTR deletion viruses by electron microscopy using immunogold labeling with HN antibody (Fig. 3B). Since complete 3′ UTR deletion alone did not alter the level of HN protein production in infected cells or its incorporation into virus particles, complete 5′ UTR deletion probably contributed to the reduced expression of HN protein in the infected cells with complete 5′ and 3′ UTR double-deletion virus. In measles virus, canine distemper virus, and rinderpest virus, deletion of

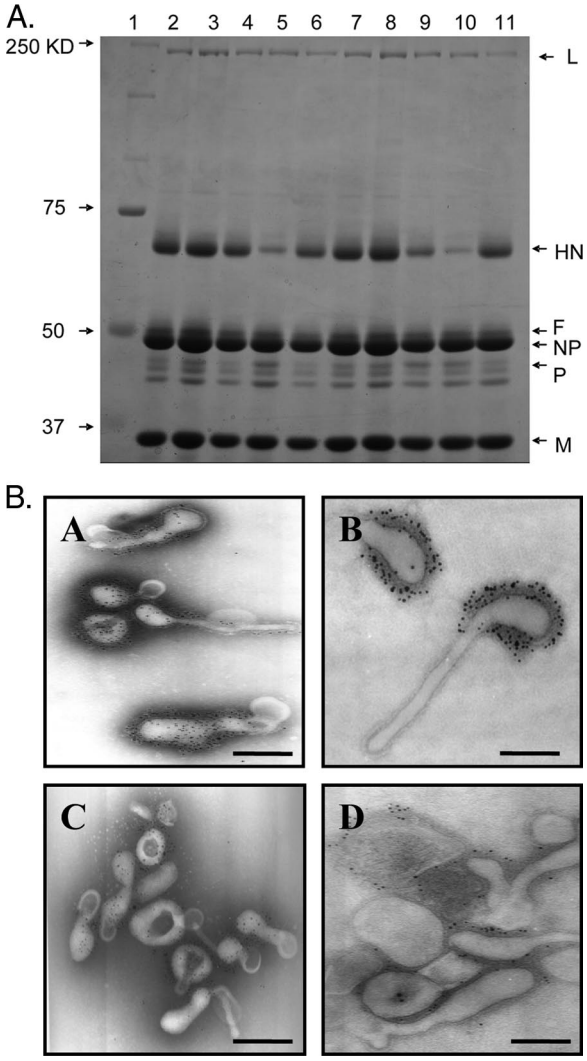


FIG. 3. Effect of UTRs of HN mRNA deletion on incorporation of HN proteins into viral particles and their surface expression. (A) Incorporation of viral proteins into virus particles. Ultracentrifuge-purified viruses from infected allantoic fluids were separated by electrophoresis on an 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel. The gel was then stained with Coomassie brilliant blue. The molecular masses and individual viral proteins are indicated. Lanes: 1, protein standard; 2, rBC; 3, rBC-5′UTRΔ1-6; 4, rBC-5′UTRΔ76-81; 5, rBC-5′UTRΔ1-78; 6, rBC-3′UTRΔ1-6; 7, rBC-3′UTRΔ161-166; 8, rBC-3′UTRΔ40-63; 9, rBC-3′UTRΔ1-162; 10, rBC-5′UTRΔ1-78/3′UTRΔ1-162; and 11, rBC-NP5′UTR/NP3′UTR. (B) Immunogold electron micrographs of parental and complete 5′ and 3′ UN UTR deletion viruses. Image A, rBC; image B, rBC-3′UTRΔ1-162; image C, rBC-5′UTRΔ1-78; image D, rBC-5′UTRΔ1-78/3′UTR Δ1-162. Bar, 100 nm.

FIG. 1. (A) Constructs of recombinant viruses containing deletions in the 5′ and 3′ UTRs of HN mRNA and Northern blot analysis of RNAs synthesized by these mutant NDVs. Solid bars represent HN mRNA of rBC, double lines represent 5′ and 3′ UTRs, dotted lines represent deletions of UTRs, and single lines represent 5′ and 3′ UTRs of NP mRNA. Briefly, rBC-5′UTRΔ1-6, rBC-5′UTRΔ76-81, and rBC-5′UTRΔ1-78 have deletions of nucleotides 1 to 6, 76 to 81, and 1 to 78 from the 5′UTR of HN mRNA, respectively. rBC-3′UTRΔ1-6, rBC-3′UTRΔ40-63, rBC-3′UTRΔ161-166, and rBC-3′UTRΔ1-162 have deletions of nucleotides 1 to 6, 40 to 63, 161 to 166, and 1 to 162 in the 3′ UTR of HN mRNA, respectively. rBC-5′UTRΔ1-78/3′UTRΔ1-162 includes deletions of nucleotides 1 to 78 in the 5′UTR of HN and nucleotides 1 to 162 in the 3′ UTR of HN mRNA. rBC-NP5′UTR/NP3′UTR has the 5′and 3′ HN UTRs replaced by the NP 5′ and 3′ UTRs. (B) For Northern blot analysis of RNAs synthesized by the mutant NDVs, DF1 cells were infected with the indicated viruses (multiplicity of infection of 5 PFU), incubated for 24 h, and harvested, and then total intracellular RNAs were extracted. The RNAs were separated by electrophoresis in a formaldehyde agarose gel, transferred onto nitrocellulose membranes, and hybridized with ^{32}P-labeled double-stranded cDNA probes specific to the HN, NP, L, and F genes. Lanes: 1, rBC-5′UTRΔ1-6; 2, rBC-5′UTRΔ76-81; 3, rBC-5′UTRΔ1-78; 4, rBC-3′UTRΔ1-6; 5, rBC-3′UTRΔ161-166; 6, rBC-3′UTRΔ40-63; 7, rBC-3′UTRΔ1-162; 8, rBC-5′UTRΔ1-78/3′UTRΔ1-162; and 9, rBC. (C and D) The mRNA ratios of HN to NP (C) and F to NP (D) were quantitated by measuring radioactivity using a phosphorimager.

TABLE 1. Replication of recombinant viruses in 2-week-old chickens[a]

Virus	Viral titer (log PFU/g) at[b]:							
	3 dpi				5 dpi			
	Brain	Lung	Trachea	Spleen	Brain	Lung	Trachea	Spleen
rBC	3.2 ± 0.07	2.8 ± 0.61	3.6 ± 0.35	2.7 ± 0.28	3.0 ± 0.05	3.0 ± 0.33	3.8 ± 0.40	2.7 ± 0.34
rBC-Δ5′UTR	ND	ND	ND	ND	ND	ND	ND	ND
rBC-Δ3′UTR	2.8 ± 0.43	ND	3.0 ± 0.94	ND	2.8 ± 0.43	3.4 ± 0.39	4.1 ± 0.29	2.7 ± 0.90
rBC-Δ5′UTR/Δ3′UTR	ND	ND	ND	ND	ND	ND	ND	ND
rBC-NP5′UTR/NP3′UTR	2.9 ± 0.34	2.6 ± 0.13	3.6 ± 0.43	ND	2.8 ± 0.16	ND	ND	ND

[a] Two-week-old chickens in groups of nine were infected with 10^6 PFU of each virus per chicken by oculonasal route. On days 3, 5, and 7, three chickens per group were sacrificed. Brains, lungs, tracheas, and spleens were collected, and virus titers were determined by plaque assay. No virus was detected in all the tissue samples from each group on day 7.

[b] Values are means ± standard errors of the means. ND, not detected.

the 5′ UTR of F mRNA was also found to have an inhibitory effect on the translation of F protein (4, 5, 8). A secondary structure predicted to form in the 5′ UTR may allow ribosomes to avoid scanning the entire region, and loss of this structure may affect translation efficiency (8). Therefore, in this study, inefficient translation of the HN protein in complete 5′ UTR deletion and 5′ and 3′ double-deletion viruses may be due to a lack of this secondary structure. It is also noteworthy that decreased incorporation of the HN protein in those mutant viruses did not increase the incorporation of the F protein into virus particles (Fig. 3A), suggesting that F and HN proteins have specific anchor sites insides the virus particles and that these sites are not interchangeable.

We further determined pathogenicity and replication of these mutant viruses in chickens. The pathogenicity of the mutant viruses in 1-day-old specific-pathogen-free chicks was determined by the intracerebral pathogenicity index (ICPI) test (1). The highest ICPI value that can be achieved by a highly virulent NDV strain is 2.00, while the avirulent strains have ICPI values approaching 0.00. Complete 5′ UTR deletion and 5′ and 3′ UTR double deletion lowered the ICPI values of mutant viruses (0.86 and 1.08, respectively) compared to that of the parental virus (1.49). The ICPI values of other mutant viruses were 1.43 (rBC-5′UTRΔ1-6), 1.24 (rBC-5′UTRΔ76-81), 1.35 (rBC-3′UTRΔ1-6), 1.33 (rBC-3′UTRΔ161-166), 1.16 (rBC-3′UTRΔ40-63), and 1.26 (rBC-NP5′UTR/NP3′UTR). These results showed greater levels of attenuation for complete HN 5′ UTR and complete 5′ and 3′ UTR double-deletion viruses than for other UTR deletion viruses. We then evaluated the replication abilities of the mutant (complete 5′ UTR deletion, complete 3′ UTR deletion, and complete 5′ and 3′ UTR double-deletion) viruses in 2-week-old chickens by inoculating 10^6 PFU of viruses per bird by the oculonasal route of infection. Viruses were detected in most of the tissues (brain, lung, trachea, and spleen) collected from three birds infected with the parental and complete 3′ UTR deletion viruses at 3 and 5 dpi (Table 1). In contrast, replication of complete 5′ UTR deletion and complete 5′ and 3′ UTR double-deletion viruses in chickens was rarely detected, indicating that complete deletion of the 5′ UTR of HN strongly impaired the replication ability and pathogenicity of NDV in chickens.

In addition to deletion of the UTRs of HN mRNA, we demonstrated that the UTRs of HN can be replaced with the UTRs of NP without affecting the functions of HN. The growth kinetics and the level of HN protein produced in the virus-infected cells and the in vivo replication of the virus were similar to those of the parental virus (Fig. 2 and 3A and Table 1). Our findings suggest that UTRs can be exchanged between NDV mRNAs without affecting the replication of the virus in vitro and in vivo. It will be interesting to determine whether the UTRs from heterologous virus mRNA can also be used to substitute the functions of UTRs of NDV mRNAs.

In general, our study provides insight into the functions of NDV UTRs. The HN UTRs may not be essential for replication of NDV in vitro, but they do play a critical role in its replication and pathogenicity in vivo. Therefore, UTR deletion would be a potential strategy for designing live attenuated recombinant NDV vaccines that would be beneficial to the poultry industry throughout the world.

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